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Activation of Extracellular Signal-regulated Kinase by Ultraviolet Is Mediated through Src-dependent Epidermal Growth Factor Receptor Phosphorylation

ITS IMPLICATION IN AN ANTI-APOPTOTIC FUNCTION*

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Ultraviolet (UV) irradiation stimulates stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK), which is a member of the mitogen-activated protein kinase (MAPK) superfamily and implicated in stress-induced apoptosis. UV also induces the activation of another MAPK member, extracellular signal-regulated kinase (ERK), which is typically involved in a growth-signaling cascade. However, the UV-induced signaling pathway leading to ERK activation, together with the physiological role, has remained unknown. Here we examined the molecular mechanism and physiological function of UV-induced ERK activation in human epidermoid carcinoma A431 cells that retain a high number of epidermal growth factor (EGF) receptors. UV-induced ERK activation was accompanied with the Tyr phosphorylation of EGF receptors, and both responses were completely abolished in the presence of a selective EGF receptor inhibitor (AG1478) or the Src inhibitor PP2 and by the expression of a kinase-dead Src mutant. On the other hand, SAPK/JNK activation by UV was partially inhibited by these inhibitors. UV stimulated Src activity in a manner similar to the ERK activation, but the Src activation was insensitive to AG1478. UV-induced cell apoptosis measured by DNA fragmentation and caspase 3 activation was enhanced by AG1478 and an ERK kinase inhibitor (U0126) but inhibited by EGF receptor stimulation by the agonist. These results indicate that UV-induced ERK activation, which provides a survival signal against stress-induced apoptosis, is mediated through Src-dependent Tyr phosphorylation of EGF receptors.

Exposure of mammalian cells to ultraviolet (UV) irradiation elicits a variety of responses including apoptosis that is morphologically characterized by cell-death patterns of DNA fragmentation, chromatin condensation, and membrane blebbing. Several intracellular molecules responsible for UV-induced cell apoptosis have recently been identified by means of gene targeting in mice. For example, cytochrome *c* (1), Apaf-1 (2), the initiator caspase 9 (3, 4), and the effector caspase 3 (5) have been demonstrated to function as apoptosis-operating machinery in the late signaling steps because murine cells lacking these genes are insensitive to UV irradiation. In addition, the stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK),[1] which is a member of the mitogen-activated protein kinase (MAPK) superfamily, appears to play an important role in UV response (6, 7). JNK-deficient murine embryonic fibroblasts exhibited a defective apoptosis in response to UV irradiation and impaired mitochondria functions (8). We have also reported that two MAPK activators, SEK1/MKK4 and SEK2/MKK7, are both required for UV-induced synergistic activation of SAPK/JNK in murine ES cells (9). Thus, a signaling pathway leading to cell apoptosis has been proposed by Davis (10) and other groups as follows: UV → SEK1 and MKK7 → SAPK/JNK → mitochondria → cytochrome *c* → Apaf-1 → caspase 9 → caspase 3.

In an early study that had been reported before the discovery of SAPK/JNK involvement in cell apoptosis, Devary *et al.* (11) observed that UV irradiation induces the activation of Src, Ha-Ras, and Raf-1 and the phosphorylation of c-Jun in HeLa cells. This finding suggested that UV response also has a cell survival function and is initiated at the cell surface membrane rather than within the nucleus. The same group (12) has recently shown that the strong SAPK/JNK activation by UV was triggered by the activation of membrane receptors for epidermal growth factor (EGF), tumor necrosis factor, and interleukin-1. Thus, the signaling pathway from UV to SAPK/JNK stimulation appeared to involve the activation of cell surface tyrosine kinase receptors.

On the other hand, the extracellular signal-regulated kinase (ERK), another member of the MAPK superfamily, is typically activated in response to growth factors such as EGF and platelet-derived growth factor (PDGF). Interestingly, recent studies (13) have revealed that UV irradiation induces the activation of

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[1] The abbreviations used are: SAPK/JNK, stress-activated protein kinase/c-Jun NH_2-terminal kinase; ERK, extracellular signal-regulated kinase; SEK, SAPK/ERK kinase; MAPK, mitogen-activated protein kinase; MKK, MAPK kinase; MBP, myelin basic protein; MCA, aminomethylcoumarin; ES, embryonic stem; EGF, epidermal growth factor; PI 3-K, phosphatidylinositol 3-kinase; PP2, 4-amino-5-(4-chloropheny)-7-(*t*-butyl)pyrazolo [3,4-D[rsqb] pyrimidine; Ab, antibody; GST, glutathione-*S*-transferase.

ERK in a number of cell types and that the UV-induced ERK activation also involves the activation of EGF receptors. However, upstream molecules responsible for the EGF receptor stimulation and the physiological role(s) of ERK activation in UV irradiation have not been fully determined.

