UV Irradiation and Heat Shock Mediate JNK Activation via Alternate Pathways*

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To elucidate cellular pathways involved in J un-NH2-terminal kinase (JNK) activation by different forms of stress, we have compared the effects of UV irradiation, heat shock, and H2O2. Using mouse fibroblast cells (3T3-4A) we show that while H2O2 is ineffective, UV and heat shock (HS) are potent inducers of JNK. The cellular pathways that mediate JNK activation after HS or UV exposure are distinctly different as can be concluded from the following observations: (i) H2O2 is a potent inhibitor of HS-induced but not of UV-induced JNK activation; (ii) Triton X-100 treated cells abolish the ability of UV, but not HS, to activate JNK; (iii) the free radical scavenger N-acetylcysteine inhibits UV- but not HS-mediated JNK activation; (iv) N-acetylcysteine inhibition is blocked by H2O2 in a dose-dependent manner; (v) a Cockayne syndrome-derived cell line exhibits JNK activation upon UV exposure, but not upon HS treatment. The significance of J un phosphorylation by JNK after treatment with UV, HS, or H2O2 was evaluated by measuring J un phosphorylation in vivo and also its binding activity in gel shifts. HS and UV, which are potent inducers of JNK, increased the level of c-Jun phosphorylation when this was measured by32Porthophosphate labeling of 3T3-4A cultures. H2O2 had no such effect. Although H2O2 failed to activate JNK in vitro and to phosphorylate c-Jun in vivo, all three forms of stress were found to be potent inducers of binding to the AP1 target sequence. Overall, our data indicate that both membrane-associated components and oxidative damage are involved in JNK activation by UV irradiation, whereas HS-mediated JNK activation, which appears to be mitochondrial-related, utilizes cellular sensors.

The response of mammalian cells to stress in the form of UV irradiation or heat shock (HS)† involves key regulatory proteins such as p53 (1), GADD45 (2, 3), WAF1/p21/cip (4, 5), NF-xB (6, 7), and c-jun (8–10). Changes in the expression and activities of the stress-modulated cellular proteins affect cell cycle distribution (11), rate of repair, and DNA replication (12). Such changes also lead to temporal growth arrest (2, 13) or apoptosis (14). While the primary sensors that trigger the stress response are not known, a subset of ras-dependent protein kinases (15–17), including Src (18), Raf (19), J un-NH2-terminal kinase (19, 20), and mitogen-activated protein kinase (21), as well as growth factor receptors (22), were shown to participate.

To understand the nature of the stress response, one needs to identify the mechanisms involved in the activation of stress-related protein kinases. Pathways that were thus far shown to mediate the stress response include cell surface receptors, such as epidermal growth factor receptor. These are capable of activating mitogen-activated protein kinase (22) through ras, Raf, MEK, and ERK, leading to the phosphorylation and activation of the transcription factors TCF/ELK1 and c-Fos (23). A second pathway includes yet unidentified cytoplasmic components that can activate JNK via its own kinases, including MKK4, (17), which leads to the phosphorylation of c-jun. Interestingly, JNK is also activated by osmotic shock as was demonstrated in Chinese hamster ovary cells (24). Upstream JNK kinases also phosphorylate p38-MpK2 (25) which is able to reconstitute osmotic response in yeast strains that lack HOG1, the yeast homologue of p38 (24). HOG1 was reported to share similarity with the mitogen-activated protein kinase activating protein 2-reactivating kinase that mediates the response to heat shock and phosphorylates small heat shock proteins (26). Augmented by ras, mitogen-activated protein kinase and JNK activities contribute to the overall phosphorylation of c-jun (20, 27). JNK phosphorylates c-jun and ATF2 on their NH2-terminal region, whereby these transcription factors are enabled to mediate transcription, replication, and transformation activities (28, 29). To exert such activities, JNK requires interaction with the d domain of c-jun (30). This key component is deleted in c-jun's oncogenic counterpart v-jun (31, 32).

In elucidating mechanisms involved in the cellular response to UV irradiation and HS treatment as two different forms of stress, we have focused on the role of JNK. Although UV and HS are potent inducers of JNK, they are expected to damage cells via alternate pathways.

