Mechanisms of Signal Transduction: Phosphorylation of c-Fos by Members of the p38 MAPK Family: ROLE IN THE AP-1 RESPONSE TO UV LIGHT

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Phosphorylation of c-Fos by Members of the p38 MAPK Family

ROLE IN THE AP-1 RESPONSE TO UV LIGHT*

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Exposure to sources of UV radiation, such as sunlight, induces a number of cellular alterations that are highly dependent on its ability to affect gene expression. Among them, the rapid activation of genes coding for two subfamilies of proto-oncoproteins, Fos and Jun, which constitute the AP-1 transcription factor, plays a key role in the subsequent regulation of expression of genes involved in DNA repair, cell proliferation, cell cycle arrest, death by apoptosis, and tissue and extracellular matrix remodeling processes. Besides being regulated at the transcriptional level, Jun and Fos transcriptional activities are also regulated by phosphorylation as a result of the activation of intracellular signaling cascades. In this regard, the phosphorylation of c-Jun by UV-induced JNK has been readily documented, whereas a role for Fos proteins in UV-mediated responses and the identification of Fos-activating kinases has remained elusive. Here we identify p38 MAPKs as proteins that can associate with c-Fos and phosphorylate its transactivation domain both in vitro and in vivo. This phosphorylation is transduced into changes in its transcriptional ability as p38-activated c-Fos enhances AP1-driven gene expression. Our findings indicate that as a consequence of the activation of stress pathways induced by UV light, endogenous c-Fos becomes a substrate of p38 MAPKs and, for the first time, provide evidence that support a critical role for p38 MAPKs in mediating stress-induced c-Fos phosphorylation and gene transcription activation. Using a specific pharmacological inhibitor for p38α and -β, we found that most likely these two isoforms mediate UV-induced c-Fos phosphorylation in vivo. Thus, these newly described pathways act concomitantly with the activation of c-Jun by JNK/MAPKs, thereby contributing to the complexity of AP1-driven gene transcription regulation.

Repeated and prolonged exposure to sunlight and hence to UV radiation causes skin damage that may induce alterations in the DNA and ultimately evolve into skin cancer. Extensive investigation of the response of mammalian cells to UV light has shown that exposure to UV light results in the rapid activation of a group of enzymes known as stress-activated protein kinases (SAPKs)† (1, 2) and the induction of expression of a set of immediate early genes (ergs) (3–6), which in turn participate in the cellular responses to this type of environmental stress.

SAPKs is the common denomination for a subgroup of highly homologous proteins, JNKS and p38s, that belong to a superfamily of serine-threonine kinases known as mitogen-activated protein kinases (MAPKs) (7–10). These kinases play an essential role in the transduction of environmental stimuli to the nucleus, as they are capable of regulating the expression of genes involved in a variety of cellular processes, including cell proliferation, differentiation, programmed cell death, and neoplastic transformation (11–13). MAPKs have been classified into at least six subfamilies, among which the Erk/MAPKs (Erk1 and -2), JNKs (JNK1, -2, and -3), and p38 kinases (α, β, γ, and δ) are the most extensively studied. Erk5 (also known as Big MAPK or BMPK) (14) and the recently identified ERK7 (15) and ERK8 (16) complete the picture. Whereas Erk1/2 and Erk5 are considered to respond to growth signals (17), JNKS and p38s are activated by cellular stresses like exposure to heat shock, protein synthesis inhibitors such as anisomycin, free radicals, ionizing radiation, and UV light (18–21). A variety of mitogens acting on cell surface cellular receptors promote the sequential activation of small GTP-binding proteins of the Ras and Rho family and a cascade of protein kinases that ultimately phosphorylate and activate each MAPK. Indeed, each MAPK is specifically regulated by MAPK kinases (MAPKKs). Despite the knowledge accumulated on agonist-induced MAPK activation, the way stresses are sensed and where and how the signals are converted into SAPK activation with the consequent triggering of nuclear responses are still open questions.

Among the immediate early genes that are rapidly turned on by UV light are the members of the AP-1 transcription factor family (22), which play a key role in normal and abnormal epithelial cell growth and differentiation (23). This transcription factor is formed by dimers of proteins encoded by the Fos

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† The abbreviations used are: SAPK, stress-activated kinase; AP-1, activator protein 1; GFP, green fluorescent protein; MAPK, mitogen-activated kinases; AU5, epitope tag peptide, AU5; HA, epitope tag hemagglutinin; GST, glutathione S-transferase; JNK, c-Jun N-terminal kinase; ERK, extracellular signal-regulated kinase; MAPKKs, MAPK kinases; MEK, MAPK/ERK kinase; MOPS, 4-morpholinepropanesulfonic acid; DTT, dithiothreitol; PMSF, phenylmethylsulfonyl fluoride; PBS, phosphate-buffered saline; wt, wild type; TAD, transcriptional activation domain; FL, full-length; mut, mutant.

