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Inhibition of Epidermal Growth Factor Receptor Tyrosine Kinase Activity in A431 Human Epidermoid Cells following Psoralen/Ultraviolet Light Treatment

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SUMMARY

One of the more prominent clinical treatments for skin diseases such as psoriasis and vitiligo involves the use of a combination of psoralens and UV light, a procedure referred to as PUVA chemotherapy. This drug regimen markedly alters epidermal cell growth and differentiation. In many cell types, an early cellular event following treatment of cells with PUVA is inhibition of binding of epidermal growth factor (EGF) to its receptor. To examine the mechanism underlying this effect, we used A431 cells, a human epidermal cell line known to express large numbers of EGF receptors. We found that exposure of A431 cells to PUVA caused a dramatic inhibition of EGF-stimulated EGF receptor tyrosine kinase activity. Inhibition required intact cells and did not appear to be mediated by protein kinase C, because this inhibition was apparent in cells in which the enzyme was down-regulated by phorbol ester pretreatment and in cells treated with inhibitors of protein kinase C. Inhibition of tyrosine kinase activity by PUVA was distinct from other inhibitors of EGF receptor function in that it was associated with a rapid increase in the amount of phosphate incorporated into serine residues of the EGF receptor. This suggested that PUVA-induced serine phosphorylation may mediate EGF receptor kinase activity. These results demonstrate that alterations in EGF receptor function may contribute to the therapeutic efficacy of PUVA in photochemotherapy.

In the skin, the combination of psoralens and UVA, also known as PUVA, is a potent modulator of epidermal cell growth and differentiation (1–3). The mechanism underlying the effects of PUVA on epidermal cells is unknown but has been postulated to be due to the DNA binding properties of these agents (4, 5). A specific, saturable, high affinity receptor is a major target for the psoralens (6, 7). This receptor, a 22,000 molecular weight protein present in membrane and cytoplasmic fractions of cells, is photoalkylated by the psoralens following UVA light treatment. It has also been shown that psoralen receptor binding and UVA light activation is associated with inhibition of EGF receptor binding (8). The EGF receptor is a 170,000 molecular weight transmembrane glycoprotein possessing intrinsic tyrosine-specific protein kinase activity that is activated by EGF (9–14). The receptor protein is phosphorylated at threonine and serine residues and, following EGF binding, at tyrosine residues through a process of autophosphorylation (15). Agents that modulate cell growth and inhibit EGF binding, including phorbol ester tumor promoters, stimulate phosphorylation of the EGF receptor and reduce its tyrosine kinase activity (16–19). Because photoactivated psoralens, like phorbol esters, inhibit EGF binding to cells, it was of interest to determine whether this group of drugs also modulated EGF receptor kinase activity. For these studies, we used A431 cells, a cell line possessing large numbers of EGF receptors (20). PUVA was found to be a potent inhibitor of EGF-induced receptor autophosphorylation. Inhibition was associated with increased serine phosphorylation of the EGF receptor. These data provide evidence that the biological actions of the psoralens may be mediated by alterations in the tyrosine kinase signal transduction pathways.

Experimental Procedures

Cell culture and [^{32}P]orthophosphate labeling. A431 cells, obtained from Dr. Sidney Pestka (UMDNJ-Robert Wood Johnson Medical School), were maintained in DMEM supplemented with 10% fetal bovine serum and 4 mM L-glutamine. For labeling studies, cells (2×10^5) were inoculated into 35-mm culture dishes. After 13 hr, the culture medium was removed and the cells were refed with 1 ml of phosphate-

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ABBREVIATIONS: UVA light, UV light of wavelength 320–400 nm; PUVA, psoralens used in combination with UVA light; TMP, 4,5′,8-trimethylpsoralen; EGF, epidermal growth factor; DMEM, Dulbecco's modified Eagle's medium; TPA, 12-*O*-tetradecanoyl-phorbol-13-acetate; H7, 1-(5-isoquinolinylsulfonyl)-2-methyl-piperazine; SDS, sodium dodecyl sulfate; TPCK,-*N*-tosyl-L-phenylalanine chloromethyl ketone; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

free DMEM supplemented with 0.5 mCi/ml [^{32}P]orthophosphate (ICN, Irvine, CA). The cells were then incubated at 37° in a humidified CO_2 incubator. After 5 hr, TMP (4.4 μM final concentration) or EGF (150 ng/ml) was added to the cultures. Five minutes later, some of the cells were irradiated with UVA light (2.8 J/cm^2) emitted from a bank of four BLB fluorescent light tubes (F40BL, Sylvania) placed approximately 10 cm above the culture plates. UVA light was monitored with an IL-SE 115 probe and 363 UVA pass filter. To down-regulate protein kinase C, cells were pretreated with TPA (100 ng/ml) for 24 hr before use in phosphorylation assays (21–25). H7 (30 μM) and sphingosine (10 μM) were used to inhibit protein kinase C (26, 27). The cells were treated with the inhibitors 50 min before and during stimulation with EGF.

Isolation of EGF receptors. EGF receptors were isolated from the cells using a polyclonal anti-EGF receptor antibody (E5) (Stuart Aaronson, National Institutes of Health) as described by Kris *et al.* (28) with some modifications. The medium from the culture plates was removed and 0.4 ml of ice-cold lysis buffer (1% Triton X-100, 10 mM Tris·HCl, pH 7.6, 5 mM EDTA, 50 mM NaCl, 30 mM tetrasodium pyrophosphate, 50 mM NaF, 100 μM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride) was added to each dish. After 10 min, the solubilized cells were scraped from the plates with a rubber policeman and transferred into 1.5-ml Eppendorf microcentrifuge tubes. The samples were then centrifuged (13,000 × *g*, 7 min) to remove cellular debris and the clear supernatants were incubated with anti-EGF receptor antibody and Protein A-Sepharose for 2 hr at 4°. The reaction was stopped by the addition of Laemmli sample buffer (0.125 M Tris·HCl, pH 6.8, 20% glycerol, 4 mM dithiothreitol, 4.6% SDS) followed by boiling. The EGF receptor was then separated on 7.5% SDS-polyacrylamide gels and visualized in the gels by autoradiography. Tyrosine-phosphorylated EGF receptors were purified from detergent extracts of labeled cells using a phosphotyrosine monoclonal antibody (1G2) coupled to Sepharose 4B, prepared as described by Frackelton *et al.* (29).

