Plasma Membrane-Targeted Raf Kinase Activates NF-κB and Human Immunodeficiency Virus Type 1 Replication in T Lymphocytes

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Increasing evidence points to a role of the mitogenic Ras/Raf/MEK/ERK signaling cascade in regulation of human immunodeficiency virus type 1 (HIV-1) gene expression. Stimulation of elements of this pathway leads to transactivation of the HIV-1 promoter. In particular, the NF-κB motif in the HIV long terminal repeat (LTR) represents a Raf-responsive element in fibroblasts. Regulation of the Raf kinase in T cells differs from findings with a variety of cell lines that the catalytic domain of Raf (RafΔ26–303) shows no activity. In this study, we restored the activity of the kinase in T cells by fusing its catalytic domain to the CAAX motif (-Cx) of Ras, thus targeting the enzyme to the plasma membrane. Constitutive activity of Raf was demonstrated by phosphorylation of mitogen-activated protein kinase kinase (MEK) and endogenous mitogen-activated protein kinase 1/2 (ERK1/2) in A3.01 T cells transfected with RafΔ26–303-Cx. Membrane-targeted Raf also stimulates NF-κB, as judged by κB-dependent reporter assays and enhanced NF-κB p65 binding on band shift analysis. Moreover, we found that active Raf transactivates the HIVNL4-3 LTR in A3.01 T lymphocytes and that dominant negative Raf (C4) blocked 12-O-tetradecanoylphorbol-13-acetate induced transactivation. When cotransfected with infectious HIVNL4-3 DNA, membrane-targeted Raf induces viral replication up to 10-fold over basal levels, as determined by the release of newly synthesized p24ag protein. Our study clearly demonstrates that the activity of the catalytic domain of Raf in A3.01 T cells is dependent on its cellular localization. The functional consequences of active Raf in T lymphocytes include not only NF-κB activation and transactivation of the HIVNL4-3 LTR but also synthesis and release of HIV particles.

Transcriptional control of human immunodeficiency virus type 1 (HIV-1) in T lymphocytes involves a complex interaction between cellular and viral regulatory proteins and their target sequences within the long terminal repeat (LTR) (15). Enhancement of HIV-1 replication can be induced by external stimuli that activate T lymphocytes, such as cytokines, or by T-cell receptor engagement, indicating that these factors can trigger cellular signaling pathways leading to viral gene expression (11). We and others have identified cellular proteins belonging to the NF-κB family of transcription factors and other NF-κB-binding proteins as important stimulating factors (3–5, 13, 15, 16, 28). Specifically, the NF-κB-binding motif in the HIV LTR is a Raf-responsive element (8, 12). Moreover, in monocytes, HIV infection activates mitogen-activated protein kinase kinase (MEK), a downstream target of Raf-1, and this activation participates in NF-κB stimulation (14). Taken together, these data suggest a direct link between the Raf/MEK/ERK intracellular signaling pathway and HIV-1 transcriptional activation.

The serine/threonine kinase Raf is a member of the mitogen-activated protein kinase pathway. This cascade transmits and amplifies signals generated by a variety of stimuli, including growth factors and phorbol esters (6, 9, 34). In lymphoid cells, Raf-1 kinase is activated upon T-cell receptor engagement, interleukin treatment, CD4 cross-linking, or binding of HIV-1 gp120 to CD4 surface receptors (25, 30, 33, 35, 36).

Activation of Raf-1 kinase is a complex multistep regulated process involving changes in phosphorylation events, subcellular localization, and protein interactions (27). Receptor tyrosine kinase signaling through Ras leads to Raf-1 activation, which in turn phosphorylates and stimulates the dual-specificity kinase MEK, which transmits the signal to mitogen-activated protein kinase (ERK). The latter has been shown to phosphorylate and to activate several proteins, including other protein kinases, transcription factors, and cytoskeletal proteins (9, 29).

