Delayed and Sustained Activation of Extracellular Signal-regulated Kinase in Human Keratinocytes by UVA

IMPLICATIONS IN CARCINOGENESIS*

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Exposure to the sun’s UV radiation appears to be the most important environmental factor involved in the development of skin cancer. UVA is the major portion of UV radiation in sunlight and is considered to be a human carcinogen. In this study, we have investigated the delayed and sustained activation of ERK MAPK by UVA exposure. In parallel, a delayed Ras activation with a similar time course was observed after UVA exposure. The activated Ras was found to be localized in endomembranes such as the Golgi apparatus instead of plasma membranes. Expression of dominant negative Ras (N17Ras) abolished ERK activation by UVA. The presence of AG1478, an epidermal growth factor (EGF) receptor (EGFR) kinase inhibitor, had no effect on ERK or Ras activation, indicating that EGFR kinase activity is not involved in ERK activation by UVA. In contrast, protein kinase C (PKC) depletion by chronic 12-O-tetradecanoylphorbol-13-acetate treatment nearly abolished UVA-induced ERK and Ras activation. The presence of the Ca²⁺-dependent PKC inhibitor Gö6976 had a similar effect. These findings suggest that ERK activation by UVA is mediated by PKC in a Ras-dependent pathway.

In addition, a gradual increase in intracellular calcium level after UVA exposure was detected by flow cytometry. The presence of the PLC inhibitor U73122 or the calcium chelator 1,2-bis(2-aminophenoxy)ethane-N,N',N''-tetraacetic acid tetrakis (acetoxymethyl ester) (BAPTA-AM) blocked both ERK and Ras activation, suggesting that both PLC and calcium are required for ERK activation. Our findings demonstrated that, different from UVC and UVB, UVA-induced delayed and sustained ERK activation is EGFR kinase activity-independent, but PLC/calcium/PKC-mediated. The delayed and sustained ERK activation provides a survival signal to human HaCaT keratinocytes, which may serve as an important mechanism for cell transformation and potential skin carcinogenesis in vivo caused by UVA exposure.

Prolonged exposure of human skin to sunlight can cause premature skin aging and skin cancer. UVA (315–400 nm) is the major component (about 95%) of the ultraviolet (UV) part of sunlight that reaches the Earth’s surface. Recently UVA has been listed as a reasonably anticipated human carcinogen. Cells damaged by UVA normally undergo apoptosis; failed apoptosis plays an important role in tumor development. Indeed, cell survival can be promoted by active suppression of apoptosis via survival signals, thus allowing potential malignant transformation. The molecular mechanisms of cell survival after UVA exposure due to escape from apoptosis are not fully understood.

An important suppressive regulator of apoptosis is extracellular signal-regulated kinase (ERK), one member of the mitogen-activated protein kinase family (MAPK). In many cells, activation of the ERK1/2 pathway suppresses apoptosis induced by a variety of apoptotic stimuli (1–5). Sustained activation of the ERK pathway has been shown to protect cells from photodynamic therapy-induced phototoxicity (6).

The ERK1/2 pathway is an evolutionarily conserved signaling cascade that plays a critical role in gene expression, cell growth, differentiation, and apoptosis through the activation of intracellular substrates, including transcription factors such as Elk-1 (7) and cytoskeletal proteins (8). Its role in apoptosis has made ERK1/2 a priority for research related to many human diseases such as cancer. In many human tumors, oncogenic Ras resulting from a mutation in Ras persistsently activates the ERK1 and ERK2 pathways, which contributes to the increased proliferative rate of tumor cells (8) and resistance to therapeutic treatment. For this reason, inhibitors of the ERK pathways are entering clinical trials as potential anticancer agents.

ERK1/2 is activated by multiple extracellular stimuli, including growth factors and phorbol esters. The mechanisms of activation by various stimuli may differ, in part to control the strength and duration of the ERK response. The best characterized is the growth factor receptor pathway (9). For example, epidermal growth factor (EGF) triggers autophosphorylation of the tyrosine on the C-terminal tail of the EGF receptor, resulting in a phosphotyrosine-dependent association of Shc and/or Grb2 with the receptor. Membrane translocation of the Grb2-SOS complex stimulates Ras-GDP to -GTP exchange, in turn

