F-actin-binding protein drebrin regulates CXCR4 recruitment to the immune synapse

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Summary

The adaptive immune response depends on the interaction of T cells and antigen-presenting cells at the immune synapse. Formation of the immune synapse and the subsequent T-cell activation are highly dependent on the actin cytoskeleton. In this work, we describe that T cells express drebrin, a neuronal actin-binding protein. Drebrin colocalizes with the chemokine receptor CXCR4 and F-actin at the peripheral supramolecular activation cluster in the immune synapse. Drebrin interacts with the cytoplasmic tail of CXCR4 and both proteins redistribute to the immune synapse with similar kinetics. Drebrin knockdown in T cells impairs the redistribution of CXCR4 and inhibits actin polymerization at the immune synapse as well as IL-2 production. Our data indicate that drebrin exerts an unexpected and relevant functional role in T cells during the generation of the immune response.

Key words: Actin cytoskeleton, CXCR4, Immune synapse

Introduction

T cell activation is a key process in the generation of the adaptive immune response. Activation of T cells during their interaction with antigen-presenting cells (APCs) requires the formation of a highly organized complex of surface receptors and intracellular signaling molecules at the site of cell-to-cell contact, known as the immunological synapse (IS) (Dustin, 2009; Monks et al., 1998). IS organization is characterized by the segregation of key molecules into different concentric structures named supramolecular activation clusters (SMAC). The central SMAC includes the T-cell receptor (TCR), CD3, and associated co-stimulatory molecules such as CD28 (Irvine et al., 2002), whereas the peripheral SMAC mainly contains adhesion receptors (integrins and intercellular adhesion molecules or ICAMs) (Cemerski and Shaw, 2006; Monks et al., 1998; Saito and Yokosuka, 2006). Subsequent studies revealed a third concentric zone, the distal SMAC, which is outside the peripheral SMAC and is enriched in actin and actin-related proteins such as Arp2/3 (Dustin, 2009; Freiberg et al., 2002; Sims et al., 2007). Formation of a mature IS thus requires a dynamic reorganization of the cellular cytoskeleton, including the formation of a tight ring of polymerized actin beneath the plasma membrane of the peripheral SMAC and distal SMAC to maintain the cell-to-cell contact (Dustin, 2009; Valitutti et al., 1995).

Several actin-binding proteins are involved in IS formation and T cell activation, including the Wiskott-Aldrich syndrome protein (WASP), HS1 and WAVE, which activate the Arp2/3 complex (Billadeau et al., 2007) promoting nucleation, extension and branching of actin filaments. Additional studies have shown that WASP-deficient T cells show defects in activation and TCR clustering during the formation of IS (Holsinger et al., 1998; Snapper et al., 1998). However, the function of other actin cytoskeleton regulatory proteins expressed by lymphocytes during the formation of the IS is currently unclear (Fuller et al., 2003).

Drebrin is an actin-binding protein, expressed mainly by neurons (Sekino et al., 2007; Shirao and Obata, 1985), which exerts an important regulatory role on actin dynamics. Two drebrin isoforms have been described: an embryonic type abundant in the cell bodies of developing neurons (drebrin E), and an adult type (drebrin A) which is mainly present in dendritic spines in adult brain. However, no clear functional differences have been described between these two isoforms (Hayashi and Shirao, 1999). Drebrin regulates neuronal actin dynamics and plasticity (Asada et al., 1994; Kobayashi et al., 2004; Sekino et al., 2007; Shiraishi-Yamaguchi et al., 2009) binding to F-actin with high affinity, thereby competing with proteins such as fascin, α-actinin and tropomyosin (Ishikawa et al., 1994; Sasaki et al., 1996). Binding of drebrin to F-actin also facilitates the recruitment of other regulatory proteins such as gelsolin. Accordingly, overexpression of drebrin dissociates tropomyosin from actin filaments, and induces dramatic changes in stress fibers (Ishikawa et al., 1994; Shirao et al., 1994). Drebrin has also been included in the actin depolymerizing factor homology (ADF-H) family of actin-binding proteins, since it contains an actin-depolymerizing factor domain (Lappalainen et al., 1998). Furthermore, drebrin interacts directly with the actin-binding protein profilin (Mammoto et al., 1998).

Chemokine receptors (CKRs) regulate critical processes in immune and inflammatory responses, including leukocyte activation and migration, immune cell development (Rossi and Zlotnik, 2000; Rottman, 1999) and angiogenesis (Salcedo and Oppenheim, 2003). The CKR CXCR4 is essential for homeostasis, cell migration,

inflammation, B-cell development and tumor metastasis (Muller et al., 2001; Proudfoot et al., 1999; Wells et al., 1998). CKR signaling induces a rapid increase in the affinity and mobility of adhesion molecules, including the integrin LFA-1, which interacts with ICAM-1 (Kinashi, 2005). Chemokines might also regulate TCR triggering, since a functional association between CXCR4 and the TCR complex has recently been reported (Kumar et al., 2006). In this regard, the redistribution of CKRs to the IS enhances T cell-APC adhesion, cytokine production and T-cell proliferation, and diminishes the sensitivity of T cells to other chemokine gradients (Molon et al., 2005).

In this study, we describe the expression and functional role of drebrin in T cells. Drebrin interacts with CXCR4, and this molecular complex is redistributed towards the peripheral SMAC and distal SMAC during IS formation. Furthermore, the interaction between drebrin and CXCR4 increases upon antigenic stimulation in the cell-cell contact area. Finally, drebrin knockdown impairs CXCR4 redistribution and actin polymerization at the IS resulting in defective IL-2 production.

Results

Drebrin associates with the chemokine receptor CXCR4

In the search for molecules able to interact with CXCR4, a GST fusion protein containing the C-terminus of this chemokine receptor (GST-CXCR4cyt) was generated, as described previously (Rey et al., 2002). We detected three protein bands specifically bound to the C-terminus of CXCR4 by pull-down assays performed with cell lysates of J77 T cells. Whereas the two higher molecular mass bands corresponded to myosin II heavy chains (Rey et al., 2002), proteomic

