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 of human immunodeficiency virus type 1 (HIV-1) is a critical factor for high virus replication and disease progression in humans and animal models (Deacon et al., 1995; Hanna et al., 1998; Kirchhoff et al., 1995). Nef has several activities: downmodulation of cell-surface molecules such as CD4 and major histocompatibility complex class I (MHC-I), alteration of cellular signalling pathways and stimulation of HIV-1 infectivity and replication (reviewed by Fackler & Baur, 2002; Federico, 2004; Peterlin & Trono, 2003). Conserved amino acid residues and motifs in Nef have been characterized for their role in specific activities and interactions (Arold & Baur, 2001; Geyer et al., 2001), but it is still unclear which one(s) of these ultimately mediate(s) the pathogenic potential of Nef. A conserved proline-rich motif (PxxP) of Nef was accredited as a Src homology domain 3 (SH3)-binding site, with its central prolines, Pro72 (P72) and P75, contacting two hydrophobic pockets in the SH3 domain, whereas P69 and P78 assist the PxxP positioning (Arold et al., 1997; Lee et al., 1996). Through PxxP, Nef binds SH3-containing signalling molecules such as Hck (Saksela et al., 1995) and Vav (Fackler et al., 1999). Although the PxxP motif has been involved in most Nef functions (Greenberg et al., 1998; Hanna et al., 2001; Iafrate et al., 1997; Mangasarian et al., 1999; Saksela et al., 1995), some studies have shown that it is dispensable for Nef activity on MHC-I (Riggs et al., 1999) or CD4 (Saksela et al., 1995) and HIV-1 replication and pathogenesis (Kawano et al., 1997). In addition, it is unclear whether the role of the PxxP motif in the various biological functions of Nef is invariably mediated by its binding to SH3-containing proteins. As for MHC-I downmodulation, P78 rather than P69/P72/P75 was shown to be crucial (Yamada et al., 2003). Besides, conflicting reports exist on the putative role of PI3-K (Blagoveshchenskaya et al., 2002; Kasper & Collins, 2003) and Hck (Chang et al., 2001; Greenberg et al., 1998; Mangasarian et al., 1999) as relevant SH3 ligands for Nef activity on MHC-I.

To further investigate the role of the PxxP motif of Nef, we performed various analyses based on an in vivo-selected mutation. We showed previously that two Nef proteins, NP5-7 and NP5-8, derived from a non-progressor patient (Casartelli et al., 2003a), were impaired in MHC-I downmodulation and partially defective in CD4 downregulation (Casartelli et al., 2003b). As both proteins contained a leucine at position 78, we restored P78 in NP5-8 by mutagenesis and tested the resulting NP5-8P78 protein for both CD4 and MHC-I downregulation activities by means of a

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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 Arol'd & 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 (Arol'd 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 titrated 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). 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 x-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). Coo massie 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 NEFRL78, respectively, in their relative Hck-binding capacity (Fig. 2c). The binding specificity was confirmed by the absence of Hck associated with GST–NEF–SH3 interaction (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 NEFRL78, respectively, in their relative Hck-binding capacity (Fig. 2c). The binding specificity was confirmed by the absence of Hck associated with GST–NEF–SH3 interaction (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, NP5-8 or NP5-8P78. Thus, the functional defect induced by L78 cannot be attributed to inefficient in vitro AP-1 binding, although we cannot exclude the possibility that L78 might interfere with the interaction between Nef and AP-1 that is relevant for MHC-I downmodulation.

To gain insights into the mechanism by which P78 regulates Nef activity on MHC-I, we examined MHC-I trafficking in Jurkat cells infected with retroviruses expressing NEF, NEF-L78 and the NEF-4E4Q mutant by a method described by Kasper & Collins (2003). In agreement with previous reports (Kasper & Collins, 2003; Larsen et al., 2004), we observed that NEF stimulated MHC-I internalization from the cell surface slightly (Fig. 3b) and reduced both transport of newly synthesized class I molecules (Fig. 3c) and recycling of internalized MHC-I to the cell surface (Fig. 3d). Interestingly, NEF-L78 displayed the same phenotype as NEF-4E4Q, being unable to reduce MHC-I recycling.

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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 down-modulate 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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