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1 The abbreviations used are: UVA, ultraviolet A (315–400 nm); AG (AG1478), inhibitor for EGFR intrinsic kinase activity; BAPTA-AM, calcium chelator (1,2-bis(2-aminophenoxy)ethane-N,N',N''-tetraacetic acid tetrakis (acetoxymethyl ester)); EGF, epidermal growth factor; EGFR, EGF receptor; EGFR-KI, EGFR-kinase inactive; ERK, extracellular signal-regulated kinase; Gö6976, calcium-dependent PKC inhibitor; MAPK, mitogen-activated protein kinase; PD98059, MEK1 inhibitor; PKC, protein kinase C; PLC, phospholipase C; RTKs, receptor tyrosine kinases; TPA, 12-O-tetradecanoylphorbol-13-acetate; U73122, PLC inhibitor; RB, Ras-binding domain; UVB, ultraviolet B (280–315 nm); UVC, ultraviolet C (200–280 nm); MEK, MAPK/ERK kinase; DMEM, Dulbecco’s modified Eagle’s medium; PBS, fetal bovine serum; PBS, phosphate-buffered saline; GFP, green fluorescent protein; ER, endoplasmic reticulum.
stimulating the Ras, MEK, and the ERK series. Another class of ERK activators is the protein kinase C (PKC) family of protein Ser/Thr kinase stimulated by the phorbol ester tumor promoter TPA. PKC stimulation activates ERK through several pathways, including Ras-dependent and -independent mechanisms, that are not well understood (10).

In the present report we have demonstrated that UVA induces ERK activation in a delayed and sustained pattern. We found that ERK activation by UVA was mediated by PKC in a Ras-dependent pathway that required phospholipase C (PLC) and calcium but not the EGF receptor kinase activity. The delayed and sustained ERK activation provides a survival signal allowing human HaCaT keratinocytes to escape from apoptosis.

**EXPERIMENTAL PROCEDURES**

**Plasmids—**Raf-1-RBD-GFP was generated by PCR amplification of the human Raf RBD domain (amino acids 51–131) using primers flanking with HindIII and KpnI cleavage sites at each end (primers 5′-AAGCTTCCCTTATGAACTACGAC-3′ and 5′-GGTACCCAGG-GGATCCCTGCGCTACG-3′). The PCR product was cloned into pCDM8-2 (Invitrogen). After digestion with HindIII and KpnI, the DNA fragment was subcloned in-frame into the pEGFP-C2 vector (Clontech). Dominant negative Ras (N17Ras) was a kind gift from Dr. John O’Bryan (NIHES, National Institute of Health). EGFR-KI (EGFR-kinase inactive) was generously provided by Dr. Sally Parsons (University of Virginia).

**Cell Culture and Transfection—**HaCaT (obtained from Prof. N. Fusieng, German Cancer Research Center, Heidelberg, Germany) and A431 and COS-7 (ATCC) cells were maintained in a monolayer culture in 95% air/5% CO2 at 37 °C in Dulbecco’s modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 31 g/ml streptomycin. Cells to be examined by Western blotting were plated and sustained ERK activity provides a survival signal allowing human HaCaT keratinocytes to escape from apoptosis.

**RESULTS**

**UVA-induced Delayed and Sustained ERK1/2 Activation—**To determine the UVA effect on ERK1/2 activation, HaCaT cells were irradiated with 24 J/cm² UVA and then incubated for varying times after exposure. Western blotting showed that there was no phosphorylation of ERK1/2 in either control cells or irradiated cells within 1 h after UVA exposure. In contrast, a marked phosphorylation of ERK1/2 was observed 3 h after exposure (Fig. 1, A and B). Interestingly, the phosphorylation of ERK1/2 was maintained at a slightly increased level up to 15 h after UVA exposure and still observed at 24 and 48 h after UVA irradiation (data not shown). When cells were irradiated with different doses of UVA (4–32 J/cm²), a weak phosphorylation of ERK was seen at 12 J/cm² and increased phosphorylation detected up to 32 J/cm² at 6 h after UVA exposure (Fig. 1, A and B). These data demonstrate that ERK is phosphorylated by UVA exposure in a time- and dose-dependent pattern. Clearly phosphorylation of ERK by UVA is delayed and sustained after exposure. To determine whether the phosphorylation of ERK can be translated into the activation of ERK, cell lysates were immunoprecipitated with anti-ERK antibody for assays of the activity of the ERK immunocomplex with Elk-1 as a substrate. In control cells, there was no ERK activity detected in the form of Elk-1 phosphorylation, whereas UVA (24 J/cm²) irradiation induced dramatic ERK kinase activity 3 h after exposure. This demonstrates that ERK is activated by UVA (Fig. 1, C and D).

Similar delayed and sustained phosphorylation of ERK after UVA exposure was detected in human A431 keratinocytes as well as monkey COS-7 kidney cells (Fig. 1E). In A431 cells,

**Determination of Activation of ERK and Caspase by Flow Cytometry—**Briefly, HaCaT cells were irradiated and fixed with 4% formaldehyde. After permeabilization with 90% methanol, cells were double stained with anti-phospho-ERK Alexa Fluor 488 (Cell Signaling) and anti-active caspase-3-PE (BD Biosciences) antibodies. The activations of ERK and caspase-3 in cells were determined by flow cytometry.

