Multiple human immunodeficiency virus type 1 Nef functions contribute to efficient replication in primary human macrophages

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The human immunodeficiency virus type 1 (HIV-1) Nef protein has been shown to accelerate viral growth kinetics in primary human T-lymphocytes and macrophages; however, the specific function(s) of Nef responsible for this phenotype in macrophages is unknown. To address this issue, mutants of a molecularly cloned macrophage-tropic isolate, HIV-1_SF162, were generated expressing single point mutations that abrogate the ability of Nef to interact with cellular kinases or mediate CD4 down-regulation. Infection of primary monocyte-derived macrophages (MDM) with these mutant viruses revealed that residues in the PX PX motif contribute to efficient replication. Interestingly, viruses expressing alleles of Nef defective in CD4 down-modulation activity retain wild-type levels of infectivity in single-round assays but exhibited delayed replication kinetics and grew to lower titres compared to the wild-type virus in MDM. These data suggest that efficient HIV-1 replication is dependent on the ability of Nef to interact with cellular kinases and remove CD4 from the surface of infected macrophages.

The Nef protein is required for high viral loads and, in most cases, progression toward disease in human immunodeficiency virus type 1 (HIV-1)-infected individuals (Deacon et al., 1996; Saksela et al., 1995; Learmont et al., 1999) and in simian immunodeficiency virus (SIV)-infected rhesus macaques (Kestler et al., 1991). Nef is a multifunctional, 25–27 kDa myristoylated protein lacking enzymic activity that mediates several biological effects in vitro through its ability to bind directly to its substrate or indirectly by stimulating an intermediate protein. The known functions of Nef identified include the down-regulation of the CD4 receptor (García & Miller, 1991; Guy et al., 1987) and the major histocompatibility complex class I (MHC-I) molecule (Schwartz et al., 1996), activation and interaction with signal transducing proteins (Baur et al., 1997; Bell et al., 1998; Collette et al., 1996; Fackler et al., 1999; Greenway et al., 1995; Howe et al., 1998; Lee et al., 1995; Nunn & Marsh, 1996; Saksea et al., 1995; Sawai et al., 1994; Smith et al., 1995; Xu et al., 1999), infectivity enhancement (Chowers et al., 1994; Miller et al., 1995) and impairment of Fas and tumour necrosis factor receptor-mediated apoptosis (Geleziuñas et al., 2001). These functions have been mapped to specific amino acid residues that are well conserved among different viral isolates. Specific residues in the polyproline type II helix of Nef (P⁶⁹, P⁷², P⁷⁵) are required for its interaction with a Ser/Thr kinase later identified as a PAK family member (Manninen et al., 1998; Nunn & Marsh, 1996; Sawai et al., 1994; Wiskerchen & Cheng-Mayer, 1996), enhancement of infectivity (P⁶⁹, P⁷², P⁷⁵) (Goldsmith et al., 1995; Wiskerchen & Cheng-Mayer, 1996) and for binding to Src family tyrosine kinases such as HCK (P⁵², P⁵³) (Saksea et al., 1995).

HIV-1-infected T-lymphocytes and the resulting destruction and impairment of these cells play a major role in the development of AIDS. Accordingly, most studies of Nef functions have focused on this cell type. However, macrophages are also a critical HIV-1 target cell, and play an important role in viral transmission and dissemination (Hirsch et al., 1998; Ignatius et al., 2002) and in the development of HIV-associated dementia (Kaul et al., 2001). Furthermore, the HIV–macrophage interaction differs in important ways from that of HIV–T-cell biology. Although virus entry into T-cells requires the CD4–CCR5 receptor–coreceptor interaction that allows fusion between Env and the plasma membrane, alternative and/or specialized entry pathways appear to be operative in macrophages (Marechal et al., 2001; von Lindern et al., 2003). Furthermore, virions were shown to accumulate in intracellular vesicles in HIV-infected macrophages (Orenstein et al., 1988) that have recently been identified as late endosomes (Pelchen-Matthews et al., 2003; Raposo et al., 2002). These properties, as well as their increased resistance to anti-retroviral therapy...
Aquaro et al., 1997) and the cytopathic effects of HIV-1 infection (Gartner et al., 1986; Ho et al., 1986; Meltzer et al., 1990), suggest that macrophages may serve as virus reservoirs (Gartner et al., 1986; Meltzer et al., 1990; Orenstein et al., 1997). Therefore, understanding the role of Nef in the context of HIV-1 infection of macrophages will be important for developing therapeutic strategies aimed at inhibiting virus replication via targeting this gene product.

