Genetic Evidence That Stress-Activated p38 MAP Kinase Is Necessary but Not Sufficient for UV Activation of HIV Gene Expression†

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ABSTRACT: We have examined the role of stress-activated p38 MAP kinase in regulating human immunodeficiency virus (HIV) gene expression in response to ultraviolet light (UV). We found that UV activated p38 in HeLa cells harboring stably integrated copies of an HIVcat plasmid to levels similar to those obtained by hyperosmotic shock. However, hyperosmotic shock resulted in one order of magnitude smaller increase in CAT activity than treatment with UV. The specific p38 inhibitor SB203580 significantly decreased (>80%) UV activation of HIV gene expression whereas PD98059, a specific MEK-1 inhibitor did not, suggesting that p38 is specifically involved in the HIV UV response and little to no contribution is provided by MEK-1 and the p42/p44 MAP kinase pathway. Whereas increased binding of NF-κB to an oligonucleotide spanning the HIV enhancer was observed after UV, as expected, this binding was not affected by SB203580. Furthermore, UV activation of HIV gene expression in cells having the *cat* reporter gene under control of an HIV promoter deleted of the enhancer (−69/+80) produced results indistinguishable from those using HIVcat/HeLa cells with an intact HIV promoter (−485/+80), suggesting that SB203580 acts through the basal transcription machinery. Northern blot analysis of steady-state RNA from HIVcat/HeLa cells revealed an almost complete inhibition of UV activation with SB203580 at the RNA level. Similarly, the UV response was almost completely obliterated at the CAT and RNA levels in HIVcat/HeLa cells stably transfected with a plasmid expressing a kinase-inactive mutant of p38 (isoform α), without affecting NF-κB activation, providing strong genetic evidence that p38, at least the α isoform, is necessary for UV activation of HIV gene expression and that NF-κB activation alone is insufficient. These results firmly establish p38 MAP kinase as a key modulator of HIV gene expression in response to UV that acts independently of NF-κB.

The mechanism by which ultraviolet light (UV) activates human immunodeficiency virus (HIV)[1] gene expression is unclear. Although transient transfection experiments have demonstrated that the HIV UV response involves the pleiotropic transcription factor NF-κB (*1*, *2*), more recent studies have indicated that neither NF-κB or the HIV enhancer nor any other single upstream promoter element is necessary for UV activation of HIV gene expression in stably transfected cells (*3*, *4*). Rather, UV may act through the basal promoter elements by an as yet unidentified transcriptional derepression mechanism (*5*−*7*), perhaps involving modulation of chromatin structure (*5*, *8*, *9*). Furthermore, NF-κB is similarly activated in response to UV and ionizing radiation (IR), whereas only UV significantly activates HIV gene expression in stably transfected cells (*7*). These findings suggest that activation of the NF-κB transcription factor is not sufficient for a full transcriptional UV response and that other factors or functions are important as well.

Protein phosphorylation is one important mediator of cell signaling which occurs in response to extracellular growth factors, mitogenic stimulation, and stress, which ultimately reaches the nucleus to regulate gene expression (*10*, *11*). At least three MAP kinase pathways operate in mammalian cells: (a) the p42/p44 MAP kinase pathway activated by hormones and growth factors, (b) the JNK pathway activated by mitogens, cellular stress, and DNA damaging agents, and (c) the p38/[RK (reactivating kinase)/SAPK-2] pathway also activated by stress and DNA damaging agents. The mammalian homologue of the yeast *HOG1* gene, p38 MAP kinase (*12*), is activated in response to cytokines such as TNF-α, and UV, as well as other DNA damaging agents and is positioned downstream of MKK3 (*12*, *13*). There are at least four isotypes (α, β, γ, δ) of the p38 MAP kinase family, and they are all triggered by agents causing cellular stress such as hyperosmotic shock and DNA damage (*14*−*17*), and their activities and functions overlapping and redundant in some respect, yet demonstrating some characteristic phenotypes (*16*, *17*).

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[1] Abbreviations: CAT, chloramphenicol acetyltransferase; EMSA, electrophoretic mobility shift assay; GST, glutathione *S*-transferase; HIV, human immunodeficiency virus; IR, ionizing radiation; JNK, c-Jun NH_2-terminal kinase; MAP, mitogen-activated protein; MBP, myelin basic protein; MEK, mitogen-activated protein kinase; PMA, phorbol 12-myristate 13-acetate; SAPK, stress-activated protein kinase.

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Recently, the importance of p38 in regulating HIV replication and UV activation of HIV gene expression was reported (*18*−*20*). However, the mechanism by which UV activates HIV transcription and the exact role of p38 in this response have not been established, and the downstream target of p38 has not been identified. One established target for p38 is MAPKAPK-2, which is able to phosphorylate the transcription factors cAMP-responsive element binding (CREB) protein (*21*, *22*), Gadd153 (*23*), ATF-2 (*24*), and most probably also ATF-1 (*21*, *22*). MAPKAPK-2 also phosphorylates the small stress protein Hsp27 in response to heat shock and oxidative stress (*25*). Furthermore, p38 phosphorylates the p38-regulated/activated protein kinase PRAK, which in turn also phosphorylatea Hsp27 (*26*).

In the present study we have examined the role of stress-activated p38 MAP kinase in UV activation of HIV gene expression. We demonstrate that activation of p38 MAP kinase, most likely the α isoform, is required but not sufficient for a full HIV transcriptional response after exposure to UV. We also show that, after UV exposure, p38 and NF-κB activation act independently of each other and NF-κB activation alone is not sufficient to activate HIV gene expression.

