Epidermal Growth Factor Receptor Down-regulation Induced by UVA in Human Keratinocytes Does Not Require the Receptor Kinase Activity*

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Activation of the epidermal growth factor (EGF) receptor by EGF, its ligand, results in receptor internalization and down-regulation, which requires receptor kinase activity, phosphorylation, and ubiquitination. In contrast, we have found here in human HaCaT keratinocytes that exposure to UVA induces EGF receptor internalization and down-regulation without receptor phosphorylation and ubiquitination. The presence of the receptor kinase activity inhibitor AG1478 increased UVA-induced receptor down-regulation, whereas it inhibited EGF-induced receptor down-regulation. These observations demonstrate that, in contrast to EGF, receptor kinase activity is not required for receptor down-regulation by UVA. Concurrent with receptor down-regulation, caspases were activated by UVA exposure. The presence of caspase inhibitors blocked receptor down-regulation in a pattern similar to poly(ADP-ribose) polymerase cleavage. Much more receptor down-regulation was observed after UVA exposure in apoptotic detached cells in which caspase is activated completely. These results indicate that UVA-induced receptor down-regulation is dependent on caspase activation. Similar to UVA, both UVB and UVC induced receptor down-regulation, in which receptor kinase activity is not required, whereas caspase activation is involved. Inhibition of EGF receptor down-regulation increased receptor activation and activation of its downstream survival signaling ERK and AKT after UVA exposure. Preventing the activation of each of these pathways enhanced apoptosis induced by UVA. These findings suggest that EGF receptor down-regulation by UVA may play an important role in the execution of the cell suicide program by attenuating its anti-apoptotic function and thereby preventing cell transformation and tumorigenesis in vivo.

The ultraviolet portion of solar radiation is divided into 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 surface of the earth. Although some UVB does reach the surface of the earth, most of it is absorbed by the ozone layer, and the UVB portion of UV in sunlight is small. The risk of UVB-induced acute and chronic damage, mainly mediated by DNA damage like UVC, can be minimized by avoiding direct exposure during midday hours, wearing protective hat/clothes, and using sunscreens (1, 2). However, UVA, constituting about 95% of ultraviolet irradiation in natural sunlight and presenting a major environmental challenge to the skin (3, 4), has been listed recently as being reasonably anticipated to be a human carcinogen (5) as was UVB and UVC. UVA has been shown to be a risk factor for melanoma in fish (6) and could be also in humans (7–9). As compared with UVB and UVC, less is known about the effect of UVA both in vivo and in vitro. Recently we have shown that UVA exposure induced apoptosis in human keratinocytes (10), which is important for the prevention of skin cancer.

The EGF receptor (EGFR), one of the receptor tyrosine kinases (RTKs), plays a pivotal role in regulating cell proliferation, differentiation, and transformation. Defective down-regulation of growth stimulatory receptors might result in malignant transformation of cells (11–13). The oncogenic action of EGFR correlates with its overexpression or deregulated activation at the plasma membrane. Removal of activated receptor molecules from the cell surface by sorting for degradation in lysosomes is expected to inhibit their oncogenic potential and maintain normal cell development.

Binding of growth factors to RTKs promotes receptor dimerization and subsequent activation, which enhances phosphorylation of RTKs, phosphorylation of numerous cellular proteins, and recruitment of adaptor molecules, which initiate signaling cascades, including receptor down-regulation (14). Mechanisms of receptor down-regulation have been best studied for RTKs, and especially EGFR.

Interaction of EGFR with the ubiquitin ligase c-Cbl leads to c-Cbl phosphorylation (12), which results in the activation of the ubiquitin ligase activity of c-Cbl. This promotes the recruitment of the ubiquitin-conjugating enzyme UbcH7 (13) and the subsequent receptor ubiquitination, which targets the receptor to lysosomal degradation (15, 16). EGFR degradation controls the strength and duration of RTK-induced signals and is critical for preventing EGFR hyperactivation, commonly associated with tumorigenesis.

