JOURNAL OF VIROLOGY, Feb. 2006, p. 1837–1849 Vol. 80, No. 4
0022-538X/06/$08.00+0 doi:10.1128/JVI.80.4.1837–1849.2006

Modulation of Cellular Protein Trafficking by Human Immunodeficiency Virus Type 1 Nef: Role of the Acidic Residue in the ExxxLL Motif

Scott H. Coleman,[1,3] Ricardo Madrid,[4] Nanette Van Damme,[3] Richard S. Mitchell,[1] Jerome Bouchet,[4] Cecile Servant,[4] Satish Pillai,[2,3] Serge Benichou,[4] and John C. Guatelli[1,3]*

Department of Medicine[1] and Division of Biology,[2] University of California–San Diego, La Jolla, California 92093-0679; San Diego Veterans Affairs Healthcare System, San Diego, California 92121[3]; and Institut Cochin, Department of Infectious Diseases, INSERM U567-CNRS UMR8104, Universite Paris V, 75014 Paris, France[4]

Received 16 June 2005/Accepted 18 November 2005

The *nef* gene contributes to the replication of primate lentiviruses by altering the trafficking of cellular proteins involved in adaptive immunity (class I and II major histocompatibility complex [MHC]) and viral transmission (CD4 and DC-SIGN). A conserved acidic leucine-based sequence (E_{160}xxxLL) within human immunodeficiency virus type 1 (HIV-1) Nef binds to the cellular adaptor protein (AP) complexes, which mediate protein sorting into endosomal vesicles. The leucine residues in this motif are required for the down-regulation of CD4 and for the up-regulation of DC-SIGN and the invariant chain of MHC class II, but the role of the acidic residue is unclear. Here, substitution of E160 with uncharged residues impaired the ability of Nef to up-regulate the expression of the invariant chain and DC-SIGN at the cell surface, whereas substitution with a basic residue was required for a similar effect on the down-regulation of CD4. All substitutions of E160 relieved the Nef-mediated block to transferrin uptake. E160 was required for the efficient interaction of Nef with AP-1 and AP-3 and for the stabilization of these complexes on endosomal membranes in living cells. Systematic mutation of the ExxxLL sequence together with correlation of binding and functional data leads to the hypotheses that AP-1 and AP-3 are major cofactors for the effect of Nef on the trafficking of transferrin, are less important but contribute to the modulation of the invariant chain and DC-SIGN, and are least critical for the modulation of CD4. The data suggest that the E160 residue plays a differential role in the modulation of leucine-dependent Nef-targets and support a model in which distinct AP complexes are used by Nef to modulate different cellular proteins.

The *nef* gene of primate lentiviruses is required for high-level viremia and the efficient pathogenesis of AIDS (12, 25, 44). These effects are at least partly due to the effect of Nef on the cellular protein trafficking environment. Nef alters the subcellular localization of a number of proteins, including CD4, DC-SIGN, transferrin receptor, tumor necrosis factor, LIGHT, CD28, class I major histocompatibility complex (MHC), and both mature and immature class II MHC (1, 27, 39, 40, 42, 43). These effects likely influence the efficiency of viral replication. For example, the down-regulation of the cell surface level of CD4 by Nef prevents the binding of the viral envelope glycoprotein (gp120) to CD4 on the surface of the virus-producing cell, preserving the infectivity of newly formed virions and potentially enhancing their release (26, 37). In contrast to CD4, Nef up-regulates the surface level of DC-SIGN, a C-type lectin expressed on dendritic cells that both allows the uptake of mannosylated antigens and serves as an adhesion molecule, facilitating the interaction of dendritic cells with T cells during antigen presentation (17, 40). Although DC-SIGN binds gp120, the human immunodeficiency virus virions internalized into dendritic cells remain infectious and are subsequently transmitted to T cells, a process that Nef may facilitate. Finally, Nef disrupts the presentation of viral antigens by down-regulating class I MHC and mature class II MHC from the cell surface, while up-regulating the surface expression of the invariant chain, which normally chaperones the immature class II complex to an endosomal compartment in which antigens derived from the extracellular space are processed (42).

The down-regulation of CD4, CD28, and transferrin receptor as well as the up-regulation of tumor necrosis factor, LIGHT, invariant chain, and DC-SIGN require two leucine residues within a C-terminal, solvent-exposed loop of the Nef protein (4, 9, 18, 27, 40, 42, 43). The leucine codons are conserved among human immunodeficiency virus type 1 (HIV-1) *nef* alleles, and their mutation leads to the complete loss of Nef's effect on these membrane proteins. The two leucines, together with the adjacent upstream sequence, conform to a class of intracellular sorting signals whose consensus sequence is E/DxxxLL. These sequences bind to the heterotetrameric adaptor protein (AP) complexes, which form part of the coat of a subset of vesicles that mediate transport within the endosomal system (21, 31). The family of AP complexes has four members: AP-1 mediates vesicular transport between the *trans*-Golgi network and endosomal compartments; AP-2 mediates transport between the plasma membrane and early/sorting endosomes; AP-3 mediates transport from the *trans*-Golgi network and early endosomes to late endosomes and lysosomes; and AP-4 may mediate transport from the *trans*-Golgi network to the basolateral plasma membrane of polarized cells.

