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CARACTERIZACIÓN DEL COMPLEJO SURFACTANTE PULMONAR: ESTUDIO DE LA ESTRUCTURA Y FUNCIÓN DE LA PROTEÍNA A (SP-A)

TESIS DOCTORAL

por

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A Miguel, por su vitalidad y mi inmortalidad.

A Lola, cuando menos mi mejor amigo.

A mi familia, por crearme y criarme hasta hoy.

A Angelina Ayuso, por su inquietud científica.

A D. José, a sus circunstancias y a las mías.

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ABREVIATURAS

C1qR	receptor de C1q
C1qR(P)	receptor de C1q y colectinas
CL-43	colectina 43
DPPC	dipalmitoil-L-α-fosfatidilcolina
InsatPC	fosfatidilcolina insaturada
K_a^{Ca2+}	constante de activación de calcio
LC	estado líquido condensado
LE	estado líquido expandido
LipNeu	lípidos neutros
LPC	lisofosfatidilcolina
LPS	lipopolisacárido
MBP	proteína ligante de manosa
NRDS	síndrome de *distress* respiratorio neonatal
P	presión
PC	fosfatidilcolina
PE	fosfatidiletanolamina
PG	fosfatidilglicerol
PI	fosfatidilinositol
POPC	β-palmitoil-γ-oleil-L-α-fosfatidilcolina
PS	fosfatidilserina
r	radio alveolar
SatPC	fosfatidilcolina saturada
SM	esfingomielina
SP-A	proteína A del surfactante pulmonar
SP-B	proteína B del surfactante pulmonar
SP-C	proteína C del surfactante pulmonar
SP-D	proteína D del surfactante pulmonar
T_m	temperatura de transición de fase
UTR	regiones no traducidas del DNA
γ	tensión superficial

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1. INTRODUCCIÓN

1.1. EL SURFACTANTE PULMONAR. IMPORTANCIA BIOLÓGICA Y CLÍNICA

La importancia de las fuerzas de tensión superficial dentro de las propiedades mecánicas del pulmón fue reconocida por primera vez hace 70 años por el fisiólogo sueco Kurt von Neergaard (von Neergaard 1929). En 1961, Clements y colaboradores (Clements *et al.* 1961) propusieron un modelo basado en la ley de Laplace para explicar las fuerzas que actúan en el interior de los alveolos pulmonares durante la respiración. Según este modelo, el pulmón se compondría de un conjunto de cámaras esféricas (los alveolos) conectadas con el exterior por una serie de conductos. La **ley de Laplace** relaciona la presión de aire necesaria para abrir estas cámaras (P) con el radio de éstas (r) y con la tensión superficial en su interior (γ) (fig. 1.1):

$$P = \frac{2\gamma}{r}$$

Según esta relación, la presión necesaria para volver a expandir los alveolos de menor radio tras una espiración sería muy alta si la comparamos con la que se precisa para aquellos otros de gran tamaño. Esto hace que los primeros tiendan a colapsarse, comprometiéndose seriamente la función respiratoria. La estabilidad mecánica del alveolo pulmonar es posible gracias al **surfactante pulmonar**, un complejo lipoproteico que se encuentra tapizando sus paredes. Gracias a sus propiedades tensoactivas, el surfactante es capaz de reducir la tensión superficial en la interfase aire/agua hasta valores inferiores a 10 mN/m al final de la espiración (el valor para el agua pura es de 72 mN/m), evitando así el colapso de los alveolos más pequeños (Hamm *et al.* 1996, Hawgood 1997).

Además de su función mecánica, se atribuyen otras propiedades al surfactante

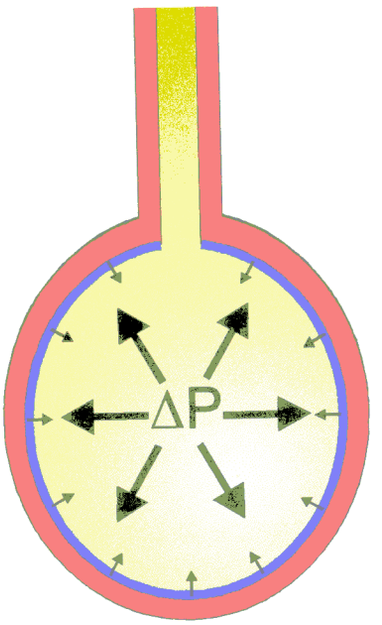

Figura 1.1. Esquema del alveolo pulmonar donde se muestran las fuerzas que actúan en el interior de los alveolos pulmonares. El agua alveolar tiende a colapsar el alveolo debido a las fuerzas de tensión superficial (flechas pequeñas). Estas fuerzas son las que el pulmón debe vencer durante la inspiración (flechas grandes).

pulmonar, como es la de evitar la transudación de líquido intersticial al espacio alveolar (Daniels *et al.* 1995, Hohlfeld *et al.* 1997). También se ha demostrado una función de defensa frente a microorganismos patógenos. Recientemente se ha comprobado que esta última propiedad se debe a las proteínas hidrofílicas (ver más adelante). Debido a sus propiedades tensoactivas, el surfactante pulmonar favorece la solvatación de las partículas pequeñas que penetran por las vías aéreas con el agua de la subfase acuosa, facilitando así su contacto con el epitelio alveolar (Schürch *et al.* 1990). Este hecho posibilitaría por tanto la fagocitosis de estas partículas por los macrófagos alveolares, si bien la solvatación también podría facilitar la interacción de

toxinas y sustancias nocivas con el epitelio alveolar. Asimismo, se ha demostrado su actividad antioxidante y la capacidad inmunorreguladora de algunos de sus componentes (Wright 1997).

Alteraciones cualitativas y cuantitativas del surfactante pulmonar aparecen asociadas a numerosas patologías pulmonares, habiéndose observado variación en la composición lipídica, disminución en la relación cuantitativa lípido/proteína y alteración de sus propiedades tensoactivas (Hamm *et al.* 1996). Entre estas patologías resulta de especial interés el **síndrome de *distress* respiratorio neonatal** (NRDS), causado por una deficiencia de surfactante debida a la inmadurez pulmonar y que es muy frecuente en prematuros (Avery y Mead 1959). Es precisamente en el tratamiento de esta patología donde más se han desarrollado las posibilidades terapeúticas de diversos surfactantes exógenos. El origen de los surfactantes utilizados en la práctica clínica es variado: lavado pulmonar de ternera, extracto orgánico de pulmón bovino o porcino troceado, y extracto de líquido amniótico humano, habiéndose puesto a punto también algunos surfactantes artificiales. Varias líneas de investigación estudian actualmente la incorporación de péptidos sintéticos que imiten las propiedades de las proteínas específicas del surfactante natural, y el uso de estos surfactantes en otras patologías pulmonares (síndrome de *distress* respiratorio del adulto, neumonía, neumonitis por hipersensibilidad, enfermedad pulmonar obstructiva) y en trasplante de pulmón (Hamm *et al.* 1996).

1.2. COMPOSICIÓN DEL SURFACTANTE PULMONAR. DESCRIPCIÓN ESTRUCTURAL DE SUS COMPONENTES

El surfactante pulmonar se compone aproximadamente de un 90% de lípidos y un 10% de proteínas en peso. En la figura 2 se representa la composición cuantitativa en peso del surfactante pulmonar según la revisión de Johansson y Curstedt (Johansson y Curstedt 1997). En esta figura no se incluyen otras proteínas, en su mayoría de origen plasmático, que suelen aparecer débilmente asociadas al surfactante en el lavado broncoalveolar y que se aislan con él.

1.2.1 COMPOSICIÓN LIPÍDICA

En las numerosas especies estudiadas, los fosfolípidos constituyen el 80-90% en peso de los lípidos del surfactante (fig. 1.2), siendo el resto lípidos neutros, principalmente colesterol (Johansson y Curstedt 1997, van Golde y Casals 1997). La **fosfatidilcolina** (PC), que es el fosfolípido mayoritario (70-80%), se encuentra disaturada (SatPC) en gran parte, principalmente ($\approx$ 40%) en forma de **dipalmitoilfosfatidilcolina** (DPPC). Entre las especies insaturadas predominan las formas monoenoicas, especialmente la 1-palmitoil-2-oleilfosfatidilcolina (POPC).

En hombre, perro y rata, entre otras especies, el segundo fosfolípido en importancia es el fosfatidilglicerol (PG), que aparece en una proporción anormalmente alta (10%), si bien en algunas especies o en determinadas fases del desarrollo de otras especies aparece sustituido por otro fosfolípido ácido, el fosfatifilinositol (PI). Otros

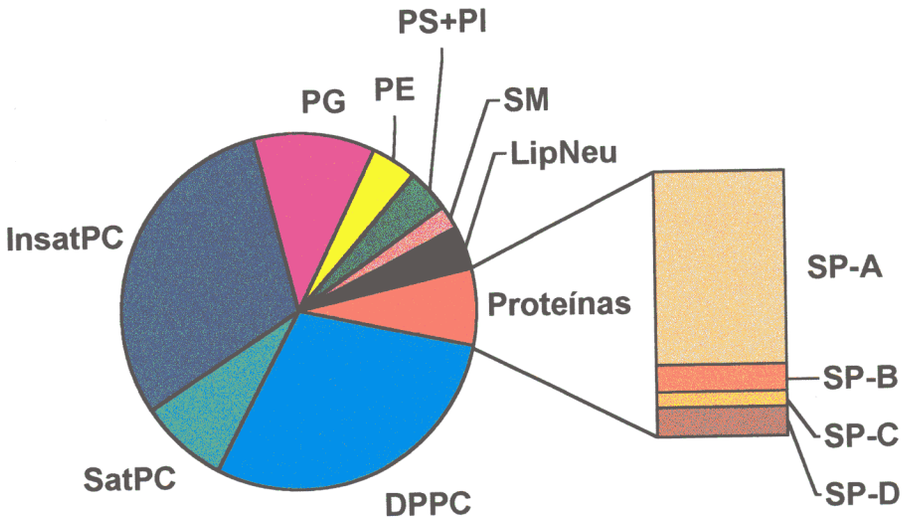

Figura 1.2. Composición cuantitativa del surfactante pulmonar. DPPC, dipalmitoilfosfatidilcolina; SatPC, fosfatidilcolina saturada; InsatPC, fosfatidilcolina insaturada; PG, fosfatidilglicerol; PE, fosfatidiletanolamina; PS, fosfatidilserina; PI, fosfatidilinositol; SM, esfingomielina; LipNeu, lípidos neutros.

fosfolípidos minoritarios son la fosfatidilserina (PS), la fosfatidiletanolamina (PE) y la esfingomielina (SM). En la composición lipídica del surfactante se han encontrado además glicolípidos, aunque aún no se conoce su función. También se han detectado cantidades importantes de plasmalógenos de colina, con un residuo de palmitoilo en la posición *sn*-2.

Se ha observado que junto a los lípidos del surfactante se secreta vitamina E, lo que explicaría al menos en parte sus propiedades antioxidantes (Rüstow *et al.* 1993).

1.2.2 COMPOSICIÓN PROTEICA

Las proteínas que se aislan con el surfactante son apoproteínas específicas aproximadamente en un 50 %; el resto lo constituyen otras proteínas inespecíficas como albúmina, lipoproteínas séricas, inmunoglobulinas (IgG e IgA), citoquinas y factores de crecimiento, en gran parte de origen plasmático (Hawgood 1997, Wright 1997). Se han detectado además varios oligopéptidos aniónicos ricos en aspartato con propiedades antibacterianas (Brogden *et al.* 1996).

Las proteínas específicas se denominan SP-A, SP-B, SP-C y SP-D, según la nomenclatura propuesta por Possmayer en 1988 (Possmayer 1988). Sus características estructurales se detallan a continuación.

SP-A

La SP-A, denominada SAP-35 hasta 1988 y descrita por primera vez en 1973 (King *et al.* 1973), es una proteína hidrofílica, con un pI de 4.4-5.6 (Benson *et al.* 1985). Su estructura cuaternaria consiste en un octadecámero de 650-700 kDa, compuesto por seis trímeros que forman hélices de tipo colagénico (Voss *et al.* 1988, Haagsman *et al.* 1989, King *et al.* 1989) (fig. 1.3). Se ha observado mediante microscopía electrónica que esta estructura es muy similar a la de la proteína C1q, presentando el aspecto de un ramo de flores con una longitud de 20 nm (Voss *et al.* 1988). Los monómeros, de 28-36 kDa,

están unidos mediante puentes disulfuro e interacciones de tipo no covalente entre sus dominios colagénicos. La variación en el pI y en la masa molecular del monómero de proteína se debe a los distintos grados de glicosilación y a las modificaciones covalentes sufridas a lo largo de su ciclo metabólico.

La SP-A pertenece a la familia de las llamadas lectinas tipo C o dependientes de Ca^{2+} y, dentro de éstas, se incluye en el grupo de las colectinas (lectinas con un dominio colagénico), junto con las proteínas ligantes de manosa (MBPs), la SP-D, la colectina 43 (CL-43) y la conglutinina. Todas estas proteínas poseen una estructura cuaternaria muy similar a la de la SP-A (fig. 1.4), en la que podemos distinguir varias regiones:

- La **región N-terminal** consta de 7-10 aminoácidos. Uno de estos aminoácidos es una cisteína implicada en la formación de un puente disulfuro intercatenario (Haas *et al.* 1991). Los puentes disulfuro formados de este modo conectan covalentemente a los seis trímeros que constituyen el octadecámero. En la posición 1 existe una Asn que se encuentra glicosilada en rata (McCormack *et al.* 1994a) y posiblemente en perro (Benson *et al.* 1985). Esta Asn no aparece en cambio en conejo (Boggaram *et al.* 1988) ni en hombre (Floros *et al.* 1986).

- A esta zona le sigue un dominio constituido por 70-73 aminoácidos que forman una **hélice de tipo colagénico** (Whitsett *et al.* 1985a, Whitsett *et al.* 1985b, Ross *et al.* 1986a, Voss *et al.* 1988, Haagsman *et al.* 1989). La secuencia de esta región está compuesta por 23-24 repeticiones de la secuencia Gly-X-Y, donde Y es hidroxiprolina en más de la mitad de ellas. Estos residuos de hidroxiprolina parecen ser importantes para mantener la estabilidad de la

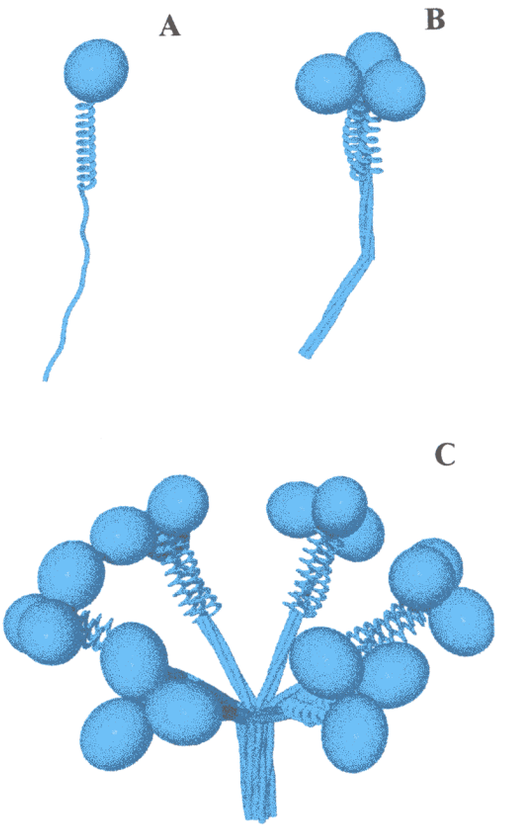

Figura 1.3. Formas monomérica (A), trimérica (B) y octadecamérica (C) de la SP-A.

hélice colagénica (McCormack *et al.* 1994a). Hacia la mitad de este dominio hay una Gly que no forma parte de ningún triplete, lo que origina una discontinuidad en la hélice. Esta discontinuidad podría funcionar como bisagra dentro de la molécula.

- Más adelante hay una zona de 30-40 aminoácidos conocida como **"cuello"**, que conecta la zona colagénica con el dominio globular C-terminal. Su secuencia se ajusta a la de una hélice anfipática, seguida de un segmento hidrofóbico. Aunque la estructura primaria de esta región no está muy conservada, la repetición periódica de héptadas donde los residuos primero y cuarto son hidrofóbicos hace pensar que la estructura tridimensional sí lo está (Weis y Drickamer 1994). El segmento N-terminal del cuello, al igual que en otras co-

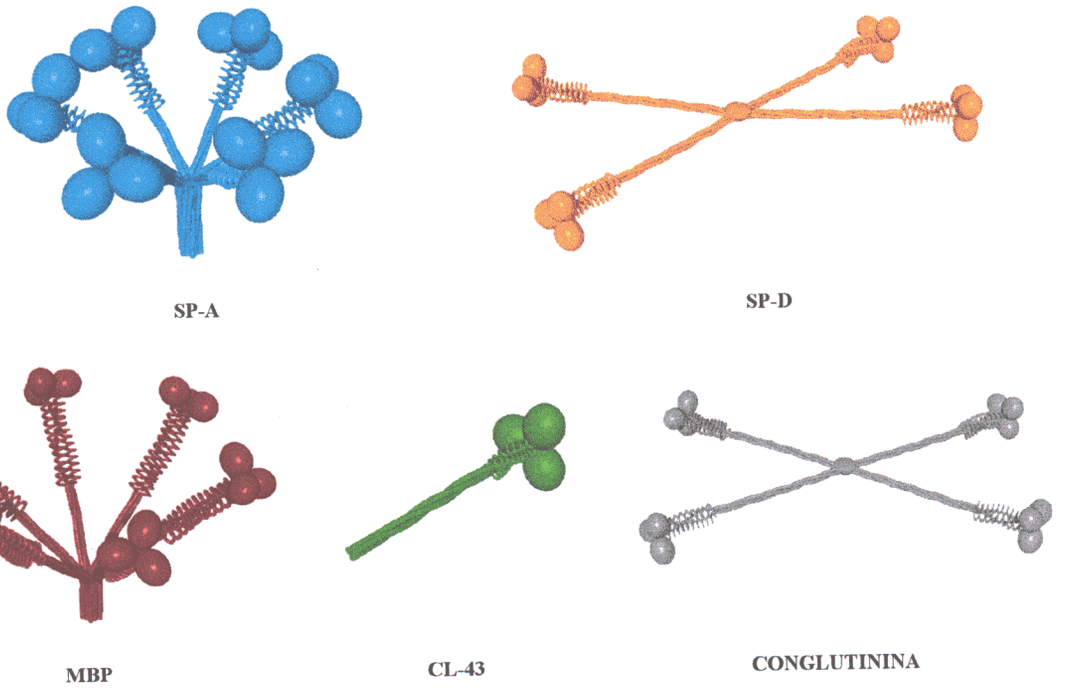

Figura 1.4. Proteínas del grupo de las colectinas. La SP-D y la conglutinina son proteínas dodecaméricas; la SP-A y la proteína ligante de manosa (MBP) son octadecaméricas; la colectina 43 (CL-43) es trimérica. En el dibujo se ha intentado mantener la proporción de tamaño de las distintas regiones dentro de cada proteína, y de las distintas proteínas entre sí.

lectinas, podría formar un "coiled-coil" compuesto por tres hélices α (Hoppe *et al.* 1994). Aunque para la SP-A no se ha demostrado aún, es posible que existan interacciones no covalentes entre el extremo C-terminal de esta región y el dominio globular.

- Finalmente encontramos un **dominio globular de tipo lectina** (de reconocimiento de carbohidratos) formado por unos 130 aminoácidos. La Asn-187 está unida covalentemente a una cadena oligosacárida rica en manosa y acabada en un residuo de ácido siálico (Whitsett 1985a, Whitsett *et al.* 1985b, Whitsett *et al.* 1985c, Phelps *et al.* 1986, Ross *et al.* 1986a). En la SP-A humana se han hallado determinantes antigénicos del grupo sanguíneo A en esta cadena (Stahlman *et al.* 1992). También hay en este dominio cuatro cisteínas que participan en dos puentes disulfuro intracatenarios (Haagsman *et al.* 1989, Ross *et al.* 1991). Estos puentes disulfuro, así como la posición de otros 13 residuos de esta región, están muy conservados en varias lectinas de mamíferos e invertebrados.

SP-B

La SP-B (fig. 1.5), que hasta 1988 aparece en la bibliografía como SPL(Phe) o SP18, es una proteína de naturaleza hidrofóbica y pI básico, cuyo monómero tiene 8.7 kDa de masa molecular (Johansson y Curstedt 1997). Su secuencia se compone de 79 aminoácidos. Seis de sus siete cisteínas forman tres puentes disulfuro intracatenarios muy conservados (Johansson *et al.* 1991b, Johansson *et al.* 1992). La existencia de estos puentes disulfuro y de otros residuos, implicados al parecer en la formación de varias hélices α anfipáticas, hace que se incluya a la SP-B en la familia de polipéptidos del tipo de la saposina (Patthy 1991, Zaltash y Johansson 1998), a la que también pertenecen la NK-

Figura 1.5. Forma monomérica de la SP-B, representada a partir de la predicción de regiones α de Garnier-Robson y de regiones α anfipáticas de Eisenberg. Se ha tomado además como modelo de plegamiento la estructura tridimensional de la NK-lisina, con la que presenta una alta homología (Liepinsh *et al.* 1997).

lisina, amebóporos, saposinas, y dominios de la esfingomielinasa ácida y de la aciloxiacilhidrolasa. Se ha observado que, tanto en presencia de lípidos como en distintas mezclas de disolventes, la SP-B tiene un contenido muy alto de hélice α (Vandenbussche *et al.* 1992b, Pérez-Gil *et al.* 1993, Morrow *et al.* 1993a, Cruz *et al.* 1995). Esta proteína se encuentra normalmente formando dímeros de 17.4 kDa constituidos por un puente disulfuro (Weaver *et al.* 1988, Johansson *et al.* 1991b, Johansson *et al.* 1992), aunque a veces aparece también en forma oligomérica.

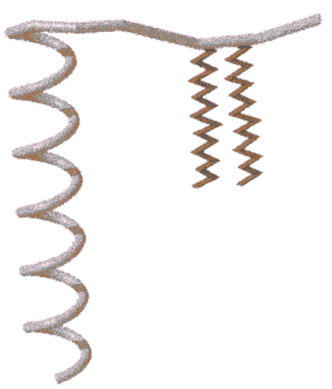

Figura 1.6. Representación de la forma dipalmitoilada de la SP-C.

SP-C

La SP-C (denominada SPL(pVal) o SP5 hasta 1988) es, al igual que la SP-B, uno de los llamados proteolípidos del surfactante (fig. 1.6). Se trata de un péptido de aproximadamente 4 kDa, aunque en condiciones no reductoras puede formar dímeros de 8 kDa (Creuwels *et al.* 1995a). Junto a la SP-C normal se han detectado también otras formas de esta proteína carentes de uno o más aminoácidos en ambos extremos de la cadena (Johansson *et al.* 1988, Simatos *et al.* 1990, Johansson *et al.* 1991a). La hidrofobicidad de esta proteína es muy elevada debido a su alto contenido en residuos de valina, isoleucina y leucina, y a la palmitoilación de sus dos cisteínas (Curstedt *et al.* 1990, Stults *et al.* 1991). Curiosamente, en perro sólo hay una cisteína, también palmitoilada (Stults *et al.* 1991, Johansson *et al.* 1991a). La función de las cisteínas aciladas aún no está clara, pero la desacilación de la proteína provoca cambios conformacionales en ésta. Así, aunque tanto en bicapas (Pastrana *et al.* 1991, Vandenbussche *et al.* 1992a) como en monocapas (Oosterlaken-Dijksterhuis *et al.* 1991b) la mayor parte de la estructura de la forma monomérica está constituida por hélice α, en el dímero, que no se encuentra acilado (Baatz *et al.* 1992, Creuwels *et al.* 1995a), predomina la estructura en lámina β (Pastrana *et al.* 1991, Baatz *et al.* 1992, Pérez-Gil *et al.* 1993, Cruz *et al.* 1995). Estos cambios estructurales podrían ser la causa de que la forma dimérica tenga propiedades distintas a las del monómero (Baatz *et al.* 1992, Creuwels *et al.* 1995a). Los aminoácidos situados entre las posiciones 13 y 35, ambas incluidas, son de naturaleza hidrofóbica. Estos aminoácidos se encuentran en el extremo C-terminal, donde forman una hélice α hidrofóbica (Johansson *et al.* 1994). La posición de la SP-C, al menos en lo relativo al ángulo de inclinación de la hélice hidrofóbica, es

diferente según se encuentre en bicapas o en monocapas (Gericke *et al.* 1997). Cuando la SP-C se reconstituye en una bicapa lipídica, la hélice hidrofóbica se dispone de forma paralela a las cadenas de acilo de los lípidos (Pastrana *et al.* 1991, Vandenbussche *et al.* 1992a). Cerca del extremo N-terminal hay dos residuos básicos que se situarían, al igual que el grupo amino del extremo N-terminal (Horowitz *et al.* 1993), cerca de la superficie (y por tanto de las cabezas polares de los fosfolípidos) cuando la proteína se encuentra insertada en una bicapa (Morrow *et al.* 1993b). La presencia de estas cargas positivas es importante, ya que su neutralización afecta a las propiedades funcionales de la proteína (Creuwels *et al.* 1995b).

SP-D

Al igual que la SP-A, la SP-D es una proteína hidrofílica que por su estructura y características funcionales se incluye en el grupo de las colectinas. Se han detectado varias isoformas con pI entre 6 y 8. En su estructura nativa, la SP-D se compone de dodecámeros cruciformes de 600 kDa, cada uno de ellos constituido por cuatro trímeros con estructura colagénica y unidos covalentemente entre sí por la zona N-terminal mediante puentes disulfuro (fig. 1.7) (Persson *et al.* 1989, Crouch *et al.* 1994, Brown-Augsburger *et al.* 1996a, Brown-Augsburger *et al.* 1996b). Cada brazo del oligómero posee una longitud de 46 nm. Los monómeros, con una masa molecular de 43 kDa, tienen varias regiones bien diferenciadas:

- Una **región N-terminal** con dos cisteínas implicadas en puentes disulfuro intercatenarios (Shimizu *et al.* 1992, Lu *et al.* 1992, Motwani *et al.* 1995).
- Un **dominio de tipo colagénico** rico en hidroxiprolinas (Persson *et al.* 1989, Lu *et al.* 1992) formado por 59 repeticiones de la secuencia Gly-X-Y (sin interrupciones, a diferencia de la SP-A). En esta región hay una secuencia consenso de N-glicosilación con un residuo de Asn al que se une un oligosacárido con ácido siálico (Shimizu *et al.* 1992, Motwani *et al.* 1995, Brown-Augsburger *et al.* 1996a). También hay carbohidratos unidos en forma de glucósidos de hidroxilisina (Persson *et al.* 1989).
- Un **"cuello"** entre la zona colagénica y el dominio globular, formado por la asociación de tres hélices α con estructura de "coiled-coil" (Hoppe *et al.* 1994, Motwani *et al.* 1995).
- Un **dominio de reconocimiento de carbohidratos**, con 4 cisteínas muy conservadas que forman dos puentes disulfuro intracatenarios (Lu *et al.* 1992, Shimizu *et al.* 1992, Motwani *et al.* 1995).

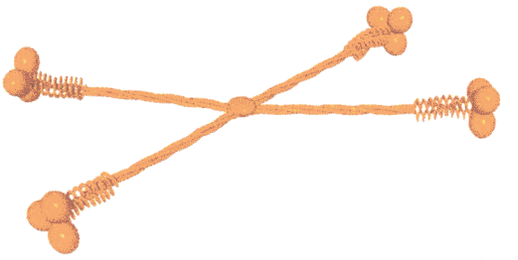

Figura 1.7. Forma dodecamérica de la SP-D.

La SP-D no se ha podido detectar dentro de los cuerpos lamelares (Voorhout *et al.* 1992b), y no precipita asociada al surfactante en condiciones en las que sí lo hacen el resto de sus componentes al centrifugar el lavado broncoalveolar. Además, no se le conoce otra función que la de defensa frente a microorganismos patógenos, lo que ha hecho que algunos autores se replanteen el incluirla entre las proteínas específicas del surfactante. Por tanto, esta proteína podría quedar incluida en el grupo de proteínas que aparecen en el lavado broncoalveolar sin ser específicas del sur-

factante. Sin embargo, se ha demostrado que la SP-D es capaz de contrarrestar el efecto inhibidor de la SP-A sobre la secreción de surfactante en los neumocitos tipo II (Kuroki *et al.* 1991c), y que su carencia provoca alteraciones en el metabolismo de los lípidos del surfactante (Botas *et al.* 1998, Korfhagen *et al.* 1998), por lo que podría desempeñar algún papel regulador específico en el ciclo metabólico del surfactante.

1.3. CICLO METABÓLICO DEL SURFACTANTE PULMONAR

La síntesis, almacenamiento y secreción de los componentes del surfactante pulmonar corren a cargo de los **neumocitos tipo II**, también llamados células tipo II, células alveolares tipo II y neumocitos granulares (fig. 1.8A). Estas células, situadas en el epitelio alveolar, poseen unos orgánulos característicos, los **cuerpos lamelares**, donde se almacena el surfactante antes de ser secretado al espacio alveolar (fig. 1.8B). En la composición de los cuerpos lamelares, además de fosfolípidos y proteínas específicas del surfactante, se incluyen una α-glucosidasa específica y otras enzimas lisosomales y proteínas, todo ello envuelto en una membrana y con un medio interno ácido y rico en Ca^{2+} (Hawgood 1997).

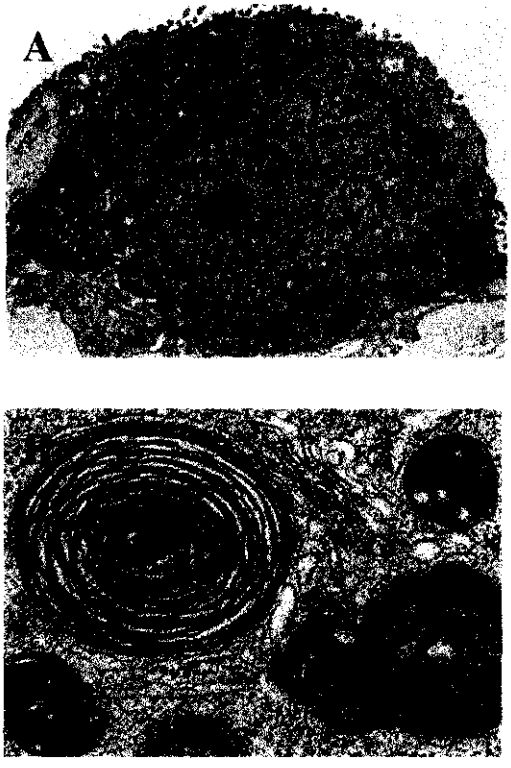

Figura 1.8. **(A)** Neumocito tipo II de pulmón humano. La cara luminal de la membrana plasmática (parte superior de la imagen) forma microvellosidades, y en ella se acumulan los cuerpos lamelares antes ser secretados. **(B)** Detalle de un cuerpo lamelar. Obsérvese el empaquetamiento de las bicapas. Imágenes extraídas de Mason R. J. y Shannon J. M. (1997). Alveolar type II cells, en *The Lung: Scientific Foundations*, 2nd edition, ed. R.G. Crystal, J.B. West, E.R. Weibel, P.J. Barnes (Philadelphia: Lippincott-Raven Publishers): 9-18.

1.3.1 SÍNTESIS

Experimentos con diversas especies animales han demostrado que la biosíntesis de los distintos componentes del surfactante pulmonar aumenta hacia el final de la gestación. Numerosos estudios, realizados mayoritariamente en modelos fetales (ya sea en animales o en cultivos celulares) han demostrado que gran parte de los factores implicados en la regulación de la síntesis del surfactante lo están también en la ontogenia del propio pulmón (van Golde y Casals 1997).

Síntesis de fosfolípidos

La síntesis de los fosfolípidos se realiza en el retículo endoplásmico de los neumocitos tipo II. Aunque ya hemos visto que la composición lipídica del surfactante es variada, sólo estudiaremos las rutas biosintéticas de la DPPC por ser el principal componente (fig. 1.9). La síntesis de novo de PC en el pulmón se produce casi exclusivamente a través de la formación de CDP-colina, siendo limitante el paso catalizado por la colinafosfato citidililtransferasa (van Golde y Casals 1997). Sin embargo, otras enzimas pueden ejercer un papel regulador en determinadas condiciones. Estas enzimas son la colina quinasa, la glicerol-3-fosfato aciltransferasa y la fosfatidato fosfatasa. En neumocitos tipo II fetales, la ácido graso sintasa puede catalizar también la etapa limitante de esta ruta sintética. La actividad de muchas de estas enzimas parece estar sometida a control hormonal, tanto en pulmón adulto como fetal.

La biosíntesis de novo da cuenta del 45 % de la DPPC del surfactante, formándose el resto de la DPPC mediante mecanismos de desacilación-reacilación a partir de especies insaturadas de PC (van Golde y Casals 1997). En esta segunda vía, la eliminación de la cadena insaturada de acilo en la posición *sn*-2 de la PC podría estar catalizada por la fosfolipasa A_2 microsomal dependiente de Ca^{2+}, o bien por la fosfolipasa A_2 citosólica independiente de Ca^{2+}. No obstante, el papel de estas enzimas en la síntesis de DPPC aún no está claro. En este sentido, se ha descrito una vía por la cual la eliminación de la cadena de acilo insaturada se produce mediante transacilación de dicha cadena a un lisofosfolípido. Finalmente, la lisofosfatidilcolina (LPC) resultante del proceso de desacilación o de transacilación sería reacilada con palmitoíl-CoA mediante la LPC aciltransferasa, obteniéndose DPPC.

Síntesis de las proteínas específicas del surfactante

La **SP-A** humana está codificada por dos genes de secuencia muy conservada,

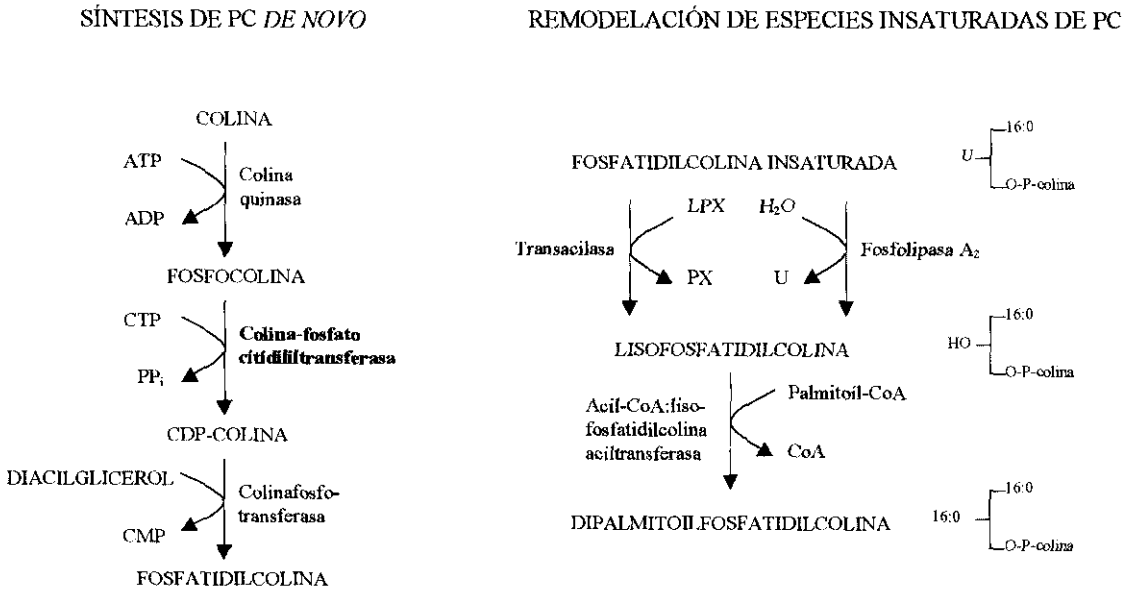

Figura 1.9. Rutas sintéticas de la fosfatidilcolina. En la síntesis *de novo* (parte izquierda de la figura) se ha resaltado con negrita la enzima limitante de esta vía. LPX = lisofosfolípido; PX = fosfolípido; 16:0 = palmitoílo; U = acilo insaturado.

SP-A1 (White *et al.* 1985) y SP-A2 (Floros *et al.* 1986, Katyal *et al.* 1992), y por un pseudogén (Korfhagen *et al.* 1991), incluidos todos en el cromosoma 10 (Bruns *et al.* 1987, Fisher *et al.* 1987, Korfhagen *et al.* 1991). Actualmente se piensa que cada trímero de SP-A (dentro del octadecámero) se compone de un producto del gen SP-A1 y dos del gen SP-A2 (Voss *et al.* 1991); el hecho de que la diferencia en la secuencia de ambos genes corresponda a la zona colagénica hace pensar que la formación de estos heterotrímeros tiene como fin aumentar la estabilidad de la triple hélice, ya que esta región es muy importante en el emsamblaje y estabilización del oligómero. En perro, conejo, rata y ratón sólo se ha encontrado un gen hasta el momento (Benson *et al.* 1985, Boggaram *et al.* 1988, Fisher *et al.* 1988a, Korfhagen *et al.* 1992), localizado en el cromosoma 14 en el caso de la última especie (Moore *et al.* 1992). Además de en neumocitos tipo II, esta proteína también se expresa en células no ciliadas del epitelio bronquiolar (células Clara) y en células del epitelio bronquial. En los últimos años se ha detectado su expresión también en intestino (Rubio *et al.* 1995, Eliakim *et al.* 1997) y células mesentéricas de rata (Chailley-Heu *et al.* 1997), y en oído medio humano (Yamanaka *et al.* 1991).

El precursor de la SP-A, de 26-31 kDa (Floros *et al.* 1985, Weaver *et al.* 1986), posee un péptido señal (17 a 20 aminoácidos, según la especie) en el extremo N-terminal (Benson *et al.* 1985, White *et al.* 1985, Boggaram *et al.* 1988), encargado de dirigir a la proteína hacia la ruta secretora de la célula y que se elimina durante su translocación al lumen del retículo endoplásmico (fig. 1.10) (Floros *et al.* 1985). En rata se ha detectado recientemente la presencia en este péptido señal de un resi-

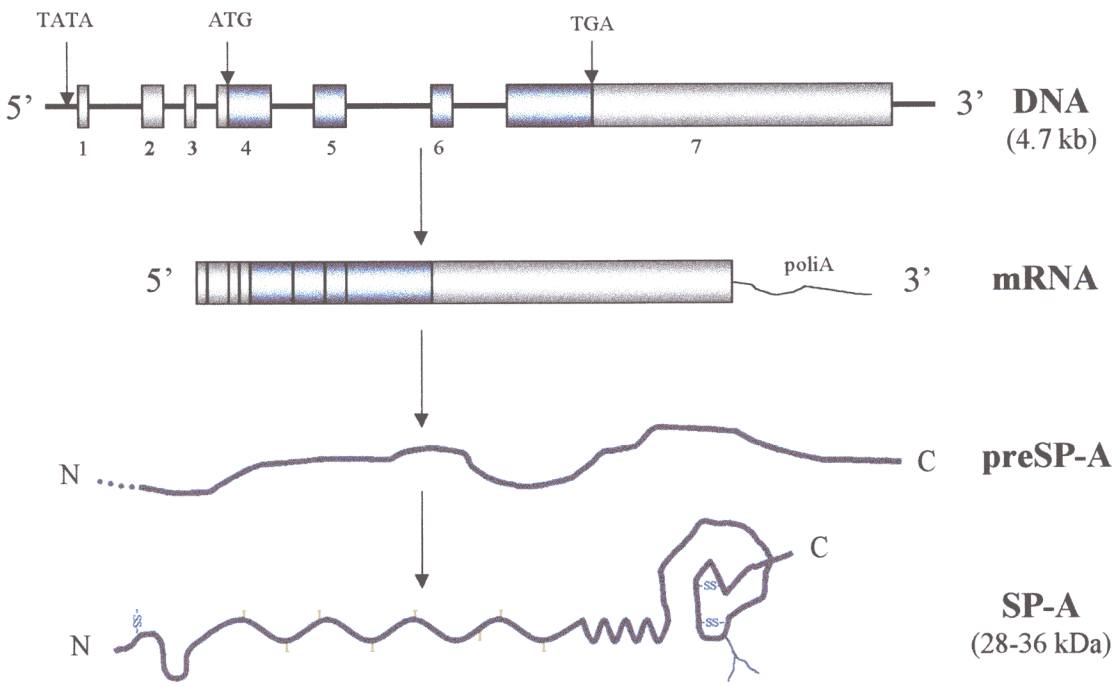

Figura 1.10. Síntesis de la SP-A. El gen de la SP-A posee 7 exones, que incluyen 4 regiones codificantes y 5 regiones UTR (regiones no traducidas). En la preproteína se ha indicado la posición del péptido señal con trazo discontinuo. En la SP-A madura (esquematizada en forma monomérica) se han representado la hidroxilación de las prolinas del dominio colagénico (I), los puentes disulfuro (-SS-) y la glicosilación del dominio globular.

duo de Cys, al parecer implicado en la formación de un puente disulfuro necesario para el correcto plegamiento de la proteína (Elhalwagi *et al.* 1997). Una vez en el retículo endoplásmico, se produce la glicosilación de la Asn-187 con un oligosacárido rico en manosa al que, ya en el complejo de Golgi, se añade un residuo de ácido siálico (Whittset *et al.* 1985c, Phelps *et al.* 1986). También se produce la hidroxilación de algunos residuos de prolina (Benson *et al.* 1985). Aunque sólo se han observado en algunas especies, en condiciones *in vitro* y por medios indirectos, se han detectado además otras modificaciones covalentes como N-acetilación de la metionina N-terminal (Floros *et al.* 1986, Weaver *et al.* 1986), sulfatación de la cadena oligosacárida (Weaver *et al.* 1987) y γ-carboxilación dependiente de vitamina K de residuos de ácido glutámico (Rannels *et al.* 1987). El correcto tráfico intracelular y secreción de la SP-A son independientes de la presencia del dominio colagénico (McCormack *et al.* 1997c) y la cadena oligosacárida (O'Reilly *et al.* 1988), aunque la velocidad de incorporación a los cuerpos lamelares es menor si se inhiben la glicosilación o el procesamiento de la cadena oligosacárida (Alcorn y Mendelson 1993).

El gen que codifica para la **SP-B** se localiza en el cromosoma 2 en humanos (Emrie *et al.* 1988, Pilot-Matias *et al.* 1989) y en el 6 en ratón (Moore *et al.* 1992). Este gen se expresa tanto en neumocitos tipo II como en células Clara. Curiosamente, también se ha detectado SP-B en intestino de rata (Eliakim *et al.* 1989).

El producto inicial de la traducción de la proteína es un polipéptido de 42 kDa, la preproSP-B, que sufre un primer procesamiento en el que pierde un péptido señal de 20-23 residuos (fig. 1.11) (Glasser *et al.* 1987, O'Reilly *et al.* 1989, Weaver y Whit-

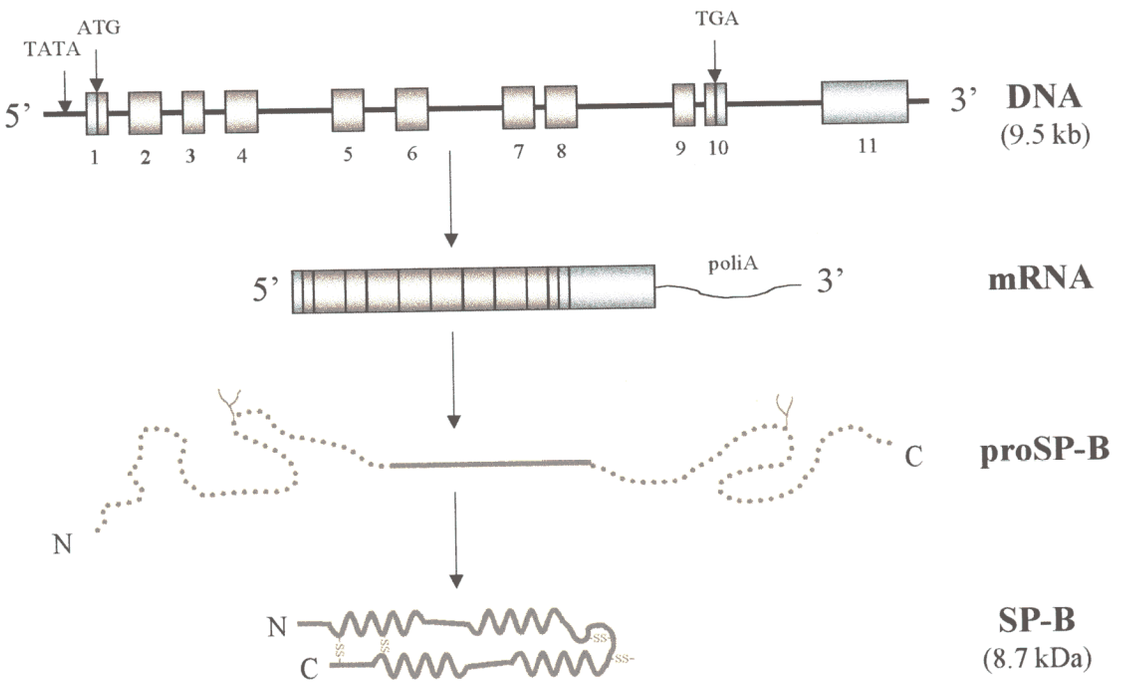

Figura 1.11. Síntesis de la SP-B. El gen de esta proteína está compuesto por 11 exones en los que hay 10 regiones codificantes y 3 regiones UTR. Los tramos discontinuos señalados en la proSP-B corresponden a los segmentos N (incluido el péptido señal) y C-terminal que se eliminan en el proceso de maduración de la proteína. Obsérvese en ambos tramos la presencia de sendas cadenas oligosacáridas. En la SP-B madura se han representado los puentes disulfuro intra e intercatenarios (-SS-).

sett 1989). El producto resultante, la proSP-B, experimenta la glicosilación de uno o dos residuos de asparragina con un oligosacárido rico en manosa y con ácido siálico (Emrie *et al.* 1989, O'Reilly *et al.* 1989, Hawgood *et al.* 1993). La proSP-B presenta un alto grado de homología con el precursor de la saposina, así como con la glicoproteína sulfatada-1 (Patthy 1991, Zaltash y Johansson 1998). Debido a ello, se cree que esta proteína, además de ser la precursora de la SP-B, podría tener alguna otra función. Finalmente se eliminan de la proSP-B sendos fragmentos de 176 y 102-107 residuos de los extremos N y C-terminal (Weaver y Whitsett 1989), respectivamente, durante su transporte desde el complejo de Golgi hasta los cuerpos lamelares en un compartimento endosómico-lisosomal (Voorhout *et al.* 1992a). El procesamiento del extremo N-terminal se lleva a cabo por la acción de una proteasa del tipo catepsina D (Weaver *et al.* 1992). La región N-terminal y la parte correspondiente al péptido maduro son necesarias para que la SP-B llegue a los cuerpos lamelares (Lin *et al.* 1996a, Lin *et al.* 1996b). Parece ser que al menos parte del procesamiento postraduccional que experimenta la SP-B se produce específicamente en neumocitos tipo II (Hawgood *et al.* 1993).

La **SP-C** humana está codificada por dos genes situados en el cromosoma 8 (Fisher *et al.* 1988b, Glasser *et al.* 1988a). Aunque son dos los c-DNA que se han detectado, esto se podría deber a una variación alélica o a variaciones en el sitio de procesamiento intrón-exón más que a la existencia de un gen duplicado (Glasser *et al.* 1988b). En conejo (Boggaram y Margana 1992) y en ratón (Glasser *et al.* 1990) sólo se ha encontrado un gen hasta el momento, situado en el cromosoma 14 en esta última especie (Moore *et al.* 1992).