In the present study, we examined the effects of various inhibitors for EGF receptors, tyrosine kinases, and phosphatidylinositol 3-kinase (PI 3-K) on the UV-induced activation of ERK and SAPK/JNK in murine ES cells and the human epidermoid carcinoma cell line A431. The contribution of UV-induced ERK activation to cell apoptosis was further investigated by measuring DNA fragmentation and caspase activity. Our present results clearly indicate that the UV-induced activation of ERK is mediated through the phosphorylation of EGF receptors by Src tyrosine kinase and that ERK activation functions as a cell survival signal rather than in apoptosis. Instead, the UV-induced activation of SAPK/JNK, which appears to contribute to the apoptotic pathway, was partially dependent on the Src-induced EGF receptor activation.

EXPERIMENTAL PROCEDURES

Cell Culture and Materials—The murine ES cell line E14K (wild type) was maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 15% fetal calf serum and leukemia inhibitory factor as described previously (14). The human cell line A431 was maintained in DMEM containing 10% fetal calf serum. The cells grown at 80% confluent were serum-starved in DMEM containing 0.1% bovine serum albumin and 20 mM Hepes-NaOH (pH 7.4) for 12 h. The cells were stimulated with UV-C type (1 kJ/m^2 for ~1 min or 0.1 kJ/m^2 for ~0.1 min) using a 254-nm wavelength DNA Stratalinker (Stratagene) in the presence or absence of inhibitors and subjected to the assays of kinase activity, Tyr phosphorylation, caspase 3 activity, and DNA fragmentation as described below.

Mouse EGF was purchased from Toyobo Co., Ltd. The EGF receptor-specific inhibitor AG1478 and the Src family-specific inhibitor 4-amino-5-(4-chloropheny)-7-(*t*-butyl)pyrazolo [3,4-D] pyrimidine (PP2) were obtained from Calbiochem. Genistein, wortmannin, and the MAPK/ERK kinase (MEK)-specific inhibitor U0126 were purchased from Wako Chemicals, Sigma, and Promega, respectively. Antibodies (Abs) specific for ERK1 (C-16), ERK2 (C-14), SAPK/JNK1 (C-17 and FL), Lyn kinase (cat. no. 44), and EGF receptor (cat. no. 1005) were purchased from Santa Cruz Biotechnology. Anti-v-Src (OP07) and anti-EGF receptor (GR01) Abs were from Calbiochem. An anti-phospho-Tyr (PY20) Ab was from BD Transduction Laboratories.

Construction of Plasmids and Transfection—A cDNA encoding a kinase-defective, dominant inhibitory form of Src (SrcK298M), with Lys-298 mutated to Met, was cloned into mammalian expression vector pME-18S. For gene expression analysis, A431 cells were plated to ~50% confluence and transfected 1 day later with expression vectors using the LipofectAMINE method (Invitrogen). The cells, after being cultured for a half-day, were serum-starved for 12 h and subsequently stimulated with UV irradiation (1 kJ/m^2). Cell extracts were prepared for immunoprecipitation and immunoblotting.

Immunoprecipitation and Immunoblotting—A431 cells (~1×10^6 cells) were suspended at 4 °C in 0.5 ml of a lysis buffer consisting of 1% Nonidet P-40, 80 mM Tris-HCl (pH 7.5), 10 mM EDTA, 100 mM NaF, 4 mM Na_3VO_4, 4 μg/ml aprotinin, and 2 mM phenylmethane sulfonyl fluoride. The cell lysates were incubated with the anti-EGF receptor monoclonal Ab (GR01) at 4 °C for 2 h, and the immunocomplexes were washed three times with the lysis buffer. The samples were analyzed by SDS-PAGE and immunoblotting. Proteins were electrophoretically transferred to a polyvinylidene difluoride membrane (Bio-Rad) and probed with the anti-EGF receptor (number 1005), anti-phospho-Tyr (PY20), and anti-v-Src (OP07) Abs. The bands were visualized by SuperSignal West Pico chemiluminescent substrate for the development of immunoblots utilizing a horseradish peroxidase-conjugated second Ab according to the manufacturer's instructions (Pierce).

Assay of SAPK/JNK and ERK Activity—MAPK proteins were immunoprecipitated at 4 °C for 2 h using the anti-SAPK/JNK (C-17) and anti-ERK (C-16 and C-14) Abs. The SAPK/JNK and ERK activities in the precipitated fractions were measured with GST-c-Jun and the myelin basic protein (MBP), respectively, as *in vitro* substrates in the presence of 60 μM [γ-^{32}P]ATP as described previously (14, 15). The amounts of the precipitated SAPK/JNK and ERK that had been monitored by immunoblotting with the anti-SAPK/JNK (FL) and anti-ERK (C-16 and C-14) Abs, respectively, were almost constant in a series of the present experiments.

Assay of Src and Lyn Activity—A431 cells were lysed in a buffer consisting of 50 mM Tris-HCl (pH 8.0), 1% Nonidet P-40, 1 mM EDTA, 100 mM NaCl, 0.2 mM Na_3VO_4, and 4 μg/ml aprotinin. Cell lysates were subjected to immunoprecipitation with the anti-Src (OP07) and anti-Lyn (cat. no. 44) Abs. The immunoprecipitants were washed twice with the lysis buffer, twice with a tyrosine kinase buffer consisting of 50 mM Hepes-NaOH (pH 7.4), 10 mM $MgCl_2$, and 3 mM $MnCl_2$ and resuspended in 30 ml of the tyrosine kinase buffer containing 2.5 μg of acid-treated enolase and 60 μM [γ-^{32}P]ATP. The reaction mixture was incubated for 10 min at 30 °C. Proteins were separated by SDS-PAGE and analyzed by autoradiography. The amounts of the precipitated Src and Lyn were normalized by immunoblotting with the anti-Src and anti-Lyn Abs.

