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Journal of Cellular Biochemistry 106:812–822 (2009)

Journal of Cellular Biochemistry

HIV-1 Nef Induces p47^{phox} Phosphorylation Leading to a Rapid Superoxide Anion Release from the U937 Human Monoblastic Cell Line

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ABSTRACT

The Nef protein of the human immunodeficiency virus type 1 (HIV-1) plays a crucial role in AIDS pathogenesis by modifying host cell signaling pathways. We investigated the effects of Nef on the NADPH oxidase complex, a key enzyme involved in the generation of reactive oxygen species during the respiratory burst in human monocyte/macrophages. We have recently shown that the inducible expression of HIV-1 Nef in human macrophages cell line modulates in bi-phasic mode the superoxide anion release by NADPH oxidase, inducing a fast increase of the superoxide production, followed by a delayed strong inhibition mediated by Nef-induced soluble factor(s). Our study is focused on the molecular mechanisms involved in Nef-mediated activation of NADPH oxidase and superoxide anion release. Using U937 cells stably transfected with different Nef alleles, we found that both Nef membrane localization and intact SH3-binding domain are needed to induce superoxide release. The lack of effect during treatment with a specific MAPK pathway inhibitor, PD98059, demonstrated that Nef-induced superoxide release is independent of Erk1/2 phosphorylation. Furthermore, Nef induced the phosphorylation and then the translocation of the cytosolic subunit of NADPH oxidase complex p47^{phox} to the plasma membrane. Adding the inhibitor PP2 prevented this process, evidencing the involvement of the Src family kinases on Nef-mediated NADPH oxidase activation. In addition, LY294002, a specific inhibitor of phosphoinositide 3-kinase (PI3K) inhibited both the Nef-induced p47^{phox} phosphorylation and the superoxide anion release. These data indicate that Nef regulates the NADPH oxidase activity through the activation of the Src kinases and PI3K. J. Cell. Biochem. 106: 812–822, 2009.

KEY WORDS: NADPH oxidase; ROS; Src family kinases; PI3K; U937 cell line

Human monocyte/macrophages release high levels of reactive oxygen species (ROS) in response to different stimuli. These cells play a crucial role in host defense against pathogens, by generating large amounts of superoxide anion ($O_2^{\cdot-}$), a phenomenon known as the respiratory burst. The main $O_2^{\cdot-}$ producer is the NADPH oxidase, a multi-component enzymatic complex catalyzing NADPH-dependent reduction of oxygen to superoxide anion [Chanock et al., 1994; Roos et al., 2003]. In resting cells, the oxidase is dormant and consists of both cytoplasmic (p47^{phox}, p67^{phox}, p40^{phox}, and Rac1/2) and integral membrane (cytochrome b_{558}, that consists of gp91^{phox} and p22^{phox}) subunits. On stimulation, the cytosolic components translocate to the inner surface of the plasma membrane by bonding with the cytochrome b_{558} [Vignais, 2002; Babior, 2004].

The impairment of both oxidative burst response and phagocytosis in monocytes/macrophages from HIV-1 patients have been widely described [Muller et al., 1990; Kimura et al., 1993; Flo et al., 1994; Pitrak et al., 1998; Koziel et al., 2000; Tachado et al., 2005]. High levels of ROS production by HIV-1 infection may contribute to the development of AIDS pathogenesis by means of the induction of an inflammatory status leading to the recruitment and activation of cells of the immune system for optimal virus spread. On the other hand, suppression or restriction of ROS production is essential to avoid irreparable damage to host tissue.

Nef is a 27–34 kDa myristoylated protein produced exclusively by Human and Simian Immunodeficiency Viruses, and is considered a major pathogenetic factor as demonstrated both in animal models [Hanna et al., 1998] and HIV-infected humans [Kestler et al., 1991].

Grant sponsor: Ministry of Health (Rome, Italy).

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Received 19 August 2008; Accepted 2 December 2008 • DOI 10.1002/jcb.22041 • 2009 Wiley-Liss, Inc.
Published online 7 January 2009 in Wiley InterScience (www.interscience.wiley.com).

Although Nef does not have a catalytic activity, it however influences cellular signaling pathways leading to the enhancement of viral replication, immune elusion, and enhanced survival in T-cells and macrophages [Piguet and Trono, 1999; Briggs et al., 2001; Fackler and Baur, 2002; Choi and Smithgall, 2004a]. This occurs through the interaction with several distinct classes of host cell proteins, including immune receptors, protein kinases, intracellular trafficking proteins, and guanine nucleotide exchange factors [Arold and Baur, 2001; Geyer et al., 2001]. Furthermore, Nef induces the secretion from primary human monocyte-macrophages of chemotactic factors like the CC-chemokines (CCL2) and (CCL4) [Swingler et al., 1999], correlating with the activation of AP-1, NF-κB, STAT1, and STAT3 transcription factors [Biggs et al., 1999; Briggs et al., 2001; Federico et al., 2001; Olivetta et al., 2003; Percario et al., 2003]. It has been reported that in microglia cells, Nef activates the Vav/Rac/p21-activated kinase (PAK) signaling pathway, leading to the increase of superoxide anion production by lowering the activation threshold of NAPDH oxidase to a variety of stimuli, like Ca^{2+}-ionophores or endotoxines [Vilhardt et al., 2002]. We previously reported that Nef regulates the NADPH oxidase activity also in the macrophagic cell line. In particular, we observed that Nef modulates the superoxide release in a bi-phasic manner, an early Nef-induced increase of superoxide release followed by a strong inhibition starting at 10 h from the onset of Nef induction. We also determined that the inhibition of superoxide release is likely to be mediated by Nef-induced soluble factor(s), where IL-10 could be part of such a mechanism [Olivetta et al., 2005].

In this study, we sought to analyze in detail the effects of Nef on the activation of the NADPH oxidase, in terms of the phosphorylation of $p47^{phox}$ and the transduction pathways involved in these processes. We found that Nef-induced superoxide release is independent of the mitogen-activated protein kinases (MAPKs) pathway, but mediated by Nef-dependent activation of the Src family kinases, and that this activity correlates with the phosphorylation of the NADPH oxidase subunit $p47^{phox}$.

MATERIALS AND METHODS

REAGENTS AND ANTIBODIES

Puromycin was from Sigma–Aldrich (Milan, Italy). GM-CSF (Biosource International, CA) was used at the concentration of 100 ng/ml, 4-hydroxytamoxifen (4-HT) (Sigma–Aldrich) was used to activate the Nef-Estrogen receptor (Nef-ER) fusion proteins at the concentration of 50 nM, and DMSO was used as vehicle. Diphenyliodonium (DPI), H89, PD98059, and LY294002 were from Sigma–Aldrich. Src family kinase inhibitor PP2 was purchased from Calbiochem (San Diego, CA). [^{32}P]orthophosphoric acid was from Perkin-Elmer (Boston, MA) and [γ^{32}P]ATP (>3,000 Ci/mmol) was obtained from DuPont NEN (Boston, MA). Antibodies used in Western blotting and immunoprecipitation were as follows: sheep polyclonal anti-Nef anti-serum, was a gift of M. Harris (University of Leeds, Leeds, UK); rabbit polyclonal anti-phospho p44/42 MAP kinase from Cell Signaling Technology, Inc. (Beverly, MA); rabbit polyclonal anti-ERK1/2 from Promega Corporation (Madison, WI); mouse monoclonal anti-actin from Amersham Pharmacia Biotech (Uppsala, Sweden); rabbit polyclonal anti-$p47^{phox}$ and mouse monoclonal anti-$gp91^{phox}$ from Upstate (Lake Placid, NY); mouse monoclonal anti-$p47^{phox}$ from BD Biosciences (San Jose, CA); rabbit polyclonal anti-Hck (N-30), anti-Lyn (44), anti-cSrc (N-16), anti-Fgr (N-47), and goat polyclonal anti-γPAK(C19) from Santa Cruz Biotechnology (Santa Cruz, CA). Immunopure trysacryl immobilized protein A from Pierce (Rockford, IL) and Protein A/G PLUS-agarose were obtained from Santa Cruz Biotechnology.