Phosphoamino acid analysis. The phosphoamino acid content of the EGF receptor was determined after partial acid hydrolysis, as described by Cooper *et al.* (30). Phosphoamino acids were separated by one-dimensional thin layer electrophoresis using 5% acetic acid and 1% pyridine, pH 3.5. For two-dimensional separation, phosphoamino acids were first electrophoresed at pH 3.5 and then at pH 1.9 (88% formic acid/glacial acetic acid/H_2O, 50:156:1749, v/v/v).

Tryptic peptide analysis. Gel slices containing the EGF receptor were incubated overnight in 1 ml of buffer containing 0.05 M ammonium bicarbonate, 1% SDS, 1 mM EDTA, and 10 μg/ml bovine serum albumin. The extracted receptor was then lyophilized and resuspended in 150 μl of ice-cold 33% trichloroacetic acid. The precipitate was washed once with trichloroacetic acid, three times with 5% perchloric acid and then three times with acetone. Extracts were then incubated with 100 μl of TPCK-trypsin (50 μg/ml in 0.05 M ammonium bicarbonate) for 16 hr at room temperature and then for 4 hr at 37°. Samples were lyophilized, washed with distilled water, and then treated for 2 hr on ice with performic acid. After lyophilization, samples were washed two times with distilled water and the phosphopeptides were separated in two dimensions using thin layer electrophoresis (acetic acid/formic acid/water, 15:5:80, v/v/v, pH 1.9) in the first dimension, followed by thin layer chromatography (butanol/pyridine/acetic acid/water, 32:25:5:20, v/v/v/v, pH 8.9) in the second dimension.

***In vitro* tyrosine kinase assay.** For kinase assays, membranes from A431 cells were prepared according to the method of Weber *et al.* (31) with some modifications. Briefly, exponentially growing cells (5 × 10^7) were suspended in 2 ml of hypotonic buffer (5 mM Tris·HCl, pH 7.9, 2 mM $MgCl_2$, 50 μM Na_3VO_4, 2 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, and 1 μg/ml aprotinin). After 10 min, the cells were homogenized with a Dounce homogenizer (20 strokes). The broken cells were layered onto 2 ml of a 41% sucrose cushion and centrifuged (1000 × *g*, 20 min, 4°) to separate nuclei. The membrane-containing fraction was centrifuged at 100,000 × *g* for 60 min at 4°. The pelleted membranes were resuspended in 1 ml of 20 mM HEPES, pH 7.4, and then mixed with an equal volume of solubilization buffer (20 mM HEPES, pH 7.4, 2% Triton X-100, 10% glycerol, 2 mg/ml bovine serum albumin). After 15 min at 25°, the membranes were diluted 5-fold in buffer containing 20 mM HEPES, pH 7.4, 10% glycerol, and 1 mg/ml bovine serum albumin and were centrifuged at 10,000 × *g* for 10 min. The supernatants were collected and assayed directly for enzyme activity. Tyrosine kinase assays were carried out in 58-μl volumes containing 20 mM HEPES, pH 7.4, 1 mM $MnCl_2$, 5 mM $MgCl_2$, 50 μM Na_3VO_4, 5 μM [γ-^{32}P] ATP (650 Ci/mmol) (ICN, Irvine, CA), and appropriate amounts of membrane extract. In some experiments, EGF (150 ng/ml) was added to the membrane extracts before their addition to the tyrosine kinase assay. Unless otherwise specified, each assay contained 0.25 μCi of [^{32}P]ATP. Assays were run for 2 min at 30°. Some of the membrane samples were pretreated with TPA (100 ng/ml), or TMP (4.4 μM), and UVA light (2.8 J/cm^2) before the addition of the solubilization buffer. The enzyme reaction was stopped by the addition of an equal volume of Laemmli sample buffer. The EGF receptor was identified as a 170,000 molecular weight phosphoprotein by SDS-polyacrylamide gel electrophoresis and the extent of phosphorylation was quantified by cutting the receptor from the gels and counting for radioactivity.

^{125}I-EGF binding assays. High affinity ^{125}I-EGF binding assays were performed as previously described by Friedman and Rosner (25).

Results

Effect of psoralens and UVA light on ^{125}I-EGF binding in A431 cells. In previous studies, we reported that psoralens and UVA light were potent inhibitors of ^{125}I-EGF binding in different mouse and human cell types (8). In initial experiments, we tested the ability of PUVA to inhibit ^{125}I-EGF binding to high affinity receptors in A431 cells. In these studies we used TMP, a psoralen analog used clinically in photochemotherapy (32). We found that pretreatment of the cells with this psoralen (4.4 μM), followed by UVA light (2.1 J/cm^2), caused a 31% inhibition of high affinity ^{125}I-EGF receptor binding, whereas TPA caused only an 18% inhibition (Table 1). Under similar conditions, we previously reported that TMP and UVA light inhibited ^{125}I-EGF specific binding to HeLa and PAM 212 epidermal cells by over 76 and 88%, respectively (8).