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¶ Institution propanesulfonic acid; DTT, dithiothreitol; PMSF, phenylmethylsulfonyl fluoride; PBS, phosphate-buffered saline; wt, wild type; TAD, transcriptional activation domain; FL, full-length; mut, mutant.
c-Fos as a Substrate for p38s

(c-Fos, FosB, Fra-1, and Fra-2) (24–28) and the Jun subfamilies (c-Jun, JunD, and JunB) (29–33). Homodimerization of Jun proteins or heterodimerization between proteins of the two subfamilies confers to the resulting AP-1 complexes the ability to recognize specific DNA sequences known as tetradecanoylphorbol acetate-responsive elements or AP-1-binding sites (34, 35), which are found in the regulatory regions of a variety of genes (36, 37), including cell cycle-related and AP-1 genes themselves (38–40). AP-1 proteins are often the final target of signal-transducing kinase cascades, and upon phosphorylation become transcriptionally active triggering the activity of AP-1-driven promoters and the expression of their corresponding regulated genes (41). The best studied example is the phosphorylation of c-Jun by UV-activated JNK, which in turn acts on AP-1 sequences present on its own promoter. Recently, it has been shown that a parallel pathway involving platelet-derived growth factor-activated Erk2 also leads to the phosphorylation of c-Fos and consequent AP-1 activation (42, 43). In addition, the involvement of the three major MAPK pathways (ERK, JNK, and p38) in the induction of the c-fos promoter has been reported (44–46). However, the activation of c-Fos proteins by MAPKs in response to stress-activated signaling pathways has not been investigated extensively.

In this study, we show that c-Fos is rapidly phosphorylated in response to UV light exposure and that this phosphorylation is mostly dependent on UV-induced p38 kinases rather than resulting from Erk1/2 or JNK activation. Moreover, we observed that the phosphorylation of c-Fos in its transactivation domain leads to c-Fos translational activation and to c-Fos-mediated AP-1 activity. In addition, by using an array of point mutations, we examined the contribution of each putative p38 target residue within the c-Fos transactivation domain to its transcriptional response. To the best of our knowledge, this is the first report that involves c-Fos as a target of UV-triggered, p38-mediated signaling pathways that influences AP-1 activity and the subsequent regulation of genes involved in cellular responses to injury caused by DNA-damaging agents.

EXPERIMENTAL PROCEDURES

Culture of Cell Lines—HEK 293T cells were maintained in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal bovine serum and penicillin/streptomycin/amphotericin B (Invitrogen). NIH 3T3 mouse fibroblasts were grown in Dulbecco’s modified Eagle’s medium containing 10% calf serum and the above antimicrobial mixture.

Transformation Assay—The BL21 E. coli strain was transformed with the plasmid described (47). Expression vectors for p38A-HA-FLAG, p38A-Myc-FLAG, and p38A-Actin-FLAG were described (48). AP (dominant negative) mutant forms of p38A, -B, -C, and -D have been provided by J. Han (see Ref. 49).

Western Blot Analysis—Cell lysates were washed with PBS twice and resuspended in Lysis buffer (25 mM HEPES, pH 7.5, 0.3 M NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 0.5 mM DTT, 1% Triton X-100, 0.1% SDS, 20 mM β-glycerophosphate, 1 mM sodium vanadate, and 1 mM phenylmethylsulfonyl fluoride (PMSF). HA-tagged kinases were immunoprecipitated from the cleared lysates by incubation with the specific antibody against HA (MMS-101R, Covance) for 1.5 h at 4 °C. Immunocomplexes were recovered with the aid of Gammabind-Sepharose beads (Santa Cruz Biotechnology) and washed three times with PBS containing 1% Nonidet P-40 and 2 mM sodium vanadate. The lysates were assayed using the Dual-luciferase Reporter System (Promega). Light emission was quantitated using the Monolight 2010 luminometer as described (40). Parallel immunoprecipitates were processed for Western blot analysis using the same antiserum as described (40).

Luciferase Assay—Cells were plated in 6-well dishes and transfected with expression vectors for HA-tagged kinases, alone or in combination with the respective upstream-activating molecules. 24 hours after transfection, cells were starved with serum-free media for 2 h, washed with cold phosphate-buffered saline, and lysed at 4 °C in a buffer containing 25 mM HEPES, pH 7.5, 0.3 M NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 0.5 mM DTT, 1% Triton X-100, 0.1% SDS, 20 mM β-glycerophosphate, 1 mM sodium vanadate, and 1 mM phenylmethylsulfonyl fluoride (PMSF). HA-tagged kinases were immunoprecipitated from the cleared lysates by incubation with the specific antibody against HA (MMS-101R, Covance) for 1.5 h at 4 °C. Immunocomplexes were recovered with the aid of Gammabind-Sepharose beads (Santa Cruz Biotechnology) and washed three times with PBS containing 1% Nonidet P-40 and 2 mM sodium vanadate. The lysates were assayed using the Dual-luciferase Reporter System (Promega). Light emission was quantitated using the Monolight 2010 luminometer as described (40). Parallel immunoprecipitates were processed for Western blot analysis using the same antiserum as described (40).

Luciferase Reporter Assays—Cells were seeded on 6-well dishes and transfected with different expression plasmids together with 0.1 μg of luciferase reporter vector and 0.01 μg of PRL-null (a plasmid expressing the enzyme Renilla luciferase from Renilla reniformis). The total amount of DNA was adjusted with pcDNAIII β-galactosidase. Cells were lysed in passive lysis buffer (Promega) for 10 min post-transfection. Cell lysates (50 μl/well) were transfected to a 96-well luminometer plate, and firefly and Renilla luciferase activities were assayed using the Dual-luciferase Reporter System (Promega). Light emission was quantitated using the Monolight 2010 luminometer as specified by the manufacturer (Analytical Luminescence Laboratory).