CELL LINES, PLASMIDS, AND TRANSFECTION

U937 (human leukemic monocyte lymphoma cell line) were maintained with RPMI 1640 medium supplemented by 10% decomplemented fetal calf serum (dFCS), L-glutamine, and antibiotics. dFCS was substituted with 0.2% of bovine serum albumin (BSA) in cell starvation procedures. The pEBB wtNef-ER-IRES-puro vector encoding the Nef-ER fusion protein was provided by Dr. Walk. The Nef-ER is part of a bicistronic gene with an internal ribosomal entry sequence (IRES) followed by the coding sequence for the puromycin resistance gene (Puro) [Walk et al., 2001]. The Nef-ER expression is driven by the elongation factor 1α (EF-1α) promoter [Lamkin et al., 1997]. The pEBB Nef-ER-IRES-puro mutant vector G2A was obtained modifying the Nef wild-type sequence by PCR, using as forward primer: 5′-GGT GGA TCC ACG CGT ATG GCC GCA AAG TGG-3′ (G2A).

Proline-rich region mutant was obtained by amplifying the sequence containing the mutation from pcDNA-3 Nef AxxA with a forward primer: 5′-GGT GGA TCC ACG CGT ATG GGT GGC AAG 3′ (AxxA).

Reverse primer used is the same in each reaction: 5′-CGG CGG CCG CAT CGA TAC TAG TGC AGT TCT TGA AGT ACT CCG GAT G-3′.

The fragments were subcloned into the pEBB vector between Bam HI and Not I sites. The sequences of the mutants were verified by sequencing.

Stably Nef expressing U937 cells were obtained by electroporating 10^7 cells with 20 μg of DNA at 250 V and 960 μFD, using a BioRad gene pulser transfection apparatus (Hercules, CA). Cells were grown for 2 weeks in a medium containing Puromycin (0.8 mg/ml). Selected populations were tested for Nef-ER expression. To induce overexpression of $p47^{phox}$, U937-Nef/$p47^{phox}$ cells were further transfected with a human $p47^{phox}$ expression plasmid, kindly provided by Prof. Enrico Avvedimento (University of Naples, "Federico II," Italy), into the U937-Nef cells. Selected cell populations were kept in media containing both 0.5 μg/ml puromycin and hygromycin.

FLOW CYTOMETRY ANALYSIS

Cells were suspended in Ca^{++}- and Mg^{++}-free phosphate-buffered saline (PBS) supplemented with 0.5% BSA and labeled with phycoerythrin (PE)-conjugated anti-CD4 monoclonal antibodies (BD Biosciences, Erembodegem, Belgium). The staining was performed for 30 min on ice using antibodies at the concentration of 1 μg/10^6 cells. The cell fluorescence was analyzed with the FACSAria (BD Biosciences). Cells incubated with appropriately fluorochrome conjugated isotype control (BD Biosciences) were used to gate non-specific fluorescence signals, and dead cells were excluded by propidium iodide staining (5 μg/ml, Sigma–Aldrich).

SUPEROXIDE RELEASE MEASUREMENT

The rate of $O_2^{\cdot-}$ formation was measured as superoxide dismutase (SOD)-inhibitable reduction of ferrocytochrome *c* [Tarpey and Fridovich, 2001]. Briefly, 10^6 cells were washed in phosphate buffer containing the metal chelator diethylenetriaminepenta acetic acid (DPTA, 0.1 mM, pH 7.4), and re-seeded in the same buffer (1 ml) containing cytochrome *c* (10 μM) in the presence or absence of SOD (10 μg/ml). After incubation at 37°C for 30 min, the reaction was monitored spectrophotometrically with Lambda 14 P UV/Vis (Perkin-Elmer, Norwalk, CT) at 550 nm ($\varepsilon = 21$/mM/cm). All the reagents in this section were from Sigma–Aldrich.

WESTERN BLOTTING ASSAY

For Western blot, cells were lysed in 1% Triton X-100 in the presence of 20 mM Tris–HCl pH 7.5, 150 mM NaCl, 5 mM EDTA, 10 mM sodium orthovanadate, 100 mM sodium fluoride, and 0.5 mM PMSF for 20 min on ice. Whole cell lysates were centrifuged at 16,000*g* for 20 min at 4°C, the supernatants were collected and the protein concentrations were determined by the BioRad protein assay. Samples of 50 μg were separated by 10% SDS–PAGE and transferred by electroblotting to a nitrocellulose membrane (Hybond, Amersham Pharmacia Biotech, Milan, Italy). For the immunoassay, the membrane was blocked in 5% non-fat dry milk in 1× PBS/0.1% Triton X-100, for 1 h at room temperature, and then incubated overnight at 4°C with specific primary antibodies. Immune-complexes were detected through horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse anti-sera (Amersham Pharmacia Biotech), followed by enhanced chemiluminescence reaction (ECL, Amersham Pharmacia Biotech).

IMMUNOPRECIPITATION PROCEDURE AND IN VITRO KINASE ASSAY

To immunoprecipitate the Src family kinase and PAK, the washed and packed cells were solubilized with 1 ml of 25 mM Tris–HCl, pH 7.5, 0.15 M NaCl, 1%, Triton X-100, 0.1% SDS, 1% (v/v) sodium deoxycholate, 0.1 mM Na_3VO_4, 1 mM PMSF, 1 μg/ml leupeptin, 1 μg/ml aprotinin (RIPA buffer) by incubation for 1 h at 0°C. Where necessary, cells were pretreated with PD98059 and LY294002 for 30 min before stimulation. After centrifugation (16,000*g* at 4°C for 10 min), the supernatants were incubated with 25 μl of 50% (w/v) protein A/G plus-agarose beads for 1 h at 4°C. The supernatants were then clarified by centrifugation and incubated overnight at 4°C in a rotating wheel with 1 μg of polyclonal antibodies previously conjugated with trysacryl immobilized protein A. The beads were collected by centrifugation, and washed twice with RIPA buffer, twice with 50 mM Tris, pH 7.5, 150 mM NaCl (TBS), and finally once in the kinase buffer (25 mM Tris–HCl pH 7.5, 10 mM $MnCl_2$, and 0.1 mM Na_3VO_4). The kinase reaction was carried out in 20 μl of kinase buffer containing 1 μCi of [γ^{32}P] ATP (>3,000 Ci/mmol) at room temperature for 10 min. The reaction was stopped by adding 10 μl of 4× loading buffer and the samples were subjected to 10% SDS–PAGE. The gels were dried and exposed to X-ray film for autoradiography. Dried gels were used for direct determination of radioactivity using a Phosphoimager Instrument (Packard Canberra, CO). For Western blot analysis, an aliquot of each sample was collected before the immunoprecipitation and solubilized in 4× loading buffer, boiled for 5 min, and resolved on SDS–PAGE. Proteins were transferred to nitrocellulose paper at 35 V overnight. Blots were washed with TBS, 0.05% Tween-20 (TTBS), and blocked with 3% BSA in TTBS for 2 h. Washed nitrocellulose filters were incubated overnight at 4°C with anti-Hck antibody (1:1000). After extensive washes in TTBS, the immunoreactive bands were detected by chemiluminescence coupled to peroxidase activity, according to the manufacturer's specifications (ECL kit, Pierce).