EXPERIMENTAL PROCEDURES

Reagents. TNF-α was obtained from GIBCO-BRL (Grand Island, NY). Bovine brain myelin basic protein (MBP), phorbol myristate acetate (PMA), protein A-conjugated agarose, β-glycerophosphate, aprotinin, leupeptin, benzamidine, and PMSF were obtained from Sigma (St. Louis, MO). The CDP-Star system was from Tropix, Inc. (Bedford, MA). Bradford protein dye was from Bio-Rad (Richmond, CA). [1,2-^{14}C]chloramphenicol was from Amersham (Arlington Heights, IL), and [γ-^{32}P]ATP, [α-^{32}P]dCTP, and GeneScreen nylon membrane were from New England Nuclear/DuPont (Boston, MA). PD98059 was purchased from Calbiochem (La Jolla, CA). The phospho-specific p38 Western blot kit was from New England Biolabs (Beverly, MA), and anti-p38 antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The HIV NF-κB oligonucleotide (*7*) was purchased from Genosys Inc. (The Woodlands, TX). Luciferase activity was measured on a luminometer (Lumat LB 9501 Berthold) using a kit from Promega (Madison, WI).

Cell Culture and Cell Treatments. The HIVcat/HeLa (−485/+80) cells, carrying the HIVcat transcriptional unit, and the enhancer$^+$ HIV(−119/+80)cat and enhancer$^-$ HIV-(−69/+80)cat HeLa cells have been described previously (*4*, *27*). Stably transfected hc-jun-luc/HeLa cells have the human *c-jun* promoter under control of the luciferase gene stably integrated in the genome (Taher et al., in preparation). Cells were maintained in DMEM (high glucose) supplemented with 10% fetal bovine serum (Irvine Scientific from Irvine, CA), penicillin (100 units/mL), and streptomycin (100 μg/mL). UV irradiations were carried out at a dose rate of 1−2.5 W/m^2 with a calibrated (Spectronic Model DM-254N) germicidal (254 nm) UV lamp. The medium was completely removed with a pipet and immediately added back to the culture dish after irradiation. CAT assays were carried out as described (*27*). 4-(4-Fluorophenyl)-2-(methylsulfonyl)-5-(4-pyridyl)-1*H*-imidazole (SB203580) and its inactive analogue SKF105809, generous gifts of Dr. John Lee, Smith Kline Beecham Pharmaceuticals (King of Prussia, PA), and the PD98059 (Calbiochem) MEK-1 protein kinase inhibitor were dissolved in DMSO at 10 mM and stored at −20 °C. Unless otherwise stated, the inhibitors were added to the cell culture medium to the indicated final concentrations 30 min before irradiation and left in the medium throughout the experiment. Scrape loading of protein A and protein A−Tat extracts was carried out with a cell scraper for 30 s in 2 mL volume, immediately adding complete DMEM medium supplemented with 100 μg/mL gentamycin.

Protein Analysis and Western Blotting. Proteins were separated by 4−20% SDS−PAGE (Novex, San Diego, CA) and transferred to PVDF membranes (Bio-Rad, Richmond, CA). The membranes were blocked with 5% dry milk in Tris-buffered saline containing 0.1% Tween-20 and probed with phospho-specific p38 antibody at 1:1000 dilution. Blots were developed using the alkaline phosphatase (1:2000)/CDP-Star system followed by exposure to X-ray film. The same blot was used to probe for equal loading of protein using antibody to nonphosphorylated p38.

Protein Kinase Assay. Protein kinase activities were determined essentially as described (*28*). Briefly, cells were homogenized in 1 mL of ice-cold buffer A (25 mM HEPES, pH 7.4, 5 mM EDTA, 5 mM EGTA, 5 mM benzamidine, 1 mM PMSF, 1 mg/mL soybean trypsin inhibitor, 40 μg/mL pepstatin A, 40 μg/mL E64, 40 μg/mL aprotinin, 1 μM microcystin-LR, 0.5 mM sodium orthovanadate, 0.5 mM sodium pyrophosphate, 0.05% sodium deoxycholate, 1% Triton X-100, 0.1% 2-mercaptoethanol). Homogenates were clarified by centrifugation at 4 °C for 10 min at 10000*g*. Antibody (2 μg, 20 μL) was added to each tube, and the tubes were incubated for 3 h at 4 °C. Clear cell lysates (0.5 mL, 1 mg total protein) were mixed with protein A−agarose using gentle agitation (2.5 h, 4 °C). The immunoprecipitate was recovered by centrifugation, the supernatant discarded, and the mixture washed (10 min) sequentially with 0.5 mL of buffer A (twice), PBS, and buffer B (25 mM HEPES, pH 7.4 at 4 °C, 15 mM $MgCl_2$, 0.1 mM sodium orthovanadate, and 0.1% 2-meracaptoethanol). The p38 immunoprecipitates were incubated with 10 μg of myelin basic protein (MBP) or GST−ATF2 in a final volume 100 μL. Reactions were initiated with 98 μL of buffer B containing 0.2 mM [γ-^{32}P]-ATP (5000 cpm/pmol) and 1 μM microcystin-LR. After 30 min, reactions were terminated with sample buffer and prepared for 10% SDS−PAGE. To quantify ^{32}P incorporation into MBP and GST−ATF2, protein bands were cut out from the gel and counted by scintillation counting.

Electrophoretic Mobility Shift Assay (EMSA). Nuclear extracts from HIVcat/HeLa cells were prepared as described (*7*). Briefly, cells from a confluent 100-mm dish were washed and scraped in PBS and collected by slow-speed centrifugation. The cells were then lysed with 500 μL of a buffer containing 20 mM Tris, pH 7.4, 140 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 1 mM EDTA, 1 mM DTT, 0.5% NP-40, 0.5 mM sodium orthovanadate, and protease inhibitors (aprotinin, leupeptin, and PMSF). The nuclei were washed once with 1 mL of the lysis buffer lacking NP-40 and resuspended in 50 μL of nuclear extraction buffer (50 mM Tris-HCl, pH 7.8, 60 mM KCl, 1 mM EDTA, 1 mM EGTA, 2 mM DTT, 1 mM PMSF, 0.5 mM sodium orthovanadate). After freeze−thawing three times the nuclear extracts were obtained by centrifugation at 10000*g* for 15 min and then