Effect of PUVA and EGF receptor tyrosine kinase activity. We next determined whether PUVA inhibition of EGF receptor binding in A431 cells was the result of interference with the biological functions of the EGF receptor. Initially, we examined the effects of PUVA on tyrosine kinase activity of the EGF receptor in the cells. For this analysis, we used a specific monoclonal antibody to phosphotyrosine (19, 29). This antiphosphotyrosine monoclonal antibody permitted us to selectively isolate tyrosine-phosphorylated EGF receptors. A431 cells, labeled with [^{32}P]orthophosphate, were pulsed with EGF (150 ng/ml) for 5 min to stimulate receptor tyrosine kinase activity. Cells were then lysed and tyrosine-phosphorylated

TABLE 1

Inhibition of ^{125}I-EGF binding to high affinity receptors in A431 cells

Each point represents the average of four samples ± standard error. High affinity EGF binding to A431 cells was performed as described by Friedman and Rosner (25). Cells were pretreated with TPA (170 nM) or TMP (4.4 μM) and UVA light (2.1 J/cm^2), as described in Experimental Procedures.

Treatment	^{125}I-EGF binding
	cpm/4 × 10^5 cells
Control	1017 ± 27
TPA	832 ± 38
PUVA	698 ± 25

EGF receptor was isolated using an immunobatch procedure (29). Fig. 1 (*right*, *lanes 1* and *2*) shows that, under the conditions used, no significant amount of tyrosine-phosphorylated EGF receptor was detectable in either control or PUVA-treated cells. In contrast, EGF treatment markedly enhanced the phosphorylation of tyrosine residues on the EGF receptor (15, 29) (Fig. 1, *lane 4*). When cells were treated with PUVA before EGF, we observed a significant inhibition of EGF receptor phosphorylation (Fig. 1, *lane 3*). Psoralen alone had no effect on phosphorylation, whereas UVA light caused a small but reproducible inhibition of receptor phosphorylation (not shown). Phosphoamino acid analysis of the purified tyrosine phoshorylated EGF receptor confirmed that PUVA treatment inhibited EGF-stimulated phosphorylation on tyrosine residues (Fig. 1, *left*). In this figure, less phosphoserine and phosphothreonine were visualized because of the decrease in tyrosine-phosphorylated receptor in the cells that occurs following PUVA treatment. Fig. 2 shows the time course of the inhibition of EGF-stimulated EGF receptor phosphorylation by PUVA. We found that inhibition occurred within 45 sec following the addition of EGF. Tyrosine kinase activity remained inhibited for at least 25 min following PUVA treatment. Inhibition of EGF-stimulated tyrosine phosphorylation was also found to be dose dependent with increasing concentrations of TMP (Fig. 3). Taken together, these data show that PUVA inhibits EGF receptor tyrosine kinase activity.

Role of protein kinase C in the action of PUVA. Phorbol ester tumor promoters are also known to be inhibitors of tyrosine kinase activity of the EGF receptor (16–19). These compounds are thought to mediate their biological actions on

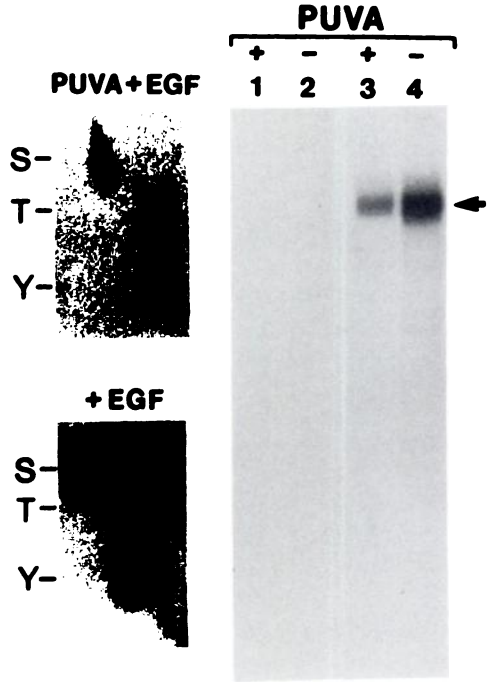

Fig. 1. PUVA-mediated inhibition of EGF receptor tyrosine kinase activity. A431 cells were prelabeled with [^{32}P]orthophosphate and then treated with PUVA followed by EGF, as described in Experimental Procedures. *Right*, *lane 1*, TMP (4.4 μM) alone; *lane 2*, control; *lane 3*, TMP plus UVA light (2.8 J/cm^2) followed by EGF (150 ng/ml); *lane 4*, EGF alone; *Left*, autoradiograph of phosphoamino acids from acid hydrolysates of EGF receptor isolated from *lanes 3* and *4*. Phosphoamino acids were resolved by thin layer electrophoresis at pH 3.5 followed by electrophoresis at pH 1.9, as described by Cooper *et al.* (30).

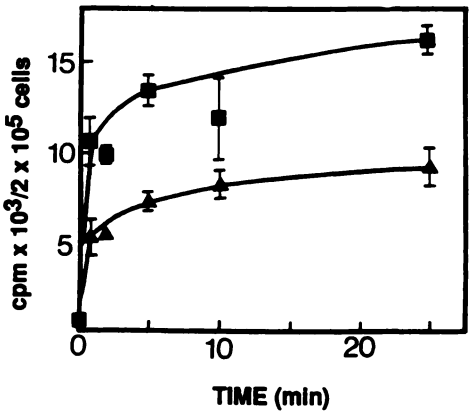

Fig. 2. Effect of PUVA on the time course of EGF-stimulated autophosphorylation of the EGF receptor in A431 cells. A431 cells labeled with [^{32}P]orthophosphate were treated with 4.4 μM TMP and UVA light (2.8 J/cm^2) and then incubated with 150 ng/ml EGF for the indicated times. EGF receptor was then isolated from the cells using an antiphosphotyrosine antibody and SDS-polyacrylamide gel electrophoresis. Phosphate incorporated into the receptor protein was quantified as described in Experimental Procedures. ■, EGF-stimulated EGF receptor phosphorylation in control cells; ▲, EGF-stimulated EGF receptor phosphorylation in cells pretreated with PUVA. Each *point* represents the average of three samples ± standard error.