SUBCELLULAR FRACTIONATION

U937Nef/p47^{phox} expressing cells (5×10^7) were incubated for 1 h with 4-HT or DMSO, collected, and suspended in a relaxation buffer (10 mM Pipes, pH 7.4, 100 mM KCl, 3 mM NaCl, 3.5 mM $MgCl_2$, 1 mM PMSF, 10 μg/ml leupeptin, and 10 μg/ml aprotinin) and then sonicated on ice for 3×15 s. The lysate was ultracentrifuged at 100,000*g* for 30 min at 4°C in a TL100 Ultracentrifuge (Beckman Coulter, Fullerton, CA). The supernatant contains the cytosolic fraction and the recovered pellet includes the plasma membrane proteins. Proteins were finally subjected to 10% SDS–PAGE and Western blotting.

IN VIVO PHOSPHORYLATION OF p47^{PHOX}

U937Nef/p47^{phox} expressing cells were incubated in phosphate-free buffer (20 mM HEPES, pH 7.4, 140 mM NaCl, 5.7 mM KCl, 0.8 mM $MgCl_2$, and 0.025% BSA) containing 0.5 mCi of [^{32}P]orthophosphoric acid/10^8 cells for 3 h. After the first 2 h 4-HT or DMSO was added to the cells. In the experiments with the inhibitors, the cells were incubated with various kinase inhibitors for 30 min before 4-HT treatment. In all cases, the reaction was stopped by adding ice-cold buffer and centrifugation at 400*g* for 7 min at 4°C. The cells were lysed in RIPA buffer. The lysate was centrifuged at 16,000*g* for 30 min at 4°C, and cleared supernatants were used for immunoprecipitation studies as described later.

IMMUNOPRECIPITATION OF p47^{PHOX}

The supernatants were incubated overnight with rabbit anti-serum anti-p47^{phox} at the 1:200 dilution and proteins were immunoprecipitated with 20 μl of resuspended volume of protein A/G, and incubated at 4°C overnight in a rotating wheel. The samples were then washed four times in 1× PBS, denatured in Laemmli sample buffer, and then subjected to 10% SDS–PAGE. The separated proteins were transferred to nitrocellulose, and the membrane was exposed to X-ray film for autoradiography. The same membrane was probed with monoclonal anti-p47^{phox} (1:1000). The intensity of the phosphorylated and total p47^{phox} bands was quantified by densitometry using the ImageJ analysis software version 1.37 from the National Institutes of Health (USA).

STATISTICAL ANALYSIS

Data are presented as mean ± standard deviation (SD). Statistical analysis was performed according to paired Student's *t*-test. *P*-value <0.05 was considered significant.

RESULTS

NEF-INDUCED ACTIVATION OF SUPEROXIDE RELEASE IS DEPENDENT ON BOTH NEF MEMBRANE LOCALIZATION AND SH3-BINDING DOMAIN FUNCTIONALITY

We have recently shown that HIV-1 Nef protein influences the release of superoxide by means of a bimodal, time dependent mechanism, with an early increase of superoxide release followed by a late strong inhibition starting 10 h from the onset of 4-HT treatment [Olivetta et al., 2005]. Here, we examine the molecular mechanisms regulating the Nef-induced NADPH oxidase activation. To identify Nef domains involved in the Nef-induced increase of the superoxide release, we stably expressed two Nef mutants in human U937 cell line: G2A, lacking the myristoylation site determining an impaired Nef membrane localization [Geyer et al., 2001], and AxxA, lacking the interaction with SH3-binding domain of cell protein partners [Saksela et al., 1995].

The inducible activation of Nef by the estrogen analogous 4-HT was verified by the down-regulation of CD4 expression. The flow cytometry analysis (Fig. 1A) indicates that wild-type Nef and AxxA mutant, in the presence of 4-HT, maintained its capacity to down-regulate CD4 while G2A was ineffective. Western blot analysis (Fig. 1B, upper panel) shows the expression of Nef in stably transfected cells, in respect to the untransfected control and (Fig. 1B, lower panel) also established that U937 cells express the Nef-ER fusion proteins at comparable level. We have already demonstrated that superoxide anion production in U937-Nefwt, U937-NefG2A, and U937 parental cells treated with 4-HT for 1 h in the presence or absence of GM-CSF stimulation required membrane localization [Olivetta et al., 2005]. We also examined the effect of the AxxA Nef mutant on ROS production. For this purpose, superoxide release was measured in the presence or absence of GM-CSF stimulation in U937-Nef cell lines, treated with 4-HT or with the 4-HT vehicle (DMSO). Figure 1C clearly shows that both NefAxxA and NefG2A mutants fail to induce an increased superoxide anion production, suggesting that the proline-rich domain of Nef is required to induce NADPH oxidase activity, similar to the Nef membrane localization. Interestingly, the Nef-induced increase of superoxide release is observed in both unstimulated and GM-CSF-stimulated cells, suggesting the possible existence of an alternative pathway independent from that induced by the growth factor.