Indirect Immunofluorescence—HEK 293T cells were seeded on glass coverslips and transfected with Lipofectamine Plus Reagents (Invitrogen) as described above. 16–18 hours post-transfection, cells were washed with 1× PBS, fixed with 1× PBS, fixed with 1× PBS, fixed with 1× PBS, and then permeabilized with 4% formaldehyde and 0.5% Triton X-100 in 1× PBS for 10 min. After washing with PBS, cells were blocked with 1% bovine serum albumin and incubated with anti-FA (Covance) as primary antibodies for 1 h. Following incubation, cells were washed three times with 1× PBS and then incubated with the corresponding secondary antibodies (1:200) conjugated with tetramethylrhodamine B isothiocyanate (Jackson ImmunoResearch). Cover-
p38γ Interacts with c-Fos in a SOS Two-hybrid Assay—In order to identify unknown p38-binding proteins that might take part in p38-mediated signaling pathways, we performed an SOS yeast two-hybrid screen (50) of a HeLa cells-cDNA library fused to an Src myristoylation signal using the human p38s as bait. In this system, the bait is attached to the coding sequence of the exchange factor for Ras, c-Fos. Thus, only proteins that bring SOS close to the plasma membrane result in GTP loading on Ras and therefore allow growth of the cdc25ts yeast strain at the restrictive temperature of 37 °C (Fig. 1A). By using the full-length p38γ cDNA as bait, we obtained several candidate clones. The sequence of one of them, clone E62-83, corresponded to amino acids 137–239 of the AP-1 member c-Fos. Fig. 1B shows that although yeast strains containing the DNA for clone E62-83 together with plasmids encoding either the bait or an empty vector grew well at 28 °C, growth at the restrictive temperature of 37 °C was only achieved when the bait was present (Fig. 1B, left panels). Positive controls using a full-length cDNA for c-Jun, a well known c-Fos-interacting protein, are depicted on the right panels.

RESULTS

p38γ Interacts with c-Fos in a SOS Two-hybrid Assay—In order to identify unknown p38-binding proteins that might take part in p38-mediated signaling pathways, we performed an SOS yeast two-hybrid screen (50) of a HeLa cells-cDNA library fused to an Src myristoylation signal using the human p38s as bait. In this system, the bait is attached to the coding sequence of the exchange factor for Ras, c-Fos. Thus, only proteins that bring SOS close to the plasma membrane result in GTP loading on Ras and therefore allow growth of the cdc25ts yeast strain at the restrictive temperature of 37 °C (Fig. 1A). By using the full-length p38γ cDNA as bait, we obtained several candidate clones. The sequence of one of them, clone E62-83, corresponded to amino acids 137–239 of the AP-1 member c-Fos. Fig. 1B shows that although yeast strains containing the DNA for clone E62-83 together with plasmids encoding either the bait or the absence of the bait. At 37 °C growth restriction is inflicted upon those clones carrying an empty plasmid instead of either the bait or the cDNA. The photograph, left panel, shows the actual data obtained with c-Fos and c-Fos, and the right panel shows a positive control using c-Fos and its AP-1 partner protein c-Jun.

Cell Fractionation and Nuclear Translocation Assay— Nuclear extracts were separated from its corresponding cytoplasmic fractions as already described above for the electrophoretic mobility shift assays. Homogenates from the nuclear extracts were obtained by incubation with hypotonic buffer as indicated above. SDS sample loading buffer was added to samples from both fractions before loading SDS-polyacrylamide gels and transferred to Immobilon-P membranes as indicated. The protein bound to the membranes was detected by Western blot with the aid of the same anti-c-Fos antibody mentioned above.

Two-hybrid Assays (Sos Rescue System)—To assay for protein-protein interaction in yeast, we employed the Sos rescue system, which takes advantage of a Saccharomyces cerevisiae strain carrying a Cdc25 allele that displays a temperature-sensitive phenotype. This cdc25-2 strain can be propagated at 28 °C but is unable to grow at 37 °C unless expressing a functional SOS protein. Protocols for growth of the yeast strains and transfection with plasmid DNAs have been described previously (50).

UV Stimulation—HEK 293 cells were transfected and starved overnight. 24 hours after transfection, the medium was removed, and the cells were irradiated with UV light and pretreated with the SB 203580 compound as indicated. Cells were washed in cold PBS and lysed in 400 μl of buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM FMSF) and rocked at 4 °C for 15 min on a shaking platform. Homogenates were centrifuged for 5 min, and the supernatants (nuclear extracts) were aliquoted and stored at −70 °C. After determining protein concentrations using protein assay (Bio-Rad Laboratories), 2 μg of protein were incubated at room temperature with 1 μg of poly(dI-dC) and 0.1 μg of salmon sperm DNA in 20 μl of binding buffer (12 mM HEPES, pH 7.8, 60 mM KCl, 2 mM MgCl2, 0.12 mM EDTA, 0.3 mM DTT, 0.3 mM PMSF, 12% glycerol) for 15 min. Complexes were analyzed on nondenaturing (4.5%) polyacrylamide gels and transferred to Immobilon-P membranes as indicated. Protein bands were visualized with 1:lakh diluted anti-c-Fos antibody mentioned above. The antigen was detected by enhanced chemiluminescence (Amersham Biosciences) and ImageQuant software (Molecular Dynamics). The intensity of the bands was calculated and used for quantification. The experiments were performed three times with similar results.