**Western Blotting—**Cells were subjected to electrophoresis followed by electrophoretic transfer to nitrocellulose membranes. Membranes were blocked in 5% nonfat milk in Tris-HCl, pH 7.4, containing 150 mM NaCl, and 0.1% Tween 20) followed by incubation with primary antibody and secondary antibody. Proteins were visualized using SuperSignal chemiluminescent substrate (Pierce).

**Determination of Apoptosis by Flow Cytometry—**Briefly, HaCaT cells were washed and fixed followed by staining with propidium iodide. The DNA content was determined by flow cytometry and the sub-G1 portion was considered to be the apoptotic cell population.

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ERK phosphorylation increased at 1 h in a sustained manner up to 6 h after UVA exposure. In COS-7 cells, an increase in ERK phosphorylation was detected at a sustained manner up to 6 h after UVA exposure. These data indicate that the delayed and sustained ERK activation by UVA is not cell type-specific.

Previous studies have reported that UVC induced rapid but transient activation of ERK (12) in A431 cells. To determine whether the distinct pattern of ERK activation by UVA from UVC is due to the different types of UV, we irradiated both HaCaT cells and A431 cells with UVC, the shorter wavelength portion of UV radiation, and also with UVB, the region that falls between UVA and UVC (Fig. 1). Upon UVC exposure, activation of ERK was detected at 15 min after exposure, whereas it decreased at 1 h and later, suggesting that UVC induced a rapid but transient activation of ERK, consistent with the previous report (12). In comparison, UVB induced activation of ERK at 15 min after exposure, and the activation was sustained up to 6 h. These results demonstrate that the distinct pattern of ERK activation is caused by different types of UV exposure.

**ERK Activation by UVA Provided an Anti-apoptotic Signal**—We have shown that UVA induces apoptosis and caspase activation in human keratinocytes (13). To determine UVA-induced apoptosis at time points parallel to the ERK activation, we performed the same experiments as shown in Fig. 1 (A and B) except that activation of caspase-3 was determined by immunoblotting. As seen in Fig. 2A, activation of caspase-3 followed a pattern similar to ERK activation, i.e. in a time- and dose-dependent manner. The activation of caspase-3 was detected at 3 h after UVA (24 J/cm²) exposure, and was maintained at a slightly increased level for up to 15 h. A slight activation of caspase-3 was observed after 12 J/cm², and increased activation was seen with a higher dose but not in a linear pattern. These findings imply that activation of ERK is concurrent with caspase-3 activation, a marker for the apoptotic process.

To determine whether the concurrent activation of ERK and caspase-3 observed in Figs. 1A and 2A is in the same or different cell populations, we double stained the cells with phospho-ERK-Alexa Fluor488 and active caspase-3-PE antibodies. UVA induced activation of both ERK and caspase-3 at 6 h after UVA (Fig. 2C, upper two panels). When the level of ERK activation in
stained with propidium iodide and apoptotic cells (sub-G1) were determined as in Fig. 1 and then incubated for 18 h. After fixation, cells were same or different cell populations.

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Alexa Fluor488 and active caspase-3-PE. Flow cytometry was used to
cells were fixed, permeabilized, and double stained with phospho-ERK-

indicated. Cell lysates were detected as in

SDS-PAGE electrophoresis and immunoblotted with anti-caspase-3 (Santa Cruz Biotechnology). The two bands shown were activated
caspase-3 with – 19- and 17-kDa molecular mass. B, cells were irradiated as in Fig. 1 and then incubated for 18 h. After fixation, cells were stained with propidium iodide and apoptotic cells (sub-G1) were determined by flow cytometry. C, 6 h after irradiation with UVA (24 J/cm2), cells were fixed, permeabilized, and double stained with phospho-ERK-Alexa Fluor488 and active caspase-3-PE. Flow cytometry was used to determine whether the activation of ERK and caspase-3 was in the same or different cell populations. C, control; U, UVA-irradiated. D, HaCaT cells were irradiated with 24 J/cm2 UVA and then incubated for 24 h. Attached and floating cell lysates were collected and subjected to immunoblotting with anti-phospho-ERK and anti-caspase-3 antibodies. E, cells were starved as in A and left intact or preincubated with PD98059 (10 or 20 µM) for 1 h. Cells were then exposed to UVA (24 J/cm2) and incubated for 3 h in the presence or absence of PD98059, as indicated. Cell lysates were detected as in A.

caspase-3-inactive cells was compared with that in caspase-3-active cells, higher activation of ERK (3.1-fold of the control) was observed in cells without caspase-3 activation after UVA exposure, as compared with cells with caspase-3 activation (1.2-fold of the control) (Fig. 2C, lower two panels). In addition, we also extracted protein from attached (surviving) and floating (apoptotic) cells 24 h after UVA exposure (Fig. 2D). In attached cells, no activation of caspase-3 was observed, whereas in floating cells the majority of caspase-3 was activated. UVA-induced activation of ERK was detected in surviving cells, whereas no ERK phosphorylation was observed in floating apoptotic cells, which confirmed the observation from flow cytometry (Fig. 2C).