DNA Fragmentation Assay—A431 cells (~1×10^6 cells), which had been attached to and detached from culture dishes after UV irradiation, were collected by means of incubation with trypsin/EDTA and centrifugation, respectively. The cells were mixed, washed twice with phosphate-buffered saline, and collected by centrifugation. After removing the supernatants, the cells were lysed in 0.33 ml of a buffer consisting of 5 mM Tris-HCl (pH 7.4), 20 mM EDTA, and 0.5% Triton X-100. The cell lysates were subjected to phenol extraction and ethanol precipitation for DNA purification. The precipitated DNA was suspended in 20 ml of H_2O and treated with 20 μg/ml of RNase for 30 min at room temperature. The DNA samples (20 μl) were subjected to electrophoresis on 1.5% agarose gels and visualized by a UV illuminator.

Assay of Caspase 3 Activity—A431 cells (~1×10^6 cells) in culture dishes were harvested as described above and washed twice with phosphate-buffered saline. The cells, after being resuspended in 50 μl of a buffer consisting of 10 mM Tris-HCl (pH 7.5), 5 mM dithiothreitol, and 1 mM phenylmethane sulfonyl fluoride, were frozen in liquid nitrogen and thawed at 37 °C three times. The cell lysates (40 μg) were incubated at 37 °C for 30 min with 20 μM DEVD-MCA in the final volume of 50 μl in 20 mM Hepes-NaOH (pH 7.4), 2 mM dithiothreitol, and 10% glycerol. The reaction was terminated by adding 450 μl of ice-cold H_2O, and substrate cleavage leading to the release of free MCA (excitation 355 nm, emission 460 nm) was monitored at room temperature.

All experiments were repeated at least three times with different batches of the cell samples, and the results were fully reproducible. Hence, most of the data shown are representative of several independent experiments.

RESULTS

Loss of UV-induced ERK Activation by Inhibition of EGF Receptor Tyrosine Kinase in ES Cells—We recently reported that UV irradiation activates not only SAPK/JNK but also ERK in murine ES cells. The former activation was more profoundly and slowly observed than the latter one (9). Moreover, activation of EGF receptors appears to be involved in UV responses (12, 13). To investigate signaling pathways leading to the activation of two MAPKs, the effect of an EGF receptor kinase inhibitor, AG1478, was first investigated in ES cells. ERK and SAPK/JNK activities in response to UV irradiation (1 kJ/m^2) were measured at 5 and 15 min, respectively, by their abilities to phosphorylate MBP and GST-c-Jun as substrates. In accordance with the previous report (9), UV irradiation induced the activation of two MAPKs in ES cells; the ERK activation (Fig. 1*A*, *lane 2*) was weaker than the SAPK/JNK activation (Fig. 1*B*, *lane 2*) but significant and reproducible. AG1478 almost completely inhibited the UV-induced ERK activation (Fig. 1*A*, *lane 3*). However, inhibition of the SAPK/JNK activation by AG1478 was partial (Fig. 1*B*, *lane 3*). When ES cells were stimulated with EGF, there was significant stimulation of ERK (Fig. 1*A*, *lane 4*), suggesting the presence of growth factor receptors in the ES cells. In contrast, EGF-induced SAPK/JNK activation was very weak compared with UV irradiation (Fig. 1*B*, *lane 4*). These results suggest that the activation of EGF receptors is essentially required for UV-induced ERK activation and partially involved in SAPK/JNK activation in ES cells.

Loss of UV-induced ERK Activation by the Inhibition of the EGF Receptor and Src in A431 Cells—The involvement of EGF receptors in UV-induced ERK activation was further investigated in A431 cells, which express a large number of the

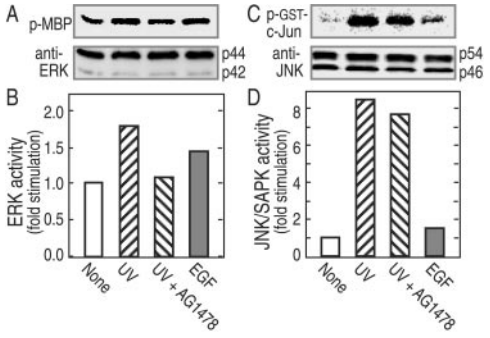

FIG. 1. **UV-induced ERK and SAPK/JNK activation in murine ES cells.** ES cells starved for 12 h were treated without (*lanes 1*, *2*, and *4*) or with (*lane 3*) AG1478 (200 nM for 20 min), and stimulated with UV-C irradiation (1 kJ/m^2; *lanes 2* and *3*) or EGF (5 ng/ml for 5 min; *lane 4*). *A* and *C*, the cells were lysed and immunoprecipitated with anti-ERK (*A*) and anti-SAPK/JNK (*C*) Abs. ERK and SAPK/JNK activities in the precipitated fractions were measured with the MBP and GST-c-Jun, respectively, as the substrates in the presence of [γ-^{32}P]ATP (*upper panels*). The amounts of the precipitated ERK (*p42* and *p44*) and SAPK/JNK (*p46* and *p54*) were monitored by immunoblotting with anti-ERK and anti-SAPK/JNK Abs, respectively (*lower panels*). *B* and *D*, the ERK and SAPK/JNK activities were expressed as the fold stimulation compared with the control levels observed without UV irradiation.

