Activation of the HIV-1 Long Terminal Repeat by Cytokines and Environmental Stress Requires an Active CSBP/p38 MAP Kinase*

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Sanjay Kumar‡§, Michael J. Orsini‡§, John C. Lee‖, Peter C. McDonnell‡, Christine Debouck¶, and Peter R. Young‡**

From the Departments of ‡Molecular Immunology, §Molecular Genetics, and ‖Cellular Biochemistry, SmithKline Beecham Pharmaceuticals, King of Prussia, Pennsylvania 19406

The human immunodeficiency virus, type 1 (HIV-1) promoter is known to be activated by proinflammatory cytokines and UV light. These stimuli also activate various members of the mitogen-activated protein kinase family, including JNK/SAPK and CSBP/p38. In HeLa cells containing an integrated HIV-1 long terminal repeat (LTR)-driven reporter, we now show that the specific p38 inhibitor, SB203580, inhibits activation of the HIV-1 LTR by interleukin-1, tumor necrosis factor, UV light, and osmotic stress. Inhibition was 70–90% in all but the case of tumor necrosis factor stimulation, where inhibition was 50%. Each of these stimulated p38, which was inhibited by SB203580 in vitro and in vivo with an IC50 (between 0.1 and 1 μM) similar to that required to inhibit transcription. In contrast, SB203580 had no effect on JNK, which was also activated by these stimuli. The NFκB sites in the HIV-1 LTR were required for a response to cytokines but not to UV, and SB203580 remained capable of inhibiting UV activation in the absence of the NFκB sites. Studies in which SB203580 was added at different times relative to UV stimulation suggested that the critical p38-mediated phosphorylation event occurred between 2 and 4 h after UV treatment. These data indicate that p38 is required for HIV-1 LTR activation but that the action of p38 is delayed, presumably due to substrate unavailability or inaccessibility.

Infection by the human immunodeficiency virus, type 1 (HIV-1) often results in a period of viral latency after the virus integrates into the host cell chromosome that is characterized by low levels of virus production (1, 2). Activation of viral gene expression can occur in response to a variety of stimuli including mitogens, cytokines, and environmental stresses such as UV light, heat shock, and oxygen radicals (3–8). The exact mechanism by which these stimuli activate HIV gene expression is not completely understood.

Translation from viral RNA to protein is not completely understood. Protein synthesis by infected cells can be regulated by various stimuli that activate or inhibit protein synthesis (9–13). Translation is regulated by the activity of several protein synthesis initiation factors, the activation of which is regulated by a variety of cellular and environmental stresses (14, 15). The integration of the HIV-1 long terminal repeat (LTR) contains two consensus NFκB binding sites (6, 7, 9). Deletions or mutations in the NFκB enhancer abolish transactivation by these stimuli.

The human immunodeficiency virus, type 1 (HIV-1) integrates into the host cell chromosome that is characterized by low levels of virus production (1, 2). Activation of viral gene expression can occur in response to a variety of stimuli including mitogens, cytokines, and environmental stresses such as UV light, heat shock, and oxygen radicals (3–8). The exact mechanism by which these stimuli activate HIV gene expression is not completely understood.

Cytokines and mitogens such as phorbol esters appear to activate HIV gene expression in part through the core enhancer present in the HIV-1 long terminal repeat (LTR), which contains two consensus NFκB binding sites (6, 7, 9). Deletions or mutations in the NFκB enhancer abolish transactivation by these stimuli.

In contrast, the mechanism of UV-induced HIV-1 expression is more complex. While UV light has been reported to increase the amount of nuclear-localized NFκB (10–13) and activate AP-1 and p53 (13–16), deletion of all 5’ promoter elements in the HIV-1 LTR, including the two NFκB sites, had no effect on the absolute level of transcription was reduced (13). It has been suggested that UV light may activate HIV gene expression through DNA damage and chromatin decondensation, which leads to increased accessibility of transcription factors to the promoter (12, 17, 18), and through growth factor receptor activation, possibly via free oxygen radicals or secreted cytokines (10, 11, 14, 19–23).

