JOURNAL OF VIROLOGY, Dec. 1996, p. 9051–9054 Vol. 70, No. 12
0022-538X/96/$04.00+0

Specific Cleavage Sites of Nef Proteins from Human Immunodeficiency Virus Types 1 and 2 for the Viral Proteases

JACQUELINE SCHORR,[1] ROLAND KELLNER,[2] OLIVER FACKLER,[3] JENS FREUND,[1] JAN KONVALINKA,[4]† NORBERT KIENZLE,[3]‡ HANS-GEORG KRÄUSSLICH,[4]§ NIKOLAUS MUELLER-LANTZSCH,[3] AND HANS ROBERT KALBITZER[1]*

Max-Planck-Institute for Medical Research[1] and German Cancer Research Center,[4] Heidelberg, Institute for Physiological Chemistry and Pathobiochemistry, University of Mainz, Mainz,[2] and Department of Virology, University of Homburg, Homburg,[3] Germany

Received 18 April 1996/Accepted 19 August 1996

Human immunodeficiency virus type 2 (HIV-2) Nef is proteolytically cleaved by the HIV-2-encoded protease. The proteolysis is not influenced by the absence or presence of the N-terminal myristoylation. The main cleavage site is located between residues 39 and 40, suggesting a protease recognition sequence, GGEY-SQFQ. As observed previously for Nef protein from HIV-1, a large, stable core domain with an apparent molecular mass of 30 kDa is produced by the proteolytic activity. Cleavage of Nef from HIV-1 in two domains by its own protease or the protease from HIV-2 is also independent of Nef myristoylation. However, processing of HIV-1 Nef by the HIV-2 protease is less selective than that by the HIV-1 protease: the obtained core fragment is heterogeneous at its N terminus and has an additional cleavage site between amino acids 99 and 100. Preliminary experiments suggest that the full-length Nef of HIV-2 and the core domain are part of the HIV-2 particles, analogous to the situation reported recently for HIV-1.

Nef (negative factor) is a 24- to 35-kDa protein unique to the primate lentiviruses human immunodeficiency virus type 1 (HIV-1), HIV-2, and SIV (5, 10). In eukaryotic cells, Nef is usually myristoylated at its N-terminal glycine residue (2, 4). It is predominantly localized in the cytoplasm associated with lipid membranes, but it is also found in the nucleus (4, 12, 14, 17, 23). The biological importance of Nef was first demonstrated in the rhesus monkey model system of simian immunodeficiency virus-induced AIDS with the finding that functional *nef* expression is required for both high viral load and disease progression (13). Recent reports show that these observations are also valid for HIV infections of humans (3, 16). Proteolytic studies in conjunction with ^{1}H nuclear magnetic resonance spectroscopy showed that the Nef protein from HIV-1 probably consists of two structural domains, the N-terminal anchor domain and the C-terminal core domain (7). Nef protein of HIV-1 contains a specific cleavage site for the HIV-1 protease that separates these two domains (8, 9). On the basis of these observations, the three-dimensional structure of the well-folded core domain has been determined recently by NMR spectroscopy (11) and X-ray crystallography (18). Freund et al. (8) suggested that processing of Nef by the viral protease may regulate its activity in vivo; it may even be possible that the two domains have independent biological functions. Recently, cleavage of HIV-1 Nef by the viral protease was also observed in HIV-1 replication in infected T cells. Both the full-length Nef and the cleaved core domain were detected in virus particles (19, 21). Our previous proteolytic experiments (8) included only unmyristoylated Nef protein from HIV-1 expressed in *Escherichia coli*. If the processing of Nef is of general biological importance, it should also occur in Nef from HIV-2 and in its prevalent myristoylated form. These questions are addressed in the present study.

Influence of the N-terminal myristoylation on the protease processing. Myristoylation of Nef might impede the cleavage of Nef or lead to a different cleavage pattern. Therefore, proteolytic experiments were performed with Nef from HIV-1 (strain pNL4-3) obtained from a baculovirus expression system, in which Nef has been demonstrated to be efficiently myristoylated (12). Figure 1 (lane 2) shows that cleavage of baculovirus-expressed Nef by the protease of HIV-1 produces a large, stable fragment. Comparison of this proteolytic fragment with the core domain of Nef from HIV-1 expressed in *E. coli* (Fig. 1) shows that the two fragments have the same electrophoretic mobility. Incubation of the myristoylated HIV-1 Nef with the protease from HIV-2 leads to a stable fragment with a similar size (Fig. 1, lane 3 [see below]). This is in line with the observations from Gaedigk-Nitschko et al. (9) on persistently HIV-1-infected human cell lines.