Fig. 1. HIV-1 SF2 E nef-mediated down-regulation of CD4 cell-surface expression. The Jurkat T-cell line was transiently transfected with the plasmid pRES2-EGFP or with constructs encoding both EGFP and either wild-type or mutant nefs expressed from a bicistronic mRNA. Vector or pGFP, expressed only the GFP; WT, wild-type Nef; Nef−, frameshift mutant; P74, P77 and P71 indicate Nefs containing alanine substitutions in specific residues of the proline motif that disrupt interactions with cellular kinases; W59 and EE156,57, are Nefs mutated in residues previously shown for T-tropic isolates to affect Nef-mediated CD4 down-regulation function. Half of the transfected cells were analysed by flow cytometry for CD4 surface expression and the remaining were subjected to Western analyses for Nef protein expression. (A) Cells were harvested 40 h post-transfection and stained with an anti-CD4 phycoerythrin-conjugated antibody and analysed using two-colour flow cytometry. The x- and y-axes represent the log of GFP and CD4 phycoerythrin fluorescence intensity, respectively. (B) The mean fluorescence intensity of CD4 staining on the GFP+ cells shown in (A) (upper right quadrant) is given. The data represent the mean and standard error of three independent experiments. (C) An equal number of cells for each sample were lysed and submitted to Western analysis for Nef expression.
To determine which function(s) of Nef contribute to efficient replication in MDM, single or double alanine point mutations in amino acid residues known to disrupt the ability of Nef to interact with PAK (Manninen et al., 1998; Sawai et al., 1995; Wiskerchen & Cheng-Mayer, 1996) or Src (Saksele et al., 1995) family kinases, and to down-regulate CD4 (Grzesiek et al., 1996; Hua et al., 1997), were introduced into a bicistronic pIRES-EGFP plasmid (Clontech) expressing nef from the M-tropic viral isolate SF162 (Cheng-Mayer et al., 1990). Before introducing the mutant genes into the proviral DNA backbone, the nef alleles were characterized for their ability to down-regulate CD4 in transient transfection assays performed using Jurkat JJK cells (D. Littman, Skirball Institute, New York, NY, USA). Approximately 6 x 10⁵ JJK cells were transfected with 20 μg of the pCEF2Nef-IRES-EGFP plasmids using the lipofection agent DMRIE-C (Invitrogen) and harvested for flow cytometry (FACS) 48 h post-transfection. Cells were washed once in PBS containing 10 mM EDTA and stained with saturating amounts of anti-CD4 antibody conjugated to phycoerythrin (PE) (CalTag) in the same buffer with 10 mM sodium azide at room temperature for 30 min. The stained cells were washed and fixed in PBS/1% formaldehyde and receptor levels were measured using a FACSCalibur flow cytometer and CellQuest analysis software (BD Biosciences). The mean fluorescence intensity (MFI) of the GFP-positive and hence the Nef-expressing cells was examined. In agreement with previous reports, the W59 mutant displayed a defect, while the proline mutants were characterized for their ability to down-regulate CD4 in transient transfection assays performed using Jurkat JJK cells (D. Littman, Skirball Institute, New York, NY, USA). Approximately 6 x 10⁵ JJK cells were transfected with 20 μg of the pCEF2Nef-IRES-EGFP plasmids using the lipofection agent DMRIE-C (Invitrogen) and harvested for flow cytometry (FACS) 48 h post-transfection. Cells were washed once in PBS containing 10 mM EDTA and stained with saturating amounts of anti-CD4 antibody conjugated to phycoerythrin (PE) (CalTag) in the same buffer with 10 mM sodium azide at room temperature for 30 min. The stained cells were washed and fixed in PBS/1% formaldehyde and receptor levels were measured using a FACSCalibur flow cytometer and CellQuest analysis software (BD Biosciences). The mean fluorescence intensity (MFI) of the GFP-positive and hence the Nef-expressing cells was examined. In agreement with previous reports, the W59 mutant displayed a defect, while the proline mutants were competent for CD4 down-regulation (Fig. 1A, B). The Nef W59 mutant that should fail to target CD4 to a degradative compartment (Piguet et al., 1999) displayed an intermediate phenotype (Fig. 1A, B). Western analyses of transfected cell lysates revealed that the mutant Nefs were all expressed at comparable levels (Fig. 1C).

