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HIV-1 Nef associates with p22-phox, a component of the NADPH oxidase protein complex

Siham Salmen [a], Melisa Colmenares [a], Darrel L. Peterson [b], Elbert Reyes [c], Jose D. Rosales [d], Lisbeth Berrueta [a,*]

[a] Institute of Clinical Immunology, University of Los Andes, Merida, Venezuela
[b] Department of Biochemistry and Molecular Biology, Virginia Commonwealth University, Richmond, VA, USA
[c] Department of Physiology, Medical School, University of Los Andes, Merida, Venezuela
[d] Laboratory of Parasite Enzymology, Faculty of Science, University of Los Andes, Merida, Venezuela

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ABSTRACT

Altered neutrophil function may contribute to the development of AIDS during the course of HIV infection. It has been described that Nef, a regulatory protein from HIV, can modulate superoxide production in other cells, therefore altered superoxide production in neutrophils from HIV infected patients, could be secondary to a direct effect of Nef on components of the NADPH oxidase complex. In this work, we describe that Nef, was capable of increasing superoxide production in human neutrophils. Furthermore, a specific association between Nef and p22-phox, a membrane component of the NADPH oxidase complex, was found. We propose that this association may reflect a capability of Nef to modulate by direct association, the enzymatic complex responsible for one of the most efficient innate defense mechanisms in phagocytes, contributing to the pathogenesis of the disease.

1. Introduction

The NADPH oxidase complex is composed of two flavocytochrome subunits located in the plasma membrane, gp91-phox and p22-phox constituting the catalytic core of the enzyme, and by three cytosolic subunits, named p47-phox, p67-phox and p40-phox. Upon stimulation, the cytosolic factors are activated and translocated to the plasma membrane and bind the flavocytochrome, thereby completing the active form of the enzyme [1]. Whereas the role of p40-phox remains undefined, there is evidence that points to a role for p47-phox as an adaptor protein between flavocytochrome *b*558 and p67-phox [2]. On the other hand, p67-phox represents an essential *activating* cofactor, which includes a domain that regulates the reduction of FAD by NADPH [3,4]. During activation, inhibitory intramolecular SH3 domain interactions in p47-phox are relieved via phosphorylation events [5], allowing the protein to make direct contact with p22-phox, thereby bringing p67-phox to the membrane where it makes direct contact with gp91-phox [6]. Nevertheless, translocation of cytosolic phox proteins to the flavocytochrome *b*558 is not enough to activate the oxidase. The presence of the small cytosolic GTPase Rac (1 or 2) is absolutely required for electron transfer reactions [7], and it has been proposed that Rac regulates electron transfer reactions of the assembled oxidase [8] via direct interactions with p67-phox and gp91-phox.

During HIV infection an impaired function of neutrophils has been demonstrated, which compromise the ability to kill bacterial and fungal pathogens predisposing patients to certain opportunistic infections [9]. These abnormalities include decreased number of neutrophils [10], accelerated neutrophil death [11], secondary to an altered oxidative stress [12], impairment of chemotaxis and phagocytosis [13]. These dysfunctional activities have been demonstrated in neutrophils from both symptomatic and asymptomatic HIV infected patients [14,15]; hence an increased basal superoxide production has been described in HIV infected patients at early stages of the disease [16].

Altered ROS production by HIV-1 infection may contribute to the development of AIDS pathogenesis by inducing an inflammatory status which may lead to recruitment and activation of immune cells [17] for optimal virus spread. Since neutrophils have not been proved to be reservoirs for HIV, they may become targets of the virus by direct contact between viral proteins and elements of the neutrophil cell membrane [18].

Nef is a 27–34 kDa myristoylated protein produced exclusively by HIV-1/2 and simian immunodeficiency virus, playing a pivotal

* Corresponding author. Address: Institute of Clinical Immunology, University of Los Andes, Avenida 16 de Septiembre, Edificio Louis Pasteur, Anexo IAHULA, P.O. BOX 566, Mérida 5101, Venezuela. Fax: +58 2742403188.
E-mail address: lberruet@ula.ve (L. Berrueta).

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role in AIDS pathogenesis. It is produced shortly after virus infection, and it is expressed throughout all stages of the disease [19]. While Nef is known to enhance virus production and infectivity, it has been recently recognized that Nef also exerts pathogenic effects independently of viral replication [20,21]. Despite its small size this protein presents a relatively large surface area for protein interaction. Binding of Nef to a multitude of host proteins including plasma membrane receptors, sorting receptors and signaling proteins has been documented [22,23].

Because it has been shown that Nef can regulate superoxide production in macrophages and microglias [24,25], we hypothesized that the altered superoxide production observed in neutrophils from HIV infected patients, could be secondary to an interaction between Nef and components of the NADPH oxidase complex. In this work, we show that Nef, was capable of increasing superoxide production in human neutrophils. Furthermore, Nef associated with the p22-phox component of the NADPH oxidase complex, from neutrophil cell lysates and to purified p22-phox. We propose that the dysfunctional activity observed in neutrophils from HIV infected patients may be explained in part by an interaction between viral proteins (such as Nef) and elements of the NADPH oxidase enzymatic complex.

