Internalization and intracellular retention of CD4 are two separate functions of the human immunodeficiency virus type 1 Nef protein

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The pathogenic Nef protein of the human immunodeficiency virus type 1 (HIV-1) downregulates CD4 by inducing its endocytosis and by inhibiting the transport of the receptor to the cell membrane. By means of in vivo-selected mutations, we show that L37, P78 and E177 residues of Nef are required for its effect on CD4 internalization and recycling but dispensable for Nef-induced retention and degradation of intracellular CD4. Of note, the function of Nef on the anterograde transport of newly synthesized CD4 molecules is irrelevant in cells with a slow constitutive CD4 turnover such as T cell lines. Moreover, we show that a mutated CD4 that is unresponsive to Nef-mediated endocytosis, CD4LL144 AA, is retained intracellularly and degraded by Nef like wild-type CD4. Thus, Nef’s abilities to enhance endocytosis and induce intracellular retention of CD4 are mediated by separate protein surfaces and occur through distinct mechanisms.

The multifunctional Nef protein of the human immunodeficiency virus type 1 (HIV-1) is a critical determinant for viral replication and pathogenesis (Deacon et al., 1995; Kestler et al., 1991; Kirchhoff et al., 1995). CD4 downregulation is Nef’s best studied activity, for which a pathogenetic role has been proposed (Lama, 2003). Nef accelerates CD4 endocytosis by acting as an adaptor that specifically connects the receptor to clathrin adaptor protein (AP) complexes. In addition, Nef inhibits CD4 recycling to the cell membrane, presumably by misdirecting internalized molecules to lysosomes for degradation (Oldridge & Marsh, 1998). More recently, a strong Nef-mediated inhibition of the CD4 anterograde pathway was also demonstrated in epithelial cells (Rose et al., 2005). Whether the activities of Nef in the CD4 endocytic and anterograde pathways are mediated by common protein surfaces and molecular interactions remains to be established. In this study, the link between the Nef-mediated mechanisms of CD4 downregulation have been investigated by means of in vivo-selected mutations of Nef.

We previously described Nef proteins defective in CD4 downregulation that were derived from HIV-1-infected patients (Casartelli et al., 2003b). Although amino acids required for Nef’s activity on CD4 were maintained in the defective proteins, some substitutions at highly conserved residues have occurred (Casartelli et al., 2003a). The RP2-7 protein presented glutamine in place of leucine at position 41 (corresponding to position 37 in Nef of the NL4-3 strain), and RP4-11 displayed lysine instead of glutamic acid at position 178 (position 177 in NL4-3 Nef). Hence, we restored the conserved amino acids by mutagenesis, creating RP2-7L41 and RP4-11E178. In addition, we introduced in vivo-selected mutations in NL4-3 Nef (NEF), generating NEFQ37 and NEFK177. The wild-type and mutated proteins were tested for their CD4 downregulation activity in HeLa-CD4 cells by a previously described retrovirus-based transduction system followed by two-colour flow cytometry (Casartelli et al., 2003b). The Q41L and E178K back-mutations fully restored the capacity of patient-derived Nefs to downregulate CD4 (Fig. 1a). In addition, NEFQ37 and NEFK177 lost activity, indicating that in vivo-selected substitutions impaired Nef function aside from the allelic background. The detrimental effect of the substitutions could not be attributed to protein instability, since they had no effect on the MHC-I downregulation activity (Casartelli et al., 2003b) and did not alter the protein’s steady-state expression, as shown by immunoblotting analysis (Fig. 1b). Therefore, two novel residues of Nef important for its activity of CD4 downregulation have been identified: L37, which resides in a conserved alpha helix (αH2) of the N-terminal flexible region (Geyer et al., 1999), and E177, which belongs to one of the two clusters of acidic residues that delimit the
C-terminal loop and likely maintain the loop flexible through their reciprocal repulsion (Geyer & Peterlin, 2001). In accordance, by simultaneous substitution of 2–3 residues with alanines, both the αH2 helix and the acid cluster have been involved in Nef-mediated CD4 down-regulation in previous studies (Greenberg et al., 1997; Iafrate et al., 1997). The introduction of the polar Q37 residue should potentially alter the structure of the αH2 helix. Since the N-terminal region of Nef is important for high-affinity CD4 binding (Preusser et al., 2001), the disruption of the αH2 helix may potentially perturb the Nef–CD4 interaction. The positively charged K residue in position 177 should alter the C-terminal loop, thus reducing the exposure of resident motifs (LL165 and ED175) critical for Nef functions in protein trafficking (Geyer et al., 2001). In pull-down assays with cellular lysates, NEFK177 associated with AP complexes or vacuolar ATPase as efficiently as wild-type Nef (data not shown). However, these assays may not reflect the capacity of Nef to form functional complexes required for effective CD4 down-regulation in intact cells.

