UV Activates Growth Factor Receptors via Reactive Oxygen Intermediates

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Abstract. Exposure of mammalian cells to UV irradiation induces rapid and transient expression of early growth response–1 gene (Egr-1) encoding a transcription factor that plays a role in cell survival. These signals from the irradiated cell surface are likely to involve more than one pathway, and we show here that an essential pathway involves activation of several growth factor receptors by reactive oxygen intermediates (ROI). UVC irradiation causes the tyrosine phosphorylation of EGF receptor (EGFR) in mouse NIH 3T3 fibroblasts and HC11 mouse mammary cells. EGFR activation by irradiation of cells is abrogated by suramin, by antioxidants, and by the presence of a dominant negative EGFR. UV induces the formation of complexes between activated EGFR and SOS, Grb2, PLCγ, and SHC that can be precipitated with antibodies to EGFR. The activation of EGFR by UV is mimicked by H_2O_2, suggesting that ROI may function upstream of EGFR activation. Our observations support the hypothesis that ROI and growth factor receptors operate in the early steps of the UV signal that lead to the enhanced expression and activity of Egr-1.

With the predicted destruction of the ozone layer by environmental pollutants, the human population is expected to be exposed to elevated doses of short wavelength UV irradiation. UV damage can have dramatic direct, indirect, and systematic effects via DNA mutations (Sachsenmaier et al., 1994*a*), activation of dormant viruses (Herrlich et al., 1992), and systemic immunosuppression (Kripke, 1994). One of the results of UV irradiation in areas that have thinner ozone layers is an increasing rate of skin cancer. UV irradiation of cells elicits complicated cellular responses involving the induction of expression of several genes (Fornace et al., 1992; Huang and Adamson, 1995). Some of these gene products are part of the DNA damaging mechanism, and some are thought to have protective effects such as DNA repair enzymes or those leading to apoptosis of damaged cells. Many of the activated genes encode transcription factors (such as AP-1, NF-κB, and Egr-1) that can lead to pleiotropic responses including inhibition of RNA and DNA syntheses and subsequently the revival of the cell cycle after repair and/or apoptosis of damaged cells (Zhan et al., 1993; Orren et al., 1995; Petersen et al., 1995; Wang and Ellem, 1994). One of the transcription factors that responds within 10 min of UV irradiation is the early growth response–1 gene (Egr-1)[1], a member of a larger family of genes mostly related through their homologous zinc-finger domains that bind the same DNA sequence (GCGG/TGGGCG) (LeMaire et al., 1988; Sukhatme et al., 1988). We have recently described the induction kinetics of the Egr-1 gene by UV (Huang and Adamson, 1995). Oxidants produced in the cell, serine/threonine and tyrosine kinases, and the c-*Ras* gene product were all intermediaries necessary to elicit Egr-1 induction. Similar findings have been described for other transcription factors (Derijard et al., 1994; Engelberg et al., 1994; Hibi et al., 1993). The Egr-1 induced by UV irradiation is part of a protective mechanism since cells that cannot express Egr-1 are subject to increased levels of apoptosis and cell cycle pausing (Huang, R–P., Y. Fan, Z. Ni, W. Matheny, and E.D. Adamson, manuscript submitted for publication).

The UV signal pathway involves a phosphorylation cascade (Devary et al., 1992; Sachsenmaier et al., 1994*a*,*b*), because the induction of Egr-1 is inhibited by tyrphostin and genistein, inhibitors of tyrosine kinases, and by H7, an inhibitor of protein kinase C (PKC) (Huang and Adamson, 1995). The exposure of 3T3 cells to the tumor promoter 12–*O*–tetradecanoylphorbol-13-acetate (TPA), down-regulates PKC activity and also inhibits the production of activated, phosphorylated Egr-1 after UV irradiation. Addition of kinase inhibitors to the medium of cells together with UV irradiation inhibit the transactivation of the Egr-1 promoter. The active form of Egr-1 protein produced by UV induction is highly phosphorylated, in contrast to the Egr-1 induced by serum, growth factors, or TPA. The nature of the phosphorylated forms of Egr-1 has not yet been analyzed, but phosphorylated forms bind to DNA more efficiently (Huang and Adamson, 1994) and transactivate the DNA binding site more strongly (Huang, R–P., Y. Fan, Z. Ni, W. Matheny, and E.D. Adamson, manuscript submitted for publication). We believe that the phosphory-

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1. *Abbreviations used in this paper:* CAT, chloramphenicol acetyl transferase; DHR, dihydrorhodamine 123; EGFR, EGF receptor; Egr-1, early growth response–1 gene; NAC, *N*-acetylcysteine; PDGFR, PDGF receptor; PDTC, pyrrolidine thiocarbamate; PKC, protein kinase C; ROI, reactive oxygen intermediate; TNFα, tumor necrosis factor α; TPA, 12-*O*-tetradecanoylphorbol-13-acetate.

lated forms of Egr-1 play roles in cell survival after UV irradiation.

The precise mechanisms for the generation of a signal from the extracellular incidence of UV and the gene responses remain largely unknown. Tyrosine kinases such as c-Src have been implicated (Devary et al., 1992), and several groups have noted that the growth factor signal pathway has elements and components in common with the irradiation pathway (Datta et al., 1992). Several groups (Sachsenmaier et al., 1994*b*; Ley and Ellem, 1992; Coffer et al., 1995) have demonstrated that UV activates tyrosine kinases by comparing the downstream pathways after UV or ligand binding. How UV irradiation generates the signal pathway(s) from the cell surface to the nucleus is the focus of the present study. We demonstrated earlier that reactive oxygen species, rather than DNA damage, appear to be major components in the induction of Egr-1 (Huang and Adamson, 1995). Here we show that reactive oxygen intermediates (ROI) are upstream of tyrosine kinase receptors in the signal pathway. Together, the evidence supports a pathway for the UV response that includes ROI, EGF receptor (EGFR), Shc/Grb2/Sos/PLCγ, Ras, and other unknown components leading to Egr-1 induction.