Several distinct MAP kinases have been shown to respond to extracellular stimuli in mammalian cells (24, 25). One of these MAP kinases, extracellular signal-regulated kinase (ERK), is primarily involved in the regulation of growth and differentiation and is activated by epidermal growth factor (EGF) and phorbol esters, but only poorly activated by stress or inflammatory cytokine stimuli (26–29). In contrast, the c-Jun N-terminal kinase (JNK, also known as SAPK) and p38 MAP kinase (also known as CSBP, RK, Mk2, or HOG1) are activated by a variety of environmental stresses, UV light, and inflammatory cytokines, but are activated only poorly or not at all by EGF and phorbol esters (30–35).

We have identified p38 as the molecular target of a class of compounds which inhibit IL-1 and TNF production in monocytes in response to bacterial lipopolysaccharides (33, 36). These compounds are highly specific inhibitors of p38 kinase activity and have been used to confirm that two in vitro substrates of p38, MAPKAP kinase-2 and MAPKAP kinase-3, are also in vivo substrates (33, 37, 38). Both of these MAPKAP kinases in turn phosphorylate the small heat shock protein hsp27 in vivo, resulting in cytoskeletal changes (39–44).

In order to dissect how cytokines and environmental stresses activate HIV-1 gene expression, we examined the effect of one of these inhibitors, SB203580, on the activation of HIV-1 LTR-directed CAT gene expression by cytokines and cellular stress. Our results suggest that p38 is involved in a pathway leading to activation of the HIV-1 LTR by cytokines and cellular stress.

MATERIALS AND METHODS

Cell Culture and Treatments—The construction of the HeLa cell line A5, which contains an integrated copy of the HIV-1 LTR driving expression of chloramphenicol acetyltransferase (CAT) has been de-
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TRneo was created by ligating the subcloned into the PCRII vector (Invitrogen). The plasmid pCDNL-(containing the neomycin and bound to protein A-agarose for 4 h at 4°C. The beads were washed twice with PBS and added at the times and concentrations indicated in the figure legends. Cells were harvested at the times indicated in the figure legends. The inhibitors SB203580 and SB202474 (33) were dissolved in dimethyl sulfoxide and added at the times and concentrations indicated in the figure legends. Cells were lysed, and CAT assays were performed as described previously (45) using equivalent amounts of protein. CAT assays were quantitated on a B603 Betascope (Betagen). Protein concentrations were determined by the bicinchoninic acid (BCA) method (Pierce).

Immunoprecipitations, Kinase Assays, and Immunoblotting—HeLa A5 cells were activated with UV light, sorbitol, TNF-α, or IL-1β treatment and pretreated with SB203580 or SB202474 as described above. The cells were washed twice in phosphate-buffered saline and solubilized in lysis buffer (20 mM tri-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 10% glycerol, 2 mM EDTA, 25 mM β-glycerophosphate, 20 mM NaF, 1 mM sodium orthovanadate, 2 mM sodium pyrophosphate, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 5 units/ml aprotinin) and centrifuged at 15,000 × g for 20 min at 4°C. Endogenous kinases were precipitated from cell lysates using anti-p38 (33) or anti-MAPKAP kinase-2 antibodies (46) (kindly supplied by Dr. Nick Morrice, University of Dundee, UK) or anti-JNK (Santa Cruz Biotechnology Inc.) bound to protein A-agarose for 4 h at 4°C. The beads were washed twice with lysis buffer and twice with kinase buffer containing 25 mM Heps, pH 7.4, 25 mM MgCl₂, 25 mM β-glycerophosphate, 100 μM sodium orthovanadate, 2 mM dithiothreitol. The immune complex kinase assays were initiated by the addition of 25 μl of kinase buffer containing 10 μg of myelin basic protein for p38, 5 μg of GST-c-Jun (kindly provided by Dr. Roger Davis, University of Massachusetts Medical School, Worcester, MA) for JNK, 3 μg of hsp27 for MAPKAP kinase-2 as substrate, and 50 μM [γ-32P]ATP (20 Ci/mmol). After 30 min at 30°C, the reaction was stopped by the addition of SDS sample buffer, and the phosphorylated products were resolved by SDS-polyacrylamide gel electrophoresis and visualized by autoradiography. The amount of radioactivity was quantitated in a B603 Betascope (Betagen). The amount of p38 present in the immunoprecipitates and its extent of activation were determined by an immunoblot using antiphosphotyrosine (PY20, Santa Cruz Biotechnology Inc.) and anti-p38 antibodies.