To reveal the role of ERK activation by UVA in apoptosis of human keratinocytes, the MEK1 inhibitor PD98059 (10 and 20 µM) was used to prevent ERK activation. As shown in Fig. 2D, inhibition of ERK activation enhanced apoptosis induced by UVA (12 J/cm2). This indicates that ERK activation serves as a survival signal to prevent apoptosis from proceeding after UVA exposure.

Ras Is Activated upon UVA Radiation—Ras is known to be upstream of ERK activation in the pathway following EGF binding to the EGFR (9). To determine whether Ras is activated in human keratinocytes after UVA radiation, we determined Ras activity at different times after UVA radiation. After UVA exposure, there was no immediate Ras activation (Fig. 3A) either at 15 min or 1 h. However, at 3 and 6 h after UVA radiation, a dramatic increase in Ras activity was detected, indicating a delayed and sustained Ras activation similar to the ERK activation (Fig. 1). In addition, the activation of Ras was dependent on UVA doses. 12 J/cm2 induced a slight increase in Ras activity, whereas 24 and 32 J/cm2 resulted in much higher Ras activation (Fig. 3B).

Recent studies have used green fluorescence protein (GFP)


fused to the Ras-binding domain of Raf1 (GFP-RBD) as a spatial probe to show that Ras is activated on and transmits signals from the endoplasmic reticulum (ER) and the Golgi apparatus as well as the plasma membrane (14, 15). We used GFP-RBD to determine whether Ras is activated in the ER/Golgi and/or the plasma membrane after UVA exposure. In control HaCaT cells, GFP-RBD was homogenously localized in the cytosol and nucleoplasm, without accumulating on any membrane structure (Fig. 3C). This pattern is similar to that of GFP expression. When HaCaT cells were irradiated with UVA, we observed a dramatic recruitment of GFP-RBD to a juxtanuclear structure 3 h after UVA exposure (Fig. 3C). Juxtanuclear GFP-RBD was colocalized with Golgi marker Golgin 97 (Fig. 3D), indicating that the perinuclear structure to which GFP-RBD is recruited is the Golgi. No increased recruitment of GFP-RBD to the Golgi was observed either 15 min or 1 h after UVA exposure (data not shown), which is consistent with the results of Ras activity determined by immunoprecipitation (Fig. 3A). In contrast, little GFP-RBD recruitment to the plasma membrane was observed. Thus, Ras is activated in response to UVA radiation mainly in the endomembranes such as the Golgi region.

To determine whether the delayed ERK activation is mediated by the Ras pathway, we transfected COS-7 cells with dominant negative Ras (N17Ras). UVA exposure induced increased activation of ERK at 6 h. However, expression of N17Ras completely abolished the increase in ERK activation induced by UVA exposure (Fig. 3E). These data indicate that ERK activation by UVA is Ras-dependent.

**Activation of ERK Is Localized in Both the Nuclear and Membrane Fractions**—To determine the location of activated ERK, HaCaT cells were irradiated with UVA. At 3 h after exposure, cytosolic, nuclear, and membrane fractions were separated and ERK phosphorylation was detected by immunoblotting. No cytosolic activation of ERK was observed, whereas dramatic ERK activation was detected in nuclear and membrane fractions (Fig. 4). It is noteworthy that the membrane fraction includes the plasma membrane and endomembranes, including the ER and Golgi membrane fractions.

**EGF Receptor Kinase Activity Is Not Required for UVA-induced ERK Activation**—The EGF receptor (EGFR) is the best-characterized pathway that can lead to ERK activation upon EGF binding. To determine whether EGFR is required for ERK activation upon UVA exposure, cells were pretreated with AG1478, a specific EGFR kinase inhibitor, and then irradiated with UVA. Without the inhibitor, an increase in EGFR phosphorylation was observed 3 h after UVA irradiation, whereas the presence of AG1478 abolished EGFR phosphorylation even at the basal level (Fig. 5A). Without AG1478, ERK was activated at 3 h after exposure; however, the presence of AG1478 failed to diminish ERK activation. In comparison, AG1478 abolished ERK activation upon EGF treatment (Fig. 5A). Expression of kinase-inactive EGFR (EGFR-KI) had no effect on UVA-induced ERK activation (Fig. 5B), consistent with the effect of AG1478. Similarly, UVA-induced Ras activation was not affected by the presence of AG1478 (Fig. 5C). In comparison, the presence of AG1478 abolished ERK activation upon UBV or UVC exposure (Fig. 5D), indicating that UBV/UVC-induced ERK activation is EGFR-dependent. These data suggest that, different from UVC and UBV, UVA-induced ERK activation does not require EGFR kinase activity.