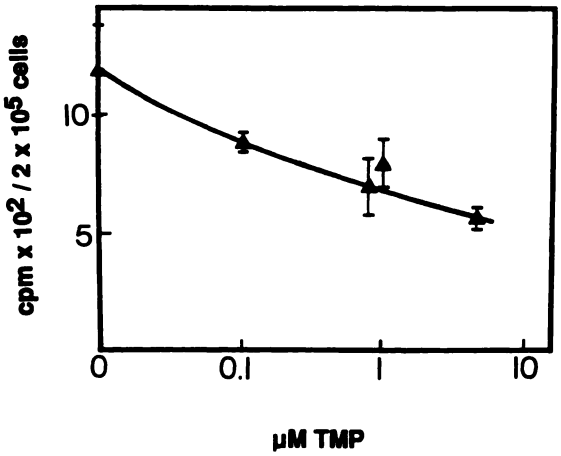

Fig. 3. Dose-dependent inhibition of EGF-stimulated autophosphorylation of the EGF receptor following PUVA treatment of A431 cells. A431 cells were labeled with [^{32}P]orthophosphate, incubated with varying concentrations of TMP, and pulsed with UVA light (2.8 J/cm^2). Cells were then incubated with EGF (150 ng/ml). After 5 min, EGF receptor was isolated from the cells using a monoclonal antiphosphotyrosine antibody and SDS-polyacrylamide gel electrophoresis. Phosphotyrosine content of the receptor was determined by partial acid hydrolysis and phosphoamino analysis followed by thin layer electrophoresis. The data represent radioactive phosphotyrosine isolated from the EGF receptor after SDS-polyacrylamide gel electrophoresis and phosphoamino acid analysis. Each point represents the average of three samples ± standard error.

cells by binding to and activating the calcium- and phospholipid-dependent enzyme protein kinase C (33–37). To determine whether protein kinase C was involved in PUVA-inhibited EGF receptor kinase, A431 cells were preincubated with TPA (100 ng/ml) for 24 hr, a treatment that has been shown to down-regulate protein kinase C in A431 cells as well as other cell types (21–25). Cells were treated with PUVA, and EGF-stimulated receptor kinase activity was then assayed. Under these conditions, we found that PUVA continued to be an effective inhibitor of EGF receptor phosphorylation (Fig. 4A). We next examined the effects of two protein kinase C antagonists, H7

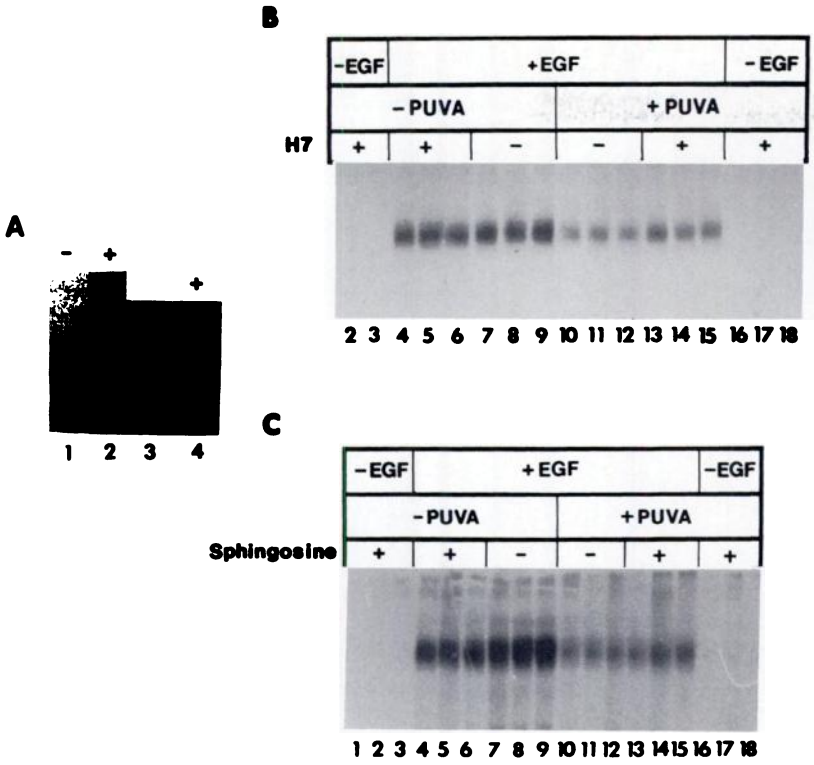

Fig. 4. Protein kinase C and PUVA-mediated inhibition of EGF receptor kinase activity. A431 cells were assayed for EGF receptor tyrosine kinase activity using the phosphotyrosine antibody, as described in Experimental Procedures section. A, Effect of PUVA on EGF receptor kinase in protein kinase C down-modulated cells. *Lane 1*, EGF-stimulated kinase activity 24 hr after treatment with TPA (170 nM); *lane 2*, EGF (150 ng/ml, 5 min)-stimulated kinase activity in protein kinase C down-modulated cells treated with PUVA (4.4 μM TMP, 2.8 J/cm²); *lane 3*, EGF (150 ng/ml, 5 min)-stimulated kinase activity in control cells; *lane 4*, EGF (150 ng/ml, 5 min)-stimulated kinase activity in control cells treated with PUVA (4.4 μM TMP, 2.8 J/cm²). *Lanes 1* and *2* were exposed to film for 4 hr. *Lanes 3* and *4* were exposed to film for 1 hr. B and C, Effect of protein kinase C inhibitors H7 (10 μM) (B) and sphingosine (30 μM) (C) on EGF receptor kinase activity. *Lanes 1–3*, protein kinase C inhibitor treatments alone without EGF stimulation; *lanes 4–6*, inhibitor treatments with EGF (100 ng/ml); *lanes 7–9*, EGF-stimulated kinase without inhibitor treatments; *lanes 10–12*, EGF-stimulated kinase following PUVA treatment; *lanes 13–15*, EGF-stimulated kinase following inhibitor and PUVA treatments; *lanes 15–17*, inhibitor and PUVA treatments without EGF stimulation.

and sphingosine, on inhibition of EGF receptor kinase by PUVA. We found that pretreatment of the cells with sphingosine had no effect on the PUVA-induced inhibition of EGF receptor tyrosine kinase activity (Fig. 4C). H7 was found to slightly antagonize the inhibitory effects of PUVA on the EGF receptor (Fig. 4B). This may have been due to the ability of H7 to inhibit other protein kinases (27). Taken together, these data suggest that protein kinase C does not directly mediate the effects of PUVA on EGF receptor tyrosine kinase activity.