Analysis of Steady State Levels of CAT mRNA—Total RNA was isolated from A5 cells by a modified guanidinium method according to manufacturer protocol (Tel-Test, Inc.). Cells were treated with UV light, SB203580, cycloheximide (10 μg/ml), or a combination of these as described in the figure legends. 5 μg of total RNA were used for oligo(dT)-primed cDNA synthesis using the Superscript RT kit (Life Technologies, Inc.) according to manufacturer directions. Semi-quantitative PCR was performed in a 20-μl volume in a Perkin-Elmer model 9600 thermal cycle using one-tenth of the cDNA product as described by the Superscript protocol according to the following program: 95°C soak for 2 min, followed by 25–30 cycles of melting at 95°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 1 min. The control primers supplied in the Superscript kit were used to amplify the CAT cDNA, and primers for β-2 microglobulin were purchased from Clontech. The PCR products were analyzed on ethidium bromide-stained gels using one-fifth to one-tenth of the PCR product.

RESULTS

The HIV-1 LTR Is Activated by Cytokines and Environmental Stress—To evaluate the activation of HIV-1 LTR-directed gene expression by the cytokines IL-1β and TNF-α, UV light, and osmotic stress, we used a HeLa cell line containing an integrated HIV-1 LTR directing expression of the CAT gene (8). Such cells have been considered to be a good model of HIV reactivation, since they can be infected with HIV upon transfection of CD4 (47, 48). As shown in Fig. 1, CAT activity was induced by IL-1β and TNF-α and UV light as previously reported (49), and also by osmotic shock, which had not been observed before. Similar data were obtained with UV stimulation of HIV-1-CAT in Jurkat cells (not shown), a T cell line, indicating that UV-mediated activation is not cell type specific.

Activation of the HIV-1 LTR by Cytokines and Cellular Stress Is Inhibited by SB203580—Since cytokines and environmental stress are known to activate both p38 and JNK MAP kinases (34), we evaluated the effect of SB203580, a specific inhibitor of p38 kinase activity (37), on the ability of cytokines or environmental stress to induce HIV gene expression. SB203580 inhibited HIV-1 LTR-directed gene expression induced by IL-1β, TNF-α, osmotic stress and UV light in a dose-dependent manner with an IC₅₀ between 0.1–1 μM (Fig. 2A-D). While activation of the LTR by UV, IL-1, and sorbitol was inhibited 70–90% by 10 μM SB203580, activation by TNF was inhibited only ~50%. In contrast, SB202474, an inactive analog of SB203580, failed to inhibit activation of the HIV-1 LTR (data not shown).

We next examined the activation of p38 in response to various stimuli by immune complex kinase assay. IL-1β, TNF-α, UV light, and osmotic shock increased p38 activity 4–6-fold over basal levels, and this could be inhibited by more than 90% by addition of 10 μM SB203580 (Fig. 3, top panel). IL-1β, TNF-α, UV light, and osmotic shock also increased JNK activity, but as shown previously (37), 10 μM SB203580 failed to inhibit JNK activity (Fig. 3, bottom panel). This suggests that activation of the HIV-1 LTR by these stimuli depends upon activation of the p38 MAP kinase pathway.