Materials and Methods

Cell Culture and Materials

NIH 3T3 cells were from a stock with no spontaneous plaque formation (obtained from Dr. C. Der, University of North Carolina, Chapel Hill). HC11 ia a clone of normal mouse mammary epithelial cells derived from COMMA-1D cells (Danielson et al., 1984) (obtained from Drs. J. Rosen and D. Medina, Baylor University, Houston, TX). The characteristics of HC11 have been described before (Ball et al., 1988; Marte et al., 1995). NIH 3T3 cells were grown in DME containing 5% calf serum at 37°C and 5% CO_2. HC11 cells were maintained in DME supplemented with 10% FBS at 37°C and 5% CO_2. UV irradiation was performed as described elsewhere (Huang and Adamson, 1995) All the chemicals described below were purchased from Sigma Chemical Co. (St. Louis, MO). Suramin was prepared as a stock solution of 50 mM in PBS before sterile filtration. TPA was made 0.25 mM in DMSO. Pyrrolidine thiocarbamate (PDTC) and *N*-acetylcysteine (NAC) were dissolved in water immediately before use. Hydrogen peroxide was obtained as a 30% solution and diluted into desired concentration immediately before use. Irradiation with UVC was applied as described before (Huang and Adamson, 1995).

Antibodies

Polyclonal antibodies to mouse EGF receptor (EGFR) were described earlier (Weller et al., 1987). Antibodies to PDGF receptor (PDGFR)β were raised in rabbits to a peptide sequence in the extracellular domain. The peptide, 423–443 in the mouse protein sequence, was linked to Keyhole limpet hemocyanin for use as the antigen, and the antiserum was shown to precipitate a polypeptide of ~180 kD from lysates of NIH 3T3 cells (data not shown). Polyclonal antibody to Egr-1 was raised against amino-terminal portion of Egr-1 as a glutathione-S-transferase-fusion protein (Huang et al., 1994*a*). MAb PY20 (ICN Pharmaceuticals, Irvine, CA) specifically recognizes the phosphorylated form of tyrosine. Anti-Grb-2, anti-PLCγ, anti-SHC, and anti-SOS are mAbs obtained from Transduction Laboratories (Lexington, KY).

Immunoprecipitation and Immunoblotting

Immunoprecipitation was carried out as described (Huang and Adamson, 1994). Essentially, cell lysates dissolved in (RIPA) buffer (20 mM Tris, pH 7.5, 0.15 M NaCl, 0.5% sodium deoxycholate, 1% NP-40, 0.1% sodium dodecyl sulfate) containing protease inhibitors and 1 mM sodium orthovanadate to inhibit phosphatases were immunoprecipitated with corresponding antibodies and formalin-fixed *Staphylococcus aureus*. The insolubilized antigens adsorbed by the bacteria were extensively washed, and the immunoprecipitated complexes were analyzed by SDS-PAGE. To detect active EGFR-associated protein, cells were lysed with solubilizing buffer, 1% Triton X-100, 0.5% deoxycholate, and 10% glycerol in Hepes-buffered saline (HBS) (consisting of 50 mM Hepes, pH 7.5, 150 mM NaCl, and 1 mM sodium orthovanadate) for 30 min. The cell lysates were then incubated overnight at 4°C with anti-EGFR IgG. The antigen complexes were precipitated using *S. aureus* for 2 h at 4°C. The immunoprecipitated complexes were then washed three times with solubilization buffer and once with HBS. Immunoblotting of the immunoprecipitates was performed as described previously (Huang et al., 1994*a*). Briefly, equal amounts of protein or immunoprecipitated complexes were analyzed by SDS-PAGE. Proteins were transferred to polyvinyline difluoride membrane (Immobilon; Millipore Corp., Bedford, MA). The specific antigens were detected by the corresponding antibodies coupled to the enhanced chemiluminescence system (ECL; Amersham Corp., Little Chalfont, UK). The intensity of the signal was measured by densitometry, and at least three results were averaged to compare the level of total Egr-1 in samples.

Cell Fractionation

Cytoplasts were isolated according to method described by Poste (1972). Basically, cells were incubated in 20 μg/ml cytochalasin B (Sigma Chemical Co.) for 4 h, washed with PBS, detached from culture dishes with 0.1% EDTA, and resuspended in Percoll (1.05 g/ml; Pharmacia Fine Chemicals, Piscataway, NJ) in PBS containing cytochalasin B (5 μg/ml). After centrifugation for 1 h at 33°C, 24,000 rpm (77,000 *g*) in a rotor (SW28; Beckman Instruments, Inc., Fullerton, CA), enucleated cells were recovered from the midpoint of the gradient, washed twice with PBS, resuspended in complete fresh medium, and replated onto 60-mm tissue-culture plates. After incubation for 1 h at 37°C, cytoplasts were treated with UV or EGF for 5 min and harvested for assay of phosphorylation of EGFR. The degree of enucleation was monitored by staining fixed intact cells and cytoplasts with Hoechst dye (Hoechst 332508; Sigma Chemical Co.) for 20 min and viewing under phase contrast with a UV filter in an epifluorescence microscope (Nikon Inc., Garden City, NY).

FACS® Analysis

The levels of ROI were determined by FACS® analysis as described before (Lo and Cruz, 1995). HC11 cells were irradiated with UVC 40 J/m^2 at different time points after incubation for 1 h in the presence of 2 μM dihydrorhodamine 123 (DHR) (Molecular Probes, Eugene, OR) with or without PDTC (0.8 μM). The intracellular DHR was irreversibly changed into the green fluorescent compound, rhodamine 123 (wavelength 500–540 nm), by the ROI generated inside the cells. Cells were fixed for 20 min in 1% formaldehyde, and the cellular rhodamine 123 fluorescence intensity of 10,000 HC11 cells was measured for each sample by flow cytometry with the excitation source at 488 nm (FACS®ort).

Transfection and Chloramphenicol Acetyl Transferase (CAT) Assay

Transfection and CAT assays were performed as described previously (Huang et al., 1994*b*). NIH 3T3 cells (2×10^5) were seeded into 60-mm tissue-culture dishes 20 h before transfection. 1 μg of pEgr-1B950CAT containing full-length Egr-1 promoter (960 bp) (Huang et al., 1994*b*) was cotransfected with a dominant negative EGFR expression vector DNA (Wu and Adamson, 1993) by calcium phosphate precipitation. 40 h after transfection, cells were harvested for CAT assays. Signals were detected and analyzed using a phosphoimager system (Bio-Rad Laboratories, Hercules, CA).

Growth Assays

Nonradioactive Cell Proliferation Assay (MTT Assay). Cells were seeded in 6-well plates at 5×10^4 cells per well and incubated at 37°C overnight. Cells were then exposed to 20 J/m^2 UVC with or without suramin (50 μM) pretreatment for 1 h. Cells were trypsinized, suspended in 1 ml of original medium, and transferred to 96-well plates (100 μl per well). After 2 d, cells were stained and processed as described by the manufacturer (Promega, Madison, WI). The values of absorbances at 600 nm were plotted as a measure of the relative number of cells. Each assay was done in quadruplicate and repeated three times.