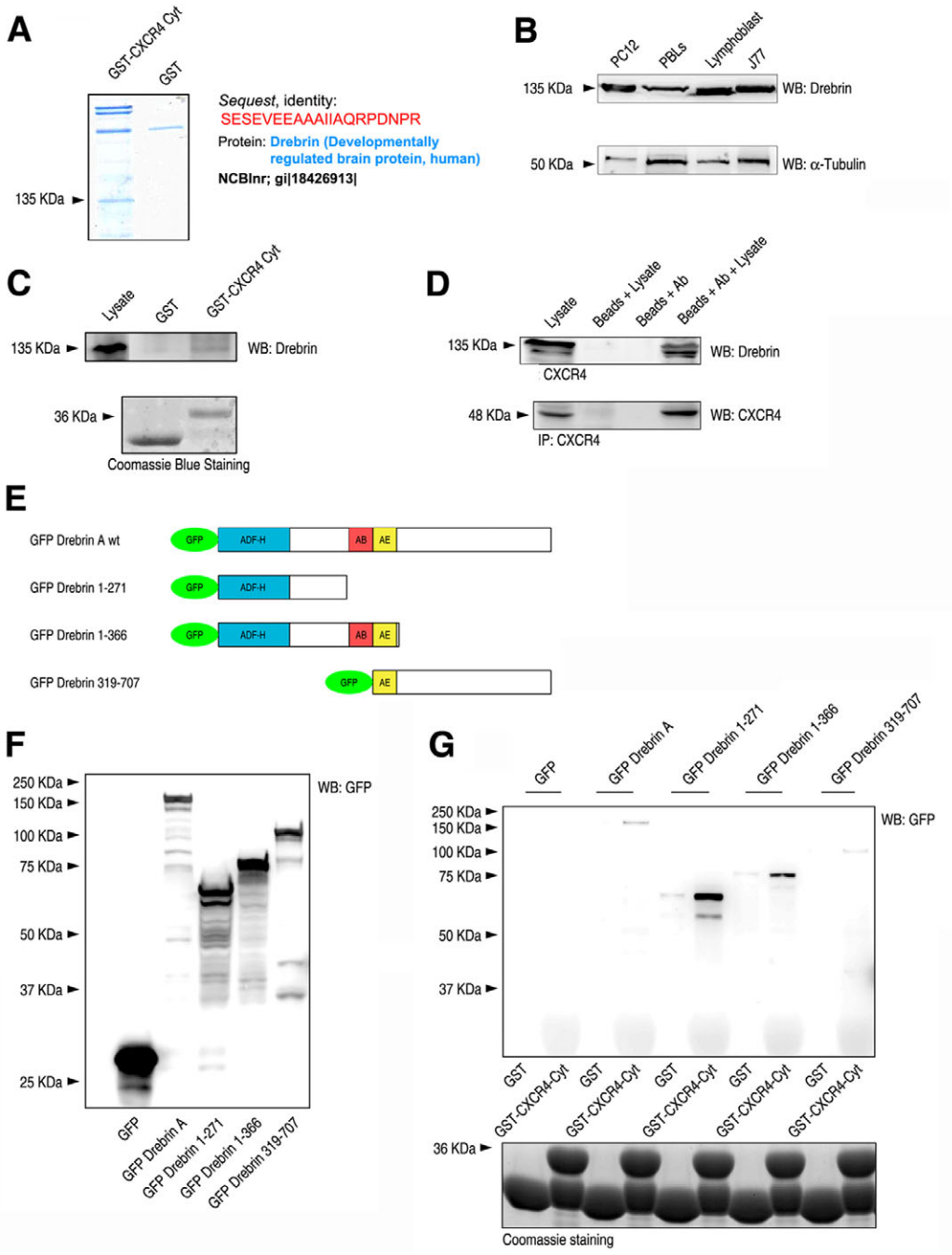

Fig. 1. Drebrin associates with the chemokine receptor CXCR4. (**A**) Proteomic identification of drebrin from J77 cell extract pulled-down with the cytoplasmic tail of CXCR4 coupled to GST (GST-CXCR4-Cyt). Database analysis confirmed the identity of the 135 kDa band as drebrin. (**B**) Drebrin immunodetection from total lysates of a neural cell line (PC12), primary peripheral blood lymphocytes (PBLs), primary T lymphoblasts and J77 T cell line. α-tubulin was used as loading control (lower panel). (**C**) GST-CXCR4-Cyt pull-down from J77 cell lysates immunoblotted to detect drebrin. Coomassie Blue staining of the GST precipitates is shown in the lower panel. After subtraction of background signal and normalization according to their loading level (Coomassie Blue staining), GST densitometry signal had a value of 0.06, and GST-CXCR4-Cyt of 1.0. (**D**) Immunoprecipitation of drebrin with anti-CXCR4 antibody. CXCR4 immunodetection is shown in the lower panel. (**E**) Scheme showing the different truncated construct of drebrin and its predicted functional domains. ADF-H, ADF homology domain; AB, actin binding domain; AE, adult specific exon. (**F**) Representative western blot detecting the GFP signal of total lysates from HEK293T cells transfected with the indicated drebrin constructs used in the pull-down experiments. (**G**) Representative pull-down experiment using GST-CXCR4-Cyt and HEK293T cells transfected with different GFP-drebrin truncated constructs. Upper panel: immunodetection of GFP; lower panel: Coomassie Blue staining.

analysis identified the third protein (135 kDa) as drebrin (Fig. 1A; supplementary material Fig. S1). The presence of drebrin in peripheral blood T cells, T lymphoblasts and Jurkat J77 cells was confirmed by western blot analysis (Fig. 1B). In addition, the association of drebrin with CXCR4 was corroborated by pull-down assays with J77 T cell lysates and GST-CXCR4-Cyt, and staining with an anti-drebrin monoclonal antibody (Fig. 1C). Association between endogenous drebrin and CXCR4 was further confirmed by co-immunoprecipitation assays, with anti-CXCR4-coupled beads, and staining for drebrin and CXCR4 in J77 T cell lysates (Fig. 1D). In these assays, two bands were detected with an anti-drebrin polyclonal antibody, corresponding to the A and E isoforms of this protein, whereas in reverse co-immunoprecipitation with beads coupled to the M2F6 monoclonal antibody only drebrin A was detected (data not shown).

To more precisely determine the interaction of drebrin with CXCR4, pull down experiments were carried out using GST-CXCR4-Cyt and lysates of HEK293T cells transfected with different truncated forms of drebrin (Fig. 1E). Expression of these constructs was checked by western blot analysis (Fig. 1F), and equivalent transfection levels were selected to perform the pull-down assay. Although a significant interaction was observed between the cytoplasmic tail of CXCR4 and full-length drebrin, a stronger association was detected using drebrin 1-271 and 1-366 (Fig. 1G). By contrast, the 319-707 truncated form barely associated with the CXCR4 tail (Fig. 1G). These results indicate that drebrin associates with CXCR4 by its N-terminal region, and that its C-terminal region seems to negatively regulate this interaction.

Drebrin is recruited to the T cell–APC contact site during IS formation

Since recent studies have described the presence of different CKRs at the IS (Kumar et al., 2006; Molon et al., 2005), we next addressed the localization of drebrin in conjugates of J77 T cells with Raji B cells, by confocal microscopy. In the absence of the superantigen E (SEE), drebrin and F-actin were evenly distributed throughout subcortical regions of the T-cell membrane and cytoplasm, and CD3 was uniformly distributed throughout the T-cell membrane (Fig. 2A). By contrast, in the presence of SEE-loaded APCs, drebrin, F-actin and CD3 accumulated at the T cell-APC contact area (Fig. 2A), and drebrin localization clearly mirrored that of F-actin surrounding CD3 staining (Fig. 2A). Quantitative analysis showed that the presence of SEE induced a significant increase in cells that relocalized endogenous drebrin towards the IS (Fig. 2B).

To further ascertain the subcellular distribution of drebrin at the IS, we assessed, by confocal microscopy, the localization of endogenous drebrin together with several markers of the peripheral and distal SMACs. We found that drebrin partially colocalized with VLA-4 integrin and talin at the peripheral SMAC and mostly overlapped with Arp2/3 and cofilin at the distal SMAC (Fig. 3A,B; data not shown and supplementary material Movie 1).

Additional experiments showed that GFP-drebrin A fully colocalized with endogenous drebrin in the presence or absence of SEE (data not shown). Accordingly, SEE stimulation induced GFP-drebrin relocation to the T-cell–B-cell contact zone, in parallel with CD3 and F-actin (supplementary material Fig. S2A). Quantitative analysis showed that GFP-drebrin A (which was expressed at its expected size; supplementary material Fig. S2B) concentrated at the IS in 75% of cell conjugates formed in the presence of SEE, whereas in the absence of superantigen only 28% of cell conjugates showed drebrin relocation (supplementary material Fig. S2C). Similar results were observed when J77 T cells were transfected with GFP-drebrin E, the embryonic isoform of this protein (supplementary material Fig. S2C).

