The HIV-1 Nef Protein Interferes with Phosphatidylinositol 3-Kinase Activation 1*

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...nef...is a human immunodeficiency virus (HIV) gene encoding a 27-kDa myristoylated protein with structural features of a signal transducing molecule, whose functions are largely unknown. We studied the interactions of Nef with the signal transduction pathways triggered by the platelet-derived growth factor (PDGF) receptor. The association of phosphatidylinositol (PI) 3-kinase with the activated receptor was severely impaired by nef expression. Conversely, PDGF-induced receptor tyrosine phosphorylation, binding to phospholipase C-γ and to Ras-GAP were not modified. Microtubule-associated protein kinase activation and intracellular calcium influx in response to PDGF were either unaffected or only slightly enhanced. Nef significantly reduced the proliferative response to the growth factor, while the chemotactic response was unchanged. These data show that Nef affects selectively the PI 3-kinase signaling pathway and suggest that this interference results in some of the HIV adverse effects on host cell functions.

Human HIV-1 is a complex retrovirus containing several genes which regulate viral replication and gene expression. nef is one of the seven nonstructural genes highly conserved in HIV-2 and SIV (for a review, see Ref. 1). Its expression is critical for maintenance of high viral titer in vivo and for disease progression in primates (2). In vitro, nef is required for optimal viral replication in growth-stimulated PBLs (3-5). nef encodes a 27-kDa N-terminal myristoylated protein which is localized at the plasma membrane (1). Myristoylation is an absolute requirement for biological activity (6-9). Nef features multiple phosphorylation sites and has been reported to associate with an intracellular serine kinase (10). Furthermore, it contains a proline-rich region that binds to high affinity the SH3 domains of the cytoplasmic tyrosine kinase hck (11).

Together these data suggest that Nef may regulate viral growth and affect host cell function by interfering with signaling pathways. Indeed, (i) purified Nef protein microinjected in peripheral blood lymphocytes inhibits the proliferative response to IL-2 (9); (ii) constitutive expression of nef inhibits signaling by IL-2 and TCR in leukemic cells (9, 12-15) and by growth factors in murine fibroblasts (16); (iii) transgenic expression in thymocytes interferes with TCR signaling and perturbs their development (8, 17). However, the biochemical mechanisms underlying such interference still wait to be elucidated. Herein, we investigated the interactions between the Nef protein and the signaling pathways triggered by the PDGF receptor, which are among the best characterized (18).

EXPERIMENTAL PROCEDURES

Reagents, Antibodies, and Cells—HIV1-neffull-length cDNA was kindly provided by Dr. Montaigner, anti-Nef and anti-PDGFR receptor antibodies by Dr. Samuel and by Dr. Heldin. Anti-p85, anti-PLC-γ, and anti-Ras-GAP were from UBI. PDGF-BB was from Amersham. Either full-length nef cDNA subcloned in the eukaryotic expression vector pZip-neo or an empty expression vector as control was introduced into NIH 3T3 by Lipofectin (Life Technologies Inc.). Cells were co-transfected with pSV2neo, selected in the presence of 0.7 mg/ml G418 (Sigma) and analyzed for the expression of Nef. Five to ten individual clones expressing a comparable amount of protein were pooled.

Immunoprecipitation, Western Blotting, and PI 3-Kinase Assay—Immunoprecipitations were carried out as described previously (19), incubating the extracts with protein A-Sepharose coupled with anti-PDGFR antibodies. Immunoprecipitates or whole cell lysates were solubilized in boiling Laemmli buffer, separated on SDS-polyacrylamide gel electrophoresis, and electrotransferred into nitrocellulose filters (Hybond, Amersham). Specific binding was detected by the enhanced chemiluminescence system (ECL™, Amersham). PI 3-kinase activity was assayed on the immunoprecipitates in the presence of [γ-32P]ATP and phosphatidylserine (Sigma), as described previously (19). In these conditions, PI 3-kinase phosphorylates preferentially PI(4,5)P2, generating PI(3,4,5)P3 (20).

Determination of Free Cytosolic Calcium Concentration—NIH-3T3 cells were cultured on glass coverslips coated with poly(ethylene). The cells were loaded with the calcium indicator fura-2/AM (21) for 45 min at 37°C. Cells were then washed twice in Tyrode's solution (154 mM NaCl, 4 mM KCl, 2 mM CaCl2, 1 mM MgCl2, 5 mM NaHepes, 5.5 mM glucose, NaOH to pH 7.4) and stimulated by switching to the same solution containing different concentrations of PDGF. Intracellular calcium levels were determined on groups of 10-15 cells as described (22).

