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Ultraviolet Irradiation-Induces Epidermal Growth Factor Receptor (EGFR) Nuclear Translocation in Human Keratinocytes

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ABSTRACT

Epidermal growth factor receptor (EGFR) plays a critical role in mediating ultraviolet (UV) irradiation-induced signal transduction and gene expression in human keratinocytes. EGFR activation results from increased phosphorylation on specific tyrosine residues in the C-terminal intracellular domain. It has recently been reported that following growth factor stimulation EGFR translocates from the surface membrane to the nucleus, where it may directly regulate gene transcription. We have investigated the ability of UV irradiation to induce EGFR nuclear translocation in human primary and HaCaT keratinocytes. UV irradiation caused rapid nuclear translocation of EGFR. Significant accumulation of EGFR in the nucleus was observed within 15 min after UV irradiation exposure. Maximal translocation occurred at 30 min post-UV irradiation, and resulted in a 10-fold increase in EGFR in the nucleus, as determined by Western blot analysis of nuclear extracts and confirmed by immunofluorescence. Inhibition of nuclear export by Leptomycin B did not alter UV irradiation-induced nuclear accumulation. EGFR tyrosine kinase inhibitor (PD169540) reduced UV irradiation-induced EGFR nuclear translocation 50%. Mutation of either tyrosine 1148 or tyrosine 1173 reduced nuclear translocation 70%, while mutation of tyrosine 1068 was without effect. In addition, over-expression of receptor type protein tyrosine phosphatase-kappa (RPTP-κ), which specifically dephosphorylates EGFR tyrosines, decreased UV irradiation-induced EGFR nuclear translocation in human keratinocytes. These data demonstrate that UV irradiation stimulates rapid EGFR nuclear translocation, which is dependent on phosphorylation of specific EGFR tyrosine residues. EGFR nuclear translocation may act in concert with conventional signaling pathways to mediate UV irradiation-induced responses in human keratinocytes. J. Cell. Biochem. 107: 873–880, 2009.

KEY WORDS: SIGNAL TRANSDUCTION; NUCLEAR TRANSLOCATION; EGFR; ULTRAVIOLET

Approximately 90% of human skin cancers, the most common human malignancies, are thought to be caused by solar UV irradiation [Koh, 1995]. In animal models, UV irradiation has been shown to be both a tumor initiator and a tumor promoter [Staberg et al., 1983; Strickland, 1986; Ananthaswamy and Pierceall, 1990]. UV irradiation can induce permanent DNA damage due to imperfect repair. UV irradiation also induces signal transduction pathways that lead to aberrant regulation of oncogenes and tumor suppressor genes. Both nuclear and non-nuclear initiated events seem to contribute to UV irradiation-induced biological effects. Elucidation of the mechanisms by which UV irradiation regulates gene expression is crucial for the understanding of UV irradiation-induced tumorigenesis in human skin.

One of the earliest cellular responses to UV irradiation is phosphorylation and activation of certain cell surface growth factor receptors [Sachsenmaier et al., 1994; Rosette and Karin, 1996]. Among these receptors, epidermal growth factor receptor (EGFR) has been demonstrated to mediate many UV irradiation-induced signal transduction pathways [Xu et al., 2006b]. EGFR (also known as ErbB1 or HER1) is a member of ErbB family of receptor protein tyrosine kinases (RPTKs). Other ErbB family members include ErbB2 (Neu, HER2), ErbB3 (HER3), and ErbB4 (HER4) [Yarden and Sliwkowski, 2001]. EGFR is composed of an extracellular ligand binding domain, a single transmembrane domain, and an intracellular kinase domain. The binding of ligand to the receptor induces receptor dimmerization/oligomerization, resulting in trans-autophosphorylation of multiple tyrosine residues at the carboxyl-

Abbreviations used: EGFR, epidermal growth factor receptor; UV, ultraviolet; CHO, Chinese hamster ovary; LMB, leptomycin B.