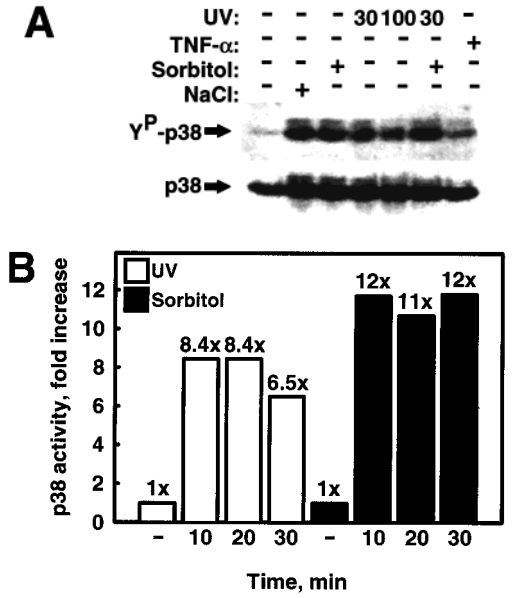

FIGURE 1: UV and hyperosmotic stress activate p38 to similar levels. (A) HIVcat/HeLa cells were treated with UV (30 or 100 J/m^2), TNF-α (10 ng/mL), NaCl (0.5 M), or sorbitol (0.5 M). After 30 min Western blot analysis was carried out using phospho-specific p38 antibody. The blot was reprobed with p38 antibody to normalize protein loading. (B) p38 activity was measured after immunoprecipitation. HIVcat/HeLa cells were incubated with 0.5 M sorbitol for 30 min and then replaced with conditioned medium. p38 activity was determined after immunoprecipitation with anti-p38 antibody and protein A-coupled agarose beads. Myelin basic protein was used as substrate for p38.

used in DNA binding assay. The NF-κB oligonucleotide was labeled with T4 polynucleotide kinase and [γ-^{32}P]ATP. Typically, the binding reaction consisted of 5 μg of protein, 5% glycerol, 1 μg of poly(dI-dC), and 0.1 ng of ^{32}P-labeled NF-κB oligonucleotide. The reaction mixture was incubated for 30 min at 30 °C. The specific protein−DNA complexes were then separated on 5% PAGE in 0.5× Tris−borate−EDTA buffer at 40 V. The gels were dried in a glycerol−ethanol mixture and exposed to X-ray film.

RNA Analysis. Total RNA was extracted with Triazol (GIBCO-BRL). The RNA was electrophoresed on a 1% formaldehyde−agarose gel and transferred to a nylon membrane by capillary transfer. The membrane was hybridized to a ^{32}P-labeled *cat* probe as described previously (*7*). After extensive washing the membranes were exposed to X-ray film with intensifying screens. Fold increases in *cat* steady-state mRNA levels were determined by densitometric scanning of ethidium bromide-stained rRNA to normalize the mRNA levels.

RESULTS

UV and Hyperosmotic Stress Activate p38 to Similar Levels. One of the best characterized stress responses in yeast and mammalian cell culture systems is that inflicted by hyperosmotic shock (*15*, *29*, *30*). To investigate the effect of UV on p38 activation and to directly compare the relative effect of UV and hyperosmotic shock on HIV gene expression, we first examined p38 activation in extracts obtained from treated HIVcat/HeLa (*27*) by Western blotting using phospho-specific p38 antibody. We found that HIVcat/HeLa cells treated with either sorbitol or NaCl (0.5 M) for 30 min resulted in a nearly 5-fold increase in phosphorylated p38 (Figure 1A). Exposure of cells to UV at 30 or 100 J/m^2 resulted in similar increases in p38 phosphorylation at 30 min (Figure 1A). When cells were treated with both UV and sorbitol, no further increase in p38 phosphorylation was seen compared to either UV or sorbitol alone. In line with previous reports, TNF-α also activated p38 in these cells (*12*, *31*), albeit at much lower levels.

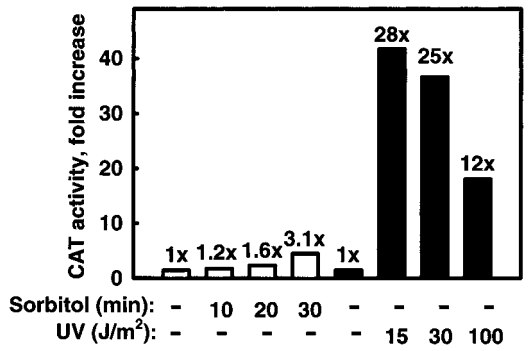

FIGURE 2: UV is more potent than hyperosmotic shock in activating HIV gene expression. Cells were treated with 0.5 M sorbitol in DMEM for 10, 20, and 30 min or with UV (15, 30, or 100 J/m^2). The sorbitol was removed after incubation, the cells were washed once with DMEM, and the conditioned medium was added back. CAT activity was measured after 2 h.

To substantiate these findings, we also performed an immunokinase assay of p38 using myelin basic protein as substrate (Figure 1B). Again, cells were treated with either sorbitol (0.5 M) or irradiated with UV (30 J/m^2), and at 10, 20, and 30 min, cytoplasmic extracts were immunoprecipitated with p38 antibody followed by kinase assay. We found that UV activated p38 ~8-fold at 10 and 20 min and then slightly declined to 6.5-fold at 30 min. Sorbitol activated p38 to slightly larger levels than UV at 10, 20, and 30 min of incubation (11−12-fold), and p38 activation appeared more sustained than after UV.

These results demonstrate that sorbitol and UV activate p38 to similar levels and kinetics without any further activation observed by combining the two treatments, suggesting that these two agents activate p38 by the same, or very similar, mechanism.

UV Is More Potent than Hyperosmotic Shock in Activating HIV Gene Expression. To investigate the effects of hyperosmotic shock on HIV gene expression, we treated HIVcat/HeLa cells with sorbitol for 10, 20, and 30 min. After 20 h, the cells were harvested, and CAT assays were carried out to determine the effect on HIV gene expression. As demonstrated in Figure 2, CAT activity increased only 1.2-, 1.6-, and 3.1-fold, respectively, by sorbitol treatment whereas UV irradiation with 15, 30, or 100 J/m^2 resulted in 28-, 25-, and 12-fold increases in CAT activity. These results demonstrate that although sorbitol treatment is effective in activating p38 (see Figure 1), this is not sufficient to activate the HIV promoter to levels seen after UV treatment. These results show that UV activates HIV gene expression one order of magnitude more effectively than hyperosmotic shock, suggesting that p38 activation alone is not sufficient for a full trancriptional response.

