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Nef-induced Alteration of the Early/Recycling Endosomal Compartment Correlates with Enhancement of HIV-1 Infectivity*

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human immunodeficiency virus type 1 (HIV-1) Nef interacts with the clathrin-associated AP-1 and AP-3 adaptor complexes, stabilizing their association with endosomal membranes. These findings led us to hypothesize a general impact of this viral protein on the endosomal system. Here, we have shown that Nef specifically disturbs the morphology of the early/recycling compartment, inducing a redistribution of early endosomal markers and a shortening of the tubular recycling endosomal structures. Furthermore, Nef modulates the trafficking of the transferrin receptor (TfR), the prototypical recycling surface protein, indicating that it also disturbs the function of this compartment. Nef reduces the rate of recycling of TfR to the plasma membrane, causing TfR to accumulate in early endosomes and reducing its expression at the cell surface. These effects depend on the leucine-based motif of Nef, which is required for the membrane stabilization of AP-1 and AP-3 complexes. Since we show that this motif is also required for the full infectivity of HIV-1 virions, these results indicate that the positive influence of Nef on viral infectivity may be related to its general effects on early/recycling endosomal compartments.

Trafficking of membrane proteins is governed by a regulated machinery that involves the vesicular transport of proteins throughout different intracellular compartments. One major regulatory mechanism is related to the function of the adaptor protein (AP)[1] complexes that assemble on donor membranes of the endocytic pathway to form transport vesicles (for review, see Ref. 1). The sorting of transmembrane proteins into these vesicles requires the recognition by the AP complexes of specific tyrosine- or leucine-based motifs contained within the cytoplasmic domains of cargo proteins (2). Four different types of heterotetrameric AP complexes (AP-1–AP-4) have been identified (3). AP-2 is specifically involved in the formation of clathrin-coated vesicles at the plasma membrane, whereas AP-1 and AP-3 mediate the formation of clathrin-coated vesicles at the levels of the trans-Golgi network (TGN) and endosomes. The function of AP-4 is less well documented, but it regulates formation of non-clathrin-coated vesicles at the TGN. The association of the AP-1, AP-3, and AP-4 complexes with TGN and endosomal membranes is regulated by ADP-ribosylation factor 1 (ARF1).

The Nef protein of HIV-1 is a 27-kDa protein that associates with the cell membranes through N-terminal myristoylation and is abundantly produced shortly after virus infection (for review, see Refs. 4 and 5). Nef is an essential factor *in vivo* for efficient viral replication and pathogenesis. *In vitro*, Nef also facilitates virus replication and enhances the infectivity of virions. Although the positive influence of Nef on viral replication and infectivity may be multifactorial, genetic evidence suggests a relationship between these virological effects and the ability of Nef to modulate the cell surface expression of multiple membrane-associated proteins. In addition to CD4 and major histocompatibility complex class I (MHC-I) molecules (6–8), the list of membrane proteins in which intracellular trafficking is affected by Nef now includes MHC class II (MHC-II) molecules, the costimulatory CD28 molecule, and the lectin DC-SIGN (9–11). These alterations likely promote an immune evasion response of infected cells and enhance the spread of viruses within the host during the natural course of HIV infection (12–15).

Mechanistically, these observations indicate that Nef exerts a general influence on the intracellular trafficking of membrane proteins. Although the molecular basis of this general effect is not fully understood, it is now evident that it relates to the ability of Nef to interact directly with vesicle coat components involved in the vesicular transport throughout the endocytic pathway, including the clathrin-associated AP complexes. The HIV-1 Nef protein recognizes preferentially AP-1 and AP-3 complexes (16–20) and selectively stabilizes the association of these complexes on endosomal membranes by an ARF1-inde-

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[1] The abbreviations used are: AP, adaptor protein; HIV, human immunodeficiency virus; ARF1, ADP-rybosylation factor 1; Tf, transferrin; TfR, transferrin receptor; TGN, trans-Golgi network; GFP, green fluorescent protein; EEA1, early endosome antigen 1; EGF, epidermal growth factor; EGF-TxR, Texas Red-conjugated EGF; EGFR, epidermal growth factor receptor; ERC, endosomal recycling compartment; PE, phycoerythrin; MHC, major histocompatibility complex; Ab, antibody; mAb, monoclonal antibody; FITC, fluorescein isothiocyanate; PBS, phosphate-buffered saline; BSA, bovine serum albumin; Cy, cyanin; WT, wild type.