Clonogenicity. Colony formation assays were carried out as previously described (Huang et al., 1995). Cells were seeded in 60-mm plates (500

cells per plate) and incubated overnight. After pretreatment with suramin (50 μM) or without drug for 1 h, cells were irradiated with 20 J/m^2 of UVC, and the same medium was replaced. 2 d later, the medium was changed, and culture was continued for 2–3 wk. Colonies were fixed in 4% formaldehyde-PBS and stained with Giemsa solution. Colonies containing >50 cells were counted, and the fraction of surviving cells was calculated. The results represent averages of three separate experiments.

Results

Involvement of Growth Factor Receptors in the UV Response

The goal of this study is to identify the origin of the UV-induced signal that activates the Egr-1 gene. The UV response by cells has been shown to include growth factor receptor activation (Ley and Ellem, 1992; Coffer et al., 1995). The cells used here represent two distinct types; NIH 3T3 are normal immortalized mouse fibroblasts (mesodermal), while HC11 are normal mouse epithelial cells of epidermal origin. Their responses to many stimuli differ, but UV irradiation stimulates the Egr-1 gene in both cell types whether quiescent or in the log phase of growth (Huang and Adamson, 1995). One approach to test for membrane receptor stimulation is to apply the drug suramin, since it is able to intervene with many kinds of extracellular interactions at the membrane without entering the cell. Suramin at nontoxic doses is known to block the activation of the EGFR, bFGF receptor, InsulinR, and PDGFR (Betsholtz et al., 1986). When suramin was applied to NIH 3T3 and to HC11 mammary cells for 2 h immediately after irradiation, the induction of Egr-1 protein was almost completely inhibited (Fig. 1, *A* and *B*; *left panels*) compared to cells treated with UV alone. In these experiments, cells in log phase of growth (*L*) were used, and their low prestimulation levels of Egr-1 are shown in Fig. 1, *A* and *B*, lanes *1*. The UV-stimulated levels are shown in Fig. 1, *A* and *B*, lanes *2* and are six- to 10-fold higher than log phase cells. In contrast, the induction of Egr-1 by TPA was not inhibited by suramin (Fig. 1, *A* and *B*, lanes *6*). To test if inhibition by suramin specifically acted through growth factor receptors, the receptors were down-regulated by pretreatment with EGF (Fig. 1, *A* and *B*, *left panel*, lanes *7* and *8*); insulin (Fig 1, *A* and *B*, *right panel*, lanes *4–6*); bFGF (lanes *7–9*); or PDGF-B (lanes *10–12*) before applying the UV stimulus. The loss of available receptors for EGF, insulin, and bFGF strongly inhibited the UV-elicited stimulation of Egr-1 levels, indicating that the growth factor receptors are necessary to generate the signal. The induced levels of Egr-1 were quantified by densitometry and expressed as the average of three experiments (Fig. 1 *D*). Down-regulation of these receptors inhibited Egr-1 induction by 50 to 60%.

Interestingly, the down-regulation of PDGFRβ had no effect on the stimulation of Egr-1 by UV. This result could be explained by the lack of PDGF receptors on mammary epithelial cells since epithelial cells are not expected to express PDGF receptors. However, HC11 cells do express PDGF receptors (Marte et al., 1995). Quiescent HC11 and NIH 3T3 cells (cultured in 0.5% FBS for 24 h) responded to the addition of PDGF-B to the medium, giving a significant transient increase in Egr-1 expression measured 1 h after growth factor addition (Fig. 1 *C*, lanes *3*). Both cell types expressed all the receptors examined here since they responded to all the growth factors by transient Egr-1 expression at 1 h (Fig. 1 *C*, lanes *3–6*), including a mixture of growth factors (Δ; Fig. 1 *C*, lanes *2*). We concluded that PDGFRβ does not respond to the UV signal although the other receptors are sensitive.

Note that, in Fig. 1, *A* and *B*, pretreatment of cells with various growth factors to down-regulate their receptors had no effect on the subsequent stimulation of Egr-1 expression by TPA. This indicates that the proximal TPA signaling mechanism is different from the UV pathway and appears not to involve growth factor receptors. We have shown that the pretreatment of cells with TPA to down-regulate PKC does interefere with the ability of UV to induce Egr-1 expression after UV irradiation (Huang and Adamson, 1995), thus implicating at least some of the same pathways from PKC stimulation in the UV response.

The mechanism of action of suramin is unknown although it has "anti-growth factor activity" (Middaugh et al., 1992). In the above experiment, suramin abrogated the UV response since Egr-1 induction was inhibited. The Egr-1 response plays a role in cell survival (Huang and Adamson, 1995), therefore, suramin treatment of cells should also diminish the survival of cells after UV irradiation. This was tested by measuring the growth rates of cells before and after irradiation using the nonradioactive proliferation MTT assay. UV irradiation alone causes cell cycle delay and cell apoptosis depending on the cell type, and only additional effects caused by suramin were measured. Fig. 2 *A* shows that suramin had a small effect (15% reduced) on the normal growth rate of NIH 3T3 cells, but after irradiation, cell numbers 2 d later were > 60% inhibited in the presence of suramin. In colony formation assays, suramin reduced the survival of irradiated cells by > 40% in NIH 3T3 and in HC11 mammary epithelial cells (Fig. 2 *B*). Thus, the loss of growth factor receptor activity significantly increased the sensitivity of both kinds of cells to UV.

Activation of EGF Receptor by UV

To test whether the EGF receptor is specifically involved in the UV response, we used a truncated EGFR expression vector that produces a "kinase dead" receptor protein (Wu and Adamson, 1993). The expression of this protein acts as a dominant negative EGF receptor in cells that have the normal receptor protein, thus inhibiting function. Transient expression of this construct, together with an Egr-1 promoter–CAT reporter gene (pEgr-1 B950), inhibited UV-induced promoter activity by 40 to 50% (Fig. 3). This result suggests that at least 40% of the Egr-1–inducing UV signal acts through the EGFR. The response and the inhibition of the Egr-1 promoter–CAT construct was specific since the empty CAT reporter activity was unchanged with or without the mutant EGFR expression vector.