We next determined whether inhibition of HIV-1 LTR activation by SB203580 occurred in the same dose range required to inhibit p38 activity. The addition of 0.4 μM sorbitol resulted in an 8-fold stimulation of p38 activity, which was inhibited in a dose-dependent manner by addition of SB203580 added in vitro
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Fig. 2. Effect of SB203580 on cytokine- and stress-activated HIV-1 LTR CAT gene expression. A5 cells were exposed to the indicated concentrations of IL-1β (A), TNF-α (B), or 40 mM sorbitol (C), or 40 J/m² UV light (D) as described in the legend to Fig. 1. The indicated concentrations of SB203580 were added to the cell medium 1 h prior to each treatment and were present throughout the experiment. Cells were harvested 48 h later, and CAT assay was measured. Fold activation is expressed relative to basal CAT activity in untreated cells (considered as 1). The experiment was performed in duplicate and the average value and standard error are shown.

Fig. 3. Cytokines and environmental stress activate p38, and activity is inhibited by SB203580. A5 cells were treated with the cytokines, UV light, and sorbitol as described in the legend to Fig. 1. Cells were harvested after 30 min and lysed, and p38 was immunoprecipitated with a rabbit antiserum raised against recombinant CSBP2. Top panel, immunoprecipitated CSBP was analyzed for its ability to phosphorylate myelin basic protein in the presence (+) or absence (−) of 10 μM SB203580 added to the kinase reactions. Bottom panel, JNK activity was determined by immunoprecipitation of JNK from cell lysates and kinase assay using the substrate GST-c-Jun in the presence (+) or absence (−) of SB203580 added to the kinase reactions. The fold activation of CSBP and JNK MAP kinases relative to control untreated cells (C) are indicated below each lane.

with an IC₅₀ of approximately 0.6 μM (Fig. 4, top panel). In contrast, the inactive analogue SB202474 had no effect on CSBP activity (Fig. 4, lane 7) or HIV gene expression.

When incubated with cells, SB203580 inhibited the activation of MAPKAP kinase-2, an in vitro substrate of p38, by 0.4 μM sorbitol with an IC₅₀ of ~0.5 μM, as measured by immune complex assay using hsp27 as substrate (Fig. 4, bottom panel). Similar results were obtained when the effect of SB203580 on p38 kinase activity was analyzed in response to cytokines and UV light (data not shown). These data show that the in vitro and in vivo concentrations of SB203580 required to inhibit p38 activity are the same as those required to inhibit HIV-1 LTR-driven CAT expression.

Kinetics of HIV-1 LTR and p38 Kinase Activation—In order to determine the time at which p38 activity was required, we examined the effect of SB203580 on HIV-1 LTR-directed gene expression when the compound was added to cells at different times relative to a single dose of UV light. HIV-1 LTR-directed CAT expression was completely inhibited by SB203580 when added before or up to 2 h after UV treatment (Fig. 5). Inhibition diminished between 2 and 4 h after UV treatment, and no inhibition was observed 8 h after UV treatment, suggesting that the key phosphorylation event mediated by p38 occurred between 2 and 4 h after UV stimulation.

To ensure that the time of action of SB203580 corresponded to a period of p38 activation, we examined the kinetics of p38 activation in response to UV light. p38 activity was stimulated 5–6-fold in as little as 5 min following UV exposure, and maintained this level for up to 2 h (Fig. 6, top panel). Approximately 60% of the maximal CSBP activity still remained 4 h after UV exposure, and activity returned to basal levels by 16 h. The tyrosine phosphorylation of p38 followed similar kinetics (Fig. 6, second panel). In contrast, MAPKAP kinase-2 activation peaked between 5 and 60 min after UV exposure and decreased to 25% of maximal activity between 2 and 4 h after UV exposure (Fig. 6, bottom panel). These results confirm that active p38 and MAPKAP kinase-2 are present in the same interval in which SB203580 inhibits activation of the HIV-1 LTR.

Analysis of CAT mRNA Levels in Response to UV Light, SB203580, and Cycloheximide—To examine whether the delayed requirement for activity of p38 was due to effects at the level of transcription or translation, we examined the kinetics of CAT mRNA expression in response to UV light by semi-quantitative RT-PCR. In the absence of UV stimulation, a low level of CAT RNA was detected (Fig. 7A), in agreement with previous reports in which integrated HIV-1 LTR reporter con-
The appearance of a downstream substrate of the p38 MAP kinase pathway may be dependent on the action of the p38 MAP kinase pathway (19). To ascertain if the appearance of the substrate required protein synthesis, we examined the effect of cycloheximide (CHX) on CAT RNA levels. Cycloheximide added 30 min prior to UV exposure blocked subsequent activation of CAT RNA (Fig. 7C, cf. lane 2 to lane 3), whereas addition of cycloheximide at any time after UV exposure had no effect. These data suggest that p38-dependent activation of the HIV-1 LTR depends on the synthesis of an “immediate early” factor.