**PKC Is Required for UVA-induced ERK Activation**—Protein kinase C (PKC) is a well known serine/threonine kinase, consisting of three groups, conventional (α, β, β2, and γ, which respond to both TPA and Ca2+ signals), novel (δ, ε, η, and θ, which respond to TPA but not Ca2+ signals), and atypical PKC (ζ and η, which respond to neither TPA nor Ca2+ signals) (16). PKC is known to be an ERK1/2 activator upon TPA stimulation. HaCaT cells contain PKCα, PKCδ, PKCε, and PKCζ/λ (data not shown). To determine whether UVA-induced ERK activation is mediated by PKC, we depleted PKC by chronic exposure of HaCaT cells to TPA (600 nM) for 18 h. As shown in Fig. 6 (A and B), UVA radiation of untreated cells induced dramatic activation of ERK. However, when PKC was depleted, UVA-induced ERK activation was nearly abolished. A similar pattern was observed with ERK activity determined by Elk phosphorylation and with Ras activation.

When we determined the levels of PKC isoforms with or without TPA chronic treatment in HaCaT cells by Western blotting, PKCα and PKCδ were completely depleted and PKCε was only partially depleted, whereas PKCβ1 and PKCδ were not affected by TPA chronic treatment (Fig. 6, C and D). This implies that PKCs, PKCδ, and/or PKCζ, the TPA-responsive isoforms, mediate ERK activation after UVA radiation. To further identify whether one or more of these three PKC isoforms is involved in the observed ERK activation, HaCaT cells were treated with G66976, a selective inhibitor for Ca2+-dependent PKC (i.e. conventional PKC), after UVA exposure. The presence of G66976 nearly abolished ERK phosphorylation induced by UVA (Fig. 6, E and F). These findings demonstrate that PKCα, responsive to both TPA depletion and Ca2+ signals, is required for UVA-induced ERK activation in HaCaT keratinocytes.

**PLC and Calcium Are Required in UVA-induced ERK Activation**—Phospholipase C (PLC) is a key enzyme in regulating the activation of PKC by generating diacylglycerol and inducing calcium mobilization from intracellular stores (17–19), thereby regulating the activation of ERK. When cells were pretreated with the specific PLC inhibitor U73122 (5 and 10 μM), the activation of ERK after UVA radiation was nearly
abolished (Fig. 7, A and B). An enhanced activation of caspase-3 was observed in the presence of U73122. These data indicate that PLC is required for UVA-induced ERK activation and that blocking ERK activation sensitized cells to UVA-induced apoptosis. When cells were irradiated with UVA, the calcium level was elevated ~3-fold as compared with a control sample at 3 h after exposure, as determined by flow cytometry following fluo-4 staining (Fig. 7C). The mobilization of calcium is a sustained process rather than a transient one. A gradual increase in intracellular calcium level was observed starting at 30 min up to 3 h after UVA exposure, whereas no significant change in calcium was seen in the dark (Fig. 7D), suggesting that UVA radiation caused a gradual calcium mobilization after UVA exposure. When cells were pretreated with BAPTA, a calcium chelator, a slight inhibition of ERK activation by UVA was observed in the presence of 5 μg/ml, whereas the presence of 10 μg/ml BAPTA almost abolished the activation of ERK (Fig. 7, E and F). Like U73122, the presence of BAPTA increased the activation of caspase-3. Thus calcium is required for the UVA-induced ERK activation that served as a survival signal. The presence of either U73122 or BAPTA blocked Ras activation (Fig. 7G), suggesting that PLC and therefore calcium mediate ERK activation in a Ras-dependent pathway.

DISCUSSION

The ultraviolet (UV) portion of solar radiation is composed of UVC (200–280 nm), UVB (280–315 nm), and UVA (315–400 nm). UVC is absorbed completely by the stratospheric ozone layer and thus does not reach the Earth’s surface. Most of the UVB is absorbed by the ozone layer, and the UVA portion of UV in sunlight is small. However, UVA, constituting about 95% of the ultraviolet irradiation in natural sunlight and presenting a major environmental challenge to the skin (20, 21), has recently been listed as reasonably anticipated to be a human carcinogen.