To distinguish between the effects of UV and HS, we have evaluated the role of oxidative stress which is induced by UV to a greater extent than by HS. Oxidative stress can induce c-jun, c-Fos, NF-xB, early growth response gene (EGR1), and heme oxygenase expression at the transcriptional level (33, 34). This induction correlates with transcriptional activities as demonstrated for J un, and NF-xB (35, 36), and it appears to be mediated via tyrosine kinases in a protein kinase C-dependent manner (37, 38). Poly-(ADP-ribosylation) was also shown to participate in AP1 activation by H2O2 (39). Hydrogen peroxide-mediated cellular changes can be suppressed by Bcl-2 through inhibitory effects on lipid peroxidation at the site of free radical generation within the mitochondria (40). Suppression of mitochondrial activities has been associated with elevated levels of oxidative damage and uncoupling of electron transport from ATP production. It is also linked to the induction of apoptosis (39, 41, 42).

The role of oxygen radicals and membrane components,
which are involved in cellular stress response, was evaluated to enable the identification of mechanisms involved in JNK activation by different forms of stress. The degree of JNK activation by various forms of stress was evaluated to enable the identification of mechanisms involved in JNK activation. The degree of JNK activation by different forms of stress was evaluated.

**MATERIALS AND METHODS**

**Cell Culture**—3T3-4A cells are a mouse fibroblast cell line (carrying an integrated copy of temperature-sensitive polyoma virus; Ref. 43) kindly provided by Dr. Claudio Basilico. These cells are maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and antibiotics. J133 are fibroblast cells, provided through the courtesy of Dr. Alan Lehman. They were obtained from skin biopsies of a patient with Cockayne syndrome. These cells were maintained in minimum Eagle’s medium, supplemented with 5% fetal bovine serum and basal amino acids. Cells that were depleted of mtDNA (143B206) were maintained in Dulbecco’s modified Eagle’s medium supplemented with 5% fetal bovine serum and uridine (50 μg/ml). Chemicals such as genistein (ICN), sodium vanadate, hydrogen peroxide, N-acetylcysteine, Triton X-100, or glutathione in oxidized or reduced forms (Sigma) were dissolved/diluted in 0.1× Hepes, pH 7.4, and added individually or in combination to the cells (as indicated under “Results”). In all cases, solutions were freshly prepared for each experiment.

**UV Irradiation and Heat Shock Treatment**—Cells were exposed to UV irradiation as described previously (44). Briefly, prior to irradiation, the cells were washed with phosphate-buffered saline, and, with the lids off, the culture dishes were placed in marked areas in the tissue culture hood. These areas were precalibrated for the dose of UV, using a germicidal lamp (254 nm) and a UV-C probe (UVP, San Diego, CA). The medium was removed before UV exposure and added again immediately after irradiation. The cells were harvested at the indicated time points. Heat shock treatments were performed by incubating the tissue cultures in a 42°C incubator for 1 h. When Triton X-100 was used in the protocol, the indicated concentration was added to the medium either before or after HS treatment, as indicated under “Results.” In all cases, cultures were washed three times before treatment.

**Oligonucleotide Synthesis**—Oligonucleotides representing a dimer of the AP1 (CTGACTCATCCGTGACTAACT) and NFκB sequences were labeled with [32P]orthophosphate. To standard procedures, the labeled DNA (0.4 ng, 4400 cpm) was added to the mixture for 1 h at 4°C, the immunocomplexes were precipitated by adding 3 M LiCl, 5 μg of protein G-sepharose, and 100 μg of rabbit IgG. The precipitate was then washed five times with 10 mM Tris-HCl, pH 7.5, 150 μM EDTA, and resuspended in 20 μl of reaction buffer. The reaction was initiated by the addition of 3 μg of protein A and antibody to c-Jun and allowed to incubate for 1 h at 4°C. The reaction was terminated by the addition of 5 μg of antibody to c-Jun and 20 μl of 10% SDS-PAGE. The gel was washed for 1 h in 100 mM Tris-HCl, 1% SDS, 8 M urea, and 15% acrylamide gel (10 μg/ml). Equal amounts of proteins (50 μg) were separated in a routine 10% SDS-PAGE. To remove SDS, the gels were washed for 4–12 h in 20 mM Hepes, pH 7.4, with 0.2 mM EDTA. The gel was then incubated in kinase buffer (see above) in the presence of 1 μCi of [γ-32P]ATP (specific activity, 6000 Ci/mmol) for 30 min. Followed by extensive washings in the presence of Dowex 2×8 (Bio-Rad), mixed with DEAE-650 (Supelco), and packed within a dialysis bag (to compete for nonspecific bound [γ-32P]ATP), the gel was silver-stained to ensure proper distribution and that equal amounts of protein substrate were loaded, dried, and autoradiographed.