De la expresión de este gen, que se produce exclusivamente en neumocitos tipo II, se obtiene un péptido de aproxima-

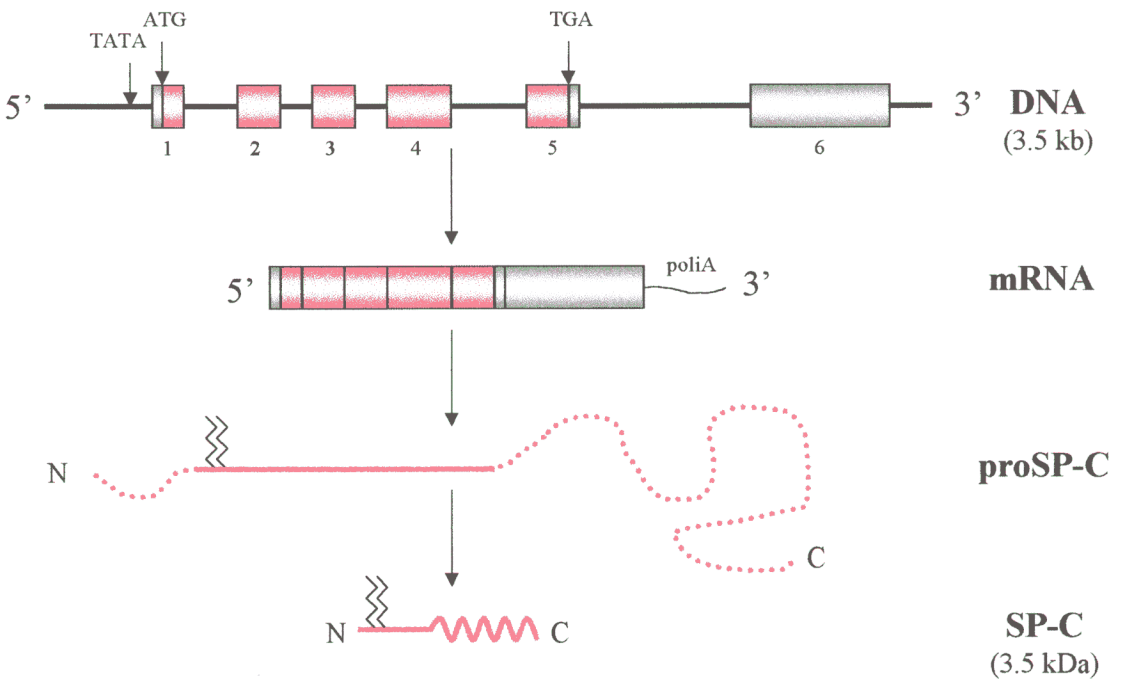

Figura 1.12. Síntesis de la SP-C. El gen de la SP-C se compone de 6 exones, en los que se incluyen 5 regiones codificantes y 3 regiones UTR. En la proSP-C se muestran con trazo discontinuo las porciones N y C-terminal que son eliminadas en el procesamiento postraduccional. Las dos líneas negras en zigzag representan los dos restos de acilo unidos a los residuos de cisteína del extremo N-terminal.

damente 21 kDa, la proSP-C (fig. 1.12) (Warr *et al.* 1987, Glasser *et al.* 1988a, Glasser *et al.* 1990, Vorbroker *et al.* 1992, Beers *et al.* 1994). Este péptido experimenta posteriormente una modificación aún sin identificar que le hace aumentar de masa (Vorbroker *et al.* 1992, Keller *et al.* 1992), y es palmitoilado en dos cisteínas (Vorbroker *et al.* 1992). Finalmente, es sometido a varias etapas proteolíticas en las que pierde residuos en ambos extremos (Glasser *et al.* 1988a, Beers *et al.* 1994, Beers y Lomax 1995). Parte de este proceso (Beers *et al.* 1994, Vorbroker *et al.* 1995b) se produce en el trans-Golgi y los cuerpos multivesiculares, y termina en los cuerpos lamelares (Beers 1996). La proSP-C carece de péptido señal, aunque se ha propuesto que parte de la secuencia de la SP-C madura podría actuar como tal y como dominio de anclaje a membranas (Keller *et al.* 1991, Keller *et al.* 1992).

Al igual que en el caso de la SP-A, el gen que codifica para la **SP-D** humana se encuentra en el cromosoma 10 (Kölble *et al.* 1993, Crouch *et al.* 1993), mientras que en ratón se sitúa en el cromosoma 14 (Motwani *et al.* 1995).

La expresión del gen se produce tanto en neumocitos tipo II como en células Clara, habiéndose descubierto recientemente que la proteína también se expresa en glándulas traqueales (Wong *et al.* 1996) y, curiosamente, en estómago, corazón, riñón y mesenterio de rata (Motwani *et al.* 1995, Fisher y Mason 1995, Chailley-Heu *et al.* 1997). Su producto inicial de traducción es un péptido de 39 kDa que experimenta glicosilación en residuos de asparragina, e hidroxilación y posterior glicosilación de residuos de Lys en el dominio colagénico (fig. 1.13) (Crouch *et al.* 1991). En la secuencia del c-DNA aparece un péptido señal (Shimizu *et al.* 1992) que desaparece

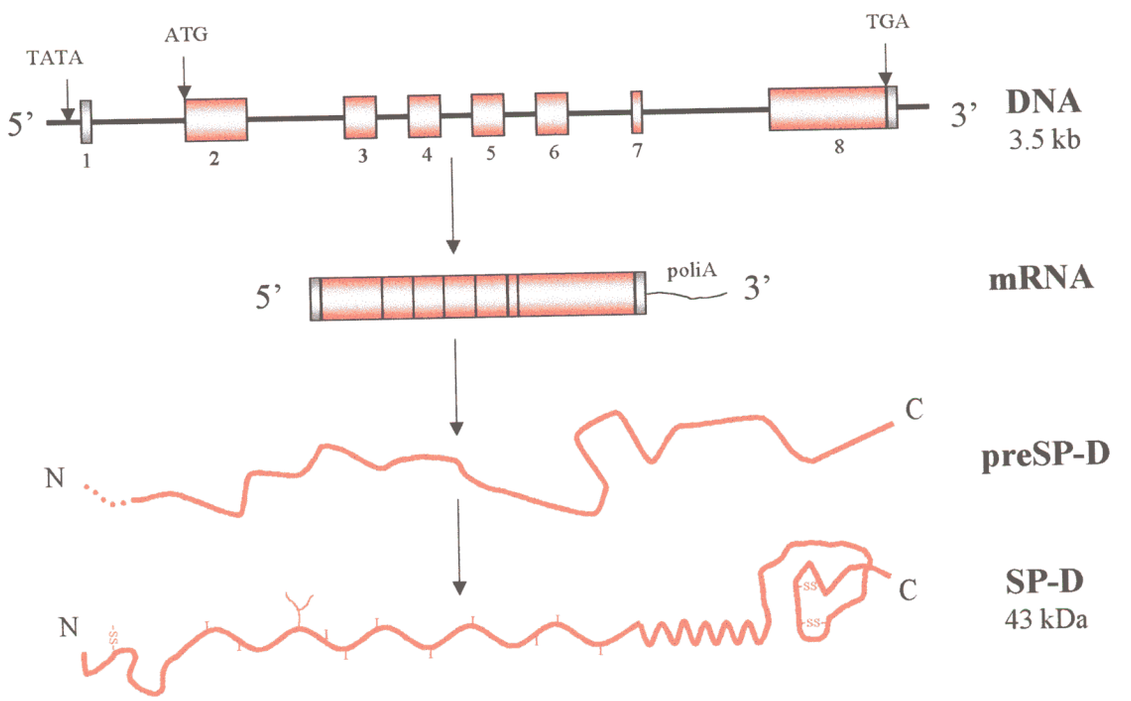

Figura 1.13. Síntesis de la SP-D. El gen de esta proteína está compuesto por 8 exones, en los que hay 7 regiones codificantes y 2 regiones UTR. En la preSP-D se ha indicado la presencia del péptido señal con trazo discontinuo. Además, en la proteína madura (esquematizada en forma monomérica) se muestran la hidroxilación de residuos de lisina y prolina del dominio colagénico (I), los puentes disulfuro (-SS-) y la glicosilación del dominio colagénico.

en la proteína madura. Para que la proteína pueda ser posteriormente secretada, es necesaria la formación de los puentes disulfuro intercatenarios que permiten la formación de los dodecámeros, siendo la estabilidad de la triple hélice de colágeno (hidroxilación de residuos de prolina y de lisina) un requisito previo para que se constituyan estas uniones (Brown-Augsburger *et al.* 1996b).

1.3.2 ALMACENAMIENTO

El surfactante pulmonar se almacena en los cuerpos lamelares antes de ser secretado al espacio alveolar. Aún no está clara la forma en que los fosfolípidos son transportados desde el retículo endoplásmico hasta los cuerpos lamelares a través del complejo de Golgi. Aunque los primeros estudios parecían indicar que el proceso de transporte se llevaba a cabo mediante proteínas transportadoras de fosfolípidos, hoy se cree más probable que se produzca por fusión de pequeñas vesículas mediada por proteínas ligantes de fosfolípidos (Hawgood 1997).

El transporte de las proteínas SP-A, SP-B y SP-C hasta los cuerpos lamelares se produce a través de la propia ruta biosintética (que al parecer es común para las tres) mediante cuerpos multivesiculares (Voorhout *et al.* 1993).

1.3.3 SECRECIÓN

La secreción del surfactante pulmonar se produce por exocitosis de los cuerpos lamelares. Iones Ca^{2+} (Haller *et al.* 1998), microtúbulos y microfilamentos (Bhandari *et al.* 1997) parecen estar implicados en el proceso. También la anexina II (Liu *et al.* 1996, Liu *et al.* 1997) y una proteína muy similar a la sinexina de las glándulas adrenales (Chander y Wu 1991) podrían participar. Sin embargo, no todos los elementos del surfactante salen al alveolo con los cuerpos lamelares. En este sentido, la SP-D no se ha logrado detectar en estos orgánulos, de lo que se ha deducido que se secreta por otra vía (Voorhout *et al.* 1992b). Igualmente, algunos autores sostienen que parte de la SP-A sintetizada en los neumocitos tipo II se podría secretar a través de la vía constitutiva, independientemente de la ruta de los cuerpos lamelares. Esta opinión se basa en que una estimulación en la secreción de PC no va acompañada de un incremento proporcional en los niveles extracelulares de SP-A, y en la aparición de SP-A marcada en el fluido alveolar antes de que se pueda detectar en los cuerpos lamelares (Ikegami *et al.* 1992, Froh *et al.* 1993, Rooney *et al.* 1993, Ikegami *et al.* 1994).

El proceso de secreción resulta estimulado por hiperventilación, debido a una acción mecánica producida por distorsión celular, a cambios en el pH extracelular o a ambos factores (Batenburg 1995, Floros y Phelps 1997). Un estudio realizado recientemente por Tolle y colaboradores muestra que la hiperoxia aumenta los niveles de SP-A, pero no los de lípidos del surfactante, indicando una regulación independiente de la secreción de ambos componentes (Tolle *et al.* 1997). También actúan como secretagogos múltiples compuestos: ésteres de forbol y diacilgliceroles, ácido araquidónico y eicosanoides, ionóforos de Ca^{2+}, derivados de piridina, y agonistas β-adrenérgicos, histaminérgicos, purinérgicos y de vasopresina (Hawgood 1997, Floros y Phelps 1997). Es posible que en este pro-

ceso haya una interacción endotelio-epitelio de tipo paracrino, pues se ha demostrado que el péptido vasoactivo endotelina-1 estimula la secreción de surfactante (Sen *et al.* 1994). En cuanto a posibles secretagogos exógenos, se ha observado que el lipopolisacárido (LPS) de *Escherichia coli* también estimula la secreción (Romero *et al.* 1995), aunque un estudio reciente muestra el efecto contrario para el LPS de *Salmonella minnesota* (Fehrenbach *et al.* 1998).

La SP-A (Rice *et al.* 1987, Dobbs *et al.* 1987) y, en menor extensión, los lípidos del surfactante (Dobbs *et al.* 1987) ejercen un mecanismo de retroalimentación negativa sobre la secreción del surfactante pulmonar. La acción inhibidora de la SP-A se produce mediante su unión a un receptor (Kuroki *et al.* 1988b, Kuroki *et al.* 1988c). Se han propuesto como posibles receptores de la SP-A proteínas de 32 kDa (Strayer *et al.* 1993, Strayer *et al.* 1996b), 170-200 kDa (Stevens *et al.* 1995) y 210 kDa (Chroneos *et al.* 1996) situadas en la membrana de los neumocitos tipo II.

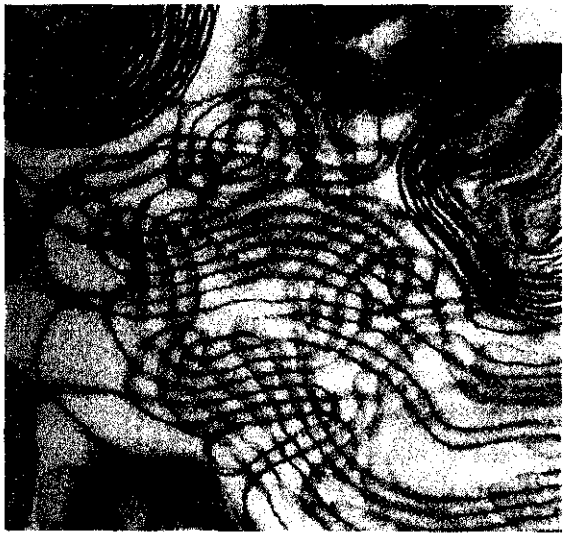

Figura 1.14. Mielina tubular. Las estructuras más apiladas que aparecen en la parte superior de la imagen son cuerpos lamelares. Imágenes extraídas de Mason R. J. y Shannon J. M. (1997). Alveolar type II cells, en *The Lung: Scientific Foundations*, 2nd edition, ed. R.G. Crystal, J.B. West, E.R. Weibel, P.J. Barnes (Philadelphia: Lippincott-Raven Publishers): 9-18.

Una vez en la luz alveolar, el surfactante procedente de los cuerpos lamelares se desempaqueta y forma una estructura altamente ordenada en forma de malla, la **mielina tubular** (fig. 1.14). Para la formación de mielina tubular es necesaria la presencia de SP-A, SP-B, DPPC, PG y Ca^{2+} (Suzuki *et al.* 1989, Williams *et al.* 1991, Poulain *et al.* 1992, Clark *et al.* 1995, Korfhagen *et al.* 1996). Las partículas observadas en las esquinas de las formas poligonales que aparecen en esta estructura son al parecer SP-A (Voorhout *et al.* 1991). En general, resulta un hecho aceptado que la mielina tubular actúa como reservorio y precursor de la monocapa que se forma posteriormente en la interfase aire/agua del alveolo pulmonar. En este sentido, se ha observado en estudios *in vitro* que la actividad de una serín-proteasa (Gross y Schultz 1990) o de la fosfolipasa D (Dhand *et al.* 1998) facilita su conversión a la forma vesicular a partir de la cual se formaría la monocapa, aunque algunos autores sostienen que esta transformación del surfactante parece estar relacionada más con la salida de materiales de la monocapa que con su incorporación a ésta (Veldhuizen *et al.* 1993, Gross *et al.* 1997). Por otra parte, estudios con surfactantes exógenos (Notter *et al.* 1986) y con ratones transgénicos que no expresan SP-A (Korfhagen *et al.* 1996) han demostrado que el surfactante no necesita transformarse primero en mielina tubular para ejercer su actividad.

Durante muchos años se ha asumido que el surfactane funcional se encuentra formando una monocapa en el alveolo. Sin embargo, esto no se ha podido demostrar aún, y es probable que este modelo sea en realidad una simplificación de las estructuras que realmente existen. Estudios recientes de microscopía electrónica y de tensión superficial apuntan más bien hacia

un sistema de varias membranas lipídicas asociadas estrechamente a la monocapa en la interfase aire/agua (Schürch *et al.* 1995) (fig. 1.15). Es posible que esta estructura en multicapas se forme en cada proceso de compresión (espiración) de la monocapa y que después, al expandirse durante la inspiración, se redisperse en la interfase aire/líquido con ayuda de la SP-C (Amrein *et al.* 1997, von Nahmen *et al.* 1997a, von Nahmen *et al.* 1997b). De cualquier forma, la estructura (o estructuras) formada se podría enriquecer en DPPC posteriormente con cada ciclo de compresión-expansión, mediante la exclusión de componentes distintos de DPPC. Este proceso está favorecido al parecer por la SP-B (Yu y Possmayer 1990) y, probablemente, por la SP-A (Yu y Possmayer 1990, Schürch *et al.* 1992, Schürch *et al.* 1995, Yu y Possmayer 1998, Cajal et al. 1998).

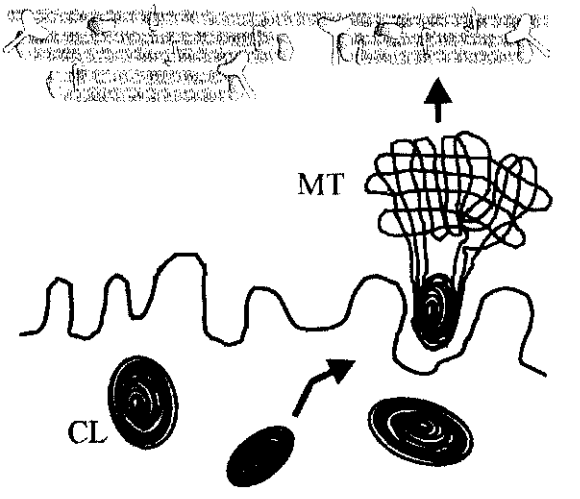

Figura 1.15. Secreción del surfactante pulmonar. CL , cuerpos lamelares; MT, mielina tubular; figuras azules, SP-A; figuras verdes, SP-B; figuras rosas, SP-C.

1.3.4 DEGRADACIÓN Y RECICLAJE

En el líquido alveolar hay estructuras vesiculares pobres en proteínas y con baja actividad superficial, conocidas en la bibliografía como "vesículas o agregados pequeños" (Batenburg 1995, Floros y Phelps 1997). Estas estructuras están constituidas probablemente el material de salida (principalmente fosfolípidos) de la monocapa enriquecida en DPPC. La formación de estos agregados pequeños en cada ciclo de compresión-expansión pulmonar parece estar relacionada con la degradación de la SP-B por parte de una serín-proteasa (Veldhuizen *et al.* 1993) denominada convertasa (Gross y Schultz 1992). Esta enzima, una glicoproteína de 72 kDa, ha sido clonada recientemente (Krishnasamy *et al.* 1998).

Una vez fuera de la monocapa, los lípidos pueden seguir varios caminos (Batenburg 1995, Hamm *et al.* 1996, Floros y Phelps 1997):

1. Transporte ciliar hacia las vías aéreas altas.
2. Captura y degradación por parte de los macrófagos alveolares. La SP-A aumenta el proceso de captación de lípidos por estas células (Wright y Youmans 1995).
3. Paso a linfa o sangre.
4. Recaptura por parte de los neumocitos tipo II para ser reutilizados o degradados en lisosomas. En este último caso, parte de los productos de degradación se aprovechan posteriormente para resintetizar PC. La recaptura por parte de los neumocitos tipo II parece ser la vía más importante de aclaramiento de los lípidos del surfactante.

Aunque un estudio reciente demuestra que la presencia de SP-A no es necesaria para la recaptura de lípidos del surfactante por las células alveolares (Ikegami *et al.*

1997), trabajos anteriores indican que la SP-A podría mediar o al menos acelerar el proceso (Wright *et al.* 1987, Rice *et al.* 1989, Tsuzuki *et al.* 1993, Bates *et al.* 1994) al favorecer la unión de aquéllos a la membrana de los neumocitos tipo II (Horowitz *et al.* 1996). Esta unión está mediada al parecer por la interacción de la proteína con un receptor (Wright *et al.* 1989, Ryan *et al.* 1989, Strayer *et al.* 1993, Stevens *et al.* 1995, Strayer *et al.* 1996b). La SP-B y la SP-C (Rice *et al.* 1989, Horowitz *et al.* 1996) también favorecen la unión a la membrana celular y la endocitosis de vesículas lipídicas. Aunque su acción no parece estar mediada por receptores, se ha observado que la SP-B asociada a lípidos se une a zonas concretas de la membrana de los neumocitos tipo II (Bates *et al.* 1992). El aclaramiento del surfactante afecta mayoritariamente a los agregados pequeños, permaneciendo los denominados agregados grandes (fracción funcional del surfactante) intactos en la luz alveolar (Horowitz *et al.* 1997b).

Al igual que los fosfolípidos, las proteínas son recapturadas por los neumocitos tipo II, aunque su recambio es más rápido que el de la SatPC (Hawgood 1997). La SP-A es endocitada posiblemente tras unirse a un receptor a través del dominio de reconocimiento de carbohidratos (Murata *et al.* 1993). Posteriormente, parte de la proteína se incorporaría de nuevo a los cuerpos lamelares vía cuerpos multivesiculares (Ryan *et al.* 1989, Young *et al.* 1993). Por otra parte, la SP-B parece fijarse a zonas determinadas de la membrana de neumocitos tipo II (Bates *et al.* 1992) para ser internalizada y, en parte, secretada de nuevo con los cuerpos lamelares (Breslin y Weaver *et al.* 1992). Otra fracción de SP-B es degradada en el interior de estas células y en macrófagos alveolares (Bates y Fisher 1993). Su reciclaje parece estar estrechamente relacionado con el de la SatPC, aunque se produce más rápidamente (Henry *et al.* 1996). También se ha observado, mediante marcaje radiactivo, que la SP-C es recapturada y vuelta a secretar al espacio intraalveolar (Baritussio *et al.* 1992, Pinto *et al.* 1993) en un proceso favorecido por la SP-A (Pinto *et al.* 1995) e inhibido por la SP-B (Horowitz *et al.* 1997a). El aclaramiento de este péptido también se produce mediante captura por macrófagos alveolares (Baritussio *et al.* 1989). Al parecer, la forma monomérica de la SP-C se elimina con mayor facilidad que la dimérica (Li *et al.* 1998). La recaptura de esta última forma se acelera al elevar los niveles de glutation en el medio, posiblemente debido a su conversión en monómero. En cuanto a la SP-D, su aclaramiento parece correr a cargo (al menos en parte) de los macrófagos alveolares, siendo independiente de la presencia de SP-A y lípidos del surfactante (Dong y Wright 1998).

Para mantener la homeostasis del surfactante pulmonar debe haber un equilibrio entre su secreción y su eliminación. En este sentido, tanto la hiperventilación como los agentes agonistas β-adrenérgicos, además de actuar como secretagogos, son capaces de activar su captura y metabolismo (Batenburg 1995, Hawgood 1997). La acción de los agonistas β-adrenérgicos se debe probablemente a que inducen un aumento de receptores para la SP-A (Chen *et al.* 1996). En contradicción con lo anteriormente dicho, Griese y colaboradores observaron que estas sustancias no tenían un efecto estimulante sobre el aclaramiento del surfactante, y que la concanavalina A, una lectina que inhibe la secreción de fosfolípidos, es sin embargo incapaz de estimular la recaptura de fosfolípidos (Griese *et al.* 1991). A la vista de estos resultados, proponen que la secreción y la eliminación del surfactante están reguladas por mecanismos distintos.

1.4. CARACTERÍSTICAS FUNCIONALES DE LOS COMPONENTES DEL SURFACTANTE PULMONAR

1.4.1 LÍPIDOS

Desde hace varias décadas se sabe que la DPPC, componente lipídico mayoritario del surfactante pulmonar, es el principal responsable de sus propiedades tensoactivas (Hawgood 1997). Cualquier suspensión de fosfolípidos en agua tiende a formar espontáneamente una monocapa en la interfase aire/agua. El desplazamiento de las moléculas de agua en la interfase por parte de esta monocapa se traduce en una disminución de la tensión superficial. La tensión superficial del agua pura es de 70 mN/m. Si ponemos un exceso de fosfolípidos en suspensión, la acumulación de éstos en la interfase se produce hasta alcanzar $\gamma \approx 25$ mN/m, que marcaría el punto de equilibrio del sistema. Aunque aumentemos la concentración de fosfolípidos en la suspensión, la tensión superficial se mantendrá en este valor. Sin embargo, si comprimimos la monocapa formada después de alcanzar este equilibrio, los fosfolípidos situados en ella pasan a ocupar un área molecular menor, quedando más empaquetados. En estas condiciones se expulsan más moléculas de agua de la interfase, y la tensión superficial se reduce aún más, pudiendo alcanzar incluso valores cercanos a los 0 mN/m, dependiendo de la especie fosfolipídica de que se trate. Sin embargo, este estado de compresión no es estable, tendiendo el sistema a recuperar la tensión superficial de equilibrio mediante la exclusión de moléculas de fosfolípido de la monocapa. Los fosfolípidos que posean cadenas de acilo insaturadas quedarán peor empaquetados al comprimir la monocapa. Por el contrario, aquellos otros con cadenas saturadas podrán dar lugar a estructuras más ordenadas, capaces de soportar mejor tensiones superficiales más bajas. Esto último es lo que ocurre con las especies disaturadas de PC, más concretamente con la DPPC. Las monocapas de DPPC pueden ser comprimidas hasta valores de γ inferiores a 1 mN/m, mostrando una marcada estabilidad frente al colapso De ahí que esta especie sea mayoritaria en el surfactante y sea la máxima responsable de su actividad como componente antiatelectásico.

Sin embargo, para que los fosfolípidos ejerzan su actividad superficial, antes deben acceder a la interfase para constituir la monocapa. El proceso de adsorción se produce más rápidamente cuando los lípidos se encuentran en estado líquido cristalino. La DPPC posee una T_m (temperatura de transición de fase gel-líquido cristalino) de 41 °C, es decir, se encuentra en estado gel a 37 °C, por lo que su adsorción es muy lenta en estas condiciones. Las especies insaturadas, en cambio, forman más rápidamente una monocapa en la interfase al tener una T_m inferior a los 37 °C. Este hecho podría justificar la presencia de estas especies en el surfactante, aunque no se descarta que puedan desempeñar otras funciones. De este modo, las especies de PC insaturadas facilitarían la adsorción del surfactante y, una vez formada la monocapa, la DPPC podría ejercer su acción reductora de la tensión superficial hasta valores de alrededor de 1 mN/m. Aunque su papel en el surfactante aún no está claro y es posible que sólo se trate de contaminantes de origen celular, ésta podría ser también la función del colesterol y de otros lípidos neutros del surfactante que modifican la fluidez de la DPPC. En este sentido, al comparar la temperatura corporal normal en dis-

tintas especies de vertebrados, se ha observado que, cuanto menor es ésta, mayor es la presencia de colesterol y de especies insaturadas en el surfactante, incluso en individuos de la misma especie (Daniels *et al.* 1995, Daniels *et al.* 1998). Una vez formada la monocapa en la interfase, ésta se iría enriqueciendo en DPPC al ir eliminándose otros fosfolípidos (principalmente los insaturados) en cada ciclo de compresión-expansión.

Se ha observado que también el PG y otros fosfolípidos ácidos favorecen el proceso de adsorción del surfactante (Meban 1981). En determinadas condiciones y en algunas especies el PI puede reemplazar al PG sin que se altere el correcto funcionamiento del surfactante (Batenburg 1995, Floros y Phelps 1997). Sin embargo, es muy probable que la carga negativa de sus cabezas polares confiera a los fosfolípidos ácidos otras funciones adicionales mediante interacción con iones Ca^{2+} o con grupos catiónicos presentes en las proteínas del surfactante (Hawgood 1997).

Los lípidos del surfactante podrían desempeñar un importante papel en la regulación de los procesos de defensa en el pulmón debido a su capacidad inmunosupresora. En este sentido, se ha demostrado que lípidos y extractos de surfactante carentes de proteínas hidrofílicas (SP-A y SP-D) son capaces de inhibir la producción de citoquinas (Thomassen *et al.* 1992, Thomassen *et al.* 1994, Kremlev y Phelps 1994b), prostanoides (Földes-Filep *et al.* 1994) e inmunoglobulinas (Kremlev y Phelps 1994b), implicados en el proceso de inflamación mediado por los macrófagos alveolares, además de bloquear también la proliferación de linfocitos inducida por mitógenos (Wilsher *et al.* 1988a, Wilsher *et al.* 1988b, Kremlev *et al.* 1994a) y la quimiotaxis de macrófagos alveolares (Tanaka *et al.* 1997). Asimismo, inhiben la capacidad de la SP-A de estimular la producción de especies reactivas de oxígeno (Földes-Filep *et al.* 1994) y la aparición de marcadores de superficie (Kremlev y Phelps 1997b) en macrófagos.

1.4.2 SP-A

La SP-A es capaz de interaccionar con lípidos de forma independiente de Ca^{2+} (King *et al.* 1983, King 1984, Casals *et al.* 1993). Aunque la unión presenta una alta especificidad para la DPPC (Kuroki y Akino 1991a, Casals *et al.* 1993), ésta es mayor con mezclas de DPPC y fosfolípidos ácidos en presencia de Ca^{2+} (King y MacBeth 1981, King *et al.* 1983, King 1984). En contra de lo observado anteriormente por algunos autores (Ross *et al.* 1986b, Kuroki y Akino 1991a), experimentos más recientes con SP-A recombinante han demostrado que la integridad del dominio colagénico no es imprescindible para que se produzca esta interacción (McCormack *et al.* 1997c). Aunque la zona del cuello de la SP-A parece participar en la interacción de la proteína con lípidos, debe haber otra región implicada (Ogasawara *et al.* 1994). Esta región, aparentemente más importante en la interacción, estaría situada en el dominio de reconocimiento de carbohidratos (Kuroki *et al.* 1994, McCormack *et al.* 1997b). En este sentido, Sano y colaboradores han demostrado que la región comprendida entre los residuos Glu-195 y Phe-228 es necesaria para la interacción con fosfolípidos (Sano *et al.* 1998). Un aumento de fuerza iónica en el medio o la adición de agentes quelantes de cationes divalentes no rompe las interacciones entre SP-A y lípidos en el surfactante nativo, lo que sugiere que las interacciones apolares desempeñan un papel importante en el proceso (King *et al.*

1983, King 1984). Se ha observado además que la asociación de SP-A y fosfolípidos se ve favorecida a temperaturas inferiores a la de transición de fase, es decir, cuando los lípidos se encuentran en estado gel (King *et al.* 1983, King 1984, King *et al.* 1986, Casals *et al.* 1993).

Además de unirse a vesículas lipídicas, la SP-A es capaz de inducir su agregación en presencia de Ca^{2+} (King *et al.* 1983, Hawgood *et al.* 1985, Efrati *et al.* 1987, Casals *et al.* 1993). El dominio de reconocimiento de carbohidratos desempeña un papel importante en la agregación de vesículas (McCormack *et al.* 1994b, Ogasawara *et al.* 1994, Kuroki *et al.* 1994, Hiraike *et al.* 1995, McCormack *et al.* 1997b), y el mantenimiento de la estructura cuaternaria es fundamental para esta actividad (Ross *et al.* 1991, Kuroki y Akino 1991b, McCormack *et al.* 1997c).

El Ca^{2+} actúa como ligando de la SP-A. Parece haber al menos dos sitios de unión. Uno de ellos, de alta afinidad, parece estar situado en el fragmento resistente a colagenasa (Haagsman *et al.* 1990, Sohma *et al.* 1992). Se ha observado que la unión de Ca^{2+} a esta región es responsable de un cambio conformacional en la proteína (Haagsman *et al.* 1990, Sohma *et al.* 1992, Sohma *et al.* 1993), y que concentraciones fisiológicas de Ca^{2+} inducen la autoagregación reversible de la SP-A (Haagsman *et al.* 1990). La existencia de residuos de ácido γ-carboxiglutámico podría estar relacionada con la unión a este catión (Rannels *et al.* 1987).

La SP-A, al igual que el resto de las lectinas de tipo C, tiene la capacidad de unirse, de forma dependiente de Ca^{2+}, a carbohidratos (Haagsman *et al.* 1987, Haurum *et al.* 1993) y glicolípidos (Childs *et al.* 1992, Kuroki *et al.* 1992b) a través del dominio de reconocimiento de carbohidratos. Entre las distintas especies de monosacáridos, la SP-A presenta una especial afinidad por manosa (Haagsman *et al.* 1987). Sin embargo, la función de la cadena oligosacárida de esta proteína no está clara. Se ha comprobado que no está implicada en el transporte ni en la secreción de la proteína (O'Reilly *et al.* 1988), y que su ausencia parcial o total no afecta ni a la capacidad de la SP-A de inhibir la secreción de surfactante ni a la unión a su receptor (Kuroki *et al.* 1988a, McCormack *et al.* 1994a). Sí se ha observado, en cambio, que la SP-A humana desglicosilada pierde la capacidad de agregar vesículas lipídicas (Haagsman *et al.* 1991), aunque un trabajo más reciente contradice estos resultados (McCormack *et al.* 1994a).

En cuanto al papel que desempeña la SP-A en el contexto del alveolo pulmonar, son varias las funciones que se le atribuyen, pudiéndose agrupar en tres apartados:

1) **Actividad biofísica del surfactante**.

- Participa en la formación de la mielina tubular a partir del surfactante secretado desde los cuerpos lamelares. Para este proceso también es necesaria la presencia de SP-B y de Ca^{2+} (Suzuki *et al.* 1989, Williams *et al.* 1991).
- Facilita la adsorción de los lípidos del surfactante a la interfase aire/agua del alveolo, aunque de forma relativamente débil en comparación con las proteínas hidrofóbicas SP-B y SP-C (King y MacBeth 1979, King y MacBeth 1981, Chung *et al.* 1989, Schürch *et al.* 1992, Yu y Possmayer 1993, Putman *et al.* 1996). Este efecto es mayor en presencia de SP-B, SP-C y Ca^{2+} (Hawgood *et al.* 1985, Hawgood *et al.* 1987, Efrati *et al.* 1987, Yu y Possmayer 1990).
- Favorece el enriquecimiento de la monocapa en DPPC (Yu y Possmayer 1990, Schürch *et al.* 1992, Yu y Possmayer 1998).
- Participa, junto con la SP-B, en el mantenimiento de los agregados gran-

des, que constituyen la fracción funcionalmente activa del surfactante en el alveolo pulmonar (Veldhuizen *et al.* 1994, Veldhuizen *et al.* 1996).

- Contrarresta la inhibición de las propiedades tensoactivas del surfactante provocada por proteínas plasmáticas como albúmina y fibrinógeno (Cockshutt *et al.* 1990, Hallman *et al.* 1991, Strayer *et al.* 1996a). Además, parece intervenir en el aclaramiento de componentes plasmáticos en los alveolos. En este sentido, se ha visto que la SP-A se une a VLDL y favorece su captura por macrófagos alveolares (Alberti *et al.* 1998).

2) **Ciclo metabólico del surfactante.**

- Interviene en la recaptura del surfactante para su reciclaje, favoreciendo al menos la unión de los lípidos a la membrana de los neumocitos tipo II (Wright *et al.* 1987, Rice *et al.* 1989, Tsuzuki *et al.* 1993, Bates *et al.* 1994).

- Inhibe la secreción del surfactante, participando en un mecanismo regulador de retroalimentación negativa (Dobbs *et al.* 1987, Rice *et al.* 1987).

- Un trabajo reciente realizado por Korutla y Strayer señala que la SP-A estimula la transcripción de los genes de SP-B y SP-C, así como la de su propio gen (Korutla y Strayer 1999). Debido a ello, los autores proponen un papel de citoquina para la SP-A, que funcionaría mediante un mecanismo autocrino.

Estas funciones relacionadas con el ciclo metabólico del surfactante están mediadas por su unión a un receptor específico situado en la membrana de los neumocitos tipo II (Kuroki *et al.* 1988b, Wright *et al.* 1989, Stevens *et al.* 1995, Chroneos *et al.* 1996, Pattanajitvilai *et al.* 1998). La integridad del dominio colagénico es necesaria para que se produzca su interacción con este receptor (Kuroki y Akino 1991b, McCormack *et al.* 1997c), posiblemente debido a que la eliminación de esta región produce una pérdida de estructura cuaternaria. Varias evidencias demuestran que esta interacción es dependiente de Ca^{2+} (Kuroki *et al.* 1988c, Wright *et al.* 1989, Chroneos *et al.* 1996) y que se produce a través del dominio de reconocimiento de carbohidratos (McCormack *et al.* 1994b, Kuroki *et al.* 1994, Hiraike *et al.* 1995). Dentro de esta región, la secuencia comprendida entre los residuos Glu-195 y Phe-228 desempeña un papel fundamental (Sano *et al.* 1998). Esto podría explicar el hecho de que otras lectinas sean capaces también de estimular la recaptura de fosfolípidos o de inhibir su secreción en los neumocitos tipo II (Griese *et al.* 1991).

3) **Defensa frente a agentes patógenos.**

- Induce quimiotaxis (Wright y Youmans 1993) y aparición de marcadores de superficie (Kremlev y Phelps 1997b) en macrófagos.

- Acelera la maduración de linfocitos (Kremlev *et al.* 1994a) e incrementa la secreción de determinadas citoquinas en monocitos, neumocitos tipo II y macrófagos alveolares (Blau *et al.* 1994, Kremlev y Phelps 1994b, Kremlev *et al.* 1997a), así como la producción de inmunoglobulinas (Kremlev y Phelps 1994b). Estos datos se contradicen sin embargo con los resultados obtenidos en otros trabajos, en los que se ha observado que la SP-A inhibe la proliferación de linfocitos y la producción de interleukina-2 (Borron *et al.* 1996) mediante su unión a un receptor (Borron *et al.* 1998).

- Incrementa la producción de especies reactivas de oxígeno de forma selectiva en macrófagos alveolares (van Iwaarden *et al.* 1990, Weissbach *et al.*

1994, van Iwaarden *et al.* 1995). Este efecto se debe al parecer a un aumento en la expresión de la NO sintasa inducible (Blau *et al.* 1997). Recientemente se ha sugerido la idea de que la SP-A, a la vez que induce la producción de estas especies en macrófagos, es capaz de proteger al surfactante frente a su acción (Amirkhanian y Merritt 1998).

- Favorece la fagocitosis de algunos virus mediante su opsonización (van Iwaarden *et al.* 1991, van Iwaarden *et al.* 1995), y neutraliza a otros (Hartshorn *et al.* 1994, Benne *et al.* 1995). La interacción podría estar mediada por la unión del residuo de ácido siálico de la cadena oligosacárida de la SP-A a una lectina de la cubierta viral (van Iwaarden *et al.* 1992b, Malhotra *et al.* 1994, Benne *et al.* 1995, Benne *et al.* 1997).

- Estimula la fagocitosis y destrucción de bacterias (van Iwaarden *et al.* 1990, Manz-Keinke *et al.* 1992, Geertsma *et al.* 1994, Pikaar *et al.* 1995, Tino y Wright 1996, Weikert *et al.* 1997, Hickman-Davis *et al.* 1998). Ni la región de reconocimiento de carbohidratos ni las cadenas oligosacáridas de la SP-A intervienen al parecer en la unión a algunos microorganismos Gram negativos, por lo que posiblemente sea la región de unión a lípidos de la SP-A la responsable de la interacción con el lípido A del lipopolisacárido (LPS) de estas bacterias (van Iwaarden *et al.* 1994). La unión a otras bacterias se realiza mediante interacción con los polisacáridos capsulares (Kabha *et al.* 1997). Se ha demostrado que, una vez en marcha la respuesta inflamatoria, el receptor de manosa de los macrófagos alveolares (capaz de unir directamente patógenos no opsonizados) se inactiva, y la afinidad de estas células por la SP-A se dispara, lo que aumentaría la importancia del papel de opsonina de la SP-A en la fagocitosis de microorganismos patógenos (Chroneos y Shepherd 1995).

- Su unión dependiente de Ca^{2+} a *Aspergillus fumigatus* también facilita la fagocitosis y destrucción de este hongo (Madan *et al.* 1997a). Además, inhibe la unión de las IgE a alergenos de este microorganismo y la liberación de histamina en basófilos, lo que le podría conferir un papel protector frente a reacciones de hipersensibilidad producidas por alergenos inhalados (Madan *et al.* 1997b).

Tabla 1. Funciones de defensa de la SP-A.

Sistemas de defensa del organismo	Macrófagos	Quimiotaxis, aparición de marcadores de superficie, producción de especies reactivas de oxígeno
	Linfocitos	Aceleración de la maduración, aumento de secreción de inmunoglobulinas y citoquinas
	Otras células	Aumento de la secreción de citoquinas
Agentes patógenos	Virus	Aumento de la fagocitosis, neutralización
	Bacterias	Aumento de la fagocitosis y destrucción
	Hongos	Aumento de la fagocitosis y destrucción
	Alergenos	Disminución en la unión a IgE, inhibición de la secreción de histamina

La unión de la SP-A a macrófagos alveolares parece estar mediada por la interacción de su dominio colagénico (Pison *et al.* 1992, Malhotra *et al.* 1993, Wright y Youmans 1993) con un receptor de alta afinidad situado en la membrana plasmática de estas células (Kuroki *et al.* 1988c, Manz-Keinke *et al.* 1991, Pison *et al.* 1992, Oosting y Wright 1994). Este receptor ha sido identificado por algunos autores como C1qR (el receptor de C1q), que podría ser común para varias colectinas (Malhotra *et al.* 1990, Malhotra *et al.* 1992, Geertsma *et al.* 1994). Sin embargo, hay que tener en cuenta que la mayoría de estos estudios están basados en la competencia de proteínas que poseen dominios colagénicos con la SP-A por su unión al receptor, y que la SP-A es capaz de interaccionar con algunas de ellas, por lo que el efecto inhibidor de estas proteínas podría deberse a su interacción con la SP-A, y no a la unión de éstas al receptor (Oosting y Wright 1994). Además, se ha observado en otros estudios que la presencia de manosa en la cadena oligosacárida del receptor o en la de la propia SP-A es fundamental para esta interacción (Wintergerst *et al.* 1989, Manz-Keinke *et al.* 1991, Ohmer-Schröck *et al.* 1993, Gaynor *et al.* 1995), y que ésta es dependiente de Ca^{2+} (Manz-Keinke *et al.* 1991, Ohmer-Schröck *et al.* 1993), lo que podría implicar a interacciones de tipo lectina en el mecanismo de unión. Integrando las dos posturas anteriormente expuestas, Ohmer-Schröck y colaboradores han propuesto que, aunque la interacción con el receptor se produce a través de su dominio de reconocimiento de carbohidratos, la SP-A podría tener además un sitio de unión para su dominio colagénico en C1qR (Ohmer-Schröck *et al.* 1993). Se necesitan por tanto más estudios para avanzar en la comprensión de este proceso. De momento, los datos publicados parecen apoyar la hipótesis de que hay distintos receptores de colectinas en las células del sistema inmunitario (Wright 1997). En este sentido, un trabajo publicado por Chroneos y colaboradores muestra que la interacción de la SP-A con macrófagos está mediada por un receptor distinto de C1qR (Chroneos *et al.* 1996). Este nuevo receptor para la SP-A, denominado C1qR(P), podría ser una proteína de membrana de 126 kDa, que ha sido clonada y analizada por Nepomuceno y colaboradores (Nepomuceno *et al.* 1997).

A la vista de las propiedades descritas de interacción con células del sistema inmunitario, se deduce que la SP-A ejerce una actividad proinflamatoria. Teniendo en cuenta las propiedades antiinflamatorias de los lípidos del surfactante comentadas anteriormente, será la relación fosfolípidos/SP-A la que determine si el surfactante pulmonar tiene en conjunto un carácter anti o proinflamatorio. La hipótesis de que la SP-A desempeña un papel importante en la defensa de las vías respiratorias estaría además apoyada por el hecho de que el interferón-γ estimula su síntesis sin alterar la producción de otros componentes del surfactante (Ballard *et al.* 1990). Asimismo, se ha observado que los niveles de SP-A en enfermos de neumonía de origen bacteriano son menores que en individuos sanos (Baughman *et al.* 1993), y que ratones transgénicos carentes de SP-A sufren con mayor facilidad infecciones por estreptococos del grupo B (LeVine *et al.* 1997) y *Pseudomonas aeruginosa* (LeVine *et al.* 1998), lo que podría establecer una relación entre los niveles de esta proteína y la susceptibilidad a infecciones. Sin embargo, y en contraposición con lo anteriormente dicho, esta proteína po-

dría actuar también como adyuvante de algunos agentes infecciosos. En este sentido, se ha observado que la SP-A podría facilitar la infección de macrófagos alveolares por *Mycobacterium tuberculosis* (Downing *et al.* 1995, Gaynor *et al.* 1995, Pasula *et al.* 1997), y del epitelio alveolar por *Pneumocystis carinii* (Zimmerman *et al.* 1992), aunque también favorece la unión de este protozoo a macrófagos alveolares, posibilitando así su fagocitosis (Williams *et al.* 1996). Su unión a las cadenas oligosacáridas de *Pneumocystis carinii* es dependiente de Ca^{2+}, y en ella está implicado el dominio de reconocimiento de carbohidratos de la SP-A (Zimmerman *et al.* 1992, McCormack *et al.* 1997a).

1.4.3 SP-B

La importancia de la SP-B en el surfactante pulmonar ha sido demostrada en varios experimentos realizados *in vivo*: anticuerpos contra esta proteína inactivan surfactantes exógenos y producen fallo respiratorio agudo en ratones y conejos (Johansson y Curstedt 1997). Además, la carencia congénita de SP-B es incompatible con la vida. Este hecho se ha demostrado al comprobar que ratones transgénicos carentes de SP-B morían al nacer (Clark *et al.* 1995, Tokieda *et al.* 1997). En ratones heterozigotos se producen graves trastornos respiratorios (Clark *et al.* 1997, Tokieda *et al.* 1997). También se ha detectado una mutación en el gen humano de la SP-B que produce fallo respiratorio irreversible en neonatos homozigotos (Nogee *et al.* 1994), y transitorio en individuos heterozigotos (Klein *et al.* 1998). Esta deficiencia está relacionada al parecer con un procesamiento incompleto de la forma inmadura de la SP-C (Vorbroker *et al.* 1995a, Clark *et al.* 1995, Klein *et al.* 1998).

Aunque aún no está clara la función de la SP-B, sus propiedades podrían arrojar alguna luz sobre su papel biológico:

- Es necesaria, junto con la SP-A y el Ca^{2+}, para la formación de mielina tubular (Suzuki *et al.* 1989, Williams *et al.* 1991).
- Acelera la adsorción de lípidos a la interfase aire/agua (Hawgood *et al.* 1987, Oosterlaken-Dijksterhuis *et al.* 1991a, Wang *et al.* 1996a).
- Gracias a la SP-B, los lípidos de la monocapa pueden producir tensiones superficiales muy bajas en el alveolo, posiblemente debido a que enriquece el contenido de la monocapa en DPPC promoviendo la expulsión de otros componentes (Mathialagan y Possmayer 1990, Yu y Possmayer 1990, Wang *et al.* 1996a).
- Aumenta la estabilidad de la monocapa (Cochrane y Revak 1991, Wang *et al.* 1996a, Lipp *et al.* 1996) y de los agregados grandes, que constituyen la fracción funcional del surfactante (Veldhuizen *et al.* 1994).
- Podría estar implicada en la recaptura de fosfolípidos por neumocitos tipo II (Rice *et al.* 1989, Horowitz *et al.* 1996) gracias a su capacidad de inducir mezcla de contenidos lipídicos entre membranas (Rice *et al.* 1989, Poulain *et al.* 1992, Poulain *et al.* 1996). Algunos autores sostienen que esta propiedad le otorga también un papel en el empaquetamiento de lípidos en los cuerpos lamelares y en el aumento en la velocidad de adsorción de fosfolípidos a la interfase aire/agua (Oosterlaken-Dijksterhuis *et al.* 1991b, Oosterlaken-Dijksterhuis *et al.* 1992).
- Se ha observado que, tras producirse el colapso de una monocapa de

DPPC al ser comprimida, la SP-B favorece la redispersión de ésta en la interfase (Taneva y Keough 1994, Lipp *et al.* 1997).

La estructura dimérica de la SP-B, que le permitiría interaccionar a la vez con dos bicapas, o con una bicapa y una monocapa, podría ayudarnos a comprender parte del mecanismo por el cual lleva a cabo sus funciones.

La SP-B se encuentra ocupando un plano paralelo a la bicapa, ubicándose en su superficie sin profundizar mucho en ella (Baatz *et al.* 1990, Vandenbussche *et al.* 1992b). La región rica en aminoácidos hidrofóbicos quedaría de esta forma interaccionando con las cadenas de acilo de los fosfolípidos mediante fuerzas de van der Waals, mientras que los residuos básicos lo harían con los grupos fosfato de las cabezas polares mediante fuerzas electrostáticas (Cochrane y Revak 1991, Vandenbussche *et al.* 1992b, Morrow *et al.* 1993a).

Estudios de anisotropía de fluorescencia (Baatz *et al.* 1990) y de resonancia de spin electrónico (Pérez-Gil *et al.* 1995) indican que hay una interacción selectiva entre la SP-B y fosfolípidos aniónicos. Estos datos apoyarían otros estudios en los que se ha observado que los efectos de mezclas de SP-B y SP-C o de SP-B sola sobre fosfolípidos son dependientes de la presencia de fosfolípidos ácidos (Shiffer *et al.* 1988) y del pH (Shiffer *et al.* 1988, Oosterlaken-Dijksterhuis *et al.* 1991a, Camacho *et al.* 1996). La abundancia de residuos básicos en el extremo N-terminal de la proteína podría ser importante en esta interacción (Longo *et al.* 1993).