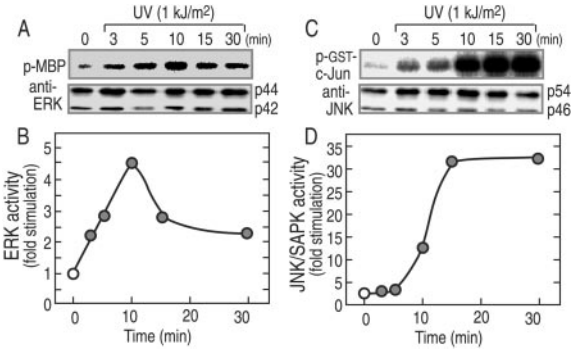

FIG. 2. **Time courses of UV-induced ERK and SAPK/JNK activation in A431 cells.** A431 cells that had been starved for 12 h were stimulated with UV irradiation (1 kJ/m^2) and incubated at 37 °C for the indicated times. ERK and SAPK/JNK activities were measured as described in the Fig. 1 legend.

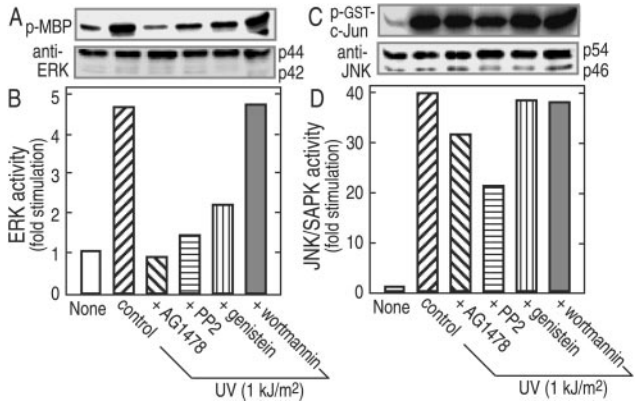

FIG. 3. **The effects of various inhibitors on UV-induced ERK and SAPK/JNK activation in A431 cells.** A431 cells were first treated without (*lanes 1* and *2*) or with AG1478 (200 nM for 20 min; *lane 3*), PP2 (50 μM for 30 min; *lane 4*), genistein (200 μM for 30 min; *lane 5*) or wortmannin (200 nM for 20 min; *lane 6*). The cells were stimulated with UV irradiation (*lanes 2–6*; 1 kJ/m^2). ERK and SAPK/JNK activities were measured as described in the Fig. 1 legend.

growth factor receptors. Fig. 2 shows the time courses of ERK and SAPK/JNK activation in response to UV irradiation in A431 cells. UV-induced ERK activation was more evident in A431 cells than in ES cells, and maximally 4–5-fold stimulation was transiently observed at 10 min (Fig. 2*B*). UV irradiation also induced marked stimulation of SAPK/JNK activity (>30-fold increase), and this response was characterized by a slow onset but sustained stimulation; the maximum level was observed at 15 min and maintained until 30 min (Fig. 2*D*).

Fig. 3 summarizes the effects of various kinase inhibitors on the UV-induced ERK and SAPK/JNK activation in A431 cells. The EGF receptor kinase inhibitor AG1478 completely inhibited the UV-induced ERK activation (Fig. 3*B*), as has been observed in ES cells. However, inhibition by AG1478 of UV-induced SAPK/JNK activation was again partial in A431 cells (Fig. 3*D*). Interestingly, a Src family kinase inhibitor, PP2, inhibited the ERK activation almost completely and also induced a 50% inhibition of SAPK/JNK activity. UV-induced ERK activation but not SAPK/JNK activation was sensitive to a non-selective tyrosine kinase inhibitor, genistein. However, a PI 3-K inhibitor, wortmannin, did not exert any influence on the ERK or SAPK/JNK activation. Thus, it is very likely that the UV-induced ERK activation is completely dependent on the activation of EGF receptors and Src or Src family tyrosine kinase(s). On the contrary, the UV-induced SAPK/JNK activation appeared to be partially dependent on the tyrosine kinases.