In summary, myristoylation appears to have no influence on the observed digestion pattern, and, furthermore, the proteases from both HIV-1 and HIV-2 produce the same or very similar fragments of Nef (see below).

Specificity of the HIV proteases. Although the experiments with baculovirus-cleaved Nef protein indicated that HIV-2 protease cleaved HIV-1 Nef at a similar if not identical position, we were interested in determining the exact cleavage site for this enzyme as well as analyzing whether differences arise because of different substrate specificities of the enzymes. These experiments were performed with (nonmyristoylated) Nef protein expressed in *E. coli* and purified as described earlier (22). Figure 2 shows that the patterns of fragments obtained after digestion with the proteases from HIV-1 and HIV-2 are not completely identical. The main product obtained from incubation with HIV-2 protease for 12 h has an apparent molecular mass of approximately 18 kDa, which is slightly smaller than that obtained with the HIV-1 protease

* Corresponding author. Mailing address: Max-Planck-Institut für Medizinische Forschung, Abteilung Biophysik, Jahnstr. 29, D-69028 Heidelberg, Germany. Phone: 49 6221 486 445. Fax: 49 6221 486 437.

† Present address: Czech Academy of Science, Prague, Czech Republic.

‡ Present address: Queensland Institute of Medical Research, Brisbane, Australia.

§ Present address: Heinrich-Pette-Institute, Hamburg, Germany.

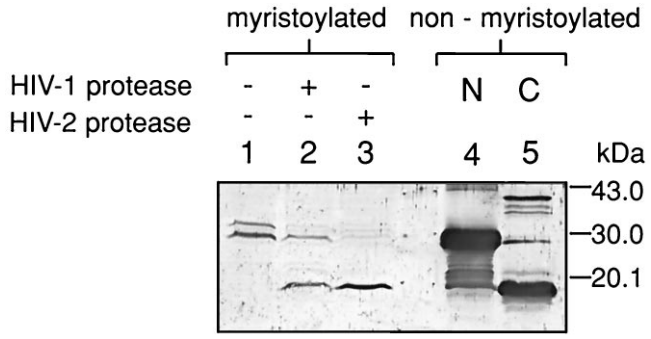

FIG. 1. Proteolytic cleavage of myristoylated and nonmyristoylated HIV-1 Nef. Results of Western blot analysis of HIV-1 Nef cleavage products are shown. The incubation mixtures were separated on a 15% polyacrylamide gel by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electroblotted onto a nitrocellulose membrane (NC BA 85; Schleicher & Schuell). Antibody dilutions were performed in 2% BLOTTO in 1× phosphate-buffered saline. For the detection of HIV-1 Nef and cleavage products, polyclonal antibodies raised against recombinant full-length Nef expressed in *E. coli* (22) were used in a dilution of 1:250. Myristoylated HIV-1 Nef (strain pNL-4) derived from insect cells was incubated with 0.2 μM HIV-1 (lane 2) or HIV-2 (lane 3) protease for 12 h at room temperature (pH 6.6). The recombinant HIV proteases were expressed in *E. coli* and purified as described in reference 20. Lane 1 shows the uncleaved Nef protein. For comparison, the nonmyristoylated, purified full-length Nef (N) from the HIV strain pNL4-3 (1) and the corresponding core domain (C) expressed in *E. coli* are also shown.

(Fig. 2, compare lanes 4 and 5). Analysis of the cleavage pattern in a Western blot (immunoblot) with three different antibodies against full-length Nef (anti-full-length Ab), the N-terminal anchor domain (anti-anchor Ab), and the C-terminal core domain (anti-core Ab) allowed us to identify the different fragments. As expected, full-length Nef is recognized by all three antibodies. Both proteases produce fragments with an apparent molecular mass of approximately 6 kDa which are recognized by anti-full-length Ab and anti-anchor Ab but not