grade transport from early/sorting endosomes to the TGN (44, 45). Second, AP-3 has been found to be associated at least in part with a TfR-positive compartment (46). Third, clathrin coats have been detected on vesicles budding from the early endosomal membranes (33). Interestingly, Tf recycling was inhibited by the fungal metabolite brefeldin A, an inhibitor of ARF1-dependent recruitment of coat proteins such as AP-1 and AP-3 (47–49). Nevertheless, the precise role of the AP-1 and AP-3 complexes within the recycling pathway remains to be defined, and Nef may constitute a powerful tool for this purpose.

Finally, what is the role of the perturbations of membrane protein trafficking induced by Nef in the HIV-1 life cycle? Interestingly, there is a striking correlation between the Nef-mediated enhancement of viral infectivity, the stabilization of AP-1 and AP-3 complexes on endosomal membranes, and the Nef-induced alterations of trafficking within the early/recycling endosomal compartment. All of these properties require the leucine-based motif in Nef. Here, we not only have confirmed the crucial role of the leucine-based motif of Nef for optimal viral infectivity (22) but have further emphasized that these effects are not a direct consequence of the down-regulation of CD4. It is noteworthy that these effects are apparent even when virus particles are produced from CD4-negative cells (see Fig. 8*D*). Although the mechanism of Nef-mediated enhancement of infectivity remains ill-defined, these observations indicate that the virological effects of Nef are related to its morphologic and functional effects on the early/recycling compartment. The precise basis of this connection between the trafficking and the virologic effects of Nef is open to hypothesis. One possibility is that by acting on the endocytic pathway, Nef may be involved in the virion assembly and/or budding processes. By inducing an expansion of multivesicular endosomal structures (17, 39, 40), Nef may promote the formation of an intracellular platform for assembly of new HIV-1 virions. In support of this hypothesis, a connection between protein sorting within the endosomal system and the budding of virions has been established, demonstrating that the endosomal sorting complexes required for transport (ESCRT) play essential roles in the budding process (50–52). Furthermore, recent findings show that virion assembly and budding can occur within the endosomal compartments (53–55). If HIV-1 uses endosomal membranes as a platform for an aspect of viral morphogenesis, then it becomes an attractive hypothesis that Nef-mediated alterations of this system facilitate assembly to yield optimally infectious viral particles.