In this study we have found that in HaCaT cells, UVA exposure caused EGFR down-regulation without phosphorylation and subsequent ubiquitination. In contrast, we found that...
EGFR down-regulation by UVA is dependent on caspase activation and plays an important role in apoptosis after UVA exposure.

EXPERIMENTAL PROCEDURES

Cell Culture—The spontaneously immortalized human keratinocyte cell line HaCaT (17), obtained from Prof. N. Fusseneg (German Cancer Research Center, Heidelberg, Germany), was maintained in monolayer culture in 95% air, 5% CO₂ at 37 °C in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 31 μg/ml penicillin, and 50 μg/ml streptomycin. For experiments, HaCaT keratinocytes were seeded in plastic Petri dishes (100 mm) and allowed to attach for 24 h. Prior to UVA treatment, subconfluent cells were given fresh DMEM containing 1% FBS and incubated overnight. For EGF experiments, cells were incubated with EGF (100 ng/ml) (Sigma) in serum-free DMEM at 37 °C.

UVA Treatment—The medium was removed, and cells were washed twice with sterile PBS (PBS-CMF, calcium/magnesium-free). After the addition of sterile PBS, the cells were irradiated with fluorescent lamps (Houvalite F20T12BL-HO PUVA, National Biological Corp., Twinsburg, OH) with the dish lid on. The UVA dose was monitored with a Goldlux UV meter equipped with a UVA detector (Oriel Instruments, Stratford, CT). Control samples were kept in the dark under the same conditions. After treatment, fresh medium containing 1% FBS was added after exposure, and the cells were incubated at 37 °C. At predetermined time points, attached and floating cells were harvested and subjected to analysis. In selected experiments, cells were preincubated with inhibitors at 37 °C prior to irradiation. Inhibitors used as follows: AG1478 and FP2 from Calbiochem; MG132 (N-Cbz-Leu-Leu-Leu-al), cycloheximide (CHX), wortmannin, and caspase inhibitors (DEVD, IETD, and LEHD) from Sigma; PD98059 from Promega (Madison, WI); bafilomycin A1 and lactacystin from Biomol (Plymouth Meeting, PA).

Immuno precipitation— Immunoprecipitations were performed as described previously (18). After treatment, cells were extracted in solubilization buffer containing 50 mM Tris, pH 7.5, 150 mM NaCl, 10% glycerol, 1% Igepal (Sigma), protease inhibitor mixture (Calbiochem), and phosphatase inhibitor mixture (Sigma). Lysates were cleared by centrifugation, and proteins in the lysate supernatants were immunoprecipitated by overnight incubation with the indicated antibodies at 4 °C, followed by protein G (Sigma) precipitation for 1.5 h. Precipitating antibodies used are as follows: anti-EGFR clone R-1 and anti-c-Cbl clone c-15 (Santa Cruz Biotechnology, Santa Cruz, CA). Immune precipitates were washed three times with HNTG buffer containing 20 mM HEPES, pH 7.5, 150 mM NaCl, 0.1% Triton X-100, and 10% glycerol and were detected by immunoblotting.

Western Blotting—After treatments, cells were harvested and lysed with M-PER mammalian protein extraction reagent (Pierce) supplemented with protease inhibitor mixture (Calbiochem) and phosphatase inhibitor mixture (Sigma). Total cell lysates were cleared by centrifugation, and protein concentrations were determined by using the BCA assay (Pierce).

Equal amounts of protein were subjected to electrophoresis on NuPAGE pre-cast gels (4–12%) (NOVEX, San Diego, CA), followed by electrophoretic transfer to nitrocellulose membranes. Membranes were blocked in 5% nonfat milk in TBST (15 mM Tris-HCl, pH 7.4, 150 mM NaCl) for 1 h at room temperature. Cells were blocked with 0.5% gelatin and 5% goat serum in PBS for 1 h. The coverslips were incubated with primary antibodies overnight at 4 °C and secondary antibodies for 1 h at room temperature. Primary antibodies used were Alexa Fluor 488 goat anti-mouse IgG and Alexa Fluor 568 goat anti-rabbit IgG (Molecular Probes, Eugene, OR) for the detection of EEA1, CD63, and EGFR. Coverslips were mounted onto glass slides using the ProLong® Antifade kit (Molecular Probes). Confocal microscopy at ×100 was carried out by using a Zeiss 410 confocal microscope.