**Immunoprecipitation and Western Blot Analysis**—Protein extracts (approximately 1×10^7 cell equivalents) were incubated with (as indicated under “Results”) were incubated with antibodies to c-Jun or the cellular extract that contains JNK, in the presence of kinase buffer (20 mM Hepes, 7.5, 1 mM EDTA, 1 mM diethiothreitol, 2 mM MgCl2, 2 mM MnCl2, 5 mM NaF, 1 mM NaVO3, 50 mM NaCl). The beads were pelleted and thoroughly washed with PBS (150 mM NaCl, 16 mM sodium phosphate, pH 7.5, 1% Triton X-100, 2 mM EDTA, 0.1% β-MeOH, 0.2 mM phenylmethylsulfonyl fluoride, and 5 mM benzamidine), before they were incubated with [γ-32P]ATP (50 cpm/ml) in the presence of kinase buffer. These steps were undertaken to ensure that c-Jun phosphorylation is carried out by JNK, which is known to exhibit high affinity to the phosphorylated c-Jun under these conditions (43, 44, 47).

Following extensive washing, the phosphorylated GST-Jun was boiled in SDS sample buffer, and the eluted proteins were run on a 15% SDS-polyacrylamide gel. The gel was dried, and phosphorylation of the Jun substrate was determined by autoradiography. The radioactive signal was quantified with a computerized radioimaging blot analyzer (AMBIS, San Diego, CA). A variation of the phosphorylation assay was performed on in vitro synthesized c-Jun. With the aid of wheat germ extract (Promega) full-length c-Jun was transcribed and translated. This product was phosphorylated with purified JNK of U937 cells, followed by its immunoprecipitation with antibodies to c-Jun and analysis on SDS-PAGE via autoradiography.

An in-gel kinase assay was performed by embedding the c-Jun NH2-terminal region that was purified from the PGE2 Jun within the acrylamide gel (10 μg/ml). Equal amounts of proteins (50 μg) were separated in a routine 10% SDS-PAGE. To remove SDS, the gels were washed for 4–12 h in 20 mM Hepes, pH 7.4, with 0.2 mM EDTA. The gel was then incubated in kinase buffer (see above) in the presence of 1 μCi of [γ-32P]ATP (specific activity, 6000 Ci/mmol) for 30 min. Followed by extensive washings in the presence of Dowex 2×8 (Bio-Rad), mixed with DEAE-650 (Supelco), and packed within a dialysis bag (to compete for nonspecific bound [γ-32P]ATP), the gel was silver-stained to ensure proper distribution and that equal amounts of protein substrate were loaded, dried, and autoradiographed.

**RESULTS**

**Selective Activation of JNK by UV but Not by HS in Cells of a CS Patient**—In studying the activation of JNK in radiation-related disorders, including melanoma, Xeroderma pigmentosum, and Cockayne syndrome, we have identified cells of a Cockayne syndrome patient that exhibited deregulation of c-Jun (47). In assaying for JNK activation, we found that the low basal activity of JNK in these CS-derived cells is induced to considerable levels following UV irradiation, in a pattern similar to that previously observed in HeLa cells (30). Surprisingly, however, heat shock was not able to activate JNK in

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2 V. Adler, L. Dolan, V. Dhar, and Z. Ronai, unpublished observations.
these CS cells (Fig. 1A).