SB203580, a Specific p38 Inhibitor, Prevents UV Activation of HIV Gene Expression. Recently, a specific inhibitor of p38, SB203580, was identified that acts by binding to the ATP binding site of p38 in a competitive fashion (*32*).

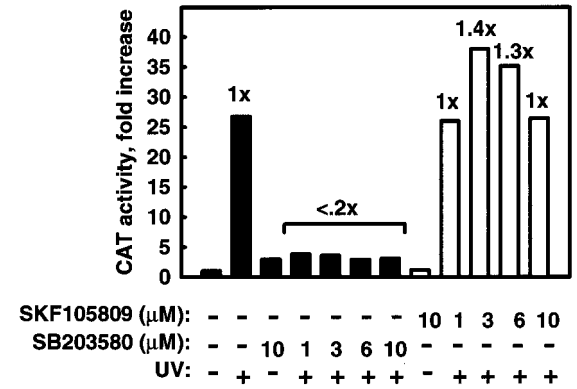

FIGURE 3: The p38 inhibitor SB203580 prevents UV activation of HIV gene expression. HIVcat/HeLa cells were preincubated for 30 min with SB203580 or SKF105809 at the concentrations indicated and then irradiated with UV (30 J/m^2). After 20 h CAT activity was measured as described in Experimental Procedures.

To examine whether SB203580 would inhibit UV activation of HIV gene expression, HIVcat/HeLa cells were preincubated with increasing concentrations of drug (1−10 μM). As a control we used the inactive analogue SKF105809, which does not show any p38 inhibitory properties (*33*). As shown in Figure 3, UV irradiation resulted in a nearly 30-fold increase in CAT activity. SB203580 and SKF105809 by themselves neither inhibited nor activated HIV gene expression significantly. However, UV activation of HIV gene expression decreased more than 80% after treatment with 1−10 μM SB203580, with 1 μM being as effective as 10 μM. On the other hand, treatment with SKF105809 did not result in any inhibition. In fact, the lower concentrations of SKF105809 appeared to slightly stimulate HIV gene expression. These results suggest that activation of p38 is essential for the HIV UV response.

UV Activation of HIV Gene Expression Specifically Involves p38 and Is Independent of the MEK-1/p42/p44 MAP Kinase Pathway. Because UV irradiation results in numerous pleiotropic effects on cells, it was of interest to examine whether the inhibition of HIV gene expression by SB203580 was specific or not. One likely possibility is that a number of signaling pathways are triggered in response to UV to increase HIV gene expression and p38 may be only one of several pathways that affects HIV gene expression. Therefore, we tested whether the HIV UV response is also transmitted through the p42/p44 MAP kinase pathways apart from the p38 pathway. Whereas UV irradiation resulted in a 14-fold increase in CAT activity, the MEK-1 inhibitor PD98059 at three concentrations tested (10, 25, and 50 μM) did not inhibit UV activation of HIV gene expression and PD98059 did not by itself significantly increase CAT activity (Figure 4A). On the other hand, PD98059 inhibited both UV and phorbol ester (PMA) activation of *c-jun* expression, using a HeLa cell clone stably transfected with a plasmid having the human *c-jun* promoter controlling the expression of the luciferase reporter gene (Taher et al., in preparation). As shown in Figure 4B, UV and PMA activated *c-jun*-luc expression 4−6-fold at 6 h. Pretreatment with 50 μM PD98059 inhibited *c-jun*-luc expression 70−80% after treatment with either UV or PMA.

These results suggest that the involvement of p38 in the HIV UV response is specific and there is no evidence for an involvement of MEK-1/p42/p44 MAP kinase in this

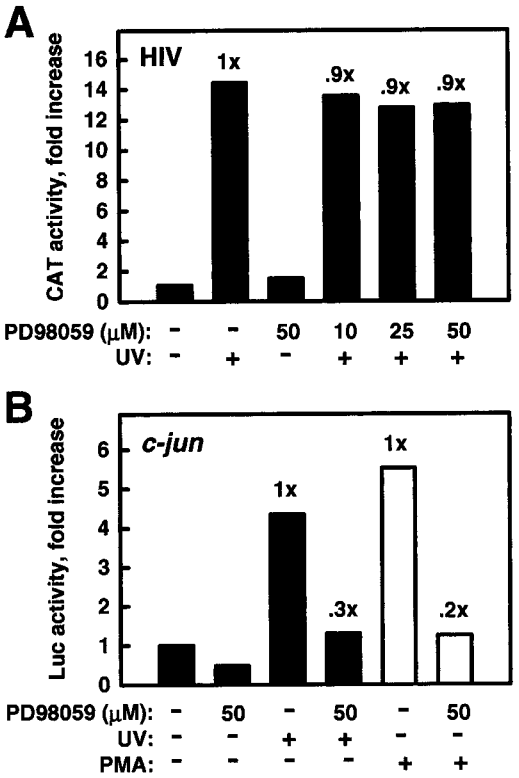

FIGURE 4: Inhibition of MEK-1 does not block UV activation of HIV gene expression. (A) HIVcat/HeLa cells were preincubated with the specific MEK-1 inhibitor PD98059 at the indicated concentrations for 1 h and then irradiated with UV (30 J/m^2). CAT assays were performed 20 h later. (B) HeLa cells harboring integrated copies of a *c-jun* promoter luciferase reporter gene (hcjun-luc/HeLa) were pretreated with PD98059 (50 μM) for 1 h and then treated with UV (30 J/m^2) or PMA (20 nM). After 6 h luciferase activity was measured as described in Experimental Procedures. Activity is expressed as relative light units.

response in HeLa cells. On the other hand, transcriptional activation of *c-jun* in response to UV is to some extent transmitted through the MEK-1/p42/p44 MAP kinase which is inhibited by PD98059 (*34*, *35*).