The activation of the EGFR can be directly observed as tyrosine autophosphorylation of an M_r 170-kD protein. NIH 3T3 or HC11 cells were UV irradiated (or treated with EGF for 10 min) and lysed 0.2, 1, and 5 min later. The lysates were immunoprecipitated with anti–mouse EGF receptor antibodies, and the washed complexes were ana-

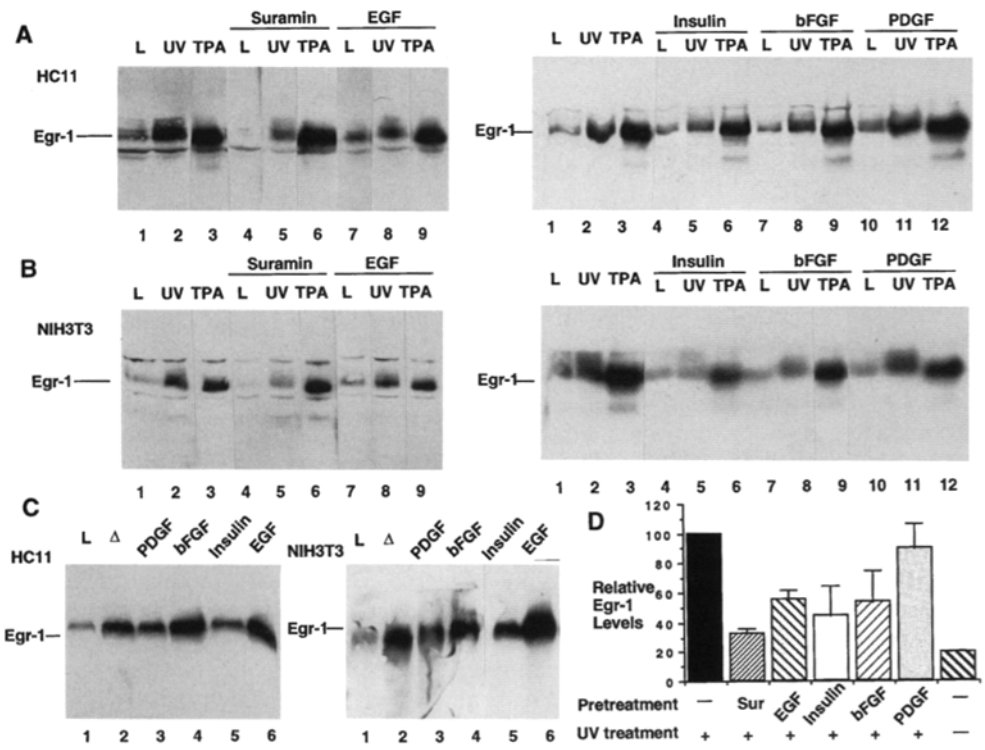

Figure 1. Immunoblot assays of Egr-1 from cells after various growth factor treatments. (*A*) HC11 cells were pretreated for 2 h with suramin (50 μM), EGF (50 ng/ml), insulin (10 μg/ml), bFGF (20 ng/ml), or PDGF (20 ng/ml), followed by a second treatment with either UV (40 J/m^2) or TPA (0.2 μM) or without treatment (*L*) as indicated. 2 h later cell lysates were analyzed for Egr-1 level with anti–Egr-1 antibody. The levels of Egr-1 observed were low if the receptors involved in the response were down-regulated by the pretreatment with ligand. (*B*) The same treatments were performed with NIH 3T3 cells. (*C*) Cells were made quiescent and then treated with PDGF-B, bFGF, insulin, EGF, or a combination of bFGF, insulin, and EGF (Δ) for 1 h. The cell lysates were assayed for the Egr-1 level by immunoblotting analysis. These results represent transient stimulation of Egr-1. (*D*) Quantitative evaluation of some of the data presented in *A* and *B*. The results are expressed as the average of both cell types. Egr-1 expression in UVC-irradiated nonpretreated cells has been set at 100%.

lyzed by SDS-PAGE and transferred to nitrocellulose as described in the Materials and Methods section. The membranes were reacted with anti-phosphotyrosine antibodies to reveal activated EGFR at 170 kD. All the samples from irradiated cells had an EGFR band similar to that produced by EGF addition (Fig. 4 *A*). Whilst UV rapidly activated the EGF receptor, it had no effect on the overall EGF receptor protein level that remained constant during this time (Fig. 4 *B*).

Since PDGF receptors did not appear to be involved in the UV response, we tested if either UV or PDGF-B would elicit an activated tyrosine-phosphorylated protein of M_r 180–190 kD. Fig. 4 *C* shows that no activated PDGFRβ was produced after UV irradiation, whilst PDGF-B was able to stimulate the tyrosine phosphorylation of PDGFRβ. This confirms the lack of effect of PDGFRβ on Egr-1 induction by UV.

According to current views, activation of EGFR by its ligands involves dimerization and autophosphorylation and leads to the tyrosine phosphorylation of the Shc proteins. Upon tyrosine phosphorylation, Shc proteins bind to Grb2 and the Ras GTP-GDP exchange protein Sos. In addition, the activation of EGFR also triggers the PLCγ pathway. Thus, the activation of EGFR brings about the formation of a large complex including EGFR, Shc, Grb2, Sos, and PLCγ (Gergel et al., 1994; Batzer et al., 1994). This was assayed after UV activation of the EGFR in HC11 cells. Lysates from cells that were UV irradiated or treated with EGF were immunoprecipitated with anti-EGFR antibody, followed by immunoblotting with antibodies against each suspected protein in the complex. Fig. 5 shows that all components, Shc, Grb2, Sos, and PLCγ, were present in the EGFR immunoprecipitates, while cyclin B, a nuclear protein irrelevant to this complex, was not present and acted as a negative control. Together, the data make a strong case for the involvement of the EGFR in the UV response pathway.

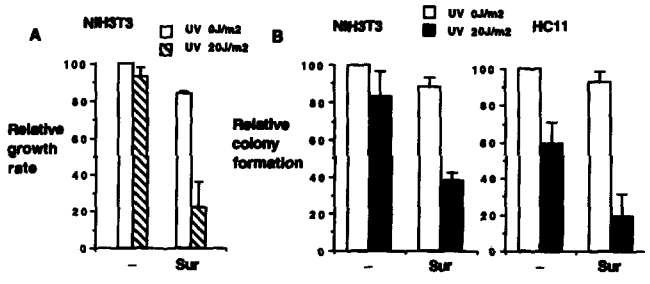

Figure 2. The growth factor receptor reactive drug, suramin (*Sur*), further reduced cell survival upon UVC irradiation. (*A*) Cell growth was measured 2 d after UVC irradiation and compared with nonirradiated cells. Inhibitor suramin (50 μM) was added 1 h before UVC exposure. (*B.*) Clonogenic growth 2 to 3 wk after UV exposure (see Materials and Methods for details).