1.4.4 SP-C

Actualmente se desconoce la función de la SP-C. Sin embargo, se sabe que acelera la adsorción de lípidos a la interfase aire/agua y que favorece su actividad tensoactiva (Warr *et al.* 1987, Yu y Possmayer 1990, Simatos *et al.* 1990, Oosterlaken-Dijksterhuis *et al.* 1991a, Oosterlaken-Dijksterhuis *et al.* 1991b, Seeger *et al.* 1991, Creuwels *et al.* 1993). Al igual que la SP-B, facilita la redispersión de DPPC en la interfase tras producirse el colapso de la monocapa durante un ciclo de compresión-expansión, permitiendo que la exclusión de fosfolípidos en cada uno de estos ciclos sea reversible (Taneva y Keough 1994). Aunque en menor grado que la SP-B, la SP-C también induce mezcla de contenidos lipídicos (Poulain *et al.* 1992), lo que podría implicarla en la recaptura de fosfolípidos del surfactante en neumocitos tipo II (Rice *et al.* 1989).

Las cargas positivas de la proteína, muy conservadas, podrían desempeñar un papel importante en la incorporación de fosfolípidos a la monocapa (Creuwels *et al.* 1995b). La capacidad de la SP-C de disminuir la T_m del fosfolípido (Simatos *et al.* 1990) y de aumentar su elasticidad y reducir su viscosidad (Pastrana *et al.* 1991) también parecen favorecer el proceso. En este sentido, se ha observado que, aunque este péptido aumenta el orden de las cabezas polares en una bicapa, altera a la vez el empaquetamiento de las cadenas de acilo de los fosfolípidos (Horowitz *et al.* 1992).

Aunque aún no se sabe qué papel desempeña la palmitoilación de los residuos de cisteína en la SP-C, es posible que esta modificación sirva de anclaje del dominio N-terminal en la membrana, ya que se ha demostrado que sí influye en la estructura de la proteína y en su interacción con lípidos (Creuwels *et al.* 1993, Pérez-Gil *et al.* 1994, Johansson *et al.* 1995), y que afecta a su capacidad de favorecer la adsorción de fosfolípidos a la monocapa y las propieda-

des tensoactivas de éstos (Wang *et al.* 1996b). Estos resultados, sin embargo, contradicen en parte los publicados por Qanbar (Qanbar *et al.* 1995).

Otro aspecto que se ha relacionado con sus propiedades funcionales es su capacidad de segregarse dentro de la bicapa a temperaturas inferiores a la T_m, separándose en dominios proteicos (Horowitz *et al.* 1993). Este hecho se debe probablemente a que la longitud de la hélice transmembranar de la proteína es menor que el grosor de la bicapa en fase gel (debido al gran empaquetamiento de las cadenas de acilo), lo que crearía en ésta irregularidades termodinámicamente desfavorables que se evitarían mediante la segregación de la SP-C en los dominios anteriormente mencionados.

1.4.5 SP-D

Al igual que la SP-A y otras colectinas, la SP-D tiene la propiedad de unirse a carbohidratos de forma dependiente de Ca^{2+} (Persson *et al.* 1990, Schelenz *et al.* 1995). Igualmente se ha observado su capacidad para interaccionar, también de forma dependiente de Ca^{2+}, con algunos lípidos, presentando especial afinidad por el fosfatidilinositol (Ogasawara *et al.* 1992, Persson *et al.* 1992, Ogasawara *et al.* 1994) y la glucosilceramida (Kuroki *et al.* 1992a). En la interacción con cada uno de los lípidos anteriormente citados intervienen al parecer zonas diferentes de la proteína (Sano *et al.* 1998). La unión de la SP-D a lípidos se produce principalmente a través del dominio de reconocimiento de carbohidratos y la zona del cuello (Ogasawara *et al.* 1994, Ogasawara y Voelker 1995a, Kishore *et al.* 1996, Sano *et al.* 1998), aunque otro trabajo ha revelado que los dominios N-terminal y colagénico podrían intervenir también en dicha unión (Ogasawara y Voelker 1995b).

La única función que se ha atribuido a la SP-D hasta el momento es la de intervenir en los mecanismos de defensa del pulmón, función para la cual resulta necesaria la integridad de la estructura dodecamérica (Brown-Augsburger *et al.* 1996a). En efecto, se ha demostrado su capacidad de unirse, agregar, neutralizar y aumentar la unión de virus a neutrófilos, acciones todas ellas mediadas por la unión a carbohidratos dependiente de Ca^{2+} (Hartshorn *et al.* 1994). Además, produce aglutinación de bacterias Gram negativas tras unirse al LPS (Kuan *et al.* 1992, Lim *et al.* 1994), y también se asocia a *Pneumocystis carinii* mediante interacciones con carbohidratos, facilitando así su unión a macrófagos alveolares, aunque no su fagocitosis (O'Riordan *et al.* 1995). En cambio, sí facilita la fagocitosis y destrucción del hongo *Aspergillus fumigatus* tras unirse a sus cadenas oligosacáridas de forma dependiente de Ca^{2+} (Madan *et al.* 1997a), y protege al organismo frente a reacciones de hipersensibilidad provocadas por los alergenos de este hongo (Madan *et al.* 1997b). A su vez estimula la producción de especies reactivas de oxígeno en macrófagos alveolares (actúa incluso a concentraciones menores que la SP-A), acción independiente de la presencia de lípidos del surfactante (van Iwaarden *et al.* 1992a). Esta última propiedad, al igual que ocurre en el caso de la SP-A, parece ser bastante selectiva para los macrófagos alveolares, ya que se han obtenido resultados negativos al trabajar con macrófagos peritoneales (van Iwaarden *et al.* 1992a). La interacción con estas células también está mediada por cadenas oligosacáridas (Kuan *et al.* 1994). Como en el caso de la SP-A, también hay receptores específicos para la SP-D en este tipo de células, que al parecer son distintos de los receptores de C1q (Miyamura *et al.*

1994). Sin embargo, la producción de ambas proteínas en respuesta a una infección no está regulada de forma coordinada (Dulkerian *et al.* 1996).

En 1991, Kuroki y colaboradores también propusieron un posible papel de la SP-D en la regulación de la secreción del surfactante, al observar que contrarrestaba la acción inhibidora de la SP-A sobre la secreción de fosfolípidos (Kuroki *et al.* 1991c). Estudios más recientes han confirmado esta hipótesis. Aunque no se ven afectadas la actividad biofísica del surfantante, la función respiratoria, ni la supervivencia de ratones homozigotos deficientes de SP-D, sí se ha observado una acumulación de lípidos y proteínas del surfactante en el espacio alveolar de estos animales, acompañada de alteraciones en los cuerpos lamelares y en los macrófagos alveolares (Botas *et al.* 1998). Korfhagen y colaboradores también observaron una acumulación de fosfatidilcolina acompañada de cambios morfológicos en el surfactante, en los macrófagos alveolares y en el epitelio alveolar, aunque no detectaron aumento de SP-B y SP-C, y sí una reducción en la expresión de SP-A (Korfhagen *et al.* 1998).

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2. OBJETIVOS

El surfactante pulmonar tiene una gran importancia clínica, ya que su carencia o alteración produce importantes patologías como el síndrome de distrés respiratorio neonatal (NRDS) o el síndrome de distrés respiratorio del adulto (ARDS). Actualmente se están empleando surfactantes de distinta procedencia en el tratamiento del NRDS, y se están ensayando en el ARDS. Los estudios sobre las características funcionales y estructurales de los diversos componentes del surfactante podrían ayudarnos a profundizar en el conocimiento de estas patologías, así como en su tratamiento.

A la vista de los antecedentes bibliográficos expuestos en la introducción, resulta por tanto de interés profundizar en el estudio de las propiedades de la SP-A con el fin de ampliar nuestra comprensión sobre el ciclo biológico y las características funcionales del surfactante pulmonar.

Aún quedan por esclarecer algunos aspectos sobre las interacciones de la SP-A con lípidos, con otras moléculas de SP-A, y con otras proteínas del surfactante. En este sentido, aún no está clara la relación que hay entre la agregación de vesículas lipídicas inducida por la proteína y la autoagregación de la propia SP-A en distintas condiciones. Por otra parte, los estudios de interacción entre SP-A y lípidos realizados hasta la fecha se han llevado a cabo en su mayor parte utilizando bicapas lipídicas. No se ha investigado, en cambio, la interacción de la proteína con monocapas lipídicas, teniendo en cuenta que esta estructura es al parecer la responsable de las propiedades tensoactivas del surfactante en el alveolo pulmonar. Además, todavía no se conoce bien cómo es el proceso de secreción de la SP-A, desde que se sintetiza en el retículo endoplásmico hasta que se secreta tras almacenarse en los cuerpos lamelares. A lo largo de la ruta de secreción, la proteína pasa por distintas condiciones de pH, permaneciendo en un ambiente iónico rico en calcio y de fuerza iónica fisiológica. En este momento quedan importantes lagunas sobre cómo van evolucionando la estructura y las propiedades funcionales de la SP-A en cada etapa de dicha ruta.

Teniendo en cuenta todos estos puntos, este trabajo se propone alcanzar los siguientes objetivos:

1. **Estudiar la interacción de la SP-A con bicapas fosfolipídicas en distintas condiciones iónicas y de pH.**

2. **Analizar la interacción de la SP-A con monocapas de fosfolípidos.**

3. **Estudiar el estado de autoagregación de la SP-A en distintas condiciones iónicas, de pH y de temperatura.**

3. INTERACCIÓN DE LA SP-A CON BICAPAS LIPÍDICAS

INTRODUCCIÓN

Desde hace más de una década se sabe que la SP-A es capaz de interaccionar con bicapas de fosfolípidos y de inducir la agregación de éstas en presencia de Ca^{2+}. Actualmente se cree que esta propiedad podría ser uno de los factores desencadenantes del reordenamiento a que se ven sometidas las estructuras lipídicas del surfactante tras su secreción y que podría facilitar su adsorción a la interfase aire/agua, donde ejercerían su actividad superficial.

No obstante, aún no está claro el mecanismo molecular que produce la agregación lipídica inducida por SP-A. Se ha sugerido que la agregación de las vesículas lipídicas podría estar mediada por la autoagregación de la propia SP-A tras la unión de ésta a dichas vesículas, posiblemente mediante interacciones de tipo lectina-lectina (Haagsman *et al.* 1991). Sin embargo, se sabe que la cadena oligosacárida de la proteína no es necesaria para su unión a lípidos (Kuroki y Akino 1991, Haagsman *et al.* 1991, McCormack *et al.* 1994a), y se ha observado que la unión de la SP-A a lípidos y la agregación de éstos inducida por la proteína parecen estar mediadas por mecanismos distintos (McCormack *et al.* 1994b).

Teniendo en cuenta lo dicho anteriormente, se realizó el primer trabajo de este capítulo, cuyo objetivo fue estudiar y comparar la agregación de vesículas lipídicas inducida por SP-A y la autoagregación de esta proteína con el fin de profundizar en el conocimiento del mecanismo que dirige el primero de ambos procesos.

Por otro lado, antes de ser secretada, la SP-A se almacena junto con otras proteínas (SP-B y SP-C) y los lípidos del surfactante pulmonar en los cuerpos lamelares, unos orgánulos en cuyo interior hay un pH ácido (≈ 5.5) (Chander *et al.* 1986) y una concentración de Ca^{2+} libre entre 2 y 10 mM (Eckenhoff 1989). Sin embargo, casi todos los estudios estructurales y de interacción de la SP-A con lípidos se han llevado a cabo a pH neutro. Se desconoce por tanto a qué cambios funcionales y estructurales se ve sometida la SP-A a lo largo de su ruta de secreción. Esta ruta comienza en el retículo endoplásmico, con un pH neutro que se va acidificando de un compartimento celular a otro hasta llegar al pH ácido de los cuerpos lamelares, para volver finalmente al pH neutro de la subfase acuosa del líquido alveolar una vez secretado el surfactante.

Durante el proceso de formación de los gránulos de secreción regulada se produce la segregación de las proteínas de estos gránulos de aquellas otras que serán secretadas por la vía constitutiva. Parece ser que en este proceso de separación podría desempeñar un papel muy importante la agregación selectiva de proteínas inducida por el pH ácido y/o la alta concentración de Ca^{2+} (Chanat y Huttner 1991, Yoo 1996, Colomer *et al.* 1996). En estas condiciones las proteínas secretadas por la vía constitutiva no agregarían, y sí lo harían las secretadas por la vía regulada.

A la vista de estos hechos, el segundo trabajo de este capítulo aborda el estudio a pH ácido de la estructura e interacción con bicapas lipídicas de la SP-A en presencia y ausencia de las proteínas hidrofóbicas del surfactante SP-B y SP-C.

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Comparison of lipid aggregation and self-aggregation activities of pulmonary surfactant-associated protein A

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1. We compared the Ca^{2+} dependence of the self-aggregation of surfactant protein A (SP-A) with that of vesicle aggregation induced by SP-A. The Ca^{2+} concentration required for half-maximal activity of lipid aggregation was 0.74 ± 0.29 μM ($n = 4$) for pig SP-A and $98 + 5$ μM ($n = 2$) for dog SP-A. In contrast, the threshold concentration of Ca^{2+} required to induce self-association of both pig and dog SP-A was 0.5 mM. The Ca^{2+} concentration needed for half-maximal self-association was 2.36 ± 0.15 mM ($n = 4$) and 0.70 ± 0.06 mM ($n = 2$) for pig and dog SP-A respectively. 2. We also compared the effect of Ca^{2+} on the trypsin sensitivity of lipid-free and membrane-bound SP-A. At 1 μM Ca^{2+}, the tryptic digestion patterns of dog and pig lipid-free SP-A were quite different. Dog SP-A was very sensitive to proteolysis, being almost completely digested by 30 min, while pig SP-A was very resistant, even after 12 h. After protein aggregation of lipid-free SP-A (at 5 mM Ca^{2+}), the accessibility of the trypsin cleavage targets of the protein depended on the SP-A species (self-aggregated pig SP-A became more sensitive to degradation than its non-aggregated form, whereas self-aggregated dog SP-A was less susceptible). In contrast, membrane-bound SP-A, from either pig or dog, was clearly protected from trypsin degradation at both low (1 μM) or high (1 mM) Ca^{2+} concentrations. The protection was slightly higher at 1 mM Ca^{2+} when the extent of lipid/SP-A aggregates was maximal. 3. On the other hand, vesicle aggregation activity of SP-A was decreased by 30–40% by removing the oligosaccharide moiety of the protein, whereas self-aggregation was not influenced by deglycosylation. The presence of mannan (at concentrations not lower than 10 μg/μl) decreased vesicle aggregation induced by dog and pig SP-A by a mechanism that is independent of the binding of mannan to the carbohydrate-binding domain of SP-A. Self-aggregation of SP-A was not affected by the presence of sugars. 4. From these results, we conclude that: (1) the process of lipid aggregation induced by SP-A cannot be correlated with that of self-association of the protein occurring at supramillimolar concentrations of Ca^{2+}; and (2) the N-linked carbohydrate moiety of SP-A and the ability of SP-A to bind carbohydrates are not involved in lipid aggregation.

INTRODUCTION

Pulmonary surfactant is a heterogeneous complex of lipid and proteins that serves to stabilize the alveoli and distal airways at low lung volumes. Dipalmitoylphosphatidylcholine (DPPC) is widely accepted to be the major lipid component of pulmonary surfactant. Besides DPPC, phosphatidylglycerol (PG) and specific protein components are required for the full biological activity of surfactant. At least three of these surfactant proteins (SP-A, SP-B and SP-C) potentiate the surface tension-reducing properties of the surfactant lipids (see [1,2] for reviews).

The most abundant surfactant-associated protein (SP-A) has a monomeric molecular mass of 30–40 kDa. Each SP-A subunit is characterized by an N-terminal region containing a collagen-like sequence and a C-terminal domain with a sequence similar to several Ca^{2+}-dependent carbohydrate-binding proteins, C-type lectins [3,4]. The functional form of SP-A is assembled through interactions in the collagen-like domain into a complex oligomer of 18 subunits [5–7]. In one of the initial steps of the assembly of SP-A, three subunits of SP-A probably form a triple-helical stem that is stabilized by interchain disulphide bonds. In the final stage of the assembly, the hexamers appear to be formed by lateral aggregation of the N-terminal half of the triple-helical stems [8].

SP-A interacts with surfactant phospholipids, such as DPPC and PG [9–11], as well as with surfactant glycosphingolipids [12]. Several studies suggest that SP-A plays a major role in the intra-alveolar surfactant phospholipid organization: (i) SP-A induces phospholipid vesicle aggregation in the presence of Ca^{2+} [9,13]; (ii) SP-A mediates the formation of large ordered tubular aggregates known as tubular myelin, when added to DPPC, PG and SP-B mixtures in the presence of Ca^{2+} [14,15]; (iii) SP-A causes a Ca^{2+}-dependent increase of the phospholipid mixing activity of SP-B [16]; and (iv) SP-A promotes the formation of a stable surface film of phospholipids cooperating with the hydrophobic surfactant protein SP-B [17].

The phospholipid vesicle aggregation activity of SP-A has been amply assessed by changes in turbidity [9,11,13,18–22], and has been seen by electron microscopy [15,23]. Typically, negatively charged vesicles were used for these studies. Some structural requirements of SP-A for lipid aggregation have been studied. Digestion with bacterial collagenase or reduction of SP-A with dithiothreitol strongly decreases lipid aggregation mediated by the protein [20] as well as the ability of SP-A to bind phospholipids [10,20], suggesting that these two processes are dependent on the integrity of the collagenous domain of SP-A (triple helix and intermolecular disulphide bond).

The requirement of the oligosaccharide moiety of SP-A for lipid aggregation is not fully clear. The carbohydrate domain of SP-A was proposed to be an important determinant in the ability

Abbreviations used: SP-A, surfactant protein A; CRD, carbohydrate recognition domain; DPPC, dipalmitoylphosphatidylcholine; DPPG, dipalmitoylphosphatidylglycerol; PG, phosphatidylglycerol; OGP, n-octyl β-D-glucopyranoside.

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of SP-A to aggregate lipids, since deglycosylated SP-A did not aggregate phospholipids [23]. However, site-directed mutagenesis of SP-A reveals that the oligosaccharide moiety is not essential for lipid aggregation [24]. On the other hand, deglycosylation of the protein does not affect the lipid binding properties of SP-A [10,23]. Interestingly, McCormack et al. [25] recently reported that phospholipid aggregation is mediated by the C-terminal region of SP-A by a mechanism that is distinct from phospholipid binding.

Concerning the mechanism involved in the vesicle aggregation phenomenon, it has been proposed that this process could be mediated by Ca^{2+}-dependent protein–protein interaction between SP-A molecules [19]. These authors found a linear correlation between the extent of vesicle aggregation induced by SP-A in the presence of calcium and the extent of self-aggregation of SP-A lacking lipids at the same concentrations of calcium. The interaction between SP-A molecules in the presence of lipids was further proposed to occur mostly between the carbohydrate-binding domains and the oligosaccharide moieties of SP-A [23].

The present study analyses the aggregation of neutral and acidic vesicles mediated by SP-A and compares lipid aggregation and self-aggregation activities of SP-A. The possible mechanisms involved in the process of vesicle aggregation induced by SP-A are discussed.

EXPERIMENTAL

Isolation and N-glycosidase F treatment of SP-A

Pulmonary surfactant was prepared from pig or dog bronchoalveolar lavage as described previously [26]. Pig or dog SP-A was purified from isolated surfactant using sequential butanol and octyl glucoside extractions as described elsewhere [11].

Surfactant protein content was estimated by the method of Lowry using BSA as standard. However, the concentration of SP-A was always determined by quantitative amino acid analysis because the SP-A concentration was overestimated when determined by the method of Lowry. The amino acid analysis of SP-A was carried out on a Beckman System 6300 High Performance amino acid analyser. The protein hydrolysis was performed with 0.2 ml of 6 M HCl, containing 0.1% (w/v) phenol in evacuated and sealed tubes at 108 °C for 24 h. Norleucine was added to each sample as the internal standard.

Electrophoretic analysis of SP-A was performed under reducing conditions (50 mM dithiothreitol) by one-dimensional SDS/PAGE as described by Laemmli [27] using stacking and running gels of 4 and 12% acrylamide respectively. Gels were stained with Coomassie Brilliant Blue R 250.

SP-A was deglycosylated by treatment with 45 units of N-glycosidase F from *Flavobacterium meningosepticum* per mg of SP-A at 37 °C for 24 h in 15 mM sodium phosphate buffer, pH 7.4, containing 12 mM EDTA, 1.5 mM PMFS, 0.02% sodium azide and 20 mM *n*-octyl β-D-glucopyranoside (OGP). Control incubations contained all components except the enzyme. After incubation, OGP or EDTA were removed by dialysis of the reaction mixture against 5 mM Tris/HCl buffer, pH 7.4, for 48 h at 4 °C. The protein content in control and enzyme-treated samples was determined by quantitative amino acid analysis. SP-A was completely deglycosylated by N-glycosidase F, as shown by the change of mobility on SDS/PAGE under reducing conditions.

SP-A self-association assay

Ca^{2+}-dependent self-association of SP-A was studied as described previously [21]. Briefly, the sample and the reference cuvettes were first filled with 0.4 ml of 5 mM Tris/HCl buffer, pH 7.4. After 10 min equilibration at 37 °C, SP-A (20 μg) was added to the sample cuvette and the change in absorbance at 360 nm was monitored at 1-min intervals over 10 min. Next, Ca^{2+} (5 mM, final concentration) was added to both the sample and the reference cuvette and the change in absorbance was monitored again. Self-association of SP-A was reversed by adding EDTA (10 mM, final concentration).

Preparation of lipid vesicles

Unilamellar vesicles of DPPC, DPPC/dipalmitoylphosphatidylglycerol (DPPG) (7:3, w/w) and DPPC/egg-PG (7:3, w/w) were used. The different lipid vesicles were prepared as described previously [11] at a phospholipid concentration of 1 mg/ml by hydrating dry lipid films in a buffer containing 150 mM NaCl, 5 mM Tris/HCl, pH 7.4, and allowing them to swell for 1 h at temperature above the phase-transition temperature of the phospholipid vesicles. Next, the lipid dispersion was sonicated at the same temperature (above 45 °C) at 240 W with 10 bursts of 30 s (15 s between bursts) in an MSE tip Sonifer. The phospholipid concentration was determined by phosphorus determination according to Rouser et al. [28]. For vesicle-size analysis in solution, quasielastic light scattering (QELS) was used according to Koppel [29] on an Autosizer IIc Photon Correlation Spectrometer (Malvern Instruments, U.K.). Measurements were performed at 25 °C and 45 °C. Vesicle diameter for DPPC and for the binary mixtures DPPC/DPPG or DPPC/PG (7:3, w/w) was around 160–200 nm with a polydispersity index of 0.2, as reported previously [11].

Phospholipid vesicle aggregation assay

SP-A-induced phospholipid vesicle aggregation assays were performed at 37 °C in a Beckman DU-640 spectrophotometer, measuring the change in the absorbance at 400 nm as described previously [22]. Briefly, phospholipid vesicles (50 μg) were added to both the sample and the reference cuvette in a total volume of 0.5 ml of 5 mM Tris/HCl buffer, pH 7.4, 150 mM NaCl, with or without 50 μM EGTA. After 10 min equilibration at 37 °C, 5 μg of SP-A from a stock solution of the protein in 5 mM Tris/HCl buffer, pH 7.4, was added to the sample cuvette and the change in absorbance at 400 nm was monitored at 1-min intervals over 10 min. Next, Ca^{2+} (typically 1 mM final concentration) was added to both the sample and the reference cuvette and the change in absorbance was monitored again at 1-min intervals over 10 min. EDTA was used for reversing vesicle aggregation induced by SP-A.

Titration experiments

The calcium requirement for vesicle or self-aggregation was studied by titration experiments in which increasing amounts of a concentrated solution of $CaCl_2$ were added to the protein solution in the absence or presence of lipids. The assay buffer contained 50 μM EGTA. The Ca^{2+} concentration without adding $CaCl_2$ (contaminant Ca^{2+} in the experimental solution system) was 5 μM as measured by atomic absorption. The free Ca^{2+} concentration in each point of the titration experiments was estimated by a computer program (CHELATOR) [30] which also permits correction for ionic strength, temperature, pH and other competing ions.

Trypsin digestion

First, dog or pig SP-A (160 μg/ml) was incubated for 20 min at

37 °C with either DPPC or DPPC/DPPG (7:3, w/w) vesicles (1.6 mg/ml) in 5 mM Tris/HCl buffer, pH 7.4, 150 mM NaCl, 50 μM EGTA, in the presence of either 1 μM or 1 mM free Ca^{2+}. In addition, SP-A was incubated without lipids in the same conditions except that NaCl was not included in the buffer and that free Ca^{2+} concentrations were 1 μM or 5 mM. Then, trypsin (Millipore, 246 units/mg; 20 μg/ml) was added to the samples and the trypsin incubation was carried out at 37 °C at the indicated times. Digestion was stopped by adding 1 μl of 1 M HCl (final pH 2.0). The mixtures were then dried under vacuum and analysed by SDS/electrophoresis under reducing conditions.

Data reported in the Figures of this paper were obtained from four different preparations of pig SP-A and from two different preparations of dog SP-A. For each preparation experiments were repeated at least twice.

RESULTS

Figure 1 shows that the addition of pig SP-A to either DPPC or DPPC/DPPG (7:3, w/w) vesicles in 5 mM Tris/HCl buffer, pH 7.4, containing 150 mM NaCl results in a marked increase in light absorbance due to lipid aggregation. After addition of Ca^{2+} (3 mM, final concentration) the light absorbance of SP-A/lipid aggregates increases by an additional 20–25 %. Interestingly, the addition of EDTA (6 mM) dissociates the aggregate completely, suggesting that vesicle aggregation occurring after addition of pig SP-A is also dependent on Ca^{2+}. In fact, the presence of 1 mM EDTA in the assay buffer prevents lipid aggregation induced by adding SP-A (Figure 1). Considering that the endogenous Ca^{2+} concentration (i.e. without adding Ca^{2+} to our experimental aqueous system) was 5 μM, as measured by atomic absorption, the results suggest that pig SP-A induces lipid aggregation at very low concentrations of Ca^{2+}. Figure 1 also shows that pig SP-A induces aggregation of DPPC/DPPG (7:3, w/w) in the presence, but not in the absence, of 150 mM NaCl.

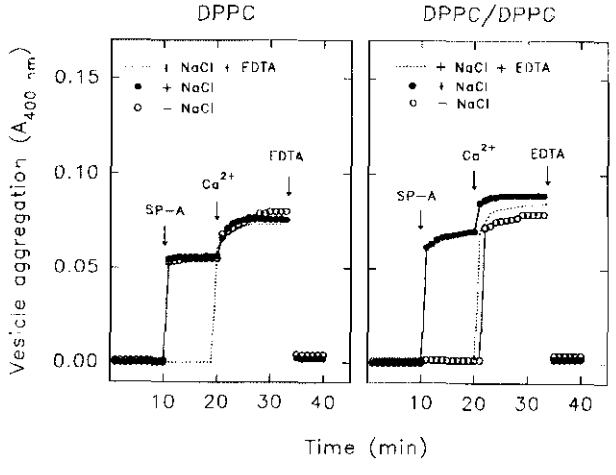

Figure 1 Physiological ionic strength is required for SP-A-induced aggregation of acidic but not neutral vesicles at low calcium concentrations

Sample and reference cuvettes were filled with 70 μg/ml of either DPPC or DPPC/DPPG (7:3, w/w) vesicles in 5 mM Tris/HCl buffer, pH 7.4, containing 150 mM NaCl (●), or 4 mM NaCl (○), or 150 mM NaCl and 1 mM EDTA (broken line). After 10 min equilibration at 37 °C, pig SP-A (final concn. 6 μg/ml) was added to the sample cuvette. Next, Ca^{2+} (final concn. 3 mM) was added to both the sample and the reference cuvette. The process was reversed by addition of 6 mM EDTA to both cuvettes. A representative experiment of four experiments is shown. Four different preparations of pig SP-A were used.

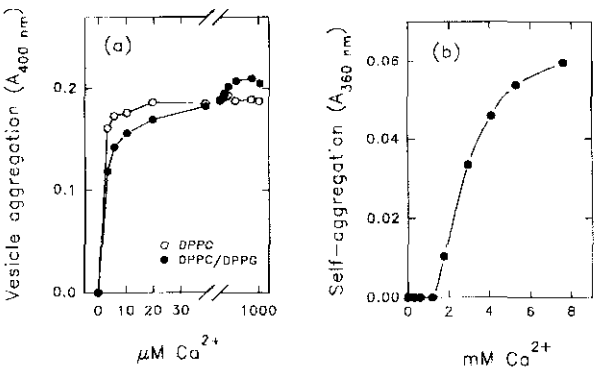

Figure 2 Ca^{2+} dependence of vesicle aggregation and self-aggregation

Experiments were done at 37 °C as described in the Experimental section. (**a**) Final concentrations of pig SP-A and phospholipids were 15 μg/ml and 150 μg/ml respectively. (**b**) Final concentration of SP-A was 44 μg/ml. A representative experiment of four experiments from four different preparations of pig SP-A is shown.

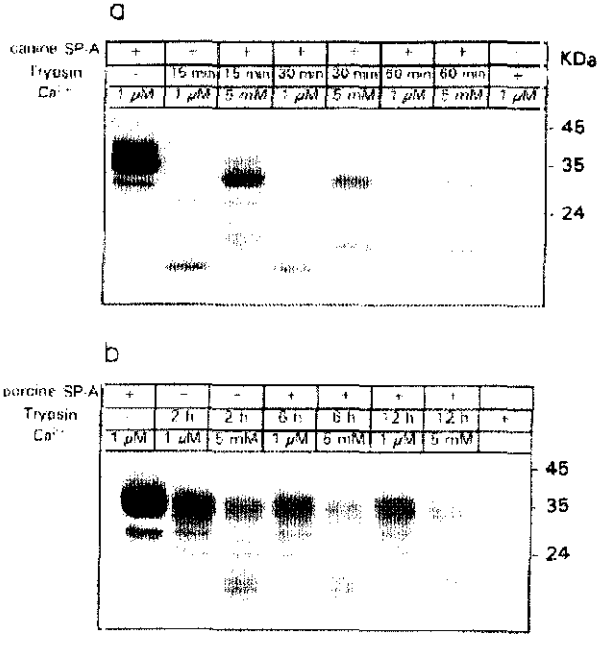

Figure 3 Trypsin sensitivity of dog (a) and pig (b) lipid-free SP-A in their non-aggregated and aggregated forms

Proteolytic digestion of SP-A was done at the indicated times as described in the Experimental section. Molecular-mass markers in kDa are indicated to the right of the gel.

However, ionic strength has no influence on the aggregation of neutral vesicles of DPPC. These results further confirm previous studies [11] which showed that the binding of negatively charged vesicles to SP-A was abrogated at low ionic strength. However, the interaction of neutral vesicles with SP-A was not dependent on the ionic strength. The lack of interaction of acidic vesicles with SP-A at low ionic strength and Ca^{2+} concentration and the subsequent abolition of vesicle aggregation is interpreted to arise from electrostatic repulsion between the negative charge of the phospholipids and the negative surface charge on the protein.

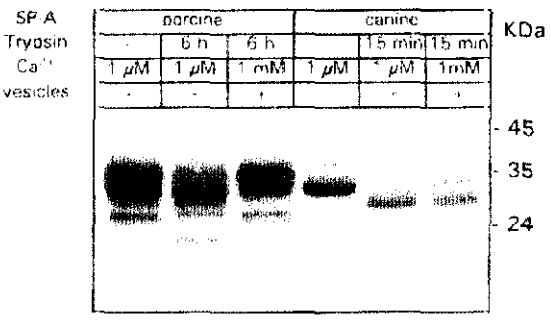

Figure 4 Trypsin sensitivity of membrane-bound SP-A

Dog or pig SP-A was first incubated with DPPC/DPPG (7:3, w/w) vesicles at 37 °C for 20 min in the presence of Ca^{2+} (1 μM or 1 mM). Then, the proteolytic digestion of either lipid bound SP-A or lipid/SP-A aggregates was done at the indicated times. Similar results were obtained with DPPC vesicles. Molecular-mass markers in kDa are indicated to the right of the gel.

The aggregation of vesicles is dependent on the lipid/protein ratio. Titration of SP-A with DPPC vesicles resulted in a hyperbolic plot which reached saturation at a lipid/protein weight ratio around 10:1. Titration of a preparation of DPPC vesicles with SP-A gave a similar saturating lipid/protein weight ratio (results not shown). The same results were obtained with negatively charged vesicles. According to these results, we used a lipid/SP-A weight ratio of 10:1 or higher in all experiments reported here.

Calcium dependence of the vesicle and self-aggregation processes

Figure 2(a) shows the calcium dependence of vesicle aggregation induced by pig SP-A. At very low concentrations of calcium, aggregation of neutral and acidic vesicles is observed, reaching a maximum level at 20 μM Ca^{2+}. The Ca^{2+} concentration required for half-maximal vesicle aggregation induced by pig SP-A ($k_a^{Ca^{2+}}$) is 0.76 ± 0.24 μM ($n = 4$) for DPPC, 0.74 ± 0.29 μM ($n = 4$) for DPPC/DPPG (7:3, w/w) and 0.74 μM ($n = 1$) for DPPC/PG (7:3, w/w). The Ca^{2+} activation constant ($k_a^{Ca^{2+}}$) for SP-A-dependent vesicle aggregation does not depend on the lipid composition of the vesicles. The extent of aggregation of DPPC/PG (7:3, w/w) or DPPC/phosphatidylinositol (7:3, w/w) vesicles, which might contain co-existing fluid and gel domains at 37 °C, was lower than that of DPPC or DPPC/DPPG (7:3, w/w) vesicles [22]. This suggests the influence of the physical state of the vesicles on the aggregation process, as previously reported elsewhere [11].

Figure 2(b) shows the calcium dependence of the self-aggregation process of pig SP-A. The Ca^{2+} concentration required for half-maximal self-association is 2.36 ± 0.15 mM ($n = 4$). This value is much higher than that for half-maximal vesicle aggregation given above. The threshold concentration of calcium required to induce self-association of pig SP-A is 0.5 mM, similar to that reported for other SP-A species (dog or human) [19]. On

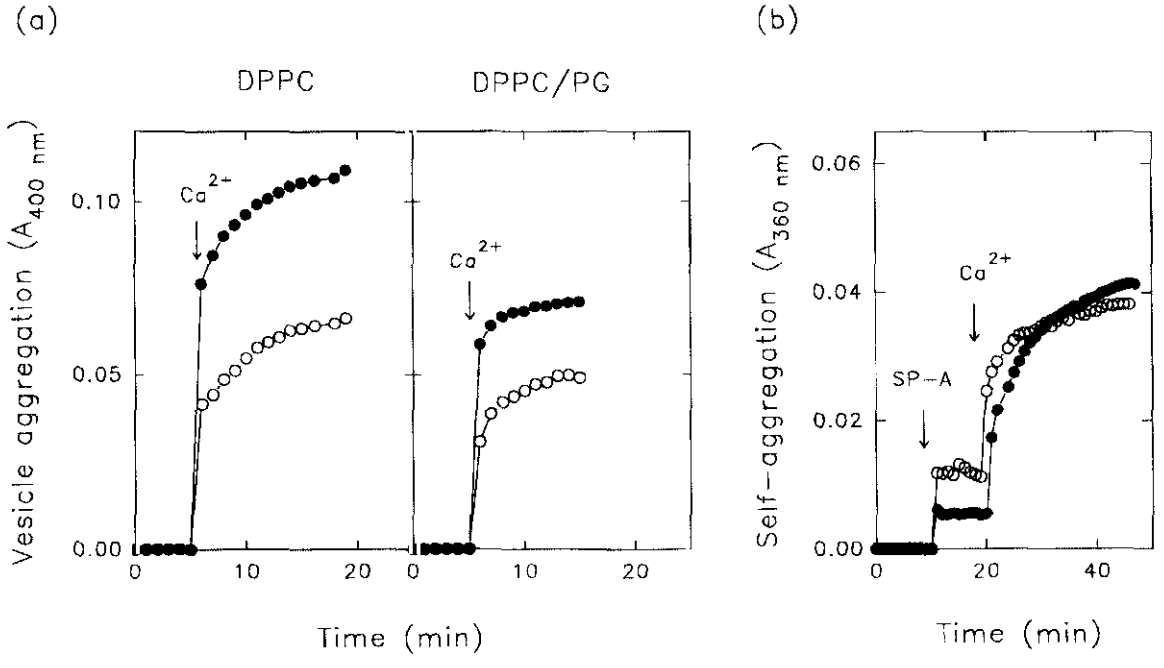

Figure 5 Effect of deglycosylation of pig SP-A on vesicle aggregation (a) and self-aggregation (b)

(**a**) Sample and reference cuvettes were filled with 70 μg/ml of either DPPC or DPPC/PG (7:3, w/w) vesicles in 5 mM Tris/HCl buffer, pH 7.4, containing 150 mM NaCl and 50 μM EGTA. After 10 min equilibration at 37 °C, 6 μg/ml SP-A was added to the sample cuvette. Next, vesicle aggregation was started by addition of Ca^{2+} (final concn. 1 mM) to both the sample and the reference cuvette. (**b**) Self-aggregation was done as described in the Experimental section. Final concentrations of pig SP-A and Ca^{2+} were 40 μg/ml and 5 mM respectively. The results shown are from a representative one of four experiments. Two different preparations of pig SP-A were used.

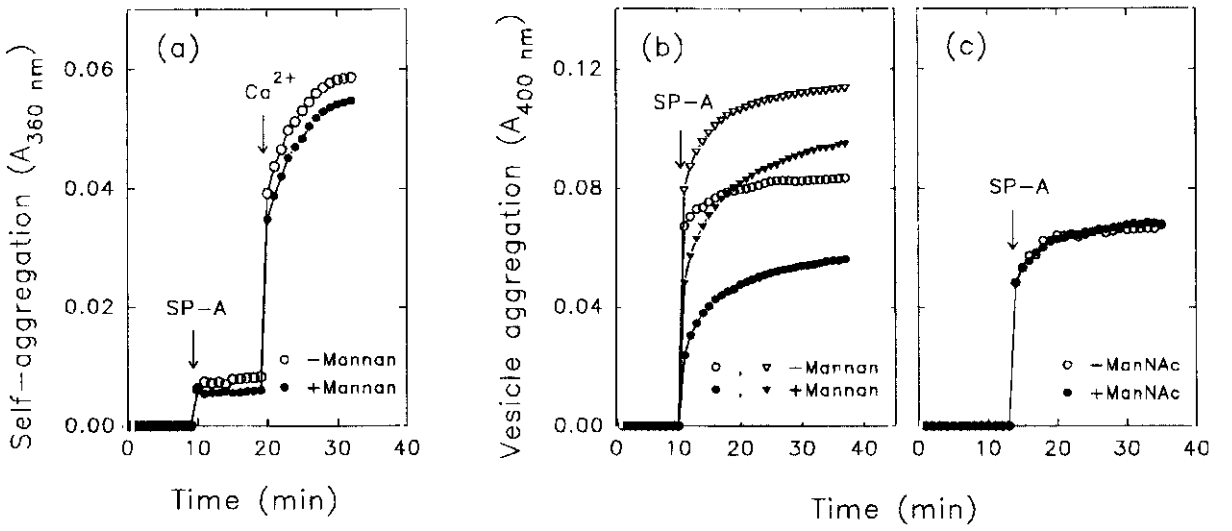

Figure 6 Effect of the presence of carbohydrates on self-association (a) and vesicle aggregation (b,c) at pH 7.4

Self association (**a**) was done as described in the Experimental section. Final concentrations of dog SP-A, Ca^{2+} and mannan were 50 μg/ml, 5 mM and 10 μg/μl respectively. For vesicle aggregation, sample and reference cuvettes were filled with either 80 μg/ml (triangles) or 40 μg/ml (circles) of DPPC/DPPG (7:3, w/w) vesicles in 5 mM Tris/HCl buffer, pH 7.4, 150 mM NaCl, 1 mM Ca^{2+}, with or without 10 μg/μl mannan (**b**) or 100 mM *N*-acetylmannosamine (**c**). After 10 min equilibration at 37 °C, vesicle aggregation was started by addition of 8 μg/ml dog SP-A. The phospholipid/protein ratio was 10:1 (triangles) or 5:1 (circles). Results presented are from a representative one of three experiments. Similar results were obtained with pig SP-A.

the other hand, the Ca^{2+} requirement for vesicle aggregation induced by SP-A is not fully clear. Ross et al. [20] reported that the threshold concentration of Ca^{2+} required for vesicle aggregation induced by dog SP-A was higher than 3 mM. That value is above the free Ca^{2+} concentration in the alveolar space of adult animals (approx. 1.5 mM) [31]. In contrast, Efrati et al. [18] found that the threshold concentration of Ca^{2+} needed for dog SP-A-induced vesicle aggregation was 0.5 mM. To determine whether the high difference between Ca^{2+} activation constants for vesicle and self-aggregation observed with pig SP-A occurred also in other SP-A species, we repeated the experiments with dog SP-A and DPPC/DPPG (7:3, w/w) vesicles. The Ca^{2+} concentration required for half-maximal vesicle aggregation induced by dog SP-A was $98 + 5$ μM ($n = 2$), whereas that required for half-maximal self-association was $0.70 + 0.06$ mM ($n = 2$). Thus independent of SP-A species, these two processes have very different Ca^{2+} requirements, showing that lipid aggregation activity of SP-A cannot be mediated by self-aggregation of the protein induced by supramillimolar concentrations of Ca^{2+}.

Trypsin sensitivity of SP-A in lipid/protein aggregates or in its lipid-free self-associated form

Figure 3 shows the tryptic digestion patterns of dog (Figure 3a) and pig (Figure 3b) lipid-free SP-A, in the presence of 1 μM or 5 mM Ca^{2+}, at the indicated times. Dog SP-A is very sensitive to proteolysis in its non-aggregated form (at 1 μM Ca^{2+}) being almost completely digested by 30 min, while pig SP-A is very resistant, even after 12 h. Interestingly, the effect of protein aggregation on the susceptibility to proteolysis of dog and pig SP-A in their self-associated forms (reached at 5 mM Ca^{2+}) is opposite. After aggregation (at 5 mM Ca^{2+}), pig SP-A becomes more sensitive to degradation than its non-aggregated form, whereas dog SP-A is less susceptible. These results suggest that aggregation seems to modify protein conformation since the accessibility of trypsin cleavage targets of SP-A changes, being more or less exposed depending on SP-A species.

Membrane-bound SP-A from either pig or dog is clearly protected from trypsin degradation at both low (1 μM) or high (1 mM) concentrations of Ca^{2+} (Figure 4). The protection is independent of SP-A species and is slightly higher when lipid/SP-A aggregates are completely formed at 1 mM Ca^{2+}.

Involvement of the carbohydrate moiety and the carbohydrate-binding domain of SP-A in vesicle aggregation and self-aggregation

To investigate whether the oligosaccharide chains of SP-A are required for vesicle aggregation and self-aggregation processes, deglycosylation of the protein was carried out using N-glycosidase F. After deglycosylation treatment, no glycosylated SP-A could be detected by Coomassie Blue staining. Deglycosylation of pig SP-A decreases aggregation of neutral and acidic vesicles by 30–40 % (Figure 5a). However, self-aggregation of the protein is not influenced by removing asparagine-linked carbohydrate (Figure 5b). Interestingly, although the extent of aggregation decreases with deglycosylated pig SP-A, the Ca^{2+} concentration required for half-maximal vesicle aggregation (K_a^{Ca2+}) is similar for the two proteins, control and deglycosylated.

To find out if the binding of mannan to the carbohydrate-binding domain of dog or pig SP-A has any influence on vesicle aggregation and self-aggregation phenomena, we studied the effect of the presence of this homopolysaccharide on these two processes. The binding of SP-A to mannan was shown to occur at pH values higher than 4.5 and at Ca^{2+} concentrations higher than 10 μM [32]. Figure 6(a) shows that the presence of mannan (at a concentration of 10 μg/μl) does not influence self-association of the protein, but decreases vesicle aggregation induced by dog or pig SP-A at pH 7.4 (Figure 6b). Lower concentrations of mannan do not influence lipid aggregation. The inhibitory

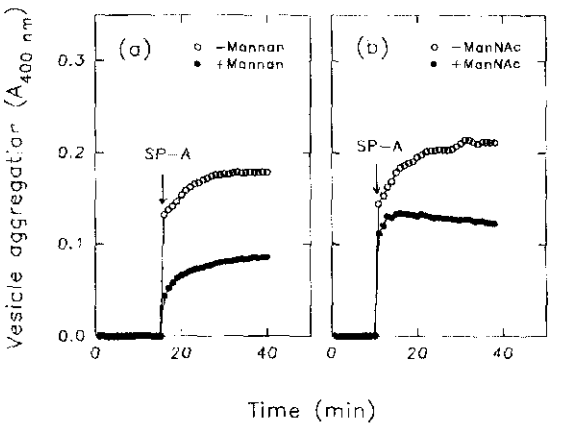

Figure 7 Effect of mannan (a) or *N*-acetylmannosamine (b) on vesicle aggregation at pH 4.0

Sample and reference cuvettes were filled with 80 μg/ml of DPPC/DPPG (7:3, w/w) vesicles in 150 mM NaCl, 1 mM Ca^{2+}, 25 mM acetate buffer, pH 4.0 with or without 10 μg/μl mannan (**a**) or 100 mM *N*-acetylmannosamine (**b**). After 10 min equilibration at 37 °C, vesicle aggregation was started by addition of dog SP-A (8 μg/ml). The results shown are from a representative one of three experiments. Similar plots were obtained with pig SP-A.

effect of mannan increases when phospholipid/protein and phospholipid/mannan weight ratios were decreased (Figure 6b). The addition of excess mannan to lipid/protein aggregates previously formed does not dissociate those aggregates (results not shown). On the other hand, the presence of an excess of *N*-acetylmannosamine (100 mM), which binds to the carbohydrate-binding domain of SP-A very efficiently [32], does not influence vesicle aggregation induced by dog or pig SP-A at pH 7.4 (Figure 6c). Higher concentrations of that monosaccharide (200 mM) are needed to cause a 20% decrease of vesicle aggregation at pH 7.4.

The possibility that the inhibitory effect of mannan on vesicle aggregation is due to the interaction of the polysaccharide with the membrane cannot be excluded. Therefore, we studied the effect of the presence of mannan and *N*-acetylmannosamine on vesicle aggregation at pH 4.0. At this pH, mannan does not bind to either SP-A or other collectins such as mannan-binding protein (MBP) or conglutinin [32]. Figure 7 shows that the presence of mannan (10 μg/μl) or *N*-acetylmannosamine (100 mM) decreases vesicle aggregation induced by dog or pig SP-A at pH 4.0. The inhibitory effect of mannan is higher at pH 4.0 than at pH 7.4. From these data we conclude that the effect of sugars on vesicle aggregation is independent of the binding of carbohydrates to the carbohydrate recognition domain (CRD) of SP-A. The effect of mannan on vesicle aggregation might be due to the interaction of carbohydrates with lipid vesicles that could lead to alterations in the physical properties of membranes [33]. Goodrich et al. [34] reported that the intercalation of carbohydrates into the interfacial region of membranes resulted in a lowering of the phase-transition temperature via expansion of the lattice and modification of the whole lipid interfacial region. We previously showed [11] that the interaction of SP-A with phospholipid vesicles requires the lipids to be in the gel phase and that SP-A-induced vesicle aggregation was strongly dependent on the physical state of the vesicles. Therefore, it is conceivable that the process of lipid aggregation would be influenced by the presence of excess mannan if the interaction between carbohydrates and phospholipid vesicles resulted in a lowering of the gel-to-liquid-crystalline phase-transition temperature. Alternatively, the intercalation of carbohydrates into the interfacial region of membranes could impede the proximity between vesicles and consequently the aggregation between them.

DISCUSSION

The ability of SP-A to aggregate phospholipid vesicles has been widely study [11,13,18–22]. It was recently reported that the CRD domain of SP-A was directly implicated in that process [25,35,36]. However, the mechanism involved in the vesicle aggregation phenomenon is poorly understood. It has been suggested that vesicle aggregation could be mediated by Ca^{2+}-dependent self-association of SP-A [19]. The results presented here prove that the process of lipid aggregation induced by SP-A cannot be correlated with that of self-association of the protein. These two processes have very different requirements of Ca^{2+}. The Ca^{2+} activation constant ($K_a^{Ca^{2+}}$) for SP-A-dependent vesicle aggregation was 0.74 ± 0.29 μM for pig SP-A and 98 ± 5 μM for dog SP-A. In contrast, $K_a^{Ca^{2+}}$ for self-aggregation of SP-A was $2.36 + 0.15$ mM and $0.70 + 0.06$ mM for pig and dog SP-A respectively. In addition, deglycosylation of the protein caused a 30–40% decrease in vesicle aggregation without influencing self-association of SP-A. Reduction of the lipid aggregation activity of deglycosylated SP-A could be due to structural modifications in the C-terminal region of SP-A caused by removing the carbohydrate moiety. It was recently shown that the C-terminal region of SP-A is critical for lipid vesicle aggregation [25,35,36]. Nothing is known about the structural domain of SP-A directly involved in the process of self-association.

