The Pro78 residue regulates the capacity of the human immunodeficiency virus type 1 Nef protein to inhibit recycling of major histocompatibility complex class I molecules in an SH3-independent manner

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The Nef protein is a crucial pathogenicity factor of human immunodeficiency virus type 1 (HIV-1) that contains a proline-rich motif consisting of four conserved prolines: Pro69 (P69), P72, P75 and P78. P72 and P75 were shown to bind Src homology domains 3 (SH3) and have been implicated in many biological functions of Nef, including downmodulation of cell-surface major histocompatibility complex class I (MHC-I). P78 is involved together with P69 in positioning of the Nef–SH3 complex and it has been shown to be essential for Nef activity of MHC-I downmodulation. It is shown here that alteration of P78 affects recycling of MHC-I molecules to the cell surface, but does not interfere with SH3 binding. In addition, it is demonstrated that P72 and P75, and thus the SH3-binding capacity, are fully dispensable for Nef activity on MHC-I.
Fig. 1. Role of P78 in Nef activity of MHC-I and CD4 downmodulation. (a) Downregulation of cell-surface MHC-I and CD4 by wild-type and mutated Nef proteins. Cells were infected as described previously (Casartelli et al., 2003b) with empty retrovirus (Pinco) or with recombinant Pinco retroviruses expressing NL4-3-derived Nef (NEF), NP5-8 or the indicated mutants. Specific mutations have been introduced into NEF and NP5-8 by standard site-directed mutagenesis based on recombinant PCR. After 48 h, cells were analysed by two-colour flow cytometry for expression of GFP encoded by the Pinco vector and of cell-surface MHC-I (Jurkat cells, top panels), human leukocyte antigen A2 molecules (HLA-A2; RMAS-A2 cells) or CD4 (HeLa-CD4 cells; Jurkat, bottom panels). HLA-A2 and CD4 stainings were performed as described previously (Casartelli et al., 2003b). For MHC-I staining, a phycoerythrin (PE)-conjugated anti-HLA-A/B/C antibody (Becton Dickinson) was used. (b) Jurkat cells were infected and analysed for expression of MHC-I (top) and CD4 (bottom) as described in (a). The geometric mean fluorescence intensities (MFI) specific for MHC-I or CD4 were evaluated in cells expressing medium levels of GFP fluorescence [gated in the R1 region in (a)]. Values are expressed as a percentage of Nef activity (empty bars) of MHC-I (filled bars) and CD4 (shaded bars) downregulation. Reported values are the means ± SD of three independent experiments. (c) Immunoblotting analysis of NEF, NP5-8 and their mutants. Lysates of Phoenix cells transfected with the indicated clones were immunoblotted with an anti-Nef antibody (upper panel; ARP3026; MRC AIDS Reagent Project) as described previously (Casartelli et al., 2003b). The arrow indicates the Nef protein bands. A cross-reacting, slower-migrating band present in the lysates is also visible. Due to their slower migration (Casartelli et al., 2003b), NP5-8 and NP5-8P78 co-migrate with the cross-reacting band. The blot was then stripped and reprobed with an anti-GFP antibody (Clontech) to evaluate transfection efficiency (lower panel). Similar results were obtained with lysates of cells shown in (a) (not shown).
retrovirus-based transduction system in RMAS-A2 (MHC-I), HeLa-CD4 (CD4) and Jurkat (CD4 and MHC-I) cells, followed by two-colour flow cytometry (Casartelli et al., 2003b). Nef proteins derived from the NL4-3 viral strain, either wild type (NEF), with P78 mutated into a leucine (NEF-L78) or mutated at residues M20 and EEEE65 [shown previously to be required for MHC-I downmodulation (reviewed by Arold & Baur, 2001; Geyer et al., 2001)] (NEF-A20 and NEF-4E4Q, respectively; shown only for Jurkat; Fig. 1a) were also tested. As shown in Fig. 1(a), the L78P substitution restored NP5-8 activity on both CD4 and MHC-I fully. The detrimental effect of L78 was unrelated to the allelic background, as NEF-L78 was also impaired (Fig. 1a, b), and was not caused by altered steady-state protein expression (Fig. 1c) or aberrant subcellular distribution (data not shown). However, in line with a previous report (Yamada et al., 2003), a Nef mutant in which P78 was substituted with alanine (NEF-A78) was defective for MHC-I downregulation, but was active on CD4, similarly to NEF-A20 and NEF-4E4Q. It is likely that, although P78 has no direct role in CD4 downmodulation, selected amino acid substitutions induce conformational changes resulting in a Nef protein defective for this activity.