Electrophoretic Mobility Shift Assays— Nuclear extracts were obtained from HEK 293 cells plated in 10-cm plates and grown to 70% confluence, starved overnight, and then treated with UV light and pretreated with the SB 203580 compound as indicated. Cells were washed in cold PBS and lysed in 400 μl of buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 1 mM EGTA, 2 mM MgCl2, 0.1 mM DTT, 1 mM FMSF). After 15 min on ice, 25 μl of 10% of Nonidet P-40 was added and vigorously vortexed for 10 s. Homogenates were centrifuged for 30 s in a microcentrifuge. The nuclear pellets were resuspended in 50 μl of ice-cold hypotonic buffer C (20 mM HEPES, pH 7.9, 0.42 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM FMSF) and rocked at 4 °C for 15 min on a shaking platform. Homogenates were centrifuged for 5 min, and the supernatants (nuclear extracts) were aliquoted and stored at −70 °C. After determining protein concentrations using protein assay (Bio-Rad Laboratories), 2 μg of protein were incubated at room temperature with 1 μg of poly(dI-dC) and 0.1 μg of salmon sperm DNA in 20 μl of binding buffer (12 mM HEPES, pH 7.8, 60 mM KCl, 2 mM MgCl2, 0.12 mM EDTA, 0.3 mM DTT, 0.3 mM PMSF, 12% glycerol) for 15 min. Complexes were analyzed on nondenaturing (4.5%) polyacrylamide gels and transferred to Immobilon-P membranes as indicated. The protein bands were visualized with 1:lakh diluted anti-c-Fos antibody mentioned above. The antigen was detected by enhanced chemiluminescence (Amersham Biosciences) and ImageQuant software (Molecular Dynamics). The intensity of the bands was calculated and used for quantification. The experiments were performed three times with similar results.

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c-Fos as a Substrate for p38s

**FIG. 2.** Assay of c-Fos phosphorylation in vitro by SAPKs. HEK 293 cells were cotransfected with expression plasmids for HA-p38α, HA-p38β, HA-p38γ, HA-JNK, or HA-Erk2 (MAPK) along with empty vectors (−) or with plasmids expressing the corresponding upstream activators (MKK6, MEKK, or MEK3EE) as indicated. The cellular lysates obtained were divided in 2 equal aliquots and immunoprecipitated using a monoclonal anti-HA antibody. Each immunoprecipitate was used to perform kinase assays using bacterially expressed GST-c-Fos TAD as substrate (upper panel) or with alternative well known substrates as positive controls (middle panels). The position and identity of each 32P-labeled substrate is indicated. In parallel, Western blot (WB) analysis was performed with anti-HA antibodies using total cell lysates to check for expression of the transfected kinases (lower panel).

vitro. Parallel samples were incubated with GST-ATF2 or myelin basic protein as controls for the activity of the different MAPKs (Fig. 2, middle panels). Expression of the transfected kinases was controlled in a Western blot of the total lysates using an anti-HA antibody (Fig. 2, lower panel). Together, these data extended previous findings suggesting that c-Fos could act as a potential target for SAPKs besides its function as an Erk2 substrate.

c-Fos Is Phosphorylated in Vivo by p38 MAPKs—In order to study whether c-Fos was also phosphorylated in vivo, we analyzed the electrophoretic mobility of c-Fos by SDS-PAGE followed by Western blot, as the appearance of slow migrating bands in c-Fos is related to its phosphorylated state (42, 43, 52, 53). Thus, HEK 293 cells were cotransfected with a full-length c-Fos (pCEFL-AU5 c-Fos FL) along with plasmids encoding different MAPKs and their corresponding activators. As depicted in Fig. 3A (upper panel), all the p38 kinases induce a mobility shift on c-Fos FL, as denoted by Western blotting using an anti-AU5 antibody. This change in mobility was strictly dependent on the activity of the kinases as it was only observed upon cotransfection with MKK6. Most interestingly, no shift was observed upon cotransfection with JNK or activated JNK, although comparable expression of all HA-tagged kinases was observed by using an anti-HA antibody on aliquots from the same samples run in parallel (Fig. 3A, lower panel). These results prompted us to ask whether the observed shift in c-Fos mobility, presumably due to its in vivo phosphorylation, involves any of the MAPK potential target residues located in the TAD of c-Fos. To answer this question we first performed the same mobility shift experiment, transfecting HEK 293 cells with the activated kinases and a plasmid coding for a GFP-tagged form of the c-Fos transactivation domain (pCEFL-GFP c-Fos TAD). Indeed, we observed that the c-Fos TAD was shifted upon conditions in which p38 MAPKs were activated by MKK6, and again no shift was induced by activated JNK (Fig. 3B). These results suggest that c-Fos acted as an in vivo target for all p38 MAPKs but not of JNK, thus providing evidence of an unexpected specificity of SAPK signaling, as not all SAPKs lead to the phosphorylation of this transcription factor.

