Proline 78 Is Crucial for Human Immunodeficiency Virus Type 1 Nef To Down-Regulate Class I Human Leukocyte Antigen

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Human immunodeficiency virus type 1 Nef down-regulates human leukocyte antigen class I (HLA-I) in T lymphocytes, and the down-regulation involves the Nef proline-rich domain (PRD) containing four prolines at positions 69, 72, 75, and 78. We used a Sendai virus vector with *nef* and examined regulation by Nef of HLA-I and CD4 in suspension cultures of cells such as T lymphocytes. Analyses of a series of PRD substitution mutants indicated that, because the substitution of Pro78 with Ala abolished down-regulation of HLA-I but not of CD4, Pro78 is important for HLA-I down-regulation in T lymphocytes.

Host immune responses against viral infection are mediated by cytotoxic T lymphocytes, which recognize viral antigens presented by human leukocyte antigen class I (HLA-I) molecules, encoded by the HLA-A, -B, and -C loci, on the surfaces of infected cells (13, 24). Some viruses escape host immune responses down-regulating HLA-I expression by their own proteins. Examples include K3 and K5 zinc finger membrane proteins of Kaposi's sarcoma-associated herpesvirus (KSHV) (15, 22, 26), and Nef of human immunodeficiency virus type 1 (HIV-1). Nef is a 27-kDa myristoylated protein of approximately 200 amino acids which is produced abundantly in early stages of the viral life cycle (16) and decreases the number of HLA-I and CD4 molecules on the surfaces of infected cells (2, 7, 28), protecting HIV-1-infected cells from anti-HIV-1 cytotoxic-T-lymphocyte attack (4, 5). Nef contains a characteristic proline-rich domain (PRD), which consists of four proline residues at amino acids 69, 72, 75, and 78. The PRD has been found to be involved in decreasing the number of HLA-I molecules by affecting HLA-I trafficking via interactions with protein sorting machinery (9, 20) and to bind to Src homology domain 3 (SH3 domain), with Pro72 and Pro75 but not Pro69 or Pro78, forming a binding facet (17, 19). Some assumed that SH3-binding activity contributed greatly to HLA down-regulation, while others assumed that it contributed less (6, 9, 20, 25). Since no studies on Ala substitution for each Pro have been available, it remains unclear which proline residue is the most important among the four proline residues (Pro69,

Pro72, Pro75, and Pro78) for Nef to down-regulate HLA-I in T cells.

In most studies, although T lymphocytes are the main target for HIV-1, adhesion cells such as human IMR90 fibroblasts and 293 cells have been used for analysis of HLA-I down-regulation by Nef because of the general difficulty of expressing *nef* in suspended cells by transfection (9, 18). To express enough Nef for studying HLA-I down-regulation in suspended rather than adherent cells, we employed a recombinant Sendai virus (rSeV) system, which has been shown to express large amounts of heterologous recombinant proteins in 24 h after infection in both suspended and adherent cells (12, 31).

To identify which proline residue among the four in the PRD contributes most to HLA-I down-regulation, we constructed a series of rSeVs that express various Nef proteins with or without substitution of alanines for prolines. The DNA constructs used in these experiments were derived from a provirus plasmid, pNL-432 (1). Mutations were introduced into *nef* by site-directed mutagenesis based on overlap extension PCR (14). Each proline residue at amino acid 69, 72, 75, or 78 in the PRD of Nef was changed to alanine (P69A, P72A, P75A, P78A), and four consecutive glutamic acids from amino acid 62 to 65 in a highly conserved cluster of acidic residues, which is reported to bind to PACS-1 to down-regulate HLA-I (23), were changed to alanines (EEEE65AAAA). The resultant plasmids were designated pNL-432-APPP (P69A), pNL-432-PAPP (P72A), pNL-432-PPAP (P75A), pNL-432-PPPA (P78A), pNL-432-PPAA (P75A and P78A), pNL-432-PAAA (P72A, P75A, and P78A), pNL-432-PPPP/AAAA (P69A, P72A, P75A, and P78A), and pNL-432-EEEE/AAAA (EEEE65AAAA). An SeV-based expression vector, pSeV(+)-18bV(-) (21), which generates antigenomic positive-strand viral RNA in which the viral V gene has been knocked out, was used in this study. The *nef* genes were amplified on wild-type and mutant pNL-432 plasmids by PCR. Based on the KSHV genomic sequence (26),