NF-κB and the HIV Enhancer Are Not Targets for SB203580. The pleiotropic transcription factor NF-κB is present in the cytosol bound to IκB in unstimulated cells. After treatment of cells with cytokines, UV, or other DNA damaging agents, IκB dissociates from the RelA(p65)/p50 NF-κB subunits by a phosphorylation/proteolysis mechanism allowing for translocation of NF-κB into the nucleus (*1*, *2*). We (*7*) and others (*1*, *2*) have reported previously that UV activates NF-κB as determined by EMSA and that UV activation of the HIV promoter does not require the enhancer region in stably transfected cells (*4*). When HIVcat/HeLa cells were exposed to SB203580 at 1 or 3 μM prior to UV irradiation, no effect on NF-κB binding was noticed (Figure 5A, compare lane 6 with lanes 9 and 10), even at a concentration as high as 10 μM (data not shown). Neither SKF105809 (lanes 2 and 3) nor SB203580 (lanes 4 and 5) by themselves or SKF105809 in combination with UV (lanes 7 and 8) influenced NF-κB binding to the oligonucleotide.

To substantiate this result using a different experimental approach, we examined whether SB203580 would affect UV activation of HIV gene expression in a panel of HeLa cell clones stably transfected with HIV promoter deletions controlling the expression of the *cat* gene (*4*). As shown in

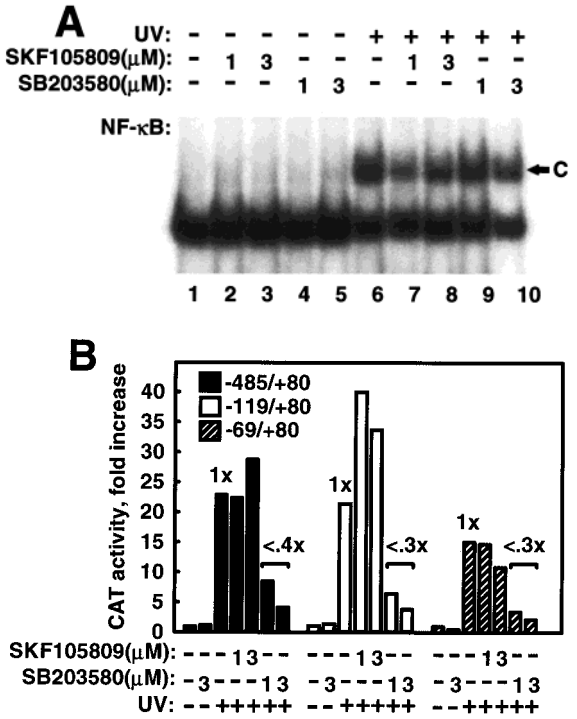

FIGURE 5: UV activation of NF-κB is not mediated by p38. (A) HIVcat/HeLa cells were preincubated with SB203580 or SKF105809 for 1 h with the indicated concentrations and then irradiated with UV (30 J/m^2). After 5 h nuclear extracts were made, and NF-κB binding activity was determined as described in Experimental Procedures. (B) Inhibition of UV activation of HIV gene expression by SB203580 does not involve the HIV enhancer or NF-κB binding. HIVcat/HeLa (−485/+80), HIV enhancer$^+$ (−119/+80), and HIV enhancer$^-$ (−69/+80) were preincubated for 30 min with SB203580 or SKF105809 at the concentrations indicated then irradiated with UV (30 J/m^2). After 20 h the CAT activity was measured as described in Experimental Procedures.

Figure 5B, UV activation of HIV gene expression was similarly blocked (60−70%) by 1 and 3 μM SB203580 in cells having a −69/+80 HIV promoter (−enhancer) driving the *cat* gene as it was in cells having the complete HIV promoter (−485/+80) or another promoter deletion construct having the enhancer (−119/+80).

These results demonstrate that SB203580 does not affect the increased binding of NF-κB to an oligonucleotide spanning the HIV enhancer after UV and the drug inhibits UV activation of HIV gene expression irrespective of whether the HIV enhancer is present in the construct or not. Therefore, NF-κB and the HIV enhancer region are not targets for SB203580. Instead, SB203580 may affect transcription through the basal transcription elements.

Establishment of an HIVcat/HeLa Cell Clone That Expresses a Kinase-Inactive p38-α. To rule out the possibility that SB203580 may have some unknown effects on normal cellular functions, we also asked the question of p38's role in the HIV UV response by taking a genetic approach using a plasmid that expresses a mutant form of p38-α with mutations affecting ATP binding ($T_{180}GY \rightarrow AGF$) (*12*). In addition, this kinase-inactive protein has a FLAG epitope fused to its amino terminus, allowing for the convenient identification of mutant p38. We supertransfected the HIVcat/HeLa parental cells with pcDNA3-FLAGp38(AGF) and selected for stably transfected cells by cotransfecting with a plasmid conferring resistance to hygromycin. One hygromycin-resistant clone expressed a FLAG protein the size of p38-α on a Western blot (Figure 6A). These cells produced reduced levels of p38 activity in immunokinase assays using either MBP (Figure 6B, top) or GST-ATF2 (Figure 6B, bottom) proteins as substrates, and the immunoprecipitated material demonstrated much reduced levels of p38 protein in the HIVcat/p38(AGF)/HeLa precipitates compared to the parental HIVcat/HeLa cells (Figure 6C). However, the FLAG epitope could only be found in the extracts from the HIVcat/

