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Functional characterization of HIV-1 Nef mutants in the context of viral infection

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Abstract

Nef is an important pathogenesis factor of HIV-1 with a multitude of effector functions. We have designed a broad panel of isogenic viruses encoding defined mutants of HIV-1_{SF2} Nef and analyzed their biological activity in the context of productive HIV-1 infection. Analysis of subcellular localization, virion incorporation, downregulation of cell surface CD4 and MHC-I, enhancement of virion infectivity and facilitation of HIV replication in primary human T lymphocytes mostly confirmed the mapping of Nef determinants previously reported upon isolated expression of Nef. However, reduced activity in downregulation of CD4, infectivity enhancement and virion incorporation of a Nef variant (Δ12–39) lacking an amphipatic helix required for binding of a cellular kinase complex and the association of Nef with MHC-I/AP-1 suggested a novel role of this N-terminal motif. The SH3 binding motif of Nef was partially required for infectivity enhancement and replication but not for receptor downmodulation. In contrast to previous results obtained using other Nef alleles, non-myristoylated SF2-Nef was only partly defective when expressed during HIV infection and was present in HIV-1 particles. Importantly, incorporation of Nef into HIV-1 virions was not required for any of the tested Nef activities. Altogether, this study provides a broad characterization and mapping of multiple Nef activities in HIV-infected cells. The results emphasize that multiple activities govern Nef's effects on HIV replication and argue against a role of virion incorporation for Nef's activity as pathogenicity factor.

Keywords: HIV Nef; Mutant virus panel; Virion incorporation; Myristoylation; Cell surface receptor modulation; Infectivity; Replication

Introduction

The accessory gene product Nef is a critical determinant for the pathogenesis of the primate lentiviruses HIV-1, HIV-2 and SIV. This is illustrated by low levels of replication in vivo achieved by viruses lacking functional *nef* genes that lead to no or delayed disease progression (Deacon et al., 1995; Kestler et al., 1991; Kirchhoff et al., 1995). Such a role for Nef as pathogenicity factor is further substantiated by a transgenic mouse model (Hanna et al., 1998) in which isolated expression of Nef causes AIDS like disease. Despite intense research efforts, the mechanisms by which Nef mediates its role as pathogenicity factor have remained obscure. Numerous activities of the viral protein, all exerted by protein interaction with cellular ligands, have been described (Arora et al., 2002; Geyer et al., 2001). In vivo, these activities are thought to mediate shielding of HIV-infected cells from the host's immune response and to directly optimize the multiplication of the virus. Immune evasion activities of Nef include the downmodulation of cell surface MHC class I and II molecules as well as the induction of apoptosis in bystander cells (Collins et al., 1998; Schwartz et al., 1996; Stumptner-Cuvelette et al., 2001; Xu et al., 1997, 1999). The optimization of the cellular environment for viral replication is achieved by the activation of signal transduction cascades leading to prolonged survival of infected cells and increased virus production (Fackler and Baur, 2002; Schrager and Marsh, 1999; Swingler et al., 2003; Wolf et al., 2001). Additionally, Nef triggers the downmodulation of

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cell surface CD4, which appears to have a profound impact on the fitness of viral progeny (Garcia and Miller, 1991; Glushakova et al., 2001; Lundquist et al., 2002). These influences lead to enhanced infectivity of HIV particles produced in the presence of Nef when assayed in a single round of replication and in increased replication kinetics over multiple rounds (Aiken and Trono, 1995; Miller et al., 1994; Schwartz et al., 1995; Spina et al., 1994). In surgery with the downmodulation of cell surface CCR5, removal CD4 also interferes with superinfection of productively infected cells (Michel et al., 2005). The individual activities of Nef appear to be largely independent of each other, and specific protein interaction surfaces have been mapped that mediate individual Nef activities (reviewed in Arold and Baur, 2001; Geyer et al., 2001). A common prerequisite to all Nef actions consists in its tethering to intracellular and plasma membranes, which depends on the N-terminal myristoylation of Nef. Possibly mediated by its association with the plasma membrane, 10–100 Nef molecules are incorporated into HIV particles (Bukovsky et al., 1997; Fackler et al., 1996; Welker et al., 1998). Whether the virion incorporation of Nef plays a role for its effects on viral replication remains to be defined.