Activation of Growth Factor Receptors by Reactive Oxygen Intermediates

Several mechanisms can be considered to explain the rapid EGF receptor activation by UV. One is that UV increases the secretion of EGF-like ligands or other factors that activate the receptor. A second possibility is that UV causes the production of ROI that trigger the UV signal cascade. Alternatively, damaged DNA produces an effect on the EGF receptor protein.

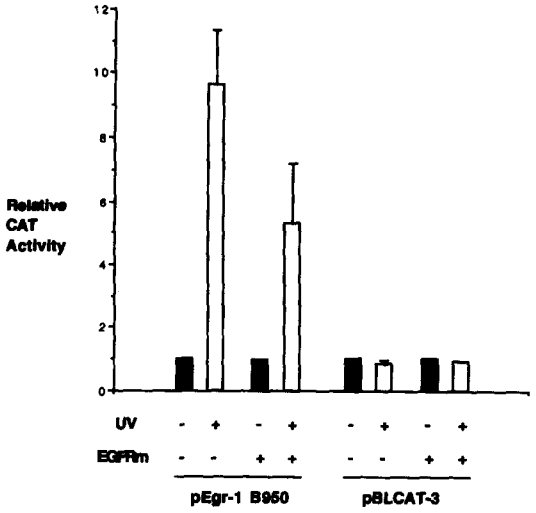

Figure 3. Dominant negative EGFR suppressed UV-induced Egr-1 promoter activity. NIH 3T3 cells were transfected with Egr-1 promoter–CAT plasmid with or without dominant negative EGFR expression vector. Transfected cells were irradiated with 100 J/m^2 UVC, and CAT assays were carried out 24 h after UVC treatment. The data represent the mean of three independent experiments.

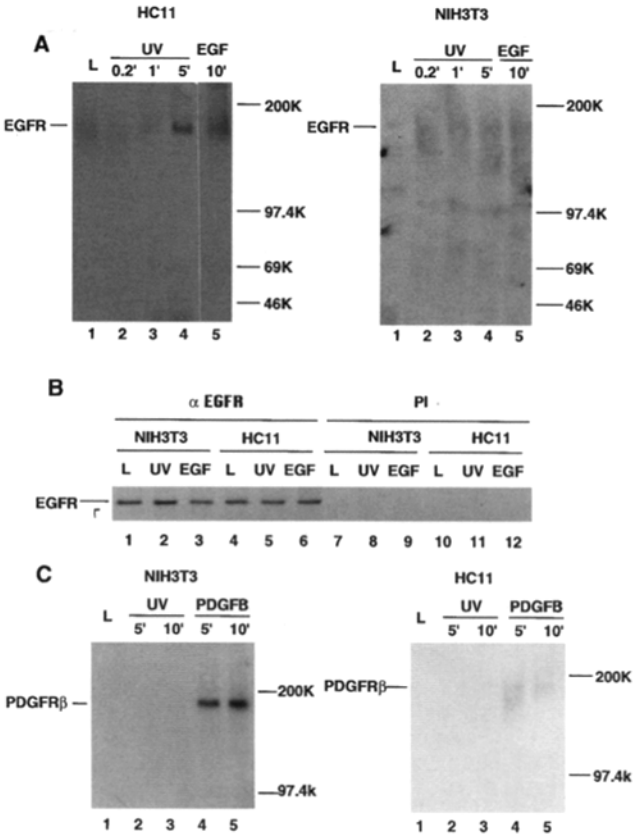

Figure 4. UVC rapidly stimulated tyrosine phosphorylation of EGFR but not PDGFRβ. (*A*) Cells were treated with 40 J/m^2 UVC or EGF. Cell lysates prepared at different time points were immunoprecipitated with anti-EGFR. The immunoprecipitated complexes were separated by 5% SDS-PAGE and visualized by immunoblotting with anti-phosphotyrosine Ab (PY20). (*B*) Cells were metabolically labeled with [^{35}S]methionine for 4 h, followed by UVC or EGF treatment. After 5 min, cell lysates were immunoprecipitated with anti-EGFR antibody and analyzed on 5% SDS-PAGE. (*C*) Cells were treated with UVC or PDGF-B (20 ng/ml). After 5 or 10 min as indicated, cells were harvested and processed for the determination of tyrosine phosphorylation of PDGFRβ by immunoprecipitation with anti-PDGFRβ antibody, followed by immunoblotting with anti-phoshotyrosine antibody (PY-20).

To test the idea that an inducing factor is released into the medium after UV irradiation, we collected the conditioned medium 5 min, 10 min, 30 min, 1 h, and 2 h after irradiation of NIH 3T3 cells and added it back to untreated NIH 3T3 cells. The endpoint in the assay was the level of Egr-1 protein induced in these cells 2 h after the conditioned medium was added. As shown in Fig. 6, there was a small but consistent effect of the conditioned medium on the stimulation of Egr-1. With a peak time of 10 to 30 min, Egr-1 expression increased threefold in the conditioned medium from UV-treated cells compared to cells whose medium was simply removed and added back. The effect of the conditioned medium was stronger from cells treated with increasingly larger doses of UV (Fig 6., lanes *9–12*), suggesting that secreted factors play a role in EGFR activation. However, the degree of the effect was insufficient to explain the 8–10-fold increase in Egr-1 expression seen after UV irradiation (Huang and Adamson, 1995).

We next assayed the effect of oxidant species on the activation of EGF receptor in UV-irradiated cells. We have previously shown that the UV stimulation of Egr-1 can be inhibited by the presence of antioxidants such as NAC and PDTC in the culture medium. Fig. 7 shows that both NAC and PDTC effectively block the tyrosine phosphorylation of the EGFR in HC11 cells after UV exposure, examined by immunoblotting with anti-phosphotyrosine after immunoprecipitation with antibodies to EGFR. Thus, UV-dependent EGFR activation depends on the presence of ROI in the cell.

To demonstrate that ROI are induced in UV-treated HC11 cells, we used a fluorescence cell sorting method. This assay is based on the ability of the substance DHR to react with oxidants in a cell and hence produce rhodamine 123 that is membrane impermeable and fluorescent in the green range of wavelengths. Cells were incubated for 1 h with DHR with or without the antioxidant PDTC and then subjected to UV irradiation. As shown in Fig. 8, UV generates ROI as little as 5 min after UV (Fig. 8, *fourth line*), seen as a shift to the right in fluorescence intensity. The ROI produced by UV is completely removed by PDTC that causes a decrease down to basal fluorescence intensity, seen as a shift to the left (Fig. 8, *fifth line*). The level of ROI in the cells 30 min after UV is even higher (Fig. 8, *sixth line*) than at 5 min, and this is efficiently reduced by PDTC (Fig. 8, *seventh line*).