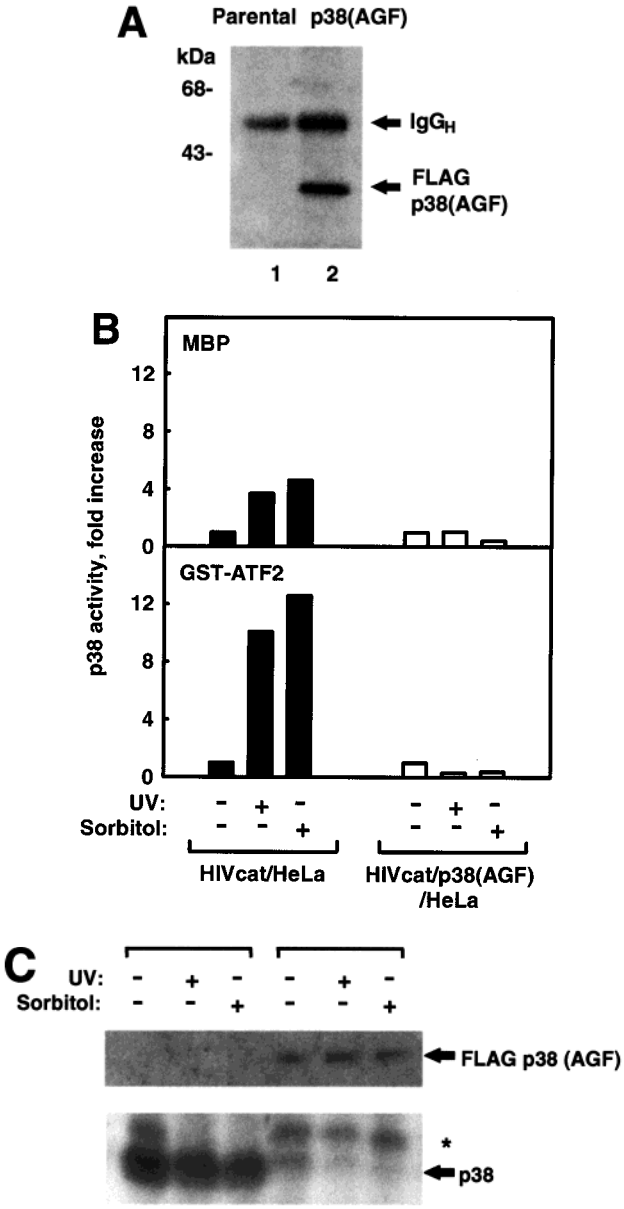

FIGURE 6: Construction of HIVcat/HeLa cells expressing kinase-inactive p38-α. (A) HIVcat/HeLa (parental) cells were stably transfected with pcDNA3-FLAGp38(AGF) [p38(AGF)] and pRS-Vhygro, and hygromycin-resistant clones were selected. An ∼40 kDa protein band was detected on a Western blot using anti-FLAG antibody. IgG$_H$, IgG heavy chain. (B) HIVcat/p38(AGF)/HeLa cells are impaired in p38 phosphorylation of MBP (top) and GST-ATF2 (bottom) in response to either UV or sorbitol as demonstrated by immunokinase assay. (C) Western blot of p38 co-immunoprecipitated protein complexes from either HIVcat/HeLa or HIVcat/p38-(AGF)/HeLa cell extracts. The top panel is probed with anti-FLAG antibody. The bottom panel is probed with anti-p38 antibody. (*) Co-immunoprecipitated protein which disappears in HIVcat/HeLa cells after UV and sorbitol treatment but not in HIVcat/p38(AGF)/HeLa cells.

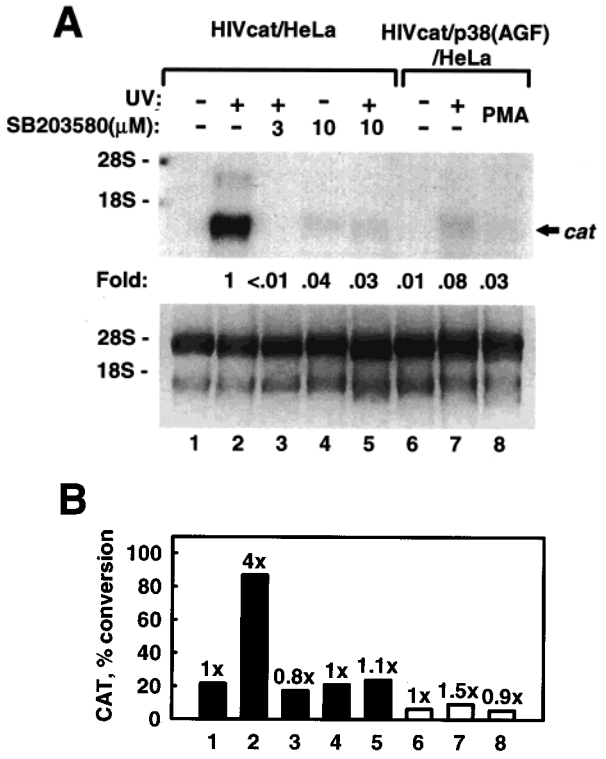

FIGURE 7: SB203580 and kinase-inactive p38-α block UV-mediated HIVcat transcriptional activitation. HIVcat/HeLa cells were pre-incubated for 30 min with SB203580 or SKF105809 with the indicated concentrations and then irradiated with UV (30 J/m^2). Similarly, HIVcat/HeLa cells stably transfected with a plasmid expressing a FLAG epitope-tagged and kinase-inactive p38-α (*12*), were treated with either UV or phorbol ester (PMA). After 5 h the cells were harvested, and total RNA was extracted. (A) Northern blot analysis using a *cat*-specific hybridization probe was carried out as described in Experimental Procedures. Fold increases were determined on the basis of densitometric scans and normalization to 28S rRNA. (B) CAT activities 5 h after UV was carried out in parallel with that done in (A).