Although UVB-induced acute and chronic effects are mainly mediated by direct DNA damage (mutations), similar to UVC damage, the underlying mechanism for UVA damage is still poorly understood. In this study we have shown that, different from UVC and UVB, UVA induces ERK activation in a delayed and sustained pattern, with a dependence on UVA dose and time after exposure. PKCα is found to be required for Ras activation and therefore ERK activation, in which phospholipase C (PLC) and calcium are also involved. However, UVA-induced ERK activation is EGFR-independent. The activation of ERK provides a survival signal that allows human keratinocytes to escape from UVA-induced apoptosis (Fig. 8).

Importantly the delayed and sustained ERK activation by UVA exposure is not HaCaT cell-specific. These findings may provide important information for the understanding of the potential transformation of skin cells by UVA radiation.

A previous study (12) has shown that UVC exposure induced activation of ERK as a survival signal. This UVC-induced ERK activation was rapid and transient in an EGFR-dependent manner. In contrast, the activation of ERK by UVA is delayed and sustained in an EGFR-independent manner, which is distinct from UVC. In comparison with UVA and UVC, ERK activation induced by UVB was rapid and sustained in both HaCaT and A431 cells in an EGFR-dependent manner. It appears that, as the wavelength of UV radiation changes from the shorter wavelengths of UVC and UVB to the longer wavelengths of UVA, ERK activation demonstrates a changing pattern: 1) rapid to delayed and 2) EGFR-dependent to EGFR-independent. This difference may imply different targets for different types of UV radiation. UVC has been shown to activate EGFR tyrosine kinase activity and its phosphorylation (12, 22), whereas no rapid EGFR phosphorylation was observed within 1 h after UVA exposure (23). The rapid activation of EGFR by UVC (12) or UVB (24) might be due to 1) the presence of chromophores, including small molecules or proteins, that absorb UVC (and UVB) but not UVA and activate EGFR via Src-dependent (12) or -independent pathways and thus the ERK MAPK (22), or 2) the fact that the absorption of UVC or UVB energy but not UVA by either membrane proteins or lipids may lead to similar changes in protein conformation and distribution as ligand binding (24).

It appears that the chromophores that absorb UVA fail to activate EGFR and thus do not engender rapid ERK activation. However, none of these chromophores has been identified and characterized, and little is known about the origin of UV signaling.

Although rapid activation of ERK1/2 can be induced by a variety of cellular stresses, including oxidative stress caused by treatment with hydrogen peroxide (25), and UVA induced immediate and rapid oxidative stress in cells (13, 21), no rapid ERK activation was detected in our study. It seems that the production of reactive oxygen species by UVA fails to induce the signaling leading to ERK activation due to multiple reactive oxygen species formed by UVA radiation such as hydrogen peroxide and singlet oxygen (26). It has been shown that singlet oxygen may initiate distinct signaling from hydrogen peroxide...
such as the activation of protein phosphatases, to attenuate ERK activation (27). Recently UVA has been shown to rapidly activate p38 and c-Jun N-terminal kinase but not ERK after exposure in fibroblasts, which is consistent with our study. Singlet oxygen generated in the cells was considered to be responsible for the signaling (28). However, ERK activation after UVA exposure was not determined at time points later than 2 h in this report (28).

Although it has been shown that PKC activation can be mediated by phospholipase C (PLC) (17–19), the mechanisms involved in UVA-induced PLC activation are not yet clear. The activation of growth factor receptors, including EGFR, upon ligand binding has been shown to induce PLC activation in a wide variety of cells (17). In addition, PLC activation has been observed in response to the activation of non-receptor protein-tyrosine kinases, in protein-tyrosine kinase-dependent and -independent pathways (29). Upon UVA radiation in HaCaT cells, ERK activation did not require EGFR intrinsic kinase activity (Fig. 5), whereas PLC was indispensable for UVA-induced ERK activation (Fig. 7). It appears that the involvement of PLC in ERK activation after UVA exposure is independent of EGFR kinase activity and its phosphorylation. The sustained increase in intracellular calcium level may serve as an indicator of sustained PLC activation and a cofactor for PKC activation after UVA radiation. The sustained calcium mobilization in the cells may be an essential upstream signaling regulator for the sustained ERK activation.