DNA Fragmentation—The pattern of DNA cleavage was analyzed by agarose gel electrophoresis as described previously (10). Briefly, after cell pellets were lysed on ice at 4 °C overnight, cell lysates were incubated with RNase A (100 μg/ml) and then with proteinase K followed by extraction with phenol/chloroform/isopropanol alcohol (25:24:1, v/v). After precipitation with ethanol, DNA samples were separated by horizontal electrophoresis on 1.5% agarose gels, stained with ethidium bromide, and visualized under UV light.

RESULTS

UVA and EGF Induce EGFR Down-regulation in HaCaT Cells—To determine the UVA effect on EGFR, HaCaT cells were irradiated with 24 J/cm² UVA and then incubated for different times after exposure. Western blotting showed there was a marked down-regulation of EGFR at 3 h after UVA exposure (Fig. 1A). Caspase-3 was activated in a similar pattern, as was the cleavage of PARP, the substrate for the active form of caspase-3. However, phosphorylated EGFR increased slightly for 6 and 9 h. There was no significant increase in EGFR phosphorylation at 1 h. Additionally, UVA-induced EGFR degradation was also seen in A431 cells and HeLa cells (data not shown).

It is known that EGFR binding to EGFR induces receptor down-regulation (14). To investigate the difference in EGFR down-regulation between UVA and EGF treatments, HaCaT cells were incubated with EGF (100 ng/ml) for different times. UVA (24 J/cm²) and EGF (100 ng/ml) induced similar EGFR down-regulation upon incubation for 3 h (Fig. 1A). However, in contrast to UVA, EGF did not cause caspase-3 activation or PARP cleavage. Unlike UVA, EGFR phosphorylation increased dramatically upon incubation with EGF for 1 h. UVA caused more EGFR down-regulation than EGF at 6 and 9 h after exposure (Fig. 1B).

To determine whether UVA-induced EGFR down-regulation involved protein synthesis, cycloheximide (CHX) (25 μg/ml) was added after UVA exposure. After 3 and 6 h the presence of CHX had no effect on EGFR levels as determined by Western blotting (Fig. 1C), indicating that UVA-induced EGFR down-regulation involves post-translational regulation initiated by UVA rather than protein synthesis. To determine whether UVB or UVC has a similar effect on EGFR levels to UVA, cells were exposed to UVB and UVC and then incubated for different times. As shown in Fig. 1D, UVB induced dramatic EGFR down-regulation and concurrent caspase-3 activation 6 h after UVB exposure (16 mJ/cm²). No EGFR degradation was observed within 2 h after UVB exposure (24 mJ/cm²). Similarly, UVC (5 and 20 mJ/cm²) also induced EGFR down-regulation and concurrent caspase-3 activation at 6 h after exposure (Fig. 1E); no EGFR degradation was detected within 2 h after UVC exposure. That UVA, UVB, and UVC caused EGFR down-regulation was also observed in A431 and HeLa cells (data not shown).

UVA Does Not Induce Tyrosine Phosphorylation of EGFR—as Efficiently as EGF—To determine whether UVA caused EGFR phosphorylation, HaCaT cells were irradiated with different doses of UVA and then incubated for 15 min. As shown in Fig. 2A, UVA up to 24 J/cm² for 15 min did not cause an increase in EGFR phosphorylation as compared with the background level. However, EGF (100 ng/ml) produced a dramatic increase in EGFR phosphorylation (Fig. 2B).