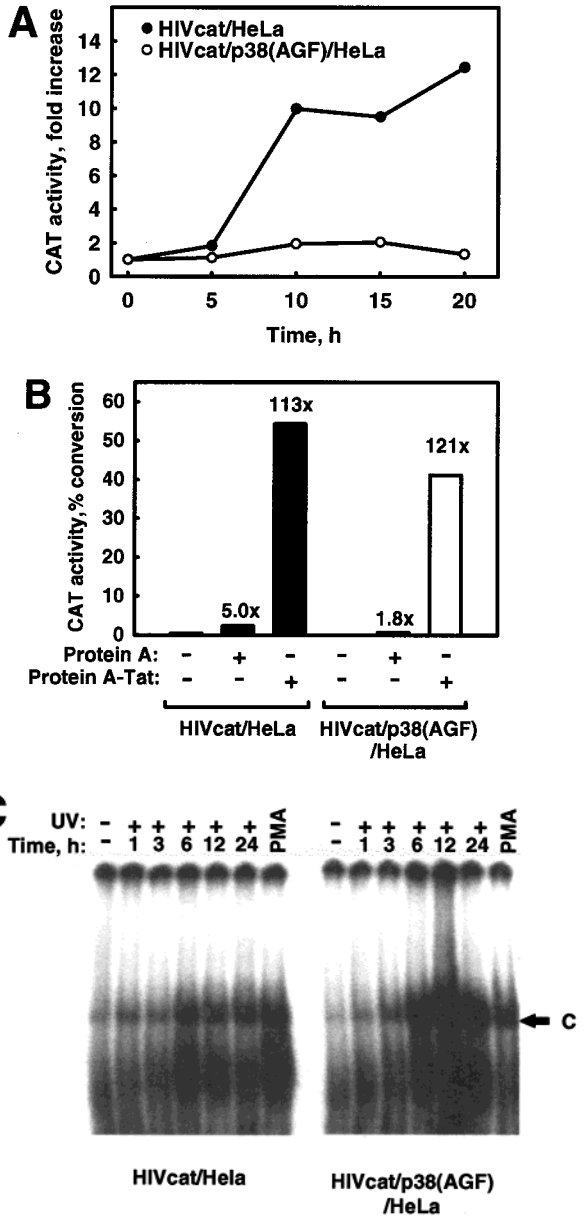

FIGURE 8: HIVcat/p38(AGF)/HeLa cells are not defective in HIV Tat-mediated gene expression or in UV activation of NF-κB. (A) HIVcat/HeLa and HIVcat/p38(AGF)/HeLa cells were irradiated with 30 J/m^2 and CAT assays performed after 5, 10, 15, and 20 h. (B) HIVcat/HeLa cells were exposed to crude protein extracts from bacterial cells expressing either protein A−Tat or protein A (*27*). The cells were exposed to the extracts and then scrape-loaded into the cells by using a cell harvest scraper for 1 min. The extracts were left in the medium with the addition of 100 μg/mL gentamycin to prevent bacterial growth. CAT assays were determined 20 h later. (C) HIVcat/HeLa and HIVcat/p38(AGF)/HeLa cells were irradiated with 30 J/m^2 or exposed to phorbol ester (PMA). After various times (1−24 h) nuclear extracts were prepared and used in EMSA with a NF-κB oligonucleotide. DNA/transcription factor complexes (C) are indicated with an arrow.

p38(AGF)/HeLa cells. Of interest is the co-immunoprecipitated protein (*) which disappears in the parental immunoprecipitations after both UV and sorbitol treatment whereas in the HIVcat/p38(AGF)/HeLa cells this protein remains associated with p38, suggesting that somehow the complex formation between p38 and this other protein is altered in the kinase-inactive cells.

p38 Acts at the Transcriptional Level during the HIV UV Response. To examine whether p38 acts at the HIV transcriptional or posttranscriptional level, we tested whether SB203580 would affect the increase in *cat* steady-state mRNA levels that occur in response to UV (*7*). HIVcat/HeLa cells were pretreated with 3 or 10 μM SB203580 30 min prior to UV irradiation (30 J/m^2), and total RNA was extracted after 5 h. We found that UV resulted in a significant increase in *cat* steady-state levels whereas treatment with 10 μM SB203580 produced no increase in mRNA levels over untreated cells (Figure 7A). Pretreatment with 3 or 10 μM SB203580 prior to UV irradiation resulted in very low levels (<3% of UV levels) of steady-state *cat* mRNA levels. To confirm these inhibitory effects of SB203580 at the transcriptional level, we also determined CAT activity levels of samples taken in parallel. Whereas UV irradiation resulted in a 4-fold increase in CAT activity at 5 h, pretreatment with SB203580 prevented this increase in CAT activity above basal levels (Figure 7B). Similarly, the HIVcat/p38(AGF)/HeLa cells were unresponsive to UV as determined by steady-state *cat* mRNA levels and CAT activity (Figure 7, panels A and B, lanes 6−8). These results suggest that p38 acts primarily at the transcriptional level to affect UV activation of HIV gene expression.

To confirm that indeed the HIVcat/p38(AGF)/HeLa cells were unable to elicit any increase of HIV CAT gene expression over longer periods of time, we irradiated the

parental HIVcat/HeLa and the HIVcat/p38(AGF)/HeLa cells with UV (30 J/m^2) and at various times determined the resulting CAT activities. As shown in Figure 8A, the HIVcat/p38(AGF)/HeLa cells produced almost no increase in CAT activity up to 20 h postirradiation whereas the parental HIVcat/HeLa cells produced 12−14-fold increases. To rule out that the reason for the lack of effect of UV on the HIVcat/p38(AGF)/HeLa cells was due to inactivation of the HIVcat transcription unit during the clone selection process, we scrape-loaded the HIV Tat protein, as a protein A fusion protein expressed in bacteria, into the parental HIVcat/HeLa cells and the HIVcat/p38(AGF)/HeLa cells. We found that both cell clones responded to HIV Tat by producing 113- and 121-fold increases in CAT activity, respectively (Figure 8B). Scrape-loading of the protein A control extract only produced a fewfold increase in CAT activity. This result suggests that the HIVcat transcription unit in HIVcat/p38-(AGF)/HeLa cells is functioning and is as equally responsive to HIV Tat transactivation as are the parental HIVcat/HeLa cells. Because of the very effective obliteration of the HIV UV response in the HIVcat/p38(AGF) cells, it was also of interest to examine the effect of UV on NF-κB activation since we did not see any effect of SB203580 in HIVcat/HeLa cells (see Figure 4A). Nuclear extracts were prepared from HIVcat/HeLa and HIVcat/p38(AGF)/HeLa cells at times between 1 and 24 h after UV exposure (30 J/m^2). We also exposed the cells separately to PMA since this treatment effectively activates NF-κB (*7*). Despite the block to p38 signaling in the HIVcat/p38(AGF)/HeLa cells, we still found that UV activated NF-κB. In fact, these cells appeared to give an even stronger response to UV than did the parental HIVcat/HeLa cells. Similarly, the response to PMA was also unaffected. These results demonstrate that the HIVcat transcription unit is intact and functioning in the HIVcat/p38(AGF)/HeLa cells and that the effect of UV on NF-κB activation in these cells is not diminished.