To test the need for a nuclear signal in the EGFR activation, cytoplasts were prepared by enucleation of cells. The cytoplasts were UV irradiated and tested for activated EGFR by immunoprecipitation and immunoblotting. HC11 cells were incubated with cytochalasin B for 4 h to enucleate the cells, and cytoplasts were collected by gradient centrifugation. The cytoplasts, free of nuclear contamination (Fig. 9, *lower panel*), were incubated, exposed to UV irra-

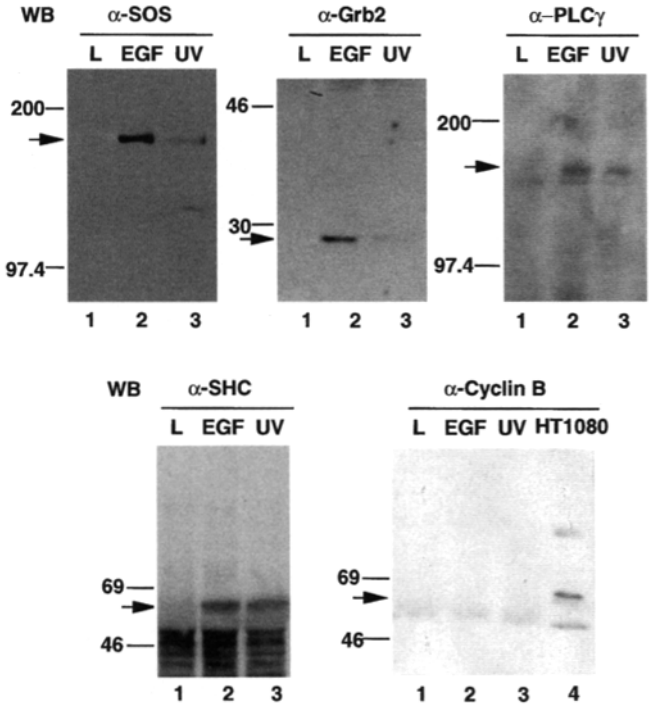

Figure 5. UVC stimulated the association of EGFR with SHC, Grb-2, SOS, and PLCγ. Cell lysates were prepared after EGF administration or UVC irradiation and immunoprecipitated with antibody raised to mouse EGFR. The immunoprecipitates were then immunoblotted with antibodies specific for SOS, Grb-2, PLCγ, SHC, and cyclin B, respectively. Lane *4* shows a positive control, a lysate of HT1080 cells analyzed as a single stage immunoblot not previously immunoprecipitated, to demonstrate that the cyclin B molecule is recognized by this antibody.

diation, and then examined for EGFR activation as before. The EGFR of the cytoplasts was still able to be UV activated in the absence of any nuclei (Fig. 9, *upper panel*).

We have shown previously that ROI such as H_2O_2 induce Egr-1 expression in NIH 3T3 cells (Huang and Adamson, 1995). Therefore, the effect of this oxidant was tested for activation of the EGF receptor in living cells. HC11 cells were treated with H_2O_2 (0.2 mM) for 5 and 10 min before immunoprecipitation with anti-EGFR and analysis by immunoblotting with anti–tyrosine phosphate antibodies, as before. The result, shown in Fig. 10, is that the ROI strongly and rapidly activated the EGF receptor, observed as tyrosine-phosphorylated 170-kD band (Fig. 10, lanes *2* and *3*). Since this activation was inhibited by the antioxidants, NAC (5 mM) and PDTC (0.8 mM), it can be concluded that ROI lead to the activation of the EGF receptor and that antioxidants are behaving in the expected manner. Activated EGFR, in turn, as shown in Fig. 1 *C*, leads to Egr-1 induction downstream.

Discussion

The Involvement of Growth Factor Receptors in the UV Response

Here we investigated the role of growth factor receptors in the UV response that leads to Egr-1 induction. We found

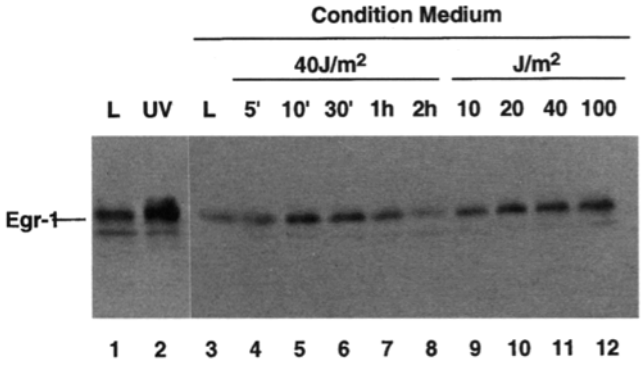

Figure 6. Conditioned media from UVC-treated NIH 3T3 cells stimulated Egr-1 expression. NIH 3T3 cells were cultured with the conditioned media from NIH 3T3 cells that were either in log phase (lane *3*) or after UVC irradiation (40 J/m²) for different times as indicated (lanes *4–8*). Conditioned medium collected from cells 30 min after treatment with different doses of UVC (lanes *9–12*). 2 h later, cells were harvested and assayed for Egr-1 levels by immunoblot analysis. Lanes *1* and *2* were the cell lysates from the log phase and UV-induced NIH 3T3 cells, showing the induction of expression of Egr-1.

that the receptors for EGF, bFGF, and insulin are involved, while the PDGFRβ is not activated, although it is expressed on the surface of NIH 3T3 fibroblasts and HC11 mammary cells. It is interesting that this receptor is excluded from making a UV response, and a literature search indicates that these receptors are not activated in any cell responses to other types of radiation. Upon UVC stimulation, specific growth factor receptors are activated, presumably by a process with some similarities to ligand binding. For EGFR, receptor activation occurs by dimerization and autophosphorylation, and this is followed several hours later by a reduction in the number of EGFR on the surface as endocytosis occurs (Ley and Ellem, 1992). Activated receptor tyrosine kinases can bind to Shc, Grb2, Sos, and PLCγ, forming large complexes and generating the downstream signal pathway leading to transcription factor induction. The similarity of the UV and growth factor sig-

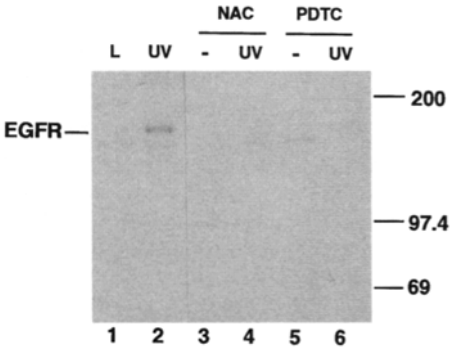

Figure 7. Antioxidants NAC and PDTC blocked the tyrosine phosphorylation of EGFR by UVC. HC11 cells treated with NAC (10 mM) or PDTC (0.8 mM) were assayed for tyrosine phosphorylation of EGFR by immunoprecipitation with anti-EGFR and immunoblotting with anti-phosphotyrosine, with and without UV irradiation. The phosphorylation stimulated by UVC was eliminated by either NAC or PDTC antioxidants.

