The exposure of mammalian cells to UV irradiation leads to the activation of transcription factors such as activated protein-1 (AP-1) and NFkB. It is postulated that epidermal growth factor (EGF) receptor, but not protein kinase C (PKC), is the major membrane mediator in UV-induced signal transduction. Since UVB is responsible for most of the carcinogenic effects of sun exposure, we investigated the role of EGF receptors and PKC in UVB-induced AP-1 activation. Our results indicated that while the down-regulation of novel PKC (nPKC) and conventional PKC (cPKC) by pretreatment of cells with 12-O-tetradecanoyl phorbol-13-acetate cannot block UVB-induced AP-1 activity, it can block 12-O-tetradecanoyl phorbol-13-acetate-induced AP-1 activity. Further, the dominant negative mutant PKC

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(compound 5 methyl ester), and 4-(3-chloroanilino)-6,7-dimethoxyquinazoline (tyrphostin AG1478) were from Calbiochem. EGF was from Collaborative Research, rabbit polyclonal IgG against PKCζ for Western blot was from Santa Cruz Biotechnology, Inc., and luciferase substrate was from Promega.

Cell Culture—The JB6 P+ mouse epidermal cell line, Cl 41, and the stable AP-1 luciferase reporter plasmid-transfected mouse epidermal JB6 P+ cell line, P'1-1, were cultured in monolayers at 37 °C with 5% CO2 using Eagle’s minimal essential medium containing 5% fetal calf serum, 2 mM l-glutamine, and 25 µg of gentamicin/ml. B82, B82L, and B82M721 murine fibroblasts were cultured in 10% FBS DMEM supplemented with 2 mM l-glutamine, 100 units of penicillin, and 100 µg of streptomycin/ml (7).

Transient Transfection of B82 and B82L—Murine fibroblasts in 10% FBS DMEM were seeded 5 x 10⁴ to each well of a 6-well plate. After being cultured at 37 °C for 12–14 h, the cells of each well were transiently transfected with 3 µg of AP-1 luciferase reporter plasmid together with 0.3 µg of β-galactosidase plasmid. After 12 h, the medium was replaced with 10% FBS DMEM and cultured for an additional 10–12 h. The cells were starved in serum-free medium for 24 h and then were or were not exposed to either UVB (1 kJ/m²) or EGF (20 ng/ml). The cells were extracted with lysis buffer 24 h later, and the luciferase activity was measured according to the manufacturer’s instructions (Promega). The luciferase activity was corrected with β-galactosidase activity, and the results were shown with relative AP-1 activity as described previously (7).

Transient Transfection of JB6 Cells—JB6 P+ cells, Cl 41, were cultured in a 6-well plate until they reached 85–90% confluence. We used 2 µg of AP-1 luciferase reporter plasmid and 0.3 µg of β-galactosidase plasmid either with or without 6 µg of dominant negative mutant of Xenopus PKCζ plasmid (CMV-Zn PKCζ mutant) and 15 µl of LipofectAMINE reagent to transfect each well in the absence of serum. After 10–12 h, the medium was replaced with 5% FBS MEM. Approximately
39–36 h after the beginning of the transfection, the cells were starved with 0.1% FBS MEM for 12 h, and cells were then either exposed or not exposed to UVB (1.5 kJ/m²) irradiation. 12 h later, the cells were extracted with lysis buffer, and the luciferase activity was measured as described above (7).

Generation of Stable Cotransfectants with AP-1 Reporter and Dominant Negative PKCα/λ Mutant—JB6 P⁺ cells, Cl 41, were cultured in 6-well plates until they reached 85–90% confluence. We used 2 μg of AP-1 luciferase reporter plasmid, 0.3 μg of CMV-neu marker vector either with or without 6 μg of dominant negative mutant of Xenopus CMV-Zn, PKCα/λ mutant, and 15 μl of LipofectAMINE reagent to transfect each well in the absence of serum. After 10–12 h, the medium was replaced with 5% FBS MEM. Approximately 30–36 h after the beginning of the transfection, the cells were digested with 0.033% trypsin, and cell suspensions were transferred to 75-ml culture flasks and cultured for 24–28 days with G418 selection (300 μg/ml). Stable transfectants were screened by assay of the luciferase activity and Western blotting with rabbit polyclonal IgG against PKCα/λ. Stable transfected cells, Xenopus PKCα/λ mutant mass₁, or AP-1 mass₁, were cultured in G418-free MEM for at least two passages before each experiment.