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FIG. 1. Flow-cytometric analysis of CD4 and HLA-I expression on cell surfaces. CEM cells were infected with each rSeV at a multiplicity of infection of 10 for 1 h. Twenty-four hours after infection, cells were treated at 4°C for 20 min with an allophycocyanin-labeled anti-CD4 antibody (Becton Dickinson Immunocytometry Systems, San Jose, Calif.), and an R-phycoerythrin-labeled anti-HLA-I antibody (W6/32; Dako, Glostrup, Denmark) against a monomorphic epitope of HLA-A, -B, and -C or an anti-HLA-C antibody (29) with an R-phycoerythrin-labeled secondary antibody (Dako). A FACSCalibur flow cytometer (Becton Dickinson Immunocytometry Systems) with CellQuest software (Becton Dickinson Immunocytometry Systems) was used for flow cytometry. Isotype-matched control antibodies were included to detect nonspecific binding to cells.

we amplified DNA containing the KSHV K3 and K5 open reading frames from genomic DNA of BCBL-1 cells (3); PCR fragments were cloned in pSeV(+)-18bV(-) (27). Unexpectedly, our K3 construct lacked 33 nucleotides spanning from nucleotide 583 to 615 in K3 ORF (26). The rSeV stocks were obtained as previously described (30) and generally had infectious titers of 10^7 to 10^9 PFU/ml. The mutant rSeVs were designated SeV-EEEE/AAAA, SeV-APPP, SeV-PAPP, SeV-PPAP, SeV-PPPA, SeV-PPAA, SeV-PAAA, SeV-PPPP/AAAA, SeV-K3, and SeV-K5.

Nef, K3, and K5 expression significantly down-regulated surface expression of HLA-I molecules compared with the mock vector, whereas the down-regulation of CD4 molecules was observed only with Nef expression (Fig. 1A). Furthermore, HLA-C molecules were down-regulated specifically by K3 and K5 but not Nef. We found that KSHV K3 and K5 down-regulated HLA-I molecules to a lower level than Nef in terms of the mean fluorescence intensities (Fig. 1B). SeV-PPPP/AAAA abolished HLA-I down-regulation strongly compared to SeV-EEEE/AAAA (Fig. 2A). Each of the four prolines in the PRD was replaced by alanine to address the question of which of the four prolines in the PRD is most critical. CD4 on CEM cells were down-regulated by infection with SeV-APPP, -PAPP, -PPAP, or -PPPA, to an extent similar to that observed with wild-type SeV. HLA-I expression was down-regulated by SeV-APPP, -PAPP, and -PPAP at a level equivalent to that of SeV-wild, but not by SeV-PPPA. Furthermore, the PPAA, PAAA, and PPPP/AAAA mutants inhibited the down-regulation of HLA-I more than the PPPA mutant. The cell lysates infected with rSeV were analyzed by Western blotting with anti-Nef mouse monoclonal antibody (Intracel, Cambridge, Mass.). Since we observed similar expression levels of Nefs in the samples (Fig. 2B), the observed extent of down-regulation seemed to be simply a function of the nature of the introduced mutations.

To ascertain whether the effects observed with Nef alone were relevant to events taking place during HIV-1 infection, selected mutants were analyzed within the context of HIV-1-infected CEM-GFP cells. CEM-GFP cells are human T-lymphoid cells that contain an HIV-1 long terminal repeat-driven green fluorescent protein (GFP) cDNA in which GFP expression is induced by Tat (8). The CEM-GFP cell line was from the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, National Institutes of Health. We used CEM-GFP cells for measuring the level of HLA-I expression on HIV-1-infected cells directly. CEM-GFP cells were infected with the different HIV-1 variants by cocultivation for 48 h with transfected HeLa cells in RPMI 10, as previously described (20). As shown in Fig. 3A, in the case of wild-type virus infection, down-regulation of HLA-I expression in GFP-positive cells was observed, and the fluorescence intensity for GFP was reversely corre-

lated with the level of HLA-I expression. In agreement with the results from analysis with rSeVs, the PPPP/AAAA mutation abolished HLA-I down-regulation strongly, compared to EEEE/AAAA. HLA-I molecules were not internalized by PPPA, although APPP, PAPP, and PPAP proteins down-regulated surface expression of HLA-I in GFP-positive cells. Furthermore, additional mutations of Pro78 of the PRD, i.e., PPAA, PAAA, and PPPP/AAAA, inhibited down-regulation of HLA-I more strongly than PPPA. By analysis of HLA-C expression, we found that HLA-C down-regulation was not induced even in the case of infection with HIV-1 carrying the wild-type *nef* gene (Fig. 3B).