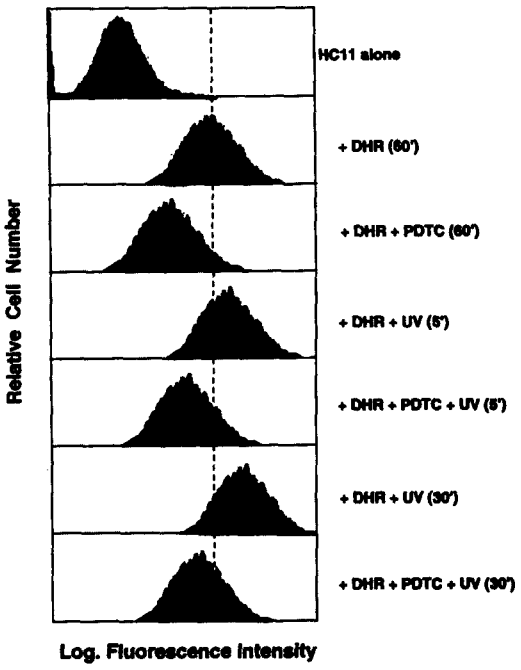

Figure 8. Antioxidant PDTC inhibited UVC-induced ROI production in HC11 cells. DHR treatment (2 μM) (*second line from top*) led to a shift in fluorescence to right compared with untreated cells (*top line*). Such endogenous ROIs were inhibited by preincubation with PDTC (0.8 mM) (*third line*). 5 (*fourth line*) or 30 min (*sixth line*) after exposure to UVC, additional increases in fluorescence intensity (shifts to the right) were observed that were also inhibited by PDTC (*fifth and bottom lines*).

naling pathways has been shown in several cell types (Coffer et al., 1995; Devary et al., 1992; Radler-Pohl et al., 1993; Schieven et al., 1994). In addition, the responsive elements in the responsive transcription factor gene have been shown to be the same for both pathways, namely the CArG boxes known to be the core elements of serum response elements that respond to growth factors (Datta et al., 1992).

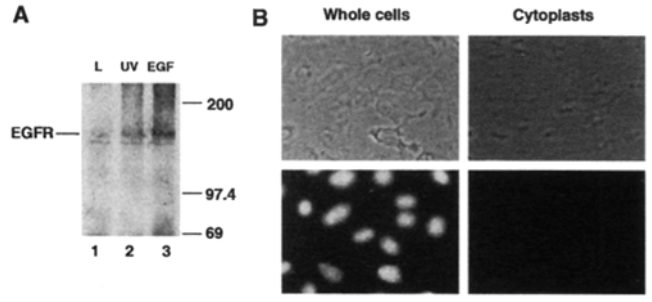

Figure 9. UVC and EGF stimulated the phosphorylation of EGFR in enucleated cells. (*A*) Cytoplasts were prepared from HC11 cells after cytochalasin B treatment and ultracentrifugation. Adherent cytoplasts were left untreated (*L*) or exposed to UVC or EGF. 5 min later, cytoplasts were assayed for the tyrosine phosphorylation of EGFR by immunoblotting. (*B*) Intact cells (*bottom left*) or cytoplasts (*bottom right*) were stained with Hoechst dye to monitor the degree of enucleation. Phase-contrast micrographs (*top*) from the same fields.

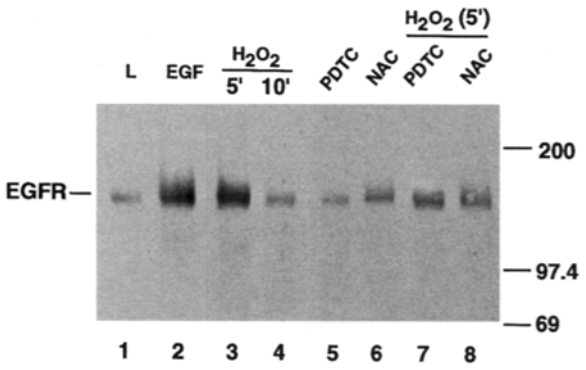

Figure 10. H_2O_2 induced the tyrosine phosphorylation of EGFR. HC11 cells were treated with H_2O_2 (200 μM) and incubated for the indicated times. Cell lysates were analyzed for tyrosine phosphorylation by immunoblotting after immunoprecipitation with anti-EGFR. After preincubation of cells with either NAC (5 mM) or PDTC (0.8 mM), EGFR phosphorylation induced by H_2O_2 (5-min treatment) was significantly reduced.

In spite of the activation of the EGFR by ROI, the most notable cellular response to UV irradiation is not growth stimulation but cell cycle pausing and apoptosis. At least some of the same intermediate steps occur in both UV and ligand activation. The differing response could be due to the presence of limiting amounts of growth-related factors or to the degree of stimulation caused by each. ROI likely lead to the induction of a different set and/or levels of transcription factors with c-Jun, NFκB, and Egr-1 being selectively and strongly induced. In addition, the phosphorylated forms of c-Jun and Egr-1 may have different functions compared to the nonphosphorylated form elicited by growth factors. It is also possible that a stimulus for mitosis before the DNA has replicated is part of the apoptotic mechanism that rids the population of damaged cells.