On the other hand, the binding of phospholipids to either dog or pig SP-A at very low concentrations of Ca^{2+} (1 μM) caused a marked protection of SP-A from trypsin degradation. At this Ca^{2+} concentration lipid aggregation did not occur (dog SP-A) or is half-maximal (pig SP-A). The protection was only slightly higher at 1 mM Ca^{2+} when the extent of lipid/SP-A aggregates was maximal. The binding of phospholipids to SP-A would lead to a reduced accessibility of the trypsin cleavage targets located in the domains in which phospholipids are bound. Both the hydrophobic region of SP-A (neck domain) and the region of the small disulphide loop in the CRD have been proved to be involved in the lipid binding properties of SP-A [35,36]. We have previously shown [11] that the interaction of phospholipids with either pig or human SP-A caused a conformational change in the protein molecule affecting tryptophan residues of SP-A, which are located in the C-terminal 38 amino acids (at positions 191 and 213) in all species studied until now. Therefore, it is conceivable that this conformational change caused by phospholipid binding, and the subsequent aggregation at higher concentrations of Ca^{2+}, led to a reduced exposure of trypsin cleavage targets located not only in the region in which phospholipids are bound but also in closed areas of the CRD. In contrast, the effect of self-association on the trypsin sensitivity of SP-A was variable depending on the SP-A species. Thus, the self-aggregated form of pig SP-A was much more sensitive to trypsin degradation than its non-aggregated form, whereas dog SP-A was less susceptible to proteolysis after self-aggregation.

Taken together, all these results show that the process of lipid aggregation is not dependent on the self-association of the protein which occurs at supramillimolar concentrations of Ca^{2+}. The question that remains is what is the mechanism involved in the vesicle aggregation phenomenon? Haagsman et al. [23] have proposed that vesicle aggregation could be mediated by Ca^{2+}-induced interactions between carbohydrate-binding domains and the oligosaccharide moieties of SP-A. This hypothesis could be

supported by two facts: (1) the low Ca^{2+} requirement for lectin activity [32], which is similar to that for vesicle aggregation; and (2) the critical requirement of the CRD for vesicle aggregation [25,35,36]. We found, however, that the carbohydrate moiety of SP-A was not critical for vesicle aggregation, in agreement with previous studies [24,37]. Furthermore, lipid/SP-A aggregates could not be dissociated by addition of excess mannan. The presence of that homopolysaccharide decreased vesicle aggregation by a mechanism that is independent of the binding of mannan to the carbohydrate-binding domain of SP-A. Reduction of vesicle aggregation occurred at both pH 7.4 and pH 4.0. However, at acidic pH the binding of mannan to SP-A or to other C-type lectins is abrogated [32]. These results exclude the involvement of interactions between oligosaccharide moieties and carbohydrate-binding sites of SP-A in vesicle aggregation.

Recent studies [25] suggested that the binding of SP-A to phospholipid vesicles and the subsequent aggregation event were separate processes, because residues Glu^{195} and Arg^{197} of the CRD of rat SP-A were critical determinants for vesicle aggregation but not for phospholipid binding. Therefore, it is possible that in the presence of Ca^{2+} and after lipid binding, additional protein-structure-related changes could occur that lead to aggregation. A possible model to explain that process would be the following: the binding of phospholipids to SP-A could induce a specific Ca^{2+}-dependent conformational change in the protein molecule which could trigger protein–protein interactions between the CRDs of SP-A. Therefore, SP-A/liposome complexes could aggregate by a mechanism of SP-A self-association that must be necessarily different from that which occurs without lipids and is triggered by supramillimolar concentrations of calcium.

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Effect of Acidic pH on the Structure and Lipid Binding Properties of Porcine Surfactant Protein A

POTENTIAL ROLE OF ACIDIFICATION ALONG ITS EXOCYTIC PATHWAY*

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Pulmonary surfactant protein A (SP-A) is synthesized by type II cells and stored intracellularly in secretory granules (lamellar bodies) together with surfactant lipids and hydrophobic surfactant proteins B and C (SP-B and SP-C). We asked whether the progressive decrease in pH along the exocytic pathway could influence the secondary structure and lipid binding and aggregation properties of porcine SP-A. Conformational analysis from CD spectra of SP-A at various pH values indicated that the percentage of α-helix progressively decreased and that of β-sheet increased as the pH was reduced. The protein underwent a marked self-aggregation at mildly acidic pH in the presence of Ca^{2+}, conditions thought to resemble those existing in the trans-Golgi network. Protein aggregation was greater as the pH was reduced. We also found that both neutral and acidic vesicles either with or without SP-B or SP-C bound to SP-A at acidic pH as demonstrated by co-migration during centrifugation. However, the binding of acidic but not neutral vesicles to SP-A led to 1) a striking change in the CD spectra of the protein, which was interpreted as a decrease of the level of SP-A self-aggregation, and 2) a protection of the protein from endoproteinase Glu-C degradation at pH 4.5. SP-A massively aggregated acidic vesicles but poorly aggregated neutral vesicles at acidic pH. Aggregation of dipalmitoylphosphatidylcholine (DPPC) vesicles either with or without SP-B and/or SP-C strongly depended on pH, being progressively decreased as the pH was reduced and markedly increased when pH was shifted back to 7.0. At the pH of lamellar bodies, SP-A-induced aggregation of DPPC vesicles containing SP-B or a mixture of SP-B and SP-C was very low, although SP-A bound to these vesicles. These results indicate that 1) DPPC binding and DPPC aggregation are different phenomena that probably have different SP-A structural requirements and 2) aggregation of membranes induced by SP-A at acidic pH is critically dependent on the presence of acidic phospholipids, which affect protein structure, probably preventing the formation of large aggregates of protein.

Pulmonary surfactant is a mixture of approximately 80% phospholipids, 10% other lipids, and 5–10% surfactant-specific proteins that lines the alveolar space and is essential for breathing (for reviews, see Refs. 1–3). The alveolar type II cell is the sole cell type in the lung that produces all components of pulmonary surfactant. The surfactant apolipoproteins (SP-A,[1] SP-B, and SP-C) and all of the surfactant phospholipids are stored intracellularly in lamellar bodies and are secreted as a complex (4–7). The release of surfactant to the alveolar lumen occurs by exocytosis of lamellar body content in response to secretagogue stimulation (8, 9). The regulated secretory pathway of the type II cell is atypical because the lamellar body not only functions as a classic secretory granule, but it also intersects with the endocytic pathway (10, 11). Like the storage granules in other secretory cells, lamellar bodies have an acidic internal environment (pH 5.5) (12) and high calcium content, bringing their intravesicular free Ca^{2+} concentration to a 2-10 mM range (13). It is well recognized that during the process of secretory granule formation, proteins of the granule content are segregated from proteins that are released from the cell by the constitutive secretory pathway. Morphological studies indicate that sorting of nascent secretory proteins into the regulated *versus* constitutive pathway occurs in the trans-Golgi network (TGN) (14) and in immature secretory granules (15). Biochemical studies indicate that selective aggregation of proteins plays an important role in sorting of proteins destined for secretory granules (16–18). This protein aggregation is triggered as proteins encounter mildly acidic pH and high calcium in the TGN lumen. Constitutively secreted proteins do not aggregate under these ionic and pH conditions and are excluded from such aggregates (17, 18).

SP-A is the major protein in the alveolar compartment (for reviews, see Refs. 2, 3, and 19). It is a multifunctional protein capable of binding several ligands including phospholipids, carbohydrates, and Ca^{2+}, and it belongs to the Ca^{2+}-dependent lectin family. SP-A is a large oligomeric protein of approximately 650 kDa, composed of 18 nearly identical subunits. Each SP-A subunit contains an amino-terminal collagen-like domain and a carboxyl-terminal carbohydrate recognition domain that are linked by a more hydrophobic domain (neck). Nearly all alveolar SP-A is complexed with phospholipids. The carbohydrate recognition domain and the neck region are important protein domains involved in SP-A/lipid interactions (20–22). The structural properties of SP-A and its interaction with phospholipids have been studied to date mainly at neutral pH. At this pH, SP-A preferentially interacts with dipalmitoylphosphatidylcholine (DPPC) (23, 24), the main surfactant

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[1] The abbreviations used are: SP-A, -B, and -C, surfactant protein A, B, and C, respectively; TGN, trans-Golgi network; DPPC, dipalmitoylphosphatidylcholine; DPPG, 1,2-dipalmitoyl-*sn*-glycero-3-phosphoglycerol; PAGE, polyacrylamide gel electrophoresis; PC, 1,2-diacyl-*sn*-glycero-3-phosphocholine; PG, 1,2-diacyl-*sn*-glycero-3-phosphoglycerol; P_{II}, poly(L-proline II)-type.

lipid (1). The interaction of DPPC with SP-A leads to a conformational change on the protein molecule (24) and a marked protection of SP-A from trypsin degradation (25). The interaction of SP-A with phospholipid vesicles induces vesicle aggregation in the presence of Ca^{2+} (25–27). SP-A also induces aggregation of liposomes containing SP-B or SP-C or both hydrophobic proteins (27, 28). The association of SP-A with lipids appears essential for the conversion of lipid aggregates from multilamellar forms present in the exocytic granule to dispersed ordered arrays known as tubular myelin (29). This physical transformation also requires the hydrophobic surfactant protein SP-B (30). SP-A enhances adsorption of lipids along the air/liquid interface in a concerted action with SP-B (31). In addition, SP-A is involved in surfactant homeostasis (2, 3, 19) and participates in lung-specific host defense (32). With respect to SP-A biosynthetic routing in alveolar type II cells, the protein is synthesized in the endoplasmic reticulum and transported together with the precursors of hydrophobic surfactant proteins SP-B and SP-C through the same pathway from Golgi complex to lamellar bodies (7). Its correct routing and secretion is independent of glycosylation (6, 33). SP-A is a protein rich in negatively charged amino acids with isoelectric points varying between pH 4.5 and 5.2 (3, 27). The question that we address here is whether the progressive decrease in pH along the exocytic pathway could influence the structure and the lipid binding and aggregation properties of SP-A.

The present study analyzes the structural properties of SP-A and its interaction with phospholipids at acidic pH in the presence and absence of the hydrophobic surfactant proteins SP-B and SP-C. We found that the secondary structure of SP-A was changed by lowering the pH and that the protein underwent a rapid aggregation at mildly acidic pH in the presence of Ca^{2+}, conditions thought to resemble those existing in the TGN and along the exocytic pathway. The binding of acidic but not neutral vesicles to SP-A, at acidic pH, affected the secondary structure of the protein. Vesicle aggregation induced by SP-A at acidic pH was critically dependent on the presence of negatively charged phospholipids in the composition of the vesicle.

EXPERIMENTAL PROCEDURES

Isolation of SP-A, SP-B, and SP-C—Pulmonary surfactant was prepared from pig bronchoalveolar lavage as described previously (34). SP-A was purified from isolated surfactant using sequential butanol and octyl glucoside extractions (31). SP-B and SP-C were isolated directly from pig lungs by minor modifications of the method of Curstedt *et al.* (35) described elsewhere (36). The purity of SP-A, SP-B, and SP-C was checked by one-dimensional SDS-PAGE (12 and 16% acrylamide for SP-A and for SP-B and SP-C, respectively) under reducing conditions (50 mM dithiothreitol). Quantification of the proteins was carried out by amino acid analysis in a Beckman System 6300 high performance analyzer. The protein hydrolysis was performed with 0.2 ml of 6 M HCl, containing 0.1% (w/v) phenol in evacuated and sealed tubes at 108 °C for 24 h. Norleucine was added to each sample as an internal standard. In the course of this work, eight different preparations of porcine SP-A and four preparations of porcine SP-B and SP-C were used. Some of the experiments were repeated with canine SP-A with identical results.

CD Measurements—CD spectra were obtained on a Jasco J-715 spectropolarimeter fitted with a 150-watt xenon lamp. Quartz cells of 1-mm path length were used, and the spectra were recorded in the far-uv region (190–260 nm) at 50 nm/min scanning speed and at room temperature. Five scans were accumulated and averaged for each spectrum. The acquired spectra were corrected by subtracting the appropriate blank runs (of water or phospholipid vesicle solutions), subjected to noise reduction analysis and presented as molar ellipticities (degrees·cm^2·dmol^{-1}), assuming 110 Da as the average molecular mass per amino acid residue. At least three independent preparations of SP-A were measured. Final SP-A concentration was 80–100 μg/ml. Measurements at acidic or neutral pH were done in 5 mM acetate, 5 mM Tris/HCl buffer, pH 4.5 or 7.2, respectively. pH titration was started at neutral pH in 5 mM Tris, 5 mM MES, 5 mM acetate buffer, pH 7.2, in the presence or absence of 5 mM $CaCl_2$. From the starting pH, SP-A was titrated to pH 4.2 by the addition of 1–2-μl aliquots of 0.05 or 0.1 M HCl solution. During titration, the medium pH was monitored with a micro-pH electrode. The spectrum of SP-A at each pH was recorded 10 min after each change of pH.

SP-A Self-aggregation Assays—Self-aggregation of SP-A induced by H^+ was studied at 37 °C by measuring the change in absorbance at 360 nm in a Beckman DU-640 spectrophotometer. Both sample and reference cuvettes were first filled with 5 mM acetate, 5 mM Tris/HCl buffer, pH 4.5. After a 10-min equilibration at 37 °C, SP-A (20 μg/ml, final concentration) was added to the sample cuvette, and the turbidity change at 360 nm was monitored at 1-min intervals over 10 min. Self-aggregation of SP-A was reversed by shifting back the pH to 7.2. pH-induced protein aggregation was also determined by centrifugation at 12,000 × *g* in a Hettich microliter centrifuge for 15 min. The entire pellet fractions and supernatants were then subjected to SDS-PAGE under reducing conditions followed by staining of the gels with Coomassie Blue. The variation of SP-A aggregation as the pH was reduced was studied at 37 °C in the presence or the absence of 5 mM Ca^{2+}. Samples containing SP-A (5.5 μg) in 350 μl of 5 mM Tris/HCl, 5 mM MES, 5 mM acetate buffer, pH 7.2, were titrated to the indicated pH values by adding 1–2-μl aliquots of 0.05 or 0.1 M HCl while vortexing. During titration, the medium pH was monitored. The absorbance at 360 nm was registered once it was stabilized after each change of pH.

Preparation of Lipid Vesicles and Liposomes Containing SP-B and/or SP-C—Synthetic phospholipids, DPPC, and 1,2-dipalmitoyl-*sn*-glycero-3-phosphoglycerol (DPPG) were purchased from Avanti Polar Lipids (Birmingham, AL); egg 1,2-diacyl-*sn*-glycero-3-phosphocholine (PC) and 1,2-diacyl-*sn*-glycero-3-phosphoglycerol (PG) were from Sigma, and their homogeneity was routinely tested on thin layer chromatography. The organic solvents (methanol and chloroform) used to dissolve lipids and to isolate and store hydrophobic surfactant proteins were high pressure liquid chromatography grade (Scharlau, Barcelona, Spain).

Throughout all experiments, unilamellar vesicles of DPPC, DPPG, or DPPC/other (7:3, w/w) were used. The different lipid vesicles were prepared at a phospholipid concentration of 1 or 3 mg/ml by hydrating dry lipid films in a buffer containing 150 mM NaCl, 0.1 mM EDTA, 25 mM Tris/HCl, 25 mM acetate, pH 7.2 or 4.5, and allowing them to swell for 1 h at a temperature above the phase transition temperature of the corresponding phospholipid. Next, the lipid dispersion (1 ml) was sonicated at the same temperature (typically above 45 °C) during 2 min at 390 watts/cm^2 (bursts of 0.6 s, 0.4 s between bursts) in a UP 200S sonifier with a 2-mm microtip. All vesicles were prepared freshly each day, just before starting the experiment. The phospholipid concentration was assessed by phosphorus determination according to Rouser *et al.* (37). For vesicle-size analysis in solution, quasielastic light scattering was used as described previously (24). Vesicle diameter at 37 °C was 135 nm for DPPC vesicles and around 60 nm for DPPC/DPPG (7:3, w/w) vesicles.

Reconstitution of SP-B or SP-C or both hydrophobic proteins in DPPC or DPPC/DPPG (7:3, w/w) vesicles was performed as described previously (38) at a protein:lipid ratio of either 1:10 or 1:20. Briefly, appropriate amounts of phospholipids dissolved in chloroform/methanol (2/1, v/v) were mixed with the desired amount of SP-B and/or SP-C stored in chloroform/methanol (2/1, v/v). Dry protein/lipid samples were hydrated in the appropriate buffer at 65 °C for 1 h with occasional mixing. Sonication was done at the same temperature under conditions described above.

Phospholipid Binding—The binding of SP-A to phospholipid vesicles at acidic pH was determined by coflotation of protein and lipids after discontinuous sucrose density gradient centrifugation. SP-A (25 μg/ml final concentration) was added to 150 mM NaCl, 25 mM acetate buffer, pH 4.5 with or without 377 μg/ml phospholipid vesicles. The mixture was incubated during 15 min at room temperature and then carefully placed in a centrifuge tube over a 1-ml cushion of 30% sucrose in the same buffer. Centrifugation was conducted at 25 °C in a Beckman SW-65 rotor at 129,000 × *g* for 2 h. The supernatants were then transferred to concentrating tubes (Millipore Microcon 100$^{(R)}$) and centrifugated in order to eliminate salts. The presence of SP-A in pellet and supernatant fractions was determined by one-dimensional SDS-PAGE, under reducing conditions. Phospholipid content in both fractions was assessed by phosphorus analysis (37). Control experiments where liposomes or SP-A were deleted from the incubation were performed. After centrifugation, more than 97% of phospholipids were recovered in the supernatant, and all SP-A sedimented in the absence of phospholipid vesicles.

Phospholipid Vesicle Aggregation Assay—The assay was performed

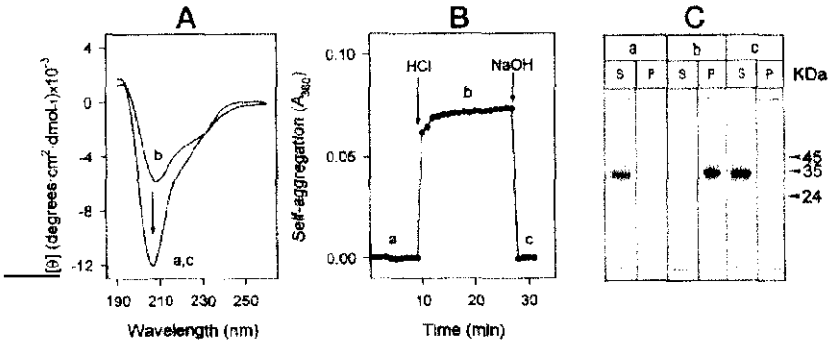

FIG. 1. **Reversible pH-dependent change of secondary structure and self-aggregation of SP-A.** *A*, Far-uv circular dichroism spectra of SP-A. *a*, spectra of SP-A (0.08 mg/ml) in 5 mM acetate, 5 mM Tris/HCl buffer, pH 7.2; *b*, spectra of the protein in the same buffer but at pH 4.5; *c*, spectra of SP-A after adding NaOH to change pH from 4.5 to 7.2. *B*, H^+-induced self-aggregation of SP-A. In this experiment, sample and reference cuvettes were first filled with 20 μg/ml SP-A in 5 mM acetate, 5 mM Tris/HCl buffer, pH 7.2 (*a*). After a 10-min equilibration at 37 °C, HCl was added to the sample cuvette to shift the pH from 7.2 to 4.5. The turbidity change at 360 nm was monitored at 1-min intervals over 20 min (*b*). When the pH was shifted back to 7.2 by the addition of NaOH, SP-A aggregates completely dissociated (*c*). *C*, SP-A is pelleted by centrifugation at acidic but not at neutral pH. SP-A concentration in the assays was 8 μg/ml. *a*, in 5 mM acetate, 5 mM Tris/HCl buffer, pH 7.2; *b*, in the same buffer after changing pH to 4.5; *c*, after the addition of NaOH to change back pH to 7.2. After centrifugation of these samples, the pellets (*P*) and the supernatants (*S*) were subjected to SDS-PAGE, and the gel was stained with Coomassie Blue. Molecular mass markers in kDa are indicated to the *right* of the gel. In *A*, *B*, and *C*, a representative one of four experiments from four different porcine SP-A preparations is shown.

at acidic or neutral pH by measuring the absorbance at 400 nm in a Beckman *DU-640* spectrophotometer at 37 °C as described previously (25). Briefly, phospholipid vesicles (40 μg) with or without hydrophobic proteins were added to both the sample and the reference cuvette in a total volume of 0.5 ml of 150 mM NaCl, 0.1 mM EDTA, 25 mM Tris, 25 mM acetate buffer, pH 7.2 or 4.5. After a 10-min equilibration at 37 °C, 4 μg of SP-A were added to the sample cuvette, and the change in optical density at 400 nm was monitored at 1-min intervals. Next, Ca^{2+} (1 mM or 50 μM, final concentration) was added to both the sample and the reference cuvette, and the change in absorbance was monitored again.

Protease Digestion of SP-A at Acidic pH—To determine the protease sensitivity of lipid-free and membrane-bound SP-A, the endoproteinase Glu-C from *Staphylococcus aureus V8* (Boehringer Mannheim GmbH, Germany) was used. This serine protease hydrolyzes peptide and ester bonds specifically at the carboxylic side of Glu at pH 4.0 or both Glu and Asp at pH 8.0–8.5. SP-A (6.7 μg) was added to 150 mM NaCl, 25 mM acetate buffer, pH 4.5, either in the presence or absence of 60 μg of phospholipid vesicles prepared in the same buffer. After 10 min of incubation at 37 °C, 13.4 μg of freshly prepared enzyme solution (2:1 enzyme/protein, w/w) were added. The final incubation volume was 110 μl. The mixture was incubated during 4 h at 37 °C, and the digestion was stopped by freezing the samples at −20 °C. Samples were analyzed by SDS-PAGE under reducing conditions followed by Western blot analysis of SP-A as described previously (39) because the electrophoretic band of V8 protease (30–35 kDa) overlapped with that of SP-A (28–36 kDa). The primary antibody was kindly supplied by Dr. J. A. Whitsett (University of Cincinnati).

RESULTS

Reversible pH-dependent Change of the Secondary Structure of SP-A and Aggregation of the Protein—CD spectra of SP-A at neutral and acidic pH are shown in Fig. 1*A*. At neutral pH, spectra were characterized by a shoulder at 220 nm and a strong negative extreme at 207 nm as previously reported (24, 40). The change of pH from 7.2 to 4.5 led to a change in the shape of SP-A spectrum and markedly reduced the contribution of the 207-nm minimum to the spectrum. This change in the CD spectra of SP-A was reversed when the pH was changed back to 7.2. On the other hand, pH-dependent aggregation of SP-A was studied by measuring H^+-induced turbidity changes (Fig. 1*B*), and the level of aggregation was determined by centrifugation (Fig. 1*C*). SP-A rapidly aggregated at pH 4.5, and all of the protein was recovered in the pellet. When the pH was changed back to 7.2, the turbidity at 360 nm vanished and all of SP-A was recovered in the supernatant fraction, indicating that pH-dependent self-aggregation of SP-A was reversible. The CD spectrum of SP-A at pH 4.5 corresponded to the H^+-induced aggregated form of the protein. To determine whether protein aggregation might affect CD measurements, CD spectra of SP-A at pH 4.5 were recorded at different protein concentrations. No significant differences were observed in the range of 40–140 μg of SP-A/ml tested.

The estimation of secondary structure fractions of porcine SP-A at neutral and acidic pH was performed by the SELCON program (the self-consistent method) recently modified by Sreerama and Woody (41). This program determines the contribution of α-helix, β-structure, β-turn, and poly(L-proline II)-type (P_{II}) conformation to the spectra of proteins. The P_{II} conformation is a left-handed extended helix with three residues per turn and is favored in proline-rich polypeptides due to the limited conformational flexibility of the proline ring (41). The P_{II} conformation was found in collagen and in short segments of some globular proteins. According to the SELCON program, porcine SP-A at neutral pH contained 19% α-helix, 26% β-sheet, 25% β-turn, 14% P_{II} conformation, and 17% unordered form. The self-aggregated SP-A at pH 4.5 contained 10% α-helix, 39% β-sheet, 22% β-turn, 12% P_{II} conformation, and 18% unordered form, indicating that at acidic pH the content of β-sheet conformation increased and that of α-helix decreased, whereas the content of P_{II} conformation did not change.

SP-A Aggregation Increases as the pH Is Reduced: This Effect Is Greater in the Presence of Ca^{2+}—Fig. 2*A* shows the effect of lowering pH on self-association of the protein in the presence and absence of 5 mM Ca^{2+}. SP-A underwent pH-dependent aggregation in the absence of Ca^{2+}. Protein aggregation started at pH below 6.0 and reached a maximum value at pH 4.5. The ionization p*K* calculated from the plot of the dependence of SP-A aggregation on the pH was 5.26 ± 0.05. In the presence of Ca^{2+}, a marked aggregation occurred in the pH range of TGN (6–6.5) and was greater as the pH was reduced. The ionization p*K* calculated in the presence of Ca^{2+} was 5.55 ± 0.15. At pH 4.5, protein aggregation was not influenced by Ca^{2+} as shown in Fig. 2*B*. At neutral pH, self-association of SP-A was absolutely dependent on 1-8 mM concentrations of Ca^{2+} (25, 42). The Ca^{2+} concentration needed for half-maximal self-aggregation of pig SP-A was 2.36 ± 0.15 mM (25).

The effect of lowering pH on the CD spectra of SP-A was analyzed in the absence (Fig. 3*A*) and the presence (Fig. 3*B*) of Ca^{2+}. Fig. 3*A* shows a progressive decrease of the negative ellipticity and a progressive shift of the negative maximum from 207 to 209 nm as the pH was reduced. Conformational analysis by the SELCON program indicated that the percent-

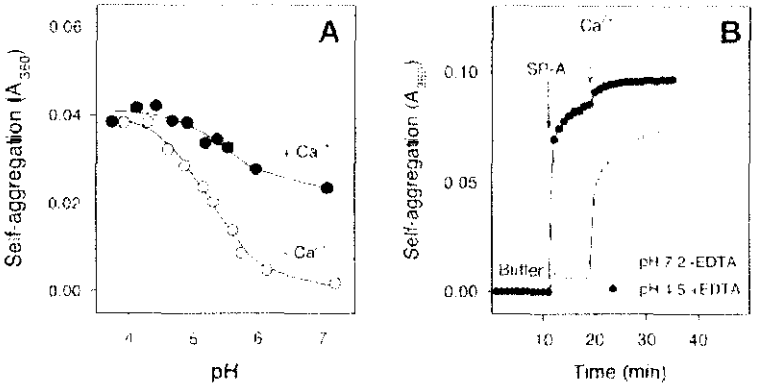

FIG. 2. **SP-A self-aggregation increases as the pH is reduced.** The experiments were done at 37 °C as described under "Experimental Procedures." *A*, pH titration started at pH 7.2 in 5 mM Tris/HCl, 5 mM MES, 5 mM acetate buffer either with or without 5 mM Ca^{2+}. A representative one of six experiments is shown. Three different preparations of porcine SP-A were used. *B*, comparison of the kinetics of self-association of SP-A at pH 4.5 and 7.2. A representative one of four experiments from two different porcine SP-A preparations is shown.

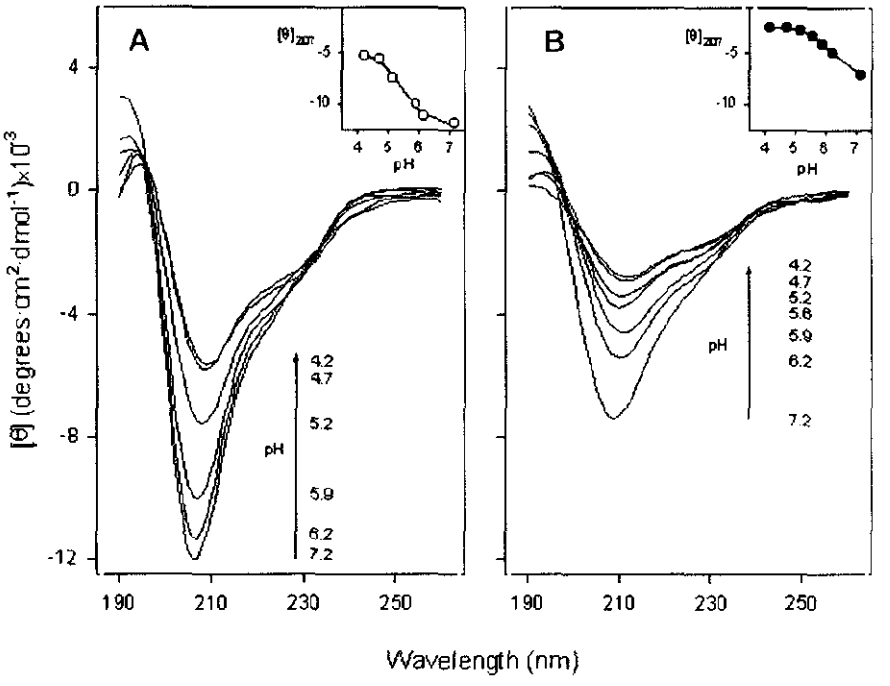

FIG. 3. **The secondary structure of SP-A changes as the pH is reduced.** pH titration experiments were performed as described under "Experimental Procedures" and started at pH 7.2 in 5 mM Tris/HCl, 5 mM MES, 5 mM acetate buffer in the absence (*A*) and the presence (*B*) of 5 mM Ca^{2+}. *Insets*, ellipticities at 207 nm of porcine SP-A as a function of pH. A representative one of three experiments from three different porcine SP-A preparations is shown.

age of α-helix progressively decreased (from 20 to 8%) and that of β-sheet progressively increased (from 26 to 41%) without significant variation in the content of P_{II} and unordered conformation. On the other hand, from data of the ellipticity at 207 nm *versus* pH (Fig. 3*A*, *inset*) we calculated a p*K* of 5.46 ± 0.1 for the H^+-induced change of the CD spectrum of SP-A. The similarity between p*K* values calculated from protein aggregation assays and CD measurements supports the concept that the change of CD spectra of SP-A as a function of the pH reflects the conformational change of SP-A at different levels of self-association.

Fig. 3*B* shows the CD spectra of the Ca^{2+}-induced aggregated form of SP-A at neutral pH. Conformational analysis estimated 11% α-helix, 38% β-sheet, 22% β-turn, 12% P_{II}, and 18% unordered form. Ca^{2+} ions at pH 7.0 seemed to produce a similar effect on the CD spectra of SP-A as that found for mildly acidic pH in the absence of Ca^{2+}. However, the changes in the CD spectra of SP-A at acidic pH were entirely reversible when the pH was shifted back to 7.0 (Fig. 1), but those changes produced by 5 mM Ca^{2+} were not reversed when the protein was dissociated by the addition of EDTA (data not shown). When the pH of samples containing Ca^{2+} was titrated from 7.2 to below 5.0, the negative ellipticity was reduced, and the negative maximum progressively shifted from 208 to 211 nm. The pH-dependent change of ellipticity at 207 nm, shown in the *inset* of Fig. 3*B*, and the pH-dependent increase of SP-A aggregation in the presence of Ca^{2+}, shown in Fig. 2*A*, were similar.

SP-A-induced Lipid Aggregation—Fig. 4 shows liposome aggregation induced by SP-A at neutral and acidic pH. The addition of SP-A to acidic vesicles (DPPC/DPPG or DPPC/PG) at pH 4.5 in the presence of EDTA resulted in a marked increase in light absorbance due to lipid aggregation. Light absorbance slightly increased after the addition of Ca^{2+}. This additional increase was completely reversed by adding EDTA. Interestingly, little or no change in light absorbance was observed after the addition of SP-A and Ca^{2+} to DPPC or DPPC/PC vesicles, indicating that SP-A-induced aggregation of neutral vesicles markedly decreased at that acidic pH. In contrast, at neutral pH, SP-A aggregated neutral vesicles in the presence of micromolar concentrations of Ca^{2+} (25). On the other hand, the extent of aggregation of disaturated phospholipid vesicles (DPPC or DPPC/DPPG), which at 37 °C were in the gel state, was higher than that of unsaturated vesicles (DPPC/PC or DPPC/PG), supporting previous results concerning the influence of the physical state of the vesicles on this process (24).

The effect of the ionic strength on lipid aggregation is shown in Fig. 5. At low or physiological ionic strength, the aggregation of DPPC at pH 4.5 is very low. To restore the extent of aggre-

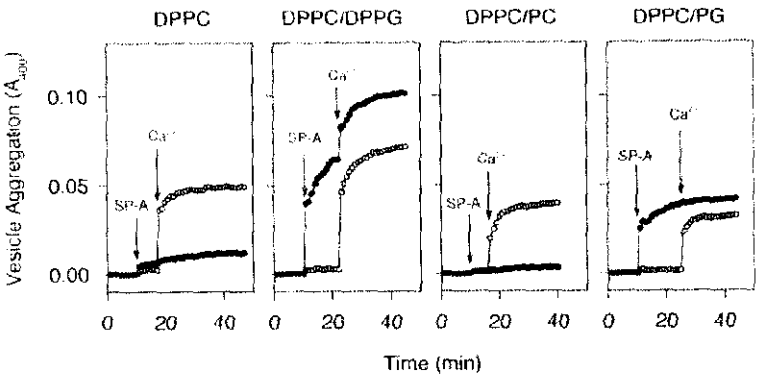

FIG. 4. **Vesicle aggregation induced by SP-A at acidic and neutral pH.** Neutral vesicles of DPPC or DPPC/PC (7:3, w/w) and acidic vesicles of DPPC/DPPG (7:3, w/w) or DPPC/PG (7:3, w/w) were prepared in 150 mM NaCl, 0.1 mM EDTA, 25 mM acetate, 25 mM Tris/HCl buffer at either pH 7.2 (○) or 4.5 (●). Sample and reference cuvettes were filled with phospholipid vesicles (80 μg/ml), and after a 10-min equilibration at 37 °C, SP-A (8 μg/ml) was added to the sample cuvette. At pH 4.5 (●), vesicle aggregation did not depend on Ca^{2+} ions and started upon SP-A addition. At neutral pH (○), SP-A-induced vesicle aggregation depended on the addition of 1 mM Ca^{2+}. These experiments were also done with 50 μM free Ca^{2+} at both neutral and acidic pH with identical results. Four different preparations of porcine SP-A were used. A representative experiment is shown.

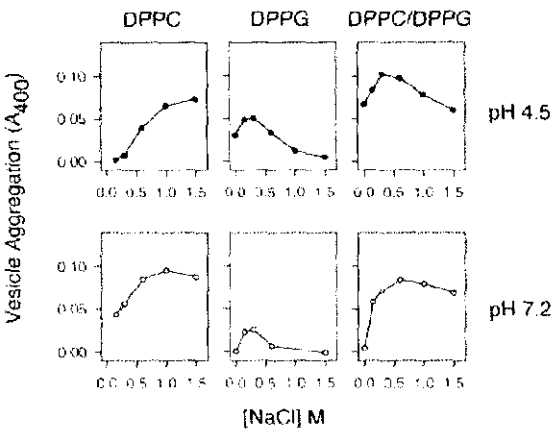

FIG. 5. **Effect of the ionic strength on vesicle aggregation induced by SP-A at acidic and neutral pH.** Concentrated vesicle suspensions (3 mg/ml) of DPPC, DPPG, and DPPC/DPPG (7:3, w/w) were prepared in 100 mM NaCl, 0.1 mM EDTA, 25 mM acetate, 25 mM Tris/HCl buffer at either pH 7.2 (○) or 4.5 (●). Vesicle aggregation experiments were done at 37 °C as described under "Experimental Procedures." Final concentrations of phospholipids, SP-A, and free Ca^{2+} were 85 μg/ml, 8.5 μg/ml, and 50 μM, respectively. Sample turbidity was continuously monitored at 400 nm. The NaCl concentration was increased in the medium by the addition of repeated small aliquots of a concentrated NaCl solution. The absorbance at 400 nm was registered once it was stabilized after each change of NaCl concentration. The experiment started at a NaCl concentration of 2.7 mM because lipid vesicles were always prepared in the presence of salts. Results presented are from a representative one of three experiments using three different preparations of porcine SP-A.

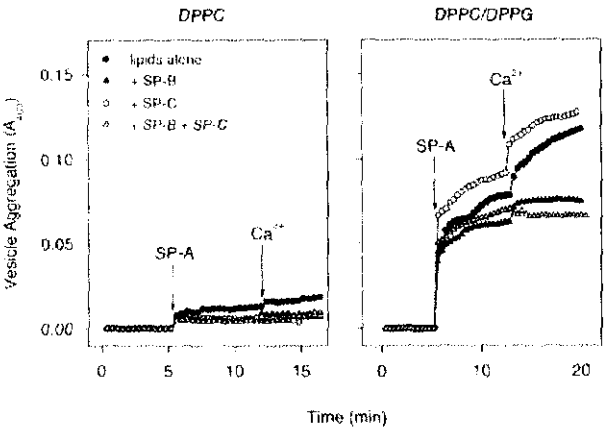

FIG. 6. **SP-A-induced aggregation of liposomes containing SP-B and/or SP-C at acidic pH.** Reconstitution of SP-B or SP-C or both hydrophobic proteins in DPPC or DPPC/DPPG (7:3, w/w) vesicles was performed, as described under "Experimental Procedures," in 150 mM NaCl, 0.1 mM EDTA, 25 mM acetate, 25 mM Tris/HCl buffer, pH 4.5. Vesicle aggregation experiments were performed as in Fig. 5. Final concentrations of SP-A, phospholipids, and Ca^{2+} were 7 μg/ml, 70 μg/ml and 1 mM, respectively. The SP-B or SP-C to lipid weight ratio was 1:20. The results shown are from a representative one of three experiments. Four experiments using vesicles containing 10% (w/w) of either SP-B or SP-C were also performed with identical results.

gation found at neutral pH, high ionic strength was needed. At pH 7.2, the aggregation of DPPC vesicles was independent of ionic strength because it occurred at very low NaCl concentrations (2.7 mM). However, the extent of aggregation was enhanced as the concentration of NaCl was increased. In the case of DPPG vesicles or vesicles containing DPPG, higher levels of vesicle aggregation occurred at very low ionic strength at pH 4.5 than at pH 7.2. At neutral pH, aggregation of acidic vesicles induced by SP-A was abrogated at very low NaCl and Ca^{2+} concentrations, probably due to electrostatic repulsion between the negative charge of phospholipids and the negative surface charge on the protein (24). However, at pH 4.5, the protonation of carboxyl groups of SP-A would reduce the negative surface charge on the protein. On the other hand, aggregation of DPPG vesicles at pH 4.5 was progressively decreased as NaCl concentration increased, suggesting that the interaction of SP-A with DPPG at acidic pH was ionogenic. Aggregation of DPPC/DPPG vesicles slightly decreased as the ionic strength increased due to the presence of DPPC in the vesicle, which might interact with SP-A by hydrophobic interactions.

SP-A is stored intracellularly in acidic lamellar bodies together with surfactant lipids and mature SP-B and SP-C (6, 7). It was of interest to determine whether the presence of SP-B or SP-C or both hydrophobic proteins modified the lipid aggregation activity of SP-A at acidic pH. Therefore, SP-B and SP-C were reconstituted in DPPC or DPPC/DPPG vesicles (protein: lipid weight ratio, 1:20 or 1:10). DPPC aggregation induced by SP-A was essentially negligible at pH 4.5 when DPPC vesicles contained SP-B, SP-C, or a mixture of both proteins (Fig. 6). DPPC/DPPG vesicles either with or without SP-B or SP-C markedly aggregated at pH 4.5 upon the addition of SP-A in a Ca^{2+}-independent manner. Aggregation of acidic vesicles containing SP-B or SP-C at acidic pH was higher than that at neutral pH (data not shown). Vesicles containing SP-B or a

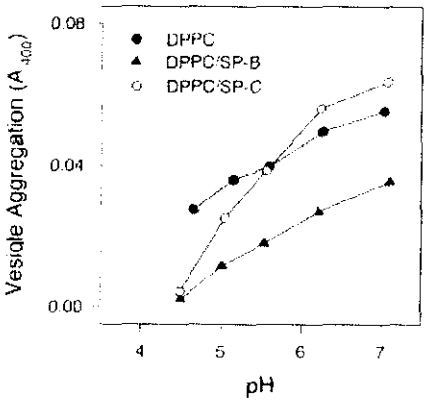

FIG. 7. **SP-A-induced aggregation of DPPC vesicles with or without SP-B or SP-C as a function of pH.** DPPC vesicles with or without SP-B or SP-C (protein:lipid weight ratio, 1:10) were prepared in 150 mM NaCl, 0.1 mM EDTA, 25 mM acetate, 25 mM MES, 25 mM Tris/HCl buffer at various pH values. Experiments were done at the indicated pH at 37 °C as described before. The final turbidity change at 400 nm after the addition of SP-A and 1 mM Ca^{2+} is shown as a function of pH. Results are from a representative one of three experiments. Two different preparations of SP-A, SP-B, and SP-C were used.

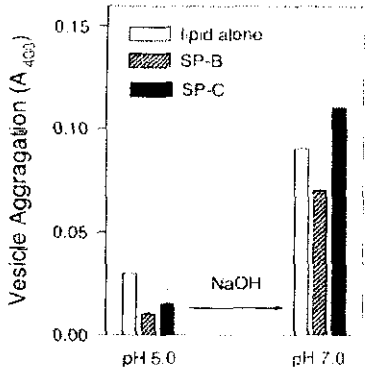

FIG. 8. **Effect of the shift of pH from 5.0 to 7.0 on SP-A-induced aggregation of DPPC vesicles containing SP-B or SP-C.** DPPC vesicles (1 mg/ml) were prepared with or without SP-B or SP-C (protein: lipid weight ratio, 1:10) in 150 mM NaCl, 0.1 mM EDTA, 25 mM acetate, 25 mM Tris/HCl buffer at pH 5.0. Sample turbidity as a consequence of DPPC aggregation at pH 5.0 was monitored at 400 nm at 37 °C. Final concentrations of SP-A, phospholipids, and Ca^{2+} were 8 μg/ml, 80 μg/ml, and 1 mM, respectively. The pH shift from 5.0 to 7.0 was done by adding a small aliquot of a concentrated solution of NaOH. After pH shift, the change in turbidity at 400 nm was monitored again over 10 min. Values are expressed as means ± S.D. of four experiments.

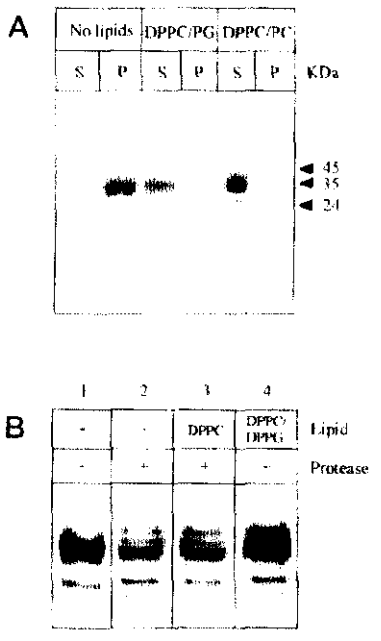

FIG. 9. **Lipid binding (*A*) and protease sensitivity (*B*) of SP-A at acidic pH.** *A*, results presented here are from vesicles of DPPC/PC and DPPC/PG (7:3, w/w) at 25 °C. Similar results were obtained from vesicles of DPPC and DPPC/DPPG (7:3, w/w) with or without SP-B and SP-C. Experiments were done as described under "Experimental Procedures." In the absence of lipids, SP-A sedimented, and all of the protein was recovered in the pellet (*P*). After incubation of SP-A with neutral or acidic vesicles, most of the protein was recovered in the supernatant (*S*) coflotating with lipids. Molecular markers in kDa are indicated to the right of the Coomassie Blue-stained SDS-PAGE gel. *B*, SP-A (6.7 μg) was digested with endoproteinase Glu-C (13.5 μg) in the absence (*lane 2*) and the presence of 60 μg of DPPC vesicles (*lane 3*) or DPPC/DPPG (7:3, w/w) vesicles (*lane 4*) in 110 μl of 150 mM NaCl, 25 mM acetate buffer, pH 4.5. A control (*lane 1*) was run with 6.7 μg of SP-A without endoproteinase Glu-C. Western blot analysis for SP-A was done as described under "Experimental Procedures." A representative one of four experiments is shown.

mixture of both hydrophobic proteins showed less aggregation than those vesicles with SP-C or without proteins.

Next, we analyzed the ability of SP-A to aggregate DPPC vesicles at various pH values (Fig. 7). DPPC aggregation increased as the pH was raised to pH 7.0. Aggregation of DPPC vesicles at pH higher than 5.0 was dependent on the presence of Ca^{2+} ions. At the pH of lamellar bodies (about 5.5), SP-A mediated aggregation of DPPC vesicles containing SP-B or a mixture of hydrophobic proteins (data not shown) is lower than that found with DPPC vesicles alone or containing SP-C.

To determine whether a rapid shift of pH from 5.0 to 7.0 was sufficient to raise the extent of DPPC aggregation, we performed pH shift experiments in the presence and the absence of Ca^{2+}. Fig. 8 shows that the addition of NaOH to a mixture of SP-A and DPPC vesicles containing SP-B or SP-C resulted in a 7-fold increase of liposome aggregation. The extent of aggregation after pH shift increased by 3-fold for DPPC vesicles without SP-B or SP-C. The presence of Ca^{2+} ions was needed for this reversion. In the absence of Ca^{2+}, the light scattering was almost negligible at pH 5.0 and after retitrating back to pH 7.0 (data not shown). These results suggest that pH changes are accompanied by changes in the SP-A/DPPC interactions, probably as a consequence of pH-dependent structural changes to the SP-A molecule. pH shift experiments were also performed with DPPC/DPPG vesicles with or without SP-B and SP-C. The shift of pH from 5.0 to 7.0 resulted in a small but significant decrease of the extent of aggregation of these vesicles (data not shown).

Lipid Binding and Protease Sensitivity of Membrane-bound SP-A—We next investigated whether the low aggregation activity of SP-A with neutral vesicles at acidic pH was a consequence of reduced ability of SP-A to bind DPPC vesicles at this pH. SP-A was coincubated at 25 °C with either DPPC/PC (7:3, w/w) or DPPC/PG (7:3, w/w) vesicles at pH 4.5, and binding was determined by sucrose density gradient centrifugation at the same temperature. Fig. 9*A* shows that most of the protein was recovered in the supernatant coflotating with DPPC/PC vesicles. In the absence of lipids, all of the protein was recovered in the pellet. At pH 4.5, SP-A hardly aggregated DPPC/PC vesicles at either 37 °C (Fig. 5) or 25 °C (data not shown), but it bound to these vesicles to nearly the same extent as to DPPC/PG vesicles (Fig. 9*A*). Similar results were obtained with DPPC and DPPC/DPPG, which at the temperature of the experiment were in the gel state. SP-A also bound to DPPC vesicles containing SP-B or SP-C (protein:lipid weight ratio, 1:10).

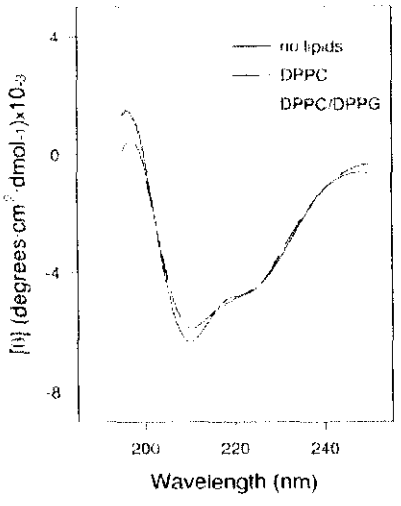

FIG. 10. **Effect of DPPC and DPPC/DPPG vesicles on circular dichroism spectra of SP-A at pH 4.5.** SP-A (0.08 mg/ml, final concentration) was added to 25 mM acetate buffer, pH 4.5, in the absence (*solid line*) or the presence of DPPC (*dashed line*) or DPPC/DPPG (7:3, w/w) vesicles (*dotted line*) (lipid:protein weight ratio, 6:1). Circular dichroism measurements were performed 10 min after the SP-A addition as described under "Experimental Procedures." CD spectra presented here are from a representative one of three experiments with different SP-A preparations.

The protease sensitivity of SP-A bound to either DPPC or DPPC/DPPG membranes at pH 4.5 was studied by means of endoproteinase Glu-C from *S. aureus V8*. Immunoblot analysis of SP-A after endoproteinase Glu-C digestion is shown in Fig. 9*B*. DPPC/DPPG-bound SP-A was more protected from protease degradation than DPPC-bound SP-A, which showed similar susceptibility to proteolysis as lipid-free SP-A. The binding of acidic vesicles to SP-A may lead to reduced accessibility of endoproteinase Glu-C cleavage targets located in the domains in which acidic phospholipids are bound. Alternatively, the reduced susceptibility to proteolysis of DPPC/DPPG-bound SP-A over DPPC-bound SP-A can be a consequence of the ability of SP-A to aggregate acidic but not neutral vesicles at acidic pH. SP-A would become more protected from proteolysis in such vesicle aggregates.