As P78 may participate in the formation of a Nef–SH3 complex (Arold et al., 1997; Lee et al., 1996), functional defects of L78 mutants might be associated with an altered SH3-binding capacity. We therefore investigated the contribution of the SH3-binding property to Nef activities by analysing two mutants in which either the central two or all four prolines of PxxP were replaced by alanines (NEF-A72A75 and NEF-AAAA, respectively). Fig. 1(a, b) shows that NEF-A72A75 was fully active on CD4 and partially impaired in MHC-I downmodulation. Of note, the steady-state expression level of the mutated protein was lower than that of the wild type (Figs 1c, 2a), in line with previous studies showing NEF-A72A75 protein instability (Craig et al., 1999; Iafrate et al., 1997). As MHC-I downmodulation requires significantly higher intracellular Nef concentrations than does CD4 downmodulation (Liu et al., 2001), low protein amounts should affect Nef activity on MHC-I preferentially. Consistently, NEF-AAAA, which was barely detectable by immunoblotting analysis (Fig. 1c), lost its activity on CD4 and MHC-I partly and completely, respectively. Thus, NEF-AAAA was disregarded for further analysis. To test whether the NEF-A72A75 defect in MHC-I downregulation was due to reduced protein expression, we titered the retroviral particles expressing NEF or NEF-A72A75 by one to four spin-infection cycles (Fig. 2a, b). We found that cells infected with the same amount of virus displayed lower MHC-I downmodulation, as well as lower Nef protein amounts, when NEF-A72A75 was expressed in place of NEF, as expected for an unstable protein. However, in cells expressing similar levels of NEF and NEF-A72A75 following two and three spin infections, respectively, the extent of MHC-I downmodulation was equivalent (Fig. 2b). Thus, NEF and NEF-A72A75 display identical MHC-I downmodulation activities, for which the SH3-binding facet of the PxxP motif is fully dispensable, at least in this experimental system. In CD4+ T cells infected with HIV-1 carrying the NEF-A72A75 mutant, MHC-I downregulation was reduced (78% of the activity of wild-type virus), concomitant with a lower Nef protein expression (36% of the wild-type Nef; unpublished data), suggesting that, also in

Fig. 2. The MHC-I downmodulation activity of Nef is independent of its SH3-binding capacity. (a, b) Jurkat cells were infected with one to four spin-infection cycles with retroviruses expressing NEF or NEF-A72A75. (a) The steady-state expression of Nef proteins was evaluated by immunoblotting cell lysates with the anti-Nef antibody mAb 158 (Fackler et al., 1997) (upper panel). Next, the blot was stripped and probed with anti-GFP antibody (lower panel). (b) An aliquot of cells was employed to assess MHC-I downregulation (columns) as described in Fig. 1(b). The downregulation efficiencies were calculated by considering as 100% the activity of NEF in cells infected by four infection cycles. Grey columns indicate similar MHC-I downmodulation activities. Grey lines indicate Nef protein levels as quantified by densitometry of blots shown in (a). Arbitrary densitometric units for Nef-specific bands were calculated by considering as 1 unit the value corresponding to the NEF-specific band in 1×-infected cells. (c) In vitro binding of Hck to NEF-R, NP5-8 and their derivatives. The upper panel shows proteins bound to GST-fusion proteins revealed by immunoblotting with an anti-Hck antibody (sc-72; Santa Cruz Biotechnology). Coomassie staining (lower panel) was used to evaluate sample loading. The value corresponding to Hck was normalized for that of GST-fusion protein in the same sample, both measured by densitometry and expressed as a percentage of the Hck protein bound to GST–NEF-R. Representative data from one of three independent experiments are shown.
primary HIV-1-infected lymphocytes, the SH3-binding surface of Nef is important for protein stability, but not for its activity on MHC-I.