The Raf protein can be subdivided into two functional domains: the kinase domain, located in the C terminus (residues 330 to 627), and a negative regulatory domain, located in approximately the first third of the protein (residues 51 to 149). Deletion of the N-terminal domain leads to a constitutively active kinase in a variety of cell lines such as fibroblasts and human embryonic kidney cells (7, 20, 22, 39); however, in T lymphocytes, such truncated versions of Raf do not exhibit catalytic activity (43). The reasons for this apparent down-regulation of Raf activity in T cells are not clear.

The N-terminal region is further distinguished by containing the elements necessary for Ras binding (44). The interaction of this region with GTP-bound p21Ras at the plasma membrane is thought to be necessary for Raf kinase activity within a cellular environment (40). This is supported by experiments where Raf was targeted to the plasma membrane by adding the farnesylation signal of p21K-Ras to the C-terminal region (37). This modified form of Raf is recruited to the plasma membrane independently of Ras and is thereby locally activated (23). Thus, this type of recruiting functions to bring Raf into close contact with its relevant physiological activators and/or substrates.
In this study, we overcome the regulation of expressed N-terminally truncated Raf in T cells by membrane targeting with the farnesyl signal of K-Ras. We used this construct to investigate the consequences of Raf/MEK/ERK signaling on NF-κB activation and stimulation of HIV-1 replication in a CD4+ T-lymphoblast cell line. In this report, we provide evidence that constitutively active Raf not only is involved in HIV-1 transactivation but also triggers κB-dependent gene expression and HIV-1 replication in T cells.

MATERIALS AND METHODS

DNA constructs and cloning. Raf22-303 and epitope-tagged (HA)-Raf22-303 have been described previously (41, 43) and carry an in-frame deletion of amino acids 22 to 303 (7, 17, 21). The construct Raf22-303-Cx (containing the C-terminal, membrane-targeting 17 amino acids of K-Ras fused to the kinase domain of Raf) was created by fusing the C-terminal part of Raf22-303-Cx to hemagglutinin (HA)-tagged Raf22-303, by using the BglII/Abl restriction sites. Raf22-303-KD and Raf22-303-KD-Cx have a K-to-W substitution at position 375 in the ATP-binding site of Raf, which abolishes kinase activity (κase-dead mutants [KD]). HA-tagged Raf-C4 is a dominant negative carboxy-terminal deletion mutant of Raf-1 and contains the Ras-binding domain (7). All cDNAs are subcloned into the multiple-cloning site of pRSPla (10). The 3×κB-Tk luciferase plasmid contains three tandem copies of the κB motif cloned upstream of a minimal thymidine kinase promoter reporter gene and was obtained from T. Wirth, University of Wuerzburg, Wuerzburg Germany. Two different NL4-3 cloned infectious HIV DNA constructs and cloning. The HIV-LTR-wt plasmid was described previously (13) and subcloned into a luciferase vector. The HIV-LTR-xplasmid contains point mutations (GGG to GCT) and (GGG to CTC) in both NF-κB sites of the HIV LTR. 

Antibodies. Monoclonal anti-p24κB antibody was obtained from the National Institutes of Health AIDS Research and Reference Reagent Program. ERK1, ERK2, and phospho-ERK specific antibodies were purchased from Santa Cruz Biotechnology, Inc. Anti-p53 rabbit antiserum was provided by Nanette Rice, Frederick, Md. Monoclonal antibodies against HA-tag (12CA5) were produced and purified by standard methods.