To investigate the mechanism of EGFR phosphorylation following UVA and EGF treatments, we compared the response to UVA and EGF in the presence of AG1478, a specific inhibitor of...
EGFR intrinsic tyrosine kinase activity, and PP2, a selective inhibitor of Src family tyrosine kinases. Fig. 2B shows that when HaCaT cells were pretreated with 10 μM AG1478, EGFR phosphorylation was completely abolished after both UVA and EGF treatments. It is noteworthy that AG1478 even abolished the background phosphorylation of EGFR. Preincubation of cells with 10 μM PP2 resulted in inhibition of EGFR tyrosine phosphorylation by EGF, but only minor inhibition was observed with UVA exposure (Fig. 2B). These results suggest that EGF-induced tyrosine phosphorylation of EGFR is because of the activation of both its intrinsic tyrosine kinase and the Src family tyrosine kinases, whereas only the activation of EGFR intrinsic tyrosine kinase is required for EGFR phosphorylation in UVA-treated cells. Additionally, UVA irradiation caused EGFR dimer formation at 3 and 6 h, and dimer level of EGFR decreased at 6 h as compared with that at 3 h after UVA (Fig. 2C).

**EGFR Intrinsic Kinase Activity and Its Activation Is Not Required for UVA-induced EGFR Down-regulation**—To find out whether EGFR kinase activity is required for EGFR down-regulation, HaCaT cells were pretreated with AG1478, a specific EGFR kinase activity inhibitor. Both UVA and EGF caused increased EGFR phosphorylation (EGF was more efficient), and EGFR down-regulation at 6 h (Fig. 3A). The pres-
ence of 1 μM AG1478 decreased EGFR phosphorylation, whereas 10 μM AG1478 abolished the phosphorylation induced by both UVA and EGF (Fig. 3, A and B). However, inhibition of EGFR phosphorylation by AG1478 had different effects on EGFR down-regulation by UVA and EGF. The presence of AG1478 decreased EGF-induced EGFR down-regulation, whereas it enhanced UVA-induced EGFR down-regulation in an AG1478 concentration-dependent pattern (Fig. 3, A and C). These results indicated that EGFR kinase activity and EGFR phosphorylation are required for EGF-induced EGFR down-regulation but not for UVA-induced EGFR down-regulation.

**UVA Fails to Enhance EGFR Phosphorylation and Ubiquitination**—Because EGF-induced EGFR down-regulation requires EGFR phosphorylation and subsequent ubiquitination (19) mediated by c-Cbl, we carried out immunoprecipitation with EGFR antibody followed by immunoblotting to identify the underlying mechanism that resulted in the different pathways for EGF and UVA-induced EGFR down-regulation. As shown in Fig. 4A, exposure to EGF dramatically enhanced EGFR tyrosine phosphorylation, which reached a maximum after 30 min of incubation with EGF and then decreased. Compared with EGF, UVA resulted in little increase in EGFR phosphorylation, whereas no such increase was seen within 60 min following exposure to UVA (Fig. 4A).

c-Cbl is the ubiquitin ligase responsible for EGF-mediated ubiquitination of EGFR upon phosphorylation (13, 20). Therefore, we investigated activation of c-Cbl in HaCaT cells upon UVA and EGF exposure. As shown in Fig. 4B, enhanced c-Cbl tyrosine phosphorylation was detected after incubation with EGF, but not after UVA radiation. These findings suggest that, unlike EGF, UVA fails to induce rapid EGFR ubiquitination within 60 min after exposure, which is due to the fact that UVA is unable to enhance EGFR phosphorylation and the subsequent c-Cbl tyrosine phosphorylation.

It has been reported that ubiquitin/proteasome is involved in EGF-induced EGFR degradation (13, 21). We therefore examined whether inhibition of proteasome would allow the accumulation of UVA-induced ubiquitination of EGFR. Cells were preincubated with or without a proteasome inhibitor, MG132,
and then exposed to UVA (Fig. 4C). In the absence of MG132, no increase in EGFR ubiquitination was detected up to 6 h after UVA exposure. In contrast, in MG132-treated cells, UVA induced accumulation of EGFR ubiquitination at 3 and 6 h after exposure (Fig. 4C). No accumulation of EGFR ubiquitination at 1 h after UVA radiation was detected in the presence of MG132. Similar results were seen with lactacystin, another proteasome inhibitor (data not shown).