To assess Nef function in the context of viral infection, the mutations described above were introduced into a macrophage-tropic full-length infectious molecular clone of HIV-1SF162. Viruses were generated by transfection of HEK 293T cells with the lipofection agent DMRIE-C. The replication of the SF162 nef mutant viruses was measured using a single-round assay (Kimpton & Emerman, 1992). HeLa P4 cells expressing the SIV CCR5 receptor (Z. Chen, Aaron Diamond Center, New York, NY, USA) were infected with 10 ng p24 of each virus in 24-well plates in triplicate and harvested 72 h post-infection for β-galactosidase gene under the control of the HIV LTR were infected overnight in the presence of 4 μg DEAE-dextran ml⁻¹ in DMEM with 10% FBS with the indicated Nef wild-type or mutant viruses in triplicate in 24-well plates. The viral transactivator Tat induces the expression of β-galactosidase in the infected target cell. At 72 h post-infection the cells were lysed and β-galactosidase activity was measured using o-nitrophenyl β-D-galactopyranoside as a substrate. The release of o-nitrophenyl was measured spectrophotometrically. The results are expressed as the percentage of wild-type activity and represent the mean and standard error of three independent experiments. (B) An in vitro kinase assay (IVKA) (top panel) or Western (bottom panel) was performed on anti-Nef immunoprecipitates from macrophages infected with the indicated wild-type or Nef mutant viruses. The phosphoproteins represent p62, PAK1/2 and PIX (p85)/p95 (Brown et al., 1999). The lower panel was probed with a polyclonal anti-Nef antibody.
complex in primary human macrophages. Monocytes were purified from the buffy coats of normal human donors (NY Blood Center) and differentiated into MDM as described previously (Brown et al., 1999). Twelve days post-infection MDM cell lysates were prepared, incubated with anti-Nef antisera and subjected to Western analyses and an in vitro kinase assay (IVKA) as described previously (Brown et al., 1999). The wild-type, P74, W59 and EE156/57 mutant viruses all interacted with the multi-molecular PAK–Pix–p95 complex (Fig. 2B) (Brown et al., 1999). In contrast, the mutant viruses Nef−, P77 and P71 failed to interact with the PAK complex (Fig. 2B). Thus, SF162 Nef interaction with the PAK–Pix–p95 complex appears to be correlated with its ability to enhance infectivity in the single-cycle MAGI-CCR5 assay.

To determine the contribution of specific Nef functions to efficient replication, primary human MDM were infected as described above and p24 capsid released into the supernatant was quantified every 3 to 4 days by ELISA (Beckman Coulter). Wild-type SF162 replicated to peak titres by 12 days post-infection in the three different MDM donors (Fig. 3). In contrast, SF162 Nef− grew with delayed kinetics and reached titres that were 4- to 12-fold lower than the Nef+ virus during the same culture period (Fig. 3). The replication phenotype of viruses containing mutations in the Nef PXXP motif, however, was more complex and dependent on the donor MDM. Viruses with the Nef P71 and P74 mutations replicated with delayed kinetics and reduced titres in two out of the three donor MDM (Fig. 3, for P71, II and III; for P74, I and II). Impaired replication and low viral titres for virus expressing the Nef P77 mutant were observed in only one out of the three donors (Fig. 3, II). Interestingly, mutations affecting the ability of Nef to directly bind CD4 and accelerate its endocytosis (W59) and degradation (EE156/57) replicated with delayed kinetics and/or produced reduced titres over the entire macrophage culture period in all MDM donors (Fig. 3).