Effect of PUVA on EGF receptor tyrosine kinase activity in A431 membranes. We next examined the effect of PUVA on EGF receptor kinase in isolated plasma membranes of A431 cells. We found that EGF readily stimulated phosphorylation of its receptor in these preparations (Fig. 5). Treatment of the membrane fractions with PUVA had no effect on EGF receptor phosphorylation, demonstrating that the psoralens do not directly inhibit the EGF receptor kinase. Similar results were observed with TPA (Fig. 5), as well as with UVA light and TMP alone (not shown).

We also analyzed EGF receptor kinase activity in membranes prepared from cells pretreated with PUVA as well as EGF or TPA *in vivo*. The results from these experiments revealed that these pretreatments altered receptor kinase activity as well as membrane phosphorylation (Fig. 6). Maximal kinase activity was observed in membranes isolated from cells pretreated with EGF *in vivo*. EGF-stimulated receptor kinase activity from membranes of PUVA-treated cells was similar to controls, whereas TPA pretreatment enhanced kinase activity. In these experiments, we identified a number of proteins in addition to the EGF receptor that were phosphorylated. Interestingly, pretreatment of cells with EGF and TPA, but not PUVA, resulted in the appearance of a unique 30,000 molecular weight phosphoprotein following EGF stimulation *in vitro*. In contrast, a 33,000 molecular weight phosphoprotein was identified only in

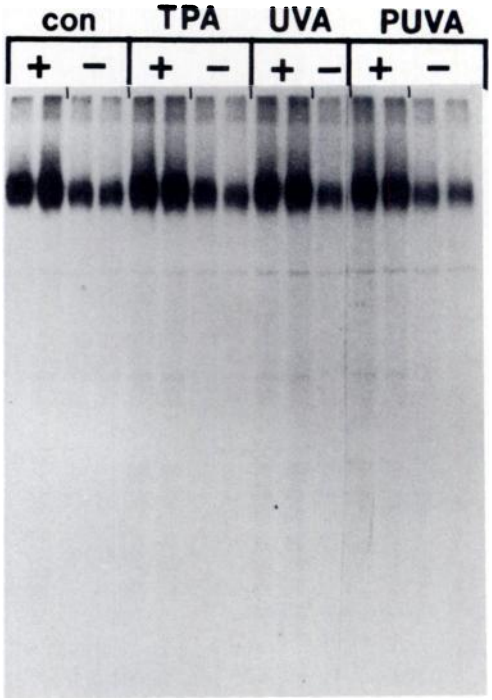

Fig. 5. Effect of TPA, PUVA, and UVA light on EGF receptor tyrosine kinase activity in A431 membranes. Membranes from A431 cells were prepared and treated with TPA (170 nM, 5 min), PUVA (4.4 μM TMP, 2.8 J/cm²), or); UVA light alone, as described in Experimental Procedures. Tyrosine kinase activity was measured in the presence (+) or absence (−) of EGF (150 ng/ml).

membranes isolated from PUVA-treated cells. The appearance of this phosphoprotein was not dependent on stimulation of the membranes with EGF *in vitro*. The fact that each treatment resulted in the phosphorylation of unique substrates provides

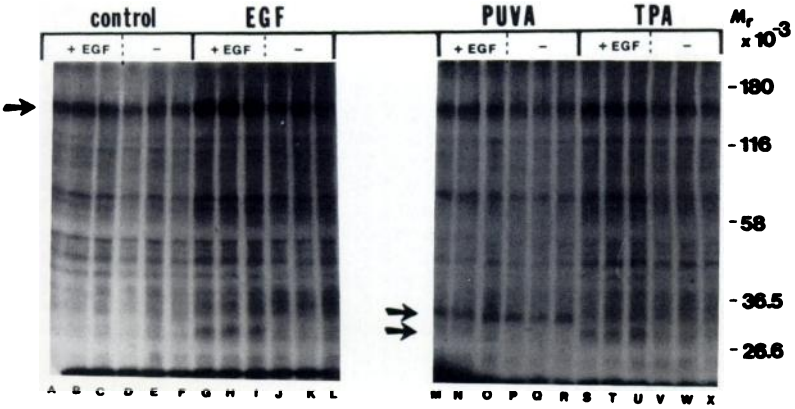

Fig. 6. *In vitro* kinase assay of membranes isolated from cells pretreated with EGF, PUVA, and TPA. Membranes were prepared from cells treated with EGF (150 ng/ml, 5 min), TPA (170 nM, 5 min), or TMP (4.4 μ) and UVA light (2.8 J/cm^2) and were used in *in vitro* kinase assays, as described in Experimental Procedures. Assays contained in 1 μCi of [γ-^{32}P]ATP. Membranes from each treatment were assayed with and without the addition of EGF (100 ng/ml) to the kinase assay. *Lanes A–C*, control cell membranes with EGF in the assay mixture; *lanes D–F*, control membranes without EGF; *lanes G–I*, EGF-treated membranes with EGF; *lanes J–cm or UVAL*, EGF-treated cells alone; *lanes M–O*, PUVA-treated membranes with EGF; *lanes P–R*, PUVA-treated membranes without EGF; *lanes S–U*, TPA-treated membranes with EGF; *lanes V–X*, TPA-treated membranes without EGF. The *arrow* on the *gel* to the *left* shows the EGF receptor. The *arrows* on the *gel* to the *right* show PUVA- and EGF-induced alterations in the expression of two phosphoproteins.

evidence that the actions of PUVA, EGF, and TPA on A431 cells are distinct.