While the molecular mechanisms of individual Nef activities are being studied intensively, their relative contribution to Nef's role as pathogenicity factor is difficult to address. Studies using Nef mutants defective in individual protein interactions allowed the correlation of some of its activities with enhanced virus replication and/or disease progression. However, direct evidence for a role of any individual Nef activity in lentiviral pathogenesis is missing, and it seems unlikely that all activities of Nef reported thus far will be relevant in vivo. Moreover, some of the in vitro phenotypes of Nef are a matter of controversy, a fact that is often attributed to the use of different alleles of the variable *nef* gene in a wide array of divergent cell lines. An additional major obstacle in revealing the pathologically relevant Nef activities consists in the use of experimental systems that rely on the in vitro overexpression of Nef in the absence of other HIV gene products. While many of Nef's effects have been observed in the context of HIV infection individually, few studies directly compared the molecular determinants in Nef for multiple of its biological activities in infected cells. Some of these studies exclusively mapped the Nef determinants for optimal HIV replication and CD4 T cell depletion (Aldrovandi et al., 1998; Stoddart et al., 2003). Others correlated effects of Nef on HIV-1 replication with Nef activities determined in overexpression systems of divergent cellular origin and used various Nef alleles instead of specific isogenic mutants (Glushakova et al., 2001) or only addressed a limited number of Nef activities in infected cells (Lundquist et al., 2002).

Here, we generated and broadly characterized a panel of isogenic viruses that expresses an array of established Nef mutants. These viruses were used to analyze Nef activities in the context of HIV infection. Our results provide evidence that CD4 downregulation correlates best with the positive effects of Nef on HIV replication in primary human T lymphocytes and reveal an unexpected role of an amphipatic helix in the N-terminal anchor domain of Nef for its activities in CD4 downregulation, infectivity enhancement and boosting of HIV replication. Furthermore, the study reveals that virion incorporation of Nef is dispensable for its activities in infectivity enhancement and optimization of HIV replication.

Results

Generation of an isogenic nef HIV-1 virus panel

We set out to generate a panel of isogenic HIV-1 proviral clones encoding for 15 established Nef mutants. We chose to use the *nef* gene from the HIV-1 strain SF2 since the corresponding Nef isoform has proven very active in all assays for Nef functions investigated thus far (Baur et al., 1994; Fackler et al., 2001; Keppler et al., 2005; Krautkramer et al., 2004). Given that the HIV-1SF2 virus does not replicate very efficiently due to difficulties of Env virion incorporation (Stamatatos and Cheng-Mayer, 1993), SF2 *nef* variants were introduced into the genetic background of the HIV-1 strain NL4-3 lacking the *nef* gene (Δ*nef*). It had been previously determined that SF2 Nef exerts key activities of Nef such as enhancement of virion infectivity and increase of virus replication in such a chimeric virus (Fackler et al., 2001; Rucker et al., 2004). The prototype chimera containing the HIV-1 NL4-3 provirus with the HIV-1 SF2 *nef* gene was designated NL4-3 SF2Nef or wild type (WT), the respective *nef* variants were all designated according to their amino acid changes in the encoded Nef protein. Nef mutants included in the panel were based on previous biochemical or structural mapping of protein–protein interaction or modification sites in Nef (Table 1). Two mutants were expected to impact on Nef's ability to associate with cellular membranes, which is thought to be vital for all of its activities. G2ANef lacks the myristoyl receptor glycine at position 2 and can therefore no longer be myristoylated by *N*-myristoyl transferase, a modification that mediates tethering of Nef to cellular membranes (Geyer et al., 2001; Hill and Skowronski, 2005). The R4A4 mutant lacks a cluster of basic amino acids described as contributing to membrane targeting of Nef (Welker et al., 1998). Another set of mutants was expected to affect Nef mediated intracellular sorting: the LLAA and EDAA mutants no longer recruit adaptor protein (AP) complexes or the catalytic subunit of V-ATPase (V1H), respectively, and are deficient in CD4 internalization (Bresnahan et al., 1998; Craig et al., 1998; Greenberg et al., 1998a, 1998b; Lu et al., 1998; Piguet et al., 1998), while NefE4A4 is blocked in the ability to associate with the PACS sorting adapter and does not downregulate MHC-I and CCR5 (Greenberg et al., 1998a, 1998b; Piguet et al., 2000; Michel et al., 2005). CD4 downregulation should also be prevented by deleting or mutating the presumed CD4 binding site in the Δ59–61 or W61A Nef mutants (Grzesiek et al., 1996; Lu et al., 1998; Mangasarian et al., 1999). Another set of mutants was designed to block individual protein interactions Nef employs to modify intracellular signal transduction. These include mutations that interfere with the association of Nef with SH3 domains of e.g. Vav or Hck (P76/79A: NefAxxA, V78A, R81A). Presumably reflecting the need for an SH3 interaction, these mutations also abrogate the association of Nef with active