We then analysed the SH3-binding capacity of NP5-8 and NP5-8P78 by testing their ability to form a complex with Hck. NEF, NEF-A72A75 and NEF-L78 were mutated by T71R substitution to optimize Nef–SH3 interaction (Saksela et al., 1995), generating NEF-R, NEF-RA72A75 and NEF-RL78. The T71R substitution did not alter CD4 or MHC-I downregulation activities (data not shown). All variants were expressed as glutathione S-transferase (GST)-fusion proteins and tested for their capacity to bind Hck from U937 cellular lysates [as described by Lee et al. (1995)]. NP5-8 and NEF-R did not differ from the corresponding mutated variants, NP5-8P78 and NEF-RL78, respectively, in their relative Hck-binding capacity (Fig. 2c). The binding specificity was confirmed by the absence of Hck associated with GST–NEF-A72A75 or GST alone. Measurement of the capacity to associate with p21-activated kinase (Krautkrämer et al., 2004), which depends strictly on the SH3-binding capacity of Nef (Manninen et al., 1998), confirmed that P78 has no role in Nef–SH3 interactions (data not shown).

As the clathrin adaptor-protein complex AP-1 is required for Nef-mediated MHC-I downmodulation (Le Gall et al., 1998; Roeth et al., 2004), we tested the ability of NP5-8 to form a complex with AP-1 by an in vitro binding assay (Fig. 3a). No difference in association with AP-1 was detected for NEF, NEF-L78, NEF-4E4Q or the Pinco empty virus. At 48 h, MHC-I endocytosis was analysed at the indicated time points as described by Kasper & Collins (2003), but with W6/32 anti-MHC-I mAb. The relative amount of cell-surface MHC-I was expressed by considering as 100 % the initial MHC-I expression of each sample. (c) Transport of newly synthesized MHC-I to the cell surface. Jurkat cells transduced as in (b) were treated or not with cycloheximide and stripped of MHC-I molecules by low-pH treatment as described by Kasper & Collins (2003). Then, cells were incubated at 37 °C and 5 % CO2 for the indicated time and stained with PE-conjugated anti-HLA-A/B/C mAb. The amount of MHC-I transported to the cell surface was determined by subtracting from each sample the corresponding MHC-I staining remaining after stripping, then subtracting the MFI of the cycloheximide-treated cells from the MFI of the untreated cells. (d) Recycling of MHC-I to the cell surface. Transduced Jurkat cells were treated as described in (c). The amount of recycled MHC-I was determined by subtracting the corresponding MFI remaining after stripping from the MFI of the cycloheximide-treated cells at each time point. Reported values are the means ± SD of duplicates from one representative experiment out of three.
Here, we provide evidence that P78 is required for Nef activity of MHC-I downmodulation without contributing to Nef interactions with SH3-containing proteins. Besides, we demonstrate that P78 mediates the capacity of Nef to inhibit recycling of MHC-I to the cell surface. Further studies are needed to investigate the role of P78 in Nef interactions with cellular cofactors regulating MHC-I retention. Of note, our results also demonstrate that the SH3-binding capacity of Nef is fully dispensable for downmodulating MHC-I. Previous studies performed with NEF-A72A75 (Blagoveshchenskaya et al., 2002; Greenberg et al., 1998; Mangasarian et al., 1999) or with dominant-negative Hck (Chang et al., 2001) suggested a role for SH3 interactions in Nef activity on MHC-I. Our results suggest that the reduced capacity of NEF-A72A75 to downmodulate MHC-I should be ascribed to a low protein amount rather than defective SH3 binding. It is likely that the association of dominant-negative Hck with the SH3-binding site of Nef inhibits Nef activity on MHC-I indirectly as a consequence of steric hindrance and/or allosteric effect. The uncoupling of Nef activity on MHC-I from SH3-binding capacity will have implications for our understanding of the cellular pathways exploited by the viral protein and for attempts to interfere therapeutically with its pathogenic functions.

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