Effect of the Lipid on the Secondary Structure of SP-A—We analyzed the effect of DPPC and DPPC/DPPG on the CD spectra of SP-A at pH 4.5 (Fig. 10). SP-A was self-aggregated at this pH in the absence of lipids and had a characteristic CD spectrum (Fig. 1). The presence of DPPC vesicles in the medium had no significant effect on the CD spectra of SP-A. Thus, DPPC bound to SP-A without modifying the secondary structure of H^+-induced aggregated form of SP-A. In contrast, the interaction of DPPC/DPPG vesicles with SP-A resulted in a change in the CD spectra of the protein. The negative ellipticity at both 207 and 222 nm increased and the negative maximum was shifted from 209 to 207 nm. These changes to the CD spectra of SP-A might be interpreted in terms of a decrease of the self-aggregation level of the protein as a consequence of the binding of SP-A to acidic vesicles. The presence of DPPC/DPPG vesicles in the medium would prevent protein self-aggregation provided lipid/protein interactions were favored over protein/protein interactions.

DISCUSSION

In exocrine, endocrine, or neuronal cells, it is well recognized that there is a progressive decrease in pH along the exocytic pathway (14). Several lines of evidence indicated that the acidification of the exocytic pathway could play a role in the regulation of sorting, transport, or proteolytic processing of proteins (16–18, 43, 44). The results presented in this paper show that a progressive decrease in pH influences the secondary structure and lipid binding and aggregation properties of SP-A.

pH-dependent Self-aggregation of SP-A—In this study, we have found that SP-A undergoes self-aggregation as the pH is reduced below 6.0. However, there is a marked aggregation of the protein in the pH range of the TGN (6–6.5) when Ca^{2+} is added. This effect is greater as the pH is reduced. There is a synergy between H^+ and Ca^{2+} on SP-A self-aggregation in the pH range of 5–6.5. Ca^{2+} has no effect on the self-aggregation of the protein when the pH is reduced toward the isoelectric pH of SP-A. Self-aggregation of the protein appears to be due to pH-induced conformational change of the protein. Conformational analysis from CD spectra of SP-A at various pH values indicates that the percentage of α-helix progressively decreases and that of β-sheet increases as the pH is reduced from 7.0 to 4.7. The increase of β-sheet content could be associated with protein/protein interactions that occurred between SP-A molecules at acidic pH.

It is of interest to note that proteins traversing the TGN and immature secretory granules, the site of sorting constitutive from regulated proteins, are exposed to mildly acidic pH and high concentrations of Ca^{2+} ions (14–16). The maintenance of an acidic pH and high Ca^{2+} milieu in the TGN and immature secretory granules is known to be important for secretory granule formation. Several individual granule content proteins from pituitary or adrenal chromaffin granules undergo pH-dependent self-aggregation *in vitro* in a Ca^{2+}-dependent (adrenal granules) (17, 18) or Ca^{2+}-independent (pituitary granules) (18) manner. Moreover, these proteins can drive co-aggregation of other granule proteins that do not have the inherent ability to self-aggregate. However, constitutively secreted proteins do not co-aggregate with pituitary or chromaffin granule content proteins (17, 18). The H^+ and Ca^{2+}-dependent aggregation property seems to be the hallmark of vesicle proteins of the regulated secretory pathway, distinguishing the regulated secretory proteins from others in the TGN. This could be the case for SP-A. We suggest that the H^+- and Ca^{2+}-dependent aggregation property of SP-A, together with its ability to bind to membranes, might be important for the segregation of this protein to secretory granules. Although selective aggregation and interaction of proteins with the membrane of the TGN are important elements in the sorting of proteins, there is evidence that suggests that aggregation alone is not sufficient to ensure the packaging of all proteins in secretory granules (45). To date, no sorting receptor has been conclusively identified. However, a putative receptor for chromogranin B may not recognize the primary sequence of the protein, but a determinant generated by higher order structure of the molecule (45). Interestingly, it has been recently reported that human pro-SP-B contains secretory granule targeting determinants in both the NH_2-terminal propeptide and the mature peptide (46).

SP-A/Lipid Interaction—The site where SP-A and surfactant phospholipids are first assembled is unknown, but it is thought to be in the lamellar body. The postulated scheme of formation and enlargement of lamellar bodies involves bulk transfer of phospholipids through budding of vesicles because synexin and other annexins promote fusion of lipid vesicles with lung lamellar bodies (47). This study was focused on the lipid binding and lipid aggregation activity of SP-A at acidic pH in the presence and absence of SP-B and SP-C.

At pH 4.5, SP-A induces massive aggregation of acidic vesicles either with or without SP-B or SP-C or both proteins. The extent of aggregation is higher at acidic than at neutral pH. Efrati *et al.* (27) previously found that SP-A aggregated a lipid

extract from surfactant at pH 4.4 in the absence of Ca^{2+}. *One* striking result we have found is that SP-A poorly aggregates DPPC or DPPC/PC vesicles at pH 4.5 in the presence of Ca^{2+}. Interestingly, aggregation of DPPC vesicles containing SP-B, SP-C, *or both proteins is abrogated at this pH. At the* pH of lamellar bodies (approximately 5.5) SP-A-induced aggregation of DPPC containing SP-B or a mixture of both proteins is very low and was Ca^{2+}-dependent. A shift of pH from 5.0 to 7.0 *results in a 7-fold increase of DPPC* aggregation when liposomes contain surfactant hydrophobic proteins and a 3-fold increase for DPPC vesicles alone. These results indicate that 1) aggregation of DPPC vesicles induced by SP-A strongly depends on pH, 2) Ca^{2+} does not have the same effect on DPPC aggregation at acidic and at neutral pH, and 3) the effect of pH on vesicle aggregation is reversible.

A possible interpretation of these results would be that the binding of SP-A to DPPC is also pH-dependent, being much lower at acidic than at neutral pH and completely abrogated at pH 4.5, when vesicles contain the positively charged hydrophobic proteins. However, we show here that SP-A binds to neutral vesicles at pH 4.5 as well as it does to acidic vesicles. SP-A also binds to DPPC liposomes, which contain SP-B or SP-C at that pH. These results suggest that DPPC binding to SP-A and DPPC aggregation induced by the protein are distinct processes, which probably have different requirements. Binding and aggregation as distinct phenomena have also been described in McCormack *et al.* (22). It is possible that the binding of acidic but not neutral vesicles to SP-A modifies the protein structure or the state of protein self-aggregation. We analyzed the effect of DPPC and DPPC/DPPG vesicles on the secondary structure of SP-A at pH 4.5. At this pH, all of the protein is in the self-aggregated form in the absence of lipids. The interaction of DPPC vesicles with SP-A under acidic conditions does not have any effect on the CD spectra of the protein, suggesting that DPPC does not modify the secondary structure of the self-aggregated form of SP-A. In contrast, the binding of DPPC/DPPG vesicles to SP-A results in a striking change of CD spectra of SP-A toward the typical CD spectra of the nonaggregated form of the protein. These experiments suggest that the presence of DPPC/DPPG in the medium prevents the rapid aggregation of the protein induced by H^+. It is possible that lipid/protein interactions are favored over protein/protein interactions at acidic pH provided that vesicles contain acidic phospholipids. We show here that SP-A markedly interacts with DPPG vesicles at acidic pH and that the interaction is ionogenic. On the other hand, SP-A massively aggregates acidic vesicles at acidic pH. We suggest here that SP-A-induced vesicle aggregation requires the protein to be in a nonaggregated form, or at least without forming large protein aggregates. This hypothesis may explain why SP-A binds to neutral vesicles but poorly aggregates them, because the amount of SP-A molecules available to aggregate liposomes is low as the protein is forming large aggregates. The idea that binding of SP-A to DPPC/DPPG but not to DPPC vesicles, at acidic pH, might lead to a decrease of protein aggregation is supported by epifluorescence microscopy studies of fluorescent Texas Red-labeled SP-A adsorbed to monolayers of either DPPC or DPPC/DPPG at pH 4.5.[2] In these experiments, SP-A was first injected in the subphase at pH 4.5, and then the monolayer was formed. Therefore, SP-A was self-aggregated before interacting with the monolayer of phospholipids. With DPPC monolayers, large aggregates of fluorescent SP-A appear at liquid condensed/liquid expanded boundary regions, indicating that SP-A aggregates

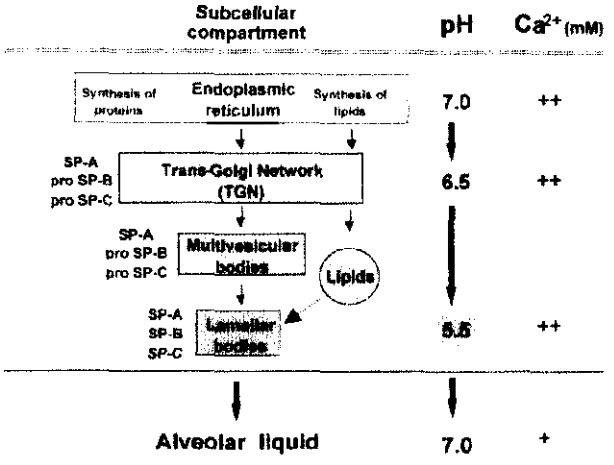

FIG. 11. **Diagrammatic representation of the exocytic pathway of pulmonary surfactant in type II cells.** *Center*, the intracellular compartments involved in posttranslational processing and secretion of surfactant apolipoproteins based upon previous data (7, 44, 48). *Right*, the internal pH of the subcellular compartments of the exocytic pathway (12, 14) and pH of alveolar fluid. These subcellular compartments are exposed to high concentrations of calcium (13, 14, 16). The Ca^{2+} concentration in the alveolar liquid is in the 1-2 mM range (25).

are segregated to the lipid packing defects in the monolayer. In contrast, with DPPC/DPPG monolayers, all fluorescent SP-A appears exclusively into the liquid expanded phase, and no aggregates of fluorescent SP-A are observed.

We also report that SP-A bound to DPPC/DPPG vesicles is more protected from endoproteinase Glu-C degradation than SP-A bound to DPPC vesicles or lipid-free SP-A. This result can be explained by the ability of SP-A to induce massive aggregation of acidic but not neutral vesicles. SP-A molecules might form bridges between vesicles and become more protected from degradation. In contrast, large aggregates of protein bind to DPPC vesicles at acidic pH but are not able to aggregate the lipid and remain more susceptible to proteolysis.

Physiological Implications—As in other exocrine cells, the exocytic pathway of type II cells shows a progressive acidification from the endoplasmic reticulum to the secretory lamellar bodies (12, 14, 44). Based upon previous published studies the exocytic pathway of surfactant components is schematically illustrated in Fig. 11. SP-A travels together with the precursors of the hydrophobic surfactant proteins SP-B and SP-C through the same pathway from the Golgi complex to lamellar bodies (7), the site where surfactant phospholipids and surfactant apolipoproteins might be first assembled. The intracellular pH gradient influences the posttranslational processing of pro-SP-C (44) and pro-SP-B (48), which involves intracellular proteolysis of these proteins in acidic subcellular compartments, and the packaging of new synthesized phosphatidylcholine in lamellar bodies (49). In contrast to SP-B and SP-C, no proteolytic events occur in the formation of SP-A except for removal of the signal peptide. However, we found that the secondary structure of the protein, its aggregation state, and its interaction with lipids change with the progressive acidification of the medium. Our results suggest that H^+- and Ca^{2+}-dependent aggregation property of SP-A, together with its ability to bind to membranes, might be important for the sorting of SP-A to secretory granules. Once in lamellar bodies, the presence of acidic phospholipids in the composition of these organelles might be needed for aggregation of membranes induced by SP-A because SP-A hardly aggregates neutral vesicles at acidic pH. This aggregation process together with the synergistic SP-A/SP-B fusion activity at acidic pH (50) could be involved in

[2] M. L. F. Ruano, K. Nag, C. Casals, J. Pérez-Gil, and K. M. W. Keough, unpublished results.

the formation of closely packed sheets of lipids in these secretory granules. In addition, the changes of SP-A/lipid interactions with pH shift suggest that the change from the acidic lamellar body to the neutral alveolar subphase could be a factor involved in the reorganization of surfactant material after secretion.

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DISCUSIÓN

A la vista de los resultados obtenidos en el primer trabajo de este capítulo, podemos concluir que la agregación de vesículas inducida por SP-A y la autoagregación de esta proteína dependientes de Ca^{2+} son procesos independientes. Esta conclusión se apoya en que los requerimientos de Ca^{2+} de ambos procesos (K_a^{Ca2+}) son distintos, siendo necesarias concentraciones mayores de Ca^{2+} para que se produzca la autoagregación de la SP-A que para la agregación lipídica inducida por estas proteínas. Hay que destacar, no obstante, que dichos procesos se han estudiado en distintas condiciones de fuerza iónica tal y como se viene haciendo en diversos laboratorios. Así, la agregación de vesículas lipídicas inducida por SP-A se estudia a fuerza iónica fisiológica, haciéndose en cambio la autoagregación de la proteína a baja fuerza iónica (en el capítulo 5 de esta tesis abordaremos de manera detallada el efecto de la fuerza iónica del medio sobre la autoagregación de la SP-A).

Teniendo en cuenta los estudios previos citados en la discusión del trabajo, se podría proponer un modelo que se ajuste a estos resultados. Según este modelo, la unión de la SP-A en presencia de Ca^{2+} a vesículas lipídicas a través del dominio CRD provocaría la agregación de las vesículas por el simple hecho de que una molécula de proteína, al ser multimérica, es capaz de unirse a varias vesículas. Esta unión, además, podría provocar cambios estructurales que disparen interacciones proteína-proteína entre distintas moléculas de SP-A, incrementando así aún más el tamaño de los agregados. Esta autoagregación sería diferente de la que se produce ante concentraciones de Ca^{2+} de orden milimolar en ausencia de NaCl.

En el segundo trabajo de este capítulo demostramos que la SP-A porcina experimenta autoagregación a valores de pH inferiores a 6, proceso que está relacionado con cambios en su estructura secundaria. Si además hay una concentración de Ca^{2+} de orden milimolar, la autoagregación de la proteína se produce a valores de pH mayores. También hemos visto que la SP-A induce, a pH ácido, la agregación de vesículas que incluyen en su composición fosfolípidos ácidos, pero no lo hace con vesículas neutras, aunque sí se une a ellas. La diferencia en la interacción con vesículas ácidas y con vesículas neutras podría estar relacionada con diferencias en la estructura secundaria de la proteína. A pH ácido, la SP-A parece tener una estructura más rica en lámina β que a pH neutro, lo que favorecería las interacciones proteína-proteína induciendo así su autoagregación. Al unirse a vesículas ácidas en condiciones de pH ácido la estructura secundaria de la SP-A cambia, indicando que la autoagregación de la proteína disminuye. En estas condiciones, las interacciones lípido-proteína predominan sobre las interacciones proteína-proteína, permitiendo que cada molécula de SP-A se una a varias vesículas (sin implicar necesariamente la desagregación total de la proteína), provocando así la agregación de éstas. Las vesículas neutras, por el contrario, son incapaces de inducir este cambio en la estructura proteica, por lo que la SP-A permanecería formando grandes agregados proteicos donde cada molécula tendría grandes dificultades para interaccionar con distintas vesículas a la vez, si bien cada agregado proteico permanecería unido en conjunto a unas pocas vesículas.

La agregación selectiva de SP-A con el pH ligeramente ácido del trans-Golgi y la presencia de Ca^{2+} podrían desempeñar un papel importante en su segregación hacia la ruta secretora regulada. Una vez en los cuerpos lamelares, la presencia de fosfolípidos ácidos sería necesaria para que la SP-A induzca la agregación de membranas que facilite el almacenamiento

del surfactante, formando las estructuras densamente apiladas de los cuerpos lamelares. Tras la secreción del contenido de los cuerpos lamelares, el pH neutro de la subfase acuosa provocaría alteraciones en las interacciones lípido-proteína que podrían estar implicadas en la reorganización del surfactante en el espacio extracelular.

4. INTERACCIÓN DE LA SP-A CON MONOCAPAS LIPÍDICAS

INTRODUCCIÓN

En el capítulo anterior estudiamos algunos aspectos de la interacción de la SP-A con bicapas lipídicas. Sin embargo, como se explicó en la introducción de esta tesis, los lípidos del surfactante presentan otras estructuras en las distintas etapas de su ciclo metabólico. Por una parte está la mielina tubular, cuya estructura (disposición de sus componentes moleculares) aún no se conoce; y por otra está la monocapa en la interfase aire/agua del alveolo, la cual es aceptada mayoritariamente en la actualidad como la estructura responsable de la actividad superficial del surfactante pulmonar en el alveolo.

Estudios previos indican que la SP-A estabiliza la monocapa del surfactante a bajas tensiones superficiales, y facilita la expulsión de lípidos distintos de DPPC durante la compresión de la monocapa (Schurch *et al.* 1992). Además, se ha observado que esta proteína es capaz de interaccionar con monocapas de DPPC a pH neutro y en ausencia de Ca^{2+} (Taneva *et al.* 1995). Con estos antecedentes, nos proponemos estudiar mediante microscopía de epifluorescencia la interacción de SP-A porcina con monocapas de DPPC, así como la influencia de la presencia de fosfolípidos ácidos (DPPG), presentes en el surfactante pulmonar, en esta interacción.

El primer trabajo incluido en este capítulo aborda el estudio de esta interacción a pH neutro, que es el pH de la subfase acuosa en el alveolo pulmonar. En el segundo trabajo el estudio se realizó a pH ácido, teniendo en cuenta que el pH afecta a la estructura, estado de agregación e interacción de la SP-A con bicapas, según se vio en el capítulo anterior.

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Differential Partitioning of Pulmonary Surfactant Protein SP-A into Regions of Monolayers of Dipalmitoylphosphatidylcholine and Dipalmitoylphosphatidylcholine/Dipalmitoylphosphatidylglycerol

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ABSTRACT The interaction of the pulmonary surfactant protein SP-A fluorescently labeled with Texas Red (TR-SP-A) with monolayers of dipalmitoylphosphatidylcholine (DPPC) and DPPC/dipalmitoylphosphatidylglycerol 7:3 w/w has been investigated. The monolayers were spread on aqueous subphases containing TR-SP-A. TR-SP-A interacted with the monolayers of DPPC to accumulate at the boundary regions between liquid condensed (LC) and liquid expanded (LE) phases. Some TR-SP-A appeared in the LE phase but not in the LC phase. At intermediate surface pressures (10–20 mN/m), the protein caused the occurrence of more, smaller condensed domains, and it appeared to be excluded from the monolayers at surface pressure in the range of 30–40 mN/m. TR-SP-A interaction with DPPC/dipalmitoylphosphatidylglycerol monolayers was different. The protein did not appear in either LE or LC but only in large aggregates at the LC-LE boundary regions, a distribution visually similar to that of fluorescently labeled concanavalin A adsorbed onto monolayers of DPPC. The observations are consistent with a selectivity of interaction of SP-A with DPPC and for its accumulation in boundaries between LC and LE phase.

INTRODUCTION

Surfactant protein A (SP-A) is the major protein associated with pulmonary surfactant, a complex lipid-protein material that covers the alveolar surface, reduces the surface tension of the air-liquid interface, and facilitates respiratory mechanics. It is widely assumed that an interfacial phospholipid monolayer enriched in dipalmitoylphosphatidylcholine (DPPC) is mainly responsible for the tensoactive properties of lung surfactant and that phosphatidylglycerol and surfactant-associated proteins SP-A, SP-B, and SP-C are also required for the formation and proper dynamics of the surfactant monolayer in the airways (Keough, 1992; Kuroki and Voelker, 1994; Johansson et al., 1994).

SP-A is a hydrophilic glycoprotein with a monomeric molecular mass of 30–40 kDa (Hawgood and Shiffer, 1991). Its N-terminal moiety has a collagen-like sequence, whereas the C-terminal portion is like a C-type lectin with binding sites for carbohydrates and Ca^{2+} (White et al., 1985; Drickamer, 1988). The native form of the protein as isolated from the alveolar spaces is assembled into a complex oligomer in which SP-A chains are organized in trimers through collagen-like triple-helices, and six trimers form a bouquet-like arrangement similar to that of the protein C1q from the complement system (Voss et al., 1988).

Studies in vitro have shown that SP-A interacts with surfactant phospholipids and glycosphingolipids both in bilayers and immobilized on silica gel plates with a selectivity for interaction with DPPC and galactosylceramide (King et al., 1983; Kuroki and Akino, 1991; Kuroki et al., 1992; Childs et al., 1992; Casals et al., 1993). SP-A induces phospholipid vesicle aggregation in the presence of Ca^{2+} (King et al., 1983; Hawgood et al., 1985; Ruano et al., 1996). These lipid-binding activities mediate the participation of SP-A in processes of surfactant metabolism in the alveolar spaces. SP-A, for instance, is required for the generation in vitro of tubular myelin (Suzuki et al., 1989; Williams et al., 1991), a structure that has been associated with highly tensoactive surfactant preparations. SP-A also participates in the regulation of surfactant secretion into and clearance from the alveolar spaces (Dobbs et al., 1987; Bates et al., 1994; Wright and Youmans, 1995) and in several activities associated with alveolar defense against pathogens (Van Golde, 1995). In addition, surfactant containing SP-A is more resistant than surfactant without SP-A to the inhibition of surfactant activity induced by plasma proteins such as fibrinogen or albumin (Cockshutt et al., 1990; Strayer et al., 1996).

Although SP-A by itself is poor at accelerating the transfer of surfactant phospholipids to the air-liquid interface, it augments rapid formation of phospholipid surface films in cooperation with the hydrophobic surfactant proteins SP-B and SP-C (Hawgood et al., 1987; Pison et al., 1990; Schurch et al., 1992). SP-A seems also to stabilize the surfactant monolayer at low surface pressures and enhance elimination of non-DPPC lipids during compression (Schurch et al., 1992). Korfhagen and co-workers (1996) have shown that SP-A knock-out mice can breathe properly and present unaltered lung morphology and function, suggesting that SP-A is not essential for respiratory function at least in the perinatal stage. However, in that study the minimal surface

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tension of monolayers formed from material extracted from SP-A-defective lungs was higher than that of surfactant from control animals. Taneva et al. (1995) have shown that at pH 7.4 in the absence of Ca^{2+}, SP-A interacts and perturbs pure DPPC monolayers, but it shows complete immiscibility with negatively charged monolayers composed of DPPG or a combination of DPPC and DPPG.

In the present study we investigated the association of SP-A from the hypophase with interfacial spread monolayers of DPPC or DPPC/DPPG by epifluorescence microscopy (Pérez-Gil et al., 1992a; Nag and Keough, 1993; Nag et al., 1996b). The use of fluorescently labeled proteins allows for the analysis of interactions of a given protein with domains or regions of the monolayer (Grainger et al., 1990; Maloney et al., 1995; Nag et al., 1996a).

EXPERIMENTAL PROCEDURES

Materials

The phospholipids, 1,2-dipalmitoylphosphatidylcholine and 1,2,dipalmitoylphosphatidylglycerol (DPPG) were purchased from Sigma Chemical Co. (St. Louis, MO). The fluorescent lipid probe 1-palmitoyl-1-{12-[(7-nitro-2-1, 3-benzoxadizole-1-yl)amino]dodecanoyl}phosphatidylcholine (NBD-PC) was from Avanti Polar Lipids (Birmingham, AL). The fluorescent-labeling chemical sulforhodamine 101 sulfonyl chloride, Texas Red™ (TR), was obtained from Molecular Probes Inc. (Eugene, OR), as was Texas Red-labeled concanavalin A (TR-conA). Chloroform and methanol were HPLC grade solvents from Fisher Scientific Co. (Ottawa, ON), and all other reagents were analytical grade chemicals from Merck (Darmstadt, Germany).

Isolation and Labeling of SP-A

Surfactant protein A was purified from pulmonary surfactant prepared from porcine bronchoalveolar lavage as previously described (Casals et al., 1989) by sequential butanol and octylglucoside extractions (Casals et al., 1993) and stored at −20°C in solutions in 5 mM Tris-HCl buffer, pH 7.4. Purity of the protein preparations was routinely checked by sodium dodecyl sulfate-polyacrylamide gel electrophoresis under reducing conditions followed by Coomassie Blue staining. SP-A was quantitated by amino acid analysis after hydrolysis of protein samples in 0.2 ml of 6 M HCl containing 0.1% (w/v) phenol in evacuated and sealed tubes at 108°C for 24 h, followed by analysis in a Beckman System 6300 high performance amino acid analyzer.

Fluorescently labeled SP-A was prepared as follows. Solutions of SP-A in 5 mM Tris-HCl buffer containing 300–500 μg of protein were adjusted to pH 8.0 by addition of 50 mM Tris, pH 8.3. The labeling reaction was started by the addition of 1 mM TR in methanol to a final SP-A/TR ratio of 5–6:1 (mol/mol). The mixture was incubated for 90 min in darkness at room temperature and then exhaustively dialyzed against 5 mM Tris-HCl, pH 7.4, to remove unreacted fluorescent reagent.

Activity of labeled TR-SP-A compared with that of the native protein was assayed by testing its ability to induce aggregation of DPPC vesicles in the presence of Ca^{2+} at 37°C as described elsewhere (Ruano et al., 1996).

Epifluorescence Experiments

Surface pressure-area measurements and microscopic observations of phospholipid monolayers were performed on an epifluorescence microscopic surface balance, the construction and operation of which have been described elsewhere (Nag et al., 1990; 1991). To form monolayers, the lipids, DPPC or the mixture DPPC/DPPG (7:3, w/w), were mixed in chloroform/methanol (3:1, vol/vol), and 1 mol % of NBD-PC was included. Monolayers were spread by depositing very small aliquots of the chloroform/methanol solutions on a subphase of 5 mM Tris-HCl, pH 7.4, and 150 mM NaCl with or without SP-A or TR-SP-A. All subphases were prepared with double distilled water, the second distillation being from dilute potassium permanganate. After spreading of a monolayer, the organic solvent was allowed to evaporate for 5 min, and, in order to facilitate SP-A adsorption to the air-liquid interface, the monolayer was compressed rapidly (707 mm^2/s) to a surface pressure of 10 mN/m and then expanded again to 0 mN/m. After a 1-h period, which would have allowed for penetration of the protein into the gas or gas-liquid expanded coexistence phases (e.g., Maloney et al., 1995), the monolayer was compressed at a slow speed (20 mm^2/s or an initial rate of 0.13 $Å^2$/molecule/s) at 23 ± 1°C. At selected surface pressures, a video recording was made for a 1-min period of both NBD and TR fluorescence by switching fluorescence filter combinations. The images obtained were analyzed with digital image processing using JAVA 1.3 software (Jandel Scientific, San Rafael, CA) as discussed elsewhere (Nag et al., 1991; Pérez-Gil et al., 1992a).

The video images were obtained with a CCD camera that records in black and white. The images presented in the figures have been false-colored to display them as they appear to the eye in the microscope.

RESULTS

Fig. 1 shows Ca^{2+}-dependent DPPC vesicle aggregation induced by native SP-A and TR-SP-A prepared under two different conditions. Incubation of SP-A with TR under mild conditions, 90 min at room temperature and pH 8.0,

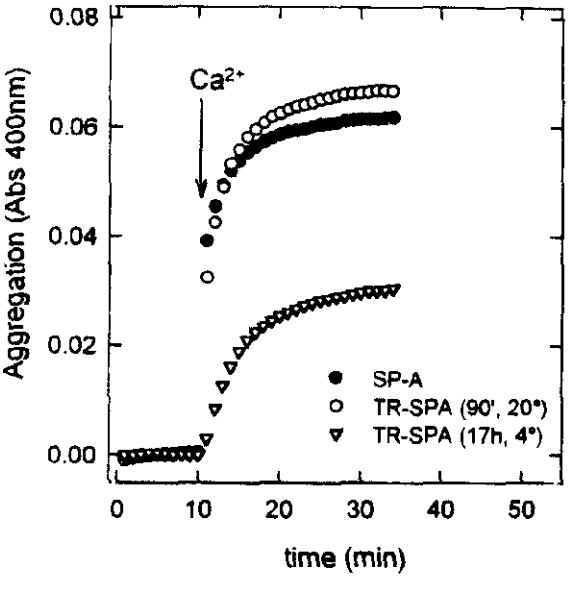

FIGURE 1 Aggregation of DPPC vesicles induced by native and TR-labeled SP-A in the presence of Ca^{2+}. DPPC vesicle suspensions (in Sample and Reference Cuvettes), native SP-A (Sample cuvette, *closed circles*) or labeled TR-SP-A (Sample cuvette, *open symbols*), and Ca^{2+} (Sample and Reference cuvettes) were sequentially added to 0.5 ml of buffer of 5 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 0.5 mM EGTA in Sample and Reference cuvettes as designated. The aggregation assay was started (*arrow*) by the addition of Ca^{2+}. Final concentrations of lipid, SP-A, and Ca^{2+} were 85 μg/ml, 8 μg/ml, and 1 mM, respectively. Two different TR-SPA batches were assayed, one labeled at 20°C for 90 min (*open circles*), and the other labeled for 17 h at 4°C (*open triangles*).

yielded a labeled protein with vesicle-aggregating activity similar to that of native SP-A. On sodium dodecyl sulfate-polyacrylamide gel electrophoresis under reducing conditions, this modified protein showed a main fluorescent band centered at 35 kDa similar to that of native SP-A (data not shown). Incubation of SP-A with TR for 17 h at 4°C produced a modified protein with higher fluorescence as observed in electrophoresis gels but having a lower ability to induce aggregation of DPPC vesicles (Fig. 1, *closed triangles*). This decrease in activity is possibly because of structural alterations in SP-A caused by extensive addition of probe molecules to amine groups of the protein. The milder conditions were selected to prepare fluorescent TR-SP-A for additional experiments. After exhaustive dialysis, spectroscopic measurements of the TR-SP-A samples prepared in this way yielded an estimate of incorporation of around 0.4 moles of probe per mole of protein monomer.

Fig. 2 shows the surface pressure — area per molecule (Π-A) isotherms of DPPC monolayers spread on subphases containing different concentrations of TR-SP-A. The isotherms showed that increasing concentrations of the protein in the subphase caused a progressive expansion of the interfacial DPPC film, suggesting that the protein is occupying some space in the interface, or, at least it is interacting with the phospholipid monolayer sufficiently to perturb the usual lipid packing. All of the monolayers showed LE (liquid expanded) to LC (liquid condensed) transitions as deduced from the plateau regions of the isotherms at surface pressures in the range of 7–9 mN/m. The effect of TR-SP-A on the Π-A isotherms of DPPC was qualitatively and quantitatively similar to the effect of native SP-A (data not shown) and analogous to the effect of native SP-A in DPPC/SP-A monolayers spread from solvents (Taneva et al., 1995).

Microscopic observations of monolayers of DPPC containing 1 mol % NBD-PC, in the absence and in the presence of TR-SP-A, showed typical LE-LC coexistence regions consisting of dark LC regions excluding the fluorescent probe and bright green areas of LE phase similar to those previously observed in lipid and lipid/protein monolayers (Nag et al., 1991; Pérez-Gil et al., 1992a; Nag et al., 1996a; 1996b). The transition of a monolayer from LE to LC phase upon compression can be quantitatively de-

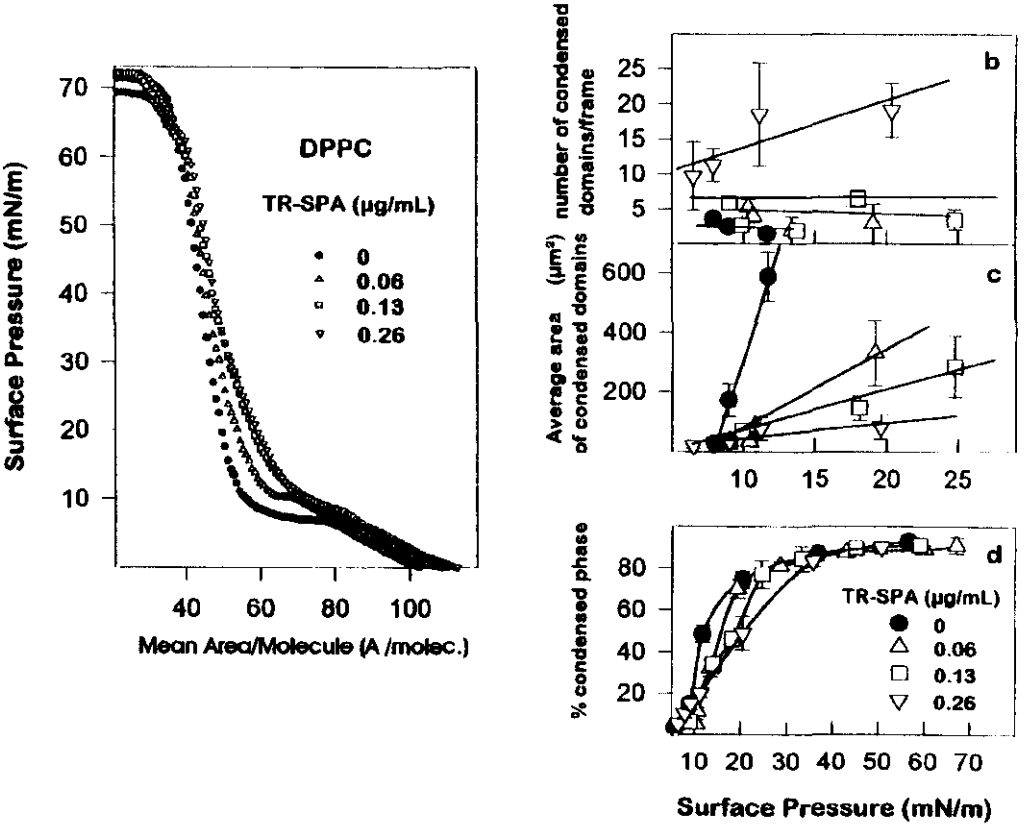

FIGURE 2 (*Left*) Typical Π-A isotherms of DPPC monolayers containing 1 mol % NBD-PC spread on subphases of 150 mM NaCl, 5 mM Tris-HCl, pH 7.4, containing the indicated amounts of TR-SP-A. (*Right*) Quantitative analysis of the effect of TR-SP-A on the condensation of DPPC monolayers containing 1 mol % NBD-PC observed during compression. Dependence of the average area (b), number of the condensed domains (c), and total percent of condensed (probe-excluding) area (*d*) on the surface pressure (Π) is plotted for different protein concentrations in the subphase. Data are given as $\bar{x} \pm$ SD for $n = 10$ images. Where error bars are not shown they are within the symbol size.

scribed by parameters such as the size and number of condensed domains and the percentage of total condensed phase as a function of the compression pressure (Peschke and Möhwald, 1987; Nag et al., 1991; Pérez-Gil et al., 1992a; Nag and Keough, 1993). Such an analysis is shown in Fig. 2 *B* for monolayers formed on subphases in the absence and in the presence of 0.06, 0.13, and 0.26 μg/ml of TR-SP-A. The data indicate that over the range of π of 5–30 mN/m, TR-SP-A 1) increases the number of condensed domains per frame, 2) decreases the average area of condensed domains, and 3) decreases the percentage of total condensed phase at lower surface pressures. Above surface pressures of about 35 mN/m, there were no differences in the total amount of condensed phase in monolayers formed in the absence or presence of TR-SP-A. This is consistent with the observation that SP-A is substantially excluded from DPPC monolayers at that surface pressure (Taneva et al., 1995).

Fig. 3 shows images obtained from a monolayer of DPPC containing 1 mol % of NBD-PC formed on a subphase containing 0.06 μg/ml TR-SP-A for fluorescence selectively coming from either NBD-PC or TR-SP-A. NBD florescence showed phospholipid LC domains with shapes similar to, but somewhat more irregular than, those of the elliptical kidney-bean-shaped condensed domains of pure DPPC (Nag et al., 1991; Pérez-Gil et al., 1992a). It is noted that the fluorescence of TR-SP-A was not observed in the monolayers at very low pressures (below 3–4 mN/m) and only was seen when LC domains began to appear. Changes in the numbers, shapes, and sizes of LC domains have been

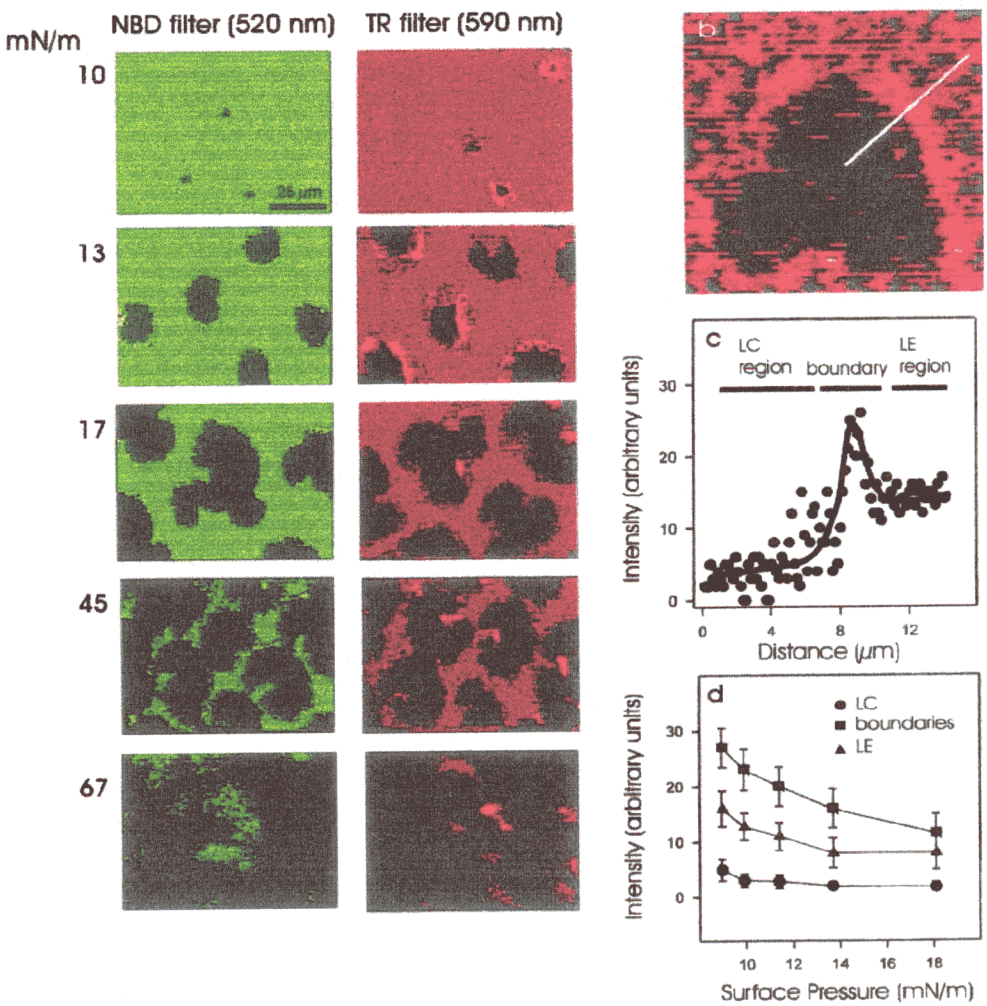

FIGURE 3 (*a*) Typical images obtained from a DPPC monolayer containing 1 mol % NBD-PC spread on a subphase containing 0.06 μg/ml of TR-SP-A at the surface pressures indicated. Images were recorded through filters selecting fluorescence coming either from NBD-PC (emission centered at 520 nm) or TR-SPA (emission centered at 590 nm). (*b*) Typical image of a single condensed domain at a surface pressure of 12 mN/m seen through the TR filter. The *white line* indicates the trajectory that TR fluorescence intensity was quantitatively analyzed in graph *c*. (*c*) Relative fluorescence intensity was evaluated in pixels along the line marked in image *b* and plotted against the distance in μm from the starting point in the dark domain. (*d*) The average fluorescence intensities of the regions defined in graph *c* plotted against surface pressure. Data are presented as $\bar{x} \pm$ SD for $n = 10$ images. Error bars not shown are within the symbol sizes.

described as one of the consequences of lipid/protein interactions in the monolayer (Pérez-Gil et al., 1992a; Nag et al., 1996a; 1996b). At surface pressures between 5 and 30 mN/m, the fluorescence from TR-SP-A showed that it associated with the fluid phase and the condensed/fluid boundaries of the monolayer. Three different regions in the monolayer can be defined according to the average intensity of fluorescence coming from TR-SP-A (Fig. 3, *A* to *C*). LC regions essentially lack any TR-SP-A fluorescence, suggesting either that SP-A does not associate directly with DPPC in LC phase or that TR-SP-A fluorescence is not accessible in the microscope when the protein is associated with the tightly packed headgroups of DPPC condensed domains. LE regions (those showing NBD fluorescence) had homogeneous TR-SP-A fluorescence intensity, suggesting a regular distribution of SP-A in the fluid areas of the monolayer. Finally, an intense ring of TR fluorescence surrounded the DPPC condensed domains in the boundaries between LC and LE regions. As the pressures increased, the TR fluorescence that accumulated at the boundaries decreased (Fig. 3 *A*). Quantitative analysis of the TR fluorescence intensity of the different regions of the monolayer (Fig. 3 *D*) suggests that the average TR-SP-A fluorescence of the LC-LE boundaries converged to the fluorescence intensity of the LE areas at surface pressures above 20 mN/m. At this pressure, however, the total amount of LE phase in DPPC monolayers is relatively small (Nag et al., 1991; Pérez-Gil et al., 1992a).

The effect of TR-SP-A on DPPC/DPPG (7:3, w/w) monolayers is shown in Fig. 4. Fig. 4 *A* shows typical Π-A isotherms of monolayers of DPPC/DPPG (7:3, w/w) containing 1 mol % NBD-PC spread on subphases containing 0, 0.06, 0.13, and 0.26 μg/ml of TR-SP-A. These isotherms are somewhat similar to those obtained for pure DPPC and DPPC/TR-SP-A systems with LE-LC plateaus also in the range 6–9 mN/m. The fluorescence coming from the lipid probe, however, showed a different effect of SP-A on DPPC/DPPG compared with the effect of SP-A on DPPC monolayers. TR-SP-A did not have any significant effect on the number and size of condensed domains or the percentage of total condensed phase of DPPC/DPPG monolayers at any surface pressure. The distribution of TR-SP-A in these phosphatidylglycerol-containing monolayers was also different from that observed for the protein in monolayers of

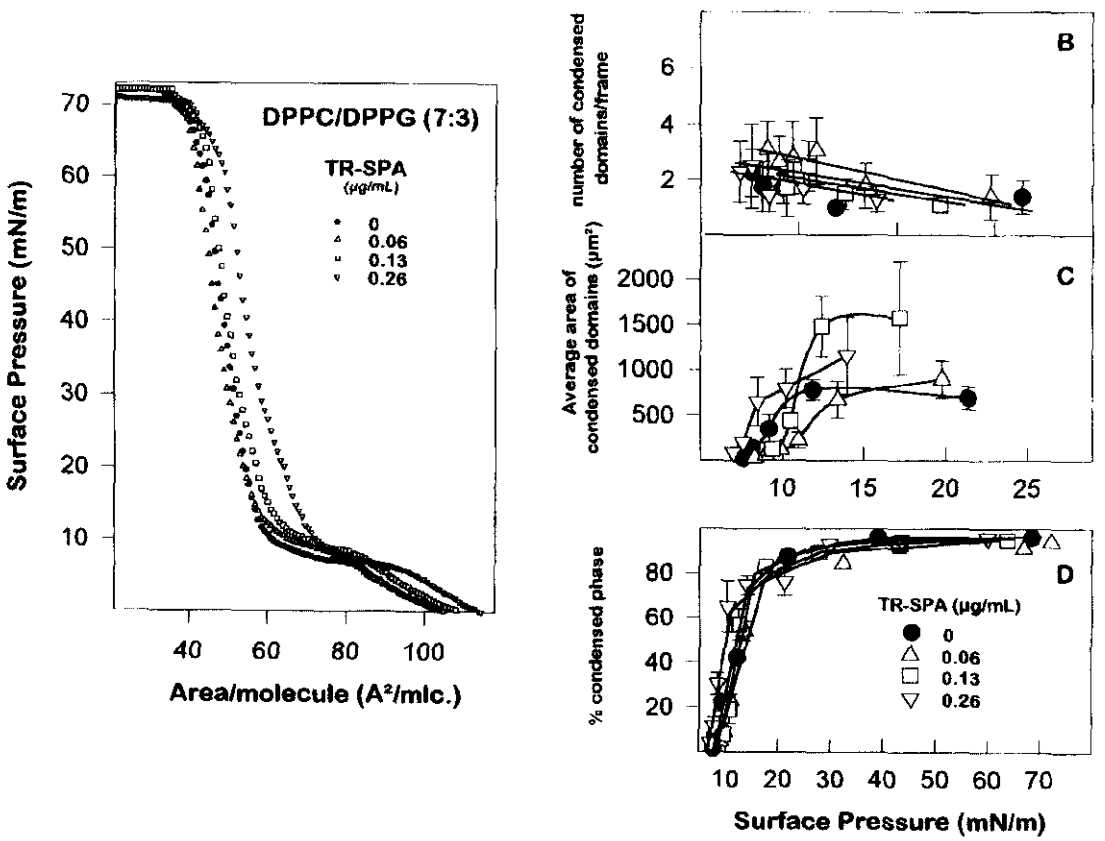

FIGURE 4 (*A*) Typical Π-A isotherms of DPPC/DPPG (7:3, w/w) monolayers containing 1 mol % NBD-PC spread on subphases 150 mM NaCl, 5 mM Tris-HCl, pH 7.4, containing the indicated amounts of TR-SP-A. Number (*B*) and average area (*C*) of the condensed domains and the percent of total condensed area (*D*) are plotted against surface pressure (Π) for various protein concentrations in the subphase. Values are $\bar{x} \pm$ SD for $n = 10$ images. Error bars not shown are within the symbol sizes.

DPPC alone. In DPPC/DPPG monolayers, TR-SP-A was located in discrete aggregates of sizes in the range of 5–10 μm at nearly all surface pressures of the isotherm (Fig. 5). Almost no TR fluorescence could be detected in either LC domains or LE regions. The fluorescent protein aggregates were also preferentially located at the boundaries between LC and LE regions as deduced from the comparison of images taken from both NBD and TR filters (Fig. 5).

To determine if the distribution of SP-A in phospholipid monolayers is specific for this protein, we studied the distribution of another fluorescently labeled glycoprotein, TR-concanavalin A, in DPPC monolayers. Concanavalin A, a tetrameric glycoprotein with a molecular mass of 104 kDa has lectin activity as does SP-A, but to our knowledge it is not a lipid-binding protein. Fig. 6 shows typical images obtained from DPPC monolayers spread on a subphase containing TR-conA at 0.26 μg/ml. Protein fluorescence from these monolayers indicated that the protein formed aggregates similar in size and shape to those formed by SP-A in DPPC/DPPG monolayers. Homogeneously distributed protein fluorescence was not detected in either LC or LE regions. The fluorescent protein patches also showed some preference for distribution next to the borders of the LC domains, although this preference seemed to be less striking than in the case of SP-A in DPPC/DPPG monolayers.

DISCUSSION

These experiments demonstrate that SP-A in the hypophase can interact and associate with interfacial monolayers formed with the main surfactant phospholipid DPPC. The observed association of SP-A with the monolayer could be a consequence of the intrinsic affinity of the protein to interact with lipids, especially DPPC, as previously demonstrated with phospholipid vesicles (King et al., 1983; Casals et al., 1993) or immobilized lipids on silica gel plates or on plastic (Kuroki and Akino, 1991; Kuroki et al., 1992; Childs et al., 1992).