Effect of PUVA on EGF receptor phosphorylation. Phorbol esters are thought to regulate EGF receptor kinase activity by inducing phosphorylation of the EGF receptor (38, 39). To determine whether the psoralens also induced EGF receptor phosphorylation, we compared [^{32}P]orthophosphate-labeled EGF control A431 cells with cells treated with PUVA or EGF. Detergent extracts of treated cells were prepared and the EGF receptor was purified by immunoadsorption to Protein A-Sepharose with polyclonal anti-EGF receptor antibody that recognizes the total EGF receptor population (28). This was followed by SDS-polyacrylamide gel electrophoresis. As shown in Fig. 7 (*right*), both EGF and PUVA (Fig. 7, *right, lanes 2* and *3*, respectively) stimulated phosphorylation of the EGF receptor over control levels (Fig. 7, *right, lane 4*). In addition, the combination of PUVA plus EGF induced greater phosphorylation of the EGF receptor than either treatment alone (Fig. 7, *right, lane 1*). UVA light alone also induced a small increase in receptor phosphorylation (see below); however, psoralen alone had no effect on the receptor (not shown). To characterize the nature of the increase in EGF receptor phosphorylation in A431 cells, we performed phosphoamino acid analysis. These studies revealed that the EGF receptor from EGF-treated A431 cells contained phosphoserine, phosphothreonine, and phosphotyrosine (Fig. 7, *left*). Similar results have been reported previously (15). In contrast, the EGF receptor from PUVA-treated cells contained only phosphoserine and phosphothreonine. In further studies, the phosphoamino acids of the EGF receptor were quantified. We found that EGF treatment resulted in an increase in the receptor content of all three phosphoamino acids, compared with controls (Fig. 8). The

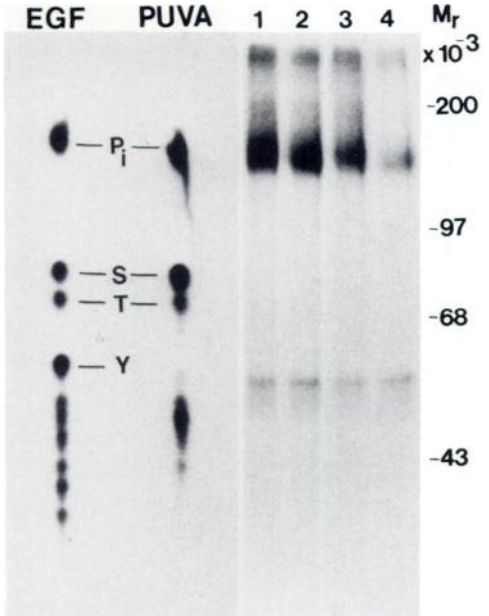

Fig. 7. Effects of PUVA on EGF receptor phosphorylation. The EGF receptor was isolated from [^{32}P]orthophosphate-labeled A431 cells using a polyclonal anti-EGF receptor antibody and was separated in 7.5% SDS-polyacrylamide gels. Right, *lane 1*, PUVA and EGF; *lane 2*, EGF; *lane 3*, PUVA; *lane 4*, control. The phosphoamino acid content of the isolated EGF receptors was determined after partial acid hydrolysis, as described in Experimental Procedures. The analysis (*left*) shows the phosphoamino acids of the EGF receptor isolated from *lanes 2* and *3*. P_i, inorganic phosphate; S phosphoserine; .G, phosphothreonine; Y, phosphotyrosine.

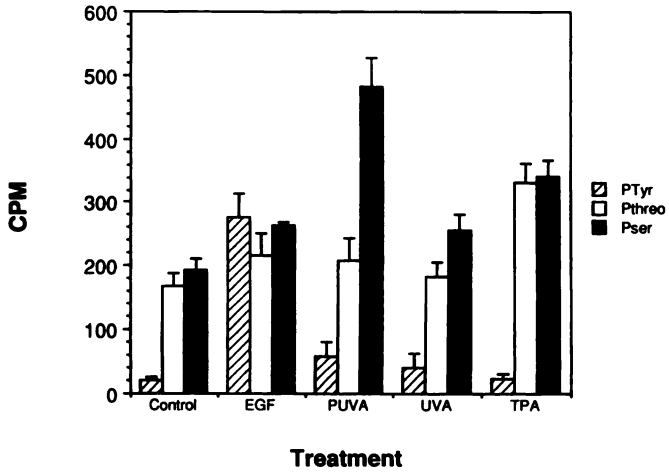

Fig. 8. Phosphoamino acid analysis of EGF receptor isolated from A431 cells. Bands from 7.5% SDS-polyacrylamide gels shown in Fig. 1, containing EGF receptor, were extracted and analyzed for phosphoamino acid content using thin layer electrophoresis. Spots containing the separated phosphoamino acids were then scraped from the chromatography plates and counted for radioactivity. Each *bar* represents the average of four samples ± standard error. Treatments: control, EGF (150 ng/ml), PUVA (4.4 μM), UVA light (2.8 J/cm^2); TPA (170 nM). PUVA treatment resulted in significant ($p < 0.01$) differences in serine phosphorylation of the EGF receptor compared with all other treatments. *PTyr*, phosphotyrosine; *Pthreo*, phosphothreonine; *Psero*, phosphoserine.

increase in phosphorylation of the EGF receptor stimulated by PUVA was found to occur largely on serine residues. UVA light alone also increased serine phosphorylation, but to a lesser extent than the combination of UVA light and psoralen (Fig. 8). EGF receptors isolated from cells treated with PUVA followed by EGF displayed similar amounts of serine phosphorylation as did cells treated with PUVA alone (not shown). In contrast, tyrosine phosphorylation in PUVA/EGF-treated cells was decreased (see further below). These data indicate that the net increase in total EGF receptor phosphorylation after the combination of PUVA and EGF (Fig. 7, *lane 1*) is the result of receptor phosphorylation induced by both treatments.