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REFERENCES

1. Robinson, M. S., and Bonifacino, J. S. (2001) *Curr. Opin. Cell Biol.* **13,** 444–453
2. Bonifacino, J. S., and Traub, L. M. (2003) *Annu. Rev. Biochem.* **72,** 395–447
3. Bonifacino, J. S., and Lippincott-Schwartz, J. (2003) *Nat Rev Mol. Cell. Biol.* **4,** 409–414
4. Janvier, K., Petit, C., Le Rouzic, E., Schwartz, O., and Benichou, S. (2000) *AIDS* **14,** Suppl. 3, S21–S30
5. Fackler, O. T., and Baur, A. S. (2002) *Immunity* **16,** 493–497
6. Piguet, V., Chen, Y. L., Mangasarian, A., Foti, M., Carpentier, J. L., and Trono, D. (1998) *EMBO J.* **17,** 2472–2481
7. Schwartz, O., Marechal, V., Le Gall, S., Lemonnier, F., and Heard, J. M. (1996) *Nat. Med.* **2,** 338–342
8. Piguet, V., Schwartz, O., Le Gall, S., and Trono, D. (1999) *Immunol. Rev.* **168,** 51–63
9. Swigut, T., Shohdy, N., and Skowronski, J. (2001) *EMBO J.* **20,** 1593–1604
10. Stumptner-Cuvelette, P., Morchoisne, S., Dugast, M., Le Gall, S., Raposo, G., Schwartz, O., and Benaroch, P. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98,** 12144–12149
11. Sol-Foulon, N., Moris, A., Nobile, C., Boccaccio, C., Engering, A., Abastado, J. P., Heard, J. M., van Kooyk, Y., and Schwartz, O. (2002) *Immunity* **16,** 145–155
12. Cohen, G. B., Gandhi, R. T., Davis, D. M., Mandelboim, O., Chen, B. K., Strominger, J. L., and Baltimore, D. (1999) *Immunity* **10,** 661–671
13. Lama, J., Mangasarian, A., and Trono, D. (1999) *Curr. Biol.* **9,** 622–631
14. Ross, T. M., Oran, A. E., and Cullen, B. R. (1999) *Curr. Biol.* **9,** 613–621
15. Stoddart, C. A., Geleziunas, R., Ferrell, S., Linquist-Stepps, V., Moreno, M. E., Bare, C., Xu, W., Yonemoto, W., Bresnahan, P. A., McCune, J. M., and Greene, W. C. (2003) *J. Virol.* **77,** 2124–2133
16. Le Gall, S., Erdtmann, L., Benichou, S., Berlioz-Torrent, C., Liu, L., Benarous, R., Heard, J. M., and Schwartz, O. (1998) *Immunity* **8,** 483–495
17. Erdtmann, L., Janvier, K., Raposo, G., Craig, H. M., Benaroch, P., Berlioz-Torrent, C., Guatelli, J. C., Benarous, R., and Benichou, S. (2000) *Traffic* **1,** 871–883
18. Janvier, K., Craig, H., Hitchin, D., Madrid, R., Sol-Foulon, N., Renault, L., Cherfils, J., Cassel, D., Benichou, S., and Guatelli, J. (2003) *J. Biol. Chem.* **278,** 8725–8732
19. Janvier, K., Kato, Y., Boehm, M., Rose, J. R., Martina, J. A., Kim, B. Y., Venkatesan, S., and Bonifacino, J. S. (2003) *J. Cell Biol.* **163,** 1281–1290
20. Greenberg, M., DeTulleo, L., Rapoport, I., Skowronski, J., and Kirchhausen, T. (1998) *Curr. Biol.* **8,** 1239–1242
21. Greenberg, M. E., Iafrate, A. J., and Skowronski, J. (1998) *EMBO J.* **17,** 2777–2789
22. Craig, H. M., Pandori, M. W., and Guatelli, J. C. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 11229–11234
23. Liu, L. X., Heveker, N., Fackler, O. T., Arold, S., Le Gall, S., Janvier, K., Peterlin, B. M., Dumas, C., Schwartz, O., Benichou, S., and Benarous, R. (2000) *J. Virol.* **74,** 5310–5319
24. Baur, A. S., Sawai, E. T., Dazin, P., Fantl, W. J., Cheng-Mayer, C., and Peterlin, B. M. (1994) *Immunity* **1,** 373–384
25. Bresnahan, P. A., Yonemoto, W., Ferrell, S., Williams-Herman, D., Geleziunas, R., and Greene, W. C. (1998) *Curr. Biol.* **8,** 1235–1238
26. Geyer, M., Yu, H., Mandic, R., Linnemann, T., Zheng, Y.-H., Fackler, O. T., and Peterlin, B. M. (2002) *J. Biol. Chem.* **277,** 28521–28529