Surprisingly, the presence of MG132 or lactacystin, the proteasome inhibitor, enhanced EGFR down-regulation at 6 h after UVA exposure (Fig. 4, D and E). Concurrently increased activation of caspase-3 and cleavage of PARP were observed in the presence of MG132. In contrast, although caspase-3 was activated by the presence of MG132 or lactacystin, EGF-induced EGFR down-regulation was inhibited slightly by the presence of MG132 or lactacystin (Fig. 4E). This indicated that UVA-induced EGFR down-regulation is proteasome-independent.

Several studies (22–24) have demonstrated that degradation of EGF-induced EGFR is mediated by lysosomal proteases. To investigate whether UVA-caused EGFR degradation is also mediated by lysosome, bafilomycin A1 and NH₄Cl were used as lysosomal inhibitors. Bafilomycin A1 is a specific inhibitor of the proton pump V-type ATPase, which prevents the acidification of the endosomal compartments required for the maturation of lysosomal proteases. NH₄Cl increases the pH in lysosomes to inhibit lysosomal degradation. As shown in Fig. 4E, the presence of bafilomycin and NH₄Cl blocked EGF-induced EGFR down-regulation completely. In contrast, bafilomycin and NH₄Cl did not prevent UVA-induced EGFR down-regulation. It should be noted that the presence of bafilomycin enhanced UVA-induced EGFR degradation and caspase-3 activation. These findings suggest that EGFR down-regulation after UVA exposure may not be mediated via the mechanisms of lysosome involvement in EGF treatment.
EGFR Down-regulation Induced by UVA

Figure 5. UVA induced EGFR internalization and colocalization of EGFR with EEA1-positive early endosomes and CD63-positive late endosomes. A, after serum-starved for 36 h, cells were preincubated with or without caspase inhibitors (10 μM) and then exposed to UVA (24 J/cm²). 20 min after exposure, cells were fixed and incubated with anti-EGFR 528 antibody and stained with Alexa Fluor 488-conjugated secondary antibody. Analysis was performed by confocal microscopy. Ctrl, control. B, cells were starved and irradiated as in A or treated with EGF (100 ng/ml). 20 min after exposure, cells were fixed and incubated with anti-EGFR 1005 (red) and anti-EEA1 (green) followed by staining with Alexa Fluor 568- and 488-conjugated secondary antibodies. Yellow shows the colocalization of EGFR and EEA1. C, cells were treated as in B, except that cells were incubated for 60 min after exposure. Cells were fixed and incubated with anti-EGFR 1005 (red) and anti-CD63 (green) followed by staining with Alexa Fluor 568- and 488-conjugated secondary antibodies. Yellow shows the colocalization of EGFR and CD63.

UVA Induces EGFR Internalization—Because EGF-induced EGFR down-regulation involves the internalization of EGFR (25), we investigated the UVA effect on intracellular localization of EGFR by confocal microscopy using antibodies to EGFR, EEA1, and CD63 (Fig. 5). In control cells, EGFR was mainly localized to the plasma membrane. 20 min after UVA exposure, EGFR was internalized, and the presence of caspase inhibitors had no effect (Fig. 5A). However, cells incubated on ice showed EGFR staining mainly at the plasma membrane regardless of treatment with either EGF or UVA (data not shown). After incubation with EGF for 20 and 60 min at 37 °C, EGFR was internalized, showing a punctate pattern throughout the cytoplasm and colocalized with EEA1-positive early endosomes and CD63-positive late endosomes (Fig. 5, B and C). As compared with EGF, UVA radiation induced partial colocalization of EGFR and the early (EEA1) and late (CD63) endosome markers in a clustered pattern (Fig. 5, B and C). The presence of caspase inhibitors did not affect the colocalization of EGFR with EEA1-positive early endosomes and CD63-positive late endosomes (data not shown).