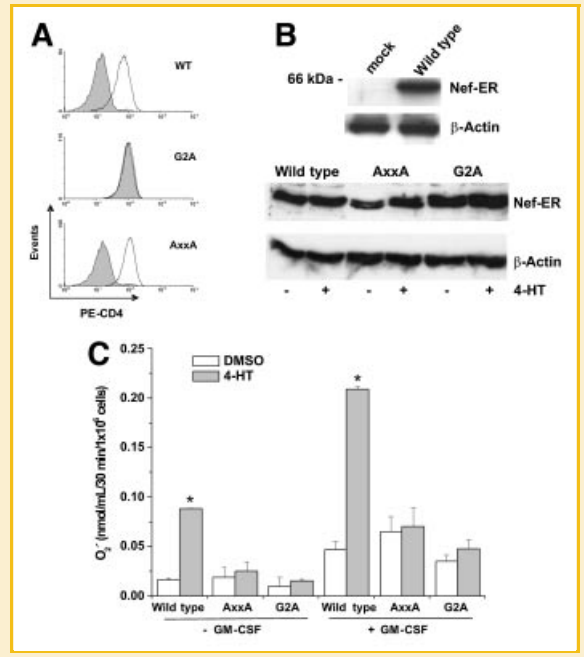

Fig. 1. Nef-induced activation of superoxide release in unstimulated or GM-CSF-stimulated U937 transfected cell lines. A: CD4 expression in U937-Nefwt, U937-NefG2A, and U937-NefAxxA transfected cells was analyzed by flow cytometry using PE-labeled anti-CD4 antibody. Fluorescence intensities in 4-HT-treated cells (solid gray histogram) were compared to DMSO-treated cells (solid line). Matched isotype was used as control of non-specific fluorescence signals and propidium iodide was used to exclude dead cells (not shown). Flow cytometry analysis is representative of at least three independent experiments. B: Western blot analysis of Nef-ER fusion protein expression in stably transfected U937 cell lines. Each lane was loaded with a 50 μg/sample. Mock, U937 parental cells; wild-type, pEBB Nef-ER-IRES-puro U937 transfected cells. The cells were treated with 50 nM of 4-HT for 1 h (upper panel). U937-Nefwt, U937-NefAxxA, and U937-NefG2A transfected cells were treated with 50 nM of 4-HT (+) or DMSO (−) for 1 h (lower panel). To ensure the presence of an equal amount of proteins, the membrane was re-probed with anti-actin antibodies. Western blots are representative of at least three independent experiments. C: U937-Nefwt, U937-NefAxxA, and U937-NefG2A stably transfected cells were cultured overnight in RPMI medium supplemented with 0.2% BSA. Later, 4-HT or DMSO was added to the cultures for 1 h. The cells were then centrifuged and resuspended in phosphate buffer for 30 min, and finally, the superoxide release was determined in the supernatants of unstimulated or GM-CSF-stimulated (100 ng/ml) cells. The NADPH oxidase activity, determined as extracellular superoxide release, was measured by the SOD-inhibitable reduction of ferricytochrome *c*, as described in the "Materials and Methods Section." These results (mean ± SD) are representative of at least five independent experiments performed in duplicate. ($^*P < 0.05$).

PI3K ACTIVITY IS REQUIRED TO ENHANCE THE NEF-STIMULATED SUPEROXIDE RELEASE

PI3K and MAPK pathways have been described as being involved in GM-CSF-induced NADPH oxidase activity [Dang et al., 1999, 2006; Mollapour et al., 2001; Hawkins et al., 2007]. In order to evaluate their role in the Nef-induced superoxide anion release, we treated unstimulated or GM-CSF-stimulated U937-Nefwt cells with PI3K and MEK-specific inhibitors. We noticed that the PI3K inhibitor LY294002 completely inhibits the superoxide release in both Nef and GM-CSF-induced cells (Fig. 2A). On the other hand, MEK-1 inhibitor PD98059 suppressed the GM-CSF-induced superoxide release while it was ineffective on the Nef-induced superoxide release. On treating Nef expressing cells stimulated with GM-CSF, with the PD98059 inhibitor, we observed only a partial reduction of the superoxide release (Fig. 2B).

To clarify the relationship between Nef and the MAPK pathway, we evaluated the ERK1/2 protein phosphorylation levels in unstimulated or GM-CSF-stimulated cells, in the presence of specific inhibitors. The results shown in Figure 2C demonstrate that Nef does not induce ERK1/2 phosphorylation. As expected, in the GM-CSF stimulated cells the MAP kinase pathway was strongly activated as demonstrated by the rapid phosphorylation of the ERK1/2 proteins. This phosphorylation is dramatically repressed by the MEK-1 inhibitor PD98059. In addition, the PI3K-specific

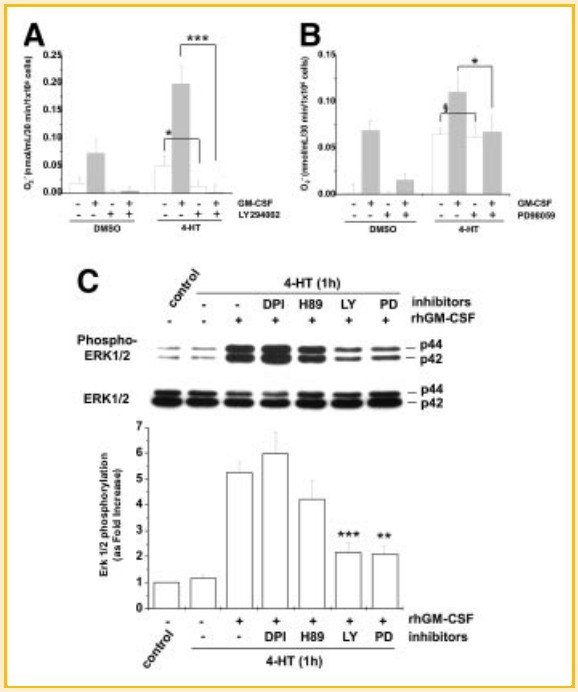

Fig. 2. Effect of Nef on superoxide release is dependent on PI3K activity. Superoxide release was determined in U937-Nefwt cells cultured overnight in RPMI medium supplemented with 0.2% BSA, treated for 1 h with 4-HT or DMSO and stimulated with GM-CSF (100 ng/ml) for 5 min. The inhibitors LY294002 (5 μM) (A) and PD98059 (5 μM) (B) were added to the cultures 30 min before 4-HT treatment. C: Representative immunoblotting, showing the expression of phosphorylated MAP kinases phospho-ERK1/2 in U937-Nefwt cells starved overnight, treated for 1 h with 4-HT or DMSO (control), and finally stimulated with GM-CSF (100 ng/ml) for 5 min. The inhibitors DPI (10 μM), H89 (10 μM), LY294002 (5 μM), and PD98059 (5 μM) were added to the cultures 30 min before 4-HT treatment (upper panel). Expression levels of phospho-ERK1/2 were normalized by re-probing the membrane with anti-ERK 1/2 antibody (lower panel). The histogram shows the mean ± SE values of ERK1/2 phosphorylation levels represented as a percentage of DMSO-treated cells (control). All the results presented in this figure (mean ± SD) are representative of at least three independent experiments. The experiments with the results given in (A) and (B) were performed in duplicate (§ = not significant, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.005$).

inhibitor LY294002 strongly inhibited ERK1/2 phosphorylation at a similar level of that shown by PD98059. On the contrary, neither the flavoproteins inhibitor DPI, nor the protein-kinase A inhibitor H89, each used as negative controls, displayed any effect (Fig. 2C). Together these results suggest that Nef activates superoxide release without affecting the MAPK pathway, probably through an alternative pathway, whereas the inhibitory effect of LY294002 may indicate that the PI3K is a crucial component in the regulation of NADPH oxidase activity.

NEF SELECTIVELY ACTIVATES THE Src FAMILY KINASES, Hck, Lyn, AND c-SRC IN A PxxP-DEPENDENT MANNER

Several reports have revealed that HIV-1 Nef interacts with a subset of the Src family kinases, via the proline-rich (PxxP) domain [Lee et al., 1995; Ye et al., 2004; Trible et al., 2006; Choi and Smithgall, 2004b]. In Figure 1C, we have shown that AxxA Nef mutant failed to induce superoxide anion release. This suggests the possible involvement of the Src family kinases on the Nef-induced NADPH oxidase activity. We thus examined the ability of Nef wild-type to induce Hck, Lyn, c-Src, and Fgr autophosphorylation analyzing the cells stimulated with 4-HT for 1 h. Figure 3A shows 4-HT-activated Nef induced autophosphorylation of Hck, Lyn, and c-Src, but not Fgr. As expected, AxxA Nef mutant failed to induce autophosphorylation activity of all the kinases analyzed.