To further elucidate this observation, we have performed in-gel kinase reactions. To this end, Jun (corresponding to the NH₂-terminal region which was eluted from the GST-Jun construct) was fixed in the acrylamide gel prior to separation of proteins prepared from control and UV- or HS-treated CS cells. As shown in Fig. 1B, a 54-kDa band appeared as a doublet; it corresponded to the M₁ of JNK2 (48, 49), and was thus identified as the kinase that phosphorylates c-jun. The lower component of this 54-kDa doublet was clearly induced, thus exhibiting greater intensity in the proteins of UV-treated cells, but substantially lower intensity in HS-treated CS cells. JNK2 was also identified in HeLa cell proteins which we used as an internal control (Fig. 1B). That equal amounts of proteins were separated on each lane was verified via silver staining of the gel before the autoradiography (not shown). The 54-kDa doublet may represent different JNK isozymes that are expressed in these cells (see below) and exhibit different kinase activities. A similar pattern of JNK activation by UV, but not by HS, treatment was observed in cells that were depleted of their mitochondrial DNA (Fig. 1B). The 54-kDa band was also noticed in HS-treated HeLa cells, whereas both 54- and 46-kDa bands (46 kDa corresponding to JNK1) were identified in HS-treated 3T3-4A cells (not shown).

JNK Isozymes Expressed and Bound to c-jun—To identify JNK isozymes that are expressed in the cells used in the present study, we have performed Western blot analysis on pGEX-Jun-unbound proteins. To this end, protein extracts prepared after UV, HS, or sham treatments were incubated with pGEX-Jun as for the kinase reaction (see "Materials and Methods"). Bound proteins were eluted with the aid of N-octyl-β-D-glucopyranoside and analyzed on Westerns, using antibodies to JNK. These antibodies were developed against JNK1 and JNK2, yet apparently they can also identify other JNK isozymes (47). We demonstrate that Jun-bound JNK in 3T3-4A cells consists of the 46- and 54-kDa proteins which correspond to JNK1 and JNK2 (Fig. 2). Comparison of Jun kinases that bind to pGEX-Jun after treatment with different types of stress reveals a similar subset of JNK isozymes (Fig. 2). When the proteins were analyzed via Western (without prior elution), a similar pattern of JNK isozymes was noticed (not shown). The 54-kDa doublet seen in 143B206 cells that were depleted of mitochondrial DNA may represent two JNK isozymes. Interestingly, it is the lower component of this doublet that is found to be induced after UV irradiation in the “in-gel” kinase assay (Fig. 1B), indicating that not all bound JNK isozymes are capable of phosphorylating c-jun. Among these two forms it is the upper component which is bound to pGEX-Jun in the Cockayne syndrome J C133 cells (Fig. 2). A similar reaction with empty pGEX (pGEX2T) did not reveal any JNK binding (not shown). When the protein extracts from HS- and UV-treated cells were first depleted of JNK (using the supernatant retained after immunoprecipitation with antibodies to JNK and protein A/G beads), there was a significant decrease in the degree of JNK activity (not shown).