Generation of Stable Transfectants with AP-1 Reporter in B82L, B82, or B82M721 Cells—B82, B82L, or B82M721 cells were cultured in a 6-well plate until they reached 85–90% confluence. We used 2 μg of AP-1 luciferase reporter plasmid and 0.3 μg of CMV-neu marker vector with 15 μl of LipofectAMINE reagent to transfect each well in the absence of serum. After 10–12 h, the medium was replaced with 10% FBS-DMEM. Approximately 30–36 h after the beginning of the transfection, the cells were digested with 0.033% trypsin, and cell suspensions were transferred to 75-ml culture flasks and were cultured for 24–28 days with G418 selection (300 μg/ml). Stable transfectants were screened by assay of the luciferase activity. The cell clones were ring-isolated and transfected to 12-well tissue culture dishes with 10% FBS MEM containing 500 μg/ml G418. Stable transfected cells were cultured in G418-free MEM for at least two passages before each experiment.

Assay for AP-1 Activity—Confluent monolayers of AP-1 reporter stable transfectants were trypsinnized, and 5 × 10⁴ viable cells suspended in 100 μl of 5% FBS MEM were added to each well of a 96-well plate. Plates were incubated at 37°C in a humidified atmosphere of 5% CO₂ and 95% gas air. 12–24 h later, cells were starved for 12–40 h prior to exposure to either UVB or EGF. The cells were exposed to either UVB or EGF at time and dose as indicated (Figs. 1–6, 8). The cells were extracted with lysis buffer and luciferase activity was measured as described previously (7). The results were expressed as either relative AP-1 activity or relative luciferase units. Relative AP-1 activity was presented as the fraction of the luciferase activity in medium

**Fig. 1.** Influence of compound 5 methyl ester and tyrphostin on UVB-induced AP-1 activity. 5 × 10⁴ JB6 AP-1 reporter stable transfected 1-1 cells suspended in 5% FBS, MEM were added to each well of a 96-well plate. After an overnight culture at 37°C, the cells were starved by replacing the medium with 0.1% FBS MEM for 12–20 h. The cells were then treated with different concentrations of compound 5 methyl ester (A) or tyrphostin AG1478 (B) as indicated for 30 min and sequentially exposed to 1 kJ/m² of UVB irradiation. After a 12-h culture, the AP-1 activity was measured by luciferase activity assay as described previously (9). The results were presented as relative AP-1 activity. Each bar indicates the mean and standard deviation of nine assay wells from three independent experiments.

**Fig. 2.** Full response of B82 and B82M721 cells to UVB in AP-1 induction. 5 × 10⁴ of murine fibroblasts in 10% FBS DMEM were seeded into each well of a 6-well plate. After being cultured at 37°C for 12–14 h, the cells of each well were transiently transfected with 3 μg of AP-1 luciferase reporter plasmid together with 0.3 μg of β-galactosidase plasmid. After 12 h, the medium was replaced with 10% FBS DMEM. The cells were cultured at 37°C overnight and starved in serum-free medium for 24 h. The cells were then exposed to UVB (1 kJ/m²) (solid bar) or EGF (20 ng/ml) (hatched bar) as indicated. The cells were extracted with lysis buffer 24 h later, and the luciferase activity was measured according to the manufacturer’s instructions (Promega). The luciferase activity was corrected with β-galactosidase activity, and the results were shown with relative AP-1 activity as described previously (9). Each bar indicates the mean and standard deviation of four assay wells from two independent experiments.

**RESULTS AND DISCUSSION**

As we studied the role of EGFR in UVB-induced AP-1 activation, we observed that cells pretreated for 30 min with Lavendustin C methyl ester (a specific EGF receptor-tyrosine kinase inhibitor (17)) did not show significant inhibition of UVB-induced AP-1 activity at noncytotoxic concentrations (doses ranging from 1 to 2 μm) in P'–1–1, which is an AP-1 luciferase reporter (Col-Luc) stable transfecant of JB6 cells (p > 0.05) (Fig. 1). Further, 4-(3-chloroanilino)-6,7-dimethoxyquinazoline, a potent selective inhibitor of EGF receptor tyrosine kinase (18) used in our study, did not show any inhibitory effects on UVB-induced AP-1 activity (p > 0.05) as it blocked EGF-induced AP-1 activity (p < 0.05) (Fig. 1). These results suggested that the EGF receptor is not involved in UVB-induced AP-1 activation.