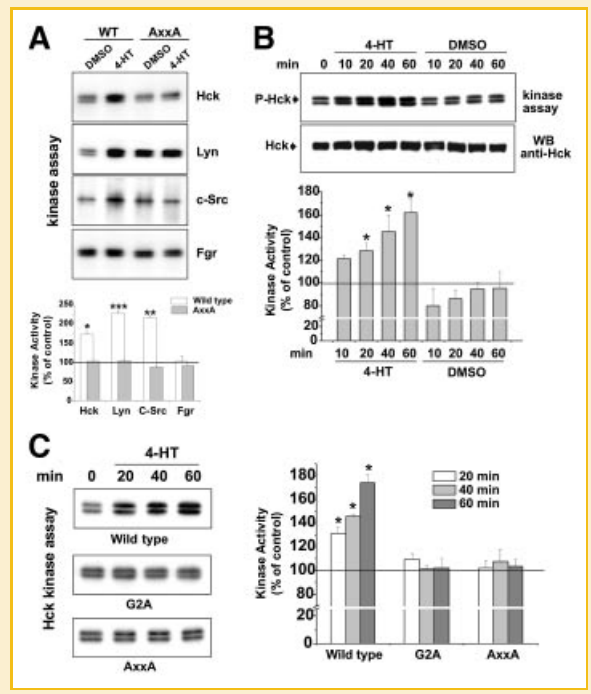

Fig. 3. Activation of the Src family kinases and time course of Nef-induced Hck autophosphorylation. A: In vitro kinase assay on U937-Nefwt and U937-NefAxxA expressing cells. Overnight, 10^7 cells were starved in RPMI medium supplemented with 0.2% BSA and treated with 4-HT or DMSO for 1 h. Hck, Lyn, c-Src, and Fgr were, respectively, immunoprecipitated from cell extracts and phosphorylated in kinase buffer in the presence of 1 μCi of [γ^{32}P] ATP, as described in the Materials and Methods Section. In the histogram is shown the densitometric quantification of autophosphorylation activity, reported as a percentage of the untreated control starved cells (black line). B: In the upper panel, U937-Nefwt cells were starved overnight in RPMI medium supplemented with 0.2% BSA and treated with 4-HT or DMSO for 10, 20, 40, and 60 min. Hck was immunoprecipitated from cell extracts and phosphorylated as shown in (A). Western blot analysis of the samples collected before the immunoprecipitation and probed with anti-Hck antibody is shown in the lower panel. Densitometric quantification of Hck autophosphorylation activity is reported as a percentage of untreated control starved cells (black lane). C: The Western blot shows the Hck autophosphorylation activities measured in cell extracts from U937-Nefwt, U937-NefG2A, and U937-NefAxxA transfected cells cultured as described in (A). The time course includes 20, 40, and 60 min of 4-HT treatment. To ensure the presence of equivalent protein recovery, the membranes of all kinase assay experiments were re-probed with the specific anti-protein kinase antibody (not shown). All densitometric analyses, show in the histogram, indicate the mean ± SE values of Hck autophosphorylation activity reported as a percentage of control (DMSO-treated cells). These results are representative of at least three independent experiments ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.005$).

Since Hck is strongly expressed in cells of the monocyte/macrophage lineage [Ziegler et al., 1987], and its SH3 domain binds Nef with a very high affinity [Briggs et al., 1997], it is possible that this kinase was involved in Nef-mediated effects. In the Figures 3B, we show the Hck autophosphorylation activity in U937-Nefwt treated at different times with HT or with DMSO as control. Nef induced a significant increase in the Hck autophosphorylation level within 10 min that peaks at 60 min from the 4-HT induction and then decreases (not shown). On the other hand, untreated cells or cells treated with DMSO did not significantly alter the levels of basal Hck autophosphorylation. In a similar experiment, we assessed the ability of different Nef mutants in inducing Hck autophosphorylation. As shown in Figure 3C, Nef mutant in the proline-rich domain (AxxA) again failed to induce Hck. Similar results were obtained with Nef mutant lacking the myristoylation site (G2A), suggesting that the membrane localization is also necessary to induce full Hck activation.

4-HT-ACTIVATED NEF INDUCES p47^{PHOX} PHOSPHORYLATION AND MEMBRANE TRANSLOCATION

The phosphorylation of the cytosolic component p47^{phox} is one of the key intracellular events associated with NADPH oxidase activation [El Benna et al., 1994]. We thus examined the effect of Nef on p47^{phox} phosphorylation. In order to increase the endogenous expression of p47^{phox}, we obtained U937 cell lines stably co-expressing both Nef and p47^{phox}. [^{32}P]labeled U937-Nefwt/p47^{phox} and U937-NefAxxA/p47^{phox} cells were incubated with 4-HT or DMSO (control) for 1 h at 37°C. The cells were then lysed and p47^{phox} was immunoprecipitated and analyzed by autoradiography. As shown in Figure 4A, the 4-HT-treatment of U937-Nefwt/p47^{phox} cells increased p47^{phox} phosphorylation levels. Conversely, no increased phosphorylation of p47^{phox} was observed in 4-HT-treated U937-NefAxxA/p47^{phox} cells, strongly suggesting the ability of Nef to interact with SH3-binding domain is critical for p47^{phox} phosphorylation.

p47^{phox} phosphorylation was involved in its translocation to the plasma membrane and its subsequent binding to the flavocytochrome b_{558} to form the functional enzymatic complex. To assess the effect of Nef protein on p47^{phox} membrane translocation, we performed a subcellular fractionation to separate cytosol and membrane fractions in U937-Nef cells overexpressing p47^{phox}. As shown in Figure 4B, the ectopic expression of p47^{phox} increased the steady-state levels of this protein in U937 cells. Notably, a large amount of p47^{phox} was bound to the membrane fraction also in the unstimulated condition. Nevertheless, 4-HT-treated U937-Nefwt/p47^{phox} potentiated the translocation of p47^{phox} while the gp91 expression, considered as a cell membrane marker, remained unmodified. Conversely, as shown by the densitometric analysis, this effect is not detectable in 4-HT-treated U937-NefAxxA/p47^{phox}, suggesting that also the p47^{phox} membrane targeting requires the integrity of Nef proline-rich domain.