To address the nature of the defects introduced by the in vivo-selected mutations, trafficking of CD4 was evaluated in Jurkat cells 48 h after transduction with retroviruses expressing Nef, NEFQ37 or NEFK177. We also tested NEFLL78, a protein with a patient-derived P78L substitution abrogating both CD4 and MHC-I downregulation (Casartelli et al., 2006), and two mutants, NEFΔ2A and NEFLL165AA, that have lost the capacity to associate with the cell membrane and AP complexes, respectively, and thus cannot downregulate CD4 (Geyer et al., 2001). In Jurkat cells, surface CD4 was reduced approximately twofold by Nef, whereas it was not affected by the mutants (Fig. 2a). As measured by a FACS-based endocytosis assay (Kasper & Collins, 2003), the expression of Nef increased the endocytic rate of residual surface CD4 (twofold higher initial rates), resulting in 80 % internalization in 60 min, rather than 40 % as observed in cells expressing NEFQ37, NEFLL78, NEFK177 or NEFΔ2A or in control cells (Fig. 2b). To evaluate the CD4 recycling rate, cells were cultivated for 3 h in the presence of a protein synthesis inhibitor, cycloheximide, then treated with trypsin (0.25 % in PBS 0.5 M EDTA for 20 min at 37 °C) until surface CD4 was completely removed (data not shown). The treated cells were washed, incubated with cycloheximide at 37 °C and the appearance of recycled CD4 on the cell surface was measured over time. The rate of CD4 recycling was slow (15 % in 180 min), and Nef expression reduced this rate almost twofold (8 % in 180 min) (Fig. 2c). On the contrary, expression of NEFQ37, NEFLL78, NEFK177 or NEFLL165AA resulted in a very modest reduction of the CD4 recycling rate (~13 % in 180 min). This assay was also performed without cycloheximide to determine the transport of newly synthesized CD4 over time (the amount of surface CD4 reappearing in cycloheximide-treated cells was subtracted from that reappearing in untreated cells). The constitutive transport of CD4 to the cell membrane was very slow (~6 % in 180 min), in cycloheximide-treated with the slow catabolism and long half-life (>24 h) of this molecule in T lymphocytes (Moller et al., 1990) and, importantly, did not vary significantly upon expression of wild-type or mutated Nef (data not shown). Next, the intracellular distribution and steady-state expression of CD4 in T cells expressing wild-type or mutated Nef proteins were analysed by immunofluorescence microscopy and immunoblotting analysis, respectively. To this aim, CEM cells were used rather than Jurkat cells because of their higher CD4 expression levels. As expected, cellular CD4 disappeared in cells expressing Nef while it was maintained upon expression of NEFQ37, NEFLL78, NEFK177 or NEFLL165AA (Fig. 2d). The intracellular CD4-specific punctate staining was not altered by either wild-
type or mutated Nef proteins. Therefore, Nef has no effect on the distribution of CD4 within T cells. As shown by immunoblotting analysis (Fig. 2e), wild-type but not mutated Nef proteins induced an approximately 30% decrease in total cell-associated CD4, in accordance with lysosomal degradation of CD4 molecules internalized by Nef. Thus, Nef-mediated CD4 downregulation in T cells can be attributed mainly to accelerated internalization and reduced recycling of CD4. Results also show that both activities are defective in Nef variants containing in vivo-selected mutations. In different cell systems such as HeLa or 293T cells transduced with CD4 expression vectors, the transport to the cell membrane of both newly synthesized and recycling CD4 molecules was shown to be strongly reduced by Nef, thus resulting in the receptor’s accumulation in perinuclear vesicles and reduced steady-state expression levels (Rose et al., 2005). In HeLa cells, the effects of Nef on CD4 endocytosis and recycling are modest (Rose et al., 2005), likely because they are masked by the rapid constitutive turnover of CD4 in these cells (Pelchen-Matthews et al., 1991). We then analysed the distribution of CD4 in transfected HeLa-CD4 cells expressing the mutated Nef proteins as GFP fusions, their wild-type counterparts or GFP alone. The construction of GFP-fusion expression vectors and transfection were performed as previously described (Fackler et al., 2000). To optimize staining of surface CD4, immunofluorescence microscopy was performed without prior permeabilization of cells. The cell-surface CD4 disappeared almost completely in cells expressing NEF–GFP or the RP2-7L41–GFP and RP4-11E178–GFP back-mutants while, in cells expressing NEFL78–GFP, RP2-7–GFP, RP4-11–GFP or NEFLL165AA–GFP, it was maintained to the same extent as in cells expressing GFP alone or not transduced (Fig. 3a). Of note, the intracellular localization of defective Nef proteins did not differ from that of wild-type Nef (mainly in perinuclear vesicles, at the plasma membrane and, to a small extent, diffused in the cytoplasm), suggesting that functional defects were not due to protein delocalization. An exception consisted in some nuclear localization and in a higher...