H₂O₂ Inhibits HS- but Not UV-mediated JNK Activation—Finding two different cell systems that fail to mediate JNK activation after HS, while exhibiting proper activation by UV (Fig. 1), prompted us to explore which cellular components are involved in the response to each of these stimuli. The approach used in the present study employed modulators of different cellular compartments that are known to play a role in the cellular stress response. These included: (i) changes of cytoplasmic components, such as (a) oxygen radicals, one of the pronounced effects of UV-irradiation (254 nm) (b) hydrogen peroxide, and (c) their respective scavengers (NAC, glutathione) and (ii) modulation of membrane components via alteration of receptor organization and anchored proteins. In utilizing this approach we have elected to study a cell system that exhibits proper JNK activation by either form of stress, UV or HS. To this end, we have characterized mouse fibroblast 3T3-4A cells that appear to express both the 46- and 54-kDa forms of JNK, representing JNK1 and JNK2, respectively (Fig. 2). When treated with various levels of H₂O₂ to inflict oxidative damage, no change in the level of JNK activity was noticed (Fig. 3A). In these experiments, cellular proteins were prepared 30 min after exposure, and JNK activity was measured via the phosphorylation of a pGEX-Jun construct which contains the NH₂-terminal region of c-jun (amino acids 5–89). Products of this kinase reaction were separated on SDS-PAGE, autoradiographed, and quantified with a radioimaging blot analyzer. H₂O₂ did not activate JNK, suggesting that not only components induced or modulated by oxidative stress, but additional factors are involved in JNK activation by ultraviolet irradiation. When cells had been treated with low doses of UV irradiation (5–10 J/m²), H₂O₂ was capable of further increasing the level of JNK activity (not shown). However, when added to cells treated with higher UV doses (40–80 J/m²), H₂O₂ did not affect the overall degree of JNK activation (Fig. 3A). The latter sug-
suggests that UV mediates JNK activation via cellular targets that are also affected by H$_2$O$_2$. Interestingly, however, when added to heat shock (42 °C for 60 min) treated cells, H$_2$O$_2$ was a potent inhibitor of JNK activity (Fig. 3B). That H$_2$O$_2$ selectively inhibits JNK activation by HS, but not by UV, substantiates the existence of a variety of cellular components as mediators of JNK activity. HS-mediated JNK activation was also noticed after shorter exposures (30 min; not shown). When cellular proteins of untreated cells were heated at viro (42 °C 1 h), a noticeable increase in JNK activity was seen (Fig. 3B). The cytotoxicity caused by HS treatment was 30%, whereas the UV irradiation doses had induced 80% toxicity after 24 h.

To quantify the degree of JNK activation noticed after HS and UV exposure, we have scanned the respective gels by a radiography blot analyzer. The counts/min obtained under each of these experimental conditions were normalized per counts measured in control (untreated) extracts, resulting in values that represent an n-fold increase in JNK activity (Fig. 3C).

While the role of tyrosine phosphorylation in JNK activation was shown previously (48, 49), we have determined the influence of tyrosine phosphorylation on JNK activity in mouse fibroblast 3T3-4A cells. One of the potent modulators of tyrosine kinases is sodium vanadate. When added to cultures of 3T3-4A cells, sodium vanadate (1 mM) itself caused a significant increase in JNK activities (Fig. 3B), suggesting that the degree of JNK phosphorylation directly contributes to its activity. Although UV is already a potent inducer of JNK activities, an enhanced effect is noted when UV is combined with sodium vanadate. Similar to the UV effect, heat shock treatment of 3T3-4A cells leads to a strong activation of JNK, which is further enhanced by sodium vanadate (not shown). Additional support for the role of tyrosine kinases in JNK activities comes from the use of genistein, a potent inhibitor of tyrosine kinase. When genistein was added to 3T3-4A cells, we have noticed a dose-dependent decrease in JNK activation after UV irradiation (not shown).

NAC Inhibits UV-mediated but Not HS-mediated JNK Activation—To further explore mechanisms involved in JNK activation by HS and UV, respectively, and in light of the ability of hydrogen peroxide to inhibit HS, but not UV-mediated JNK activation, we have used NAC, a potent scavenger of free radicals (6). As shown in Fig. 4A, NAC itself had no significant effects on JNK activity, yet, it strongly inhibited UV-mediated JNK activation. This inhibition was reversible; in fact, adding H$_2$O$_2$ to the cells restored UV-mediated JNK activation in a dose-dependent manner (Fig. 4A). This emphasizes the role of oxidative damage in JNK activation by UV irradiation. In contrast, when NAC was added to HS-treated cells, no significant changes in the levels of JNK activation were noticed (Fig. 4B). However, NAC was capable of restoring H$_2$O$_2$-mediated inhibition of JNK activation after HS. Similar observations were also made when lower doses of NAC (20 mM) were used (not shown). Thus, while oxidative stress is contributing to JNK activation by UV, it is an inhibitory component of HS-mediated JNK activation.