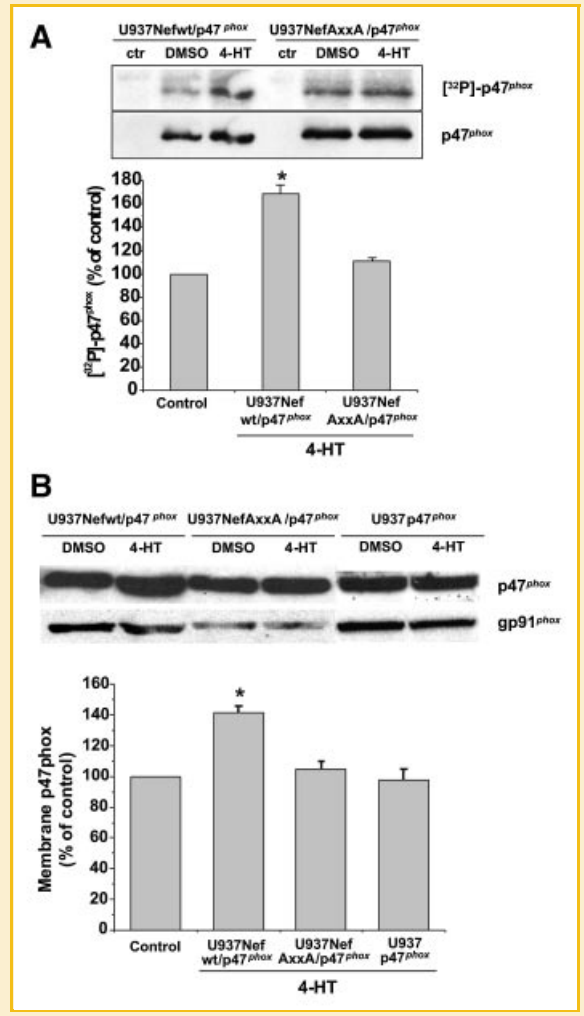

Fig. 4. Effect of 4-HT-activated Nef on p47^{phox} phosphorylation and translocation to the plasma membrane in U937 cells. A: [^{32}P]orthophosphate-labeled U937-Nefwt/p47^{phox} and U937-NefAxxA/p47^{phox} cells were incubated with 4-HT or DMSO at 37°C for 1 h. The p47^{phox} was then immunoprecipitated with rabbit polyclonal anti-p47^{phox} Ab, subjected to SDS-PAGE, blotted on nitrocellulose and detected by autoradiography ([^{32}P]-p47^{phox}, upper panel) or with mouse monoclonal anti-p47^{phox} Ab (p47^{phox}, lower panel) to confirm equal protein precipitation. Aliquots of the cell lysates from [^{32}P]orthophosphate-labeled U937-Nefwt and U937-NefAxxA cells were immunoprecipitated with rabbit serum as non-specific binding control (Ctr). In the histogram, the results are expressed as a percentage of control cells treated with DMSO. The results (mean ± SD) are representative of at least three independent experiments ($^*P < 0.05$). B: Subcellular fractionating analysis. U937-Nefwt/p47^{phox}, U937-NefAxxA/p47^{phox}, and U937-p47^{phox} cell lines were incubated for 18 h in medium containing 0.2% BSA and before harvesting, cells were incubated for 1 h with 4-HT and DMSO. Membrane fractions were analyzed for Western blot using anti-p47^{phox} (upper panel) and anti-gp91 as membrane marker (lower panel). The histogram shows the mean ± SE values of membrane p47^{phox} levels represented as a percentage of DMSO-treated cells and the results are representative of at least three independent experiments ($^*P < 0.05$).

TRANSDUCTIONAL PATHWAYS INVOLVED IN NEF-MEDIATED PHOSPHORYLATION OF p47^{PHOX}

Previous studies have clearly established that Nef binds with high affinity to the SH3 domain of Hck [Lee et al., 1995; Choi and Smithgall, 2004b; Ye et al., 2004; Trible et al., 2006], suggesting the hypothesis that this kinase could represent a key intermediate in Nef-induced superoxide anion release.

To investigate the relationships among Nef, Hck, PI3K, and the MAPK pathway, the Src family kinases inhibitor PP2, MEK-specific

inhibitor PD98059, and PI3K-specific inhibitor LY294002 were added to U937-Nefwt cells 30 min before 4-HT-treatment during the [^{32}P]orthophosphate labeling. Cells treated with DMSO and cells stimulated with GM-CSF represent, respectively, the basal level and the positive control of phosphorylated form of p47^{phox} subunit. As shown in Figure 5A, PP2 significantly reduced the Nef-induced p47^{phox} phosphorylation, whereas the MEK-specific inhibitor PD98059 did not interfere with p47^{phox} phosphorylation, in agreement with the results shown in Figure 2. Interestingly, the PI3K-specific inhibitor LY294002 inhibited the Nef-induced p47^{phox} phosphorylation, although at a lesser extent with respect to PP2 inhibitor, suggesting that this kinase has a role in the NADPH oxidase activation.

To establish the hierarchical action of the Src family kinases and PI3K in the Nef-activated pathway, we evaluated the Hck autophosphorylation activity in 4-HT treated U937-Nefwt in the presence of both PI3K-specific inhibitor LY294002 or, as negative control, the MEK-specific inhibitor PD98059. We observed that LY294002 did not affect the Nef-induced Hck autophosphorylation, strongly suggesting a downstream location of PI3K (Fig. 5B), in agreement with the data reported in the literature [Kim et al., 2006].

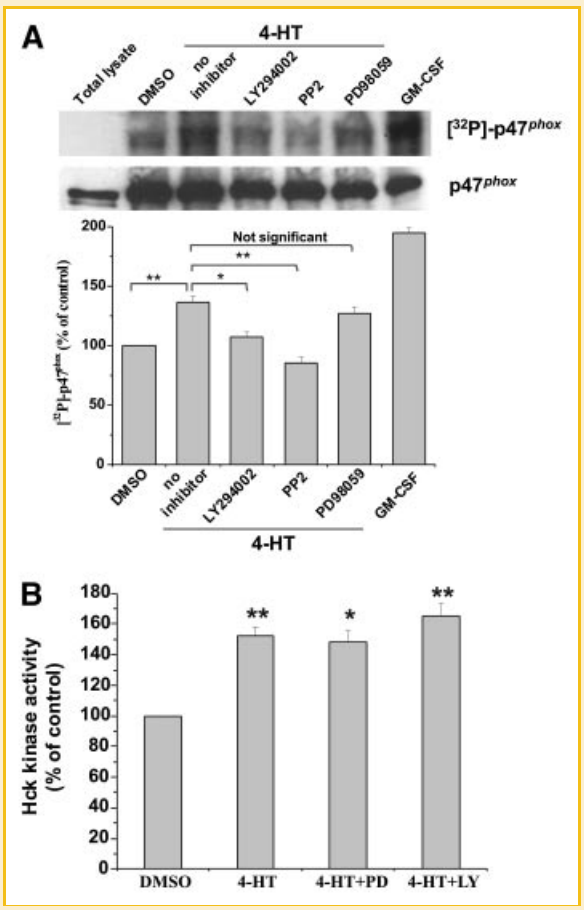

Fig. 5. Signaling pathways involved in p47^{phox} phosphorylation and Hck activation induced by Nef in U937 cell line. A: [^{32}P]orthophosphate-labeled U937-Nefwt/p47^{phox} cells were treated with the inhibitors PP2 (30 μM), LY294002 (5 μM), and PD98059 (5 μM) 30 min before 4-HT treatment. p47^{phox} was then detected by autoradiography ([^{32}P]-p47^{phox}, upper panel), or with anti-p47^{phox} Ab (lower panel). As control of p47^{phox} phosphorylation, a sample was stimulated with GM-CSF 30 min before cell protein extraction (GM-CSF). Total cell lysates from U937-Nefwt/p47^{phox} cells were used as control of p47^{phox} protein electrophoretic mobility (total lysate). The results shown in histogram are expressed as a percentage of the control cells treated with DMSO. The results (mean ± SD) are representative of at least three independent experiments and ($^{*}P < 0.05$; $^{**}P < 0.01$). B: In vitro kinase assay on Hck was performed as shown in Figure 3. The inhibitors LY294002 (5 μM) and PD98059 (5 μM) were added to the U937-Nefwt/p47^{phox} cell cultures 30 min before 4-HT or DMSO treatment. The results (mean ± SD) are representative of at least three independent experiments and ($^{*}P < 0.05$; $^{**}P < 0.01$).

4-HT-ACTIVATED NEF INDUCES PAK ACTIVITY

A clear association between Nef and PAK has been extensively described [Wolf et al., 2001; Linnemann et al., 2002]. Furthermore, Pak-induced phosphorylation of several serine residues in the C-terminus of p47^{phox} has also been described [Martyn et al., 2005]. To investigate the role of PAK in the pathway of Nef-induced NADPH oxidase we first demonstrated that PAK is activated during Nef stimulation. As expected, a robust activation of PAK in Nefwt was observed, whereas the AxxA Nef mutant had no significant effect on PAK activation (Fig. 6A). To determine whether such activation depends on Hck, we performed in vitro kinase assays on Pak immunoprecipitates in the presence of either PD98059 or PP2 kinase inhibitors. We showed that the addition of PP2 significantly reduced the Nef-induced autophosphorylation, indicating that Hck localizes upstream to the signaling cascades including the activation of PI3K and PAK. On the other hand, no effects of PD98059 were detected (Fig. 6B).