UV-induced Src Activation Is Located Upstream of EGF Receptors—To identify the Src family member responsible for the EGF receptor activation, we next investigated whether Src activity is stimulated by UV irradiation in A431 cells. Lyn kinase activity was also investigated because it has been reported that tyrosine kinase is involved in DNA damage-induced activation of SAPK/JNK (16). The kinase activities were measured with enolase as the substrates in fractions immunoprecipitated with specific antibodies. Fig. 4 shows the time courses of Src and Lyn kinase activities after UV irradiation. UV-induced Src activation was apparently observed at 1 min, and there was a progressive increase in the kinase activity as the incubation time was increased. This time course was comparable with that of UV-induced ERK activation. However, no activation of Lyn was observed during the 10-min incubation. We also questioned the effect of AG1478 on the UV-induced Src activation (Fig. 4, *right column*). In contrast to the UV-induced ERK activation, the EGF receptor kinase inhibitor did not exert any influence on the Src activation. These results suggest that UV-induced Src activation may be located upstream of EGF receptors.

We also investigated whether UV irradiation induces Tyr phosphorylation of EGF receptors in comparison with the action of the receptor agonist. The Tyr-phosphorylated form of EGF receptors was measured with a monoclonal Ab (PY20) that specifically recognized phosphorylated Tyr residues. As shown in Fig. 5*A*, EGF receptors were rapidly Tyr-phosphorylated after UV irradiation. The UV-induced phosphorylation was completely abolished in the presence of AG1478. In addition, PP2 and genistein, which inhibited UV-induced ERK activation, had inhibitory effects on the receptor phosphorylation. However, wortmannin failed to inhibit the UV-induced receptor phosphorylation. The effects of these inhibitors on the phosphorylation of EGF receptors by the native agonist EGF were also examined (Fig. 5*B*). As expected, AG1478 markedly reduced the EGF-induced receptor phosphorylation. In contrast, PP2 and genistein did not inhibit the action of EGF. These results clearly excluded the possibility that the loss of UV-induced receptor phosphorylation observed in the presence of PP2 or genistein resulted from the impairment of EGF receptor/kinase integrity. Thus, it is very likely that Src is located upstream of EGF receptors and that the tyrosine kinase may

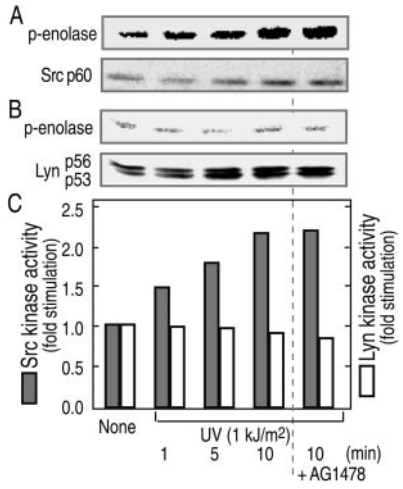

FIG. 4. **The effect of UV irradiation on Src family tyrosine kinases in A431 cells.** A431 cells were first treated without (*lanes 1–4*) or with (*lane 5*) AG1478 (200 nM for 20 min). The cells were stimulated with UV irradiation (*lanes 2–5*; 1 kJ/m^2) and further incubated at 37 °C for the indicated times. *A* and *B*, the cells were lysed and immunoprecipitated with anti-Src (*A*) and anti-Lyn (*B*) Abs. Src and Lyn kinase activities in the precipitated fractions were measured with enolase as their substrates in the presence of [γ-^{32}P]ATP (*upper panels*). The amounts of the precipitated Src (*p60*) and Lyn (*p53* and *p56*) were monitored by immunoblotting with their Abs (*lower panels*). *C*, the activities are expressed as the fold stimulation compared with the control levels observed without UV irradiation.

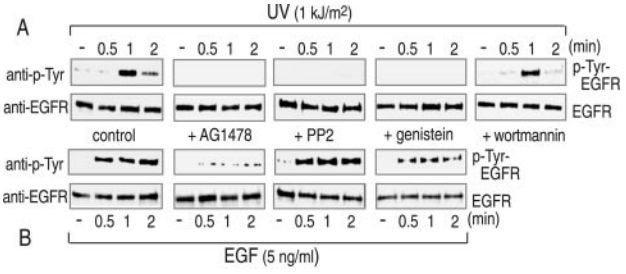

FIG. 5. **The effects of various inhibitors on UV- and agonist-induced EGF receptor phosphorylation in A431 cells.** A431 cells were first treated without (*control*) or with AG1478 (200 nM for 20 min), PP2 (10 μM for 30 min), genistein (200 μM for 30 min) or wortmannin (200 nM for 20 min). The cells were stimulated with UV irradiation (*A*, 1 kJ/m^2; *lanes 2–4*) or EGF (*B*, 5 ng/ml; *lanes 2–4*) and further incubated at 37 °C for the indicated times. The cells were lysed and immunoprecipitated with an anti-EGF receptor Ab. Tyrosine-phosphorylated EGF receptors were determined by an anti-phospho-Tyr Ab (*upper panels*), and the amounts of the precipitated EGF receptors (*EGFR*) were monitored by immunoblotting with the Ab (*lower panels*).