Cell culture, DNA transfection, and reporter gene assays. A3.01 T cells were maintained in RPMI 1640 (Gibco BRL) supplemented with 10% heat-inactivated fetal calf serum, 2 mM l-glutamine, streptomycin, and penicillin. The cells were cultured routinely to a density of 0.5 × 10^6 to 1.0 × 10^6 cells per ml. Briefly, cells (3 × 10^5 to 6 × 10^5 per six-well dish) were transfected with 2.0 to 0.2 μg of pRSPla expressing Raf kinases by a modified transfection procedure from GIBCO. For these assays, T cells were transfected with combinations of 0.4 μg of reporter construct, 0.25 to 1.5 μg of HIV LTR, 0.5 to 1.5 μg of pRSPla expressing Raf kinases, and 0.1 to 0.05 μg of pRSPla-HIV-tat expression vector as indicated in the figure legends. The cells were incubated for 5 h in an incubator at 37°C in 5% CO2 in the presence of the DMRIE-C reagent (GIBCO-BRL) nucleic complexes, and 1.5 ml of growth medium was added. For luciferase assays, total-cell extracts were prepared 24 to 48 h later. Briefly, cells from each well were harvested in 100 μl of lysis buffer (50 mM sodium morpholinopropane-sulfonate [pH 7.8], 50 mM Tris-HCl [pH 7.8], 10 mM dithiothreitol, 0.2% Triton X-100). The crude cell lysates were cleared by centrifugation, 50 μl of precleared cell extract was added to 50 μl of luciferase assay buffer (125 mM sodium MES [pH 7.8], 25 mM magnesium acetate, 20 μM of ATP per ml), and the activity was measured after injection of 50 μl of 1 mM NAD-luciferin (Appli-Chem) in a Berthold Lumat luminometer. The total protein concentration was measured by the Bradford technique (Bio-Rad). Results are presented as luciferase units normalized to protein concentration and mock transfection with empty expression vectors. Each experiment was done in triplicate and is representative of at least three different sets of experiments.

Immunocomplex kinase assay and immunoblotting. For immunocomplex kinase assays, the membranes were incubated in blocking buffer (nonfat dry milk)-Tris-buffered saline and Tween 20 (TBST) and washed in TBST as described previously (13). As a secondary antibody protein A-peroxidase (Amersham) was used. This step was followed by the standard enhanced chemiluminescence reaction.

RESULTS

Plasma membrane-targeted Raf22-303 in T cells phosphorylates MEK in vitro. To detect the effects of Raf activation in A3.01 T cells, we first established a system to measure Raf kinase activity by using transient transfection with epitope-tagged (HA) versions of the catalytic domain of Raf (Raf22-303). In this experiment, we also measured the consequences of membrane targeting of Raf22-303 by fusing the C-terminal CAAX domain of K-Ras to this protein (Raf22-303-CX). These C-terminal amino acids of Ras are sufficient to target a heterologous cytoplasmic protein to the plasma membrane (23, 37). Figure 1 shows that Raf22-303-CX expression in T lymphocytes is catalytically active toward its substrate MEK in vitro kinase assays. In contrast, Raf22-303 without the membrane-targeting signal has no detectable activity in the same cell system. Stimulation of transfected cells with phorbol ester induces enhanced kinase activity of both versions of Raf22-303-ATP-binding-site mutants (375W) of Raf22-303, either Raf22-303-KD or Raf22-303-KD-CX, showed no kinase activity when used as a negative control (Fig. 1). These results indicate that only membrane-targeted Raf22-303-CX represents a constitutively active kinase in the A3.01 T-lymphocyte environment. We next investigated the functional consequences of this activity with regard to the mitogenic signaling cascade, NF-κB activity, and HIV-1 replication.

Raf22-303-CX leads to phosphorylation of endogenous ERK in T cells. Western blot analysis of whole lysates of transfected A3.01 cells with a phospho-ERK-specific antibody demonstrates that 12-O-tetradecanoylphorbol-13-acetate (TPA) stimulation of these cells induces the phosphorylation of proteins with molecular sizes of 44 and 42 kDa (Fig. 2A, right side). By using Western blot analysis, these proteins were identified as ERK1 and ERK2, respectively (Fig. 2B, right side). Raf22-303-CX, either membrane-targeted or not, caused a significant phosphorylation of endogenous ERKs by phorbol esters, in accordance with previously published data (39). Similar to our results on MEK phosphorylation, only Raf22-303-CX is able to induce endogenous ERK1/2 activation in the absence of TPA stimulation (Fig. 2A, left side). Neither Raf22-303-KD-CX, Raf22-303 nor

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Raf26–303-KD showed any catalytic activity. These findings demonstrate that activation of MEK by transfection of membrane-targeted Raf26–303 is transmitted to endogenous ERK.