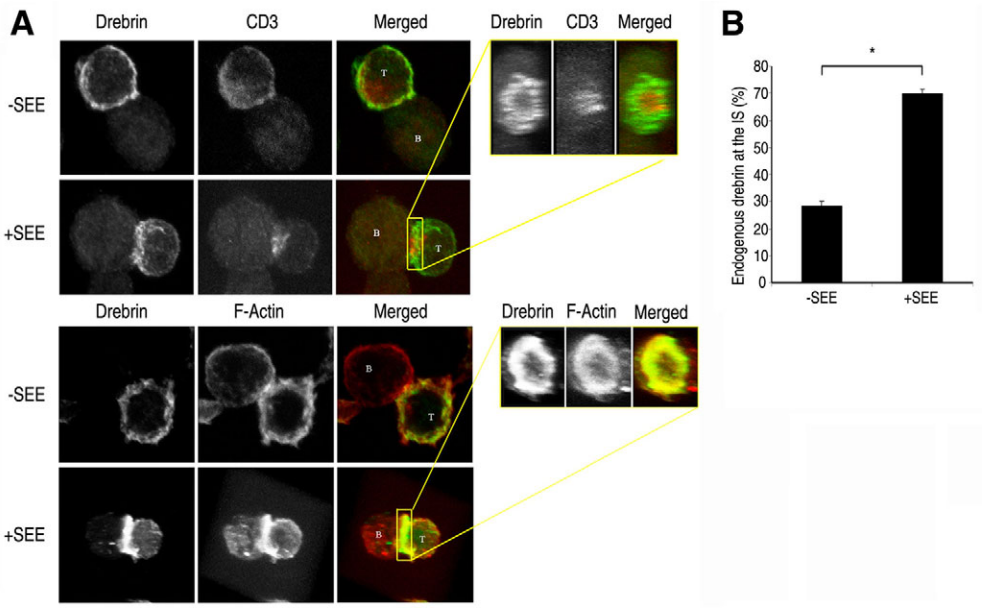

Fig. 2. Drebrin is recruited to the T cell-APC contact site during IS formation. (**A**) Confocal immunofluorescence analysis of endogenous drebrin localization in J77 T cells (T) conjugated with Raji cells loaded (+SEE) or not (–SEE) with SEE. Upper panel set shows staining for drebrin (green) and CD3 (red) and the lower panel set shows drebrin (green) and F-actin (red). Three-dimensional reconstructions of the SMAC structures are shown in the magnified panels. (**B**) Quantification of drebrin relocalization in the presence or absence of SEE stimulation. Data are presented as the means ± s.d. of the percentage of cell conjugates with endogenous drebrin relocated to the IS in four independent experiments (*P<0.05, Mann-Whitney U-test).

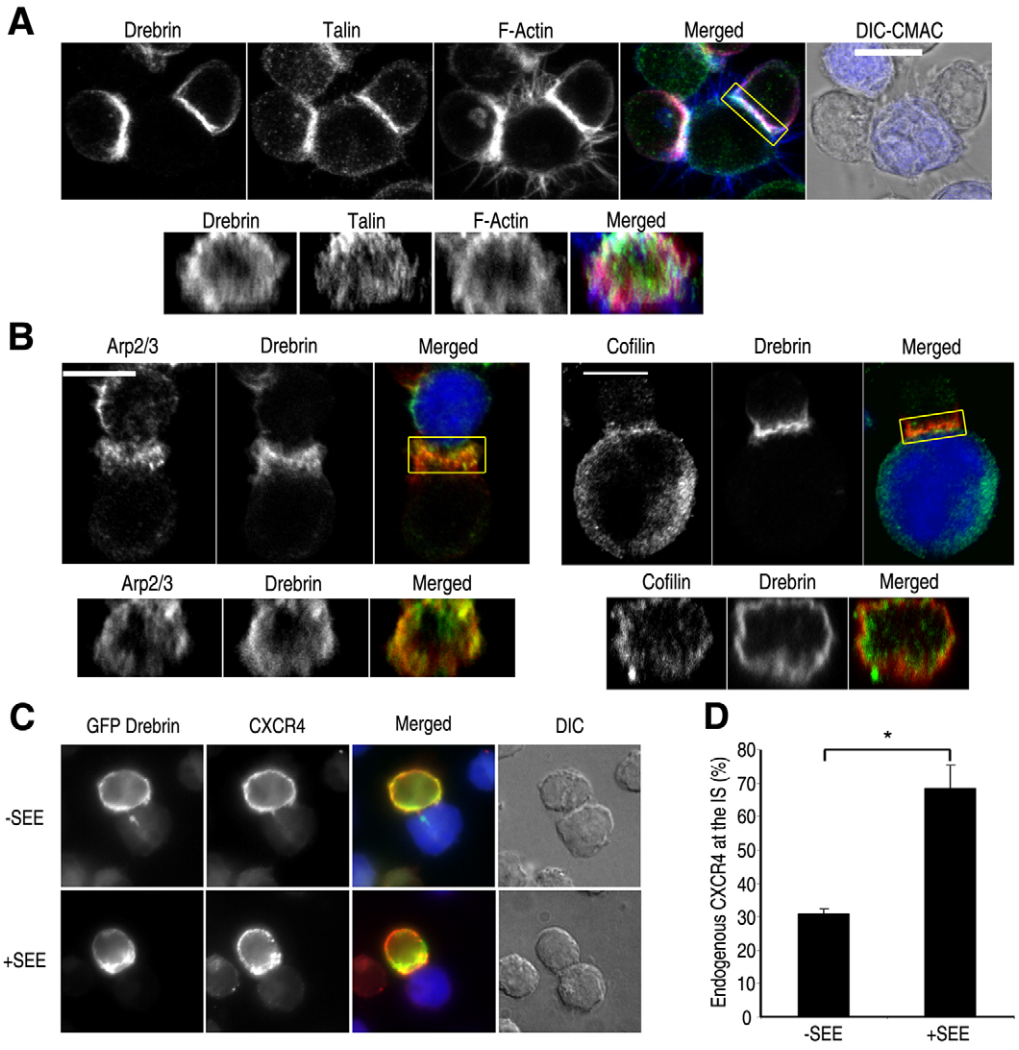

Fig. 3. Drebrin is recruited to the peripheral SMAC and distal SMAC and colocalizes with CXCR4 in the T cell-APC contact site during IS formation. (**A**) Confocal immunofluorescence analysis of endogenous drebrin localization in J77 T cells conjugated with Raji cells loaded with SEE (blue in the DIC-CMAC image) in relation to the peripheral SMAC marker talin. The upper panel set shows staining for drebrin (red), talin (green) and F-actin (blue). The coronal projections of the boxed contact is shown in the magnified panels below. (**B**) Confocal immunofluorescence analysis of endogenous drebrin localization in J77 T cells conjugated with Raji cells loaded with SEE in relation to several markers of distal SMAC. Left panel set shows staining for drebrin (red) and Arp2/3 (green), and the right panel set shows drebrin (red) and cofilin (green). The coronal projections of the contacts are shown in the magnified panels below. (**C**) Fluorescence image of J77 T cells transfected with GFP-drebrin (green) conjugated with Raji cells (blue) loaded (+SEE) or not (–SEE) with SEE. Endogenous CXCR4 staining is shown in red. In the presence of SEE, drebrin and CXCR4 colocalize at the IS. (**D**) Quantification of J77-Raji conjugates showing CXCR4 localization at the IS. J77 T cells were conjugated with Raji B cells loaded (+SEE) or not (–SEE) with SEE. Data are the percentage (± s.d.) of conjugates showing endogenous CXCR4 localized to the IS of four independent experiments (*P<0.05, Mann-Whitney U-test).

When J77 cells transfected with GFP-drebrin and conjugated with Raji B cells, in the absence of SEE, were stained for endogenous CXCR4, both drebrin and the CKR were evenly distributed along the cell membrane. By contrast, when cell conjugates were formed in the presence of SEE, GFP-drebrin and CXCR4 were redistributed towards the IS (Fig. 3C,D).

CXCR4 and drebrin dynamically redistribute to the IS

The localization of CXCR4 and drebrin at the IS was further analyzed. Three-dimensional image reconstruction of the IS formed by J77 T cells and SEE-pulsed Raji B cells, showed that CD3 was distributed at the central SMAC, as previously described (Monks et al., 1998), whereas GFP-drebrin showed the ring-shape distribution typical of the peripheral and distal SMAC (Fig. 4A). Additional analysis of conjugates formed between SEE-pulsed Raji B cells and J77 cells co-transfected with CXCR4-YFP and CFP-drebrin showed that both molecules were localized at the peripheral and distal SMAC (Fig. 4B). CXCR4 showed a punctate distribution, whereas drebrin formed a continuous ring.