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terminus of the receptor by intrinsic tyrosine kinase activity of the receptor. These phospho-tyrosines provide docking sites for downstream effector molecules such as PLC-γ1, Shc, Grb-2, and Gab1 [Jorissen et al., 2003].

Activation of EGFR signal transduction pathway by UV irradiation mimics activation induced by ligand, although it is ligand-independent in human keratinocytes [Knebel et al., 1996; Xu et al., 2006a]. UV irradiation-induced EGFR activation is mediated by oxidative inhibition of EGFR phosphatase activity, and therefore differs from that of ligand induced activation [Xu et al., 2006b]. We have previously shown that EGFR activation is a key initiator of many cellular responses of keratinocytes to UV irradiation [Xu et al., 2006a].

Activation of EGFR promotes cell survival, motility, proliferation, and tumorigenesis [Raymond et al., 2000]. Numerous studies have established the link between up-regulation of EGFR with tumorigenesis in both human and animals [Kelloff et al., 1996]. For example, aberrant regulation of EGFR signaling pathway has been found to be associated with a high percentage of tumors in the breast, ovary, head and neck, bladder, colon, esophagus, cervix, prostate, and lung [Fry, 1999]. Therefore, further understanding of EGFR signaling pathway and its contribution to tumorigenesis may ultimately provide improved preventive and therapeutic means in the fight against cancer.

Cell surface receptor protein tyrosine kinases such as the ErbB family members transmit extracellular stimuli via assembly of signaling molecules at the plasma membrane. However, several reports have described nuclear localization of EGFR and/or its ligand [Gusterson et al., 1985; Kamio et al., 1990; Lipponen and Eskelinen, 1994; Tervahauta et al., 1994; Holt et al., 1995; Zimmermann et al., 1995]. Understanding of the mechanism and biological functions of EGFR nuclear localization remains lacking. It has been reported [Lin et al., 2001] that ligand induces EGFR nuclear translocation and the carboxyl-terminus of EGFR contains a strong transcriptional activation domain when fused to GAL-4 DNA-binding domain. In addition, nuclear EGFR bound and activated AT-rich consensus sequence-dependent transcription and associated with cyclin D1 promoter in intact cells. Recently, EGFR nuclear translocation has been shown to be induced by ionizing radiation and implicated in DNA repair processes [Ditterman et al., 2005]. Another ErbB family member, ErbB4, has also been shown to translocate to the nucleus and regulate gene transcription following ligand stimulation [Ni et al., 2001]. ErbB3, which harbors a nuclear translocation signal, has been shown to shuttle between nuclear and non-nuclear compartments in response to ligand stimulation [Offterdinger et al., 2002]. Fibroblast growth factor receptor (FGFR1) has also been reported to translocate to the nucleus where it functions to regulate ligand-stimulated cell proliferation [Reilly and Maher, 2001].

In this report, we demonstrate that UV irradiation rapidly induces EGFR nuclear translocation in human keratinocyte HaCaT cells and primary human keratinocytes in a time and dose–dependent manner. The mechanism of UV irradiation induction of EGFR nuclear translocation is dependent on specific tyrosines in the C-terminus of EGFR and independent of nuclear export machinery.

MATERIALS AND METHODS

MATERIALS

HaCaT cells were generously provided by Dr. N. E. Fusenig (German Cancer Research Center, Heidelberg, Germany). Chinese hamster ovary (CHO) cells were purchased from ATCC. Dulbecco's modified Eagle's medium (DMEM), heat inactivated fetal bovine serum (FBS), Dulbecco's phosphate-buffered saline (PBS), and trypsin 2.5% were obtained from Invitrogen-Gibco (Grand Island, NY). EGFR antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), Lab Vision Corporation (Fremont, CA) and Transduction Laboratory/PharMingen (Los Angeles, CA). Phospho-EGFR antibodies were purchased from Upstate Biotechnology (Lake Placid, NY) and Cell Signaling Technology (Beverly, MA). Leptomycin B was purchased from Sigma Chemical Co. (St. Louis, MO). PD169540, a highly specific irreversible inhibitor of EGFR tyrosine kinase, was provided by Dr. David Fry (Pfizer, Ann Arbor, MI).

