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Rac1 and Toll-IL-1 Receptor Domain-Containing Adapter Protein Mediate Toll-Like Receptor 4 Induction of HIV-Long Terminal Repeat

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Opportunistic infections, common in HIV-1-infected patients, increase HIV replication; however, the intracellular signaling mechanisms involved are not clearly known. We have shown that Toll-like receptor 2 (TLR2), TLR4, and TLR9 mediate microbial Ag-induced HIV-long terminal repeat (HIV-LTR) trans-activation and HIV-1 replication, and that LPS-induced HIV-LTR trans-activation is mediated through myeloid differentiation adapter protein. Recently, Toll-IL-1R domain-containing adapter protein (TIRAP) has been identified as an adapter molecule that mediates responses to TLR2 and TLR4 ligands, and TIRAP was suggested to provide signaling specificity for different TLRs. Rac1, a small GTP-binding protein that is activated upon LPS stimulation of macrophages, activates phosphatidylinositol 3-kinase and Akt and leads to NF-κB activation. The roles of Rac1 and TIRAP in LPS activation of HIV replication is not known. In the present study we show that LPS stimulation of human microvesSEL endothelial cells leads to Rac1 activation. Constitutively active Rac1 (Rac1V12) simulated the effect of LPS to activate HIV-LTR, whereas the expression of dominant negative Rac1 (Rac1N17) partially blocked LPS-induced HIV-LTR trans-activation. Rac1V12-induced HIV-LTR activation was independent of myeloid differentiation adapter protein, and dominant negative TIRAP blocked Rac1V12-induced HIV-LTR trans-activation. In this study we show for the first time that activation of Rac1 leads to HIV-LTR trans-activation, and this is mediated through TIRAP. Together these results underscore the importance of Rac1 and TIRAP in TLR4 activation of HIV replication and help delineate the signaling pathways induced by TLRs to mediate microbial Ag-induced HIV replication and HIV pathogenesis. The Journal of Immunology, 2004, 172: 7642–7646.

Human immunodeficiency virus-1-infected patients are frequently coinfected with multiple opportunistic and pathogenic microorganisms that lead to increased HIV-1 replication (1, 2). The presence of an opportunistic infection is an independent predictor of HIV disease progression (3); however, the molecular mechanisms involved in this process are not clearly known.

HIV-1 replication is regulated through HIV-long terminal repeat (HIV-LTR), and although HIV-LTR alone can serve as its own promoter, early mRNA transcription relies primarily on binding of cellular transcription factors, including NF-κB, to the LTR (4–7). The predominant complex binding to the LTR-κB sites in activated cells is NF-κB p50-p65 heterodimer. In unstimulated cells, NF-κB is restricted to the cytoplasm through its interaction with inhibitor proteins of the IκB family. Activation of NF-κB occurs through phosphorylation and proteolysis of the IκB inhibitor, with subsequent translocation of active NF-κB into the nucleus, where it can bind to its cognate binding sites (8, 9). Regulation of NF-κB activation is also dependent on the inducible phosphorylation and trans-activation activity of p65 (10).

We have recently shown that Gram-negative bacterial LPS induces HIV-LTR trans-activation via innate immune system receptor, Toll-like receptor 4 (TLR4) and IL-1R-associated signaling molecules (i.e., myeloid differentiation protein (MyD88), IL-1R-activated kinase (IRAK), TNF receptor-associated factor-6 (TRAF6), and NF-κB-inducing kinase, which lead to IκBα phosphorylation and NF-κB activation (11).

Rac1 has recently been shown to induce NF-κB activation through both IκBα phosphorylation (12) and phosphatidylinositol 3-kinase-regulated p65 trans-activation (13). We have previously shown that chemical inhibition of phosphatidylinositol 3-kinase partially blocked the LPS-induced HIV-LTR trans-activation (11). Arbibe and colleagues (14) have shown that Rac1 played role in TLR2-mediated NF-κB activation. Currently, the role of Rac1 in LPS/TLR4-induced HIV-LTR trans-activation is unknown.