Time-lapse live-cell imaging experiments confirmed the relocalization of GFP-drebrin during the formation of the IS, and showed a clear enrichment of GFP-drebrin at the T cell-APC contact site, starting after about 5 minutes, reaching a maximum at 10 minutes, and remaining at the IS for at least 18 minutes (Fig. 4C; supplementary material Movie 2). We further assessed

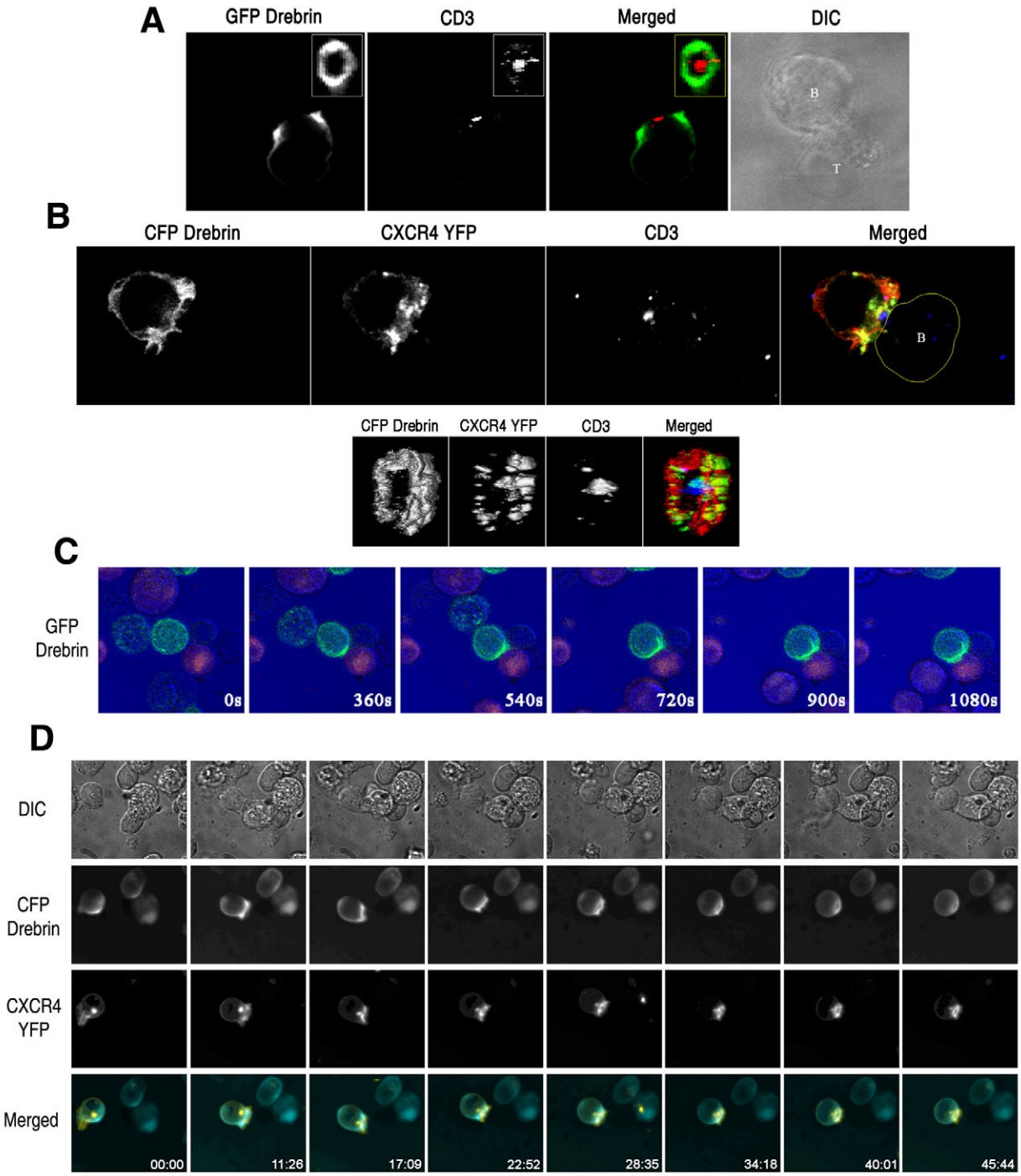

Fig. 4. Drebrin and CXCR4 dynamically redistribute at the IS. (**A**) 3D confocal images of SMACs in conjugates of J77 T cells expressing GFP-drebrin (green) with SEE-Raji. CD3 staining is shown in red. (**B**) Representative confocal image of J77 T cell coexpressing CFP-drebrin (red) and CXCR4-YFP (green) conjugated with a SEE-loaded Raji B cell. CD3 staining is shown in blue. The perimeter of the Raji cell is outlined in yellow in the merged image. The lower panel shows the 3D rendering of the T cell-APC contact area, illustrating the localization of drebrin and CXCR4, surrounding the central CD3 cluster. (**C**) Confocal time series (images acquired every 30 seconds) from J77 T cells transiently transfected with GFP-drebrin (green), contacting Raji cells (red) loaded with SEE. Time is shown in seconds. (**D**) Time-lapse fluorescence microscopy of J77 T cells transiently co-transfected with CFP-drebrin and CXCR4-YFP, contacting Raji B cells loaded with SEE. Images were acquired every 5 minutes. Time is shown in minutes and seconds in the lowest row.

the dynamic behavior of drebrin and CXCR4 molecules during IS formation. Dynamic studies revealed that the relocation kinetics of CFP-drebrin and CXCR4-YFP were similar; both molecules were rapidly recruited to the T cell-APC contact site after conjugate formation, reaching a maximum around 11 minutes after cell-cell contact and remaining at the peripheral SMAC for at least 45 minutes (Fig. 4D; supplementary material Movie 3).

