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Nef enhances c-Cbl phosphorylation in HIV-infected CD4$^+$ T lymphocytes

Polung Yang, Andrew J. Henderson*

Integrated Bioscience Graduate Program in Immunobiology, Department of Veterinary Science, Immunology Research Laboratories, 115 Henning Building, Pennsylvania State University, University Park, PA 16802, USA

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Abstract

The multifunctional HIV-1 protein Nef possesses several motifs that interact with signaling molecules in infected T cells. In order to determine whether Nef influences T cell activation, cells were infected with Nef-positive and Nef-negative clones of HIV. CD28 expression and changes in tyrosine phosphorylation were monitored. We observed no Nef-dependent changes in CD28 expression or function. However, infection with Nef-positive virus led to changes in tyrosine phosphorylation. This Nef-induced phosphorylation was observed in unstimulated cells, and c-Cbl was identified as one of the proteins whose phosphorylation was upregulated by Nef. Furthermore, Lck is required for Nef-mediated c-Cbl tyrosine phosphorylation. These results suggest that Nef modifies T cell signaling in the absence of T cell receptor engagement and co-stimulation.

Keywords: HIV-1; Nef; Cbl; T cell signal transduction

Introduction

HIV-1 Nef is a 27- to 34-kDa myristoylated protein that has been demonstrated to be critical for viral pathogenesis in part by promoting aberrant T cell function (Fackler and Baur, 2002; Renkema and Saksela, 2000). Nef is expressed throughout the HIV replication cycle and packaged in mature virions (Kotov et al., 1999; Pandori et al., 1996; Wu and Marsh, 2001). In infected CD4$^+$ T lymphocytes, expression of Nef downregulates CD4 and MHC class I molecules by targeting endocytosis and post-translation sorting, respectively (Aiken et al., 1994; Blagoveshchenskaya et al., 2002; Chen et al., 1996; Garcia and Miller, 1991; Greenberg et al., 1998; Schwartz et al., 1996). Reduced MHC I expression allows evasion of CTL surveillance, while decreased CD4 levels enhance virion release by reducing gp120–CD4 interactions during budding (Cohen et al., 1999; Collins et al., 1998). Nef has also been shown to influence apoptosis, protecting infected CD4$^+$ T cells against apoptosis while elevating CD95L expression to induce apoptosis of bystander cells (Geleziunas et al., 2001; Greenway et al., 2002; Wolf et al., 2001; Xu et al., 1999).

Several structural motifs and functional properties of Nef suggest it has a role in modulating signal transduction (Renkema and Saksela, 2000). Myristoylation at the N terminus anchors Nef to the inner leaflet of the plasma membrane, where it associates with lipid rafts and physically interacts with CD3-ζ of the TCR complex (Wang et al., 2000; Xu et al., 1999). The ability of Nef to modify TCR signaling is suggested by recent studies in which overexpression of Nef induced a transcriptional response in CD4$^+$ T cells that was similar to CD3 signaling (Simmons et al., 2001). Moreover, Nef possesses a proline-rich motif, which mediates interac-

Abbreviations: TCR, T cell receptor; HIV-1, human immunodeficiency virus type 1; LTR, long terminal repeat; CHO, Chinese hamster ovary; PBS, phosphate-buffered saline; BSA, bovine serum albumin; PI3K, phosphatidylinositol 3′-kinase; PP1, 4-amino-5-[4-methylphenyl]-7-[t-butyl]pyrazolo[3,4-d]pyrimidine; FBS, fetal bovine serum; FACS, fluorescence-activated cell sorting; MFI, mean fluorescence intensity; PLAP, placental alkaline phosphatase; CSF-1, colony-stimulating factor-1; PDGFR, platelet-derived growth factor receptor; EGFR, epidermal growth factor receptor; NFAT, nuclear factor of activated T cells; AP-1, activator protein 1; SH2, Src-homology 2; SH3, Src homology 3; NF-κB, nuclear factor κB; FITC, fluorescein isothiocyanate; GEF, guanine nucleotide exchange factor; PMA, phorbol myristate acetate.

* Corresponding author.
E-mail address: ajh6@psu.edu (A.J. Henderson).

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tions with SH3-possessing proteins such as PI3K, the guanine nucleotide-exchange factor Vav, and the Src family kinase Lck (Baur et al., 1997; Collette et al., 1996; Fackler et al., 1999; Saksela et al., 1995). The Nef–Vav complex recruits p21-activated kinases (PAK1/2) through the Arg–Arg (RR) motif of Nef and small G proteins Rac/cdc42 (Renkema et al., 2001), inducing cellular responses ranging from cytoskeletal reorganization to respiratory burst (Fackler et al., 1999, 2000; Vilhardt et al., 2002). Taken together, Nef targets multiple biochemical events that directly impact HIV replication and the survival and function of infected host cells.