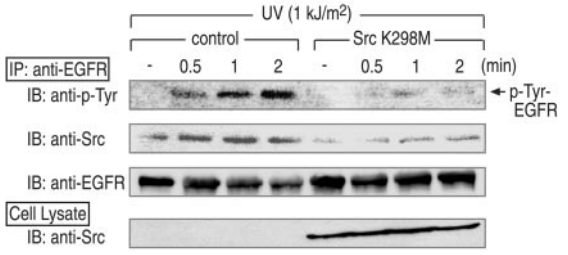

FIG. 6. **The effects of kinase-dead Src, SrcK298M, on UV-induced EGF receptor phosphorylation in A431 cells.** A431 cells (~1 × 10^6 cells) were transfected with 3 μg of pME-18S vector alone (*control*) or pME-18S SrcK298M vector. The transfected cells, after being starved for 12 h, were stimulated with UV radiation (1 kJ/m^2) and further incubated at 37 °C for the indicated times. The cells were lysed and immunoprecipitated with an anti-EGF receptor Ab. Tyrosine-phosphorylated EGF receptors were determined by an anti-phospho-Tyr Ab, and the amounts of the precipitated Src kinases or EGF receptors (*EGFR*) were monitored by immunoblotting with their specific Abs. The cell lysates were also analyzed by immunoblotting with an anti-v-Src Ab.

phosphorylate the growth factor receptors directly or indirectly. Furthermore, we examined the effect of kinase-dead Src, SrcK298M, on the UV-induced phosphorylation of EGF receptors. As shown in Fig. 6, the UV-induced phosphorylation was completely abolished by an overexpression of SrcK298M. Interestingly, UV-induced association of Src with EGF receptor-immunocomplex was also decreased by the expression of SrcK298M. These results clearly show that Src is involved in the UV-induced phosphorylation of EGF receptors and suggest that the translocation of Src into the EGF receptor complex triggers the phosphorylation.

Anti-apoptotic Role of UV-induced ERK Activation—To elucidate the physiological role of UV-induced ERK activation, we finally examined whether the inhibition or stimulation of ERK activation exerts its influence on UV-induced apoptosis. A431 cells were first treated with AG1478 or an ERK kinase inhibitor, U0126, and subjected to weak UV irradiation (0.1 kJ/m^2). DNA fragmentation was monitored by electrophoresis, and caspase 3 activity was also measured with DEVD-MCA as a substrate. As shown in Fig. 7*A*, DNA fragmentation observed with weak UV irradiation was markedly enhanced by the prior treatment of A431 cells with AG1478 or U0126. Potentiation by AG1478 or U0126 was also observed in terms of UV-induced caspase 3 activation. As shown in Fig. 7*B*, the time-dependent caspase activation in response to UV irradiation was significantly enhanced by the kinase inhibitors. These results indicate that Src and EGF receptor-dependent ERK activation is implicated in an early protective role against UV irradiation.

The above idea predicted that stimulation of EGF receptors with the agonist might protect cell apoptosis from UV irradiation. To test this prediction, A431 cells were pretreated with EGF before UV irradiation. As shown in Fig. 7*C*, UV-induced activation of caspase 3 was dramatically inhibited by the prior treatment (3 or 6 h) of the cells with EGF (5 ng/ml). The protective effect of EGF became more apparent as the pretreatment time was prolonged (Fig. 7*C*) or the concentration of EGF was increased (data not shown). These results again indicate that EGF receptor-dependent ERK activation plays a protective role in UV-induced cell apoptosis.

DISCUSSION

Recent reports (6, 7, 11–13) indicate that UV irradiation produces a variety of early responses such as activation of cell surface receptors for EGF, tumor necrosis factor, and interleukin-1 and stimulation of Src, SAPK/JNK, and ERK. The UV responses may be divided into two separate signaling pathways leading to SAPK/JNK and ERK activation: 1) UV → cell surface receptor tyrosine kinase(s), etc. → SAPK/JNK activation → apoptosis and 2) UV → EGF receptors → ERK activation. However, it remained to be determined how Src activation contributes to the UV-induced signaling pathways. Furthermore, the physiological role of the ERK activation was not apparent in stress-induced cell apoptosis. Our present experiments basically confirmed and further extended the above signaling pathways as follows (Fig. 8).

First, UV-induced ERK activation in A431 cells (and ES cells) appeared to be mediated through Src-dependent Tyr phosphorylation of EGF receptors. UV-induced ERK activation was completely abolished in the presence of the EGF receptor kinase inhibitor AG1478 and the Src inhibitor PP2 (Figs. 1 and 3). UV irradiation induced Src activation in a manner similar to the ERK activation, but AG1478 failed to inhibit the Src activation (Figs. 2 and 4). Lyn tyrosine kinase, which is responsible for DNA damage-induced SAPK/JNK activation (16), was not stimulated by UV irradiation at the early stages (Fig. 4).