In the present study we demonstrate that LPS stimulation of human dermal microvascular endothelial cells activates Rac1, and that coexpression of dominant negative (DN) Rac1 partially blocks the LPS-induced HIV-LTR trans-activation. Furthermore, expression of a constitutively active Rac1 construct induces HIV-LTR trans-activation in HMEC. We also show that constitutively active
activated GTP-Rac1 then associates and activates PAK-1 (26). GST fusion proteins corresponding to the p21-binding domain of human PAK-1 (PBD; residues 67–150) were used to precipitate the activated Rac-GTP from cell lysates using a Rac1 activation assay kit (Upstate Biotechnology) according to the manufacturer’s instructions. Briefly, confluent HMEC were stimulated with 50 ng/ml LPS and incubated at 37°C for various periods of time. Cells were then harvested into lysis buffer (20 mM HEPEs (pH 7.4), 0.5% Nonidet P-40, 10 mM MgCl₂, 10 mM β-glycerophosphate, 10% glycerol, 10 μg/ml leupeptin, and 10 μg/ml aprotinin) at the various time points by scraping. The same amount of total protein from clarified lysates was incubated with GST-PBD to precipitate GTP-bound Rac1 according to the manufacturer’s instructions (Upstate Biotechnology). Precipitated GTP-bound Rac1 was resolved on a 4–20% SDS-PAGE and immunoblotted using a Rac1 mAb (Upstate Biotechnology). Six percent of the cell lysate was also resolved in a 4–20% SDS-PAGE and immunoblotted to measure the total amount of Rac1. The nonspecific ~31-kDa band seen in the Western blot was reported by Upstate Biotechnology to be an uncharacterized protein cross-reacting with the Ab.

Statistics

In transfection experiments data shown are the mean ± SD of three or more experiments and are reported as a percentage of LPS-stimulated HIV-LTR promoter activity. Student’s t test was used to assess statistical significance.

Results

LPS stimulation of HMEC activates Rac1

Rac1 has also been shown to mediate Gram-positive Staphylococcus aureus (TLR2 ligand) activation of NF-κB in THP-1 human monocytic cell lines and TLR2-transfected 293 cells (14). We have previously shown that in the HMEC system, stimulation of TLR4 with LPS leads to HIV-LTR trans-activation and viral replication (11). To assess whether LPS stimulation of HMEC activates Rac1, confluent cells were stimulated with LPS (50 ng/ml) for different periods of time (0, 5, 15, and 30 min), and Rac1 activation was assessed by immunoprecipitating GTP-bound Rac1 using GST-PBD and Western blotting for Rac1. LPS-induced a rapid and transient Rac1 activation, starting at 5 min after stimulation and peaking at 15 min (Fig. 1). As Rac1 plays a key role in cell movement and phagocytosis, the transient nature of LPS-induced Rac1 activation may be an innate mechanism of the cell to direct its movement to the target pathogen. These results confirm that LPS stimulation of HMEC leads to Rac1 activation.

Rac1 mediates LPS-induced HIV-LTR trans-activation

Next, we assessed the role of Rac1 in LPS-induced HIV-LTR trans-activation by cotransfecting HMEC with HIV-LTR-luciferase and β-galactosidase reporter vectors and either with dominant negative Rac1 (Rac1N17) or empty vector constructs at various concentrations before LPS stimulation. Cells were lysed after 5 h of stimulation, and luciferase activity was measured. The results

![FIGURE 1](http://www.jimmunol.org/...512)
were normalized for transfection efficiency using a colorimetric galactosidase assay as described previously (18). We observed that expression of Rac1N17 inhibited LPS-induced HIV-LTR trans-activation in a dose-dependent manner; and the reduction at highest concentration of Rac1N17 (1 µg) was ~50% (Fig. 2). At this concentration, Rac1N17 construct did not induce cell death, as assessed by lactate dehydrogenase release (data not shown). These data suggest that Rac1 is involved in LPS-induced HIV-LTR trans-activation.

**Constitutively active Rac1 induces HIV-LTR trans-activation**

To further define the involvement of Rac1 in LPS-induced HIV-LTR trans-activation, we assessed whether the expression of a constitutively active form of Rac1 (Rac1V12) could mimic the effect of LPS treatment and lead to enhanced HIV-LTR trans-activation. As shown in Fig. 3, infection of HMEC with adenovirus vector expressing Rac1V12 for 20 h led to a dose-dependent increase in HIV-LTR luciferase activity compared with adenov-GFP control. Rac1V12 at MOI 200 and 400 led to 50 and 100% increases in HIV-LTR activation, respectively, above that induced by LPS (50 ng/ml for 5 h). Importantly, Rac1V12-induced HIV-LTR trans-activation was achieved in the absence of LPS stimulation, and infection of HMEC with adenov-GFP did not increase luciferase activity (data not shown). These observations together with those observed by the DN-Rac1 experiments suggest that Rac1 participates in the signaling pathways involved in the induction of HIV-LTR trans-activation.