To examine more directly whether or not EGFR or EGFR tyrosine kinase is involved in UVB-induced AP-1 activation, we transiently transfected an AP-1 reporter plasmid (Col-Luc) together with β-galactosidase plasmid into the well characterized

**Cell lines**

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**Statistical Analysis—**Data were analyzed by Student’s t test.
Murine fibroblast variants (B82, B82L, and B82M721 cells). B82 cells are murine fibroblasts devoid of endogenous EGFR (19, 20). The variant cell lines B82L and B82M721 stably express either wild-type human EGFR (B82L) or a kinase-deficient human EGFR (B82M721) (20). The results show that high levels of AP-1 activity were induced by UVB in B82L, B82M721, and B82 cells (p < 0.05). EGFinduced high levels of AP-1 activity in B82L cells and low levels in B82M721 cells, but no apparent induction occurred in parental B82 cells (p > 0.05) (Fig. 2). These results suggested that there is no role for EGFR in UVB-induced AP-1 activity. To further confirm our data, we established 10 single cloned, stable AP-1 reporter (Col-Luc) transfectants from B82, B82L, and B82M721 cells, respectively. To avoid any bias from the pressure of cell selection, we also generated Col-Luc “mass stable clones.” After transfecting cells with Col-Luc in a 100-mm dish and selecting with G418, more than 30 colonies per dish were grown. Instead of ring cloning these cells, all colonies in the dish were grown as a “mass stable culture.” The results observed from these stable transfectants are consistent with those from transient transfectants. The UVB-induced AP-1 activity in B82L and B82M721 stable transfectants showed similar fold induction of AP-1 activity with B82L stable transfectants (p > 0.05) (Fig. 3). These data further support the conclusion that EGFR is not involved in UVB-induced AP-1 activation.

The UVB-induced AP-1 activity is mediated both by induction of c-Jun and c-Fos expression and by post-translational modification of c-Jun (21–24). Shah et al. (25) reported that UVB induced an immediate early response of c-fos that is downregulated within 2 h and a strong second phase of fos expression with a maximum at 8 h that only returned to control levels after 24 h. Similar prolonged dynamic changes were observed in UVB-induced AP-1 activity in JB6 cells. To be sure that the EGFR-independent manner of UVB-induced AP-1 activity was not due to our study of one time point and one UVB dose, we performed a time course and dose response of UVB-induced AP-1 activation in AP-1 reporter stable transfectants. The UVB-induced AP-1 activity in B82 cells was not significantly different from that of B82L cells at different time points (p > 0.05) (Fig. 4A). The fold induction of AP-1 activity by different doses of UVB was similar among B82, B82L, and B82M721 cells (p > 0.05) (Fig. 4B). All of these results argue strongly that UVB-induced AP-1 activity is not dependent on either EGFR or tyrosine kinase of EGFR.

Down-regulation of PKC by pretreatment of cells with TPA blocks TPA-induced signal transduction but not UVC-induced signal transduction (9). This has been interpreted as key evidence supporting the concept that PKC is not involved in the UV-induced signal transduction pathway; however, this point of view requires further testing. Isozymes of the PKC family can be grouped into at least three subfamilies: conventional PKC (cPKC = α, β, βI, βII, and γ), novel PKC (nPKC = δ, ε, η, and θ), and atypical PKC (aPKC = λ, μ,ζ) (26, 27). cPKCs exhibit a strict requirement for phospholipids and Ca²⁺ for their activities, while nPKCs do not need Ca²⁺ for their activities. Diacylglycerol or phorbol esters bind with cPKC and nPKC and cause activation, while aPKC does not bind with diacylglycerol or phorbol ester. Phorbol ester can only down-regulate cPKC or nPKC and not aPKC (26, 27). Pretreatment of JB6 cells with 20 ng/ml of TPA for 24–36 h did not block UVB-induced AP-1 activity (p > 0.05) while it completely blocked TPA-induced AP-1 activity (p < 0.05) (Fig. 5). This result agrees with an-
other report that UVC-induced signals cannot be blocked by pretreatment of TPA (9). Also, previous results indicate that the ability of UV to induce membrane association of PKC is weak when compared with TPA, and that c-fos induction by UV and TPA is additive (28). The data suggested that inductions of AP-1 activity by TPA and UV are through different mediators. Taken together, all of these experiments can rule out the involvement of cPKC and nPKC in UVB-induced AP-1 activation but cannot rule out the role of aPKC or other unknown phorbol ester-insensitive PKCs in UVB-induced AP-1 activation.