The roles of these individual AP complexes in the effects of Nef on protein trafficking are unclear. The rate of internalization of CD4 from the cell surface is increased by Nef, suggesting involvement of AP-2 (1). In further support of a role for AP-2, Nef has been detected in clathrin-coated pits at the plasma membrane by electron microscopy (16), and a domi-

* Corresponding author. Mailing address: University of California, San Diego, 9500 Gilman Dr., La Jolla, CA 92093. Phone: (858) 552-7439. Fax: (858) 552-7445. E-mail: jguatelli@ucsd.edu.

S. Crowe, A. Maerz, S. Sonza, J. Learmont, J. S. Sullivan, A. Cunningham, D. Dwyer, D. Dowton, and J. Mills. 1995. Genomic structure of an attenuated quasi species of HIV-1 from a blood transfusion donor and recipients. Science **270:**988–991.

13. **Donaldson, J. G., D. Finazzi, and R. D. Klausner.** 1992. Brefeldin A inhibits Golgi membrane-catalysed exchange of guanine nucleotide onto ARF protein. Nature **360:**350–352.
14. **Dugast, M., H. Toussaint, C. Dousset, and P. Benaroch.** 2005. AP2 clathrin adaptor complex, but not AP1, controls the access of the major histocompatibility complex (MHC) class II to endosomes. J. Biol. Chem. **280:**19656–19664.
15. **Erdtmann, L., K. Janvier, G. Raposo, H. M. Craig, P. Benaroch, C. Berlioz-Torrent, J. C. Guatelli, R. Bernarous, and S. Benichou.** 2000. Two independent regions of HIV-1 Nef are required for connection with the endocytic pathway through binding to the μ1 chain of AP1 complex. Traffic **1:**871–883.
16. **Foti, M., A. Mangasarian, V. Piguet, D. P. Lew, K.-H. Krause, D. Trono, and J.-L. Carpentier.** 1997. Nef-mediated clathrin-coated pit formation. J. Cell Biol. **139:**37–47.
17. **Geijtenbeek, T. B., D. S. Kwon, R. Torensma, S. J. van Vliet, G. C. van Duijnhoven, J. Middel, I. L. Cornelissen, H. S. Nottet, V. N. KewalRamani, D. R. Littman, C. G. Figdor, and Y. van Kooyk.** 2000. DC-SIGN, a dendritic cell-specific HIV-1-binding protein that enhances trans-infection of T cells. Cell **100:**587–597.
18. **Greenberg, M., L. DeTulleo, I. Rapoport, J. Skowronski, and T. Kirchhausen.** 1998. A dileucine motif in HIV-1 Nef is essential for sorting into clathrin-coated pits and for downregulation of CD4. Curr. Biol. **8:**1239–1242.
19. **Greenberg, M. E., S. Bronson, M. Lock, M. Neumann, G. N. Pavlakis, and J. Skowronski.** 1997. Co-localization of HIV-1 Nef with the AP-2 adaptor protein complex correlates with Nef-induced CD4 down-regulation. EMBO J. **16:**6964–6976.
20. **Hao, M., and F. R. Maxfield.** 2000. Characterization of rapid membrane internalization and recycling. J. Biol. Chem. **275:**15279–15286.
21. **Hirst, J., and M. S. Robinson.** 1998. Clathrin and adaptors. Biochim. Biophys. Acta **1404:**173–193.
22. **Honing, S., I. V. Sandoval, and K. von Figura.** 1998. A di-leucine-based motif in the cytoplasmic tail of LIMP-II and tyrosinase mediates selective binding of AP-3. EMBO J. **17:**1304–1314.
23. **Janvier, K., H. Craig, D. Hitchin, R. Madrid, N. Sol-Foulon, L. Renault, J. Cherfils, D. Cassel, S. Benichou, and J. Guatelli.** 2003. HIV-1 Nef stabilizes the association of adaptor protein complexes with membranes. J. Biol. Chem. **275:**8725–8732.
24. **Janvier, K., Y. Kato, M. Boehm, J. R. Rose, J. A. Martina, B. Y. Kim, S. Venkatesan, and J. S. Bonifacino.** 2003. Recognition of dileucine-based sorting signals from HIV-1 Nef and LIMP-II by the AP-1 gamma-sigma1 and AP-3 delta-sigma3 hemicomplexes. J. Cell Biol. **163:**1281–1290.
25. **Kestler, H. W., III, D. J. Ringler, K. Mori, D. L. Panicali, P. K. Sehgal, M. D. Daniel, and R. C. Desrosiers.** 1991. Importance of the *nef* gene for maintenance of high virus loads and for development of AIDS. Cell **65:**651–662.
26. **Lama, J., A. Mangasarian, and D. Trono.** 1999. Cell surface expresion of CD4 reduces HIV-1 infectivity by blocking Env incorporation in a Nef- and Vpu-inhibitable manner. Curr. Biol. **9:**622–631.