UVA Causes Concurrent EGFR Down-regulation and Caspase-3 Activation—To elucidate the underlying regulation mechanism for EGFR down-regulation, we irradiated the HaCaT cells with 24 J/cm² UVA and then incubated the cells for different times. As shown in Fig. 6A, EGFR was down-regulated in a time-dependent pattern. EGFR down-regulation was observed at 3 h after UVA exposure. This is consistent with the data shown in Fig. 1A. There was no change in control cells. Concurrently caspase-3 was activated in a time course that paralleled EGFR down-regulation. Similar results were observed for the cleavage of PARP, the substrate for active caspase-3. When we irradiated HaCaT cells with different doses of UVA from 4 to 32 J/cm², a clear dose-dependent EGFR down-regulation was observed, concurrent with caspase-3 activation and PARP cleavage (Fig. 6B). These results suggest that EGFR down-regulation after UVA radiation is associated with caspase activation.

EGFR Down-regulation Induced by UVA Is Dependent on Caspase Activation—To determine whether caspase activation is required for EGFR down-regulation after UVA exposure, we pretreated HaCaT cells with inhibitors DEVD, IETD, and LEHD for caspases-3, -8, and -9, respectively, and then irradiated the cells with UVA. After 6 h, UVA alone induced EGFR down-regulation as mentioned above (Fig. 7A). The presence of the caspase-3 inhibitor DEVD up to 50 μM showed an inhibitory effect but failed to block EGFR down-regulation completely (Fig. 7, A and B). In contrast, the presence of IETD or LEHD (10 μM), caspase-8 or -9 inhibitors, appeared to be more efficient in preventing UVA-induced EGFR down-regulation, whereas inhibition of both caspases-8 and -9 produced additive effects as compared with each alone. As expected, when HaCaT cells were pretreated with a combination of DEVD, IETD, and LEHD, EGFR down-regulation was blocked more than 80%. In parallel, pretreatment with these caspase inhibitors also inhibited PARP cleavage in a manner similar to that for EGFR down-regulation. Lower concentrations of these inhibitors (5 μM) showed similar protective effect on receptor down-regulation (data not shown).

When we collected the attached cells and floating cells separately 24 h after UVA radiation, we found that in attached cells there was slight caspase-3 activation and EGFR down-regulation. However, in floating cells, caspase-3 was completely activated, and the remaining EGFR level was much lower than attached cells (Fig. 7C). These results indicate that EGFR down-regulation is dependent on caspase activation caused by UVA exposure, and the caspase cascade activation upon UVA exposure triggers the EGFR down-regulation.

To identify whether EGFR is fully degraded or cleaved into smaller fragments, antibody recognizing both the N and C termini of EGFR Ab12 was used for Western blotting. As shown in Fig. 7D, EGFR down-regulation by either UVA or EGF was detected by immunoblotting with anti-EGFR Ab12; however, no smaller fragments were observed for either UVA exposure or EGF treatment. Similarly, no cleavage fragment was observed with either anti-EGFR Ab14 or 1005, recognizing the N and C termini of EGFR, respectively. It appears that the receptor was fully degraded after UVA exposure.

To determine whether UVB and UVC-induced EGFR down-regulation is mediated by the similar mechanism to UVA, cells were preincubated with AG1478 or caspase inhibitors. In a similar pattern to UVA, the presence of AG1478 enhanced EGFR degradation, whereas caspase inhibitors blocked EGFR degradation by either UVB or UVC (Fig. 7E).

In contrast, the presence of individual inhibitor of caspase-3, -8, -9, or the combination of these inhibitors had no effect on EGF-induced receptor down-regulation (Fig. 7F). It should be noted that the presence of these inhibitors did not affect EGFR levels in the control samples. These findings suggest that EGF-induced EGFR down-regulation is independent of caspase activation.