The aPKC family is composed of PKC\(\lambda/\iota\) and PKC\(\zeta\) (29–31). The PKC\(\lambda/\iota\) and PKC\(\zeta\) show a high degree of homology in both the catalytic and regulatory domains in different species (32). Dominguez et al. (34) had cloned cDNA of an atypical PKC isotope from *Xenopus laevis* by using the regulatory domain of rat PKC\(\zeta\) as a probe. This cDNA encoding a protein was shown to be highly homologous to rat PKC\(\zeta\); therefore, this *Xenopus* cDNA was renamed *Xenopus PKC\(\zeta\)* in previous reports (13–16). A very recent report from the same group indicates that the sequence originally named as *Xenopus PKC\(\zeta\)* shows the highest degree of homology with PKC\(\lambda/\iota\), with an overall 90% identity at the amino acid level; thus, this cloned cDNA is actually *Xenopus PKC\(\lambda/\iota\)* (33). Due to the near identity of catalytic doming of the PKC\(\lambda/\iota\) and PKC\(\zeta\), these two isotypes of aPKC showed a similar stimulation effect on the \(k_B\)-dependent promoter activity. Interestingly, Diaz-Meco et al. (33) demonstrated that using either the dominant negative mutant of PKC\(\lambda/\iota\) or the dominant negative mutant of PKC\(\zeta\) could block the tumor necrosis factor-\(\alpha\)-induced \(xB\)-dependent promoter activity. Microinjection of a peptide with the sequence of aPKC isotypes but not of PKC\(\alpha\) or PKC\(\epsilon\) dramatically inhibited maturation (34) and NF\(\kappa B\) activation (35) in *X. laevis* oocytes.

**Fig. 4.** The time course and dose-response of UVB-induced AP-1 activity. 5 × 10³ cells from AP-1 reporter stable transfectants (B82-mass\(_2\), B82L-mass\(_2\), or B82M721-mass\(_2\)) were seeded into each well of a 96-well plate. After overnight culture at 37 °C, the cells were starved for 36 h by replacing medium with serum-free DMEM. A, for the time course studies, the cells were or were not exposed to UVB (1 kJ/m²), and the AP-1 activity was determined in time as indicated. B, for the dose response study, cells were treated with the indicated doses of UVB and incubated for 24 h before assaying for AP-1 activity. The results were presented as described in the legend to Fig. 2. Each bar indicates the mean and standard deviation of eight assay wells from two independent experiments.
Previous studies have shown that PKC\(\lambda\) is critically involved in many cellular functions such as cell proliferation and *Xenopus* maturation (13, 34). Platelet-derived growth factor can induce the interaction and association of PKC\(\lambda\) (previously reported as PKC\(\zeta\)) with Ras in mouse fibroblasts (16). Expression of an active mutant of PKC\(\lambda\)/\(\zeta\) activated mitogen-activated protein kinase, and the activation of mitogen-activated protein kinase by tumor necrosis factor-\(\alpha\) can be blocked by a kinase-defective dominant negative PKC\(\lambda\)/\(\zeta\) (36). Akimoto et al. (29) reported that EGFR- or platelet-derived growth factor-induced AP-1 activation is through PKC\(\lambda\) (35). To test the role of aPKC in the UVB-induced AP-1 activation, we transiently transfected a well characterized dominant negative mutant construct of *Xenopus* PKC\(\lambda\)/\(\zeta\) (13–16, 38) into JB6 cells. This dominant negative mutant contains the lysine 275 to tryptophan mutation. As shown in Fig. 6, transient transfection of *Xenopus* dominant negative mutant PKC\(\lambda\)/\(\zeta\) blocks UVB-induced AP-1 activation completely (\(p < 0.05\)) while inhibiting only 13.8% of serum-induced AP-1 activity. This result suggested that aPKC is involved in UVB-induced AP-1 activity. To further confirm this finding, we established mass stable cultures of *Xenopus* PKC\(\lambda\)/\(\zeta\) dominant negative mutant cells. A high level of PKC\(\lambda\)/\(\zeta\) dominant negative mutant protein was detected by antiserum against PKC\(\lambda\)/\(\zeta\) in *Xenopus* PKC\(\lambda\)/\(\zeta\) mass, cells (Fig. 7). The results observed from these stable transfectants are consistent with those from transient transfectants at all doses and times studied (Fig. 8). We have performed these experiments with rat PKC\(\zeta\) dominant negative mutant (39), and the results indicated that the PKC\(\zeta\) dominant negative mutant also inhibits UVB-induced AP-1 activity (data not shown). All of these data suggested that aPKC is involved in UVB-induced AP-1 activation.