c-Fos Phosphorylation Potentiates Its Transcriptional Activity—To analyze whether phosphorylation of the transactivating domain of c-Fos by p38 MAPKs in vivo can modulate the transactivating functions of c-Fos, we employed a heterologous system, in which the c-Fos TAD was expressed as a fusion protein with the DNA binding domain of the yeast transcription factor GAL4. The protein encoded by the chimeric plasmid pGBDX c-Fos TAD (GAL4 c-Fos TAD) was tested by its ability to stimulate transcription from a luciferase reporter plasmid controlled by GAL4-binding motifs (pGAL4-Luc) upon conditions in which p38 is activated by MKK6. Transfection of NIH 3T3 cells with these plasmids along with vectors that express different p38 MAPKs and MKK6 was performed. As shown in Fig. 4A, cotransfection of activated p38γ, p38β, and p38α along with pGAL4 c-Fos TAD stimulated luciferase activity by 9-, 16-, and 20-fold, respectively, when compared with samples from cells transfected with GAL4-c-Fos TAD alone taken as a reference. Remarkably, the activity of the c-Fos TAD was only slightly stimulated by activated p38β, whereas no stimulation was observed when activated JNK was present, the latter in line with the data obtained testing in vivo phosphorylation. All these results indicated that activated p38γ, p38β, and p38α were sufficient to transactivate c-Fos but not necessarily helped to understand which endogenous p38 is involved downstream of the p38 MAPKs. To analyze this point, we employed MEK3EE, a constitutive active mutant MAPKK for p38α (39) which, because of mutations in its own activation domain, has a strong kinase activity toward the p38α. In fact, this activated molecule activates the GAL4-c-Fos TAD without the need of cotransfected wild type p38s. As depicted in Fig. 4B, when MEK3EE was coexpressed with the AF mutant form of p38α that acted as dominant negatives for endogenous p38s (49), MEK3EE-induced c-Fos transcriptional activity was inhibited to different degrees, with p38α, -β, and -δ being the most potent inhibitors. Taken together, these data suggest that the transcriptional activity of c-Fos can be differentially controlled by...
phosphorylation by specific members of the p38 group of SAPKs within the MAPK superfamily.

**Transactivation of c-Fos by p38 MAPKs Induces AP-1 Activity**—Based on our results, we tested whether the transactivating effect of p38 MAPKs on c-Fos resulted in a greater AP-1 activity, as c-Fos can dimerize with Jun proteins and activate promoters that contain AP-1-binding sites. Thus, we used a reporter plasmid that carries a luciferase gene under the control of seven tandem repeats of an AP-1-response element (pAP-1-Luc). We cotransfected NIH 3T3 cells with pAP-1-Luc, pCEFL AU5 c-Fos FL, and pRNull (10 ng/each), together with pCEFL HA-p38α, HA-p38β, HA-p38γ, HA-p38δ, or HA-JNK, with or without upstream p38 or JNK activators (MKK6 or MEKK, respectively). 24 hours after transfection, cells were harvested, and dual luciferase activities were determined. Data shown correspond to the average of duplicates from a representative experiment of three performed.  

**UV Light Induces c-Fos Phosphorylation by Serine-Threonine Kinases**—Extracellular stimuli that induce cellular stress are strong activators of p38 and JNK activity and can trigger c-Jun phosphorylation (5, 11). In view of our data, we explored the *in vivo* phosphorylation of c-Fos FL, when cells were exposed to UV radiation. HEK 293 cells transfected with pCEFL AU5 c-Fos (100 ng/each), and pRNull (10 ng/each), along with pCEFL HA-p38α, HA-p38β, HA-p38γ, HA-p38δ, and the p38 MAPK activator pCEFL-GST-MKK6 as indicated in the figure. 24 hours after transfection cells were harvested and dual luciferase activities were determined. Data shown correspond to duplicates that are from a representative experiment of three performed.
that c-Fos is indeed a target of phosphorylation events induced by cellular stress.

**UV Light Induces c-Fos Phosphorylation in Specific Serine/Threonine Residues through SAPKs of the p38 Family**—In view of our observations, we decided to dissect the role of different p38s in the pathways that lead to UV-induced c-Fos phosphorylation. We took advantage of the fact that at 5 min of treatment, UV light or anisomycin induced no significant shift on c-Fos mobility (Fig. 5A and data not shown). Thus, we treated HEK 293 cells transfected with AU5-c-Fos FL alone or along with the HA-tagged forms of p38α, p38β, p38γ, or JNK, and we analyzed the phosphorylation status of c-Fos in total cell lysates by Western blot using an anti-AU5 antibody. Co-transfection of all p38 family members, which alone did not display any demonstrable effect, dramatically enhanced the effect of UV light on the mobility shift of c-Fos after 5 min of treatment, whereas JNK or Erk2 did not (Fig. 5B, upper panels and data not shown). Treatment with anisomycin under the same conditions gave nearly identical results (data not shown).