EGFR Down-regulation Decreases Anti-apoptotic Signaling—It is known that EGFR down-regulation serves to attenuate the survival and proliferation signaling from EGFR (26). To determine the biological significance of EGFR down-regulation mediated by the UVA-activated caspase cascade, we de-
determined in parallel experiments the effect of caspase inhibitors on phosphatidylinositol 3-kinase/AKT and ERK activation, the downstream signaling components of EGFR, which provide anti-apoptotic and survival signals (27–29). As shown in Fig. 8A, phosphorylation of EGFR and ERK increased while AKT phosphorylation decreased 6 h after UVA exposure. The presence of the caspase inhibitors DVED, IETD, and/or LEHD, which inhibited EGFR down-regulation, increased the phosphorylation of EGFR and EGFR-dependent activation of AKT and ERK after UVA exposure. It should be noted that the changes in ERK and AKT activation after UVA exposure may involve both EGFR-dependent and EGFR-independent pathways (30), and EGFR down-regulation by UVA may only affect EGFR-dependent ERK and AKT responses to UVA.

However, when we pretreated the cells with AG1478, PD98059, or wortmannin, inhibitors for EGFR kinase activity and ERK and AKT activation, respectively, UVA-induced apoptosis was enhanced (Fig. 8B). It can be seen that AG1478 is more efficient than PD98059 and wortmannin in enhancing apoptosis in HaCaT cells. These observations suggest that UVA-induced EGFR down-regulation serves to attenuate the anti-apoptotic signal provided by EGFR activation.

**DISCUSSION**

In the present study we have investigated the effect of UVA exposure on EGFR, one of the receptor tyrosine kinases (RTKs). Surprisingly we found that UVA exposure results in EGFR internalization and down-regulation in a pattern that is similar to EGF. However, the underlying mechanism for UVA-induced EGFR down-regulation is different from the known EGFR down-regulation caused by excessive ligand binding (11, 13, 31, 32). EGFR kinase activity is not required for EGFR internalization and down-regulation induced by UVA. In addition, inhibition of lysosome activity, which blocked EGF-induced EGFR completely, did not prevent UVA-induced EGFR down-regulation. In contrast, we found that EGFR down-regulation is dependent on caspase activation upon UVA radiation.

Our confocal microscopy study has shown that UVA radiation, like EGF, induces EGFR internalization. Unlike EGF, however, UVA does not induce significant EGFR phosphorylation within 1 h after exposure, as determined by Western blotting and immunoprecipitation (Fig. 4). Consequently phosphorylation of c-Cbl, the ubiquitin ligase responsible for the EGF-mediated ubiquitination of EGFR upon phosphorylation (13, 20), is not induced by UVA exposure but is induced by EGF treatment (Fig. 4). As a result, no EGFR ubiquitination was observed from immunoprecipitation followed by immunoblotting. These results indicate that EGFR phosphorylation and ubiquitination (33, 34) do not seem to be involved in UVA-induced EGFR internalization. Similar results have been observed with UVC-induced EGFR internalization in HeLa cells (35). It has been suggested previously that direct absorption of UVC and UVB energy causes a conformational change in EGFR (36, 37). Previously Greenfield and coworkers (38) have reported that EGF binding results in a conformational change in the external domain of its receptor, which has been suggested to be important for ligand-induced internalization of the receptor (39). It is possible that an EGFR conformational modification induced by UVA in some way mimics the receptor modification seen after ligand binding, which provides the internalization signal. EGFR internalization is observed as early
as 20 min after UVA exposure, whereas its down-regulation occurs between 1 and 3 h after UVA. This indicates that it is the full-length receptor that is internalized and EGFR down-regulation follows its internalization.