Since NAC acts as a potent scavenger of free oxygen radicals, we have tested how glutathione in its oxidized and reduced forms contributes to JNK activation. When tested by itself, neither form induced JNK activation. Yet, when added to UV-treated cells, the oxidized form of glutathione had an additive
effect on JNK activity. The reduced form of glutathione had an inhibitory effect on UV-mediated JNK activation. When added to HS-treated cells, the oxidized form of glutathione was a potent inhibitor of JNK, whereas the reduced form increased JNK activation (not shown). These observations further support our former experiments and suggest that HS utilizes a different cellular component than UV in mediating JNK activation.

Triton X-100 as Selective Inhibitor of UV-mediated JNK Activation—To further explore mechanisms that may be involved in UV- and HS-mediated JNK activation, we have tested the effect of non-ionic detergent which, at low concentrations, alters membrane components and reorganizes its associated proteins, including receptors (50–52) and p21ras protein (53). To this end we have pretreated 3T3-4A cells with various concentrations of Triton X-100 for 5 min, before applying either UV or HS treatment. Proteins were then prepared from the cells and tested for JNK activation. While JNK activation by HS was not affected by pretreatment of the cells with Triton X-100 (Fig. 5A), the JNK-activating capacity of UV was inhibited in a dose-dependent manner (Fig. 5B). A dose of 0.032% Triton X-100 caused a 60% inhibition, whereas a dose of 0.004% Triton yielded 10% inhibition of the JNK activation by UV irradiation (Fig. 5C). Interestingly, the effect of Triton on JNK activation by UV was seen only in cells that were pretreated with the detergent in culture. When Triton was added to the cells after UV or HS treatment, only minor changes in JNK activity were noticed (Fig. 5D). When protein extracts prepared from either UV- or HS-treated cells were incubated with Triton X-100 in vitro, prior to the JNK assay, no change in JNK activation was seen (not shown). To examine possible changes in membrane that may have caused leakage upon Triton X-100 exposure we have measured uptake of [α-32P]dCTP (1 μCi/assay) into 3T3-4A cells (3 × 10^6) after either 5- or 10-min incubation. Under normal growth conditions the amount of radioactive material that was incorporated into the cells was similar to that measured in cells that were preincubated with 0.04% Triton X-100 (which causes about 50% inhibition of JNK activity after UV). A significantly higher incorporation (100-fold) was observed when cells were pretreated with 0.25% of Triton X-100 (not shown). These experiments suggest that the concentration proven effective in inhibiting UV-mediated JNK activities do not allow penetration of nucleotides and therefore is not expected to cause any leakage/changes in cellular ATP content either. Triton X-100, at the concentration of up to 0.04%, may affect phospholipids or other membrane associated components which are required to mediate UV, but not heat shock, response. Similarly, Nonidet P-40 was as potent as Triton X-100 in the selective inhibition of UV-mediated JNK activation; whereas SDS was significantly less effective (not shown). Further support for changes in membrane-associated components came from the finding that Triton X-100 inhibits the ability of sodium vanadate to activate JNK (Fig. 5D). The kinase-activating ability of sodium vanadate had previously been attributed to changes at the membrane receptor level (54). Sodium vanadate-mediated JNK activation was inhibited to a greater degree than UV-mediated JNK activation (Fig. 5D). The latter suggests that UV can mediate JNK activation via other components while sodium vanadate effects are entirely dependent on alteration of membrane components by Triton X-100. This is supported by the finding that a combination of UV and sodium vanadate exerts an additive effect on JNK activity (not shown).

Effects of UV, HS, and H2O2 Treatments on c-Jun Phosphorylation in Vivo and on DNA Binding Activity—The next set of experiments was designed to evaluate whether JNK phosphorylation contributes to c-Jun unactivities. Since previous studies had documented the effects of different forms of stress on the level of c-Jun transcripts, we have measured the level of these transcripts as a control for the efficiency of the treatments administered in the present study. Northern blot analysis with RNA prepared 1 h after treatments revealed a noticeable increase in the level of c-Jun transcripts after UV and H2O2, but not after HS, treatment (not shown).