Ras is activated in a delayed pattern after UVA exposure, which is consistent with the time pattern of ERK activation. Interestingly the delayed activation of Ras was observed predominantly in the endomembrane such as the Golgi apparatus upon UVA irradiation, while no immediate Ras activation was detected. In comparison, both immediate Ras activation at the plasma membrane and delayed Ras activation on the Golgi apparatus were detected with distinct kinetics upon EGF stimulation (30, 31). Activation on the plasma membrane was rapid and transient, whereas activation on the Golgi was delayed (10–20 min) and sustained. In contrast, Ras activation on the Golgi by UVA stimulation was delayed and occurred only between 1 and 3 h, not within 1 h, after exposure. The sustained

**Figure 7.** UVA-induced ERK activation required PLC and calcium mobilization. A, HaCaT cells were starved overnight in DMEM medium containing 1% FBS and then left intact or preincubated with U73122 (5 or 10 µM) for 1 h. Cells were exposed to UVA (24 J/cm²) and then incubated in DMEM medium containing 1% FBS in the presence or absence of the inhibitor for 3 h. Phosphorylation of ERK and activation of caspase-3 were determined as in Figs. 1 and 2. B, the optical densities of the corresponding phospho-ERK and caspase-3 (19 kDa) bands in A were quantified. C, HaCaT cells were irradiated with 24 J/cm² UVA and then incubated for 3 h. Cells were labeled with Fluoro-4 (3 µM) followed by flow cytometry determination. D, HaCaT cells were irradiated with 24 J/cm² UVA and then incubated for different times. Cells were labeled with Fluoro-4 (3 µM) followed by flow cytometry determination. The mean fluorescence intensity was used as the indicator for calcium level. E, HaCaT cells were starved overnight in DMEM medium containing 1% FBS and then left intact or preincubated with BAPTA (5 or 10 µM) for 1 h. Cells were exposed to UVA (24 J/cm²) and then incubated in DMEM medium containing 1% FBS in the presence or absence of the inhibitor for 3 h. Phosphorylation of ERK and activation of caspase-3 were determined as in Figs. 1 and 2. F, the optical densities of the corresponding phospho-ERK and caspase-3 (19 kDa) bands in E were quantified. G, cells were preincubated with U73122 (10 µM) or BAPTA (10 µM), exposed to UVA (24 J/cm²), and then incubated in DMEM medium containing 1% FBS in the presence or absence of those inhibitors for 3 h. Ras activity was determined as described under “Experimental Procedures.”

**Figure 8.** Mechanism and role of UVA-induced ERK activation.

(25), such as the activation of protein phosphatases, to attenuate ERK activation (27). Recently UVA has been shown to rapidly activate p38 and c-Jun N-terminal kinase but not ERK after exposure in fibroblasts, which is consistent with our study. Singlet oxygen generated in the cells was considered to be responsible for the signaling (28). However, ERK activation after UVA exposure was not determined at time points later than 2 h in this report (28).

Although it has been shown that PKC activation can be mediated by phospholipase C (PLC) (17–19), the mechanisms involved in UVA-induced PLC activation are not yet clear. The
Ras activation mediated the sustained ERK activation.

The functional role of ERK signaling on endomembranes such as Golgi is not yet clear. The duration and location of activated Ras in the endomembrane and activated ERK in the nuclear and endomembrane fractions determine their biological roles. Artificially restricting Ras to the Golgi or the ER resulted in the transformation of murine fibroblasts (30) by activating the ERK MAPK pathway. UVA-induced sustained activation of ERK was localized in both the nuclear and endomembrane fractions. Activated Ras transmits its signal to ERK in the endomembrane, and part of the activated ERK may translocate into the nucleus. Ras activation in the endomembrane is considered to be involved in cell survival (30). Suspected ERK activation in survived cells after UVA exposure implied that ERK activation may be involved in promoting damaged cells to escape from apoptosis (1, 32). Cells whose ERK activity was inhibited by PD98058, especially live cells with higher ERK activation level, were sensitized to apoptosis after UVA exposure. ERK activation mediated by Ras activation in the endomembrane plays a key role in providing a survival signal.

Activation of ERK has been shown to inhibit the activation of caspase-8 and Bid, and inhibition of MAPK signaling markedly sensitized the cells to CD95-mediated apoptosis in Jurkat T cells (1). Recently Allen and coworkers (32) found that ERK activation inhibits caspase-9 activity by direct phosphorylation at Thr125, which is sufficient to block caspase-9 processing and subsequent caspase-3 activation. Inhibition of caspases-8 and -9 by ERK promotes cell survival and may contribute to tumorigenesis when the ERK MAPK pathway is constitutively activated (32).

In summary, our results demonstrate that, in contrast to UVB or UVC radiation, exposure of HaCaT keratinocytes to UVA induces delayed and sustained ERK activation without involving EGFR kinase activity. PLC activation and calcium mobilization are required for ERK activation, and PKCo-mediated ERK activation, in a Ras-dependent pathway. ERK activation plays an important role in providing a survival signal to allow cells to escape from apoptosis after UVA exposure and increases the potential for malignant transformation and tumor development in vivo.

