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

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Andrea Graziani, Francesco Galimi, Enzo Medico, Erika Cottone, Daniela Gramaglia‡, Carla Boccaccio, and Paolo M. Comoglio§

From the Institute for Cancer Research and Treatment (IRCC), University of Torino Medical School, 10126 Torino, Italy

***nef* is a human immunodeficiency virus (HIV) gene encoding a 27-kDa myristoylated protein with structural features of a signal transducing molecule, but 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 HIV1[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 HIV2, 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 at 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-nef_{BRU} full-length cDNA was kindly provided by Dr. Montaigner, anti-Nef and anti-PDGF 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 precoupled with anti-PDGF-R antibodies. Immunoprecipitates or whole cell lysates were solubilized in boiling Laemmli buffer, separated on SDS-polyacrylamide gel electrophoresis, and electrotransferred onto 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-^{32}P]ATP and phosphoinositides (Sigma), as described previously (19). In these conditions, PI 3-kinase phosphorylates preferentially PI(4,5)P_2, generating PI(3,4,5)P_3 (20).

Determination of Free Cytosolic Calcium Concentration—NIH-3T3 cells were cultured onto glass coverslips coated with poly(D-lysine). 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).

Proliferation and Transwell Migration Assay—Cells were seeded in 24-well plates (10^5 cells/well) and incubated in DMEM/1% FCS alone or containing either increasing concentrations of PDGF or 10% fetal calf serum. After 4 days, cells were fixed with 11% glutaraldehyde, stained with 0.1% crystal violet, 20% methanol, and counted. Stimulation of chemotaxis was determined using Transwells™ (6.5 mm, Costar), with two compartments separated by an 8-μm pore polycarbonate membrane. 10^5 cells in 200 μl of DMEM/1% FCS were placed in the upper compartment. One ml of DMEM/1% FCS, alone or containing the stimulating factor, was added to the lower compartment. Plates were incubated at 37 °C in a humidified 5% CO_2 atmosphere for 6 h. Cells migrated to the lower side of the filter were fixed, stained as above, and solubilized in 10% acetic acid. Absorbance was measured at 590 nm.

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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‡ Recipient of an AIDS-Fellowship from the Istituto Superiore di Sanitá.

§ To whom correspondence should be addressed: Institute for Cancer Research (IRCC), corso Massimo d'Azeglio 52, 10126 Torino, Italy. Tel.: 39-11-670-7739; Fax: 39-11-650-9105.

[1] The abbreviations used are: HIV-1, human immunodeficiency virus 1; 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; PIP_2, 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.

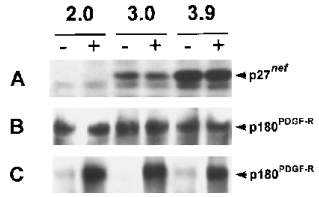

FIG. 1. **Expression of Nef protein and PDGF receptor in NIH-3T3 fibroblasts.** Control NIH-3T3 fibroblasts (*line 2.0*) and stable transfectants (*lines 3.0* and *3.9*), either unstimulated (−) or treated with PDGF-BB 100 ng/ml (+) for 15 min, were solubilized, separated by SDS-polyacrylamide gel electrophoresis, and analyzed by Western blot with anti-Nef (*A*), anti-PDGF receptor (*B*), and anti-phosphotyrosine antibodies (*C*).

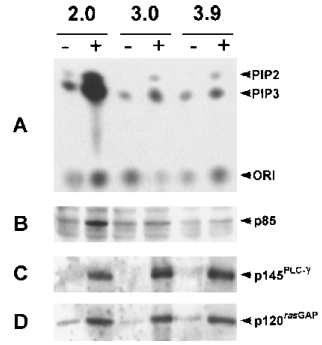

FIG. 2. **Effects of *nef* expression on association of PI 3-kinase, PLC-γ, and Ras-GAP to the PDGF-receptor.** Control (line 2.0) or *nef*-expressing cells (*lines 3.0* and *3.9*), unstimulated (−) or stimulated with 100 ng/ml PDGF-BB (+), were lysed and immunoprecipitated with anti-PDGF receptor antibodies. The immunocomplexes were analyzed for PI 3-kinase enzymatic activity (*A*) or by Western blot with anti-p85 (*B*), anti-PLC-γ (*C*), and anti-Ras-GAP (*D*) antibodies.

moter, and transfected in murine NIH-3T3 fibroblasts expressing physiological levels of PDGF receptors. Stable transfectants were selected by G418 resistance, cloned and analyzed for *nef* expression by Western blot of whole cell lysates. Clones expressing the $p27^{nef}$ protein were combined in two pools according to the level of *nef* expression (*line 3.0*, low; *line 3.9*, high; Fig. 1*A*). Clones transfected with an empty vector (*line 2.0*) were pooled together and used as control cell line.

In cells expressing either low or high levels of Nef, the amount of PDGF receptors was unchanged (Fig. 1*B*). Moreover, upon ligand stimulation, the receptor was tyrosine-phosphorylated to the same extent (Fig. 1*C*). Nef did not affect the kinetics of the receptor kinase in response to varying concentrations of ligand (data not shown). These data indicate that Nef does not interfere with the cell surface expression or the tyrosine kinase activity of the PDGF receptor.