2. Materials and methods

2.1. Media and reagents

HRP-conjugated anti-goat (2020), mouse anti-GST (B-14), goat anti-HIV-Nef (vA-19), rabbit anti-human-p22-phox (FL-195), goat anti-human-p40-phox (N-20), goat anti-human-p67-phox (C-19), goat anti-human-p47-phox (C-20), rabbit anti-human-Rap1/Krev (sc-65), rabbit anti-human-Rac1 (C-14), mouse anti-his (H-3) and rabbit anti-human-Rac2 (C11), were all purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Mouse anti-human-gp91-phox was kindly provided by Dr. Paul G Heyworth from Schering-Plough Biopharma, Palo Alto, CA. Dihydrorhodamine-123 (DHR-123) was purchased from Molecular Probes, (Eugene, OR). HRP-conjugated anti-mouse and anti-rabbit antibodies were purchased from Pierce, Rockford, IL. Diphenyleneiodonium (DPI) was purchased from Calbiochem, La Jolla, CA. pGEX-Nef (HIV-1 NL) expression plasmid, was kindly provided by Dr. Matija Peterlin from Rosalind Russell Medical Research Center, Department of Medicine, Microbiology and Immunology, University of California at San Francisco. pGEX-4T1-p22-phox, was kindly provided by Dr. Katrin Rittinger, from the Division of Molecular Structure, National Institute for Medical Research, London.

2.2. Human blood samples and isolation of PMNs

Peripheral blood samples were obtained from 10 healthy subjects. The experimental protocol was approved by the Ethics Committee of The University of Los Andes, and written informed consent was obtained from all the subjects. PMNs were obtained as previously described [12].

2.3. Fusion protein expression and purification of GST-Nef and GST-p22-phox

The production and purification of GST, GST-Nef and pGEX-4T1-p22-phox fusion proteins in *Escherichia coli* was performed following the manufacturer's protocol (Amersham Pharmacia Biotech). The *E. coli* strain BL21 was transformed with pGEX-Nef and pGEX-4T1-p22-phox was used to transform *E. coli* BL21-CodonPlus-RIPL. Protein expression was induced from cultured positive cells in the presence of 0.1 mM isopropyl-1-thio-β-galactopylanoside. Proteins were purified by adsorption to glutathione–sepharose beads (Amersham Biosciences) from bacteria cell lysates. Protein purity was confirmed by SDS–PAGE and Western blot. Purified proteins were kept frozen at −80 °C until use.

2.4. Cloning and expression of Nef-His

The Nef-His fragment was obtained by PCR from HIV delta R 8.2 pPTK, which contains HIV-1 genes. The following primers were used: Forward [5′-CCATGGgtggcaagtggtcaaaaaagaa-3′] engineered to contain the NcoI restriction site while retaining the start codon, and a reverse primer [5′-AAGCTTtcagcagtctttgaagtactccgg-3′], that was designed to contain a stop codon and the HindIII restriction site. The PCR product was cloned into the pET21d, which contained a C-terminal hexahistidine tag, was referred as pET21d-Nef. Expression of Nef-His Domain was performed by transforming *E. coli* BL21-CodonPlus-RIPL, and for protein purification a sepharose Ni/nitrilotriacetic acid (NTA) column (BioRad), was used. The presence of Nef-His was monitored by Western blot. The recombinant purified protein was submitted to three consecutive Triton X-114 extraction steps and extensively dialyzed, to eliminate any trace of endotoxin in the final product, as previously described [26].

2.5. Superoxide production

Flow cytometry analysis of neutrophil respiratory burst activity was measured using a modification of a previously published method [27]. Briefly, freshly isolated neutrophils (1×10^6 cells/ml) were preloaded with DHR-123 (1 μmol/L) at 37 °C for 15 min. Afterwards, cells were incubated with each of the following stimulus: Nef-His, GST-Nef, His or GST protein (between 0.01 and 1 μg/ml final concentration) [28,29], during 60 min at 37 °C. For some experiments cells were pre-treated with DPI (5 μM) during 15 min. Cells were analyzed on a FACScan flow cytometer (Becton Dickinson, San Jose, CA). A total of 10,000 events were collected from each sample.

2.6. GST pull down and immunoprecipitation assays

Once purified, neutrophils at a concentration of 2×10^7 cells, were lysed on ice for 15 min in 1 ml of buffer A (50 mM Tris–HCl, pH 8, 1% Triton X-100, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1 μg/ml leupeptin/aprotinin, 1 mM sodium orthovanadate) and centrifuged at 14,000*g* for 10 min to yield cell lysates. For GST pull down experiments, GST alone or GST-Nef fusion proteins coupled to glutathione–sepharose beads were added to cell lysates and incubated for 2 h at 4 °C. Cell lysates and precipitated proteins were separated by SDS–PAGE and transferred to membranes (PVDF; Millipore) and probed with different antibodies. Precipitated proteins were detected by enhanced chemiluminescense (ECL) (SuperSignal, Pierce, Rockford, IL).

Pull down experiments using GST-p22-phox were performed as follow: GST alone, or GST-p22-phox proteins coupled to glutathione–sepharose beads, were incubated for 2 h at 4 °C, with 1 ml of buffer A, to which 20 μg of Nef-His were added. The beads were then washed five times in buffer A, solubilized in SDS sample buffer, and analyzed by SDS–PAGE and Western blot.

For immunoprecipitation, protein G-sepharose beads (Pierce) were precoated with anti-His monoclonal antibody or anti-p22-phox polyclonal antibody (1 μg/ml). Antibody coupled beads were incubated for 2 h at 4 °C, with the neutrophil cell lysate, to which Nef-His was previously added (20 μg/ml) and, finally washed five times with buffer A. Proteins were solubilized in SDS sample buffer separated by SDS–PAGE, transferred to membranes and probed with each of the antibodies.