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REFERENCES
1. Holmstrom, T. H., Schmitz, I., Soderstrom, T. S., Poukkula, M., Johnson, V. L., Chow, N. C., Krammer, P. H., and Eriksson, J. E. (2000) EMBO J. 19, 5418–5428
2. Stadheim, T. A., Xiao, H., and Eastman, A. (2001) Cancer Res. 61, 1533–1549
3. Erhardt, P., Schremmer, E. J., and Cooper, G. M. (1999) Mol. Cell. Biol. 19, 5308–5315
4. Buckley, S., Driscoll, B., Barsky, L., Weinberg, K., Anderson, K., and Warburton, D. (1999) Am. J. Physiol. 277, L159–L166
5. Jost, M., Huggert, T. M., Kari, C., and Redock, U. (2001) Mol. Biol. Cell 12, 1519–1527
6. Tong, Z., Singh, G., and Rainbow, A. J. (2002) Cancer Res. 62, 5528–5535
7. Chang, L., and Karin, M. (2001) Nature 410, 37–40
8. Johnson, G. L. and Lapadat, R. (2002) Science 298, 1911–1912
9. Marshall, C. J. (1995) Cell 80, 179–185
10. Cobb, M. H., and Goldsmith, E. J. (1995) J. Biol. Chem. 270, 14483–14486
11. Princen, K., Hatse, S., Vermeire, K., De Clercq, E., and Schols, D. (2003) Cytochemistry 51A, 35–45
12. Kitagawa, D., Tanemura, S., Ohata, S., Shimizu, N., Seo, J., Nishitai, G., Watanabe, T., Nakagawa, K., Kishimoto, H., Wada, T., Tzukuz, T., Yamamoto, T., Nishina, H., and Katada, T. (2002) J. Biol. Chem. 277, 366–371
13. He, Y. Y., Huang, J. L., Ramirez, D. C., and Chignell, C. F. (2003) J. Biol. Chem. 278, 8058–8064
14. Bivona, T. G., and Philips, M. R. (2003) Curr. Opin. Cell Biol. 15, 136–142
15. Hancock, J. F. (2003) Nat. Rev. Mol. Cell. Biol. 4, 373–384
16. Newton, A. C. (2003) Biochem. J. 370, 361–371
17. Ibe, S. G., and Bae, Y. S. (1997) J. Biol. Chem. 272, 15045–15048
18. Berridge, M. J., and Irvine, R. F. (1984) Nature 312, 315–321
19. Nishinaka, Y. (1988) Nature 334, 661–665
20. de Gruijl, F. R. (1999) Eur. J. Cancer 35, 2003–2009
21. Pourzand, C., and Tyrrell, R. M. (1999) Photochem. Photobiol. 76, 380–390
22. Cofer, P. J., Burgering, B. M., Peppelenbosch, M. P., Bos, J. L., and Kruijver, W. (1999) Oncogene 18, 410–410
23. He, Y. Y., Huang, J. L., Gentry, J. B., and Chignell, C. F. (2003) J. Biol. Chem. 278, 42457–42465
24. Rosette, C., and Karin, M. (1996) Science 274, 1184–1197
25. Yoshizumi, M., Abe, J., Haendeler, J., Huang, Q., and Berk, B. C. (2000) J. Biol. Chem. 275, 11706–11712
26. Gretzberger, S., Benizzi, G., Schmitt-Brenden, H., Felsner, I., Timmer, A., Sies, H., Johnson, J. P., Piette, J., and Kretzmann, J. (2000) EMBO J. 19, 5793–5800
27. Zhuang, S., Ouedraogo, G. D., and Kocheva, I. E. (2003) Oncogene 22, 4415–4424
28. Kist, L. O., Pellieux, C., Briviba, K., Pierlot, C., Aubry, J. M., and Sies, H. (1999) Eur. J. Biochem. 260, 917–922
29. Jones, G. A., and Carpenter, G. (1993) J. Biol. Chem. 268, 20845–20850
30. Chiu, V. K., Bivona, T., Hach, A., Sajous, J. B., Silletti, J., Wiener, H., Johnson, R. L., and Cox, A. D., and Philips, M. R. (2002) Nat. Cell Biol. 4, 343–350
31. Bivona, T. G., Perez De Castro, I., Ahearn, I. M., Grana, T. M., Chiu, V. K., Lockyer, P. J., Cullen, P. J., Pellieux, C., Cox, A. D., and Philips, M. R. (2003) Nature 424, 684–688
32. Allan, L. A., Morrice, N., Brady, S., Magee, G., Pathak, S., and Clarke, P. R. (2003) Nat. Cell Biol. 5, 647–654