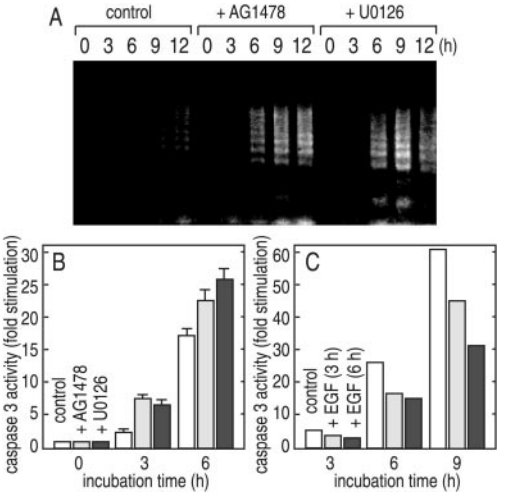

FIG. 7. **Modification of UV-induced cell apoptosis by EGF receptor activation and inhibition.** *A* and *B*, A431 cells were first treated without (*control*) or with AG1478 (1 μM for 20 min) or U0126 (20 μM for 20 min). The cells were stimulated with UV irradiation (0.1 kJ/m^2) and incubated at 37 °C for the indicated times. The cells were lysed and subjected to assays for DNA fragmentation (*A*) and caspase 3 activity (*B*). Caspase 3 activity was determined as the fold activation compared with the control level observed in non-treated A431 cells; the data with *bar*s were means ± S.D. from four independent experiments. *C*, A431 cells were treated without (*control*) or with 5 ng/ml of EGF for 3 or 6 h and stimulated with UV irradiation (0.1 kJ/m^2). Caspase 3 activity was measured and expressed as described in *B*.

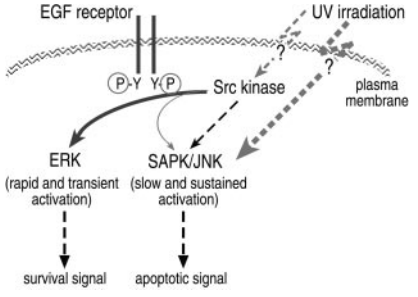

FIG. 8. **A proposed model for UV-induced signaling pathways.** See "Discussion" for an explanation of this model.

PP2 and AG1478, together with the expression of kinase-dead Src SrcK298M, inhibited UV-induced Tyr phosphorylation of EGF receptors (Figs. 5 and 6). In contrast, UV-induced activation of SAPK/JNK, which appears to be responsible for apoptotic responses, was partially inhibited by AG1478 and PP2 (Figs. 1 and 3). This indicates that a UV-induced pathway(s) other than the EGF receptor activation may also contribute to SAPK/JNK activation. One of these would be a pathway activated by PP2-sensitive Src (or Src-like tyrosine kinase) but not mediated by EGF receptor phosphorylation (see Fig. 8). Second, UV-induced ERK activation played a survival role against apoptosis. DNA fragmentation and caspase 3 activation induced by UV irradiation were markedly enhanced by AG1478 and the ERK kinase inhibitor U0126 (Fig. 6, *A* and *B*). On the contrary, UV-induced caspase 3 activation was significantly reduced by the stimulation of EGF receptors (Fig. 7*C*).

It has been reported (17, 18) that a reactive oxygen species (ROS) is generated in response to UV irradiation and hydrogen peroxide (H_2O_2) in various cell types. Quite recently, Chen *et al*. (19) reported that SAPK/JNK activation by H_2O_2 also involves Src-dependent EGF receptor activation in human endothelial cells. However, their results are somewhat different from ours in the following points. JNK activation by H_2O_2 in the endothelial cells is completely inhibited by high concentrations of AG1478 and PP2. These authors discussed the possibility that the upstream Src rather than EGF receptor itself may be the target of AG1478 used at a high concentration. However, the inhibition of H_2O_2-induced ERK activation by the inhibitors was partial in the endothelial cells (19). These findings suggest that signaling pathways induced by UV irradiation may not be totally dependent on the actions of H_2O_2, although Src-dependent EGF receptor activation is commonly observed in stress-activated cells.

In addition, Fritz and Kaina (20) reported that the PI 3-K inhibitor wortmannin specifically blocked the UV-induced JNK activation but did not affect UV-induced ERK activation in NIH3T3 cells. However, we did not observe any inhibitory effect of wortmannin on UV-induced JNK activation or EGF receptor phosphorylation in A431 cells (Figs. 3 and 5). Chen *et al*. (19) have also described no inhibitory effect of the PI 3-K inhibitor LY294002 on H_2O_2-induced JNK activation in human endothelial cells. These results suggest that stress-induced signaling pathways may be different among the types of stress signals and cells. Further experiments concerning molecular mechanisms for the stress-sensing early steps (see Fig. 8) would be required for understanding how these stress signals display different sensitivities to various inhibitors.

In this regard, it is very interesting to note here that Src-dependent EGF receptor activation is also observed upon stimulation of G protein-coupled receptors (21, 22). Stimulation of lysophosphatidic acid and adrenergic α2 receptors by the agonists activated a Ras-dependent MAPK pathway (21). This MAPK activation was mediated through the phosphorylation of EGF receptors by Src, and the phosphorylation appeared to have resulted from the action of the $\beta\gamma$ subunits of G_i-type G proteins. Thus, it is tempting to speculate that UV-induced Src and EGF receptor activation may arise from cell surface membrane components such as receptor-like molecules or G protein subunits. Indeed, a recent report (23) suggested that G protein α subunits are directly activated by reactive oxygen species.