Fig. 3. Nef mutants conserve the ability to retain and degrade CD4 that is independent from CD4 endocytosis. (a, b) HeLa-CD4 cells expressing GFP alone or the indicated GFP-tagged Nef proteins, either without (a, no perm.) or with permeabilization (b, perm.), were stained and analysed by microscopy as described in Fig. 2(d). Nuclei were stained with DAPI. Either basal planes (a) or 3D-reconstructions of all planes (b) are shown. Individual channels corresponding to GFP, CD4 and DAPI or merged GFP/CD4 images (only in b) are shown. Bar, 10 μm. (c) Lysates of HeLa-CD4 cells infected as in Fig. 1(a) and expressing the indicated proteins were analysed by immunoblotting as described in Fig. 2(e). (d) HeLa-CD4LLAA cells transfected with NEF–GFP expression plasmid were stained as in (a, b). Images were acquired by a SPOT-2 CCD digital camera (Diagnostic Instruments). (e) HeLa-CD4LLAA cells were infected with empty or NEF-expressing retrovirus as described in Fig. 1(a), lysed and analysed by immunoblotting with anti-CD4, anti-GFP and anti-Nef antibodies. The level of CD4 was expressed by considering 100% that observed in Pinco-infected cells. Data are representative of three independent experiments.
diffuse cytoplasmic distribution of NEFL<sub>78</sub>–GFP and NEFL<sub>165AA</sub>–GFP. The intracellular CD4 localization was analysed in permeabilized cells (Fig. 3b). Compared to control cells, the CD4-specific staining was less dispersed throughout the cytoplasm and accumulated in a perinuclear area in cells expressing NEF–GFP. The NEF- and CD4-specific fluorescence largely overlapped, indicating that the majority of CD4 co-localized with Nef in a vesicular compartment. The nature of NEF–GFP + vesicles, either CD4+ or not, is heterogeneous since few expressed markers of early endosomes (EEA1) or Golgi compartment (giantin), most corresponded to recycling compartments (as determined by transferrin uptake over time), and none was positive with staining specific for multivesicular bodies (CD63) or lysosomes (LysoTracker; Invitrogen) (data not shown). Surprisingly, the same accumulation of CD4 in GFP + perinuclear vesicles was observed in cells expressing NEFL<sub>78</sub>–GFP, RP2-7–GFP, RP4-11–GFP or NEFL<sub>165AA</sub>–GFP, indicating that mutants defective for CD4 downregulation conserved the capacity of wild-type Nef to retain CD4 within these cells. Next, we analysed by immunoblotting the steady-state expression of CD4 in HeLa-CD4 cells expressing Nef, NEFQ<sub>137</sub>, NEFL<sub>78</sub> or NEFK<sub>177</sub>. Both wild-type and mutated Nef induced an approximately 20 % decrease in total cellular CD4 (Fig. 3c), suggesting that retained CD4 molecules are in part redirected to a degradation pathway. Thus, L37, P78 and E177 residues of Nef are required for its effect on CD4 endocytosis and recycling, but dispensable for the protein’s capacity to induce the retention and degradation of intracellular CD4. These results suggest that to induce CD4 retention Nef uses mechanisms distinct from those used to internalize surface CD4. This hypothesis was confirmed by analysing the role of Nef on the receptor’s distribution and steady-state expression in HeLa-CD4LLAA cells that stably express a CD4 mutant in which leucines 143 and 144, required for Nef-mediated endocytosis, have been substituted with alanines (Aiken et al., 1994; Bentham et al., 2003). Although its surface levels were not altered, CD4LL<sub>144</sub>AA accumulated in a perinuclear region upon NEF–GFP expression (Fig. 3d). In addition, the amounts of cell-associated CD4LL<sub>144</sub>AA were reduced by 20 % when Nef was expressed (Fig. 3e). Therefore, Nef-induced CD4 endocytosis does not contribute to retention and degradation of intracellular CD4 that results from an independent activity of Nef. It is conceivable that, during its anterograde pathway, nascent CD4 encounters and binds Nef in some vesicular compartment in which, consequently to the formation of a CD4–Nef complex, it is retained and eventually redirected to a degradation pathway. In agreement with this model, the LL<sub>144</sub>AA mutation of CD4, that does not interfere with the formation of a CD4–Nef complex (Bentham et al., 2003), had no impact in the Nef-mediated block of CD4 transport.

Taken together, these results show that Nef uses distinct surfaces and mechanisms to enhance endocytosis and inhibit transport of CD4. Apparently, the effects of Nef on CD4 endocytosis and transport are readily detected in cells with a slow (e. g. T cells) and fast (e. g. HeLa-CD4) constitutive CD4 turnover, respectively. Since the function of Nef on CD4 transport was not detected in T cell lines, its relevance as well as its impact on HIV-1 spread should be further investigated, preferably in primary T lymphocytes that have physiological CD4 expression. The molecular dissection of Nef-mediated CD4 downregulation, an HIV-1 function implicated in pathogenesis (Lama, 2003), may help the development of new strategies for the treatment of AIDS.

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