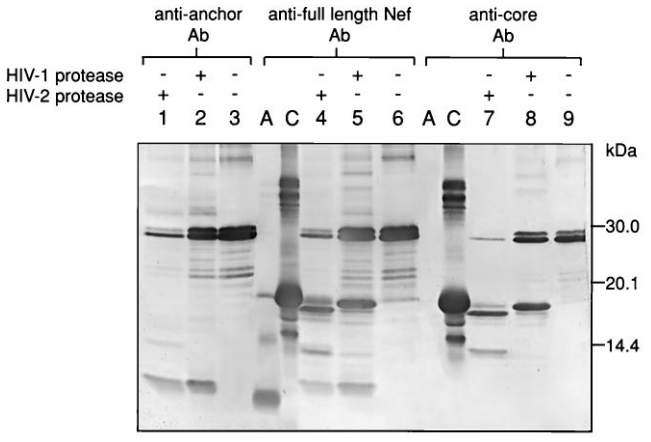

FIG. 2. Proteolytic cleavage of purified HIV-1 Nef. A total of 11 μM purified, unmyristoylated HIV-1 Nef (strain pNL4-3), dissolved in a buffer designed to simulate intracellular ionic conditions (6) (55 mM K acetate, 29.3 mM K_2HPO_4, 18.7 mM KH_2PO_4, 8 mM $NaHCO_3$, 7 mM K_2SO_4, 2 mM $MgSO_4 \cdot 7H_2O$, and 2 mM KCl), was incubated with 0.2 μM HIV-1 or HIV-2 protease for 12 h at room temperature (pH 6.0). The Nef protein was expressed in *E. coli* and purified as described previously (22). Purified recombinant core domain (C) and synthetic anchor domain (A) were used as controls. The Western blot analysis was performed as described in the legend to Fig. 1. For the detection of the Nef protein and the cleavage products, three different antibodies (Ab) raised against full-length Nef, the isolated core domain, and the isolated anchor domain were used.

by anti-core Ab. This means that the protease of HIV-2 produces the anchor domain as already described for the protease of HIV-1 (8). Both proteases produce an 18-kDa fragment that is recognized by anti-full-length Ab and anti-core Ab but not by anti-anchor Ab. This fragment represents the core domain of Nef as defined earlier (8). However, the protease of HIV-2 produces two additional fragments with apparent molecular masses of 14 and 17.5 kDa which are recognized by the two antibodies. The same products may also be generated by the HIV-1 protease, albeit at much lower levels (Fig. 2, lane 4). Closer inspection of the fragments in the 17.5- to 18-kDa range shows the occurrence of an additional fragment at low concentration with an intermediate mass; that is, the HIV-2 protease creates three slightly different digestion products in this mass range. The proteolytic products were sequenced as described in reference 7; sequencing of the three fragments obtained after cleavage with the HIV-2 protease yielded the sequences LEAQE, QEEEE, and EEVGF, corresponding to cleavage sites between amino acids 57 and 58, 60 and 61, and 63 and 64, respectively. The first of these cleavage sites represents the main cleavage site for the HIV-1 protease (8, 9). The observation of time-dependent concentration changes (data not shown) suggests that first the longest fragment is created, and then it is successively shortened by three amino acids over the time course of the incubation. (These shortened core fragments could also be generated by N-terminal trimming by contaminating bacterial exopeptidases. However, this is unlikely because such a trimming is not observed after the cleavage with HIV-1 protease which initially generates the same initial core fragment.) In addition, the incubation with the HIV-2 protease resulted in a smaller, 14-kDa fragment recognized by anti-full-length Ab and anti-core Ab but not by anti-anchor Ab. Sequencing of this fragment gives the N-terminal sequence LIHSQR, indicating that the cleavage site is located between Gly-98 and Leu-99.

In summary, under identical experimental conditions, the protease from HIV-1 produces almost exclusively two well-defined fragments, the core and the anchor domain. In contrast, the protease of HIV-2 appears to cleave HIV-1 Nef more efficiently and less specifically. In addition to the fragments produced by the protease from HIV-1, a smaller C-terminal fragment is produced. However, this smaller fragment was not observed upon cleavage of baculovirus-derived Nef protein (compare with Fig. 1), which may be due to incomplete digestion or the lack of accessibility for this cleavage site in the myristoylated protein.