The NFκB Sites in the HIV-1 LTR Not Required for Stimulation by UV Light—To analyze whether p38 acted through the NFκB sites that comprise the HIV-1 LTR enhancer, we infected HeLa cells with constructs containing either a wild-type LTR or an LTR containing mutations in both NFκB sites, indicating that the drug does not affect a pathway that signals through NFκB (Fig. 8B).

**Fig. 5.** Effect of SB203580 added at different times relative to activation of the HIV-1 LTR by UV light. A5 cells were stimulated with 40 J/m² UV at t = 0. 10 μM SB203580 was added at the indicated times relative to UV exposure and was present continuously thereafter. Cells were harvested at 24 h after UV exposure, and CAT activity was determined. The fold activation is expressed relative to activity in unstimulated A5 cells, which was set to 1.0.

**Fig. 6.** Time course of UV light-stimulated p38 kinase activation. A5 HeLa cells were exposed to a single dose (40 J/m²) of UV and harvested at the indicated times after exposure. Immune complex kinase assays were performed as described in the legend to Fig. 3 (first panel), and the fold activation of p38 relative to unstimulated cells is shown below each lane. Phosphorylation of p38 on Tyr was assayed by immunoblotting using anti-PY20 antibody. The amount of immunoprecipitated p38 in each reaction (third panel) was determined by immunoblotting using a rabbit antiserum raised against recombinant p38. Arrowheads indicate the position of the IgG heavy chain. Fourth panel, activation of MAPKAP kinase-2 was also assayed from the same cell lysates by immune complex kinase assay using a rabbit antiserum raised against a peptide from MAPKAP kinase-2 using hsp27 as a substrate. The fold activation of MAPKAP kinase-2 relative to unstimulated cells is indicated beneath each lane.

The delayed increase in CAT RNA and the late time at which SB203580 can be added to block expression suggest that the action of the p38 MAP kinase pathway may be dependent on the appearance of a downstream substrate of the p38 MAP kinase pathway (19). To ascertain if the appearance of the substrate required protein synthesis, we examined the effect of cycloheximide (CHX) on CAT RNA levels. Cycloheximide added 30 min prior to UV exposure blocked subsequent activation of CAT RNA (Fig. 7C, cf. lane 2 to lane 3), whereas addition of cycloheximide at any time after UV exposure had no effect. These data suggest that p38-dependent activation of the HIV-1 LTR depends on the synthesis of an “immediate early” factor.

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FIG. 8. NFκB binding sites are required for cytokine activation but are dispensable for UV activation of the HIV-1 LTR. A, HeLa cells were transiently transfected with 3 μg of wild-type (WT) HIV-1 LTR or κB(−) HIV-1 LTR and exposed to 40 or 100 J/m² UV 24 h later. These experiments were performed in duplicate, and the mean value and average error are shown. B, cells were transfected with κB(−) HIV-1 LTR 24 h later, cells were split, and 24 h later equal parts were exposed to UV in the presence (solid bar) or absence (open bar) of 10 μM SB203580 as indicated. Cells were harvested 24 h after UV exposure, and CAT activity was quantitated. Fold activation was normalized to basal CAT levels (considered as 1) present in transfected and unstimulated cells.