PLASMIDS

Plasmids encoding wild-type EGFR (pRK5 EGFR) and point mutations (tyrosine to phenylalanine) of EGFR were kind gifts from Dr. Axel Ullrich (Max-Planck Institute for Biochemistry, Germany) and Dr. Frank-D. Böhmer (Friedrich Schiller University, Jena, Germany), respectively. pShuttle RPTP-κ and RPTP-ξ plasmids have been described previously [Xu et al., 2005].

CELLS AND CELL CULTURE

Human epidermoid A431 cells and human keratinocyte HaCaT cells were cultured in DMEM containing 10% fetal bovine serum (FBS) under 5% CO_2 at 37°C. Chinese hamster ovary (CHO) cells were cultured in F12 media supplemented with 10% FBS under 5% CO_2 at 37°C. Human primary keratinocytes were cultured in modified MCDB153 media (EpiLife, Invitrogen-Cascade Biologics) under 5% CO_2 at 37°C.

UV SOURCE AND IRRADIATION

Cells were irradiated with 40 mJ/cm^2 UV source with 40% UVB, 27% UVA1, 19% UVA1, and 14% visible light as analyzed by Spectroradiometry OL 754 system (Optronic Laboratories, Orlando, FL), using a lamp containing six FS24T12 UVB-HO bulbs (Davvlin, Bryan, OH). A Kodacel filter (Kodak, Rochester, NY) was used to eliminate wavelengths below 290 nm (UVC). Irradiation intensity was monitored with an IL1443 phototherapy radiometer and a SED240/UVB/W photodetector (International Light, Newbury, MA).

NUCLEAR EXTRACT PREPARATION

The method by Lin et al. [2001] was used to prepare nuclear extracts. Briefly, after treatment, cells were rinsed twice with ice-cold PBS, and scraped from culture dishes in nuclear extract buffer (NEB) (20 mM Hepes, pH 7.2, 10 mM KCl, 2 mM $MgCl_2$, and 0.5% NP-40, supplemented with 10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml pepstatin A, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 1 mM sodium orthovanadate). Cells were disrupted in a Dounce homogenizer (Kontes, Vineland, NJ). Cell homogenate (1 ml) was transferred to microcentrifuge tubes and microfuged at 4,200 rpm for 5 min. The supernatant was used as non-nuclear fraction. The

pellet was resuspended and washed in NEB three times. Nuclear proteins were extracted by resuspending the pellet in 100 μl of NEB containing 0.5 M NaCl. After microcentrifugation at 13,200 rpm for 10 min, supernatants containing nuclear extract were used for analyses. Protein content of nuclear and non-nuclear fractions was determined by Bio-Rad protein assay.

WESTERN BLOT

Non-nuclear and nuclear fractions were subjected to SDS–polyacrylamid gel electrophoresis and transferred to Immobilon-P filter paper (Millipore, Bedford, MA). Immunoreactive proteins were visualized by enhanced chemifluorescence (ECF) according to manufacturer's protocol (GE Healthcare). Quantification of the intensities of immunoreactive bands was performed using a STORM PhosphorImager (Molecular Dynamics, Sunnyvale, CA). For estimation of the percentage of total EGFR that translocated to the nucleus (Fig. 1a), non-nuclear and nuclear fractions were separately normalized to total protein content, and analyzed side-by-side on the same Western blot. The relative proportion of nuclear translocation was calculated based on EGFR band intensities and total volume of the non-nuclear and nuclear fractions (10:1). For the remaining studies, EGFR levels in the non-nuclear fractions were used to verify equal cell number among treatment groups (similar to housekeeping protein used as internal loading control). For this purpose, non-nuclear and nuclear fractions were analyzed on separate gels, under conditions of different primary antibody concentrations, and development times to deliberately reduce the relatively strong non-nuclear signal. Therefore, EGFR band intensities of the two fractions do not directly reflect the proportion of total EGFR that translocated to the nucleus.