When the conditioned medium of UV-irradiated cells was added to untreated cells, an Egr-1 inductive response occurred, and therefore, the medium contains stimulatory factors released by the irradiated cells. It is possible that some growth factors are released into the medium. For instance, TGFα has been shown to be released in an active form after UV induction of melanoma (Chenevix-Trench et al., 1992), human skin (Murphy et al., 1991), and Hela cells (Brown et al., 1993). Interleukin-1 and bFGF are also released after UV irradiation and are responsible for at least some of the signal cascade (Kramer et al., 1993). The cytokine, tumor necrosis factor α (TNFα), has been shown to be released into the medium of UV-irradiated cells (Goossens et al., 1995). Further, TNFα and bFGF were able to induce ROI and c-*fos* expression by a mechanism not involving nitric oxide, although the effect was inhibited by antioxidants (Lo and Cruz, 1995). However, the secretion of growth factors in significant amounts is a slow process that can only account for later responses. The release of growth factors and the subsequent activation and down-regulation of receptors occurs over hours and probably involves both release of preexisting growth factors and new synthesis and secretion. In contrast, the immediate response to UV involving EGFR autophosphorylation occurred in 0.2 to 5 min. Another reason to separate the

early and late mechanisms of EGFR activation is based on the form of Egr-1 produced. Growth factor stimulation of cells leads to faster-migrating underphosphorylated forms of Egr-1, while UV rapidly produces slower-migrating, phosphorylated forms (Huang and Adamson, 1995). Notably, H_2O_2 induces the phosphorylated forms of Egr-1 (Huang and Adamson, 1995), supporting a predominantly oxidative pathway for UV. In summary, while the signal contribution from released growth factors on EGFR is significant, it is too little and too late to explain the observed kinetics. Although it is a problem to explain why suramin has such a strong negative effect on the UV signal, the simplest reason is that suramin has other effects at the membrane in addition to the inhibition of growth factor/receptor interactions. For instance, suramin could inhibit the activity of ROI that were secreted into the medium after UV irradiation.

The Role of Oxidative Stress in the Initiation of UV Signaling

An important and much-debated question addresses the identities and sites of the molecules absorbing UVC. Our previous studies demonstrated that oxidative stress rather than DNA damage is involved in the mediation of the UV response of Egr-1 (Huang and Adamson, 1995). This conclusion is further strengthened by the present study and by the similar pathway described for NFκB stimulation (Schreck et al., 1992). Here we not only show that antioxidant reagents could block the phosphorylation of EGFR, but also provide direct evidence that antioxidants abrogate the ROI generated by UV irradiation. In addition, ROI such as H_2O_2 also efficiently induced phosphorylation of EGFR. Furthermore, in an enucleated cell system, UV was still capable of stimulating the phosphorylation of EGFR. ROI can also activate such signal intermediates as mitogen-activated protein kinase (MAPK) and upstream activator kinases (MEK) (Fialkow et al., 1994; Stevenson et al., 1994). Other studies have confirmed that radical scavengers block UV-induced c-Jun expression (Hibi et al., 1993) and UV-activated NF-κB and jun kinase (JNK) in enucleated cells (Derijard et al., 1994). For some systems, however, the contribution of signals originating from DNA damage has been demonstrated (Herrlich et al., 1994; Sachsenmaier et al., 1994*a*; Stein et al., 1989).

One of the arguments against ROI as a cause of UV activation of a cellular signal concerns the differences observed between the responses to different wavelengths of UV radiation. Longer wavelengths, such as UVA (320–400 nm) and UVB (290–320 nm), generate much more ROI compared to UVC (200–290 nm) (Repine et al., 1981; Godar et al., 1993) and, in general, induce the UV response much less efficiently. However, in the case of Egr-1, UVA and UVB do indeed induce this transcription factor even more strongly than UVC (our unpublished data), and therefore, there is a strong positive correlation between level of ROI and level of Egr-1 induction. The discrepancy for other gene products may be specific to each gene and the cell type, the timing of the response, or a combination of these factors. To our best knowledge, this study provides the first indication that ROI as second messengers activate growth factor receptors in the UV response.

Some important questions still remain. How do ROI stimulate receptor phosphorylation and which oxidant species are involved? Our candidates include H_2O_2, O_2^-, and OH^-, but perhaps not nitric oxide. Nitric oxide–producing substrates have been shown to inhibit Egr-1 synthesis in monocyte/macrophage cells (Henderson et al., 1994), but nitric oxide could work in the later stages of cellular response when Egr-1 expression is down-regulated. Growing evidence (Rao et al., 1993; Schenk et al., 1994; Schreck et al., 1991) for many cell types suggests that ROI may serve as second messengers: (*a*) ROI at low doses may function as physiological mediators of cellular responses. It has been shown that H_2O_2 mimics the activity of insulin in glucose transport and in lipid synthesis in rat adipocytes (Mukherjee et al., 1980*a*,*b*; May and de Haen, 1979). H_2O_2 plays an important role in cellular iron metabolism by reducing the synthesis of the intracellular iron storage protein, ferritin, and by stimulating transferrin receptor expression (Pantopoulos and Hentze, 1995). ROI have been implicated in apoptosis (Hockenbery et al., 1993). ROI stimulate the expression of a wide range of immediate early response genes such as Egr-1 (Huang and Adamson, 1995; Ohba et al., 1994), c-*jun*, c-*fos*, and c-*myc* (Luna et al., 1994). Radical scavengers block the activation of NF-κB and AP-1 (Lo and Cruz, 1995; Mohan and Meltz, 1994; Devary et al., 1993). (*b*) ROI have been implicated in growth regulation, such as the regulation of Egr-1 expression by TGFβ (Ohba et al., 1994), the modulation of NF-κB activity by TNFα (Muroi et al., 1994), and the induction of c-*fos* expression by cytokines and growth factors (Lo and Cruz, 1995). Our results showed that ROI induce the phosphorylation of growth factor receptors such as EGFR. (*c*) ROI are found in every cell type, and their intracellular levels can be precisely and rapidly regulated by multiple enzymes such as superoxide dismutase, glutathione peroxidase/glutathione, and by catalase and peroxidases. (*d*) ROI are small, diffusible, and ubiquitous molecules that are rapidly metabolized. Therefore, the hypothesis that ROI may function as second messengers is attractive and appropriate.

The evidence suggests that the UV signal pathway to Egr-1 gene induction may involve a number of separate branches, but an essential pathway appears to be the production of ROI that activate growth factor receptors and hence the rest of the receptor tyrosine kinase pathway. We cannot exclude a significant contribution from a pathway that involves the secretion of growth factors upon UV reaction at the membrane, possibly through oxidative effects on membrane lipids. There may also be a direct effect of UV on receptor conformation, as well as a contribution from UV damage to DNA and subsequent intranuclear signals to the Egr-1 gene. The present study addressed the first and main pathway. Many questions remain, and using the UV-activated Egr-1 model, we hope to address these unknown areas.

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