Short Communication

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).
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 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
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, 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 in vivo 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, NEF-4E4Q or with 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.

Fig. 3. Role of P78 in Nef capacity to bind AP-1 (a) and regulate MHC-I trafficking (b–d). (a) In vitro binding of AP-1 to GST–Nef fusion proteins. GST alone, GST–NEF, GST–NP5-8 and GST–NP5-8P78 were produced and tested for their capacity to bind AP-1 from Jurkat cell lysates as described previously (Casartelli et al., 2003b). Relative AP-1-binding activity was calculated as the amount of AP-1 γ subunit detected by immunoblotting (top panel) normalized for the amount of GST-fusion protein detected by Coomassie staining (bottom panel) and expressed as a percentage of the value measured for GST–NEF. (b) Rates of MHC-I internalization. Jurkat cells were infected with retroviruses expressing NEF, NEF-L78, NEF-4E4Q or with 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.

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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 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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