Cbl family proteins regulate antigen-dependent activation of thymocytes and circulating T lymphocytes (Lupher et al., 1999; Thien and Langdon, 2001). The proto-oncogene *cbl* encodes a 906-amino acid, multi-domain protein, c-Cbl, whose primary function is to regulate tyrosine kinases (Donovan et al., 1994; Loreto et al., 2002; Ota and Samelson, 1997). In addition to c-Cbl, there are two mammalian homologues: Cbl-b, which is 982 amino acids and mainly expressed in peripheral T lymphocytes, and the truncated Cbl-3, which is 474 amino acids (Keane et al., 1995; Thien and Langdon, 2001). The three proteins share tyrosine kinase-binding (TKB), linker, and RING finger domains. The TKB domain consists of a four-helix bundle (4H), a Ca^{2+}-binding EF hand (EF), and Src homology region 2 (SH2) that is connected to a RING finger by the linker domain (Thien and Langdon, 2001). The SH2 domain has been demonstrated to bind to receptor tyrosine kinases, including CSF-1 receptor, EGFR, and PDGFR (Lee et al., 1999; Levkowitz et al., 1998; Miyake et al., 1999), as well as non-receptor tyrosine kinases such as Syk and ZAP-70, which associates with CD3-ζ of the TCR complex (Fournel et al., 1996; Lupher et al., 1996; Ota and Samelson, 1997; Rao et al., 2001). The RING finger E3 ubiquitin ligase domain recruits ubiquitin-conjugating enzymes (E2s) that catalyzes multiple ubiquitination of Cbl-interacting proteins, targeting them for proteosome degradation and thus negatively regulating T cell signaling (Levkowitz et al., 1999; Naramura et al., 2002; Panigada et al., 2002). C-Cbl and Cbl-b also interact with SH3-possessing Src family kinases Lck and Fyn, as well as adaptor proteins Nck and Grb2 through C-terminal proline-rich motifs (Andoniou et al., 2000; Donovan et al., 1996; Feshchenko et al., 1998; Ojaniemi et al., 1997). Src kinase-dependent tyrosine phosphorylation of Cbl proteins facilitates the recruitment of additional factors such as Vav and PI3K, which are required for T cell activation (Lupher et al., 1999; Marengere et al., 1997; Miura-Shimura et al., 2003). As HIV-1 Nef has also been shown to physically and functionally interact with Vav and PI3K, it is possible that direct or indirect interactions between Nef and Cbl proteins impact T cell activation and influence HIV replication.

In order to understand the effects of Nef on $CD4^+$ T cell signaling, we examined tyrosine phosphorylation patterns in HIV infected Jurkat T cells. We report that Nef enhances tyrosine phosphorylation of c-Cbl. This upregulation of c-Cbl tyrosine phosphorylation is induced by endogenous expression of Nef in HIV-1-infected cells and requires the Src tyrosine kinase Lck.

Results

HXB2 Nef does not adversely affect CD28 expression and function

Nef interacts with integral components of the TCR/CD28 signaling network in $CD4^+$ T cells, including Fyn, Lck, Vav, and PI3K. It has been previously reported that Nef down-regulates surface expression of CD28, thus altering CD28 signaling and T cell activation (Swigut et al., 2001). Since we were interested in determining if T cell signaling pathways, including those emanating from CD28, are targeted by Nef in the context of viral infection, we assessed CD28 surface expression and function in the absence and presence of HIV infection. $CD4^+$ Jurkat T cells were infected with HXBnPLAP-IRES-N+ (HIV-Nef+) (Chen et al., 1996) and examined for changes in CD28 expression. This molecular clone has a PLAP-encoding cDNA inserted in the restored HXB2 *nef* open reading frame (ORF). HXB2 Nef expression is driven by an internal ribosomal entry site from the encephalomyocarditis virus (EMCV) inserted 3′ to the PLAP ORF (Chen et al., 1996). PLAP, a surface protein not found in T cells, serves as a positive marker for HIV infection as determined by flow cytometry (Fig. 1A).

To confirm that physiological levels of Nef were being expressed in HXBnPLAP virus-infected cells, we compared Nef expression in HIV-Nef+-infected cells with Jurkat T cells infected with NL4-3 and PMA-stimulated ACH-2 T cells, which harbor inducible provirus (Clouse et al., 1989). As shown in Fig. 1B, comparable levels of Nef protein were observed in the HIV-Nef+ and NL4-3 HIV-infected cells as well as the activated ACH-2 cell line, suggesting that the levels of Nef protein expressed by the HXBnPLAP clone are representative of those found in cells actively replicating HIV.

The ability of Nef to down-regulate surface CD28 and alter its functions was examined. We used flow cytometry to monitor PLAP and CD28 expression on the surface of HIV-infected cells. HIV-infected ($PLAP^+$) cells expressed CD28 at levels that were comparable to mock-infected Jurkat T cells or uninfected Jurkat T cells exposed to virus ($PLAP^-$ cells) (Fig. 1C). To determine if CD28 signaling was affected by Nef, we co-transfected Jurkat T cells with HXB2 Nef and HIV-LTR luciferase reporter to determine if Nef influenced CD28 signaling. As reported previously, CD28-dependent induction of HIV transcription required cross-linking of CD3 and CD28, whereas cross-linking CD28 was not sufficient to induce LTR activity and CD3 alone resulted in only a modest induction of LTR activity (Fig. 1C; Cook et al., 2003). The synergistic activation of the HIV LTR by co-stimulation with