Expression of constitutively active, membrane-targeted Raf26–303 stimulates NF-κB activity in T cells. Previously, we and others have shown that active Raf transactivates κB-containing promoter elements in NIH 3T3 cells (8, 12). Since only membrane-targeted Raf26–303 is constitutively active in T cells, we investigated whether this catalytic activity is sufficient to trigger further downstream signaling effects, including NF-κB activation. Therefore, we used a 3×κB-luciferase reporter construct in transactivation assays. Figure 3A shows that stimulation with TPA resulted in an approximately 20-fold increase in luciferase gene expression compared to unstimulated A3.01 T cells. Transfection of Raf26–303-Cx alone, but not its kinase-dead mutant (Raf26–303-KD-Cx) or wild-type Raf26–303, transactivates a κB-dependent promoter ninefold over that shown for control transfected T cells. Moreover, expression of dominant-negative Raf-C4 reduced TPA-stimulated NF-κB activation by more than half. To further strengthen these results, we prepared nuclear extracts from untransfected or transfected A3.01 T cells and performed electrophoretic mobility shift assays with an NF-κB oligonucleotide as a probe. TPA stimulation for 30 min resulted in an increased binding of NF-κBp65 compared to that for untreated, mock, or unstimulated Raf26–303-KD-Cx- or Raf26–303-transfected cells (Fig. 3B). In the absence of TPA treatment, enhanced p65 binding to the NF-κB probe was apparent only in Raf26–303-Cx-transfected cells (Fig. 3B). The specificity of p65 binding was demonstrated by using excess unlabeled NF-κB probe as a competitor or by using p65-specific antibodies in supershift experiments (Fig. 3B). Taken together, these data demonstrate that Raf26–303-Cx is constitutively active and induces downstream signaling events in T cells, including ERK phosphorylation and transactivation of specific promoter elements.

Consequences of Raf activation for HIVNL4-3 promoter transactivation and viral replication. We next investigated how constitutively active Raf kinase acts on induction of the HIV-1 promoter in A3.01 T cells. To mimic the situation in HIVNL4-3-infected T lymphocytes, we used an amplified region of the HIVNL4-3 LTR spanning from nucleotides −150 to +70 including the TAT-responsive region and the NF-κB element (Fig. 4B) (19). Figure 4A shows that stimulation with phorbol ester resulted in a sevenfold stimulation of HIVNL4-3 LTR-driven gene expression compared to that in unstimulated T cells. Similar to the experiments investigating κB-dependent promoter activity, only Raf26–303-Cx was able to induce the transcriptional activity of the HIV-1 promoter (Fig. 4A). Transfections with Raf26–303-KD-Cx or Raf26–303 showed no detectable HIVNL4-3 LTR transactivation, and expression of dominant negative Raf-C4 (Fig. 4A) or dominant negative ERK (data not shown) impaired phorbol ester-stimulated HIV LTR activity. Interestingly, point mutations in both NF-κB sites of the HIV LTR reduced both the TPA and Raf26–303-Cx HIV LTR-driven gene expression (Fig. 4C). These data indicate that constitutively active Raf26–303-Cx stimulates HIV-1 promoter activity in A3.01 T cells and that functional NF-κB sites in the HIV LTR are important for this effect.