Together these results suggest that the Nef-induced p47^{phox} phosphorylation and associated NADPH oxidase activation is mediated by Hck bypassing the MAPK pathway. In such a scheme (Fig. 7A,B), the PI3K has been localized downstream of Hck, and appears essential to the induction of NADPH oxidase activity. As demonstrated in human neutrophils [Martyn et al., 2005], PAK activity is required for efficient superoxide release, and our data show that Nef-mediated Hck activation is also involved in Pak phosphorylation although a direct link between PAK and activation of NADPH oxidase remains to be established.

DISCUSSION

Altered levels of ROS production contribute to the development of HIV-1 pathogenesis. A large amount of ROS induces an inflammatory status that leads to the recruitment and activation of cells of the immune system fostering HIV infection, and has been implicated in neuropathogenesis such as HIV-associated dementia [Shiu et al., 2007]. HIV-1 infection impairs oxidative burst in monocytes/macrophages in response to various stimuli, although contradictory results indicating normal, enhanced, or diminished ROS production have been reported [Muller et al., 1990; Kimura et al., 1993; Flo et al., 1994; Pitrak et al., 1998; Koziel et al., 2000; Tachado et al., 2005]. These divergences are possibly due to the different stages of HIV-1 disease progression, to different methodological procedures used, or both. Our previous data [Olivetta et al., 2005] suggest that

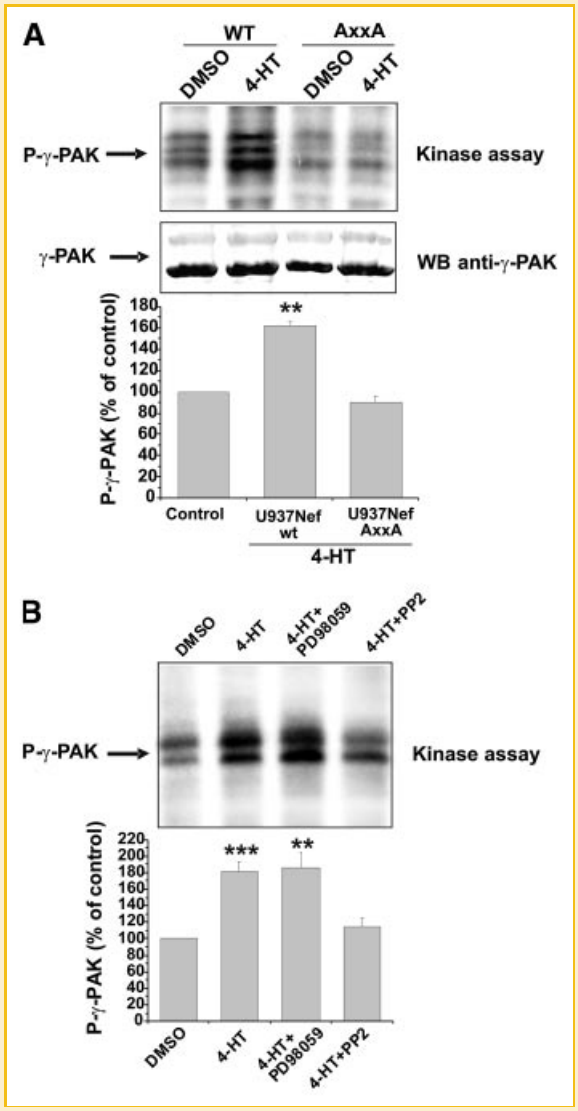

Fig. 6. Autophosphorylation of PAK by Nef is dependent on Hck activation. In vitro kinase assay on U937-Nefwt and U937-NefAxxA expressing cells. 10^7 cells were starved overnight in RPMI medium supplemented with 0.2% BSA and treated with 4-HT or DMSO for 1 h. PAK was immunoprecipitated from cell extracts and phosphorylated in kinase buffer in the presence of 1 μCi of [γ^{32}P] ATP. The reaction was stopped with sample buffer. The samples were then resolved by SDS–PAGE and gel was dried and exposed for autoradiography (A, upper panel). Equivalent recovery of PAK in each immunoprecipitate was verified on an anti-PAK immunoblot (A, lower panel). In the histogram, the results are expressed as a percentage of control cells treated with DMSO. In (B), U937-Nefwt starved cells were incubated in the presence of PP2 (30 μM) and PD98059 (5 μM) 30 min before the 4-HT treatment. PAK was immunoprecipitated from cell extracts and phosphorylated in kinase buffer in the presence of 1 μCi of [γ^{32}P] ATP as previously mentioned. Equivalent recovery of PAK in each immunoprecipitate was verified on an anti-PAK immunoblot (not shown). In the histogram, the results are expressed as a percentage of control cells treated with DMSO (lower panel). All the given results (mean ± SD) are representative of at least three independent experiments ($^{**}P < 0.01$, $^{***}P < 0.005$).

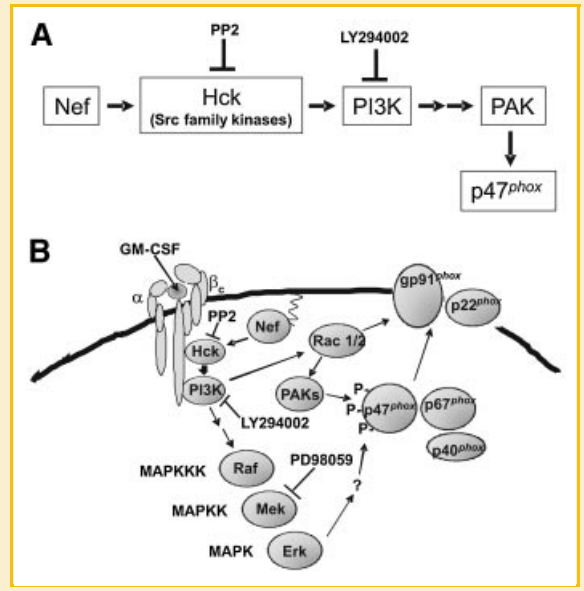

Fig. 7. Schematic models of molecular mechanisms triggered by Nef inducing NADPH oxidase activity. A: Nef may regulate superoxide anion release through the activation of the Src family kinases. Nef-induced NADPH oxidase activation could be mediated by Hck-dependent $p47^{phox}$ phosphorylation. In this pathway, PI3K has a critical role, as demonstrated by experiments performed in the presence of the inhibitor LY294002, capable of preventing $p47^{phox}$ phosphorylation as well as in strongly reducing Nef-induced superoxide anion release. Furthermore, we define the hierarchy existing between Hck and PI3K, localizing the latter downstream to the Hck. In this molecular pathway, we also assign an important role for PAK, demonstrating the dependence of Nef-induced PAK activation by the Src family kinases. The two arrows indicate the likely presence of intermediate(s), as no evidence of direct interaction between PI3K and PAK was demonstrated. B: This model, described as GM-CSF and Nef, share a common upstream signaling pathway, even though Nef seems to work mainly through Hck activation, using multiple kinases including PAK, to ensure a rapid and efficient phosphorylation of $p47^{phox}$. In this model, the MAPK cascade, usually involved with the pro-inflammatory or inflammatory cytokines as GM-CSF, could be bypassed by Nef.