To determine whether c-Jun phosphorylation in vivo reflects
H2O2 was not. The degree of phosphorylated c-Jun is even
phosphorylation by JNK in vitro on 3T3-4A cells in [32P]orthophosphate for 60 min after their treatment.
The patterns noticed in the JNK assays, we have incubated 3T3-4A cells in [32P]orthophosphate for 60 min after their exposure to HS, H2O2, or UV treatment. [32P]Orthophosphate was added to 3T3-4A cells that were grown in 150-mm dishes in phosphate-free medium. After 60-min incubation (unless otherwise indicated) proteins were prepared, immunoprecipitated with antibodies to c-Jun, and separated on SDS-PAGE, which was then subjected to autoradiography. The molecular mass of the band shown was calculated to be 39 kDa. On the same gel (a few lanes apart that were spliced out) in vitro translated, and immunoprecipitated c-Jun was also separated as an internal control. B. binding to AP1 target sequence after HS, H2O2, or UV treatment. Proteins prepared from mouse fibroblast 3T3-4A cells 1 h after exposure to HS, H2O2, or UV irradiation were incubated (5 μg) with a [32P]-labeled AP1 target sequence in the presence of nonspecific DNA and DNA binding buffer (lane marked with ). Competition experiments were performed using a 50-fold excess of the respective nonlabeled target sequence and using AP1 or NFκB as indicated in the second and third lanes of each panel. Upon their separation on a 5% PAGE, protein complexes were visualized via autoradiography. The first three lanes from the left represent free probes; the arrow points to the position of the major complex. The patterns noticed in the JNK assays, we have incubated 3T3-4A cells in [32P]orthophosphate for 60 min after their exposure to UV, HS, or H2O2. Following extensive washing, proteins were prepared and the c-Jun protein was then immunoprecipitated with the aid of antibodies to c-Jun and with protein A/G beads. Immunoprecipitated material was separated on SDS-PAGE and autoradiographed. As shown in Fig. 6A, clear differences were noticed in the overall level of phosphorylated c-Jun after these treatments. Both UV and HS were potent inducers of c-Jun phosphorylation in vivo, whereas H2O2 was not. The degree of phosphorylated c-Jun is even lower after H2O2 treatment when compared with that precipitated from control proteins. The doublet seen in c-Jun after H2O2 treatment indicates a phosphorylated form, although the overall level of phosphorylation was substantially lower than that found after UV or HS treatment, as seen in shorter exposures (not shown). The pattern seen here is in agreement with that observed in vitro after using pGEX-c-Jun (NH2-terminal region) as a substrate (Figs. 3 and 4). Longer incubation periods did not affect the overall level of phosphorylation (Fig. 6A), suggesting that the immediate response of JNK is the key determinant in c-Jun phosphorylation. The position of c-Jun was confirmed by both the molecular mass of 39 kDa and by the use of in vitro translated c-Jun which was subjected to phosphorylation by JNK in vitro and separated in parallel on SDS-PAGE (Fig. 6A).

As a measure for transcriptional activities that are mediated by proteins which interact with the AP1 target sequence, we have performed EMSA. In these assays we have compared the binding of proteins prepared after HS, UV, or H2O2 treatments. As demonstrated in Fig. 6B, whereas binding activities have increased after each of these treatments, proteins derived after HS and H2O2 treatments were more potent than those obtained after UV exposure. The specificity of this reaction was demonstrated through the use of AP1 and NFκB as specific and nonspecific competitors, respectively (Fig. 6B). While AP1 was capable of inhibiting binding to the AP1 target sequence, NFκB had no effects. A similar pattern was observed when the NFκB target sequence was used (not shown).