27. **Lama, J., and C. F. Ware.** 2000. Human immunodeficiency virus type 1 nef mediates sustained membrane expression of tumor necrosis factor and the related cytokine LIGHT on activated T cells. J. Virol. **74:**9396–9402.
28. **Le Gall, S., L. Erdtmann, S. Benichou, C. Berlioz-Torrent, L. Liu, R. Benarous, J.-M. Heard, and O. Schwartz.** 1998. Nef interacts with the μ subunit of clathrin adaptor complexes and reveals a cryptic sorting signal in MHC 1 molecules. Immunity **8:**483–495.
29. **Madrid, R., K. Janvier, D. Hitchin, J. Day, S. Coleman, C. Noviello, J. Bouchet, A. Benmerah, J. Guatelli, and S. Benichou.** 2005. Nef-induced alteration of the early/recycling endosomal compartment correlates with enhancement of HIV-1 infectivity. J. Biol. Chem. **280:**5032–5044.
30. **Marks, M. S., L. Woodruff, H. Ohno, and J. S. Bonifacino.** 1996. Protein targeting by tyrosine- and di-leucine-based signals: evidence for distinct saturable components. J. Cell Biol. **135:**341–354.
31. **Mellman, I.** 1996. Endocytosis and molecular sorting. Annu. Rev. Cell Dev. **12:**575–625.
32. **Ooi, C. E., E. C. Dell'Angelica, and J. S. Bonifacino.** 1998. ADP-ribosylation factor 1 (ARF1) regulates recruitment of the AP-3 adaptor complex to membranes. J. Cell Biol. **142:**291–402.
33. **Piguet, V., Y.-L. Chen, A. Mangasarian, M. Foti, J.-L. Carpentier, and D. Trono.** 1998. Mechanism of Nef-induced CD4 endocytosis: Nef connects CD4 with the μ chain of adaptor complexes. EMBO J. **17:**2472–2481.
34. **Pond, L., L. A. Kuhn, L. Teyton, M.-P. Schutze, J. A. Tainer, M. R. Jackson, and P. A. Peterson.** 1995. A role for acidic residues in di-leucine motif-based targeting to the endocytic pathway. J. Biol. Chem. **270:**19989–19997.
35. **Roeth, J. F., M. Williams, M. R. Kasper, T. M. Filzen, and K. L. Collins.** 2004. HIV-1 Nef disrupts MHC-I trafficking by recruiting AP-1 to the MHC-I cytoplasmic tail. J. Cell Biol. **167:**903–913.
36. **Rose, J. J., K. Janvier, S. Chandrasekhar, R. P. Sekaly, J. S. Bonifacino, and S. Venkatesan.** 2005. CD4 down-regulation by HIV-1 and simian immunodeficiency virus (SIV) Nef proteins involves both internalization and intracellular retention mechanisms. J. Biol. Chem. **280:**7413–7426.
37. **Ross, T. M., A. E. Oran, and B. R. Cullen.** 1999. Inhibition of HIV-1 progeny virion release by cell surface CD4 is relieved by expression of the viral Nef protein. Curr. Biol. **9:**613–621.
38. **Sandoval, I. V., S. Martinez-Arca, J. Valdueza, S. Palacios, and G. D. Holman.** 2000. Distinct reading of different structural determinants modulates the dileucine-mediated transport steps of the lysosomal membrane protein LIMPII and the insulin-sensitive glucose transporter GLUT4. J. Biol. Chem. **275:**39874–39885.
39. **Schwartz, O., V. Marechal, S. Le Gall, F. Lemonnier, and J.-M. Heard.** 1996. Endocytosis of major histocompatibility complex class I molecules is induced by the HIV-1 Nef protein. Nat. Med. **2:**338–342.
40. **Sol-Foulon, N., A. Moris, C. Nobile, C. Boccaccio, A. Engering, J. P. Abastado, J. M. Heard, Y. van Kooyk, and O. Schwartz.** 2002. HIV-1 Nef-induced upregulation of DC-SIGN in dendritic cells promotes lymphocyte clustering and viral spread. Immunity **16:**145–155.
41. **Stamnes, M. A., and J. E. Rothman.** 1993. The binding of AP-1 clathrin adaptor particles to Golgi membranes requires ADP-ribosylation factor, a small GTP-binding protein. Cell **73:**999–1005.
42. **Stumptner-Cuvelette, P., S. Morchoisne, M. Dugast, S. Le Gall, G. Raposo, O. Schwartz, and P. Benaroch.** 2001. HIV-1 Nef impairs MHC class II antigen presentation and surface expression. Proc. Natl. Acad. Sci. USA **98:**12144–12149.
43. **Swigut, T., N. Shohdy, and J. Skowronski.** 2001. Mechanism for down-regulation of CD28 by Nef. EMBO J. **20:**1593–1604.
44. **Tobiume, M., M. Takahoko, T. Yamada, M. Tatsumi, A. Iwamoto, and M. Matsuda.** 2002. Inefficient enhancement of viral infectivity and CD4 down-regulation by human immunodeficiency virus type 1 Nef from Japanese long-term nonprogressors. J. Virol. **76:**5959–5965.