Nef Selectively Inhibits Association of PI 3-Kinase with PDGF Receptor—It is known that, upon ligand stimulation, the PDGF receptor binds PI 3-kinase via the p85 regulatory subunit (18). In *nef* transfectants, the amount of PI 3-kinase activity associated with the immunoprecipitated receptor upon PDGF stimulation was about 10-fold lower, compared to mock-transfected cells (Fig. 2*A*). This was confirmed measuring the physical association between the PDGF receptor and p85 by immunoprecipitation and Western blot (Fig. 2*B*). Interestingly, *nef* expression also resulted in a significant reduction of the basal PI 3-kinase activity associated with the receptor in unstimulated cells (Fig. 2*A*). The inhibition on the formation of the PI 3-kinase·receptor complex correlated with the amount of *nef* expression (*cf.* Figs. 1*A* and 2, *A* and *B*). The viral protein did not affect the total cellular content of PI 3-kinase, measured by probing whole cell lysates with anti-p85 antibodies (data not shown).

The possibility that Nef inhibits PI 3-kinase/receptor association by direct binding to p85 or to the receptor was ruled out by measuring PI 3-kinase activity in anti-Nef immunoprecipitates and by probing anti-p85 or anti-PDGF receptor immunoprecipitates with anti-Nef antibodies (data not shown). Moreover, no evidence of Nef tyrosine phosphorylation upon PDGF stimulation was obtained (data not shown).

In order to establish whether the Nef inhibition on PI 3-kinase association is a selective event or a consequence of a general interference with the binding between the receptor and cytosolic transducers, PLC-γ and Ras-GAP were studied. After ligand stimulation, both transducers did bind to the PDGF receptor with the same efficiency in cells either mock-transfected or expressing low or high amounts of Nef (Fig. 2, *C* and *D*).

Nef Does Not Interfere with Ca^{2+} Channels or MAP Kinase—We then investigated whether *nef* expression could interfere with other signaling pathways, downstream to the receptor. PDGF stimulation increases intracellular calcium concentration by a dual mechanism: (*a*) a rapid release from intracellular stores and (*b*) a slow influx from the extracellular environment (23). *nef* expression did not affect the calcium response when high concentrations of PDGF were used (100 ng/ml; Fig. 3*A*). At low concentrations of PDGF (1 ng/ml), only the rapid release was slightly enhanced.

PDGF also triggers an intracellular serine kinase cascade leading to activation of MAP kinase. This activation follows phosphorylation of MAP kinase at serine and tyrosine residues, which results in a mobility shift that can be observed in Western blots (24). Expression of *nef* did not interfere with MAP kinase activation in all cell lines tested (Fig. 3*B*). A modest enhancement of MAP kinase phosphorylation in resting cells was observed.

Nef Selectively Inhibits PDGF-induced Cell Proliferation—In NIH-3T3 fibroblasts, PDGF-BB elicits a dual biological response, consisting of cell growth and chemotaxis (25). The growth response was severely impaired in *nef* transfectants, even in those expressing low levels of the viral protein. Inhibition was still evident even at high PDGF concentrations (up to 100 ng/ml, Fig. 4*A*). Cellular growth in the presence of serum was not affected, indicating that Nef acts as inhibitor of PDGF signaling rather than as inhibitor of cell proliferation *per se*. On the other hand, when cell migration toward a gradient of PDGF was evaluated, no difference in cell motility was detected between *nef* transfectants and controls (Fig. 4B). These data indicate that among the signal transduction pathways triggered by PDGF receptor, Nef inhibits selectively those leading to cell proliferation.

DISCUSSION

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

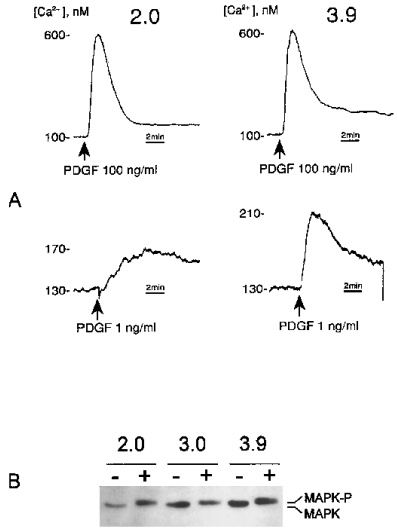

FIG. 3. **Effects of *nef* expression on PDGF-induced intracellular calcium increase and MAP kinase activation.** *A*, intracellular calcium concentration in response to PDGF stimulation, in control (*line 2.0*) and *nef*-expressing cells (*line 3.9*). The ligand concentrations are indicated. *B*, Western 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 (+).

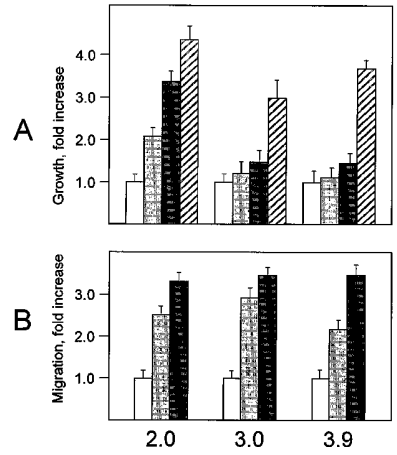

FIG. 4. **Effects of *nef* expression on PDGF-induced proliferation and chemotaxis.** PDGF-induced proliferation (*A*) and chemotactic migration (*B*) of control (*line 2.0*) and *nef*-expressing cells (*lines 3.0* and *3.9*). Cells were incubated in DMEM/1% FCS, either alone (*open bar*) or containing 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.

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-γ 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* transfectants, 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 know 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 redirectioning 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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