Protease cleavage sites in Nef protein from HIV-2. If processing of Nef protein by the viral protease and the creation of two independent domains have a physiological significance, these events should also be observed for Nef from HIV-2. To address this question, Nef derived from the HIV-2 strain BEN was expressed as a recombinant nonfusion protein in insect cells infected with the Nef baculovirus vPIBF as reported recently (15). It was also expressed as a fusion protein in insect cells with the Nef baculovirus vPUBF with the additional amino acids MPARS in front of the first methionine, thus preventing myristoylation. As described earlier (15), myristoylated Nef of HIV-2 migrates with an apparent molecular mass of 39 kDa, which is much larger than was predicted from its amino acid sequence. As reported previously (15), this 39-kDa Nef product is recognized by antibodies specific for Nef from HIV-2 (data not shown). After incubation with 0.2 μM HIV-2 protease for 12 h, the Nef protein in the cell extracts is cleaved to a well-defined product with a mass of approximately 30 kDa (Fig. 3, lane 2). This cleavage does not occur in the absence of HIV-2 protease or if a specific inhibitor of the protease is used

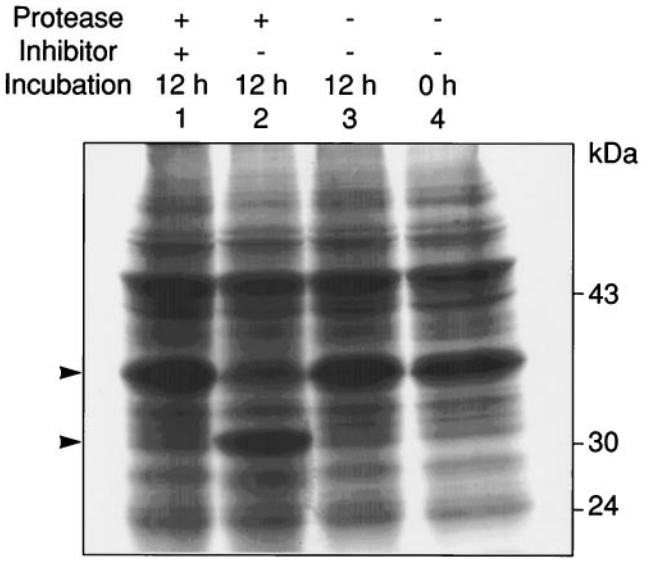

FIG. 3. Proteolytic cleavage of myristoylated HIV-2 Nef. Results of sodium dodecyl sulfate–15% polyacrylamide gel electrophoresis of an insect cell extract containing myristoylated HIV-2 Nef followed by Coomassie blue staining are shown. Recombinant HIV-2 Nef (strain BEN) was expressed in a baculoviral system (15). The cell extract was prepared by freezing and thawing 2×10^7 cells in 400 μl of protease inhibitor solution twice; unsoluble components were removed by centrifugation at 30,000 rpm (TLA 100.3-rotor) at 4°C for 30 min. The inhibitor cocktail, designed to inhibit all proteases except aspartyl proteases such as the HIV protease, contained 4 mM benzamidine, 4 mM phenanthroline, 50 mM EDTA, 2 mM NaN_3, 5 mM indoleacetic acid, 5 mM phenylmethylsulfonyl fluoride, and 0.02 μM aprotinin. The cell extracts of insect cells containing myristoylated Nef were incubated with the HIV-2 protease (0.2 μM) in the presence and absence of the specific HIV protease inhibitor $(CH_3)_3C$-O-CO-Phe[CH(OH)CH_2]Phe-Gln-Phe-NH_2 (5 μM) for 12 h at room temperature. As a control, one sample was frozen immediately after preparation (lane 4). The positions of full-length Nef and the core domain are indicated by arrowheads. Their identity was also confirmed by amino acid sequencing of the corresponding bands.

in addition to the enzyme (Fig. 3, lanes 1 and 3), implying that the HIV-2 protease is responsible for the cleavage of Nef, rather than possible, unspecific proteolytic activity in the HIV-2 protease preparation. Microsequencing of this fragment resulted in the N-terminal sequence SQFQEE, indicating a cleavage between Tyr-39 and Ser-40 of the Nef protein of HIV-2. The cleavage site recognized by the protease from HIV-2 has the sequence GGEY-SQFQ. Cleavage of nonmyristoylated HIV-2 Nef derived from baculovirus vPUBF-infected cells yielded a similar cleavage pattern, indicating that as in the case of HIV-1, Nef myristoylation has no major influence on the proteolytic cleavage.