Using M-tropic viruses encoding specific mutants incapable of down-regulating CD4 or interaction with cellular kinases, we found that both of these Nef functions contribute to efficient replication in primary MDM. The findings that Nef-mediated CD4 down-regulation is not required for single-round infectivity but for efficient replication in primary CD4+ T-cells (Lundquist et al., 2002), in human lymphoid tissues (Glushakova et al., 2001) and, as shown here, for growth of HIV-1 in primary macrophages, demonstrates the importance of this Nef function to virus spread. CD4 downmodulation by Nef in T-cells has been suggested to prevent interactions between the virus receptor and Env that would inhibit incorporation of Gp160 into budding virions (Lama et al., 1999), and to allow for efficient release of mature viral particles from the cell surface (Cortes et al., 2002; Ross et al., 1999). Although the location of HIV-1 assembly in macrophages (Nguyen et al., 2003; Pelchen-Matthews et al., 2003; Raposo et al., 2002) appears to differ from that of T-cells (Gelderblom et al., 1987), the cellular machinery and processes responsible for budding are likely to be the same (Freed, 2003; Marsh & Thali, 2003; Pornillos et al., 2002). Thus, Nef-mediated CD4 down-regulation

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**Fig. 3.** Replication of SF162 Nef mutant viruses in primary human macrophages. MDM were infected 7 days post-differentiation with wild-type or the indicated Nef mutant viruses. Every 3–4 days, culture supernatants were collected, clarified and stored at −70 °C until analysed. The production of virus p24 antigen was quantified by ELISA (Beckman Coulter). I, II and III represent replicate experiments performed on three independent MDM donors. The symbols represent (B), SF162 wild-type; (J), Nef−; (H) and dashed line P74; (1), P71; (3), P77; (E) and dashed line, W59; (C), EE156,57.
function will also be required to allow efficient virus spread in macrophages.

In contrast to the findings with the CD4 down-regulation mutants, the replication pattern of viruses harbouring mutations in the Nef PXXP motif appeared to be more variable and dependent on the donor macrophages. Furthermore, whereas a correlation between PAK kinase association and enhancement of infectivity was seen in single-cycle assays (Fig. 2), this was absent in infected MDM (Fig. 3). This discordance between single-round MAGI and multi-round replication assays in assessing the requirement for specific residues with the PXXP motif of Nef was also reported for primary T-cells, perhaps reflecting the more variable activation states of primary cells in culture (Lundquist et al., 2002). In T-cell and transgenic mouse models, Nef can cause T-lymphocyte activation (Alexander et al., 1997; Skowronski et al., 1993) and induce a gene transcription profile that resembles that of T-cells stimulated through CD3 (Simmons et al., 2001). The PXXP motif of Nef has been implicated in interactions with MAP (Greenway et al., 1995) and tyrosine kinase signalling pathways (Collette et al., 1996; Saksela et al., 1995). In addition, the P^22 and P^27 residues (P^22 and P^27 NL4-3 numbering), respectively, are required for interaction with Hck or with both Hck and a PAK-containing complex (Renkema & Saksela, 2000). These kinases and associated signalling proteins may be present or activated to a varying extent at different stages of macrophage differentiation, explaining the donor-dependent requirement for residues within the PXXP motif for efficient replication in this cell type. Indeed, similar lack of consensus on the requirement for specific PXXP residues for efficient HIV-1 replication in T-cells (Craig et al., 1999; Lundquist et al., 2002; Saksela et al., 1995), and in different mouse (Hanna et al., 2001; Kawano et al., 1997; Stoddart et al., 2003) and rhesus macaque (Khan et al., 1998; Lang et al., 1997) models, has also been reported. Further studies are required to understand the biochemical and/or molecular pathways that regulate Nef function in primary macrophages.

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