The presence of SP-A in monolayers of DPPC produced similar perturbations in the condensation of the monolayer under compression as those described for other proteins, for instance SP-C (Pérez-Gil et al., 1992a). The magnitude of the perturbation caused by SP-A appears relatively small on the basis of mass of protein but could be significant on the basis of the molar amount of protein associated with the monolayer in comparison with a smaller protein such as SP-C (Pérez-Gil et al., 1992a). As discussed previously (Pérez-Gil et al., 1992a), the influence of the protein to produce more smaller-condensed domains than are seen in the pure lipid likely reflects a compromise of forces between the perturbing influence of protein in the monolayers

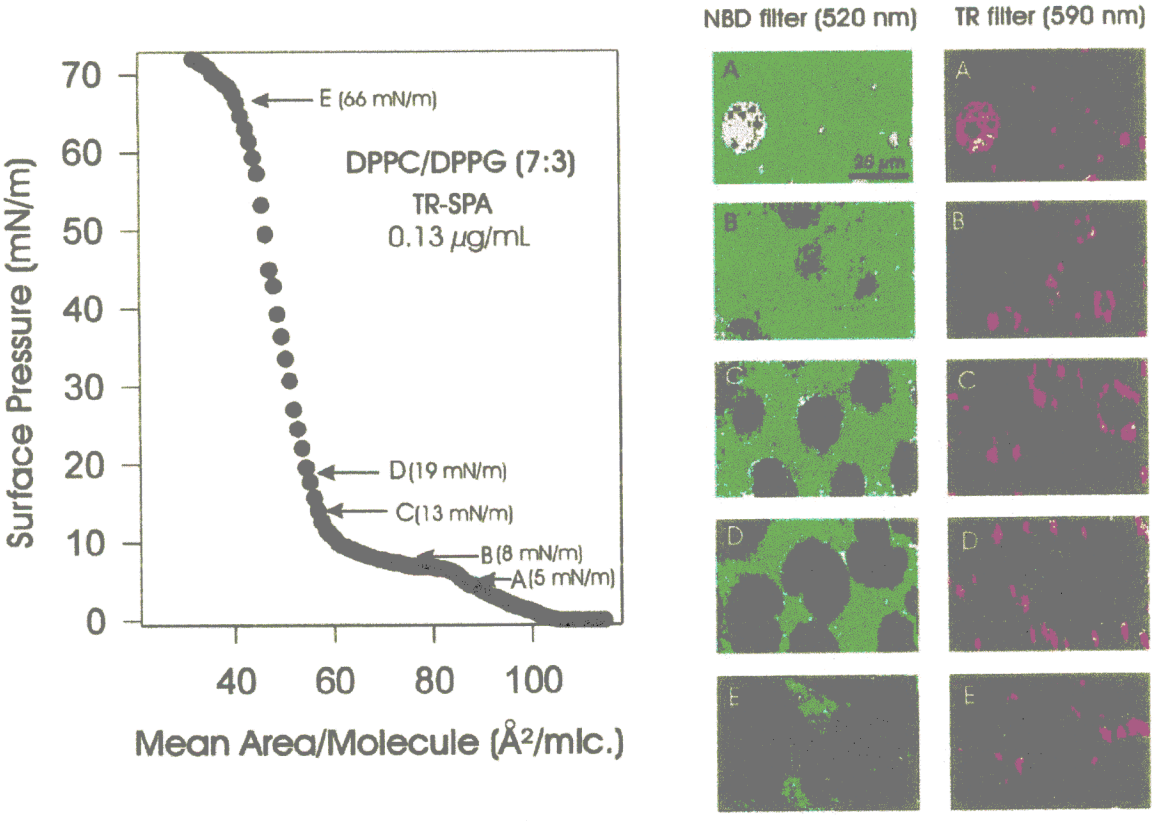

FIGURE 5 Isotherms (*left*) and typical images (*right*) obtained from a DPPC/DPPG (7:3, w/w) monolayer containing 1 mol % NBD-PC spread on a subphase containing 0.13 μg/ml of TR-SP-A. Images were recorded through filters selecting fluorescence coming either from NBD-PC or TR-SP-A.

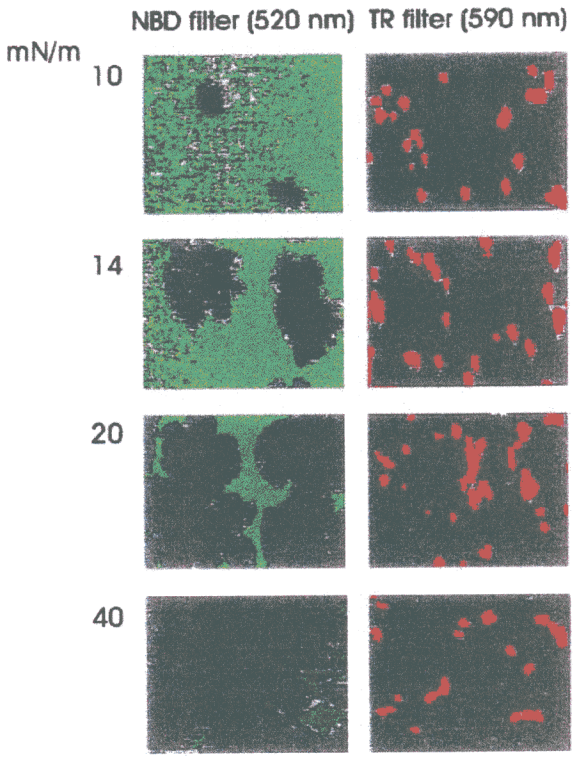

FIGURE 6 Typical images obtained from a DPPC monolayer containing 1 mol % NBD-PC spread on a subphase containing 0.26 μg/ml of TR-conA at the indicated surface pressures. Images were recorded through filters selecting fluorescence coming either from NBD-PC or TR-conA.

resisting condensation and the increasing applied pressure that promotes that condensation. The perturbing influence of SP-A is seen only in the range of π of about 5–30 mN/m (Fig. 2). Above that pressure, SP-A is primarily squeezed-out of DPPC monolayers (Taneva et al., 1995) or into small residual SP-A aggregates in or near the surface (Fig. 2). It is possible that all the proteins with ability to interact with phospholipids in monolayers will show similar effects on the condensation. In terms of its preferential accumulation at the boundaries of LC domains, however, SP-A is dramatically different from SP-C, for example, which shows uniform distribution in the LE phase.

SP-A is a water-soluble protein that can interact with selected lipids. Some of the work on the visual properties of interactions between water soluble protein and monolayer films has been summarized in Ahlers et al. (1990) and Mohwald (1990). For example, a fluorescently labeled analog of pancreatic phospholipase A_2 showed selective association with solid phase domains in DPPC monolayers, especially the edges of the solid domains (Grainger et al., 1990). A fluorescent analogue of the hydrophobic surfactant protein SP-C inserts into the fluid LE regions of DPPC and DPPC/DPPG monolayers (Nag et al., 1996a), consistent with exclusion from gel phase domains of different phospholipid bilayers (Horowitz et al., 1993; Horowitz, 1995). Fluorescently labeled SP-B, also a hydrophobic protein, also reposes in LE regions of spread phospholipid monolayers (Nag et al., 1997). The results presented here show that SP-A is present in LE fluid regions of DPPC monolayers, and it accumulates close to the boundaries between LE and LC domains. Two possible mechanisms could be suggested for this distribution: 1) preferential interaction of SP-A with the monolayer in packing defects at the LC-LE boundaries followed by diffusion of the protein to the fluid regions or 2) direct interaction of SP-A with the lipids in the fluid LE regions and subsequent segregation and accumulation of protein at the LE-LC boundaries. Given that TR-SP-A was not observed in the monolayers at very low surface pressures, alternative 1) seems more likely. The fact that the accumulation of SP-A at the LC-LE boundaries in DPPC monolayers decreases with the extent of compression is most likely because of exclusion of the protein toward the hypophase. It could be possible that some protein denaturation leads to accumulations at the phase boundaries but we note also that active phospholipase A_2 accumulates and acts at such boundaries (Grainger et al., 1990).

The effect of SP-A in DPPC/DPPG monolayers is different compared with that seen in DPPC monolayers. The protein showed almost no effect on the condensation parameters of DPPC/DPPG monolayers. Homogeneous TR-SP-A fluorescence was not detectable in LE regions, but discrete patches close to the LE-LC boundaries were observed. This would be consistent with very little or no interaction of SP-A with negatively-charged monolayers. Similar differential behavior of SP-A was found in solvent-spread monolayers of DPPC and DPPC/DPPG (Taneva et al., 1995), and SP-A interacts more strongly with DPPC than with DPPG or DPPC/DPPG bilayers (Casals et al., 1993). Interaction of SP-A with negatively charged phospholipid bilayers was completely abrogated at low ionic strength (Casals et al., 1993; Ruano et al., 1996), which is consistent with electrostatic incompatibility between protein and acidic lipids at neutral pH. Electrostatic repulsion between DPPG and protein molecules in the monolayer could drive exclusion of SP-A to the observed large protein aggregates into the boundaries between LC and LE domains, regions of the monolayers that are irregularly packed that could act as "sinks" in which lipid-immiscible molecules could most easily accumulate.

Interaction of SP-A with DPPC in the monolayer is consistent with recent results suggesting that SP-A promotes accumulation of DPPC in the surface, perhaps from a sort of DPPC-rich reservoir below the air-water interface (Yu and Possmayer, 1996).

In spite of the immiscibility of SP-A with acidic phospholipids, TR-SP-A still goes into the interfacial monolayers. This could be a consequence of intrinsic surface active properties of the protein itself. Several proteins, particularly glycoproteins, have been reported to possess such intrinsic tensoactive characteristics and can form protein interfacial monolayers by adsorption from the subphase (Ahlers et al., 1990; Heckl et al., 1985; 1987; Mohwald, 1990). We have observed that conA, an acidic glycoprotein that is not

known to interact with phospholipids, also adsorbs into DPPC monolayers with similar aggregation and distribution behavior to TR-SP-A in DPPC/DPPG monolayers. This similarity suggests that the aggregation and accumulation in the LE-LC boundary dislocation is a nonspecific property and that only the different distributions of SP-A in DPPC is specific to these compounds.

Little TR-SP-A fluorescence was observed in interiors of the dark condensed domains of the DPPC monolayers, and some fluorescence was seen in the liquid-expanded regions. King et al. (1983; 1986) have observed that the binding of SP-A to liposomes was greater when the constituent lipids were in the gel state than when they were in the liquid crystalline form. It is noteworthy that there was no appearance of the SP-A molecules at the DPPC monolayer aqueous interface when the lipid is strictly in the liquid expanded phase, which is up to pressures of 3–4 mN/m. Only when condensed domains began to appear did the TR-SP-A appear in the interface. This is consistent with its preferential interaction with gel phase in bilayers or the need for the condensed phase in monolayers. Whereas condensed phase in monolayers, gel phases in bilayers, and lipid-expanded and liquid-crystalline phases are often considered to be roughly equivalent, they are not completely so. The pressures for equivalence, for example, remain under discussion. It seems that the SP-A may be attracted to the dislocations in the condensed-expanded domain and in gel-liquid crystal boundaries, as would any protein with nonspecific binding (e.g., Netz et al., 1996). King et al. (1983) found that binding appeared highest in gel state systems in which dislocations in packing may have been anticipated. The binding of SP-A to DPPC is specific in that it has a different appearance entirely than the binding of SP-A to DPPC/DPPG or of conA to DPPC.

In conclusion, these studies demonstrate that SP-A interacts and perturbs DPPC monolayers, partitioning into the liquid-expanded fluid phase of the phospholipid, and accumulating at the LE-LC boundaries. SP-A causes no effects in monolayers of DPPC/DPPG, the protein being excluded from both LC and LE regions and being accumulated as large protein aggregates in the LE-LC boundaries of these monolayers.

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Interactions of Pulmonary Surfactant Protein A with Phospholipid Monolayers Change with pH

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ABSTRACT The interaction of pulmonary surfactant protein A (SP-A) labeled with Texas Red (TR-SP-A) with monolayers containing zwitterionic and acidic phospholipids has been studied at pH 7.4 and 4.5 using epifluorescence microscopy. At pH 7.4, TR-SP-A expanded the π-A isotherms of film of dipalmitoylphosphatidylcholine (DPPC). It interacted at high concentration at the edges of condensed-expanded phase domains, and distributed evenly at lower concentration into the fluid phase with increasing pressure. At pH 4.5, TR-SP-A expanded DPPC monolayers to a slightly lower extent than at pH 7.4. It interacted primarily at the phase boundaries but it did not distribute into the fluid phase with increasing pressure. Films of DPPC/dipalmitoylphosphatidylglycerol (DPPG) 7:3 mol/mol were somewhat expanded by TR-SP-A at pH 7.4. The protein was distributed in aggregates only at the condensed-expanded phase boundaries at all surface pressures. At pH 4.5 TR-SP-A caused no expansion of the π-A isotherm of DPPC/DPPG, but its fluorescence was relatively homogeneously distributed throughout the expanded phase at all pressures studied. These observations can be explained by a combination of factors including the preference for SP-A aggregates to enter monolayers at packing dislocations and their disaggregation in the presence of lipid under increasing pressure, together with the influence of pH on the aggregation state of SP-A and the interaction of SP-A with zwitterionic and acidic lipid.

INTRODUCTION

The collectin surfactant protein A (SP-A) is, by weight, the major protein associated with pulmonary surfactant, a lipid-protein material that lines the respiratory epithelium, the main biophysical function of which is to reduce the surface tension at the air-liquid interface of alveoli and thereby stabilize the respiratory surface. Most of the surface active properties of surfactant are associated with its phospholipid components. The major lipid, dipalmitoylphosphatidylcholine (DPPC) is responsible for the ability of this material to produce very low surface tensions at low lung volumes (for a recent review, see Johansson and Curstedt, 1997). Other phospholipid species of surfactant, including phosphatidylglycerol (PG), as well as some specific hydrophobic proteins have a role in modulating the physical properties of DPPC to allow for rapid and continuous formation of DPPC-enriched monolayers at the air-liquid interface of alveoli. SP-A improves the adsorption to the surface of preparations composed of the main surfactant phospholipids and the hydrophobic surfactant proteins (Hawgood et al., 1987). SP-A promotes interfacial adsorption of lipid suspensions in the presence but not in the absence of the surfactant proteins SP-B and SP-C (Schürch et al., 1992). In addition, SP-A is necessary for the formation of tubular myelin (Suzuki et al., 1989; Williams et al., 1991; Korfhagen et al., 1996), a unique structure of surfactant in the alveolar spaces, whose presence can be correlated with high surface activity. SP-A-defective surfactant isolated from SP-A knock-out mice had impaired properties compared with normal SP-A-containing surfactant when assayed under limited concentration conditions (Korfhagen et al., 1996). This fact suggests that SP-A could improve surface activity of surfactant under certain circumstances such as pathologically limited availability of surfactant or the presence of inhibitory compounds in the airways. Yu and Possmayer (1996) have suggested that SP-A promotes formation of DPPC-enriched reservoirs attached to the surface that would provide a mechanism of monolayer formation during respiratory mechanics. SP-A and the other collectin present in surfactant, SP-D, possess several activities related to primary host-defense mechanisms in the alveolar spaces (van Golde, 1995; Wright, 1997).

By using epifluorescence microscopy we have recently observed the interaction of SP-A monolayers of DPPC, DPPC/dipalmitoylphosphatidylglycerol (DPPG), and surfactant lipid extracts (Ruano et al., 1998a; Nag et al., 1998). This technique allows direct observation of structural transitions in monolayers such as between liquid-expanded and liquid-condensed states and of fluorescently labeled proteins in regions of the monolayer (Möhwald, 1990; Pérez-Gil et al., 1992; Nag et al., 1996–1998). SP-A associates with fluid regions of DPPC monolayers, but accumulates at boundaries between fluid and condensed domains under conditions of phase coexistence (Ruano et al., 1998a). In DPPC/DPPG monolayers SP-A is effectively excluded even from fluid regions (Taneva et al., 1995; Ruano et al.,

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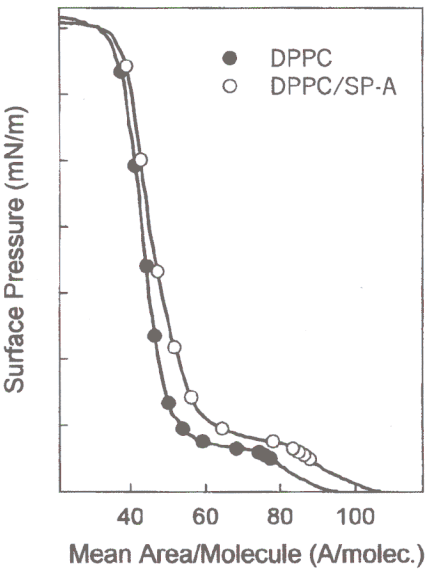

FIGURE 1 Typical Π-A isotherms of DPPC monolayers containing 1 mol % NBD-PC spread on subphases of 150 mM NaCl, pH 4.5 in the absence (*closed symbols*) or presence (*open symbols*) of TR-SP-A at 0.13 μg/ml.

1998a), accumulating in aggregates at boundaries between fluid and condensed regions. This immiscibility has been explained as resulting from electrostatic repulsion between lipids and proteins at physiological pH (Ruano et al., 1998b). Preferential interaction of SP-A with DPPC in comparison to acidic phospholipids has also been detected in phospholipid bilayers (Casals et al., 1993). The preference for DPPC could be the basis for SP-A-directed mechanisms of interfacial DPPC enrichment.

During inter and extracellular trafficking SP-A is likely to encounter milieus in which the pH might vary from ~5.5 to ~7. Changes in the environment from neutral to acidic pH would be expected to potentially modulate SP-A interactions with lipids because of effects on protein change. A recent study has shown that an acidic environment induced changes in the structure and lipid-binding properties of SP-A in bilayers (Ruano et al., 1998b) with the maximal effects being reached at pH 4.5. At that pH, the protein was able to bind to both neutral and acidic phospholipid vesicles, but the occurrence of SP-A-induced membrane aggregation was critically dependent on the presence of negatively charged lipids. To better understand the nature of and to

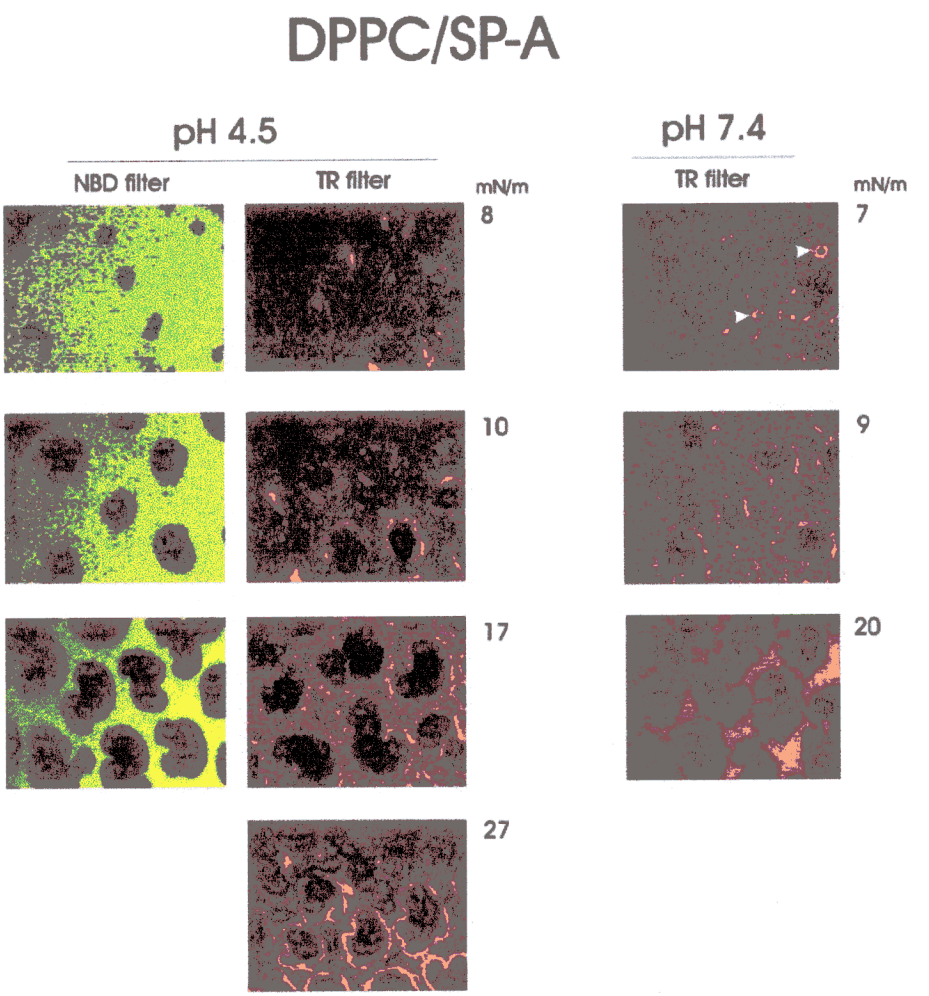

FIGURE 2 Typical images obtained from DPPC monolayers containing 1 mol % NBD-PC spread on a subphase containing 0.13 μg/ml TR-SP-A at pH 4.5 (*left*) or 7.4 (*right*). Images were recorded through filters selecting fluorescence from either NBD-PC (*green*, emission maximum at 520 nm) or TR-SP-A (*red*, emission maximum at 590 nm) at the indicated surface pressures. For pH 7.4, only the TR images are given, but the condensed domains are clearly visible.

visualize the interactions involved in the association of SP-A with surfactant films, the forces driving formation of SP-A domains at the interface and the nature of pH and lipid composition on SP-A self-aggregation, we have studied the interaction of SP-A with surfactant phospholipid monolayers at acidic pH by analyzing the association with and the location of the protein in zwitterionic (DPPC) or acidic (DPPC/DPPG) monolayers using epifluorescence microscopy. This would allow direct visual examination of the hypothesis (Ruano et al., 1998b) that association of SP-A with anionic lipids at low pH could reduce its aggregation state. Because the maximal effects of such changes were seen at pH 4.5 in the previous work with bilayers (Ruano et al., 1998b), we have selected that pH along with pH 7.4 (Ruano et al., 1998a) to make relevant comparisons. It is noted also that interactions in the extracellular milieu are important. While the pH of the aqueous lining layer or hypophase in the alveoli is neutral (Nielson et al., 1981), it can become very acidic in some pathological conditions, such as in hydrochloric acid aspiration trauma (Eijking et al., 1993).

EXPERIMENTAL

Materials

The lipids used in the experiments of this study, 1,2- dipalmitoylphosphatidylcholine (DPPC) and 1,2-dipalmitoylphosphatidylglycerol (DPPG), were from Sigma Chemical Co. (St. Louis, MO). The fluorescent lipid probe 1-palmitoyl-1-{12-[(7-nitro-2–1,3-benzoxadizole-1-yl)amino]dodecanoyl} phosphatidylcholine (NBD-PC) was from Avanti Polar Lipids (Birmingham, AL). The fluorescent labeling chemical sulforhodamine 101 sulfonyl chloride, Texas Red (TR), was obtained from Molecular Probes Inc. (Eugene, OR). Chloroform and methanol were HPLC grade solvents from Fisher Scientific Co. (Ottawa, ON) and all other reagents were analytical grade chemicals from Merck (Darmstadt, Germany).

Isolation and labeling of SP-A

SP-A was purified from pulmonary surfactant prepared from porcine bronchoalveolar lavage as previously described (Casals et al., 1989), by sequential butanol and octylglucoside extractions (Casals et al., 1993), and stored in solution of 5 mM Tris-HCl buffer, pH 7.4, at −20°C. Purity of the protein preparations was routinely checked by SDS-PAGE under reducing conditions followed by Coomassie Blue staining. SP-A was quantitated by amino acid analysis after hydrolysis of protein samples in 0.2 ml of 6 M HCl containing 0.1% (w/v) phenol in evacuated and sealed tubes at 108°C for 24 h followed by analysis in a Beckman System 6300 High Performance amino acid analysis. Fluorescently labeled SP-A was prepared as previously described (Ruano et al., 1998a).

Epifluorescence experiments

Surface pressure-area measurements and microscopic observations of monolayers were performed on an epifluorescence microscopic surface balance, the construction and operation of which have been described elsewhere (Nag et al., 1990, 1991). Phospholipid monolayers including 1 mol % NBD-PC were formed on subphases that did or did not contain SP-A as previously described (Ruano et al., 1998a). Monolayers were spread by depositing aliquots of chloroform/methanol solutions of the lipids on subphases containing 150 mM NaCl, adjusted to pH of either 7.4 or 4.5, in the absence or presence of TR-SP-A 0.13 μg/ml. All subphases were prepared with double-distilled water, the second distillation being from dilute potassium permanganate. After spreading a monolayer the organic solvent was allowed to evaporate for 5 min, and to facilitate SP-A adsorption to the air-liquid interface, the monolayer was compressed rapidly (707 mm^2/s) to a surface pressure of 10 mN/m and then expanded again to 0 mN/m. After a 1-h period allowing for observation of penetration of the protein into the gas or gas-liquid expanded coexistence phases (e.g., Maloney et al., 1995), compression of the monolayer at slow speed (20 mm^2/s or an initial rate of 0.13 $Å^2$/molecule/s) at 23 ± 1°C was begun. At selected surface pressures, a visual recording was made on videotape for a 1-min period for both NBD and TR fluorescence by switching fluorescence filter combinations to select fluorescence emission in the proper wavelength range. The images obtained were analyzed with digital image processing using JAVA 1.3 software (Jandel Scientific, San Rafael, CA) as discussed elsewhere (Nag et al., 1991; Pérez-Gil et al., 1992).

The video images were obtained with a CCD camera, which records in black and white. The images presented in the figures have been false-colored to display them as they appear approximately to the eye in the microscope.

RESULTS

Fig. 1 shows typical compression π-A isotherms of DPPC monolayers spread on subphases of 150 mM NaCl, adjusted to pH of 4.5, in the presence or absence of TR-SP-A at 0.13 μg/ml. As observed at neutral pH (Ruano et al., 1998a), SP-A expanded the isotherms of DPPC at pH 4.5, indicating that either protein insertion at the air-liquid interface or perturbation of lipid packing by the protein, or both, had occurred. Protein-containing monolayers also displayed clear plateaus in the range of 7–9 mN/m, indicative of liquid-expanded to liquid-condensed phase transitions.

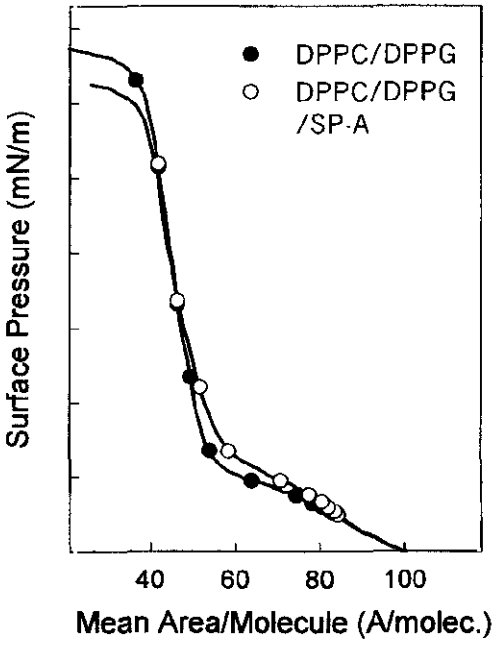

FIGURE 3 Typical Π-A isotherms of DPPC/DPPG (7:3, w/w) monolayers containing 1 mol % NBD-PC spread on subphases of 150 mM NaCl, 5 mM Tris-HCl, pH 4.5 in the absence (*closed symbols*) or presence (*open symbols*) of TR-SP-A at 0.13 μg/ml.

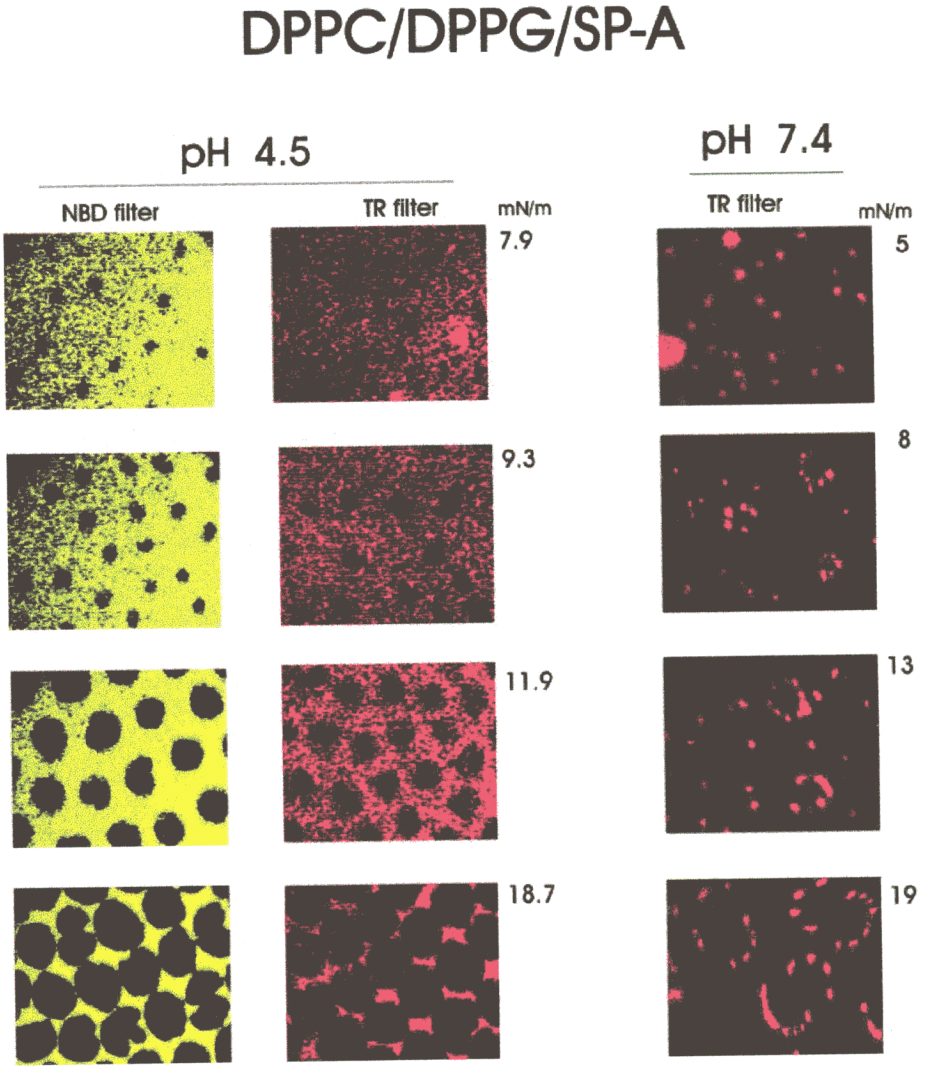

FIGURE 4 Typical images obtained from DPPC/DPPG (7:3, w/w) monolayers containing 1 mol % NBD-PC spread on a subphase containing 0.13 μg/ml TR-SP-A at pH 4.5 (*left*) or 7.4 (*right*). Images were recorded through filters selecting fluorescence coming either from NBD-PC (*green*) or TR-SP-A (*red*) at the indicated surface pressures.

Microscopic images of DPPC monolayers containing 1 mol % NBD-PC in the presence of 0.13 μg/ml TR-SP-A in the subphase at acidic pH, compared with those at neutral pH, are shown in Fig. 2. Fluorescence coming from either the lipid probe or the fluorescently labeled protein was selectively recorded from the same monolayers by switching the filters. Our apparatus does not allow, however, for instantaneous recording of images at two wavelengths. At low pressures there is considerable movement of the monolayer so that fields change more rapidly than filters can be changed. Even at higher pressures the time needed to change filters, possibly refocus, and collect visual data for processing means that fields will have changed somewhat for sequential images. The times involved for the changes are in the range of 5–15 s. The association and correlation of regions of fields can be made more rapidly by eye almost immediately upon changing the filters, but images at the two wavelengths are not superimposable because of monolayer movement between the acquisition of the images.

The association of TR-SP-A with DPPC monolayers at acidic pH is similar to that observed at neutral pH at low surface pressures (Fig. 2). At both pH values the protein starts to interact with the monolayer when condensed solid domains of lipid begin to nucleate (in the range of 6–8 mN/m). This observation has been interpreted as a requirement for gel-like condensed regions or, likely, condensed/fluid coexistence for the interaction of TR-SP-A with monolayers to occur (Ruano et al., 1998a). At surface pressures up to 15 mN/m, TR-SP-A fluorescence appeared at acidic pH in fluid-expanded regions with increased intensity at condensed/fluid boundaries. However, in contrast with the behavior observed at pH 7.4, TR-SP-A accumulation at the boundaries of the DPPC condensed domains never decreased under compression at acidic pH. Compression up to 27 mN/m produced monolayers in which nearly all protein fluorescence accumulated at the perimeters of the solid regions, forming fluorescent rings with the shape of the condensed areas.

Fig. 3 presents Π-A isotherms of DPPC/DPPG 7:3 (w/w) monolayers spread on subphases of NaCl 150 mM, at pH 4.5, in the absence or presence of TR-SP-A 0.13 μg/ml. In contrast with the behavior observed at neutral pH (Ruano et al., 1998a), TR-SP-A hardly expanded the isotherms of DPPC/DPPG monolayers at acidic pH, suggesting that at pH 4.5 the protein does not insert very much into the negatively charged interface. Microscopic images of those monolayers show dramatic differences in the distribution of TR-SP-A in acidic phospholipid-containing monolayers (Fig. 4) compared with the zwitterionic ones (Fig. 2). The distribution of TR-SP-A into DPPC/DPPG monolayers at acidic pH was dramatically different to that observed at neutral pH. At pH 4.5, TR-SP-A appeared to homogeneously distribute into the fluid or expanded phospholipid at any surface pressure up to 25 mN/m, while it was excluded from the interior of the condensed domains. No selective accumulation of TR-SP-A was detected at the solid/fluid boundaries or at any other location in the interface. By contrast, at neutral pH, TR-SP-A was present in DPPC/DPPG monolayers as discrete aggregates, more or less regular in size and distributed preferentially around the condensed lipid domains at any surface pressure. Virtually no protein fluorescence was detected in both condensed domains and fluid regions, indicating complete immiscibility of TR-SP-A and acidic phospholipid at neutral pH. The effect of TR-SP-A on the condensation of the monolayer under compression at acidic and neutral pH was quantitated by measuring the effect of the presence of the protein on the percent of total condensed area of the films. Fig. 5 shows the amount of condensation upon compression of DPPC and DPPC/DPPG monolayers at pH 7.4 or 4.5 in the absence or presence of TR-SP-A. The protein only produced a significant decrease in the total condensation when interacting with DPPC monolayers at neutral pH and at surface pressures in the range 10–30 mN/m. This effect has been attributed to protein-induced perturbation of lipid packing, which reduces condensation of lipid (Pérez-Gil et al., 1992; Nag et al., 1997). Similarly, Fig. 6 shows that TR-SP-A only produced a clear increase in the number of condensed domains in DPPC monolayers at pH 7.4. Similar effects of decreasing the total amount of condensed area while increasing the number of condensed domains have been found for hydrophobic proteins SP-B and SP-C in DPPC and

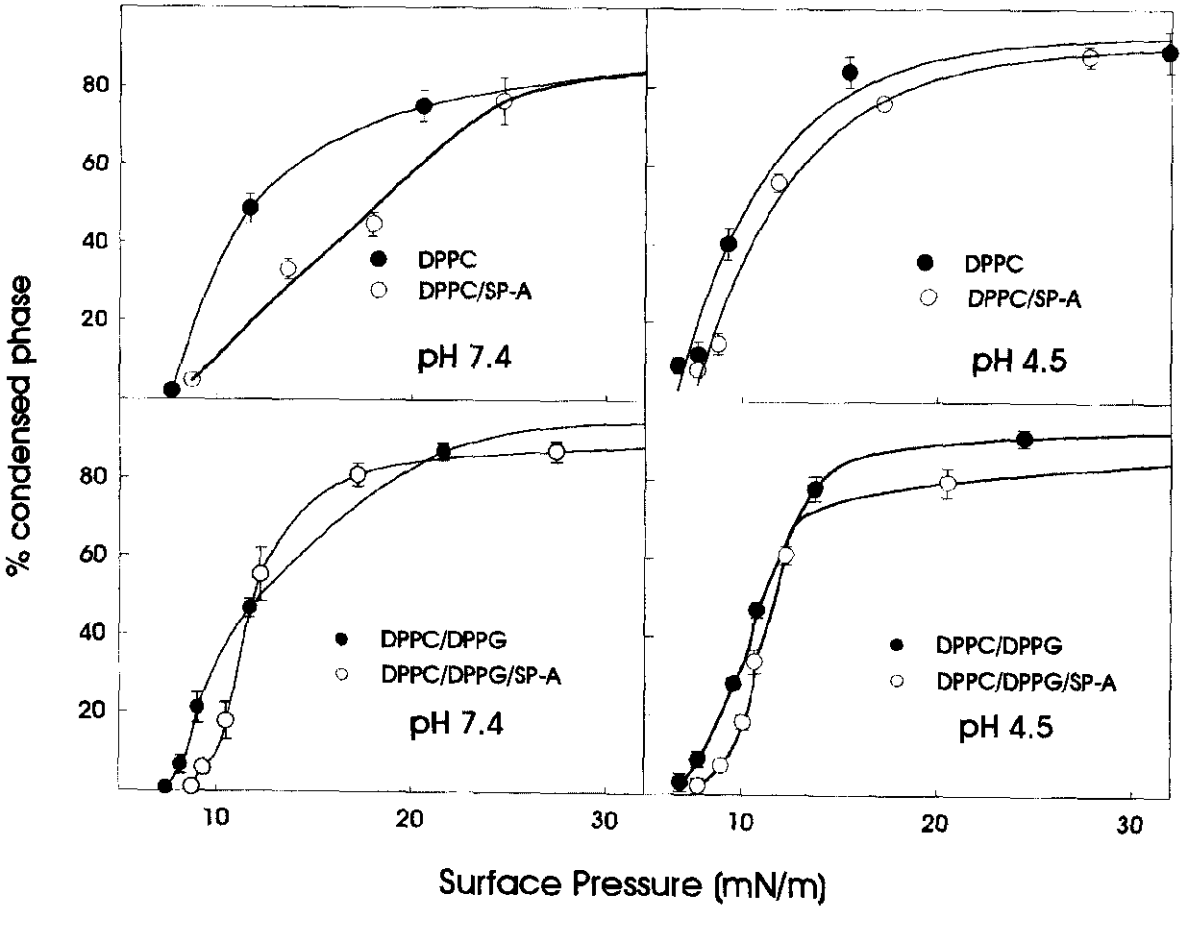

FIGURE 5 Percent of total condensed area plotted against surface pressure for different monolayers in the absence (●) and presence (○) of TR-SP-A at 0.13 μg/ml. Values are $x \pm$ SD for $n = 10$ images. Error bars not shown are within the symbol sizes.

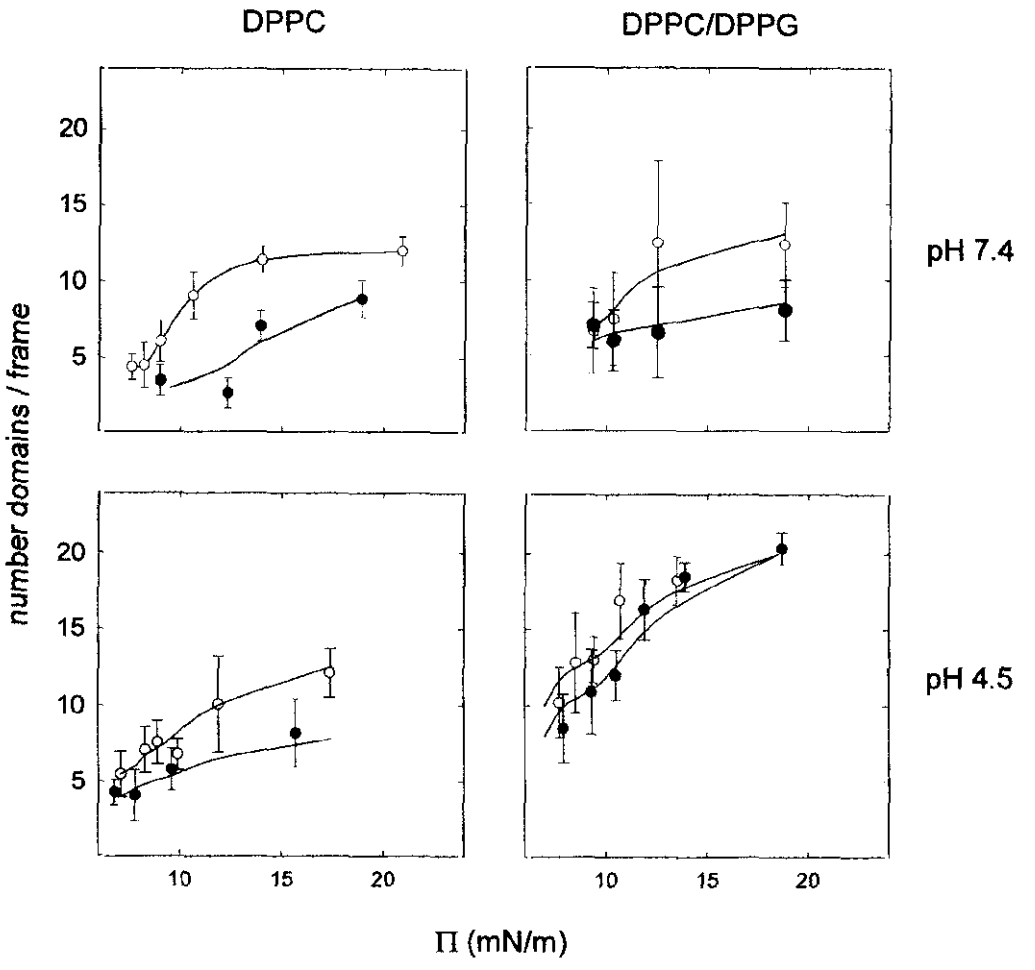

FIGURE 6 Dependence of the number of condensed domains per frame on the surface pressure for different monolayers in the absence (●) and presence (○) of TR-SP-A at 0.13 μg/ml. Values are $x \pm$ SD for $n = 10$ images. Error bars not shown are within the symbol sizes.

DPPG monolayers (Pérez-Gil et al., 1992; Nag et al., 1996, 1997).

DISCUSSION

We have previously found that fluorescently labeled SP-A associates with spread phospholipid monolayers at neutral pH (Ruano et al., 1998a). Features of the interactions of SP-A with DPPC or DPPC/DPPG monolayers at neutral pH correlated with previous observations on the interaction of SP-A with phospholipid bilayers and monolayers (King et al., 1986; Casals et al., 1993; Taneva et al., 1995; Ruano et al., 1996).

In the present work we have explored the effect of pH on both SP-A distribution in, and SP-A modifications of, the condensation during compression of monolayers of DPPC or DPPC/DPPG. In a recent paper we showed that the secondary structure and aggregation state of SP-A and its interaction with neutral and negatively charged phospholipid bilayers were dependent on pH (Ruano et al., 1998b). It was observed that SP-A bound to both neutral and negatively charged vesicles at acidic pH. The binding of negatively charged vesicles to SP-A led, however, to a change in the secondary structure of the protein, which indicated that the level of SP-A self-aggregation decreased after the interaction of the lipid and protein.

In DPPC monolayers at neutral and acidic pH, aggregates of fluorescent SP-A appear at the liquid condensed/liquid expanded boundary regions, indicating that SP-A aggregates are segregated to the lipid packing defects in the monolayer (Ruano et al., 1998a). SP-A accumulation at condensed/fluid boundaries of DPPC monolayers persists even at pressures up to 30 mN/m. These results suggest that protein/protein interactions under such conditions are stronger than lipid/protein interactions, preventing mixing of SP-A with phospholipids. Results with DPPC monolayers correlate with those previously reported with DPPC bilayers, where we showed that the binding of DPPC vesicles to SP-A at pH 4.5 did not have any effect on the state of self-aggregation of the protein (Ruano et al., 1998b). However, the insertion of aggregates of protein in condensed/fluid boundaries of DPPC monolayers at pH 4.5 caused

almost no effect in both amount of total condensation and number and size of condensed domains. This would also be a consequence of nearly total segregation of lipid and protein at acidic pH.

As previously observed the distribution of SP-A in DPPC/DPPG monolayers at neutral pH indicated that protein was aggregated and there was, effectively, natural exclusion of protein and lipid (Ruano et al., 1998a). For DPPC/DPPG monolayers at pH 4.5, the distribution of SP-A was remarkably different from that at pH 7.4, the SP-A being fairly homogeneously disbursed in the liquid-expanded phase of the monolayers, suggesting that the interactions at lower pH came about through a more dispersed form of SP-A and the lipid. This would have occurred because lowering the pH would have reduced negative charge on the protein (and lipid), reducing mutual exclusion. Since the maximal self-aggregation of SP-A occurred at pH 4.5 (Ruano et al., 1998b), other forces, however, must also be dominating the distribution of SP-A in DPPC/DPPG at pH 4.5. These findings are consistent with the view that the adsorption of TR-fluorescent SP-A to DPPC/DPPG, but not to DPPC, monolayers reverses protein self-aggregation. Taken together, these results show different modes of interaction of SP-A with monolayers of DPPC and DPPC/DPPG at pH 4.5 and reinforce the results of previous studies on the interaction of SP-A with DPPC and DPPC/DPPG bilayers at the same pH. SP-A is able to decrease the amount of condensation upon compression and to increase the number of condensed domains in monolayers of DPPC, but not in those of DPPC/DPPG, at neutral pH. At acidic pH SP-A did not affect the amounts or numbers of domains of either type of monolayer. These observations are consistent with a selective interaction of SP-A with DPPC, which is pH-dependent. Perturbation of lipid packing was also reported for the hydrophobic surfactant protein SP-C, which decreased the total amount of condensed area and produced more, smaller condensed domains in monolayers of either DPPC or DPPC/DPPG (Pérez-Gil et al., 1992; Nag et al., 1996). The perturbing influence of SP-A in monolayers is more selective, since it is only observed in DPPC monolayers at neutral pH. Under such conditions, globular "headgroups" of SP-A (comprising lipid binding domains) would interact with acyl chains of phospholipid monolayers sufficiently to perturb the usual lipid packing. The perturbation of lipid packing induced by SP-A could be important for the selective insertion of DPPC into the monolayer at low compression rates (Schürch et al., 1992; Yu and Possmayer, 1996). Yu and Possmayer (1996) suggested that SP-A could form bridges between the interfacial monolayer and subphase bilayers, contributing to the establishment of a surfactant surface reservoir. In addition, it was recently showed that SP-A mediates transfer and exchange of phospholipids between the outer monolayers of membranes (Cajal et al., 1998). Taking into account that SP-A selectivity interacts with DPPC-rich bilayers (Casals et al., 1993) and monolayers (Ruano et al., 1998a) at neutral pH, it would be possible that contacts mediated by SP-A between the interfacial monolayer and subphase bilayers are important for replenishment of DPPC in the monolayer at physiological pH. The pH dependence of the interactions of SP-A with lipids may also have impact on the processing of surfactant in the endocytic pathway (Beers, 1996).

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DISCUSIÓN

Los experimentos realizados demuestran que, a pH neutro, la SP-A porcina se une a monocapas de DPPC y altera su estado de condensación. Su distribución en la monocapa es distinta a la de las proteínas hidrofóbicas del surfactante SP-B y SP-C (Nag *et al.* 1996, Nag *et al.* 1997), ya que tiende a acumularse en los bordes entre los dominios LC (líquido condensado) y LE (líquido expandido). Esta distribución parece deberse a que la SP-A interacciona con la monocapa preferentemente en aquellas zonas donde hay defectos de empaquetamiento, como los que se forman entre los dominios LC y LE al comprimir la monocapa. Posteriormente, la proteína difundiría desde estos bordes a las regiones LE.

La presencia de DPPG en la monocapa, en cambio, hace que la SP-A no produzca cambios significativos en el estado de condensación de los lípidos. En este caso, la distribución de la proteína es diferente. En efecto, ahora no se detecta SP-A en las regiones LE, sino que aparece formando grandes agregados próximos a los bordes LC/LE. Es posible que la repulsión electrostática entre la SP-A (con carga neta negativa a pH neutro) y la DPPG empuje a la proteína hacia los bordes LC/LE, zonas en las que, debido a su empaquetamiento irregular, se acumularían más fácilmente las moléculas inmiscibles con los lípidos de la monocapa. Esta distribución podría tener un carácter inespecífico, ya que hemos observado el mismo efecto para otra proteína hidrofílica como es la concanavalina A.

La interacción específica de la SP-A con DPPC en monocapas y bicapas (Casals *et al.* 1993) podría facilitar la acumulación de este lípido en la interfase aire/agua, favoreciendo las propiedades tensoactivas del surfactante pulmonar. Es posible que esta acumulación se produzca a partir de un reservorio rico en DPPC situado debajo de la superficie, como ya han sugerido otros autores (Yu y Possmayer 1996).

En los ensayos realizados a pH ácido hemos podido comprobar que el patrón de distribución de la SP-A en monocapas de DPPC es similar al que se observa a pH neutro. En este caso, sin embargo, el estado de condensación de los lípidos de la monocapa no se ve afectado por la presencia de la proteína. Esto podría deberse a que la SP-A se encuentra en estado agregado a pH ácido,como vimos en el capítulo anterior.

A pH 4.5, la SP-A tiene carga neta neutra o positiva, con lo que desaparece la repulsión electrostática con las moléculas de DPPG en monocapas de DPPC/DPPG. Así, a este pH vemos que la distribución de la SP-A cambia radicalmente: en vez de formar los grandes agregados proteicos observados a pH neutro, encontramos que la SP-A se distribuye homogéneamente en la fase LE. Es posible que esto se deba a que, en presencia de fosfolípidos ácidos, las interacciones lípido-proteína prevalezcan sobre las interacciones proteína-proteína, como vimos que ocurría en el capítulo anterior con bicapas de igual composición. En este caso, además, tampoco se aprecia una expansión importante de la isoterma de DPPC/DPPG en presencia de SP-A, lo que sugiere que la interacción de la proteína con los lípidos de la monocapa es principalmente de naturaleza polar, con escasa penetración de la SP-A. Esto último también apoya la observación hecha en el capítulo anterior de que la interacción entre SP-A y DPPG es de carácter electrostático.