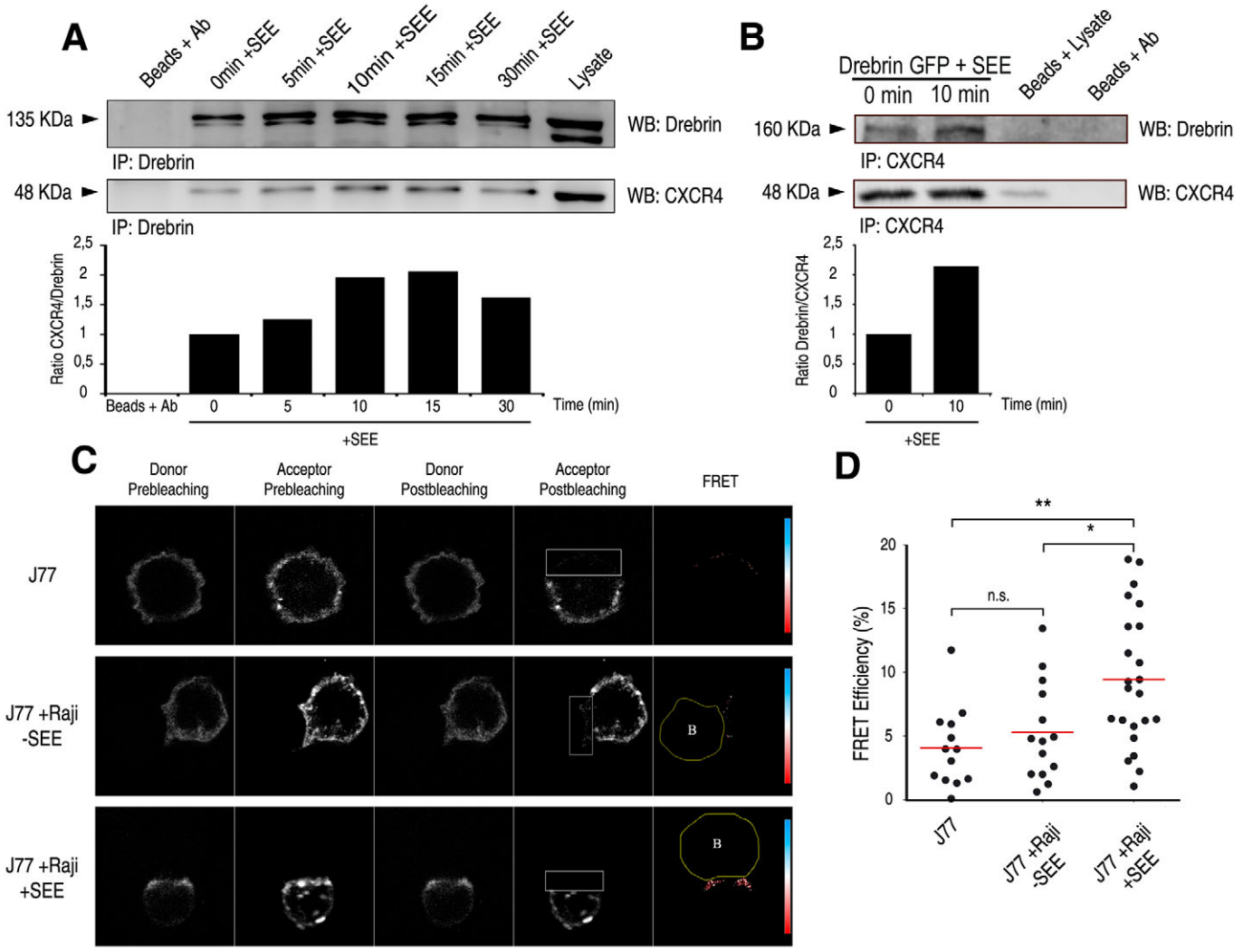

Fig. 5. Drebrin-CXCR4 interaction increases at the IS upon stimulation with SEE. (**A**) Kinetic analysis of CXCR4-drebrin interaction by immunoprecipitation with drebrin antibody in SEE-stimulated J77 cells at different times. Quantification of the CXCR4/drebrin ratio is presented in the lower panel. (**B**) Immunoprecipitation of drebrin with CXCR4 antibody in J77 cells transfected with GFP-drebrin and stimulated with SEE. Quantification of drebrin/CXCR4 ratio is presented in the lower panel. (**C**) FRET analysis of CXCR4-drebrin association. J77 cells co-transfected with CFP-drebrin and CXCR4-YFP unstimulated or conjugated with Raji cells with and without SEE. FRET efficiency was analyzed at the cell to cell contact. The regions of interest for FRET analysis are boxed. The perimeter of the Raji cells is outlined in 'FRET' panels. (**D**) FRET efficiencies for the experimental conditions in C. Horizontal red bars indicate the arithmetic mean. Data were analyzed by a parametric ANOVA with Bonferroni's post-hoc multiple comparisons test. **P*<0.05; ***P*<0.01; n.s., non-significant. *P*-value of ANOVA=0.0019.

Antigenic stimulation enhances CXCR4 and drebrin interaction at the IS

The interaction between drebrin and CXCR4 was assessed by time-course co-immunoprecipitation experiments in cell lysates from J77-Raji B cells conjugates. These assays showed that although the interaction between CXCR4 and drebrin occurs in conjugates with unstimulated Raji cells, this interaction was significantly higher in conjugates with SEE-stimulated Raji cells, reaching a maximum at approximately 10 minutes after T cell-APC contact (Fig. 5A,B).

To ascertain whether drebrin and CXCR4 directly interact, and to determine the subcellular location of this interaction, FRET experiments were performed in J77 T cells co-transfected with CFP-drebrin and CXCR4-YFP. Confirming the biochemical data, we found that the energy transference between the two molecules was increased by superantigen stimulation (Fig. 5C,D). In addition, the FRET signal was maximal at the T-cell-B-cell contact area, demonstrating that direct interaction between drebrin and CXCR4 increased at the IS.

Drebrin knockdown impairs CXCR4 recruitment to the IS

We next examined the potential role of drebrin in the subcellular localization of CXCR4 during the formation of the IS. Knockdown of drebrin by siRNA (Fig. 6A) did not modify the level of expression of CXCR4 or of other molecules involved in IS formation, including CD3, CD4, TCR, VLA-4, ICAM-3 and ICAM-1 (Fig. 6B; supplementary material Fig. S3). However, drebrin-depleted T cells were unable to properly relocate CXCR4 to the IS (Fig. 6C). Quantitative analysis showed that CXCR4 was relocated to the IS in 75% of SEE-stimulated J77 cells transfected