We next compared changes in receptor phosphorylation induced by PUVA and EGF with those induced by the phorbol ester TPA. TPA was also found to stimulate phosphorylation of the EGF receptor on serine residues, although not as effectively as PUVA (Fig. 8). However, the effects of TPA on A431 cells appeared to be distinct from those of PUVA, in that TPA also stimulated threonine phosphorylation of the EGF receptor. In these studies, psoralen without UVA light had no effect on EGF receptor phosphorylation or on its phosphoamino acid content (not shown).

To further distinguish the effects of PUVA, TPA, and EGF on phosphorylation of the EGF receptor, we performed two-dimensional tryptic peptide mapping. We found that all three agents stimulated phosphorylation of EGF receptor peptide fragments over control levels (Fig. 9). In control cells, at least two major peptide fragments were found to be phosphorylated (Fig. 9A, *fragments 1* and *2*), whereas, in EGF-treated cells, several additional phosphorylated peptide fragments were visualized (Fig. 9B). The phosphorylation of fragments 1 and 2, as well as several additional peptides migrating above the origin (Fig. 9, B–D), was enhanced by EGF as well as TPA and PUVA treatment (Fig. 9, B–D). It is apparent from this figure that multiple sites are phosphorylated only following EGF treatment (Fig. 9D, *fragments 3–7*) (13, 38, 39). We found that there were several peptide fragments migrating near and above the origin where phosphorylation was increased following PUVA treatment. These data further show that alterations in the EGF receptor induced by PUVA are distinct from those induced by TPA and EGF.

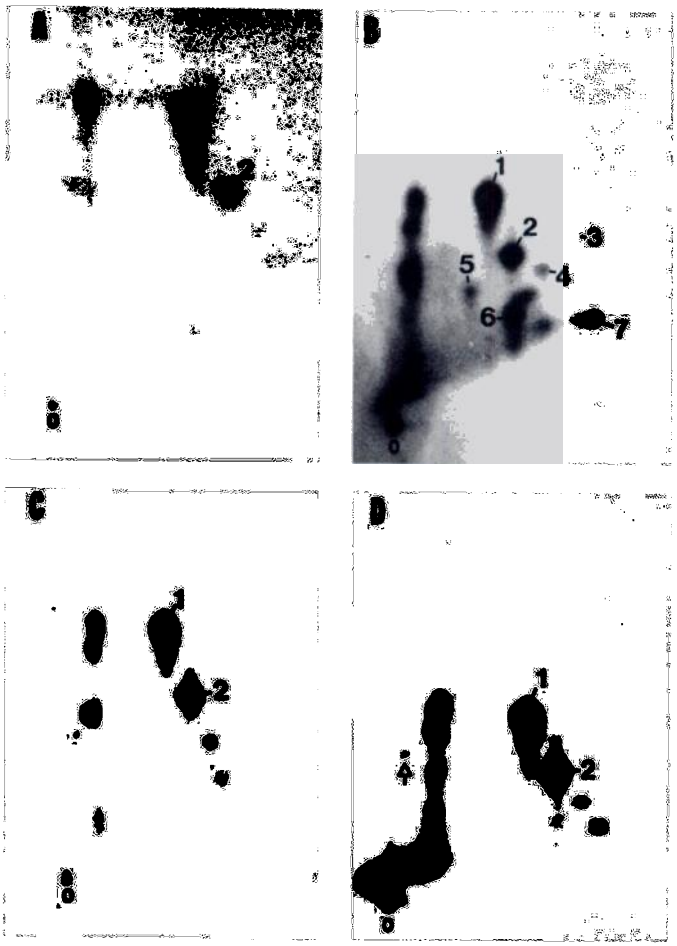

Fig. 9. Two-dimensional tryptic phosphopeptide mapping of EGF receptors from A431 cells. A431 cells were prelabeled with [^{32}P]orthophosphate and the EGF receptors were isolated using polyclonal anti-EGF receptor antibodies and peptide mapped, as described in Experimental Procedures. A, EGF receptor isolated from untreated cells; B, EGF receptor from cells treated with EGF (100 ng/ml); C, EGF receptor from cells treated with TPA (170 nM); D, EGF receptor from cells treated with TMP. (4.4 μM) and UVA light (2.8 J/cm^2). *O*, origin. Note that a number of highly phosphorylated peptide fragments were observed in PUVA-treated cells near the origin of the chromatogram. *Arrows* point out unique phosphorylated peptide fragments.

Discussion

Psoriasis is a common skin disorder characterized by increased epidermal proliferation and altered differentiation. A number of reports have suggested that abnormalities in the regulation of EGF receptor function may contribute to the pathogenesis of this disease. Thus, Nanney and co-workers (40) have demonstrated alterations in EGF binding and receptor distribution in psoriatic epidermis. In addition, Gentleman *et al.* (41) have shown that tyrosyl kinase and phosphotyrosyl phosphatase activities are elevated in psoriatic lesions. Furthermore, it has been reported that psoriatic epidermis contains elevated levels of mRNA and protein for transforming growth factor α, a structural analog of EGF that binds to the EGF receptor (42). Overexpression of transforming growth factor α with subsequent activation of the EGF receptor may be responsible for initiating and maintaining psoriatic lesions.