DISCUSSION

We have shown that the p38 MAP kinase plays a critical role in the transcriptional activation of HIV-1 LTR transcription in HeLa cells in response to UV, IL-1, TNF, or high osmolarity. While it has been observed previously that overexpression of MAP kinase kinase-3 and p38 can activate the HIV LTR (51), this did not prove that p38 is used during physiological stimulation since overexpression can give erroneous results, as has been observed with MAP or ERK kinase 1 activation of the extracellular signal-regulated kinase pathway (52). In contrast, by showing that a specific inhibitor of p38, SB203580, inhibits HIV-1 LTR driven transcription in the same dose range that p38 kinase was inhibited in vivo provides strong evidence for the direct role of p38 during physiological stimulation by UV, IL-1, TNF, and high osmolarity. These results suggest that p38 will also be important in the activation of HIV itself, and indeed, SB203580 inhibits HIV-1 replication in response to IL-1 and TNF in a chronically infected human monocytic cell line.2

One particular advantage of using a specific p38 inhibitor has been our ability to show that the key role of p38 in HIV-1 transcription activation occurs some 2–4 h after UV treatment, a somewhat surprising result given the rapid activation of p38. This delay is most likely due to the late appearance of an immediate or downstream substrate of the p38 MAP kinase pathway either through de novo synthesis, post-translational modification or localization. In support of de novo synthesis is the finding that UV stimulated HIV-1 transcription is inhibited by cycloheximide, a protein synthesis inhibitor. While cycloheximide also induces p38, it did not prevent further activation of p38 by UV,3 so that the action of cycloheximide cannot be attributed to down-regulation of the p38 pathway. It is also unlikely that cycloheximide is inhibiting the synthesis of a secreted autocrine factor that stimulates the appearance of the p38 pathway substrate through a posttranslational mechanism. Previous studies have shown that UV activation of c-fos transcription in HeLa cells depends on activation of growth factor receptors, such as EGF (19, 53–56), but this happens immediately after UV stimulation, which is too soon to allow substantial contribution from secreted factors. In support of this, we found that suramin, a polyamionic inhibitor of growth factor receptor signaling, completely inhibited both p38 activation and HIV-1-directed transcription if added prior to UV stimulation, but only partially inhibited both (40–60%) if the suramin was added 1 or 2 h after UV (data not shown). This indicates that substantial growth factor receptor stimulation occurs immediately after UV stimulation, and only part of the stimulation occurs at later times where the autocrine factor might be involved. Hence we conclude that the delay in the requirement for activated p38 is most likely due to the time required for UV to stimulate synthesis of a substrate of this pathway.

Although it is clear that p38 regulates transcription, the identity of the target transcription factor is not yet known. In the case of UV stimulation, our data show that SB203580 does not inhibit NFκB activation. Furthermore, previous studies with integrated HIV-1 LTR-CAT constructs in HeLa cells have shown that deletion of other upstream regulatory elements, including the NFκB and AP-1 sites, had no effect on UV induction (13) with the exception of the SPI sites, which had a partial effect, and the TATA box, which was essential for induction. These data argue that p38 affects a basal transcriptional component of the HIV-1 LTR promoter and not any of the previously described potential transcriptional targets of the p38 MAP kinase pathway, such as ATF2, Elk1, or CHOP (51, 57).

While activation of the HIV-1 LTR by IL-1 and TNF directly does depend on NFκB, recent data suggests that the p38 pathway may affect a similar transcriptional component. Beyaert et al. (58) showed that TNF-induced IL-6 production could be replicated by a minimal NFκB promoter CAT construct and could be inhibited by SB203580. However, SB203580 did not affect the TNF-induced DNA binding ability of NFκB or the phosphorylation of its p50 or p65 subunits, leading the authors to conclude that p38 regulated a component of the basal transcriptional machinery that interacts with NFκB. As a precedent, it was recently reported that UV can regulate the activity of the TFIIH complex (59) although there is no data to indicate whether this is responsible for the effects we are observing. It is also important to point out that the levels of other mRNAs, such as β2 microglobulin, were not affected by p38 inhibition, arguing that the p38 cascade target is unlikely to be a general transcription factor but rather one involved in the regulated expression of HIV-1 promoter, such as a coactivator. Identification of this factor will help us to understand the role of p38 in stress-activated regulation of transcription.

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2 L. Shapiro and C. A. Dinarello, personal communication.
3 S. Kumar, unpublished observations.