HIV-1 Nef protein regulates the NADPH oxidase ROS production in different ways. In particular, we observed a bi-phasic effect of Nef, represented by a prompt activation of the enzymatic activity of NAPDH oxidase followed, within 10 h, by a strong inhibition. We also demonstrated that the delayed inhibition effect is depended on de novo protein synthesis and is apparently mediated by soluble factor(s). We provide evidence that Nef, through the interaction with Hck, may induce phosphorylation and membrane translocation of $p47^{phox}$ and activation of superoxide anion release bypassing the typical pathway stimulated by the pro-inflammatory cytokine GM-CSF. We observed that MEK inhibitor PD98059 is ineffective on Nef-induced superoxide release, whereas only a partial effect is observed in GM-CSF-stimulated cells. In contrast, a complete inhibition of superoxide release was observed in the presence of PI3K inhibitor LY294002 in stimulated or non-stimulated cells. This revealed the importance of this kinase in mediating or supporting the Nef-induced superoxide release. Unfortunately, the Src family kinase inhibitor PP2 dramatically impairs the superoxide release assay making it difficult to evaluate the role of Hck and other Src kinase

family members in this phenomenon. Nonetheless, the U937-NefAxxA, lacking the proline-rich motif essential for SH3 binding domain, fails to induce superoxide anion release. The interaction between Nef protein and Hck has been widely described in both biochemical and structural studies [Lee et al., 1996]. It involves the SH3 domain of Hck and the highly conserved Nef proline-rich motif leading to constitutive Hck kinase activation [Suh et al., 2005; Trible et al., 2006]. Although Hck seems to be essential in GM-CSF-mediated cell survival/proliferation in macrophages as well as in microglia [Kedzierska et al., 2001; Suh et al., 2005], its role in modulating the NADPH oxidase activity is still unclear. Therefore, Hck is required for M-tropic HIV replication in macrophages [Komuro et al., 2003], as well as for Nef-driven AIDS-like disease in the transgenic mice model [Hanna et al., 2001]. Nef-dependent increase of superoxide anion release has already been described by Vilhardt et al. [2002] in Ra2 microglia mouse astrocytic cell line expressing Nef by means of a lentiviral expression vector. They reported that Nef fails to activate NADPH oxidase by itself, but it is capable of increasing the levels of the activated Rac1 fraction, leading to a type of priming effect of Nef that induces strong NADPH oxidase activity following the stimulation by the Ca^{2+} ionophore ionomycin, fMLP, or endotoxins. In our study, we found increased levels of superoxide anion release in the absence of specific stimuli as well as in the presence of GM-CSF. This discrepancy may be explained by the tight control of Nef activity in our cellular system permitting a more sensitive evaluation of superoxide anion release. Furthermore, the U937 are cells of myeloid lineage derivation committed toward a monocytic differentiation pathway and, based on a combination of proteomics, transcriptomics and principal component analysis (PCA) [Verhoeckx et al., 2004], these cells represent a suitable model system in order to study the macrophages differentiation process. Although the integrity of Nef proline rich domain appears critical in experiments of $p47^{phox}$ phosphorylation and membrane translocation, data enforcing Hck involvement in the induction of superoxide release were obtained in the presence of specific inhibitors. In these experiments, we show that activated Nef induces increase $p47^{phox}$ phosphorylation that may be prevented by adding the Src family kinase inhibitor PP2. It is remarkable that the PI3K-specific inhibitor LY294002 also inhibits the Nef-induced $p47^{phox}$ phosphorylation as well as the superoxide anion release demonstrating the essential role of this kinase in the regulation of NADPH oxidase activity. The specific role of PI3K activity in the NADPH oxidase components assembly and activation is still unclear, but there is emerging evidence of its involvement in $p47^{phox}$ phosphorylation through the protein kinase B (Akt) activation [Hoyal et al., 2003], Vav/Rac/PAK signaling pathway, or both [Martyn et al., 2005]. More recently, the class IA PI3K was described as a nodal point regulating CD11b/CD18-integrin-dependent PMN adhesion and activation of NADPH oxidase leading to oxidant production at PMN adhesion sites contributing to lung inflammation and microvascular injury [Gao et al., 2007]. In addition, there are many findings that imply a direct binding of the PI3K products, PtdIns(3,4)P_2 and PtdIns3P, to the PX (Phox homology) domains of the core oxidase components $p47^{phox}$ and $p40^{phox}$, respectively [Kanai et al., 2001]. Recently, a direct interaction between Nef and the regulatory p85α subunit of class IA PI3K, leading to activation of PAK and increased HIV replication has been reported [Linnemann et al., 2002], while a direct binding between Nef and PAKs is still controversial. Conversely, there is clear evidence that Nef may activate PAK kinases in a Rac/Cdc42-dependent manner [Wolf et al., 2001]. To assemble all these data, Linnemann et al. [2002] proposed an interesting functional representation of Nef as a "signalosome," recruiting different components of cellular signaling pathways including the Src family kinases, exchange factors for the small GPTase of the Rho family, PI3K and PAKs, mainly through its proline-rich motif. Our data reveal the importance of some Src kinase family members, including the protein kinases Hck, and PI3K in mediating the Nef-dependent NADPH oxidase activity. The results obtained in the presence of the MEK inhibitor PD98059 demonstrated that this cascade of molecular signals is independent of the MAP kinase pathway that is usually involved with pro-inflammatory or inflammatory cytokines such as GM-CSF. On the other hand, the strong inhibition of NADPH oxidase activity by the specific PI3K inhibitor LY294002 in either GM-CSF-stimulated and non-stimulated cells suggests the existence of a common upstream signaling pathway shared by Nef and GM-CSF inducing superoxide anion release. In this model Hck, and PI3K seem to be closely associated and, as reported in literature, Hck may be located upstream to the PI3K [Suh et al., 2005]. The molecular model in which Nef, mainly through Hck activation, "uses" this cascade of kinases, including PAK, to ensure an efficient phosphorylation of $p47^{phox}$ leading to a rapid superoxide anion release, could be taken into consideration in future studies (Fig. 7).

In summary, these results together with our previous observations [Olivetta et al., 2005] suggest that HIV-1 Nef protein may control the NADPH oxidase superoxide production through a multi-faceted process. The analysis of the Nef mutants indicates that the integrity of proline-rich domain appears essential to mediate the activation of the NADPH oxidase even though it seems dispensable for the inhibitory activity of Nef. These observations re-enforce the suggestion, already expressed [Olivetta et al., 2005], that the two activities, activation and inhibition of superoxide release, could be uncoupled. This apparently divergent behavior could be functional for the HIV-1 infection by increasing, in the first instance, the levels of ROS production responsible for the inflammatory status. This induces the recruitment and activation of the immune-system cells, creating a favorable condition for virus spread. On the other hand, subsequent suppression or restriction of ROS production is necessary to avoid irreparable cell and tissue damage favoring the development of HIV reservoirs.

Finally, future studies will clarify whether the kinases involved as proximal effectors of Nef signaling in macrophages can be considered as potential targets for anti-HIV drugs discovery.

ACKNOWLEDGMENTS

This work was supported by grants from the AIDS project of the Ministry of Health, Rome, Italy. We are indebted to Gianfranco Macchia of the Department of Cell Biology and Neurosciences, Istituto Superiore di Sanità, Rome, Italy, for his helpful technical support. We also wish to thank Maurizio Di Santolo for his assistance in the linguist revision.

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