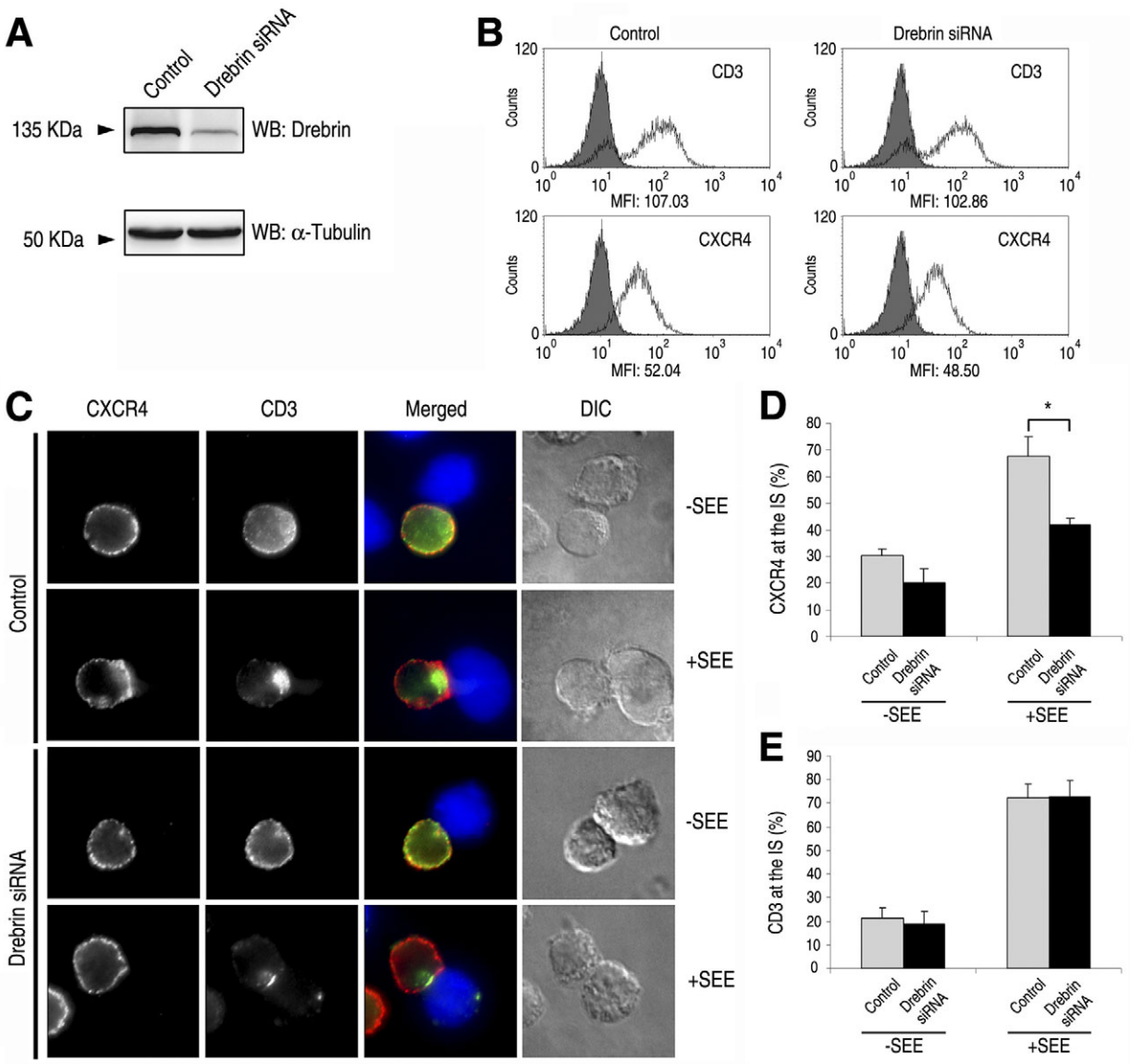

Fig. 6. Drebrin knockdown impairs CXCR4 recruitment to the immune synapse: (**A**) Western blot showing drebrin expression in control or drebrin-interfered J77 cells. α-tubulin was used as loading control. (**B**) Representative flow cytometry histograms of CD3 and CXCR4 surface expression from J77 T cells transfected with control or drebrin siRNA. Expression of both molecules was unaltered by Drebrin knockdown. MFI, mean fluorescence intensity. (**C**) Fluorescence images of J77 T cells transfected with a negative control or drebrin siRNA and conjugated with Raji B cells loaded (+SEE) or not (–SEE) with SEE. CXCR4 is shown in red, CD3 in green and B cells in blue. (**D**) Quantification of J77-Raji conjugates showing CXCR4 localization at the IS. J77 T cells transfected with control or drebrin siRNA were conjugated with Raji B cells loaded (+SEE) or not (–SEE) with SEE. Data represent the percentage (± s.d.) of four independent experiments (**P*<0.05, Mann-Whitney *U*-test. (**E**) Quantification of J77/Raji conjugates showing CD3 localization at the IS. T cells transfected with control or drebrin siRNA were conjugated with Raji B cells loaded (+SEE) or not (–SEE) with SEE. Data represent the percentage (± s.d.) of three independent experiments (**P*<0.05, Mann-Whitney *U*-test).

with control siRNA, whereas the figure for SEE-stimulated J77 cells transfected with drebrin siRNA was 46% (Fig. 6D). By contrast, CXCR4 relocation in conjugates formed with unpulsed Raji B cells was similar in control and drebrin-siRNA-treated T cells (Fig. 6D). In addition, drebrin silencing did not affect the clustering of CD3 at the central SMAC (Fig. 6E). These results highlight the specificity of the action of drebrin on superantigen-induced CXCR4 recruitment towards the IS. In addition, these data indicate that CD3 (and probably other molecules) is able to relocate to the IS despite a diminished polymerization of actin at this structure.

Silencing of drebrin downregulates actin accumulation at the IS

Since an actin cytoskeleton is necessary for T-cell activation during IS (Billadeau et al., 2007), and drebrin is involved in the regulation of actin polymerization in neurons (Hayashi et al., 1996; Sekino et al., 2007; Shirao, 1995), we decided to assess the effect of drebrin knockdown on actin dynamics in J77 T cells stimulated with SEE-loaded Raji B. We found that drebrin knockdown (Fig. 6A) significantly diminished actin polymerization at the IS contact area (Fig. 7A,B). Accordingly, these T cells showed reduced IL-2 mRNA levels and a diminished synthesis of this cytokine compared with

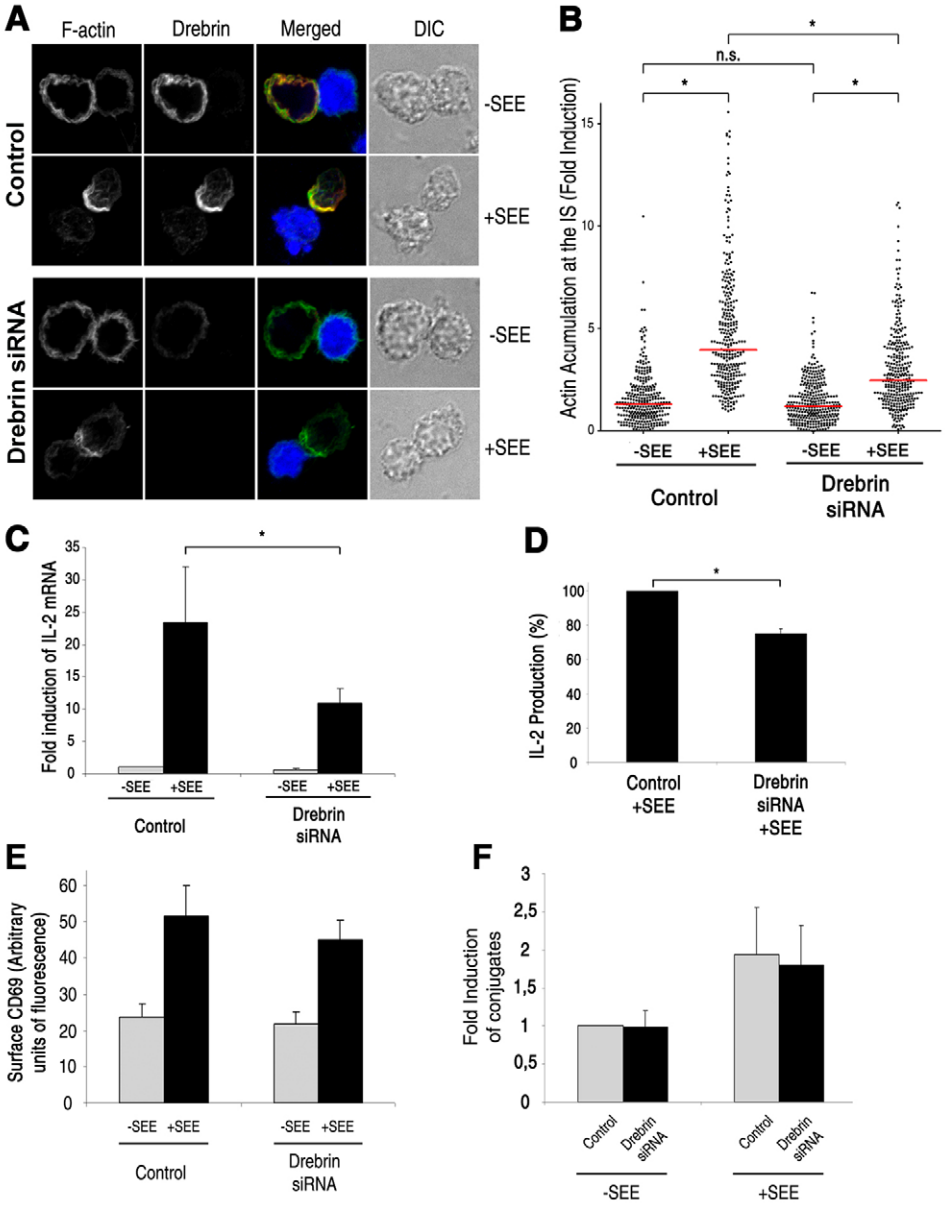

Fig. 7. Drebrin regulates T cell actin polymerization at the IS. (**A**) Confocal immunofluorescence showing F-actin (green) and drebrin (red) accumulation in control or drebrin-interfered J77 conjugated with SEE-loaded (+SEE) or unloaded (–SEE) Raji (blue). (**B**) Quantification of F-actin accumulation at the IS in control or drebrin-siRNA-transfected J77 cells stimulated with SEE or unstimulated. Medians (indicated by the red lines) of three independent experiments (*P<0.05, Mann-Whitney U-test). (**C**) Quantitative RT-PCR analysis of IL-2 mRNA expression by control or drebrin-interfered J77 SEE-stimulated or unstimulated. Data show the mean ± s.d. relative to the production by unstimulated control T cells of four independent experiments performed in duplicates (*P<0.05, Mann Whitney test). (**D**) IL-2 production detected in culture supernatants by ELISA in control or drebrin-siRNA-transfected J77 cells stimulated with SEE. Data show the mean ± s.e.m. relative to the production by SEE-stimulated control T cells of six independent experiments performed in duplicate (*P<0.05, Wilcoxon signed rank test). (**E**) Flow cytometry analysis of CD69 surface expression by drebrin-siRNA-transfected and control J77 cells, stimulated with SEE or unstimulated. Values are the arithmetic mean and s.d. of arbitrary units of fluorescence from three independent experiments. No significant differences were detected. (**F**) Fold induction of conjugate formation in control or drebrin-siRNA-transfected J77 cells stimulated with SEE or unstimulated. Values are the arithmetic mean and s.d. relative to the conjugate formation by unstimulated control T cells from three independent experiments. No significant differences were detected.

cells treated with a negative control oligonucleotide (Fig. 7C,D). By contrast, surface CD69 expression, an early marker of T cell activation, or the number of Jurkat T-cell-Raji-cell conjugates were not affected by drebrin knockdown (Fig. 7E,F).