The analysis of the primary structure of the TAD in the c-Fos protein reveals the existence of four putative MAPK phosphorylation sites displaying the consensus sequence of serine/threonine followed by a proline, Thr-232, Thr-325, Thr-331, and Ser-374. To confirm that the shift induced in c-Fos was due to phosphorylation by p38 MAPKs in any of these sites, cells were transfected with a c-Fos mutant that has these four key residues mutated to alanines along with constructs expressing the same HA-tagged kinases. Upon UV stimulation no mobility shift in the position of bands developed by the c-Fos antibody was observed when using this mutant (Fig. 5B, lower panels), which indicated that changes in c-Fos mobility were due to the presence of these MAPK target residues. These results indicate that this shift was most likely due to phosphorylation exerted by UV light-activated p38 kinases.

To confirm the activation of SAPKs in these cells under our treatment conditions, we analyzed the same total lysates by Western blot using an anti-phospho-p38 antibody capable of recognizing the phosphorylated state of all four isoforms or an anti-phospho-JNK antibody. As expected, Fig. 5C (upper panels) shows that all these kinases were activated under our experimental conditions by UV light or anisomycin exposure. Similar expression levels of the transfected kinases were assessed using an anti-HA antibody (Fig. 5C, lower panels). These results indicate that, most likely, all the p38 MAPKs but not JNK can be involved in stress-induced c-Fos phosphorylation on specific serine and threonine residues.

In view of the role of the p38s in UV-induced c-Fos phosphorylation, we used an additional approach, and we confirmed the data in cells in which endogenous p38 signaling is deterred either by using a specific p38 pharmacological inhibitor, SB 203580 (54), or by expression of dominant negative forms of p38. Although p38γ and -β are refractory to the effect of SB 203580, and there are no specific inhibitors for these kinases, the drug allows us to score at least the participation of endogenous p38α and -β in the various effects of UV light on c-Fos. Thus, we pretreated AU5 c-Fos FL-transfected cells with the SB 203580 compound, and we compared its effect with that of the JNK and MEK inhibitors, SP600125 (55) and U0126 (56), respectively, followed by treatment with UV light or anisomycin for 30 min. Most interestingly, only SB 203580 was able to reduce the stress-induced mobility shift in c-Fos, whereas the other compounds had no significant effect on it as denoted by Western blots developed using an AU5 antibody (Fig. 6A, upper panel, and data not shown). To take this a step forward and
c-Fos as a Substrate for p38s

Although overexpressed c-Fos is mainly in the cytoplasm under basal conditions (Fig. 7B), a small fraction of endogenous c-Fos remains in the nucleus, and the ratio between nuclear and cytosolic protein is around 0.4. Upon UV stimulation, c-Fos migrated from the cytosol to the nucleus. However, the p38 kinase inhibitor prevented the nuclear translocation of c-Fos. Similar results were obtained by expressing a dominant negative form of the p38 signaling pathway intermediate, MEK3AA (39). As shown in Fig. 7C, GFP c-Fos localized mainly in the cytosol of NIH 3T3 cells, but upon UV stimulation, a significant fraction translocated to the nuclear region. Moreover, this UV-induced translocation was strongly inhibited when cells were cotransfected with MEK3AA. Together, these data indicate that c-Fos is a substrate of the α and/or β isoforms of p38 kinases and depends on this phosphorylation to translocate to the nucleus as the result of their activation induced by UV light.

UV-induced AP1-DNA Binding Complexes Require p38 Activity and Contain c-Fos—As UV radiation results in p38-mediated c-Fos phosphorylation and nuclear translocation, we sought to examine the presence of this factor in UV-induced AP1-DNA binding complexes. Electromobility shift assays were performed on labeled AP-1 oligonucleotides preincubated with nuclear extracts coming from untransfected HEK 293 cells treated or not with the SB 203580 compound prior to UV exposure. As shown in Fig. 8, incubation of labeled oligonucleotides with nuclear extracts from cells in basal conditions rendered the assembly of an AP1-DNA complex as indicated. As expected, UV light induced a stronger AP1-DNA binding activity as denoted by the presence of a band of higher intensity (Fig. 8, 1st and 2nd lanes), which was prevented by pretreatment with the inhibitor SB 203580 (3rd lane). To determine the presence of c-Fos in these complexes, we incubated nuclear extracts from UV-stimulated cells with an antibody against the C-terminal portion of c-Fos protein, which resulted in the presence of a band of slower mobility corresponding to the heavier antibody-AP1-DNA complex (Fig. 8, 5th lane). All these data combined suggest that in cells stimulated by UV light, AP1-DNA binding activity is enhanced, and this is dependent on p38 activity and c-Fos, thus supporting a critical role for p38α and/or -β in mediating stress-induced c-Fos phosphorylation, nuclear translocation, and gene transcription activation in response to UV radiation.