As recently shown (19, 21), cleavage of HIV-1 Nef also occurs during viral replication in infected T cells and is mediated by the viral protease. Interestingly, both the full-length Nef and the core fragment are detected in the virus particles with a total of 5 to 10 copies of Nef per particle. These results indicate that the cleavage of Nef observed in vitro (references 8 and 9 and this study) is also relevant in virus replication. Therefore, it was of interest whether virion incorporation and proteolytic cleavage of Nef protein are also found during HIV-2 replication, as our data would suggest. Preliminary experiments indicate that HIV-2 Nef is indeed incorporated into virus particles and at least partially cleaved in the virion preparation. Nef-specific proteins with masses of 36 and 30 kDa corresponding to full-length Nef and the cleaved core domain were detected in HIV-2 fractions (Fig. 4, lane 3) and were

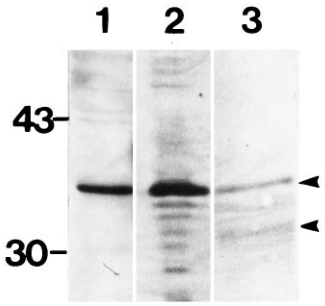

FIG. 4. Analysis of HIV-2 Nef incorporation into virus particles and Nef's cleavage. HIV-2-infected Jurkat cells (lane 2) and virus particles pelleted from the cell culture supernatant after centrifugation at 100,000 × *g* for 3 h at 4°C (lane 3) were lysed and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Nef-2-HOM was detected with the polyclonal rabbit serum anti-TrpE-Nef-2 HOM 473. The specificity of the recognition of the Nef protein by the anti-Nef serum was verified by competition experiments with recombinant Nef protein (data not shown). The positions of full-length Nef and the putative core domain in the virus pellet are indicated by arrowheads. As a control, lane 1 shows recombinant HIV-2 Nef expressed in B cells.

specifically competed for by incubation of the antiserum with recombinant Nef. Only the 36-kDa Nef protein was detected in HIV-2-infected cells (Fig. 4, lane 2), indicating that, as in the case of HIV-1, proteolytic cleavage occurs predominantly or exclusively within the virus particle.

This work was supported by the Bundesministerium für Bildung, Forschung und Technologie and the Boehringer Ingelheim Fonds.

We thank P. Ellison for carefully reading the manuscript.

REFERENCES

1. **Adachi, A., H. E. Gendelman, S. Koenig, T. Folks, R. Wiley, A. Rabson, and M. A. Martin.** 1986. Production of acquired immunodeficiency syndrome-associated retrovirus in human and nonhuman cells transfected with an infectious molecular clone. J. Virol. **59:**285–291.
2. **Allan, J. S., J. E. Coligan, T. H. Lee, M. F. McLane, J. E. Groopman, and M. Essex.** 1985. A new HTLV-III/LAV encoded antigen detected by antibodies from AIDS patients. Science **230:**810–813.
3. **Deacon, N. J., A. Tsykin, A. Solomon, K. Smith, M. Ludford-Menting, D. J. Hooker, D. A. McPhee, A. L. Greenway, A. Elett, C. Chatfield, V. A. Lawson, 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.
4. **Franchini, G., M. Robert-Guroff, J. Ghrayeb, N. T. Chang, and F. Wong-Staal.** 1986. Cytoplasmic localization of the HTLV-III 3′ orf protein in cultured T cells. Virology **155:**593–599.
5. **Francki, R. I. B., C. M. Fauquet, D. L. Knudson, and F. Brown.** 1991. Classification and nomenclature of viruses. Fifth report of the International Committee on Taxonomy of Viruses. Arch. Virol. **2:**290–299.
6. **Freund, J., and H. R. Kalbitzer.** 1995. Physiological buffers for NMR spectroscopy. J. Biomol. NMR **5:**321–322.
7. **Freund, J., R. Kellner, T. Houthaeve, and H. R. Kalbitzer.** 1994. Stability and proteolytic domains of Nef protein from human immunodeficiency virus (HIV) type 1. Eur. J. Biochem. **221:**811–819.
8. **Freund, J., R. Kellner, J. Konvalinka, V. Wolber, H.-G. Kräusslich, and H. R. Kalbitzer.** 1994. A possible regulation of negative factor (Nef) activity of human immunodeficiency virus type 1 by the viral protease. Eur. J. Biochem. **223:**589–593.
9. **Gaedigk-Nitschko, K., A. Schön, G. Wachinger, V. Erfle, and B. Kohleisen.** 1995. Cleavage of recombinant and cell derived human immunodeficiency virus 1 (HIV-1) Nef protein by HIV-1 protease. FEBS Lett. **357:**275–278.
10. **Gelderblom, H. R., M. Gentile, A. Scheidler, M. Özel, and G. Pauli.** 1993. HIV structure and function: established data, new aspects and open questions. AIDS-Forsch. **5:**231–242.
11. **Grzesiek, S., A. Bax, G. M. Clore, A. M. Gronenborn, J. S. Hu, J. Kaufman, I. Palmer, S. J. Stahl, and P. T. Wingfield.** 1996. The solution structure of HIV-1 Nef reveals an unexpected fold and permits delineation of the binding surface for the SH3 domain of hck tyrosine protein-kinase. Nat. Struct. Biol. **3:**340–345.
12. **Harris, M., and K. Coates.** 1993. Identification of cellular proteins that bind to the human immunodeficiency virus type 1 nef gene product in vitro: a role