In summary, the present results indicate that UV-induced ERK activation is mediated through EGF receptor phosphorylation by Src tyrosine kinase and that this signaling pathway plays a critical role in the rapid anti-apoptotic response by inducing gene expression. It is quite reasonable that UV-irradiated cells, which are not damaged enough to undergo apoptosis, survive by utilizing this anti-apoptotic pathway. Conversely, UV activation of this pathway may enhance the survival of mutated cells, thereby promoting cancer initiated by the stress signal.

REFERENCES

1. Li, K., Li, Y., Shelton, J. M., Richardson, J. A., Spencer, E., Chen, Z. J., Wang, X., and Williams, R. S. (2000) *Cell* **101**, 389–399
2. Yoshida, H., Kong, Y. Y., Yoshida, R., Elia, A. J., Hakem, A., Hakem, R., Penninger, J. M., and Mak, T. W. (1998) *Cell* **94**, 739–750
3. Hakem, R., Hakem, A., Duncan, G. S., Henderson, J. T., Woo, M., Soengas, M. S., Elia, A., de la Pompa, J. L., Kagi, D., Khoo, W., Potter, J., Yoshida, R., Kaufman, S. A., Lowe, S. W., Penninger, J. M., and Mak, T. W. (1998) *Cell* **94**, 339–352
4. Kuida, K., Haydar, T. F., Kuan, C. Y., Gu, Y., Taya, C., Karasuyama, H., Su, M. S., Rakic, P., and Flavell, R. A. (1998) *Cell* **94**, 325–337
5. Woo, M., Hakem, R., Soengas, M. S., Duncan, G. S., Shahinian, A., Kagi, D., Hakem, A., McCurrach, M., Khoo, W., Kaufman, S. A., Senaldi, G., Howard, T., Lowe, S. W., and Mak, T. W. (1998) *Genes Dev.* **12**, 806–819
6. Derijard, B., Hibi, M., Wu, I. H., Barrett, T., Su, B., Deng, T., Karin, M., and Davis, R. J. (1994) *Cell* **76**, 1025–1037
7. Kyriakis, J. M., Banerjee, P., Nikolakaki, E., Dai, T., Rubie, E. A., Ahmad, M. F., Avruch, J., and Woodgett, J. R. (1994) *Nature* **369**, 156–160
8. Tournier, C., Hess, P., Yang, D. D., Xu, J., Turner, T. K., Nimnual, A., Bar-Sagi, D., Jones, S. N., Flavell, R. A., and Davis, R. J. (2000) *Science* **288**, 870–874
9. Wada, T., Nakagawa, K., Watanabe, T., Nishitai, G., Seo, J., Kishimoto, H., Kitagawa, D., Sasaki, T., Penninger, J. M., Nishina, H., and Katada, T. (2001) *J. Biol. Chem.* **276**, 30892–30897
10. Davis, R. J. (2000) *Cell* **103**, 239–252

11. Devary, Y., Gottlieb, R., Smeal, T., and Karin, M. (1992) *Cell* **71**, 1081–1091
12. Rosette, C., and Karin, M. (1996) *Science* **274**, 1194–1197
13. Coffer, P. J., Burgering, B. M., Peppelenbosch, M., Bos, J. L., and Kruijer, W. (1995) *Oncogene* **11**, 561–569
14. Nishina, H., Fischer, K. D., Radvanyi, L., Shahinian, A., Hakem, R., Rubie, E. A., Bernstein, A., Mak, T. W., Woodgett, J. R., and Penninger, J. M. (1997) *Nature* **385**, 350–353
15. Nishina, H., Bachmann, M., Oliveira-dos-Santos, A. J., Kozieradzki, I., Fischer, K. D., Odermatt, B., Wakeham, A., Shahinian, A., Takimoto, H., Bernstein, A., Mak, T. W., Woodgett, J. R., Ohashi, P. S., and Penninger, J. M. (1997) *J. Exp. Med.* **186**, 941–953
16. Yoshida, K., Weichselbaum, R., Kharbanda, S., and Kufe, D. (2000) *Mol. Cell. Biol.* **20**, 5370–5380
17. Black, H. S. (1987) *Photochem. Photobiol.* **46**, 213–221
18. Cerutti, P. A., and Trump, B. F. (1991) *Cancer Cells* **3**, 1–7
19. Chen, K., Vita, J. A., Berk, B. C., and Keaney, J. F., Jr. (2001) *J. Biol. Chem.* **276**, 16045–16050
20. Fritz, G., and Kaina, B. (1999) *Mol. Cell. Biol.* **19**, 1768–1774
21. Luttrell, L. M., Rocca, G. J. D., van Biesen, T., Luttrell, D. K., and Lefkowitz, R. J. (1997) *J. Biol. Chem.* **272**, 4637–4644
22. Daub, H., Wallasch, C., Lankenau, A., Herrlich, A., and Ullich, A. (1997) *EMBO J.* **16**, 7023–7044
23. Nishida, M., Maruyama, Y., Tanaka, R., Kontani, K., Nagao, T., and Kurose, H. (2000) *Nature* **408**, 492–495