DISCUSSION

Our results demonstrate very clearly by using the specific p38 inhibitor SB203580 at concentrations that do not affect normal cellular functions and, most importantly, by genetic modulation of stably transfected cells that p38 MAP kinase is necessary for UV activation of HIV gene expression. We provide strong evidence that the ubiquitous transcription factor NF-κB, although activated by UV treatment, is not involved in the signal transduction pathway where p38 plays an essential role. However, it is possible that NF-κB plays an amplifying role in this UV response.

Extracellular stress, UV radiation, and other genotoxic agents activate the nonreceptor protein tyrosine kinases (PTKs) of the *src*- and Syk/ZAP-70 family (*36*−*38*), which signals through the cytoplasmic compartment and eventually affects nuclear transcription of receptive genes, such as HIV and many immediate-early genes (*10*, *39*). Devary et al. reported that UV triggers the activation of *src* tyrosine kinases (*2*, *40*), which requires Ras and Raf, to activate HIV gene expression as a result of NF-κB release from its association with IκB in the cytoplasm (*2*). Our studies show that UV activation of HIV gene expression is not inhibited by PD908508 treatment, indicating that the MEK-1 and p42/p44 MAP kinase pathway is not involved in this process in these cells. On the other hand, UV activation of *c-jun* reporter gene expression was blocked by PD908508, supporting the notion that the UV response for *c-jun* transcriptional activation is somewhat different from HIV and may be mediated through Ras/Raf/MEK-1. However, p38 appears to be important in *c-jun* transcriptional activation by UV, because SB203580 inhibits reporter gene activity from a *c-jun* promoter as well (*41*) (Taher et al., in preparation).

We show further that activation of p38 is not sufficient to achieve a full HIV transcriptional response, because agents that are potent activators of p38 alone, such as hyperosmotic shock, only activate HIV gene expression about 1/10 of those seen after UV. One likely possibility is that p38 and NF-κB in combination are most effective for a full response. However, p38 plays a dominant role; without p38 activation there is little to no effect of NF-κB on HIV gene expression.

Recently, it was demonstrated that SB203580 inhibited UV activation of HIV gene expression in a NF-κB-independent fashion (*18*). However, no data were presented to indicate that NF-κB binding to an HIV oligonucleotide was affected by SB203580 (*18*). The genetic data we provide here that expression of kinase-inactive p38-α obliterates the HIV UV response is particularly strong evidence that p38 is required in this response. However, it is possible that the activities of other p38 isoforms are also affected by p38-(AGF) expression in these cells, and we can therefore not rule out that other p38 isoforms are also involved in the HIV UV response.

UV activates both NF-κB and p38 in HeLa cells. However, activation of NF-κB does not depend on p38, as SB203580 or kinase-inactive p38-α do not inhibit UV-mediated NF-κB activation. It was recently reported that SB203580 did not affect NF-κB activation or phosphorylation of NF-κB subunits, suggesting that NF-κB is not a direct target for the p38 pathway (*31*). As we show here, UV activation of HIVcat gene expression appears to require p38 activation, but this is not sufficient for a full UV response and additional levels of regulation are also required. Furthermore, p38 does not appear to act through NF-κB and the HIV enhancer because NF-κB gel shifts are not affected by SB203580, UV activation of HIV enhancer deletion constructs are still inhibited by SB203580, and in cells stably transfected with a kinase-inactive form of p38-α, NF-κB activation is not affected. Therefore, the most likely target for p38 is a factor interacting with the basal transcription elements and not the enhancer region of the HIV LTR. This conclusion is supported by previous work from our laboratory demonstrating that deletion of the NF-κB elements from the enhancer region by point mutations was ineffective in blocking UV activation of HIV gene expression (*4*). In agreement with these results, it was demonstrated that p38 and NF-κB act independently of each other also in response to antioxidants, osmotic stress, and TNF-α (*42*).

The importance of p38 in the HIV life cycle was recently demonstrated (*19*). It is clear that HIV requires p38, but exactly for what process is not clear. Apparently, transactivation of HIV gene expression by Tat does not require p38 as we show here because the HIVcat/p38(AGF)/HeLa cells are fully competent in Tat transactivation. This was also alluded to, but not shown, by Kumar et al. using SB203580 treatment (*18*). During HIV infection, it is possible that p38 is required at some point immediately after infection (*19*, *43*). However, p38 is not activated by inactivated HIV virions

binding to receptors on the cell surface (*19*) and can therefore not be attributed to a stress response generated by the penetration of virus particles through the plasma membrane. One possibility is that p38 is involved in negative regulation of HIV gene expression through nontranscription factor-associated mechanisms, such as changes in chromatin structure and derepression of transcriptional elongation (*6*). Furthermore, p38 is required in T- and B-cell proliferation responses that go through IL-2 and CD-40 (*44*, *45*), both of which activate HIV transcription.

Our work has not yet identified the downstream target(s) of p38 and the mechanism by which HIV gene expression is affected. A number of p38 substrates have been identified including several isoforms of MAPKAPK, MAPKAPK-2 which phosphorylates the transcription factor CREB, Gadd153 (CHOP), ATF-2, ATF-1, and the small heat-shock protein Hsp27, and PRAK which also phosphorylates Hsp27. It remains to be determined which one of these factors, if any, is involved in UV activation of HIV gene expression.

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