After establishing the effect of constitutively active Raf kinase on the transcriptional activity of the HIVNL4-3 LTR, we used two different molecular clones of HIVNL4-3 to study viral replication in the same cell environment. After transfection, viral production of either clone was demonstrated by intracytoplasmic staining with anti p24 monoclonal antibodies, detection of p24 particles released into the supernatant, and syncytium formation. Titer determinations revealed that both HIVNL4-3 DNA con-
centrations. In the absence of phorbol esters, only expression of Raf\(_{22–303}\)-Cx enhanced HIV-1 replication in cotransfected cells. This effect was both time and dose dependent, with an optimal p24 antigen production response up to 10-fold over basal levels (Fig. 5). At later time points, the effect of membrane-targeted Raf was less significant, mostly probably due to secondary infections (data not shown). At 64 h posttransfection, these results were confirmed by fluorescence-activated cell sorter flow cytometric experiments measuring intracytoplasmic p24\(^{\text{core}}\) expression (Fig. 6). These data clearly show that activation of the mitogenic pathway stimulates HIV-1 replication in infected CD4\(^+\) T cells.

**DISCUSSION**

In this study, we investigated the functional consequences of Raf activation in T cells with regard to the classical mitogenic signaling cascade, stimulation of NF-κB activation, and HIV-1 replication. Truncated versions of Raf-1 that lack the N-terminal regulatory domain (Raf\(_{22-303}\)) are inactive when expressed in Jurkat T cells (43). Additional signals like phorbol ester or anti-CD3 stimulation appear to be necessary for Raf kinase activation in this cell type. We overcame this cell-specific regulation of Raf-1 kinase activity in T cells differing from other cell types. The mechanism for this phenomenon remains to be determined. We propose that translocation of cytoplasmic Raf at the plasma membrane appears to specifically trigger the MEK/ERK pathway, since neither the SAPK/JNK nor the p38 activating pathway was affected (data not shown). These observations support the notion that membrane targeting of Raf resembles the physiological situation in which Raf activation is achieved mainly at the plasma membrane. As discussed above, regulation of Raf-1 kinase activity in T cells differs from other cell types. The mechanism for this phenomenon remains to be determined. We propose that translocation of cytoplasmic Raf at the plasma membrane dissociates the kinase from cytoplasmic (down)regulating proteins, which are connected to downstream effectors like the nuclear shuttle protein ERK.

We and others have reported that Raf-1 stimulates NF-κB-dependent reporter gene expression in fibroblasts (8, 12). Furthermore, in situ immunofluorescence studies with NIH 3T3 cells have shown that the expression of active v-raf leads to elevated nuclear levels of the p65 subunit of NF-κB (24). In the present study, we demonstrate, using NF-κB-dependent reporter gene analysis and electrophoretic mobility shift assays, that NF-κB activity is induced in CD4\(^+\) T cells only by membrane-targeted Raf\(_{22–303}\), taking place under conditions where JNK/SAPK activity is unaffected. We observed that the binding of p65 in Raf\(_{22–303}\)-transfected cells is consider-
ably lower than that observed in TPA-stimulated cells. Whether this observation is due to the low transfection efficiency in T cells (approximately 20% [data not shown]) or whether active Raf is less efficient than TPA in stimulating NF-κB activity is not clear. Nevertheless, our data clearly show that targeting Raf-1 to the plasma membrane is sufficient to stimulate NF-κB promoter activity in T cells.

NF-κB activation is known to play a major role in HIV-1 transactivation as well as in replication (2, 16). Stimulation of NF-κB occurs through a variety of stimuli, which include signals through cytokine and growth factor receptors (3). Some of these cytokines, for example, interleukin-2 and tumor necrosis factor alpha, upregulate transcription of the HIV-1 provirus (11) and stimulate the mitogenic signaling cascade through Raf. Thus, activation of the Ras/Raf/MEK/ERK pathway may further contribute to the first round of transcription of HIV-1 provirus. To test this hypothesis, we investigated whether stimulation through Raf alone was sufficient to promote HIV transactivation and replication.