Discussion

Cytoskeletal rearrangements in T cells drive cell polarization, migration and antigen presentation, and disruption of F-actin or interference with cytoskeletal regulators impairs T cell activation (Billadeau et al., 2007; Vicente-Manzanares and Sanchez-Madrid, 2004). During the formation of the IS, the redistribution of the molecules involved is faster than would be predicted on the basis of simple diffusion (Moss et al., 2002), indicating that the actin cytoskeleton drives the rapid rearrangement of receptor and signaling molecules to the IS. However, the complex array of cytoskeletal proteins associated with F-actin that make possible its role in T-cell activation has not been completely elucidated. In this work, we obtained data that reveal that drebrin exerts a novel and interesting role in the activation of T cells during the formation of the IS. Moreover, our data indicate that drebrin associates with CXCR4 and regulates its relocation to the IS. Therefore, our data suggest that drebrin may link the actin cytoskeleton with CKRs, exerting a functional and regulatory role in T cells.

Here, we demonstrate that the actin-binding protein drebrin localizes to the IS in human T cells. Previous works on drebrin had been mainly focused on its expression and function in neurons; in the brain, drebrin appears to regulate neuronal synapse plasticity (Hayashi et al., 1996; Kobayashi et al., 2001) and neurite morphology and outgrowth (Hayashi and Shirao, 1999; Ivanov et al., 2009; Takahashi et al., 2003; Toda et al., 1999). Drebrin has recently been detected in non-neural tissues, including stomach, kidney epithelial and mesangial cells and keratinocytes (Keon et al., 2000; Luna et al., 1997; Peitsch et al., 2005; Peitsch et al., 2003), being enriched at protrusive structures and at plaques of adherent junctions (Peitsch et al., 1999; Peitsch et al., 2001). Several studies suggest that drebrin, which bears an ADF-H domain (Lappalainen

et al., 1998), is involved in the regulation of actin dynamics (Asada et al., 1994; Ikeda et al., 1996; Ikeda et al., 1995; Shirao et al., 1994; Shirao et al., 1992). Other studies showed that drebrin can directly bind actin and interact with several actin-binding molecules (for example, Cdc42, fascin, tropomyosin and myosins) (Cheng et al., 2000; Hayashi et al., 1996; Ishikawa et al., 1994; Mammoto et al., 1998; Sasaki et al., 1996). Consistent with these findings, our results demonstrate that drebrin plays a major role in the regulation of actin dynamics in T cells during the key process of antigen recognition. Specific drebrin knockdown revealed that this protein is required for both the massive F-actin accumulation that occurs at the IS, and for the subsequent synthesis of IL-2. Interestingly, HIP-55/Abp1, another member of the drebrin/Abp1 family, has been shown to play a role in T-cell activation and proliferation through its interaction with molecules involved in TCR signaling, such as HPK1 and ZAP-70 (Han et al., 2005). Moreover, HIP-55 is also redistributed at the contact area between T cells and APCs, playing a regulatory role during the formation of the IS (Le Bras et al., 2004). However, unlike drebrin, HIP-55 does not seem to be involved in actin polymerization at the IS (Le Bras et al., 2004).

Little is known about the association of drebrin with non-cytoskeletal molecules (Butkevich et al., 2004), and our data on CXCR4 provide the first evidence for the interaction of drebrin with a chemokine receptor. Our data indicate that this interaction is increased by antigenic stimulation during T-cell-APC interaction, as demonstrated by biochemical, fluorescence microscopy and FRET analysis. Several CKRs have been shown to accumulate at the IS, where they seem to exert a functional role (Molon et al., 2005). In this regard, it has been described that CXCR4 interacts with the TCR-CD3 complex and induces the recruitment of ZAP-70 and SLP-76, leading to the activation of the ERK MAP kinase pathway and the synthesis of cytokines by T cells (Kremer et al., 2003; Kremer et al., 2007; Kumar et al., 2006; Ticchioni et al., 2002). Our results thus reveal that drebrin is an important participant in the complex process of T-cell activation during IS formation, regulating the relocation of CXCR4 to the IS and F-actin reorganization. Whether or not other members of the drebrin/Abp1 family, such as HIP-55, also associate with CXCR4 and regulate its distribution at the IS remains to be determined.

The binding of chemokines to their receptors triggers various signaling cascades, including the activation of G proteins and the PI3K, Jak/STAT, Rho-p160 ROCK and MAPK signaling pathways. In addition, CKRs induce the activation of the actin polymerization machinery, remodeling cell shape and promoting cell locomotion (Ganju et al., 1998; Mellado et al., 1998; Vicente-Manzanares et al., 2002; Vicente-Manzanares et al., 1999; Vicente-Manzanares et al., 2003; Wu et al., 1993). It is known that CKRs are able to interact with β-arrestins and G-protein-coupled receptor kinases (Orsini et al., 1999), JAK/STAT proteins (Liu et al., 1998) as well as with actin and the motor protein myosin IIA (MIIA) (Rey et al., 2002). CXCR4 has also been identified as a component of a multimolecular complex involved in receptor internalization that also contains MIIA and β-arrestin (Rey et al., 2007). Since CXCR4 binds drebrin and MIIA, it is conceivable that drebrin is included in this chemokine-receptor-associated molecular complex, linking CXCR4 to the actin cytoskeleton. In this regard, it has been described that other actin-binding proteins, such as filamin A, also bind to CXCR4 (Jimenez-Baranda et al., 2007).

In summary, our results point to drebrin as a new important modulator of CXCR4 and the actin cytoskeleton in T cells, affecting key physiological processes during antigen presentation.

Materials and Methods

Cells, antibodies and reagents

The Jurkat-derived human T cells lines J77 $V\beta_8^+$ J77cl20 and HxBc2, the lymphoblastoid B Raji cells, and the HEK293T cells, were grown in RPMI 1640 medium supplemented with 10% FBS (Cambrex Bioscience). The PC12 rat pheochromocytoma cells were obtained from ATCC (ATCC number CRL-1721^{TM}) and grown in F-12K medium supplemented with 10% FBS and 5% horse serum (Gibco). Primary peripheral blood lymphocytes (PBLs) and lymphoblasts were obtained and cultured as previously described (Vicente-Manzanares et al., 2003). A biotinylated monoclonal antibody against CXCR4 was from Becton-Dickinson Pharmingen; rabbit polyclonal anti-CXCR4 and that against the N-terminal end of CXCR4 were from Santa Cruz and Sigma, respectively. Mouse monoclonal antibody anti-drebrin (clone M2F6) was from MBL and rabbit polyclonal antibody anti-drebrin was from Sigma. Mouse monoclonal antibody anti-α-tubulin unlabeled or FITC-labeled were from Sigma. Mouse monoclonal antibody anti-TCR Vβ8 was from BD Pharmingen, rabbit polyclonal antibody anti-Arp2/3 was form Upstate and rabbit anti-cofilin was form Abcam. Goat polyclonal antibody anti-talin was kindly provided by K. Burridge (University of North Carolina, NC). Mouse monoclonal antibodies: HP2/19 (anti-ICAM-3), TP1/55 (anti-CD69) (Montoya et al., 2002), T3b (anti-CD3), HP2/1 (anti-α4 integrin) and CD28.2 (anti-CD28) (Mittelbrunn et al., 2004), HP2/6 (anti-CD4) (Barrero-Villar et al., 2009) have been described previously; Hu5/3 (anti-ICAM-1) was provided by E. A. Wayner (Fred Hutchinson Cancer Research Center, Seattle, WA); and rabbit polyclonal antibody 448 (anti-CD3) was provided by B. Alarcon (Centro de Biología Molecular, Madrid). The fluorescent trackers CMAC and CM-TMR, and all secondary antibodies and phalloidins conjugated with different fluorochromes were from Molecular Probes. HRP-conjugated secondary antibodies were from Pierce. Human fibronectin, poly-L-lysine and human γ-globulin were from Sigma, and the superantigen staphylococcal enterotoxin E (SEE) was from Toxin Technology. Primer sequences were as follows: GAPDH (forward) 5′-GAA GGT GAA GGT CGG AGT C-3′, (reverse) 5′-GAA GAT GGT GAT GGG ATT TC-3′; IL-2 (forward) 5′-AAG TTT TAC ATG CCC AAG AAG G-3′, (reverse) 5′-AAG TGA AAG TTT TTG CTT TGA GC-3′.