Los resultados obtenidos en estos trabajos refuerzan la idea de la existencia de diferentes modos de interacción entre la SP-A y los lípidos en función del pH, lo que podría afectar a:

1. El tráfico, almacenamiento, secreción y adsorción a la interfase aire/agua de los componentes del surfactante pulmonar.

2. Las propiedades de la SP-A en determinadas patologías en las que el pH alveolar se encuentra disminuido, como por ejemplo en el trauma por aspiración de ácido clorhídrico (Eijking *et al.* 1993).

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5. ESTUDIO DE LAS DISTINTAS FORMAS DE AUTOAGREGACIÓN DE LA SP-A

INTRODUCCIÓN

En los dos capítulos anteriores hemos centrado el estudio de la SP-A principalmente en su interacción con los lípidos del surfactante pulmonar. Sin embargo, también hemos visto que las interacciones proteína-proteína podrían estar implicadas en características funcionales de la SP-A tales como la inducción de agregación de vesículas lipídicas o su acumulación en gránulos de secreción. Este tipo de interacciones pueden complementarse o bien competir con las interacciones de tipo lípido-proteína. Lo primero ocurre por ejemplo en el mecanismo propuesto en el capítulo 3 para la agregación lipídica: tras unirse la SP-A a los lípidos, la autoagregación de las moléculas de proteína provocaría la agregación de las vesículas. El segundo caso es el que parece darse a pH ácido, donde la presencia de vesículas de DPPC es incapaz de disminuir el estado de alta autoagregación de la SP-A, mientras que la presencia de vesículas de fosfolípidos ácidos hace que las interacciones de tipo lípido-proteína prevalezcan sobre las de tipo proteína-proteína responsables de la autoagregación.

En este último capítulo de resultados se han investigado las características de los diferentes modos de autoagregación de la SP-A, estudiando la influencia de la concentración de Ca^{2+}, la fuerza iónica, el pH y la temperatura en este fenómeno.

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SELF-AGGREGATION OF SURFACTANT PROTEIN A[†]

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ABSTRACT: Environmental factors of physiological relevance such as pH, calcium, ionic strength and temperature can affect the state of self-aggregation of surfactant protein A (SP-A). We have studied the secondary structure of different SP-A aggregates and analyzed their fluorescence characteristics. We found that self-aggregation of SP-A can be: a) Ca^{2+}-dependent. The concentration of Ca^{2+} needed for half-maximal self-association ($K_a^{Ca\,2+}$) depended on the presence of salts. Thus, at low ionic strength K_a^{Ca2+} was 2.3 mM whereas at physiological ionic strength K_a^{Ca2+} was 2.35 μM. Circular dichroism and fluorescence measurements of Ca^{2+}-dependent SP-A aggregates indicated that those protein aggregates formed in the absence of NaCl are structurally different than those formed in its presence. b) pH-dependent. Self-aggregation of SP-A induced by H^+ was highly influenced by the presence of salts, which reduced the extent of self-association of the protein. The presence of both salts and Ca^{2+} attenuated even more the effects of acidic media on SP-A self-aggregation. c) Temperature-dependent. At 20°C, SP-A underwent self-aggregation at physiological but not at low ionic strength, in the presence of EDTA. All of these aggregates were dissociated by either adding EDTA (a) or increasing pH to neutral pH (b) or increasing temperature to 37°C (c). Dissociation of Ca^{2+}-induced protein aggregates at low ionic strength was accompanied by an irreversible loss of both SP-A secondary structure and SP-A-dependent lipid aggregation properties.. On the other hand, temperature-dependent experiments indicated that a structurally intact collagen-like domain was required for either Ca^{2+}- or Ca^{2+}/Na^+-induced SP-A self-aggregation but not for H^+-induced protein aggregation.

Of the four known surfactant proteins, SP-A[1] is the most abundant. It is a multifunctional protein capable of binding several ligands including phospholipids, carbohydrates and Ca^{2+} and it belongs to the Ca^{2+}-dependent lectin family (for reviews, see Refs.1 and 2). SP-A is a large oligomeric protein of approx. 650 kDa, composed of 18 nearly identical subunits. Each SP-A subunit contains an amino-terminal collagen-like domain and a carboxyl-terminal carbohydrate recognition domain (CRD) that are linked by a more hydrophobic domain (neck). This monomeric form is about 228 amino acid long with an approximate molecular weight of 28,000. Several post-translational modifications, including glycosylation, sulfation and hydroxylation of prolines, result in a monomeric protein of heterogeneous molecular mass ranging from 30 to 40 kDa. The functional form of SP-A is assembled through interactions in the collagen-like domain into a complex octadecameric structure, which resembles a floral bouquet, where the amino-terminal and part of collagen-like regions form a common stalk.

Nearly all alveolar SP-A is complexed with phospholipids. The CRD and the neck region are important protein domains involved in SP-A-lipid interactions (3-4). SP-A preferentially interacts with dipalmitoylphosphatidylcholine (DPPC) (5-7), the main surfactant lipid (1). The interaction of DPPC with SP-A leads to a conformational change on the protein molecule (6) and a marked protection of SP-A from trypsin degradation (8). The interaction of SP-A with phospholipid vesicles induces vesicle aggregation in the presence of Ca^{2+} or H^+ (6, 8-10). SP-A also induces aggregation of liposomes containing SP-B or SP-C or both hydrophobic proteins (9). The association of SP-A with lipids appears essential for the conversion of lipid aggregates from multilamellar forms present in the exocytic granule to dispersed ordered arrays known as tubular myelin (11). This physical transformation also requires the hydrophobic surfactant protein SP-B and Ca^{2+} (12). SP-A enhances adsorption of lipids along the air/liquid

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[1] Abbreviations: CD, circular dichroism; CRD, carbohydrate recognition domain; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; DPPG, 1,2-dipalmitoyl-*sn*-glycero-3-phosphoglycerol; K_{SV}, Stern-Volmer dynamic quenching constant; K_a^{Ca2+}, calcium activation constant; SDS/PAGE, polyacrylamide gel electrophoresis in the presence of sodium dodecylsulfate; SP-A, -B, -C, and -D, surfactant protein A, B, C, and D, respectively.

interface in a concerted action with SP-B (13). In addition, SP-A as well as SP-D, another water-soluble surfactant protein, participates in lung-specific host innate defense (14, 15).

The solubility of lipid-free SP-A in stock solutions changes significantly with the buffer conditions. SP-A is insoluble in physiological saline at room temperature, but solubilizes readily in low ionic strength buffers (16). Others and we previously found that Ca^{2+} induced a rapid self-aggregation of pig, dog or human SP-A at neutral pH and in low ionic strength buffers (8, 16). The threshold concentration of Ca^{2+} required to induce self-aggregation of SP-A from different species was 0.5 mM (8, 16). However, the presence of trace amounts of Ca^{2+} were enough to induce SP-A self-aggregation in physiological ionic strength buffers at 37°C (17). In addition, we recently found that SP-A could undergo pH-dependent self-aggregation in the absence of Ca^{2+} and salts in the pH range of 4.5-6.0 (9). The state of self-aggregation of SP-A may have important effects on its functions. Soluble SP-A did not stimulate macrophage production of reactive oxygen but did stimulate when SP-A was adhered to a surface resulting in a multivalent presentation (18, 19).

To better characterize the process of self-aggregation of SP-A we have investigated the effect of ionic strength, Ca^{2+}, pH and temperature on this phenomenon. The structural characteristics of different protein aggregates were studied by circular dichroism and fluorescence spectroscopy.

EXPERIMENTAL PROCEDURES

Isolation of SP-A. Pulmonary surfactant was prepared from pig or human bronchoalveolar lavage as described previously (20). SP-A was purified from isolated surfactant using sequential butanol and octyl glucoside extractions (13). The purity of SP-A was checked by one-dimensional SDS/PAGE in 16% acrylamide under reducing conditions (50 mM dithiothreitol). Quantification of SP-A was carried out by amino acid analysis in a Beckman System 6300 High Performance analyzer. The protein hydrolysis was performed with 0.2 ml of 6 M HCl, containing 0.1% (w/v) phenol in evacuated and sealed tubes at 108°C for 24 h. Norleucine was added to each sample as internal standard. Experiments presented in this paper were performed with at least four different preparations of porcine SP-A. Some experiments were repeated with human SP-A from multiple organ donor lungs.

SP-A Self-aggregation Assays. Self-aggregation assays were performed at 37°C (unless otherwise stated) by measuring the change in protein absorbance at 360 nm in a Beckman DU-640 spectrophotometer, induced by the presence of calcium ions, H^+ or low temperature. Ca^{2+}-dependent self-aggregation experiments were done as described previously (8). The calcium requirement for self-aggregation was studied by titration experiments in which increasing amount of a concentrated solution of $CaCl_2$ were added to the protein solution (40 μg/ml, final concentration) in 50 μM EDTA, 5 mM Tris/HCl buffer, pH 7.4, either with or without 150 mM NaCl. The absorbance at 360 nm was registered once it was stabilized after each change of Ca^{2+} concentration. The free Ca^{2+} concentration in each point of the titration experiments was estimated by a computer program (CHELATOR) (21), which also permits correction for ionic strength, temperature, pH and other competing ions. pH-dependent self-aggregation experiments were done as previously described (9) The variation of SP-A self-aggregation as the pH was reduced was studied at 37°C in the presence or absence of 5 mM Ca^{2+} and either with or without salts (150 mM NaCl or 150 mM KCl). pH titration was started at neutral pH in 5 mM Tris, 5 mM MES, 5 mM acetate buffer, pH 7.2, in the presence or the absence of $CaCl_2$ and/or salts. From the starting pH, SP-A was titrated to pH 4.2 by addition of 1-2 μl aliquots of 0.05 M or 0.1 M HCl solution. During titration, the medium pH was monitored with a micro-pH electrode. The absorbance at 360 nm at each pH was registered once it was stabilized after each change of pH. To find out if SP-A was able to undergo self-aggregation at neutral pH by lowering temperature, the sample and reference cuvette were filled with 0.1 mM EDTA, 5 mM Tris/HCl, pH 7.4 either with or without 150 mM NaCl. After 10-min equilibration at 20°C, SP-A (30 μg/ml, final concentration) was added to the sample cuvette and the absorbance was monitored at 1-min intervals over 10 min. Next, the temperature was raised to 37°C and the absorbance was recorded again. In other set of experiments, self-aggregation of SP-A was studied as a function of temperature. The temperature was lowered from 40°C to 15°C or raised in reverse order. The absorbance at 360 nm was registered once it was stabilized after each temperature change.

Phospholipid Vesicle Aggregation Assay. DPPC vesicles were prepared in a buffer containing 150 mM NaCl, 5 mM Tris/HCl, pH 7.4 by sonication as described previously (8, 9). DPPC vesicle diameter at 37°C, determined by quasielastic light scattering, was around 130 nm.

The vesicle aggregation assay was done as a continuation of Ca^{2+}-dependent self-aggregation experiments after dissociation of protein aggregates by addition of EDTA. Then, 1,2-dipalmitoylphosphatidylcholine (DPPC) vesicles (200 μg/ml) (lipid/SP-A weight ratio 10:1) were added to both the sample and the reference cuvette and the turbidity change at 360 nm was monitored at 1-min intervals over 10 min.

Afterwards, Ca^{2+} (1 mM, final concentration) was added to both cuvettes and the change in absorbance was monitored again. EDTA was used again for reversing vesicle aggregation.

CD Measurements. CD spectra were obtained on a Jasco J-715 Spectropolarimeter fitted with a 150 W xenon lamp (9). Quartz cells of 1-mm path length were used and the spectra were recorded in the far-u.v. region (190-260 nm) at 50 nm/min scanning speed and at the indicated temperature. Four scans were accumulated and averaged for each spectrum. The acquired spectra were corrected by subtracting the appropriate blanks, subjected to noise-reduction analysis and presented as molar ellipticities (degrees·cm^2·$dmol^{-1}$) assuming 110 Da as the average molecular mass per amino acid residue. At least 3 independent preparations of SP-A were measured. Final SP-A concentration was 80 μg/ml. Measurements at acidic or neutral pH were done in 5 mM acetate buffer, pH 4.5, or in 5 mM Tris/HCl buffer, pH 7.2, respectively with or without either NaCl or Ca^{2+}.

Fluorescence measurements. All fluorescence experiments were carried out on a Perkin Elmer MPF-44E spectrofluorimeter operated in the ratio mode as in (6). Cells of 0.2 cm optical-path were used. The slit widths were 7 nm and 5 nm for the excitation and emission beams, respectively. Fluorescence spectra of SP-A were measured at 20°C or 37°C in 0.3 ml of 5 mM Tris/HCl buffer, pH 7.4, in the absence or the presence of either 5 mM Ca^{2+} or 5 μM Ca^{2+}/150 mM NaCl. The final protein concentration of SP-A was in the range 6.7 to 10 μg/ml. The blanks and protein samples were excited at 275 nm for measuring the total protein fluorescence spectrum or at 295 nm to preferentially excite tryptophan residues. Emission spectra were recorded from 300 to 400 nm.

The change in fluorescence emission intensity of SP-A upon addition of millimolar concentrations of Ca^{2+} was done as follows: first, the fluorescence spectrum of SP-A (2 μg) at an excitation wavelength of 275 nm was recorded in 0.3 ml of 5 mM Tris/HCl buffer, pH 7.4, 5 μM Ca^{2+}, either with or without 150 mM NaCl. Subsequently, the titration experiment was started by adding increasing amounts of a concentrated solution of $CaCl_2$ to the protein solution in the cuvette. The fluorescence intensity readings were corrected for the dilution caused by aliquot addition. The change in fluorescence emission intensity of SP-A on addition of NaCl was analyzed as described before in 5 mM Tris/HCl, pH 7.4, 5 μM Ca^{2+} either with or without 0.1 mM EDTA.

Quenching experiments by acrylamide were performed at an excitation wavelength of 295 nm to preferentially excite tryptophan residues and to reduce the absorbance by acrylamide. The quenching experiments were carried out as follows: SP-A (7 μg/ml) was added to the cell containing 5 mM Tris/HCl buffer, pH 7.4, with or without either 2 mM Ca^{2+} or 5 μM Ca^{2+}/150 mM NaCl. After 10 min equilibration at 20°C the spectrum of SP-A was recorded. Afterwards aliquots from a stock solution of acrylamide in water were added to the protein solution and fluorescence spectra were recorded. The values of fluorescence intensity at 330 nm were corrected for dilution and the scatter contribution derived from acrylamide titration of a blank. In addition, an inner filter correction for the absorbance by acrylamide was done. This correction factor was determined according to the equation ($F_c = F_m \cdot 10^{(Aex+Aem)/2}$) (22) in which F_c is the corrected fluorescence intensity, F_m is the measured fluorescence intensity after correction for scattering, and A_{ex} and A_{em} are the absorbances measured at the excitation wavelength (295 nm) and at the emission wavelength (330 nm), respectively. The absorbance of the samples was measured after each addition of acrylamide by using a Beckman DU-640 spectrophotometer.

Quenching studies were analyzed according to the classical Stern-Volmer equation for collisional quenching (23): $F_o/F = 1 + K_{sv}[Q]$, where F_o and F are the corrected emission intensities in the absence and presence of the quencher [Q], and K_{sv} is the Stern-Volmer dynamic quenching constant. K_{sv} values were calculated by the regression of the initial linear portion of the Stern-Volmer plot.

RESULTS

Effect of ionic strength on Ca^{2+}-dependent self-aggregation of SP-A. Figure 1 A shows that addition of SP-A (50 μg/ml) to 5 mM Tris/HCl buffer, pH 7.4 containing 150 mM NaCl and micromolar concentrations of Ca^{2+} (5 μM) resulted in a marked increase in light absorbance due to self-aggregation of the protein. Light absorbance did not change after a further addition of Ca^{2+} (5 mM, final concentration). The addition of EDTA (10 mM) reversed completely the process. Furthermore, the presence of EDTA (1 mM) in the buffer prevented the self-aggregation of SP-A occurring at physiological ionic strength and micromolar concentrations of Ca^{2+} (data not showed). Figure 1 A also shows that in the absence of NaCl, SP-A did not aggregate unless Ca^{2+} was added at millimolar concentrations. The extent of Ca^{2+}-dependent self-aggregation reached at low ionic strength is higher than that reached at physiological ionic strength. Figure 1 B and C show the calcium dependence of the self-aggregation process of pig SP-A in the absence (B) and the presence (C) of NaCl. The Ca^{2+} activation constant (K_a^{Ca2+}) for self-aggregation of pig SP-A depended on the presence of salts. At low ionic strength the concentration of Ca^{2+}

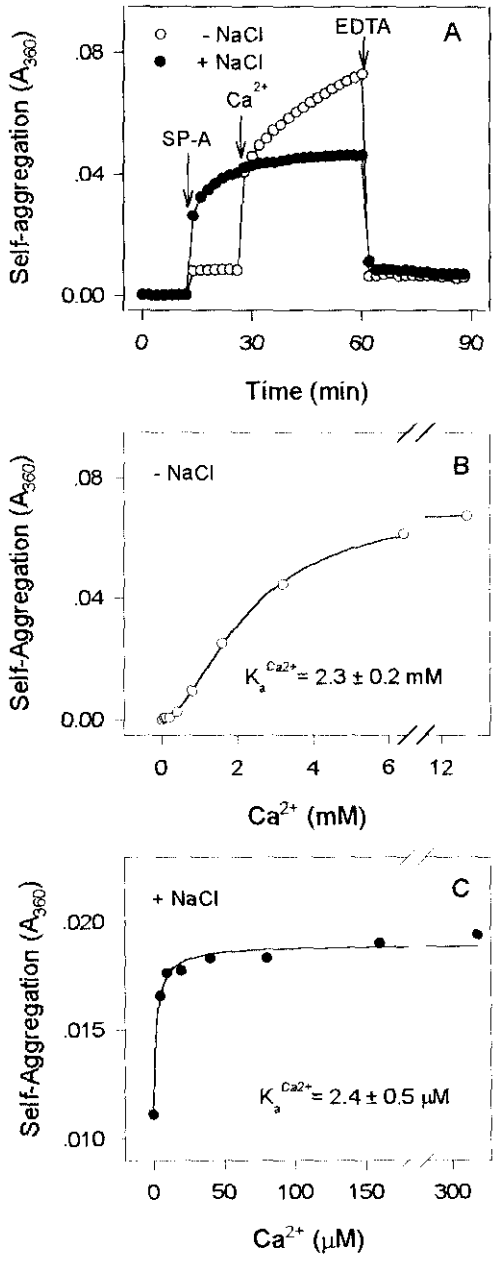

FIGURE 1: Ca^{2+}-dependent self-aggregation of porcine SP-A in the presence and the absence of NaCl. *A*, Kinetics of Ca^{2+}-dependent self-aggregation of SP-A. SP-A (50 µg/ml) was added to the sample cuvette filled with 5 µM Ca^{2+}, 5 mM Tris/HCl buffer, pH 7.4, either with (solid circles) or without (open circles) 150 mM NaCl. The turbidity change at 360 nm was monitored at 37°C at 1-min intervals. After stabilization, 5 mM Ca^{2+} (final concentration) was added to both the sample and reference cuvette and the turbidity changes monitored again. Addition of EDTA (10 mM, final concentration) completely dissociated SP-A aggregates induced by either Ca^{2+} or Ca^{2+}/NaCl. *B*, Calcium dependence of SP-A self-aggregation in the absence of NaCl. *C*, Calcium dependence of SP-A self-aggregation in the presence of 150 mM NaCl. In *B* and *C*, the final concentration of SP-A was 40 µg/ml. Experiments were done at 37°C. A representative experiment of four experiments from four different preparations of porcine SP-A is shown.

required for half maximal self-association was 2.3 ± 0.2 mM as previously reported (8). In contrast, at physiological ionic strength, K_a^{Ca2+} was 2.4 ± 0.5 µM. Thus, the presence of salts drastically decreased the concentration of Ca^{2+} required to set out this process. To find out whether the effect of ionic strength on Ca^{2+}-dependent self-aggregation of pig SP-A occurred also in other SP-A species, we repeated the experiments with human SP-A isolated from multiple organ donor lungs. The Ca^{2+} concentration for half-maximal self-aggregation of human SP-A was 3 ± 0.1 mM at low ionic strength and 12 ± 1.8 µM at physiological ionic strength.

Figure 2 A shows fluorescence emission spectra of porcine SP-A in its non-aggregated form and in its aggregated form induced by either 5 mM Ca^{2+} at low ionic strength or 5 µM Ca^{2+} plus 150 mM NaCl. The fluorescence emission spectrum of SP-A on excitation at 275 is characterized by two maxima at about 326 and 337 nm as previously reported for porcine and human SP-A (6). The two maxima are blue-shifted in comparison to that of free tryptophan model systems, revealing a partially buried character for these fluorophores. Self-aggregation of the protein induced by either 5µM Ca^{2+}/150 mM NaCl or 5 mM Ca^{2+}/0 mM NaCl led to a decrease in fluorescence emission intensity without any shift in the wavelength of the emission maxima. The observed decrease in fluorescence intensity can be explained in terms of changes in the tryptophan environment, which probably implies a higher quenching of the tryptophan fluorescence by polarizable groups in the proximity of these residues. The decrease in fluorescence emission intensity caused by 5 mM Ca^{2+}/0 mM NaCl was stronger than that caused by 5µM Ca^{2+}/150 mM NaCl, indicating a more drastic change in the tryptophan environment when the protein is self-aggregated at millimolar concentrations of Ca^{2+} in the absence of salts.

An additional criterion for revealing structural changes in the protein after self-aggregation is the analysis of the accessibility of SP-A fluorophores to acrylamide, an efficient neutral collisional quencher of indole derivates, capable of permeating the protein matrix (24). Figure 2 B shows Stern-Volmer plots of the effect of acrylamide on fluorescence emission intensity of porcine SP-A, measured in the presence and absence of either 5µM Ca^{2+}/150 mM NaCl or 2 mM Ca^{2+}/0 mM NaCl. Self-aggregation of SP-A markedly increased the accessibility of SP-A fluorophores to acrylamide. SP-A in its non-aggregated form

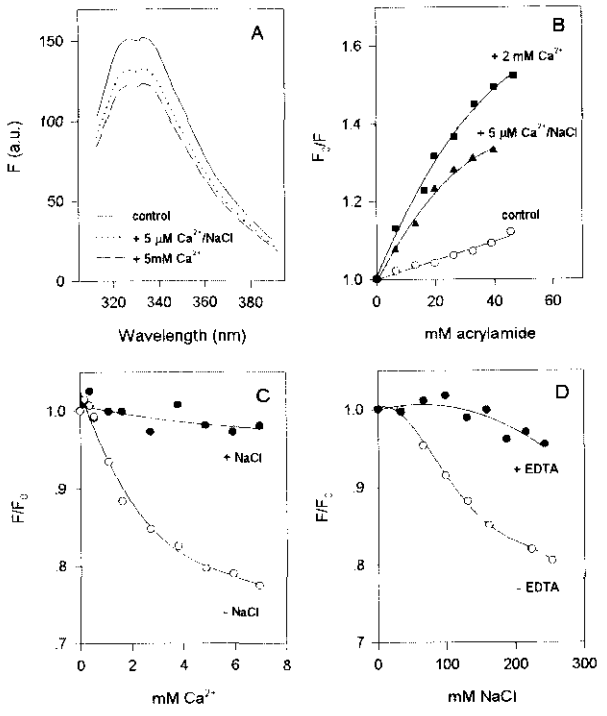

FIGURE 2: Fluorescence measurements of non-aggregated and aggregated forms of SP-A induced by either Ca^{2+} or Ca^{2+}/NaCl. *A*, Fluorescence emission spectra of SP-A on excitation at 275 nm in the absence or the presence of either 5 mM Ca^{2+} or 5 μM Ca^{2+}/150 mM NaCl. Fluorescence (F) is expressed in arbitrary units. *B*, Stern-Volmer plots of aqueous quenching by acrylamide of SP-A. F_o and F are the corrected emission intensities at 330 nm in the absence and the presence of acrylamide, respectively. Experiments were done in 5 mM Tris/HCl buffer pH 7.4 (control, open circles) with or without either 2 mM Ca^{2+} (solid squares) or 5 μM Ca^{2+}/150 mM NaCl (solid triangles). *C*, Changes in fluorescence emission intensity of SP-A on addition of $CaCl_2$. F and F_o are the corrected fluorescence emission intensities at 330 nm in the presence or the absence of $CaCl_2$, respectively. SP-A (6.7 μg/ml) was initially in 5 μM Ca^{2+}, 5 mM Tris/HCl buffer, pH 7.4, with (solid circles) or without (open circles) 150 mM NaCl. *D*, Changes in fluorescence emission intensity of SP-A on addition of NaCl. F and F_o are the corrected fluorescence emission intensities at 330 nm in the presence or the absence of NaCl, respectively. SP-A (6.7 μg/ml) was initially in 5 μM Ca^{2+}, 5 mM Tris/HCl buffer, pH 7.4, with (solid circles) or without (open circles) 0.1 mM EDTA. A representative one of four experiments is shown.

showed a lower acrylamide quenching constant (K_{SV} = 2.3 M^{-1}) than in its self-aggregated form induced by either 5μM Ca^{2+}/150 mM NaCl (K_{SV} = 10.5 M^{-1}) or 2 mM Ca^{2+}/0 mM NaCl (K_{SV} = 16.2 M^{-1}). From these results it is clear that the quenching of tryptophan residues by acrylamide was more pronounced when the protein underwent Ca^{2+}-dependent self-aggregation in the absence than in the presence of salts.

Figure 2 C shows the fluorescence emission intensity of SP-A at 330 nm as a function of Ca^{2+} concentration. At low ionic strength, the fluorescence emission intensity of SP-A started to decrease at concentrations of Ca^{2+} higher than 0.5 mM. The change in the fluorescence emission intensity of SP-A as a function of Ca^{2+} might reflect the conformational change of SP-A at different levels of self-aggregation. Figure 2 C also shows that at physiological ionic strength and micromolar concentrations of Ca^{2+} the fluorescence of SP-A did not change as the Ca^{2+} concentration increased in the millimolar range. These results support the concept that once SP-A was self-aggregated by micromolar concentrations of Ca^{2+} in the presence of salts (Figure 1 A, C) the addition of further amounts of Ca^{2+} does not induce changes in both the self-aggregation state and fluorescence emission intensity of the protein.

Figure 2 D shows the fluorescence emission intensity of SP-A at 330 nm as a function of NaCl concentration in the presence of micromolar concentrations of calcium. SP-A fluorescence progressively decreased as NaCl concentration increased reflecting a conformational change of SP-A at different levels of self-aggregation induced both by micromolar concentrations of calcium an NaCl. When the Ca^{2+} present in the buffer solution (5 μM) was chelated by addition of EDTA, the fluorescence emission intensity of SP-A was little affected by increasing ionic strength.

Figure 3 shows the change in the circular dichroism spectrum of SP-A as a function of Ca^{2+} concentration in the absence of salts (Figure 3 A) and as a function of NaCl concentration in the presence of 20 μM Ca^{2+} (Figure 3 B). Addition of Ca^{2+} at concentrations higher than 0.5 mM to the protein solution led to a marked decrease of the negative ellipticity and a shift of the minimum from 207 nm to 210 nm (Figure 3 A). CD spectra of SP-A at Ca^{2+} concentrations higher than 0.5 mM must reflect the secondary structure of SP-A at different levels of self-aggregation. At concentrations of Ca^{2+} lower than 0.5 mM, there was a small but significant decrease of the contribution of the 207-nm minimum to the spectrum without any shift of this minimum. At these Ca^{2+} concentrations the protein was not self-aggregated. The molar ellipticity at 207 nm (θ_{207}) as a function of the Ca^{2+} concentration is shown in the inset of Figure 3 A. The Ca^{2+}-dependent decrease of θ_{207} reached a maximum at around 5 mM. On the other hand, Figure 3 B shows a decrease of the negative ellipticity as the concentration of NaCl increased in the presence of 20 μM Ca^{2+}. However, that decrease in the CD signal of SP-A was much less pronounced than that induced by millimolar concentrations of Ca^{2+} in the absence of salts (Figure 3 A). The molar ellipticity at

207 nm (θ_{207}) as a function of the Na^+ concentration is shown in the inset of Figure 3 B.

Figure 4 A shows that those changes in the secondary structure of porcine SP-A produced by 5 mM Ca^{2+}/0 mM NaCl were not reversed when aggregates of protein were dissociated by addition of EDTA. Removing Ca^{2+} produced additional important changes in the CD spectrum of SP-A. Figure 4 C shows that the addition of DPPC vesicles to this non-aggregated form of porcine SP-A after chelating Ca^{2+} with EDTA (Figure 4 B) resulted in a marked increase in light absorbance due to lipid aggregation. That vesicle aggregation induced by this form of SP-A at 37°C was completely independent of Ca^{2+} and irreversible. Thus,

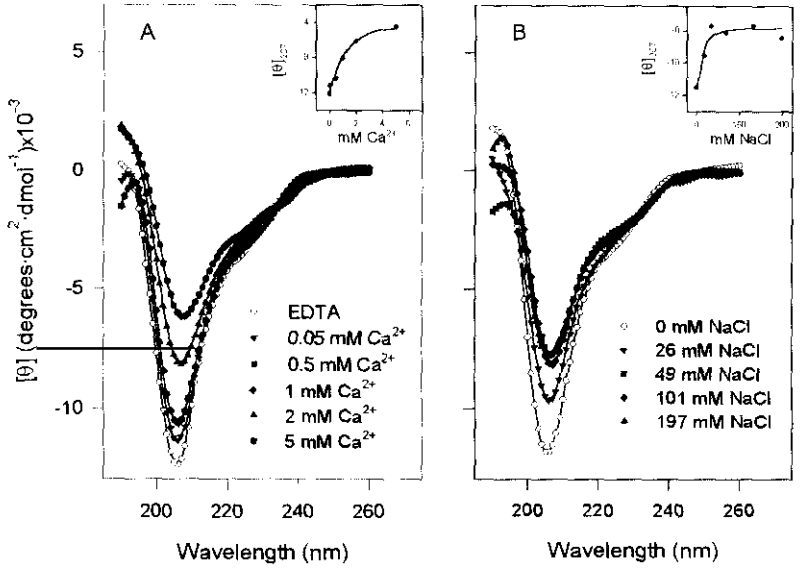

FIGURE 3. Changes in the circular dichroism spectrum of porcine SP-A as a function of Ca^{2+} (A) or NaCl concentration (B). Experiments of Ca^{2+} or NaCl titration were performed at 37°C. *A*, SP-A (80 μg/ml) was in 5 mM Tris/HCl buffer, pH 7.4, in the absence of NaCl. *B*, SP-A (80 μg/ml) was in 20 μM Ca^{2+}, 5 mM Tris/HCl buffer, pH 7.4. Insets: ellipticity at 207 nm of SP-A as a function of Ca^{2+} (A) or NaCl (B) concentration. A representative one of three experiments is shown.

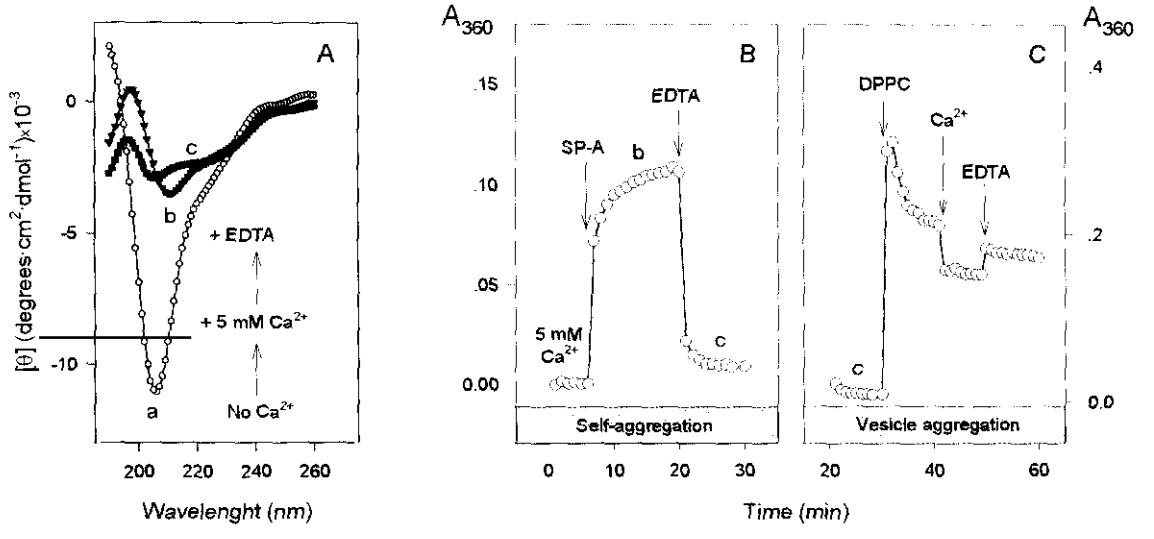

FIGURE 4: Changes in the secondary structure of SP-A and in its lipid vesicle aggregation activity after dissociation of Ca^{2+}-induced protein aggregates by addition of EDTA. *A*, Circular dichroism spectra of porcine SP-A (80 μg/ml) at 37°C under different conditions: a) 5 mM Tris/HCl buffer, pH 7.4, containing 0.1 mM EDTA (open circles); b) after addition of $CaCl_2$ (5 mM free Ca^{2+}) (solid triangles); and c) after addition of EDTA (10 mM final concentration) (solid squares). *B*, Self-aggregation (absorbance change at 360 nm) of porcine SP-A at 37°C. The protein (40 μg/ml final concentration) was added to the sample cuvette containing 5 mM Ca^{2+}, 5 mM Tris/HCl buffer, pH 7.4 (b). After stabilization, self-aggregation was reverted with EDTA (10 mM) (c). *C*, SP-A-induced vesicle aggregation assay was performed as a continuation of SP-A self-aggregation experiment given in *B*. Thus, SP-A was in a non-aggregated form after dissociation of protein aggregates by addition of EDTA (c). Vesicle aggregation was performed as described under "Experimental Procedures". A representative one of four experiments is shown.

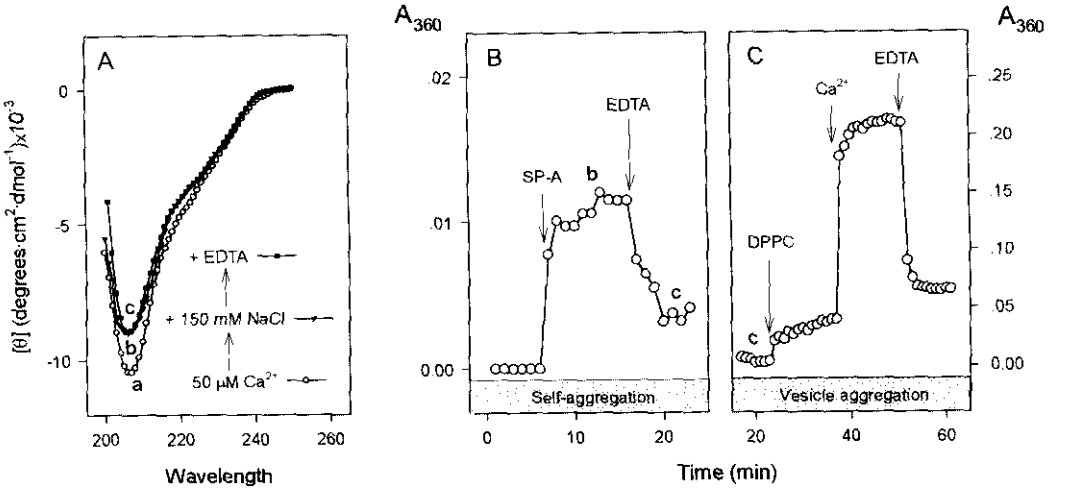

FIGURE 5. Effect of dissociation of Ca^{2+}/NaCl-induced SP-A aggregates by addition of EDTA on the secondary structure of the protein and on its lipid vesicle aggregation activity. Circular dichroism spectra of porcine SP-A (80 μg/ml) at 37°C under different conditions: a) 50 μM Ca^{2+}, 5 mM Tris/HCl buffer, pH 7.4 (open circles); b) after addition of NaCl (150 mM, final concentration) (solid triangles); and c) after addition of EDTA (solid squares). *B*, Self-aggregation (absorbance change at 360 nm) of porcine SP-A at 37°C. The protein (40 μg/ml final concentration) was added to the sample cuvette containing 50 μM Ca^{2+}, 150 mM NaCl, 5 mM Tris/HCl buffer, pH 7.4 (b). Once the absorbance was stabilized, self-aggregation was reverted with EDTA (0.5 mM) (c). *C*, SP-A-induced vesicle aggregation assay was done as a continuation of SP-A self-aggregation experiment given in *B*. Vesicle aggregation was performed as described under "Experimental Procedures". A representative one of four experiments is shown.

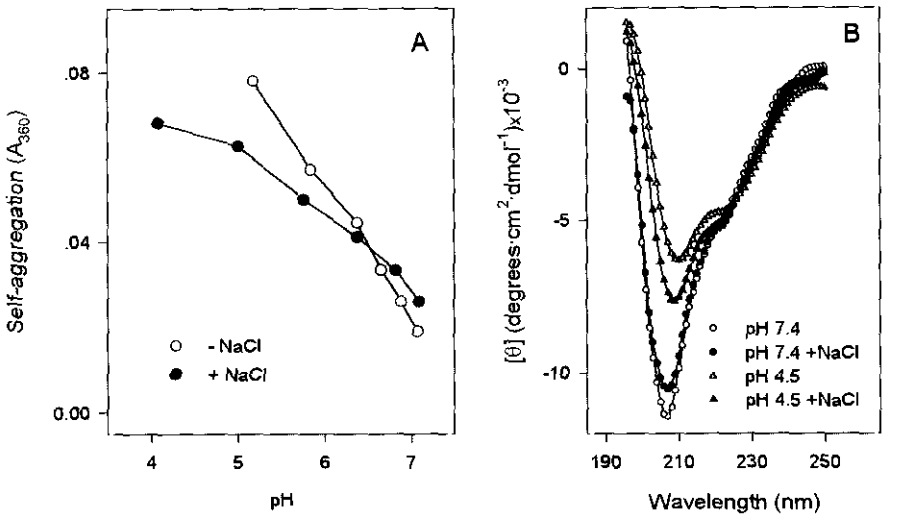

FIGURE 6. Effect of NaCl on SP-A self-aggregation (*A*) and SP-A secondary structure (*B*) at acidic and neutral pH in the absence of Ca^{2+}. *A*, pH titration started at pH 7.4 in 5 mM Tris/HCl, 5 mM MES, 5 mM acetate, 0.1 mM EDTA buffer either with (solid circles) or without (open circles) 150 mM NaCl. From the starting pH, SP-A (30 μg/ml) was titrated by addition of 1-2 μl aliquots of 0.05 or 0.1 M HCl solution. During titration, the medium pH was monitored with a micro-pH electrode. The absorbance at 360 nm was registered at 37°C once it was stabilized after each change of pH. *B*, Far-uv circular dichroism spectra of SP-A (80 μg/ml) in 5 mM acetate, 5 mM Tris/HCl buffer at neutral (circles) or acidic (triangles) pH, either with (solid symbols) or without (open symbols) 150 mM NaCl. A representative one of four experiments is shown.

removing Ca^{2+} also affected the SP-A property to aggregate lipid vesicles in a Ca^{2+}-dependent manner.

In contrast, Figure 5 shows that addition of EDTA to porcine SP-A aggregates formed in the presence of 50 μM Ca^{2+}/150 mM NaCl caused dissociation of protein aggregates (Figure 5 B) with little change in the CD spectrum of SP-A (Figure 5 A). This non-aggregated form of SP-A obtained by dissociation of SP-A aggregates with EDTA was able to mediate the process of Ca^{2+}-dependent vesicle aggregation, which

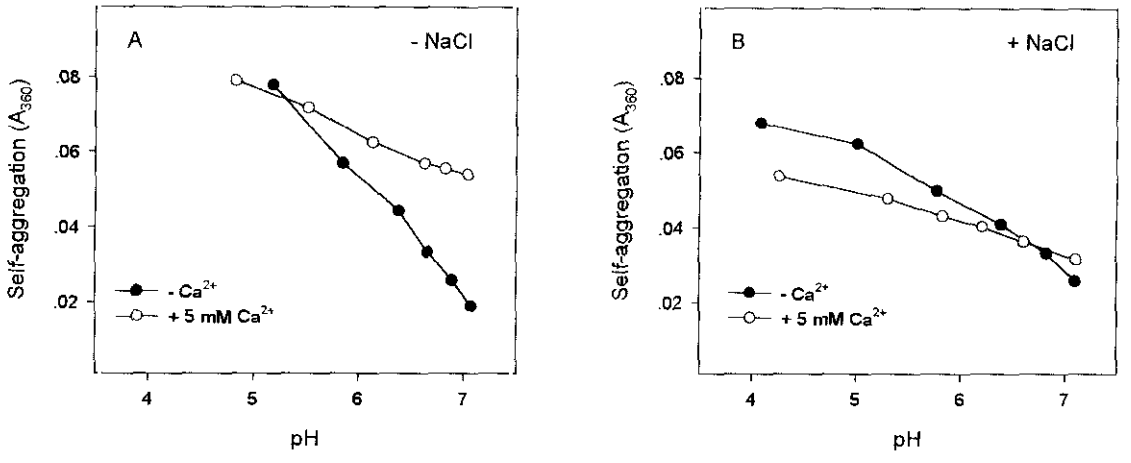

FIGURE 7. Effect of NaCl and $CaCl_2$ on SP-A self-aggregation at different pH. pH titration was done at 37°C as described in Fig. 6 either in the absence (*A*) or in the presence (*B*) of 150 mM NaCl. The same results were obtained with KCl. Solid circles, no calcium. Open circles, 5 mM $CaCl_2$. A representative one of four experiments is shown.

could be reversed by addition of EDTA (Figure 5 C). These results indicated that aggregation of SP-A induced by Ca^{2+}/NaCl was reversible and that after dissociation of protein aggregates, SP-A retained its ability to induce phospholipid vesicle aggregation in the presence of Ca^{2+} and NaCl.

Effect of the ionic strength on pH-dependent self-aggregation of SP-A. Figure 6 A shows the effect of lowering pH on self-aggregation of the protein in the presence or the absence of 150 mM NaCl. At pH below 6.0 protein aggregation was lower in the presence than in the absence of salts. CD spectra of SP-A at neutral and acidic pH, in the presence of EDTA and either with or without NaCl are shown in Figure 6 B. At neutral pH and at room temperature, the presence of NaCl in the medium slightly reduced the negative ellipticity. The change of pH from 7.4 to 4.5 led to a drastic change in the CD spectra of SP-A characterized by a marked decrease of the negative ellipticity and a shift of the minimum from 207 to 209 nm as previously reported (9). In the presence of NaCl the change of pH from 7.4 to 4.5 led to a less pronounced decrease of the negative ellipticity. This is consistent with the lower extent of pH-dependent self-aggregation of SP-A in the presence of salts.

Figure 7 A shows that at low ionic strength there was a marked aggregation of the protein in the pH range of 6-6.5 provided Ca^{2+} was present in millimolar concentrations in agreement with previous results (9). Protein aggregation was greater as the pH was reduced. Thus, there was a synergy between H^+ and Ca^{2+} on self-aggregation of SP-A in the pH range of 5-6.5. Interestingly, we show in Figure 7 B that at physiological ionic strength there was an antagonism between H^+ and Ca^{2+} below pH 6.5. Therefore, the extent of pH-dependent self-aggregation of SP-A was lower in the presence than in the absence of Ca^{2+} provided physiological concentrations of either KCl or NaCl were present in the medium.

Effect of temperature on self-aggregation of SP-A. Figure 8 A shows that SP-A underwent self-aggregation at 20°C at neutral pH in the presence of EDTA. This aggregation process required the presence of NaCl and was reversed by increasing temperature to 37°C. We found that self-aggregation of SP-A progressively increased as the temperature was decreasing from 40°C to 20°C provided NaCl was present in the medium (data not shown). The extent of this type of aggregation is much lower than that induced by Ca^{2+} or H^+. Figure 8 B shows the effect of decreasing temperature on CD spectra of SP-A. In the presence but not in the absence of NaCl there was a small but progressive decrease of the negative ellipticity without any shift of the minimum at 207 nm.

Figure 9 A shows that heat treatment of porcine SP-A resulted in a pronounced decreased of the negative band at 207 nm without any shift of this band as previously reported for canine and human SP-A (25, 26). The molar ellipticity at 207 nm (θ_{207}) as a function of temperature is shown in the inset of Figure 9 A. On the other hand, we have calculated the molar fraction of native and denatured collagen components of CD spectra of porcine SP-A as a function of temperature (inset of Figure 9A) using an algorithm developed by Perczel et al. (27), called Convex Constraint Analysis (CCA). Deconvolution of SP-A CD spectra obtained at different temperatures by CCA reveals that the change in ellipticity from 37°C to 60°C can be fitted to a structural transition between two components, which closely follows the denaturation profile of collagen.

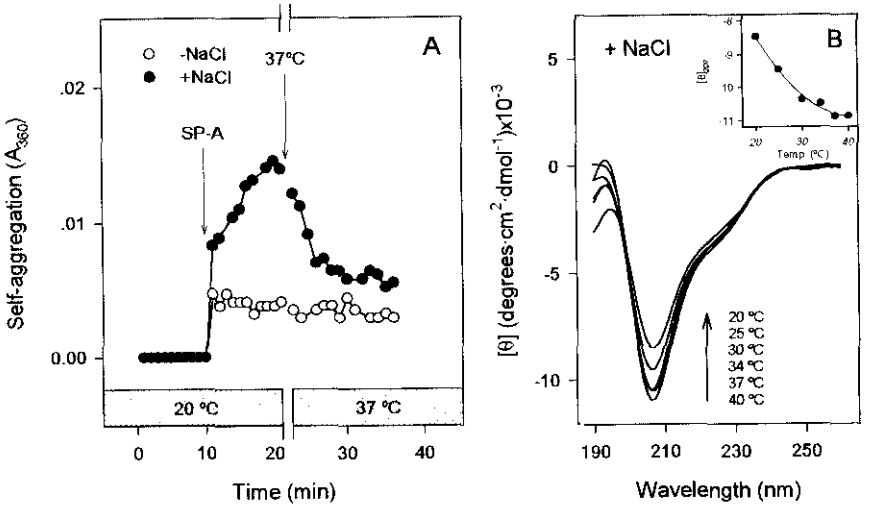

FIGURE 8. Effect of low temperature on SP-A self-aggregation (A) and SP-A secondary structure (B) in the presence of NaCl and EDTA. *A*, Self-aggregation experiments were done as described under "Experimental Procedures". *B*, Far-uv circular dichroism spectra of SP-A (80 μg/ml) were recorded from 40°C to 20°C in 150 mM NaCl, 0.1 mM EDTA, 5 mM Tris/HCl buffer, pH 7.4. Inset: molar ellipticity at 207 nm as a function of temperature.