Our finding that EGFR was down-regulated after UVA exposure without phosphorylation and was independent of receptor kinase activity was surprising. Moreover, the presence of AG1478, EGFR kinase inhibitor, has a distinct effect on EGFR down-regulation induced by UVA and EGF (Fig. 3). AG1478 enhances UVA-induced EGFR down-regulation but inhibits EGF-induced EGFR down-regulation. This is consistent with previous studies (13, 20, 21, 32, 40) that EGF-induced receptor down-regulation does require its kinase activity and phosphorylation and the subsequent c-Cbl phosphorylation and ubiquitination. In contrast, the presence of proteasome inhibitor MG132 allowed the accumulation of ubiquitinated EGFR at 3 and 6 h after UVA exposure, whereas EGFR down-regulation was enhanced at 6 h by MG132. These findings support the idea that EGFR phosphorylation and ubiquitination are not involved in receptor down-regulation after the receptor internalization. In addition, lysosome inhibitors, which blocked EGFR-induced receptor degradation completely, failed to block EGFR degradation after UVA exposure, suggesting that lysosomes may not be involved in UVA-induced EGFR degradation via the mechanism in EGF treatment.

Our finding that EGFR down-regulation is associated with the caspase cascade that occurs upon apoptosis induction in the cytosol (41) was supported by several observations. 1) UVA induced concurrent receptor down-regulation and caspase activation after exposure. 2) The presence of AG1478 enhanced EGFR down-regulation by increasing caspase activation. 3) The presence of proteasome inhibitor lactacystin or MG132 increased EGFR down-regulation by increasing caspase activation. 4) The presence of caspase inhibitors blocked EGFR down-regulation in a pattern similar to the cleavage of PARP, the caspase substrate (Fig. 7). 5) Apoptotic cells (floating cells) showed much more EGFR down-regulation and caspase activation than attached cells. These observations indicate that UVA-induced EGFR down-regulation in HaCaT cells is dependent on caspase activation but is independent of proteasome. In contrast, EGF-induced EGFR down-regulation is independent of caspase activation based on the following observations. 1) EGF did not induce caspase activation in HaCaT cells. 2) The presence of AG1478, lactacystin, or MG132 induced caspase activation, whereas EGFR down-regulation was inhibited. 3) Caspase inhibitors had no effect on the receptor degradation. These observations indicate that UVA induced distinct caspase-dependent EGFR down-regulation from EGF.

Recently, Bae and coworkers (42) have shown that active caspases of class II, including caspases-3 and -7, are able to cleave EGFR in vitro. In our study, the presence of caspase-3 inhibitor alone up to 50 μM did not block EGFR down-regulation as efficiently as a combination of caspases-3, -8, and -9 inhibitors, because caspases-8 and -9 are initiators and upstream of effectors caspases-3 and -7 (41). This is consistent with the previous in vitro study (42). It seems likely that after internalization upon UVA radiation, EGFR may act as the direct or indirect substrate of activated caspases like PARP and is cleaved by those apoptosis executors (41) in the absence of receptor phosphorylation and ubiquitination, followed by further complete degradation. Although our work cannot provide detailed biochemical mechanisms of caspase-dependent EGFR degradation induced by UVA, our studies point out the distinct pathways in the degradation of the receptor after UVA irradia-
CaT keratinocytes to UVA radiation, activation of caspases and separated on 1.5% agarose gel.

However, inhibition of EGFR or its downstream ERK or AKT signaling increased apoptosis in HaCaT cells after UVA exposure (Fig. 8). These results indicate that in the response of HaCaT keratinocytes to UVA radiation, activation of caspases downstream-regulates EGFR to attenuate its signaling, by functioning in an “executive” role to turn off protective pathways and turn on downstream activities, which in turn lead to cellular destruction (41).

In summary, our results demonstrate that exposure of HaCaT keratinocytes to UVA induces EGFR down-regulation without requiring EGFR kinase activity, phosphorylation, and ubiquitination. Caspase activation is responsible for the receptor down-regulation to decrease EGFR and its downstream anti-apoptotic signaling. This allows apoptosis to proceed and may serve to prevent cell transformation and skin cancer in vivo.

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