The drebrin GFP fusion proteins GFP-drebrin A wt, GFP-drebrin E wt, GFP-drebrin 1-271, GFP-drebrin 1-366, GFP-drebrin 319-707 (Hayashi et al., 1999), CFP-drebrin (Butkevich et al., 2004) and CXCR4-YFP fusion proteins (Hernanz-Falcon et al., 2004) have been described previously. The cytoplasmic tail of CXCR4 fused to GST (GST-CXCR4Cyt) has been described previously (Rey et al., 2002). The EGFP vector was from Clontech and the GST vector was from Amersham Biosciences. Negative control siRNA was from Eurogentec and the specific siRNA against drebrin was from Dharmacon.

Cell transfection assays

J77 cells (2×10^7) were electroporated in cold Optimem (Gibco-BRL) with DNA (20 μg) or with siRNA (1.25 μM) using a GenePulser II electroporator from Bio-Rad. Fluorescent proteins expression and siRNA knockdown were tested by flow cytometry and western blot analysis, respectively, at 24 hours.

T-B cell conjugate formation, immunofluorescence and time-lapse videomicroscopy

Conjugate formation was performed as previously described (Montoya et al., 2002). Conjugates were stained with the indicated specific antibodies followed by an Alexa-labeled specific secondary antibody, and examined with a DMR photo-microscope (Leica) using Leica QFISH 1.0 software; with a TCS-SP1 or with a TCS-SP5 Leica confocal microscopes. Three-dimensional reconstructions of confocal sections (0.118 μm separation on the vertical axis) were assembled with the Leica confocal software, Photoshop CS (Adobe Systems) and ImageJ. Video microscopy experiments were performed as previously described (Montoya et al., 2002) with a Leica TCS-SP1 confocal microscope or a Leica Multi Dimensional Workstation.

GST preparation, proteomic analysis and pull-down assays

The GST and GST-CXCR4Cyt production method, and proteomic analysis have been described previously (Rey et al., 2002). For pull-down assays, J77 cells (10^8) or HEK293T cells (10^7) were lysed in 1.2% Nonidet P-40 in TBS with a cocktail of protease inhibitors, and centrifuged at 14,000 ***g*** for 10 minutes at 4°C. The supernatant was incubated with glutathione-Sepharose (GE Healthcare) for 2 hours, and then with glutathione-Sepharose coupled to GST or GST-CXCR4Cyt for 1 hour. After pull-down, glutathione-coupled Sepharose beads were washed with lysis buffer and resuspended in Laemmli buffer. Samples were separated by SDS-PAGE and processed for mass spectrometry or transferred to a nitrocellulose membrane for immunoblot analysis with anti-drebrin antibodies.

Immunoprecipitation and western blot

J77 lymphocytes (6×10^7) were lysed in 1.2% NP-40 in PBS or 1.5% CHAPS buffer supplemented with a cocktail of protease inhibitors. Lysates were centrifuged at 14,000 ***g*** for 10 minutes at 4°C, and cell lysate supernatants were incubated for 2 hours at 4°C with cyanogen bromide (CNBr) beads blocked with BSA, then incubated with the indicated antibody covalently-coupled to CNBr beads. Pellets were washed with lysis buffer and resuspended in Laemmli buffer. Samples were separated by SDS-PAGE and transferred to nitrocellulose membranes and incubated with the indicated

antibodies. Co-immunoprecipitation experiments were performed by pre-incubating the T cells at 37°C with SEE-loaded Raji cells for the indicated times before the lysis.

Analysis of T cell activation

J77 cells (10^5) were transiently transfected with siRNA oligonucleotides, and then incubated with Raji cells (10^5) in the presence or absence of SEE (0.3 μg/ml) for 17 hours at 37°C. CD69 expression and IL-2 production were then determined as described previously (Montoya et al., 2002). For real-time PCR, RNA was isolated from cell lysates using Ultraspec RNA reagent (Biotecx). Total RNA was converted into cDNA. The reverse transcriptase (RT) reaction was carried out using 2 μg of RNA and ImProm-II Reverse Transcriptase (Promega GmbH). Gene expression was analyzed by SYBR Green real-time PCR (Roche Diagnostics) in a DNA LightCycler rapid thermal cycler (Roche). Results for each gene were normalized to GADPH expression and measured in parallel in each sample.

Flow cytometry

Cells were incubated with human γ-globulin (100 μg/ml) in PBS for 20 minutes and then with primary monoclonal antibody for 30 minutes. After washing, cells were incubated with a FITC-conjugated secondary antibody and analyzed in a FACScan flow cytometer (Becton Dickinson). Data were analyzed with Cell-Quest Pro software (Becton Dickinson).

Analysis of actin polymerization at the T-APC contact area

Conjugates were formed between drebrin-silenced or control J77 cells and SEE-loaded or unloaded APCs. The conjugates were fixed with paraformaldehyde and images acquired by confocal imaging as described above. Polymerized actin was detected by staining with Alexa-conjugated phalloidin, and images were analyzed with ImageJ software (http://rsbweb.nih.gov/ij). By using ROIs (region of interest) of the same area for all measurements, we quantified the signal generated by the T-APC contact area (IS), an area of APC membrane not in contact with the T cell (B), an area of T cell membrane not in contact with the APC (T), and the background. We first subtracted the background signal from all the all other measurements, and then measured actin accumulation at the IS in relation to the rest of the T cell, according to the formula [IS-B]/T. We assumed that actin accumulation was homogeneous in the APC, with no additional accumulation at the contact zone with the T cell.

Fluorescence resonance energy transfer (FRET) analysis

J77 cells (10^5) were co-transfected with CFP-drebrin and CXCR4-YFP and then left alone or mixed with unpulsed or SEE-pulsed Raji cells (10^5) loaded with CMAC, and incubated at 37°C for 15 minutes. Cells were plated onto slides coated with poly L-lysine (50 μg/ml), incubated for 15 minutes at 37°C, and fixed in 4% paraformaldehyde. Images were acquired with a TCS-SP5 confocal scanning laser microscope and analyzed with the FRETcalc Version 3.0 plugin (http://rsb.info.nih.gov/ij/plugins/fret/fret-calc.html) for FRET by acceptor photobleaching of the ImageJ software and Photoshop CS.

Statistical analysis

Data were tested for normality using the Kolmogorov-Smirnov test, and for homoscedasticity by the Bartlett test. Differences between two means were tested using Student's *t*-tests or Mann-Whitney *U*-tests. Differences among three or more means were tested by one-way ANOVA with Bonferroni's post-hoc multiple comparisons test. IL-2 secretion data were analyzed with the Wilcoxon signed rank-test. Differences were considered significant when $P<0.05$.

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