The mechanism behind the tumor-promoting ability of UV is not well understood. This is especially true in the study of UVB, which is very active and is responsible for most of the carcinogenic effect of sun exposure (12). Most of our understanding of the UV-induced signal transduction is based on the studies of UVC using HeLa cells, a fully transformed, cervix-derived carcinoma cell line (9). The UVC-induced signal transduction pathway is believed to originate at the cell membrane and is mediated by EGFR and other growth factor receptors but not by PKC (9, 10). The signal is then subsequently transferred to the nucleus via a signaling cascade involving Src-like tyrosine kinase, Ras/Raf kinase, mitogen-activated protein kinase in-
PKCa/l or PKCζ is known to be activated by lipid second messengers such as phosphatidic acid, phosphatidylinositol 3,4,5-P³, and ceramide (32, 35, 36, 40–42). In terms of inflammatory cytokines such as interleukin-1 or tumor necrosis factor-α (43, 44), signaling involves the binding and activation of PKCa/l and PKCζ by ceramide. Birt et al. (45, 46) have reported that PKCζ may play certain roles in the process of tumor promotion. Very recently, Verheij et al. (47) demonstrated that UVC irradiation rapidly induces an increase in ceramide in a dose-dependent manner and that exposure of cells to C2-ceramide induced concentration-dependent c-Jun N-terminal kinase activation, which is well known as a key component in UVC-induced signal cascades, to as much as 40-fold of control. Moreover, several groups demonstrated that PKC isotypes have a different substrate specificity in vitro (33, 37, 45). Whether these substrates are involved in UVC-induced signal transduction in cells has yet to be tested. Further study on the precise mechanism by which UV irradiation triggers signal transduction pathways should help us in understanding the basis of UVC-induced skin diseases such as cancer and aging.

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Fig. 8. Inhibition of UVB-induced AP-1 activity by stable overexpression of dominant negative Xenopus PKCa/l.

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Inhibition of UVB-induced AP-1 activity by stable overexpression of dominant negative Xenopus PKCa/l. 5 x 10⁶ JB6 P⁵ CI41 cells from AP-1 reporter stable transfectant (AP-1 mass ) or AP-1 reporter and Xenopus PKCa/l stable transfectants (Xenopus PKCa/l -mass₈) were seeded into each well of a 96-well plate. After overnight culture at 37 °C, the cells were starved for 12 h by replacing medium with 0.1% FBS MEM. For the dose response study (A), cells were treated with the indicated dose of UVB and incubated for 24 h before assaying for AP-1 activity. For the time course study (B), the cells were or were not exposed to UVB (1.5 kJ/m²). The AP-1 activity was determined in time as indicated, and the results were presented as described in the legend to Fig. 2. Each bar indicates the mean and standard deviation of ten assay wells from three independent experiments.

including c-Jun N-terminal kinase, resulting in the activation of transcription factors such as AP-1 and TCF/Elk-1 (21–24). Evidence supporting this model has been derived mainly from pharmacological inhibitors and dominant negative mutants of EGFR, Src, Ras, and Raf (9, 21–24). In this study we have provided the first clear and strong evidence that the pathway whereby UVB activates AP-1 activity requires aPKC but not the EGF receptor. Though the upstream effector of aPKC in the UVB signal transduction cascade is not known, there are a number of known activators of PKCa/l and PKCζ. Native
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