Individual c-Fos Phosphorylation Sites Are Differential Targets for p38 MAPKs—After determining the role of UV-induced, p38-mediated phosphorylation on the c-Fos TAD transactivation and the relevance of the MAPK target sites on it, we decided to study the contribution of each of the four residues to this response. For these studies, we employed a series of c-Fos TAD variants designed to keep only one phosphorylation site intact, while replacing the rest of them by nonphosphorylatable alanine residues. We expressed these mutant proteins as GST fusion chimeras in bacteria and used them as substrates for in vitro p38 kinase assays. Fig. 9A shows a Coomassie Blue staining of equivalent amounts of each mutant protein used in the assays. As depicted in Fig. 9B, c-Fos TAD phosphorylation by activated p38α, -β, and -δ was abolished when all four MAPK target residues are mutated (GST c-Fos TAD MUT), which was aligned with the fact that this mutant does not present any apparent shift when cells are exposed to UV light and cotransfected with different p38s (Fig. 5B). According to this evidence, p38γ appeared to induce phosphorylation on a non-MAPK target site as the c-Fos TAD MUT was still weakly phosphorylated. Analyzing each site in particular, it was interesting to note that Thr-325 seemed to be the only site that could be significantly phos-
phorylated when left alone on the TAD, suggesting that this residue may represent a preferential target for these kinases in vitro. On the other hand, the phosphorylation of Thr-232 and Ser-374 by p38/H9251 was almost undetectable, whereas all other kinases had a marginal effect (considering that the band that appeared in the lane corresponding to p38/H9253 was also present with similar intensity in the c-Fos TAD MUT). Thr-331 was just slightly phosphorylated by p38/H9251 and -/H9252, and no detectable phosphorylation was induced by p38/H9253 or -/H9254. These results suggest that each site can be phosphorylated in vitro with different effectiveness by a distinct set of p38 MAPKs, displaying a certain pattern of specificity and trans-activating potential.

Multiple MAPK Phosphorylation Sites on the c-Fos Transactivation Domain Are Required to Achieve p38-induced Transcriptional Activity—As the in vitro phosphorylation of each MAPK target site within the c-Fos TAD by p38s is different, we explored the participation of each site in c-Fos transcriptional activity in vivo using the GAL 4 Luc reporter system. We transfected NIH 3T3 cells with the different TAD mutants subcloned as Gal4-TAD chimeras together with the different p38 kinases and MKK6. Fig. 9C shows that removing all four MAPK sites abolished transcriptional activation of the chimera, which is coincident with the fact that the c-Fos TAD MUT is not phosphorylated in vitro. Adding back only one particular site at a time did not restore the transcriptional activity of c-Fos in response to any of the p38 MAPKs. These data indicate that despite the fact that some sites can be phosphorylated in vivo when present alone on the TAD, multiple sites are required to achieve maximal phosphorylation and consequent transcriptional activity.

DISCUSSION

Activation of early genes is a common feature to the cellular response to both cell-growth promoting agents and cellular stressors. Particularly, the level of expression of members of the AP-1 transcription factor family, such as c-Jun and c-Fos, has been shown to increase shortly after the exposure of cells to...
either mitogens or UV light (23, 60, 61). However, in order to exert its transcriptional activation effect on target genes that may ultimately be responsible for the onset of cellular responses, these proteins require modification by the addition of phosphate groups. Thus, MAPK cascades play an important role in both the activation of the early gene promoters and the transactivation of pre-existing and newly synthesized proteins. Among them, phosphorylation of c-Jun by JNK has received considerable attention. On the other hand, the effect of signaling cascades on c-Fos phosphorylation is much less understood. A variety of proteins have been reported as putative c-Fos kinases in the past (43, 52, 53, 62, 63). In addition, c-Fos has been shown recently to be a target for MAPK activity upon mitogenic stimulus (42). In this study we provide evidence that exposure of cells to UV light triggers the activation of members of the p38 MAPK family, which in turn phosphorylate c-Fos in its transcriptional activation domain, leading to its enhanced activity as a transcription factor.

Searching for putative p38 binding partners by a double-hybrid strategy, we identified c-Fos as an insert in various clones that were rendered positive. In order to analyze the biochemical and biological consequences of the c-Fos/p38 interaction, we initially performed in vitro assays to corroborate the function of c-Fos as a substrate for p38. We found that the four p38 isoforms immunoprecipitated from cultured cells effectively transfer phosphate groups from ATP to bacterially expressed c-Fos proteins. As phosphorylation of a given substrate by a partner protein kinase in vitro does not necessarily reflect a functional interaction in vivo, we tested the mobility of c-Fos proteins by Western blots of extracts from cultured cells expressing active kinases, as changes in mobility are considered to be indicative of alterations in the phosphorylation state of c-Fos (42, 53). We observed a remarkably slower mobility of full-length c-Fos when cells were cotransfected with all the different p38s and its activator MKK6. The same shift was observed when using only the c-Fos TAD, confirming that in vivo phosphorylation by p38s is most likely to occur on its C-terminal region.