We demonstrate that membrane-targeted Raf<sub>D<sub>22–303</sub>-Cx is constitutively active with respect to HIV-1 promoter transactivation in T cells. Furthermore, as a specific activator of the mitogenic signaling cascade, Raf<sub>D<sub>26–303</sub>-Cx also enhanced viral replication. The HIV NL4-3 molecular clone used in the experiments is well characterized and established as a model for the study of HIV replication in T cells (1). We used A3.01 CD4<sup>+</sup> T cells for two reasons. First, a derivative line, ACH-2, which represents an established cell line carrying a latent HIV-1 provirus, which can be released upon phorbol ester or TNF stimulation, is available (31, 32). Second, these cells produce mature viral particles after transfection of HIV-1 DNA or infection. HIV-1 DNA titer determination and time course experiments were performed to define the baseline of p24<sub>gag</sub> release in our cell system. By using large amounts of HIV-1 DNA and/or long time points, HIV<sub>NL4-3</sub> alone leads to the release of p24<sub>gag</sub> due to viral replication and secondary infections. By using phorbol ester as a known trigger of HIV-1 transactivation, we found that Raf<sub>D<sub>22–303</sub>-Cx is sufficient to activate HIV<sub>NL4-3</sub> LTR-dependent transcription. (A) A3.01 cells were cotransfected with 0.4 μg of HIV<sub>NL4-3</sub> LTR luciferase construct together with either Raf<sub>D<sub>22–303</sub>-Cx</sub>, Raf<sub>D<sub>26–303</sub>-Cx</sub>, or empty expression vector as a control (mock). At 24 h posttransfection, the cells were harvested and luciferase assays were performed as described above. A3.01 cells, stimulated with TPA (10 ng/ml) for 16 h or left untreated, were used as a positive control. (B) Sequence of the HIV<sub>NL4-3</sub> LTR spanning the region from nucleotides −150 to +70 of the HIV<sub>NL4-3</sub> promoter. NF-κB, SP1 binding sites, and the Tat-responsive region (TAR) are indicated. (C) Point mutations in both NF-κB sites of the HIV LTR impaired Raf<sub>D<sub>26–303</sub>-Cx</sub> as well as phorbol ester-stimulated HIV LTR transactivation. A3.01 cells were cotransfected with Raf<sub>D<sub>26–303</sub>-Cx</sub> expression vectors together with either 0.4 μg of wild-type (wt) or κB-mutant (κBmt) HIV LTR luciferase constructs.
replication in T cells, viral release was significantly increased in a dose- and/or time-dependent fashion compared to basal p24
levels, an effect which was detectable as soon as 24 h posttransfection. The laboratory strain HIVNL4-3 also replicates much more effectively in A3.01 T cells overexpressing the membrane-targeted version of RafD
26–303. This observation indicates that induction of the mitogenic signaling pathway not only transactivates the HIV LTR but also enhances viral replication. We correlate this with ERK phosphorylation and NF-kB transactivation as indicators of Raf-1-induced signaling processes. Interestingly, a recent report has shown that reverse transcriptase activity is significantly greater in HIV-infected Jurkat T cells which have been stably transfected with RafD
22–303 (33). Since RafD
22–303 is silent with respect to ERK phosphorylation and induction of NF-kB activity in our cell environment, these data would suggest that Raf-induced increases in HIV replication are regulated in at least two ways depending on the cellular localization of the catalytic domain of the kinase. This is supported by previous data demonstrating that RafD
22–303 targets GA-binding protein, an ets-like transcription factor, which binds the NF-kB element to transactivate the HIV-1 promotor in transfected NIH 3T3 cells (13).

In conclusion, our data demonstrate that the classical mitogenic signaling cascade plays an important role in NF-kB induction and HIV replication in T cells. Our findings also underline the importance of cellular localization for Raf kinase activity. Since CD4+ T cells are the predominant location of viral replication, studying T-cell-specific regulation of Raf kinase is crucial to define connections between signal transduction elements and HIV propagation.

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