for myristylation. J. Gen. Virol. **74:**1581–1589.
13. **Kestler, H. W., 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 the development of AIDS. Cell **65:**651–662.
14. **Kienzle, N., M. Bachmann, W. E. G. Müller, and N. Mueller-Lantzsch.** 1992. Expression and cellular-localization of the nef protein from human immunodeficiency virus-1 in stably transfected B-cells. Arch. Virol. **124:**123–132.
15. **Kienzle, N., M. Enders, M. Buck, H. Siakkou, S. Jahn, G. Petzold, E. K. Bachmann, W. E. G. Müller, and N. Mueller-Lantzsch.** 1992. Expression of the HIV-1-Nef protein in the baculovirus system: investigation of anti-Nef antibodies response in human sera and subcellular localization of Nef. Arch. Virol. **126:**293–301.
16. **Kirchhoff, F., T. C. Greenough, D. B. Brettler, J. L. Sullivan, and R. C. Desrosiers.** 1995. Brief report—absence of intact nef sequences in a long-term survivor with nonprogressive HIV-1 infection. N. Engl. J. Med. **332:** 228–232.
17. **Kohleisen, B., M. Neumann, R. Herrmann, R. Brack-Werner, K. J. E. Krohn, V. Ovod, A. Ranki, and V. Erfle.** 1992. Cellular localization of Nef expressed in persistently HIV-1 infected low producer astrocytes. AIDS **6:**1427–1436.
18. **Lee, C. H., K. Saksela, U. A. Mirza, B. T. Chait, and J. Kuriyan.** 1996. Crystal-structure of the conserved core of HIV-1 nef complexed with a src family SH3 domain. Cell **85:**931–942.
19. **Pandori, M. W., N. J. S. Fitch, H. M. Craig, D. D. Richman, C. A. Spina, and J. C. Guatelli.** 1996. Producer-cell modification of human immunodeficiency virus type 1: Nef is a virion protein. J. Virol. **70:**4283–4290.
20. **Pichova, I., P. Strop, J. Sedlacek, F. Kapralek, V. Benes, M. Travnicek, L. Pavlickova, M. Soucek, V. Kostka, and S. Foundling.** 1991. Isolation, biochemical characterisation and crystallisation of the p15gag proteinase of myeloblastosis associated virus expressed in *E. coli*. Int. J. Biochem. **24:**235–242.
21. **Welker, R., H. Kottler, H. R. Kalbitzer, and H.-G. Kräusslich.** 1996. Human immunodeficiency virus type 1 nef protein is incorporated into virus particles and specifically cleaved by the viral proteinase. Virology **219:**228–236.
22. **Wolber, V., H. Rensland, B. Brandmeier, M. Sagemann, M. Hoffmann, H. R. Kalbitzer, and A. Wittinghofer.** 1992. Expression, purification and biochemical characterisation of the human immunodeficiency virus 1 *nef* gene product. Eur. J. Biochem. **205:**1115–1121.
23. **Yu, G., and R. Feldsted.** 1992. Effect of myristoylation on p27 Nef subcellular distribution and suppression of HIV-LTR transcription. Virology **187:**46–55.