Our data show that a clinically important drug regimen used in the treatment of psoriasis is effective in suppressing EGF receptor function. Exposure of A431 cells to PUVA resulted in phosphorylation of the EGF receptor and inhibition of EGF receptor tyrosine kinase activity. It should be noted that it is unlikely that the psoralens mediate their effects by direct binding to the EGF receptor. We have previously shown that psoralens bind to unique receptors and do not compete with EGF for binding (6). With the exception of the psoralen receptor, these compounds display low affinity for proteins and do not photoalkylate the EGF receptor (7). Furthermore, as shown in the present studies, no direct effect of PUVA on the EGF receptor kinase was observed using purified membranes from A431 cells.

We have previously shown that PUVA is a potent inhibitor of EGF receptor ligand binding in a number of mouse and human cell lines (8). In HeLa cells, which have both higher and lower affinity receptors for EGF, PUVA specifically inhibited high affinity ligand binding. In A431 cells, we also found that PUVA inhibited ^{125}I-EGF binding to high affinity receptors. Friedman and Rosner (25) demonstrated that various tumor promoters appear to exclusively inhibit EGF binding to

the high affinity EGF receptor population in A431 cells. These investigators have suggested that this event is related to the loss of EGF-stimulated tyrosine phosphorylation (19, 43). Our data support the model that high affinity EGF binding and stimulated kinase activity reside in the same receptor population (44).

The mechanisms underlying the effects of PUVA and tumor promoters on tyrosine kinase activity appear to be distinct. TPA is thought to act by binding to and activating protein kinase C, the putative receptor for this compound (33–36). We have previously demonstrated that the psoralen receptor and protein kinase C are distinct with respect to size and associated ligand binding specificity (7, 36). Moreover, psoralens and phorbol esters do not compete with one another for receptor binding (8) and, unlike the phorbol ester (33–36), the psoralens do not directly activate protein kinase C.[1] Protein kinase C is both a serine and threonine kinase, and the EGF receptor is known to be a substrate for this enzyme (37, 38, 45). Phosphorylation of Thr654 on the EGF receptor by protein kinase C has been suggested to lead to inhibition of EGF receptor ligand binding and tyrosine kinase activity. Our findings that PUVA-inhibited receptor kinase activity in protein kinase C down-modulated cells and that the effects of PUVA on the EGF receptor were not blocked by protein kinase C inhibitors suggest that the biological actions of the psoralens are not mediated by this enzyme. The fact that PUVA selectively stimulates serine phosphorylation (see further below), and at the same time inhibits EGF-stimulated tyrosine kinase activity, indicates that sites other than Thr654 are also important in regulating EGF receptor function. Recently, Friedman *et al.* (46) reported that two agents, the calcium ionophore A23187 and the non-phorbol ester tumor-promoting agent thapsigargen, inhibit EGF receptor binding and kinase activity through mechanisms independent of protein kinase C. Similar results have been reported using the non-phorbol ester tumor promoter palytoxin (43). Using thapsigargen, Friedman *et al.* (46) also showed that sites other than Thr654 could negatively regulate EGF receptor function.

As indicated above, phosphorylation of the EGF receptor has been reported to regulate ligand binding, as well as receptor kinase activity (15, 46–48). PUVA, TPA, and EGF all differentially alter EGF receptor kinase activity and induce EGF receptor phosphorylation. Our data clearly demonstrate that each of the compounds differentially phosphorylates serine, threonine, and tyrosine residues of the EGF receptor. Similar results have been reported previously with TPA and EGF (9, 15). Tryptic peptide mapping revealed that phosphorylation of the EGF receptor by PUVA occurred on several different phosphopeptide fragments, suggesting that there are multiple sites of phosphorylation. The fact that several phosphopeptide fragments were observed in PUVA-treated receptors and not in EGF-treated receptors indicates that PUVA stimulates phosphorylation at distinct sites on the EGF receptor.

Phosphorylation of proteins in cells following treatment with EGF is an important early event in the regulation of cellular proliferation. We noted several differences in the substrates phosphorylated in isolated A431 membranes from cells pretreated with PUVA, TPA, or EGF. At the present time, it is not known whether the appearance of these phosphoproteins is due to alterations in substrate specificity of the EGF receptor kinase or to the appearance of other protein kinases and/or substrates in the membranes. The functions of these phosphoproteins in the biological actions of PUVA, TPA, and EGF are also not known. The psoralen-induced 33,000 molecular weight membrane protein may be a substrate for a kinase activated by PUVA (see below). The 30,000 molecular weight phosphoprotein induced by TPA and EGF may be related to one of a number of proteins that have been identified in cells that are important in metabolism and growth and are known to be substrates for the EGF receptor (49–51).

Serine phosphorylation of the EGF receptor is one possible mechanism by which PUVA mediates its inhibitory effects on EGF binding and tyrosine kinase activity. We hypothesize that photoactivation of the psoralen receptor is linked to increased incorporation of phosphate into serine residues of the EGF receptor. We have previously shown that, following UVA light treatment, psoralen alkylates its receptor (7). It is possible that this process activates the psoralen receptor protein. One could also speculate that serine kinase activity is intrinsic to the activated psoralen receptor. Alternatively, the activated psoralen receptor may be indirectly coupled to the regulation of EGF receptor serine phosphorylation. This may lead to activation of some alternative serine kinase or to the inhibition of a phosphatase.

Our discovery that psoralens induce biological effects at the level of the plasma membrane through alterations in EGF receptor structure and function represents a novel mechanism of action for this class of drugs. A number of studies have shown that the EGF receptor is an allosteric protein with several regulatory domains (15, 48–52). Identification of the domain(s) modified by the activated psoralen receptor are necessary to better understand the cellular mechanisms controlling EGF-mediated alterations in cell growth. Further characterization of the psoralen receptor and its interaction with growth regulatory molecules following PUVA photochemotherapy will provide important information on its role in the control of epidermal proliferative disorders.

[1] T. V. Abidi, F. H. Mermelstein, and J. D. Laskin, unpublished results.

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