27. Piguet, V., Wan, L., Borel, C., Mangasarian, A., Demaurex, N., Thomas, G., and Trono, D. (2000) *Nat. Cell Biol.* **2,** 163–167
28. Zerial, M., and McBride, H. (2001) *Nat. Rev. Mol. Cell Biol.* **2,** 107–117
29. Hao, M., and Maxfield, F. R. (2000) *J. Biol. Chem.* **275,** 15279–15286
30. Futter, C. E., Pearse, A., Hewlett, L. J., and Hopkins, C. R. (1996) *J. Cell Biol.* **132,** 1011–1023
31. Sorkin, A., and Von Zastrow, M. (2002) *Nat. Rev. Mol. Cell Biol.* **3,** 600–614
32. Wiley, H. S., and Burke, P. M. (2001) *Traffic* **2,** 12–18
33. van Dam, E. M., ten Broeke, T., Jansen, K., Spijkers, P., and Stoorvogel, W. (2002) *J. Biol. Chem.* **277,** 48876–48883
34. van Dam, E. M., and Stoorvogel, W. (2002) *Mol. Biol. Cell* **13,** 169–182
35. Lin, S. X., Grant, B., Hirsh, D., and Maxfield, F. R. (2001) *Nat. Cell Biol.* **3,** 567–572
36. Sheff, D., Pelletier, L., O'Connell, C. B., Warren, G., and Mellman, I. (2002) *J. Cell Biol.* **156,** 797–804
37. Wilcke, M., Johannes, L., Galli, T., Mayau, V., Goud, B., and Salamero, J. (2000) *J. Cell Biol.* **151,** 1207–1220
38. Blagoveshchenskaya, A. D., Thomas, L., Feliciangeli, S. F., Hung, C. H., and Thomas, G. (2002) *Cell* **111,** 853–866
39. Sanfridson, A., Hester, S., and Doyle, C. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94,** 873–878
40. Stumptner-Cuvelette, P., Jouve, M., Helft, J., Dugast, M., Glouzman, A.-S., Jooss, K., Raposo, G., and Benaroch, P. (2003) *Mol. Biol. Cell*, **77,** 10548–10556
41. Mellman, I. (1996) *Curr. Opin, Cell Biol.* **8,** 497–498
42. Johannes, L., Pezo, V., Mallard, F., Tenza, D., Wiltz, A., Saint-Pol, A., Helft, J., Antony, C., and Benaroch, P. (2003) *Traffic* **4,** 323–332
43. Kirchhausen, T. (1999) *Annu. Rev. Cell Dev. Biol.* **15,** 705–732
44. Mallard, F., Antony, C., Tenza, D., Salamero, J., Goud, B., and Johannes, L. (1998) *J. Cell Biol.* **143,** 973–990
45. Meyer, C., Zizioli, D., Lausmann, S., Eskelinen, E.-L., Hamann, J., Saftig, P., von Figura, K., and Schu, P. (2000) *EMBO J.* **19,** 2193–2203
46. Dell'Angelica, E. C., Ohno, H., Ooi, C. E., Rabinovich, E., Roche, K. W., and Bonifacino, J. S. (1997) *EMBO J.* **16,** 917–928
47. Robinson, M. S., and Kreis, T. E. (1992) *Cell* **69,** 129–138
48. Helms, J. B., and Rothman, J. E. (1992) *Nature* **360,** 352–354
49. Donaldson, J. G., Finazzi, D., and Klausner, R. D. (1992) *Nature* **360,** 350–352
50. Garrus, J. E., von Schwedler, U. K., Pornillos, O. W., Morham, S. G., Zavitz, K. H., Wang, H. E., Wettstein, D. A., Stray, K. M., Cote, M., Rich, R. L., Myszka, D. G., and Sundquist, W. I. (2001) *Cell* **107,** 55–65
51. Martin-Serrano, J., Zang, T., and Bieniasz, P. D. (2001) *Nat. Med.* **7,** 1313–1319
52. Pornillos, O., Higginson, D. S., Stray, K. M., Fisher, R. D., Garrus, J. E., Payne, M., He, G.-P., Wang, H. E., Morham, S. G., and Sundquist, W. I. (2003) *J. Cell Biol.* **162,** 425–434
53. Raposo, G., Moore, M., Innes, D., Leijendekker, R., Leigh-Brown, A., Benaroch, P., and Geuze, H. (2002) *Traffic* **3,** 718–729
54. Pelchen-Matthews, A., Kramer, B., and Marsh, M. (2003) *J. Cell Biol.* **162,** 443–455
55. Sherer, N. M., Lehmann, M. J., Jimenez-Soto, L. F., Ingmundson, A., Horner, S. M., Cicchetti, G., Allen, P. G., Pypaert, M., Cunningham, J. M., and Mothes, W. (2003) *Traffic* **4,** 785–801