TRANSIENT TRANSFECTION OF CHO CELLS

Mammalian expression vectors harboring wild-type EGFR (pRK5 EGFR) or single mutation of C-terminal tyrosine residues of EGFR were transiently transfected by Lipofectamine 2000 method into CHO cells according to manufacturer's protocol (Invitrogen Corporation, Carlsbad, CA). Cells were analyzed 24 h post-transfection.

IMMUNOFLUORESCENCE

Cells cultured in eight-well chamber slides were washed once with ice-cold PBS, fixed with 2% paraformaldehyde for 20 min at room temperature. After TBST (Tris based Saline with 0.1% Triton X-100, pH 7.4) wash, cells were incubated with methanol for 5 min and washed with TBST. Slides were then blocked with 5% normal goat serum for 30 min and then incubated with primary antibody for 1 h at room temperature and washed with PBS. After 30 min incubation with fluorescein isothiocyanate-labeled secondary antibody and subsequent washes, slides were visualized using a fluorescence microscope.

ADENOVIRUS INFECTION OF HUMAN KERATINOCYTES

Construction of RPTP-κ expressing adenovirus has been previously described [Xu and Fisher, 2005]. HEK293 cells were used for production and purification of adenovirus. Human primary keratinocytes were infected with either empty or RPTP-κ adenovirus 24 h prior to experimental treatments.

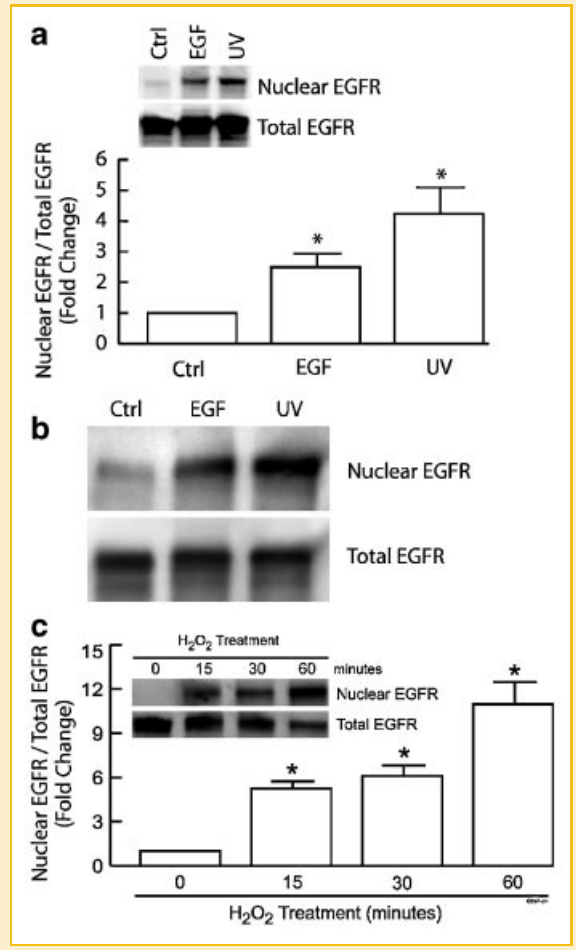

Fig. 1. EGFR nuclear translocation induced by EGF, UV irradiation, or H_2O_2 in human keratinocytes and A431 cells. (a) Human keratinocyte HaCaT cells and (b) A431 cells were sham irradiated (Ctrl), treated with 20 ng/ml EGF, or exposed to 40 mJ/cm^2 UV irradiation. Non-nuclear and nuclear fractions were prepared 30 min post-treatment. (c) Human primary keratinocytes were treated with 5 mM H_2O_2 for the indicated times. Non-nuclear and nuclear fractions were separately normalized to equal protein content and subjected to SDS–PAGE followed by Western blot probed with EGFR antibody. Quantification was performed using STORM PhosphorImager. The amount of EGFR protein present in nuclear fraction was normalized with total EGFR protein and expressed as fold change relative to levels in sham-irradiated, untreated controls (Ctrl). Data are mean ± SEM, N = 5, $^*P < 0.05$, compared to Ctrl.