Our rSeV system proved to be competent in expressing Nef in a T-cell line, CEM, and primary T cells (data not shown), since Nef expressed by rSeV was biologically functional in terms of HLA-I down-regulation. Using this system, we determined that Pro78 in the PRD contributed most to HLA-I down-regulation but not to CD4 down-regulation, in suspension-cultured cells. Evidence that rSeVs expressed biologically functional Nef is found in the fact that in both rSeV (Fig. 2A) and HIV-1 (Fig. 3A) systems, Nef down-regulated HLA-I similarly and that HLA-I-A and -B molecules were down-regulated selectively (Fig. 1B and 3B). Evidence that Pro78 played a crucial role in down-regulation of HLA-I is that rSeV-mediated introduction of mutant *nef* genes inhibited down-regulation of HLA-I but not CD4 in CEM cells (Fig. 2A) and that HIV-1 mutants showed similar results (Fig. 3A). A detailed study on HIV-1 Nef not only will lead to a better understanding of molecular mechanisms of HLA-I down-regulation but also may provide novel means for developing Nef inhibitors. The use of rSeV will significantly facilitate these analyses on Nefs.

Our results showed that Pro78 was the most critical residue for HLA-I down-regulation, while Pro72 and Pro75 were much less critical. Since the latter proline residues have been shown to form an SH3-binding surface in nuclear magnetic resonance analysis and in X-ray crystallography (10, 11, 17), SH3-mediated signaling pathways may not be required for HLA-I down-regulation by Nef. A substitution of only one single amino acid, such as Pro to Ala, may result in a dramatic conformational change. Thus, our results may indicate that Pro78Ala destroyed the Nef structure thoroughly whereas Pro69Ala, Pro72Ala, and Pro75Ala resulted in little conformational change (Fig. 2A and 3A). In other words, Pro78 might be responsible primarily for holding a certain fixed structure and secondarily for down-regulation of HLA-I. However, we prefer the explanation that Pro78 plays a primary role in HLA-I down-regulation, since all the mutant Nefs including PPPA down-regulated CD4 as well as the wild-type. This suggests that their conformational change in mutant Nefs, if any, is minimal.

FlowJo software (Tree Star, San Carlos, Calif.) was used to make configurations. (A) Cells were analyzed for fluorescence intensity in the cell population gated as shown by a circle in each upper panel. The vertical axis represents signal intensity obtained with anti-HLA-I or anti-HLA-C. The horizontal axis represents signal intensity obtained with an anti-CD4 antibody. (B) Histograms of signal intensity obtained with anti-HLA-I or anti-HLA-C. Histograms are overlaid with a dotted-line histogram of an immunoglobulin isotype control. Values are mean fluorescence intensities (MFI) of HLA-I and HLA-C on the cell surface. The data were reproduced in three independent experiments. mock, SeV without inserted genes; Nef, rSeV with wild-type *nef* gene.

FIG. 2. Analysis of HLA-I down-regulation in CEM cells infected with a series of rSeVs carrying mutant *nef*. CEM cells were infected with each rSeV at a multiplicity of infection of 10 for 1 h. (A) Flow-cytometric analysis of CD4 and HLA-I expression on the cell surface 24 h after infection. The vertical axis represents signal fluorescent intensity obtained with an anti-HLA-I antibody. The horizontal axis represents signal intensity obtained with an anti-CD4 antibody. (B) Cellular expression of Nef proteins by infection with rSeVs. CEM cells (10^6) were infected with each rSeV at a multiplicity of infection of 10. One-tenth of the cell lysates was separated by electrophoresis in a sodium dodecyl sulfate–10% polyacrylamide gel. The separated proteins were blotted to a nitrocellulose membrane and stained with an anti-Nef antibody. The arrow indicates bands of about 27 kDa, corresponding to the Nefs shown at the top. The positions of size markers are shown on the left.

FIG. 3. Flow-cytometric analysis of HIV-1-infected CEM-GFP cells. (A) The vertical axis represents signal intensity obtained with an anti-HLA-I. The horizontal axis represents signal intensity obtained with GFP fluorescence; GFP expression is achieved by HIV-1 infection. The wild-type and mutant Nefs were expressed in the context of HIV-1_{NL432}. (B) Surface levels of HLA-C of the cells infected with the wild-type HIV-1_{NL432} were assayed. The vertical axis represents signal intensity obtained with an anti-HLA-C antibody. The horizontal axis represents signal intensity of GFP fluorescence. The results are representative of three independent experiments. mock, non-HIV-1-infected CEM-GFP cells.

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