RESULTS

nef Stable Expression in NIH-3T3 Fibroblasts—The nef gene was cloned into a eukaryotic cell expression vector, under the control of the murine mammary tumor virus constitutive pro-

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1The abbreviations used are: HIV-1, human immunodeficiency virus; PI 3-kinase, phosphatidylinositol 3-kinase; PDGF, platelet-derived growth factor; PLC-γ, phospholipase C-γ; Ras-GAP, Ras GTPase activating protein; MAP, microtubule-associated protein; SIV, simian immunodeficiency virus; PBL, peripheral blood cells; TCR, T cell receptor; PIP2, phosphatidylinositol bisphosphate; SH2, Src homology region 2; SH3, Src homology region 3; IL-2, interleukin 2; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum.
This work shows that the HIV protein Nef interferes with signaling pathways in a model system generated by stable transfection of the nef gene in NIH-3T3 fibroblasts. Nef protein inhibits the complex formation between PI 3-kinase and the tyrosine-phosphorylated PDGF receptor. The biochemical mechanisms underlying such inhibition are unclear. Nef may compete with p85 (the docking subunit of PI 3-kinase) for the binding with specific phosphorylated tyrosines of the receptor (18). On the other hand, through its proline-rich motif Nef might bind the p85 SH3 domain. However, these hypotheses were not supported by co-precipitation experiments. Alternatively, as Nef is involved in a specific endocytic cellular pathway (1), it may sequester PI 3-kinase, making the enzyme unavailable for receptor binding. Finally, the viral protein may inhibit the interaction, by modifying either the PDGF receptor or the PI 3-kinase. PDGF receptor phosphorylation on tyrosine was unaffected. Nef, however, has been reported to form a...
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PLC-\(\gamma\)-rylation leads to negative modulation of PI 3-kinase (26). Interaction of the PI 3-kinase complex with a cellular serine kinase (10), and serine phosphorylation leads to negative modulation of PI 3-kinase (26).

Whatever the mechanism, the effect of Nef on PI 3-kinase is selective, as the association between the PDGF receptor and PLC-\(\gamma\) or Ras-GAP was not perturbed. Similarly, no significant alterations were observed when downstream events of the signaling cascade, such as \(Ca^{2+}\) increase and MAP kinase activation, were analyzed. These results do not confirm a previous study reporting Nef-mediated inhibition of intracellular calcium mobilization in PDGF or bombesin-stimulated fibroblasts; intriguingly, no inhibition of PLC-mediated PIP\(_2\) hydrolysis was detected (16). The authors suggest that Nef may act on IP\(_3\) receptor regulation or expression, rather than directly on signal transduction. The conflicting results reported here may depend on the different Nef alleles transfected. Indeed, either increased or decreased calcium responses were observed in murine T cells expressing different Nef alleles (8, 13).

In NIH-3T3 fibroblasts, PDGF-BB elicits a dual biological response, stimulating cell growth and chemotaxis (25). It is known that the signaling pathways involved are distinct, although partially overlapping (27). We show that Nef selectively inhibits PDGF-dependent proliferation, without affecting chemotactic migration. As activation of PI 3-kinase is essential for both responses, the apparent paradox can be explained by a different threshold of PI 3-kinase activation required for proliferation or chemotaxis (27, 28). In Nef transfecants, the low signal transduced by the residual receptor-PI 3-kinase complex could be sufficient to elicit the chemotactic response. A “threshold effect” has also been invoked to explain how epidermal growth factor and nerve growth factor elicit different biological responses in PC12 cells by activating the same signaling pathway to a different extent (29). Recently, a rare SIV-nef allele has been reported to act as an oncogene in NIH-3T3 fibroblasts (30). However, the growth-stimulating properties of the SIV-nef allele depend on two closely spaced tyrosine-containing specific sequences (31) which are not present in HIV1-nef.

Recent evidence suggests that nef is a major determinant in AIDS pathogenesis, by enhancing viral replication in host cells stimulated by mitogens (1). It is not known whether the uncoupling of PI 3-kinase from growth factor receptors is at all involved. However, it is intriguing to speculate that nef may promote viral growth by redirection of proliferative signals, arisen from growth factor receptors, to the viral replication machinery. Indeed, the binding of Nef to SH3 domains is required to achieve optimal viral replication in infected cells (11). Finally, besides affecting viral replication, nef may contribute to HIV-1 pathogenesis by altering T cell responses to antigenic stimulation (8, 13, 15). PI 3-kinase activation is in fact involved in the CD28-mediated stimulation by antigen presenting cells (32).

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FIG. 3. Effects of nef expression on PDGF-induced intracellular calcium increase and MAP kinase activation. A, intracellular calcium concentration in response to PDGF stimulation, in control (line 2.0) and nef-expressing cells (line 3.9). The ligand concentrations are indicated. B, Western blot with anti-MAP kinase antibodies on whole cell lysates from control (line 2.0) or nef-expressing cells (lines 3.0 and 3.9), unstimulated (–), or stimulated with 100 ng/ml PDGF-BB (+).

FIG. 4. Effects of nef expression on PDGF-induced proliferation and chemotaxis. PDGF-induced proliferation (A) and chemotactic migration (B) of control (line 2.0) and nef-expressing cells (lines 3.0 and 3.9). Cells were incubated in DMEM/1% FCS, either alone (open bar) or containing PDGF (shaded bar = 10 ng/ml, filled bar = 100 ng/ml) or in DMEM/10% FCS (hatched bar). Values are expressed as fold increase of cells/field (A) or of absorbance at 590 nm (B). In A, the value 1 represents 65, 70, and 74 cells/field for 2.0, 3.0, and 3.9 cells, respectively. Each value is an average of quadruplicates.
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