**TIRAP, but not MyD88, mediates Rac1 activation of HIV-LTR**

LPS stimulation of TLR4 has been known to induce both MyD88-dependent and MyD88-independent signaling cascades to activate NF-kB (29), and TIRAP has recently been suggested to control activation of MyD88-independent signaling pathways downstream of TLR4 (16). However, this was recently disputed by Yamamoto et al. (15), who suggested that TIRAP had a crucial role in the MyD88-dependent signaling pathway shared by TLR2 and TLR4. Rac1 has recently been shown to associate with the IL-1R complex via interactions with both MyD88 and the IL-1R accessory protein, and a pathway emanating from MyD88 and involving IRAK1, TRAF-6, and Rac1 was shown to be involved in the trans-activation of gene expression by the p65 subunit of NF-kB in response to IL-1 (30). To examine the roles of MyD88 and TIRAP in Rac1-induced HIV-LTR trans-activation we cotransfected HMEC with HIV-LTR-luciferase, Rac1V12, and β-galactosidase cDNA and empty vector pcDNA3, DN-TIRAP, or DN-MyD88 constructs at various concentrations using FuGene6 overnight. The cells were then stimulated with LPS (50 ng/ml) or medium for 5 h, and HIV-LTR activation was assessed by luciferase assay. As expected, both DN-TIRAP (Fig. 4A) and DN-MyD88 (Fig. 4B) blocked LPS-induced HIV-LTR trans-activation; however, only the expression of DN-TIRAP blocked the constitutively active Rac1 (Rac1V12)-induced HIV-LTR trans-activation (Fig. 4). Similar to the findings of Jefferies et al. (30), we observed that DN-MyD88 did not block Rac1V12-induced HIV-LTR, which suggests that Rac1 may be downstream of MyD88. We propose that both MyD88 and TIRAP play roles in HIV-LTR trans-activation, and Rac1-induced HIV-LTR trans-activation is TIRAP dependent.

**Discussion**

HIV infection is characterized by progressive immune dysfunction, leading to AIDS and opportunistic infections by a wide variety of microorganisms, which is an independent risk factor for death in the HIV-infected population (31). The innate immune activation-driven HIV replication may in part explain the aggressive course of HIV infection in individuals coinfected with opportunistic organisms, such as those in sub-Saharan Africa, where coinfections are common and often continuous (32, 33). Currently the molecular mechanisms involved in enhanced HIV replication during opportunistic infections are not well understood. Therefore, delineating the molecular mechanisms that regulate activation of latent HIV is of great clinical significance to develop novel modalities to control HIV replication during opportunistic infections.

We have shown that LPS interaction with TLR4 induces a signaling cascade, including MyD88, IRAK1, and TRAF6, which
leads to NF-κB activation and HIV-LTR trans-activation (11), and that costimulation of TLR4 with either TLR2 or TLR9 induces HIV replication in an additive manner (34). In this study we show that in addition to the IL-1R signaling cascade, Rac1 and TIRAP play roles in HIV-LTR trans-activation.

The Rho family of small GTPases, Rac1, is known to regulate critical cellular functions, such as cell growth, apoptosis, cytoskeleton organization, and development (35, 36). Rac1 is also implicated in different aspects of host defense against bacteria, including leukocyte chemotaxis, pathogen phagocytosis, production of oxygen radicals, and activation of multiple stress response cascades (35-41). Currently, the information on the role of Rac1 in HIV replication is limited to the potentiation effect of Rac1 on HIV negative factor-induced HIV replication and HIV disease progression in infected cells (42). In addition, CD28-dependent activation of HIV-1 transcription has recently been shown to require GTPase activity of Rac1 in T lymphocytes (43).

Rac1 has recently been shown to be a part of the IL-1R complex and TLR2 (14) and associates with MyD88, IRAK1, and TRAF6 to mediate p65 and NF-κB trans-activation (30). Currently there are no data on TLR4 activation of Rac1 and its role in HIV replication. Our data suggest that LPS stimulation of TLR4 induces Rac1, which may, in turn, lead to HIV-LTR trans-activation.

TLR signaling is initiated by the TIR domain, which recruits cellular adapter proteins that contain TIR domains. Four such adapter proteins have been discovered to date, and have been named MyD88, Mal (MyD88 adapter-like; also known as TIRAP (TIR domain-containing adapter protein)), TIR domain-containing adapter inducing IFN-β (44), and TIR domain-containing adapter inducing IFN-β-related adaptor molecule (45). The adapter proteins are suggested to confer specificity to TLR signaling; however, the molecules involved in TIRAP signaling have yet to be identified. Rac1 has been shown to associate with Myd88 (30). Our data suggest that Rac1 is also involved in TIRAP signaling; however, immunoprecipitation experiments did not show association of Rac1 with TIRAP (data not shown). This may also be due to unstable nature of Rac1-TIRAP association upon activation of cells. Another possibility is that TIRAP acts downstream of Rac1 to induce HIV-LTR trans-activation.

Our results emphasize the role of Rho GTPase Rac1 and TIRAP in HIV-LTR trans-activation. These results suggest that novel therapeutic agents targeting Rac1 or Rac1-activated signaling molecules may potentially be developed to control HIV-1 replication.

References