Phosphorylation of the c-Fos/TAD promoted c-Fos-mediated transcriptional activation, as we show by using a GAL4-c-Fos TAD, assayed upon conditions in which p38s are activated. In turn, suppression of endogenous p38 signaling by dominant negative p38 resulted in limited activity of the GAL4 luciferase reporter. These data were confirmed using a full-length c-Fos and an AP-1 reporter system. Although every p38 seemed to promote transcriptional activation of the c-Fos protein, differences in the intensity of the effect became evident, with p38α and p38β being the strongest activators, whereas p38γ and p38δ had a much more modest effect, even when they phosphorylate the c-Fos TAD in vivo and in vitro. Taken together these results suggest that the capacity of the different p38s to regulate c-Fos may be attributable not only to the ability of these kinases to mediate c-Fos phosphorylation but also to the possibility that p38s may recruit additional components to the transcriptional machinery that, depending on which p38 is involved, might be critical for changes in transcriptional activity. In addition, the cellular localization of these molecules can be a determinant of the resulting differential transcriptional activation. For example, after UV exposure, HA-tagged p38α and -γ are increased in the nucleus where they localized along with c-Fos. Most interestingly, p38β was found in the nucleus and colocalizing with c-Fos even before UV treatment, whereas p38δ did not colocalize with c-Fos in the nucleus but in the cytosol upon stimulus (data not shown). The fact that p38α, -β, and -γ localized to the nucleus after stimulation and p38δ remained cytosolic may also help to explain the differences observed in the p38-induced c-Fos transactivation potential as evidenced by the reporter assays. For instance, although p38γ can phosphorylated c-Fos, this does not lead to c-Fos transcriptional activation because the protein does not go to the nucleus (Figs. 2–4). This also helps to explain why p38δ AF (dominant negative form) can still inhibit MEK3EE-induced c-Fos transactivation, as most likely the AF mutant also sequesters c-Fos in the cytosol. This is not surprising because although there are many similarities between p38 family members, there are also some important differences that suggest that they may regulate specific functions (12). This last point is evidenced by the fact that different p38 isoforms have opposite effects on AP-1-dependent transcription through the regulation of c-Jun (49).

Most interestingly, the phosphorylation of c-Fos in response to stress-activating pathways and the simultaneous overexpression of each of the p38 family members, but not by JNK, indicate that only UV-activated p38 kinases can mediate this event. In line with this, pretreatment of cells with the p38 inhibitor SB 203580 (54) prevented the UV-induced mobility shift and AP-1 complex assembly, whereas the MEK inhibitor U0126 (56) produced no effect in the position of the c-Fos bands in UV-treated cells (not shown) indicating that, in contrast to what is observed upon activation of tyrosine kinase receptors, the Erk1/2 signaling pathway may not be involved in the c-Fos response to UV light. Similarily, cotransfection with Erk2 or Erk5, both shown to phosphorylate c-Fos upon platelet-derived growth factor stimulation or activating mutations, respectively, did not induce any changes in the apparent molecular weight of the transcription factor upon UV treatment (data not shown) (42, 43, 53). Although the inhibitor SB 203580 does not allow us to score the participation of p38γ or p38δ, the fact that p38α and p38β are the strongest activators of c-Fos transcriptional activity and that the effect of UV light on the endogenous c-Fos phosphorylation, nuclear translocation, and AP1-DNA transcription factor.
binding activity is almost abolished by the SB 203580 most likely indicates that the latter isoforms play a predominant role in the UV-stimulated signaling pathway. On the other hand, the fact that the UV-induced c-Fos mobility shift was reverted by phosphatase treatment and was not seen when using a mutant that has been depleted of critical serines and threonines indicates that these changes were the consequence of the primary addition of phosphate groups on these residues. However, due to the severity of the shift, we cannot discard the possibility that further post-transcriptional modifications may also occur on the c-Fos TAD following phosphorylation. Regarding the analysis of the participation of each of these sites on the transactivation of c-Fos, the situation appears to be quite more complex than in the case of c-Jun phosphorylation by JNK where only two sites, serine 63 and 73, are the critical residues. Thr-325 appears to be the only target site when left alone in the TAD for p38 kinases in vivo. However, despite this phosphorylation, c-Fos does not regain transcriptional activity in vivo after restoration of serines or threonines one by one, which indicates that none of the sites seem to be sufficient by themselves to exert transcriptional activation, and that more than one site is required to achieve maximal phosphorylation and consequent transcriptional activity. Particularly interesting is the fact that p38γ still can induce the phosphorylation of the TAD in the mutant that has the four putative MAPK phosphorylation sites mutated to alanines as denoted by the presence of a strong band. This might be the consequence of the phosphorylation on a non-MAPK target residue, most likely in an indirect fashion through another associated kinase brought down during the immunoprecipitation step. As the activating or repressing nature of this phosphorylation has not been established, one could speculate that this can affect the fact that although p38γ phosphorylates c-Fos and localizes to the same cellular compartment upon activation, it nonetheless only a weak inducer of its transcriptional activity.

Although the effects of UV-induced p38 on the c-fos promoter...
have already been reported (44-46), our data show for the first time that p38 promotes the phosphorylation of the c-Fos transcription factor affecting its cellular localization and transcriptional activity. In summary, our findings support a model by which UV stimulation leads to c-Fos phosphorylation through p38s, and in turn multiple putative phosphorylation sites on the c-Fos TAD are required for p38-mediated transcriptional activation of c-Fos (Fig. 10). Indeed, we can envision that UV irradiation triggers multiple signaling pathways that stimulate the activation of the promoters for c-Jun and c-Fos (5) and enhances the transcriptional activities of AP-1 through the concomitant phosphorylation of c-Jun by JNK, as reported previously (3, 21, 64), and c-Fos by p38 family members as shown in this study.

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