**DISCUSSION**

The present study provides direct evidence for the existence of alternate pathways in JNK activation by different forms of stress, as shown for UV and HS. The distinction of these alternate pathways lies in the requirement for membrane-associated components for UV-mediated, but not for HS-mediated, JNK activation. This can be concluded on the basis of the ability of Triton X-100 to block JNK activation by both vanadate and UV, when these are administered before, but not after, UV irradiation. As vanadate has been shown to mediate its effects via cell membrane receptors (54), changes in such receptors are expected to abolish its activities as was observed in our experiments (Fig. 5). Moreover, it has been previously demonstrated that Triton X-100 alters membrane organization and modulates the ability of p21ras to mediate mitogen-activated protein kinase activation (53). Similar detergents were shown to alter the ability of the epidermal growth factor receptor to dimerize and autophosphorylate (50–52). It is likely, therefore, that due to the effect of Triton X-100 on receptor organization, the receptor can no longer dimerize to transphosphorylate and thus fail to activate downstream protein kinases. Furthermore, we have recently shown the association between p21ras and JNK (55). We, therefore, cannot exclude the possibility that membrane-anchored components, such as p21ras, were also altered, so that they could not associate/activate JNK. Accordingly, HS-mediated JNK activation may utilize ras-independent pathways which were previously documented (25, 56). We conclude, therefore, that UV requires cellular membrane signaling, whereas HS does not. It is, therefore, surmised that different kinases are involved in JNK activation in response to alternate forms of stress as shown here for UV and HS.

UV-mediated JNK activation also involves oxidative damage, as demonstrated by the inhibition of this response through the radical scavenger NAC. In contrast, oxidative damage by H2O2 inhibits HS-mediated JNK activation, and we have shown that NAC is unable to inhibit HS-mediated JNK activation. That oxygen radicals, such as H2O2, inhibit the HS-mediated pathway suggests that cellular components other than catalase, superoxide dismutase, or glutathione peroxidase (which are involved in the response to UV and its oxidative damage) are playing an important role in the regulation of JNK activation. That there are different cellular sensors for UV and HS damage can also be deduced from the observation that in vitro HS treatment of cellular extracts can induce JNK (Fig. 3B), whereas UV-mediated JNK activation requires a complete and precise in vivo setting of membrane components as demonstrated in the Triton X-100 experiments. The different responses to HS and UV are not mediated by different JNK isoforms, as both the 46-kDa and the 54-kDa proteins representing JNK1 and JNK2, respectively, were equally capable of binding to the NH2-terminal region of Jun.

Both UV and HS were potent inducers of JNK activation
leading to increased c-Jun phosphorylation in vivo and to a corresponding increase in binding to the AP1 target sequence. The degree of JNK activity and, in turn, c-Jun phosphorylation is expected to be tightly regulated by specific protein phosphatases, which are also induced by external stress, as shown for HS. For example, protein phosphatase 2C is induced by HS in Schizosaccharomyces pombe (57). Similarly, PAC1, a mitogen-induced nuclear protein with tyrosine phosphatase activity, is also induced by HS (58).

Interestingly, in the cell system studied here, H$_2$O$_2$ was neither capable of activating JNK, nor was it able to increase in vivo labeling of c-Jun; however, it was as potent as UV or HS in forming complexes with AP1 target sequence DNA. It is thus possible that although complexes of similar size were formed in all cases, the binding observed in our EMSA may have been mediated not only by c-Jun proteins, but by other members of the AP1 or ATF families that can interact with the AP1 target sequence and have a M$_r$ similar to that of c-Jun. However, the overall transcriptional signal is expected to be different in the case of H$_2$O$_2$ than in that of UV or HS.

A possible clue as to the nature of the different cellular components involved in JNK activation evolved from the reactions with different cell lines. We have shown this for CS cells which mediate selective activation of JNK2 by UV but not by HS. A similar observation was made in cells that lack mitochondria DNA and exhibit JNK activation after UV exposure but not after HS treatment (Fig. 1B and Ref. 2). These observations suggest that HS activation of JNK requires mitochondria-related components, which are not available upon deregulation of the mitochondria. Impaired mitochondrial function has been widely documented for aged cells (41). The latter coincides with our observations that higher levels of oxidative stress inhibit HS-mediated JNK activities and that aged cells abort heat shock response (59–61).

Overall, that alternate pathways mediate JNK activation by different forms of stress indicates the existence of multiple cellular sensors which are triggered by the respective stimuli. UV-induced JNK activation is dependent on membrane-associated components and free oxygen radicals (our present study) and DNA damage per se (47), whereas mitochondria-related components appear to be involved in the HS response.

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Different Pathways in JNK Activation by UV and Heat Shock