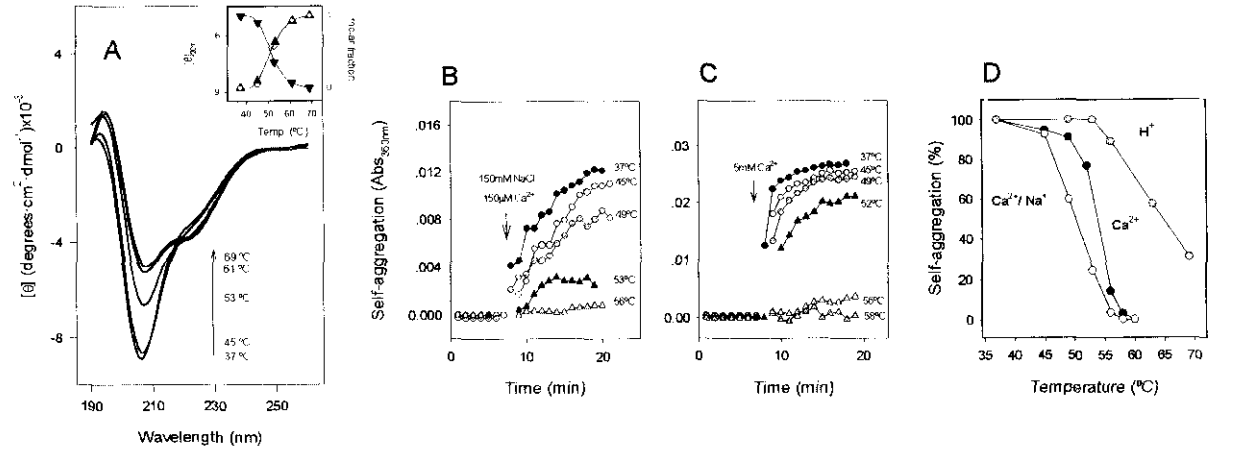

FIGURE 9. Effect of heat treatment of SP-A on the secondary structure of the protein and on its self-aggregation activity induced by either Ca^{2+}/Na^+ or Ca^{2+} or H^+. *A*, Far-uv circular dichroism spectra of SP-A (80 μg/ml) were recorded from 37°C to 69°C in 5 mM Tris/HCl buffer, pH 7.4. Inset: Molar ellipticity at 207 nm as a function of temperature (open circles). The molar fraction of native (inverted solid triangles) and denatured (solid triangles) collagen components of CD spectra of SP-A at different temperatures is also given. *B*, Comparison of the kinetics of Ca^{2+}/Na^+-induced self-aggregation of SP-A at 37°C using different protein preparations previously heated for 10 min at different temperatures in 5 mM Tris/HCl buffer, pH 7.4. SP-A was cooled to 37°C before assessing self-aggregation at 37°C. *C*, Comparison of the kinetics of Ca^{2+}-induced self-aggregation of heated SP-A as indicated in *B*. *D*, Self-aggregation activity induced by either Ca^{2+}/Na^+ or Ca^{2+} or H^+ at 37°C performed with different SP-A preparations heated for 10 min at different temperatures as indicated in *B*. The rate of self-aggregation was determined with each SP-A preparation and expressed as a percentage of the rate observed at 37°C. A representative one of three experiments is shown.

This suggests that there is no additional contribution of changes affecting other structural components in that range of temperature. Inset of Figure 9 A indicates that the midpoint transition "melting" temperature of porcine SP-A was 51°C. Comparable values were found for canine and human SP-A (26).

Figure 9 B and 9 C show kinetics of Ca^{2+}/Na^+- and Ca^{2+}-dependent protein self-aggregation at 37°C respectively, using porcine SP-A previously heated for 10 min at different temperatures and then cooled to 37°C before assessing self-aggregation activity. Both, Ca^{2+}- and Ca^{2+}/Na^+-dependent SP-A self-aggregation was completely abrogated when the protein was pre-heated at 56°C indicating that a structurally intact collagen-like domain is required for both processes. The denaturation temperature at which SP-A showed half-maximal Ca^{2+}-dependent self-aggregation was 50°C in the presence of NaCl and 54°C in its absence (Figure 9D). These data suggest that Ca^{2+}/Na^+-induced SP-A self-aggregation is more susceptible to partial unfolding of the collagen-like triple helical domain of SP-A than Ca^{2+}-induced self-aggregation. Interestingly, H^+-induced SP-A self-aggregation was not affected by partial or total unfolding of the collagen-like domain of SP-A (Figure 9D).

DISCUSSION

Ca^{2+} and H^+-dependent self-aggregation of SP-A. It is well established that SP-A self-aggregates to form higher-order structures in the presence of Ca^{2+} (8, 16) or under mildly acidic pH (9). The biological significance of this process is unknown. Ca^{2+}-dependent self-aggregation of SP-A has typically been studied in buffers without physiological saline (150 mM NaCl) (8, 16). Under these conditions, this process requires mM concentrations of Ca^{2+} (8, 16). The Ca^{2+} concentration required for half-maximal self-association is 2.3 $\pm$ 0.2 mM for porcine SP-A and 3 $\pm$ 0.1 mM for human SP-A. These values are above the free Ca^{2+} concentration in the alveolar space of adult animals (approx. 1.5 mM) (28). The Na^+ and Cl^- concentrations of the lung liquid are similar to plasma (29). Therefore, we have studied here the Ca^{2+}-dependent self-aggregation of SP-A in the presence of physiological saline (150 mM NaCl). The Ca^{2+} concentration required to set out self-aggregation of SP-A is highly reduced at physiological ionic strength. Under these conditions, the concentration of Ca^{2+} required for half-maximal self-aggregation is 2.4 $\pm$ 0.5 μM for porcine SP-A and 12 $\pm$ 1.8 μM for human SP-A. In the presence of 150 mM NaCl, addition of higher concentrations of Ca^{2+} (in the mM range) neither increases the extent of protein aggregation nor changes the fluorescent characteristics of SP-A. Addition of EDTA completely reverses the process of Ca^{2+}/Na^+-dependent self-aggregation. The presence of EDTA in the medium prevents that process at 37°C. Therefore, NaCl does not promote self-association of the protein in the absence of Ca^{2+} at 37°C.

We previously reported that the phospholipid vesicle aggregation activity of SP-A also required μM concentrations of Ca^{2+} for canine and porcine SP-A (8). This finding was confirmed for other SP-A species (30). Vesicle aggregation studies were done at physiological ionic strength. We suggest that Ca^{2+}/Na^+-dependent lipid vesicle aggregation is likely mediated by Ca^{2+}/Na^+-induced self-aggregation of the protein on different vesicles. Haagsman et al., (16) suggested that the process of lipid aggregation induced by SP-A could be correlated with that of self-association of the protein occurring at supramillimolar concentrations of Ca^{2+} and low ionic strength. However, these two processes have very different requirements of Ca^{2+} (8) unless physiological concentrations of NaCl are present in the medium.

Circular dichroism and fluorescence measurements of Ca^{2+}-dependent SP-A aggregates indicate that those protein aggregates formed in the absence of NaC l are structurally different than those formed in its presence. Changes in the secondary structure of SP-A, in the fluorescence emission intensity of the protein and in the accessibility of SP-A tryptophan residues to acrylamide are much more pronounced when the protein undergoes Ca^{2+}-induced self-aggregation in the absence than in the presence of NaCl. In addition, the extent of Ca^{2+}-dependent self-aggregation of SP-A is much higher in the absence than in the presence of NaCl. Transmission electron microscopy studies (31) indicate that SP-A octadecamers become compacted (closed bouquet) and the stem becomes more detectable in the presence of millimolar concentrations of Ca^{2+} and in the absence of NaCl. Interestingly, when calcium and NaCl are present in the medium, the SP-A octadecamer retains more of an opened-bouquet structure and the stem is less detectable. Thus, the effect of Ca^{2+} on SP-A structure appears to be modified by the presence of NaCl. Compacted SP-A conformation induced by millimolar concentrations of Ca^{2+} in the absence of NaCl could contribute to the formation of larger protein aggregates in comparison to a more opened-bouquet structure.

Addition of EDTA to Ca^{2+}-dependent SP-A aggregates causes dissociation of those aggregates formed either in the presence or in the absence of NaCl. However, when the protein is in the absence of NaCl, addition of EDTA leads to a marked loss of ellipticity. Furthermore, SP-A loses its ability to aggregate lipid vesicles in a Ca^{2+}-dependent manner. In contrast, when EDTA is added to Ca^{2+}/Na^+-induced protein aggregates, the resulting non-aggregated SP-A hardly changes its secondary structure and retains its vesicle aggregation properties. All together, these data indicate the strong effect of the ionic strength on Ca^{2+}-dependent self-aggregation process of SP-A.

On the other hand, SP-A undergoes a marked self-aggregation as the pH is reduced below 6.5. The protein encounters a mildly acidic pH along its exocytic/endocytic pathway (9, 32). Acidic compartments along this pathway have millimolar concentrations of Ca^{2+} and physiological ionic strength. SP-A becomes immunologically unreactive under these conditions (33) and its lipid binding properties changed (9, 34). We show here that the presence of salts in the medium is a regulatory element defining the level of pH-dependent self-aggregation. In the presence of salts (either 150 mM NaCl or 150 mM KCl), the extent of self-aggregation as a function of pH is reduced. The effect of salts is also visible in the change in the secondary structure of the protein. When both NaCl (or KCl) and $CaCl_2$ are present in the medium the level of self-aggregation decreases even more as the pH is reduced in the range 4.2-6.5, suggesting different effects of Ca^{2+} and H^+ ions on the protein in the presence of salts. H^+ could induce self-aggregation of the protein by protonation of the carboxyl groups, which would reduce the negative surface charge on the protein. The action of

Ca^{2+} on the protein in the presence of salts might be more specific and not simply related to neutralization of the negative charged carboxyl groups on the protein.

Temperature effect on self-aggregation of SP-A. At physiological temperature, SP-A self-aggregation can be induced either by Ca^{2+} or by H^+. Interestingly, at low temperature SP-A undergoes self-aggregation in the presence of EDTA at neutral pH provided physiological saline is present in the medium. Increasing temperature to 37°C or 40°C reverses this process. In the absence of NaCl, the negative surface charge on the protein would likely prevent protein aggregation at low temperatures unless charge stabilization occurred in the presence of NaCl. The negatively charged CRD regions of SP-A would approach each other more easily when charge-stabilization occurs. The same process would happen as the pH is reduced. The head groups of SP-A would bring closer to each other. The effect of low temperature on SP-A self-aggregation can be explained by a decrease in solubility. Purified lipid-free SP-A is soluble in a presumably native octadecameric form in very low ionic strength buffers and neutral pH. Other conditions affecting ionic strength, pH, temperature or even the presence of trace amounts of calcium in the protein solution could lead to some type of self-aggregation of the protein in the aqueous phase. The state of SP-A self-aggregation could account for some differences in the results from different laboratories.

Interestingly, both Ca^{2+}-and Ca^{2+}/Na^+-dependent self-aggregation activity of SP-A is completely inhibited by unfolding of the collagen-like domain of porcine SP-A at 56°C. Haagsman et al. (1990) reported that lipid aggregation induced by canine SP-A, in the presence of Ca^{2+} and salts, decreased as temperature increased up to 60°C, indicating that an intact collagen domain may be required for specific SP-A-induced lipid aggregation. The similar temperature dependence of both, self-aggregation and lipid aggregation processes also supports the concept that Ca^{2+}-dependent self-aggregation and SP-A-induced lipid aggregation are related phenomena.

Fully assembled SP-A is a hexamer of trimers. Strong noncovalent intermolecular forces in the N-terminal segment and the first part of the collagen-like region of SP-A, and not interchain disulfide bonds, are the major determinants of SP-A assembly into supratrimeric oligomers (35). Deoligomerization is expected to lead to loss or alteration of biological functions of SP-A (36). Because interactions between collagen triple helices are important in SP-A oligomerization, the formation of smaller forms of SP-A after mild heat treatment could lead to inhibition of self-aggregation and lipid-aggregation activities of SP-A. However, it can not be ruled out the possibility that collagen-like domain is involved in the process of protein-protein aggregation induced by Ca^{2+}.

Interestingly, self-aggregation induced by H^+ is not affected by unfolding of the collagen-like domain indicating that neither this domain nor oligomerization of SP-A is absolutely required for this type of self-aggregation. This finding strongly suggests that H^+ and Ca^{2+} ions induce SP-A self-aggregation by different mechanisms. This idea is additionally supported by the finding that the presence of Ca^{2+}/NaCl attenuates the effect of H^+ ions on protein self-aggregation.

Physiological Implications. SP-A aggregation mediated by Ca^{2+} and NaCl might have physiological significance since alveolar hypophase contains Na^+ (150 mM) and Ca^{2+} (1.5 mM) ions. The interaction of SP-A with pathogens or with its receptors on macrophages or on type II cells or the ability of SP-A to aggregate lipids has been studied under these ionic conditions. Thus, SP-A could interact with different ligands in a Ca^{2+}/Na^+-dependent self-aggregated form, which would retain an opened-bouquet structure (31). We suggest that SP-A bound to lipids, carbohydrates or cell surfaces in the alveolar subphase is in a self-aggregated form induced by physiological concentrations of Ca^{2+} and NaCl resulting in a multivalent presentation of SP-A to its ligands.

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DISCUSIÓN

Previamente habíamos comprobado que la SP-A experimenta autoagregación en presencia de calcio o a pH ácido (ver capítulo 3). En este capítulo abordamos el estudio de la autoagregación de la SP-A en condiciones fisiológicas de fuerza iónica. En el trabajo presentado en este capítulo, hemos observado que, a 37 °C en un medio con baja fuerza iónica, la SP-A necesita concentraciones de Ca^{2+} de orden milimolar para que se dé este fenómeno. Sin embargo, en presencia de NaCl 150 mM, el requerimiento de Ca^{2+} para que esto ocurra baja hasta 2-15 μM. Además, los agregados de SP-A inducidos por Ca^{2+} difieren estructuralmente de aquéllos inducidos por Ca^{2+}/Na^{+}, como lo demuestran los experimentos de fluorescencia y de dicroísmo circular. Parece ser, por tanto, que la presencia de NaCl modifica el efecto del Ca^{2+} sobre la SP-A. Estos resultados apoyan las observaciones de Palaniyar y colaboradores (Palaniyar *et al.* 1998a), que observaron mediante microscopía electrónica de transmisión que la molécula de SP-A adopta en presencia de Ca^{2+} una conformación más compacta (más estirada) que cuando hay Ca^{2+} y NaCl en el medio.

La autoagregación inducida por Ca^{2+}/Na^{+} es reversible. La disociación del agregado por EDTA da lugar a una proteína con estructura secundaria semejante a la que tenía antes de la autoagregación, y con capacidad de inducir agregación de vesículas lipídicas de forma dependiente de Ca^{2+}. Sin embargo, aunque la autoagregación inducida sólo por Ca^{2+} se revierte también con EDTA, la SP-A resultante pierde dicha actividad agregante de vesículas dependiente de Ca^{2+}.

La autoagregación de la SP-A que se produce en presencia de Ca^{2+} y NaCl podría tener una gran importancia fisiológica, ya que en el alveolo pulmonar se encuentran estas especies iónicas en concentraciones similares a las ensayadas (Nielson *et al.* 1981, Nielson y Lewis 1988). Además, el requerimiento de Ca^{2+} para la autoagregación en presencia de NaCl, de orden micromolar, es similar al necesario para inducir la agregación de vesículas lipídicas (Ruano *et al.* 1996), con lo cual es posible que haya una correlación entre ambos procesos, en el sentido de que la agregación lipídica esté mediada por la autoagregación de la proteína después de unirse a las vesículas. De esta manera, la autoagregación en estas condiciones podría ser responsable, al menos en parte, de las transformaciones estructurales que experimenta el surfactante tras su secreción desde los cuerpos lamelares al espacio alveolar. Por otra parte, los estudios de unión de la SP-A a sus receptores en neumocitos tipo II y macrófagos y su interacción con algunos patógenos han sido abordados bajo estas condiciones iónicas, por lo que podría ser la forma autoagregada la forma responsable de estas propiedades.

Las sales también modifican la autoagregación de la SP-A que se produce en condiciones de pH ácido. La presencia de NaCl 150 mM produce diferencias en el espectro de dicroísmo circular de la proteína a pH ácido, y hace que la extensión de la autoagregación sea menor (menor absorbancia a 360 nm), lo que podría deberse a la existencia de interacciones de tipo electrostático implicadas en la autoagregación a valores bajos de pH. La presencia simultánea de NaCl y Ca^{2+} también hace que la extensión de la autoagregación al ir disminuyendo el pH sea menor que en ausencia de estas sales. Este hecho se podría deber a que dichas sales producen un efecto más específico en estas condiciones de pH que el que correspondería sólo a la neutralización de residuos ácidos por parte de los iones H^{+} del medio. Todo esto podría condicionar las propiedades de la SP-A dentro de los cuerpos lamelares si tenemos en cuenta que estos orgánulos, ade-

más de poseer un pH ácido, poseen Ca^{2+} y fuerza iónica fisiológica en su interior.

Por otra parte, la autoagregación inducida por Ca^{2+} (pH neutro) depende de la integridad del dominio colagénico (Haagsman *et al.* 1990), lo que no sucede con la autoagregación inducida por pH ácido. Esto indica que ambos fenómenos están regidos por mecanismos diferentes, en los que probablemente estén implicadas partes distintas de la molécula.

En resumen, hemos visto que la autoagregación de la SP-A se produce en diversas circunstancias siguiendo mecanismos distintos, y que la presencia de NaCl afecta a la formación de estos agregados. Esto se puede deber a que los iones Na^+ y Cl^- neutralizan las cargas de la proteína, eliminando de esta forma la repulsión electrostática entre las moléculas de SP-A. Así, la presencia de NaCl a pH neutro facilitaría la autoagregación de la proteína al disminuir su solubilidad (por ejemplo al disminuir la temperatura, según hemos visto en este capítulo), y también permitiría que los iones Ca^{2+} o H^+ produzcan cambios estructurales y funcionales más específicos en la SP-A.

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6. DISCUSIÓN GENERAL E IMPLICACIONES FISIOLÓGICAS

A lo largo de esta tesis se ha profundizado en el conocimiento de algunas características estructurales y funcionales de la SP-A. El estudio de estas propiedades, que rigen su interacción con los lípidos y otras proteínas del surfactante, podría aclarar algunos aspectos sobre el papel que desempeña la SP-A en las distintas fases del ciclo metabólico del surfactante.

Tras ser sintetizada, la SP-A entra en la ruta de secreción de los neumocitos tipo II. Se sabe que, al menos en parte, es secretada al espacio alveolar desde los cuerpos lamelares a través de la vía secretora regulada. En el primer y tercer capítulos de resultados demostramos que la SP-A experimenta autoagregación en presencia de calcio, así como en condiciones de pH ácido. Este tipo de autoagregación se produce también con otras proteínas que son exocitadas a partir de gránulos de secreción por la vía regulada (Orci *et al.* 1987, Kuliawat y Arvan 1994). La capacidad de la SP-A de experimentar autoagregación en estas condiciones podría estar relacionada con su segregación hacia la vía regulada, separándose así de las proteínas que son secretadas por vía constitutiva (fig. 6.1). En efecto, el ambiente que halla la SP-A en su camino hacia el espacio al-

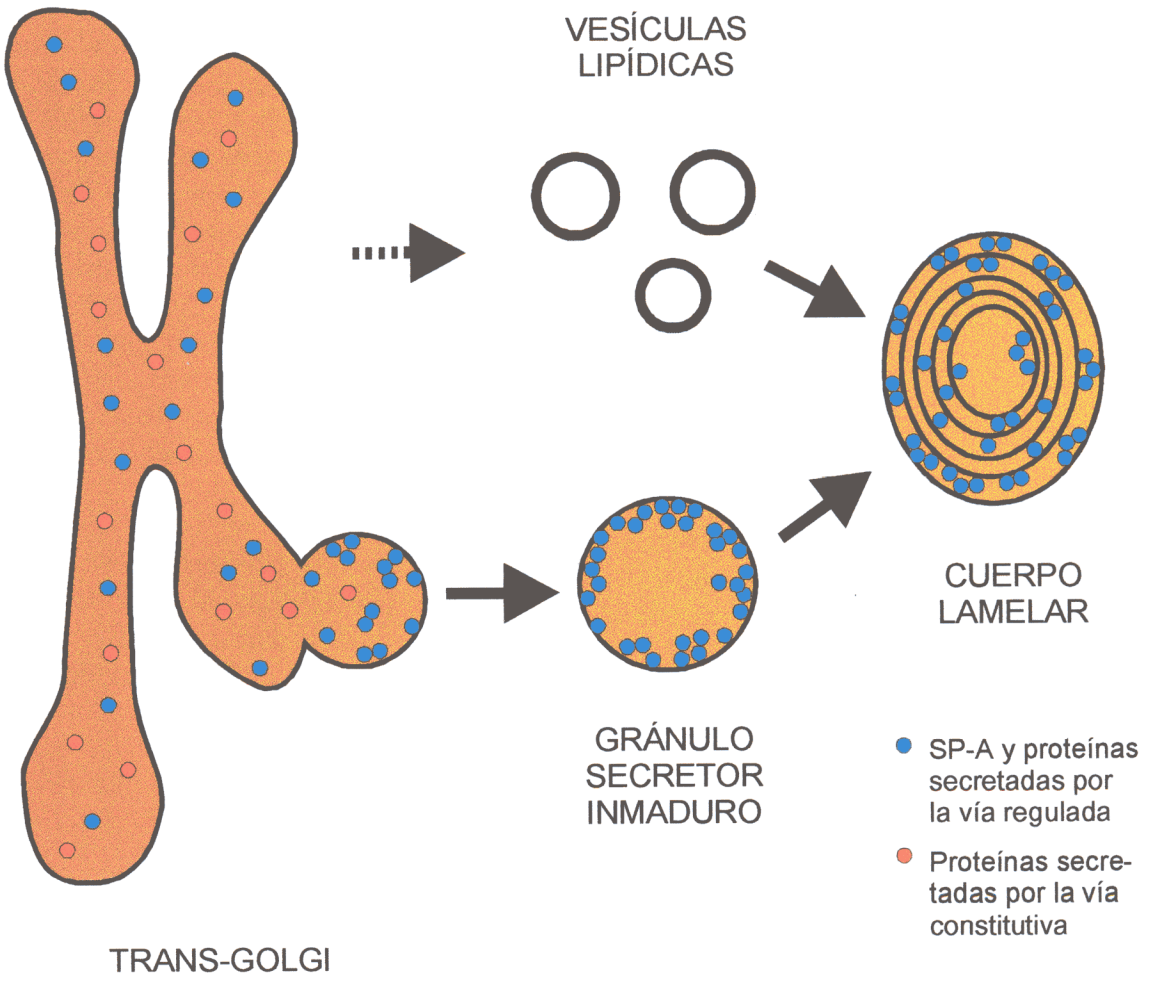

Figura 6.1. Esquema de la posible ruta de secreción de la SP-A. Al salir del trans-Golgi, la SP-A se segrega de las proteínas que serán secretadas por la vía constitutiva. El pH del medio en el que se encuentra la SP-A a lo largo de esta ruta de secreción se va acidificando, hasta llegar al pH alrededor de 5 de los cuerpos lamelares.

veolar se va acidificando desde el retículo endoplásmico (pH ≈ 7.0) hasta los cuerpos lamelares (pH ≈ 5.5) (Chander *et al.* 1986). Además, la concentración de calcio en el interior de estos compartimentos es relativamente alta (2-10 mM) (Eckenhoff 1989). Es posible que además la SP-A interaccione con algún elemento de la membrana de los compartimentos por los que pasa a lo largo de su ruta de secreción, lo que podría contribuir a su segregación de aquellas proteínas que serán secretadas por la vía constitutiva. El estado de autoagregación y los cambios conformacionales que experimenta la SP-A con el pH y el ambiente iónico del interior de estos compartimentos podría estar relacionado con su posible interacción con estos elementos de membrana. La fuerza iónica del compartimento también podría modular este fenómeno; según vimos en el último capítulo de resultados, la presencia de NaCl o de KCl 150 mM afecta al cambio estructural y a la extensión de la autoagregación producidos a pH ácido.

Por otra parte, hemos visto que la SP-A tiene la capacidad de inducir la agregación de bicapas lipídicas a pH ácido siempre que éstas contengan fosfolípidos ácidos; esta interacción con dichos fosfolípidos disminuye el estado de agregación de la SP-A. Es posible que esta propiedad esté implicada en el mecanismo que permite el denso apilamiento de membranas lipídicas dentro de los cuerpos lamelares. En este sentido, el PG, cuya función en el surfactante se desconoce actualmente, podría facilitar el empaquetamiento de las membranas del surfactante (dependiente de la SP-A y las proteínas hidrofóbicas SP-B y SP-C) dentro de estas estructuras de almacenamiento. En efecto, a un pH ácido similar al existente en los cuerpos lamelares, vimos que la SP-A, aunque se une a bicapas de DPPC, no induce su agregación, lo que sí ocurre si en la bicapa hay fosfolípidos ácidos. La presencia de PG podría por tanto favorecer e incluso ser necesaria para el empaquetamiento de membranas fosfolipídicas en los cuerpos lamelares. La especial interacción de la SP-A a pH ácido con mezclas lipídicas que contienen fosfolípidos ácidos podría otorgar un importante papel a estos fosfolípidos en la fase pre-secretora del ciclo del surfactante.

Una vez secretados los componentes del surfactante al espacio alveolar, las interacciones entre ellos varían al cambiar las condiciones de pH. Al pasar al pH neutro de la subfase acuosa del alveolo, la proteína experimenta cambios estructurales. En las condiciones iónicas presentes en el medio extracelular alveolar (NaCl 150 mM, Ca^{2+} 1.8 mM, pH 7.0) la SP-A interacciona con otras moléculas de SP-A, y además se incrementa su afinidad por DPPC e induce la agregación de vesículas compuestas por este fosfolípido. En estas condiciones, la SP-A no induce la agregación de vesículas de DPPG, a no ser que haya DPPC en la composición de la vesícula. Todos estos cambios podrían estar implicados en el reordenamiento de las membranas fosfolipídicas recién secretadas, y por tanto en la formación de mielina tubular y la posterior creación de la monocapa de DPPC y otros fosfolípidos por adsorción a la interfase de parte del material secretado (fig. 6.2).

El papel de la mielina tubular en el ciclo metabólico del surfactante pulmonar no está aún claro. Se sabe que su presencia no es necesaria para el correcto funcionamiento del surfactante como agente tensoactivo (Notter *et al.* 1986, Korfhagen *et al.* 1996). Sin embargo, se ha observado que la existencia de mielina tubular aparece asociada a una adsorción más rápida de sus componentes a la interfase aire/líquido (Korfhagen *et al.* 1996). El que la SP-A intervenga y sea necesaria para su formación se correlaciona con el hecho experimental de que la SP-A facilite la adsorción de fosfolípidos a la interfase (Hawgood *et al.* 1987, Yu y Possmayer 1990), aunque para ello necesita la ayuda de SP-B, proteína que también es imprescindible

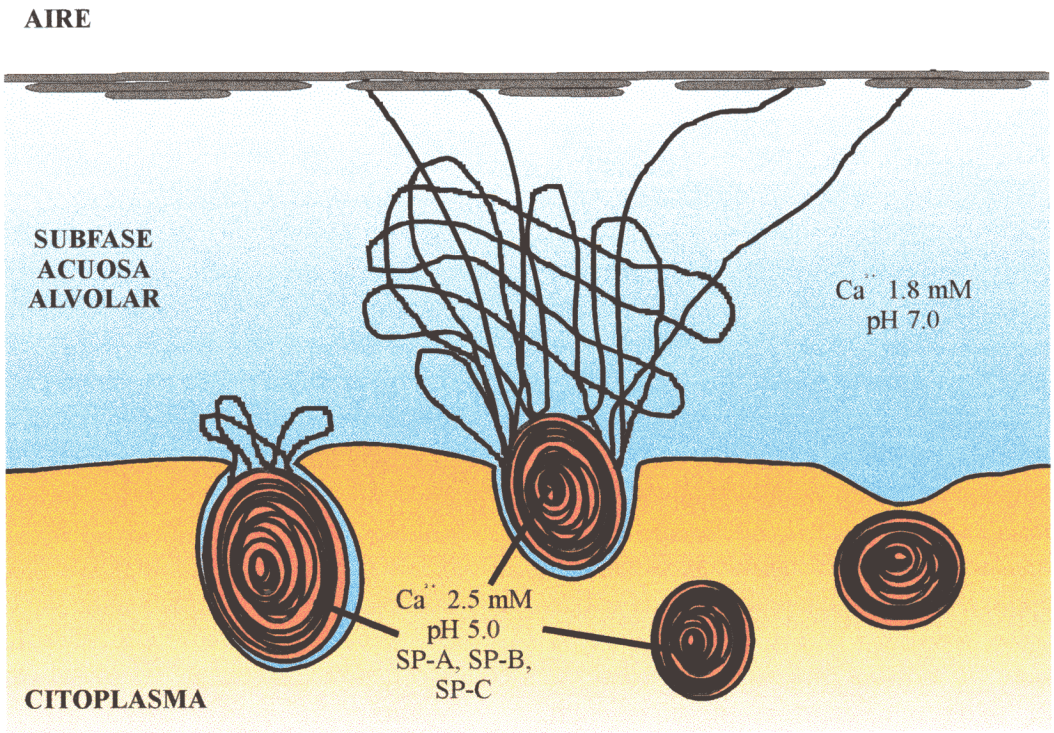

Figura 6.2. Secreción del surfactante pulmonar al espacio alveolar y formación de la monocapa. Al pasar del pH ácido de los cuerpos lamelares al pH neutro de la subfase acuosa en el espacio alveolar, los componentes del surfactante experimentan cambios en su organización que desembocan en la formación de la mielina tubular primero, y de la monocapa en la interfase después. Asociado a la monocapa permanece un reservorio de material lipoproteico.

para la formación de mielina tubular. En las condiciones iónicas existentes en la subfase acuosa del espacio alveolar (pH neutro, fuerza iónica fisiológica y calcio), la SP-A induce la agregación de vesículas lipídicas (King *et al.* 1983, Hawgood *et al.* 1985, Casals *et al.* 1993). La actividad de agregación de vesículas lipídicas de la SP-A se correlaciona con su capacidad para acelerar la adsorción interfacial de membranas que contengan SP-B, y probablemente con su participación en la formación de mielina tubular junto con la SP-B (Suzuki *et al.* 1989, Williams *et al.* 1991). Sin embargo, aún no se conoce el mecanismo por el que la SP-A induce esta agregación de vesículas lipídicas. Se ha propuesto que dicho fenómeno se podría dar como consecuencia de la autoagregación de la propia proteína mediante interacciones de tipo lectina (dependientes de calcio) entre los dominios CRD (dominios de reconocimiento de carbohidratos) de distintas moléculas de SP-A (Haagsman *et al.* 1991). Sin embargo, los resultados recogidos en el primer capítulo de esta tesis indican que la desglicosilación de la SP-A o la inhibición de interacciones de tipo lectina no inhibe la agregación de vesículas, lo que descarta la participación de dichas interacciones en este fenómeno. En el tercer capítulo proponemos que la agregación de vesículas inducida por la SP-A en presencia de Ca^{2+}/Na^{+} estaría mediada por la autoagregación de SP-A unida a lípidos en las mismas condiciones iónicas. Dos hechos experimentales apoyan esta hipótesis:

a) El requerimiento de Ca^{2+} (K_a^{Ca2+}) para ambos procesos en estas condiciones (pH neutro, fuerza iónica fisiológica) es muy similar (de orden micromolar).

b) La integridad del dominio colagénico es necesaria también para que se den tanto la autoagregación de la proteína como la agregación de las vesículas inducida por la SP-A (Haagsman *et al.* 1990; ver también el capítulo 5).

El mecanismo propuesto en el párrafo anterior, según el cual la agregación de vesículas lipídicas estaría mediada por la autoagregación de la SP-A, podría ayudarnos a comprender cómo se forma la mielina tubular. La mielina tubular es una estructura tridimensional. Para su formación, por tanto, es necesaria la presencia de algún elemento que sea capaz de distribuir los lípidos en distintos planos en el espacio. Este elemento podría ser la SP-A, si tenemos en cuenta que, en su estructura nativa, es un octadecámero organizado en seis trímeros. Cada uno de los trímeros puede interaccionar con lípidos en un plano distinto (agregación lipídica), y cada octadecámero puede interaccionar con otros octadecámeros (autoagregación), lo que contribuiría a formar la estructura tridimensional de la mielina tubular.

Una vez formada la monocapa en la interfase, la SP-A sigue interaccionando con los lípidos situados en ella. Como vimos en el segundo capítulo de resultados, cuando la monocapa está expandida (baja presión superficial o alta tensión superficial), la proteína tiende a situarse en la interfase, penetrando en la monocapa por los "huecos" existentes debido a defectos de empaquetamiento lipídico entre los dominios LC (líquido condensado) y LE (líquido expandido). La distribución de la SP-A en la monocapa depende de la composición de ésta: la presencia de fosfolípidos ácidos (PG) hace que se segregue en agregados proteicos (situados entre los dominios LC y LE) debido a la repulsión electrostática entre la SP-A (con carga superficial negativa a pH neutro) y el PG; en cambio, la ausencia de fosfolípidos ácidos (DPPC sola) permite su interacción

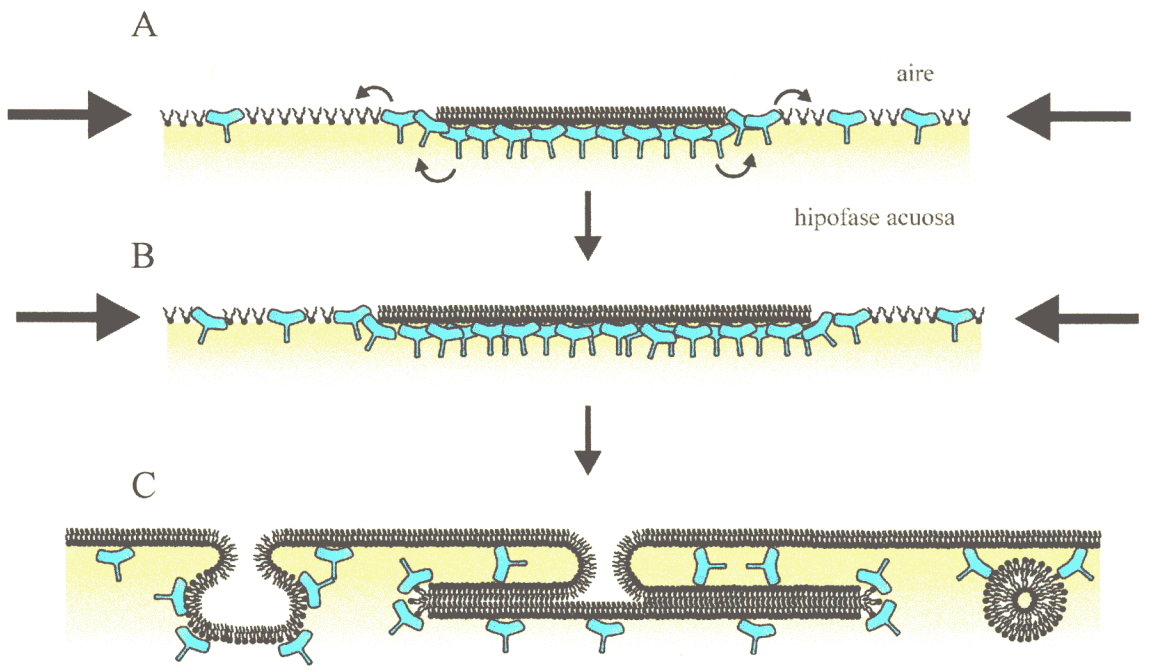

Figura 6.3. Compresión de la monocapa de surfactante. La SP-A procedente de la subfase acuosa se asocia con las zonas de la monocapa más empaquetadas, distribuyéndose posteriormente al resto de la monocapa desde estas regiones (A). Al aumentar el estado de compresión de la monocapa, aumenta el empaquetamiento de sus componentes (B) hasta alcanzar el colapso (C). Tras el colapso, los lípidos del surfactante permanecen asociados a la interfase aire/líquido gracias a la SP-A.

con los dominios LE, y posiblemente su penetración en ellos. Se cree que la monocapa de surfactante situada en la interfase aire/líquido del alveolo está muy enriquecida en DPPC, por lo que la interacción de la SP-A con los dominios LE no estaría impedida por repulsiones electrostáticas. Una consecuencia de dicha interacción podría ser un aumento en la estabilidad de la monocapa. Después, durante la compresión, la SP-A es expulsada de la monocapa, aunque es posible que siga asociada a ella de alguna manera formando puentes entre ésta y un reservorio de lípidos situado en la subfase (Yu y Possmayer 1996), lo que permitiría la reinserción de la proteína durante la siguiente expansión del alveolo (fig. 6.3). En esta reinserción podría arrastrar de nuevo hacia la monocapa parte de la DPPC que fue expulsada al alcanzarse el colapso de la monocapa durante la compresión, favoreciendo así que se mantenga más o menos constante la disponibilidad de DPPC en la interfase.

Por otra parte, la SP-A de la subfase acuosa (no asociada a la monocapa) podría interaccionar con los lípidos (DPPC y otros) que han sido expulsados de ésta durante la compresión, causando la agregación de éstos. La agregación de los elementos eliminados de la monocapa podría facilitar su captura por neumocitos tipo II y macrófagos alveolares (mediada por la unión de la SP-A a sus receptores situados en la membrana de estas células) para su posterior destrucción o reutilización. Aunque la interacción de la SP-A con monocapas es menor cuando hay fosfolípidos ácidos (ver segundo capítulo de resultados), cuando se trata de bicapas con este tipo de fosfolípidos la extensión de la agregación llega a ser incluso mayor que cuando las vesículas están compuestas sólo por DPPC en las condiciones del alveolo pulmonar (Casals *et al.*, 1993), por lo que la variada composición (incluidos fosfolípidos ácidos) de las vesículas salientes de la monocapa podría favorecer su agregación inducida por la SP-A.

La subfase acuosa del alveolo pulmonar tiene un pH neutro. En determinadas patologías, como en el síndrome de aspiración gástrica, el pH puede volverse ácido. Con los estudios realizados en esta tesis, hemos visto que una disminución del pH alveolar podría alterar el funcionamiento del surfactante, en tanto en cuanto afecta a la interacción entre sus distintos componentes. Así, en el primer capítulo de resultados demostramos que la SP-A es capaz de inducir la agregación de vesículas lipídicas con fosfolípidos ácidos, pero no de vesículas de DPPC a pH ácido. Por otra parte, esta proteína tampoco interacciona de igual forma con monocapas que poseen DPPG a pH neutro que a pH ácido (ver segundo capítulo de resultados); en el primer caso, la carga negativa del fosfolípido ejerce una repulsión electrostática sobre la proteína debido al pI ácido de ésta, mientras que a pH suficientemente ácido parte de sus residuos se protonan, permitiendo así la interacción entre el fosfolípido ácido y la proteína.

En resumen, el estudio de la estructura que adopta la SP-A en las diversas condiciones a las que se ve sometida durante su paso por los distintos compartimentos intra y extracelulares, así como el de sus propiedades funcionales en dichas condiciones, nos pueden ayudar a comprender los mecanismos moleculares que rigen los procesos de almacenamiento, secreción, adsorción y reciclaje del surfactante pulmonar. Sin embargo, aún es necesario ampliar más el conocimiento de estas propiedades, especialmente en lo referente a la interacción de la SP-A con las otras proteínas del surfactante (principalmente la SP-B y la SP-C), y estudiar qué partes de la proteína están implicadas en cada una de sus funciones.

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7. CONCLUSIONES

Los resultados concluyentes de esta memoria se exponen en el orden de presentación de los capítulos de acuerdo con los objetivos planteados.

A. Interacción de la SP-A con bicapas fosfolipídicas.

A1. pH neutro.

1. La SP-A se une e induce la agregación de vesículas que contengan DPPC (DPPC, DPPC/PC, DPPC/DPPG, etc.) a pH neutro. El proceso de agregación de vesículas dependiente de SP-A requiere concentraciones de Ca^{2+} de orden micromolar, siendo K_a^{Ca2+} = 0.74 ± 0.29 μM para la SP-A porcina, y 98 ± 5 μM para la SP-A canina. La extensión de la agregación de vesículas de fosfolípidos disaturados (DPPC, DPPC/DPPG), que a 37 °C se encuentran en estado de gel ondulado ($P_{\beta'}$), es mayor que la de vesículas formadas por DPPC/PC o DPPC/PG, lo que indica la influencia del estado físico de las vesículas en dicho proceso.

2. La agregación de vesículas que contienen DPPC no disminuye al aumentar la fuerza iónica del medio, lo que indica una importante contribución de las interacciones hidrofóbicas en la asociación SP-A/DPPC. La agregación de vesículas de DPPC/DPPG no ocurre a baja fuerza iónica debido a la repulsión electrostática entre la carga negativa de los fosfolípidos y la carga superficial negativa de la proteína.

3. La SP-A unida a membranas fosfolipídicas es menos susceptible de ser degradada por tripsina. La protección es ligeramente superior a concentraciones de Ca^{2+} en las que la extensión de los agregados vesícula lipídica/SP-A es máxima.

4. La capacidad de la SP-A de unirse a carbohidratos en presencia de Ca^{2+} no está implicada en el proceso de agregación lipídica inducida por esta proteína. Por otra parte, la eliminación de la cadena oligosacárida de la SP-A reduce sólo parcialmente su actividad agregante de vesículas. Estos resultados se oponen y refutan el mecanismo propuesto para explicar la agregación de vesículas inducida por SP-A (Haagsman *et al.* 1991) en el que se postula que dicha agregación podría estar mediada por interacciones lectina-lectina inducidas por Ca^{2+} entre dos moléculas de SP-A unidas a lípidos, de manera que la cadena oligosacárida de una de ellas interaccionaría con el dominio de unión a carbohidratos de la otra.

5. Se refuta también la hipótesis de que los complejos SP-A/vesícula agregan mediante el proceso de autoagregación de la SP-A caracterizado hasta fechas recientes como un proceso dependiente de concentraciones de Ca^{2+} de orden milimolar y baja fuerza iónica (Haagsman *et al.* 1990). Nuestra objeción a dicha hipótesis se basa en que el fenómeno de agregación de vesículas mediado por SP-A y la autoagregación de la SP-A tienen distintos requerimientos de Ca^{2+} (de orden micromolar para la agregación de vesículas y milimolar para la autoagregación de la proteína) y fuerza iónica. Además, ambos procesos se ven afectados de forma diferente por la acción de proteasas, la presencia del homopolisacárido manano, y la desglicosilación de la SP-A en presencia de N-glicosidasa F.

A2. pH moderadamente ácido (4.5-6.0).

1. A pH moderadamente ácido, la proteína SP-A se une tanto a vesículas neutras (DPPC) como a vesículas ácidas (DPPC/DPPG). Sin embargo, sólo es capaz de inducir una marcada agregación de vesículas que contengan fosfolípidos ácidos, mediante un proceso independiente de Ca^{2+}. La agregación de vesículas de DPPG a pH 4.5 inducida por SP-A decrece con el aumento de la concentración de NaCl en el medio, indicando que la interacción de SP-A con DPPG a pH ácido tiene lugar por interacciones electrostáticas. La agregación de vesículas de DPPC/DPPG a pH ácido disminuye sólo ligeramente con el incremento de la fuerza iónica del medio debido a la presencia de DPPC.

2. La unión de vesículas ácidas, pero no neutras, a la SP-A produce: a) un marcado cambio en la estructura secundaria de la proteína, y b) una significativa protección de la proteína frente a la degradación por la endoproteinasa Glu-C.

3. La unión de la proteína SP-A a vesículas fosfolipídicas y la agregación de dichas vesículas inducida por SP-A son fenómenos diferentes que probablemente tienen distintos requerimientos estructurales de la SP-A. Los estudios de dicroísmo circular y de epifluorescencia llevados a cabo en esta tesis indican que la interacción de vesículas ácidas con la SP-A impide la autoagregación masiva de la proteína que tiene lugar a pH ácido debido a la protonación de los grupos carboxilo de la SP-A, y por tanto a la reducción de su carga superficial negativa.

4. La agregación de vesículas de DPPC inducida por la SP-A en presencia de Ca^{2+} es altamente dependiente del pH, decreciendo progresivamente al disminuir éste. El efecto del pH sobre la agregación de vesículas de DPPC inducida por la SP-A es reversible, de manera que se produce un drástico incremento de la agregación de vesículas de DPPC inducida por SP-A en presencia de Ca^{2+} cuando el pH del medio cambia de ácido a neutro. Estos resultados son aún más marcados cuando las bicapas de DPPC contienen una o ambas proteínas hidrofóbicas del surfactante (SP-B y/o SP-C).

B. Interacción de la SP-A con monocapas de fosfolípidos a pH neutro y ácido.

1. La SP-A interacciona y perturba el empaquetamiento de monocapas de DPPC a pH neutro. La SP-A fluorescente se distribuye en la fase LE del fosfolípido y se acumula en la interfase entre los dominos condensados y fluidos (LC-LE) bajo condiciones de presión superficial en las que hay coexistencia de ambas fases.

2. La perturbación del empaquetamiento lipídico de monocapas de DPPC inducida por la SP-A se pone de manifiesto por la formación de dominios LC más pequeños y numerosos a una presión dada, y por la disminución de la cantidad total de área condensada. Dicha perturbación de la monocapa es similar a la producida por la proteína hidrofóbica del surfactante pulmonar SP-C, e indica que la SP-A debe interaccionar con las cadenas de acilo de la monocapa de fosfolípidos de manera suficiente como para perturbar el habitual empaquetamiento de lípidos.

3. La influencia perturbadora de la SP-A es específica para monocapas de DPPC a pH neutro, ya que no se observa en monocapas de DPPC/DPPG. Además, la fluorescen-

cia de la SP-A marcada con Texas Red® no se detecta en las regiones LE de monocapas de DPPC/DPPG, observándose sólo en la interfase LC-LE parches discretos de fluorescencia que se interpretan como acúmulos de SP-A agregada segregados en dicha interfase. La exclusión de la SP-A de las regiones LE se debe a la repulsión electrostática entre la proteína y los fosfolípidos ácidos (DPPG) a pH neutro. Las propiedades tensoactivas intrínsecas de la SP-A le hacen acceder a la monocapa desde la hipofase acuosa a pesar de su inmiscibilidad con fosfolípidos ácidos.

4. El tipo de interacción de la SP-A con monocapas de DPPC o DPPC/DPPG varía con el pH, debido a los cambios estructurales que experimenta la proteína a pH moderadamente ácido. Los agregados de SP-A, formados en la hipofase a pH ácido, se insertan en monocapas de DPPC en la interfase LC-LE. Dicha inserción no produce ningún efecto ni en la cantidad total de dominios condensados ni en el número y tamaño de éstos, lo que indica una total segregación del lípido y la proteína a pH ácido.

5. La distribución de SP-A marcada con Texas Red® en monocapas de DPPC/DPPG a pH ácido es notablemente diferente a la observada a pH neutro. En medio ácido, la SP-A se distribuye de manera casi homogénea en la fase LE. La interacción de la SP-A con monocapas de DPPC/DPPG impide y revierte la agregación de la proteína en medio ácido. Dicha interacción es de carácter electrostático y no induce perturbaciones en el empaquetamiento de fosfolípidos en la monocapa.

C. Interacciones proteína-proteína.

1. La SP-A experimenta autoagregación a pH neutro en presencia de Ca^{2+}. La concentración de Ca^{2+} requerida para dicho proceso depende de la fuerza iónica del medio. A baja fuerza iónica la K_a^{Ca2+} para el proceso de autoagregación de la SP-A es 2.3 ± 0.2 mM para la SP-A porcina, y 3.0 ± 0.1 mM para la SP-A humana. Sin embargo, a fuerza iónica fisiológica las K_a^{Ca2+} son 2.4 ± 0.5 μM y 12.0 ± 1.8 μM para la SP-A porcina y humana, respectivamente.

2. Estudios de dicroísmo circular y de fluorescencia sobre la autoagregación de la SP-A dependiente de Ca^{2+} indican que los agregados de proteína formados en ausencia de NaCl son estructuralmente distintos de aquéllos formados en presencia de esta sal.

3. Ambos tipos de agregados (los dependientes de Ca^{2+} y de Ca^{2+}/NaCl) se disocian mediante la adición de EDTA. Sólo la proteína resultante de la disociación de agregados inducidos por Ca^{2+}/NaCl mantiene su estructura secundaria y su actividad biológica agregante de vesículas lipídicas.

4. Experimentos realizados a temperaturas inferiores y superiores a la temperatura de desnaturalización de la región colagénica de la SP-A permiten concluir que esta región es necesaria para la autoagregación de la SP-A inducida por Ca^{2+} o Ca^{2+}/NaCl. Las interacciones entre las triples hélices de colágeno son importantes para la oligomerización de la SP-A y el mantenimiento de su estructura cuaternaria.

5. La SP-A experimenta una marcada autoagregación a pH moderadamente ácido en ausencia o presencia de Ca^{2+}. Dicho proceso se acompaña de un cambio en la estructura secundaria de la proteína que es totalmente reversible al neutralizar el pH.

6. La autoagregación de la SP-A aumenta en función de la disminución del pH. Sin embargo, dicho proceso está modulado por la presencia de fuerza iónica fisiológica y Ca^{2+}, factores que reducen la extensión de esta autoagregación. Estas condiciones iónicas son semejantes a las que existen a lo largo de la ruta exocítica de la SP-A.

7. En contraste con la autoagregación de la SP-A dependiente de Ca^{2+}, la autoagregación de la SP-A dependiente de pH no se ve afectada por el desplegamiento del dominio de colágeno, lo que indica que ni la integridad de este dominio ni la oligomerización de la SP-A son condiciones necesarias para este tipo de agregación proteica.

8. El proceso de autoagregación de SP-A dependiente de Ca^{2+}/NaCl y el proceso de agregación de vesículas inducido por SP-A y dependiente de Ca^{2+}/NaCl parecen estar relacionados. Ambos procesos tienen K_a^{Ca2+} similares y requieren un dominio colagénico intacto en la SP-A. Proponemos que en determinadas condiciones iónicas, de pH y de temperatura la asociación entre moléculas de SP-A unidas a lípidos desencadena la agregación entre vesículas a su vez unidas a la SP-A.

Las implicaciones fisiológicas de todos estos resultados se analizan en la discusión general.