RESULTS

STIMULATION OF NUCLEAR TRANSLOCATION OF EGFR BY UV IRRADIATION

Human keratinocyte HaCaT cells were treated with either 20 ng/ml EGF or exposed to 40 mJ/cm^2 UV irradiation. Nuclear and non-nuclear fractions were analyzed by Western blot. Both UV irradiation and EGF treatment significantly stimulated EGFR nuclear translocation (Fig. 1a). The amount of total cellular EGFR remained constant. Interestingly, induction of EGFR nuclear translocation by UV irradiation was 1.7-fold greater ($P < 0.05$) compared to induction by EGF treatment (Fig. 1a). Taking into account the relative proportion of each fraction that was analyzed

(nuclear: non-nuclear = 10:1), the amount of EGFR translocated to the nucleus in response to UV irradiation represented approximately 2% of total cellular EGFR. To examine whether UV irradiation-induced EGFR nuclear translocation differs among cell types, we performed similar experiments with human epidermoid carcinoma A431 cells, which express relatively high levels of EGFR. Results shown in Figure 1b indicate that both UV irradiation and EGF treatment induced EGFR nuclear translocation in A431 cells, to similar extents seen in HaCaT cells.

Similiar to UV irradiation, hydrogen peroxide (H_2O_2) has been reported to induce EGFR tyrosine phosphorylation, in a ligand independent manner [Rao, 1996; Huang et al., 2001]. Therefore, we examined whether H_2O_2 also stimulates EGFR nuclear translocation. Figure 1c demonstrates time-dependent increase of nuclear EGFR following treatment of primary human keratinocytes with H_2O_2. The extent of EGFR nuclear localization, observed at 30 min after treatment, was similar to that observed following UV irradiation (Fig. 1a).

In order to confirm the above results, immunofluorescence experiments were performed to localize EGFR after UV irradiation in HaCaT cells. In control cells, EGFR was primarily localized at the cell surface. Upon UV irradiation (40 mJ/cm^2), nuclear staining of EGFR was clearly detected (Fig. 2a), using the same antibody used in Western blot analysis (Fig. 1). Immunofluorescence experiments with another EGFR antibody (Transduction Laboratory Cat #E12020) revealed a similar staining pattern (Fig. 2b). UV irradiation also induced EGFR nuclear translocation in primary adult human keratinocytes (Fig. 2c).

Kinetics of UV irradiation induction of EGFR nuclear translocation are shown in Figure 3a. No increase of nuclear EGFR was detected 5 min after UV irradiation. EGFR nuclear translocation became apparent within 15 min, peaked at 30 min and started to decline at 60 min post-UV irradiation exposure. These data reveal the transient nature of EGFR nuclear translocation induced by UV irradiation. Dose dependent UV irradiation induction of EGFR nuclear translocation in human keratinocyte HaCaT cells is shown in Figure 3b. EGFR nuclear translocation was detectable at 20 mJ/cm^2, and increased with dose of 40 mJ/cm^2. No further increase was observed at higher dose.

INHIBITION OF NUCLEAR EXPORT MACHINERY DOES NOT ALTER EGFR NUCLEAR TRANSLOCATION INDUCED BY UV IRRADIATION

Nuclear accumulation of EGFR could involve increased nuclear entry and/or decreased nuclear export. Leptomycin B (LMB) is a

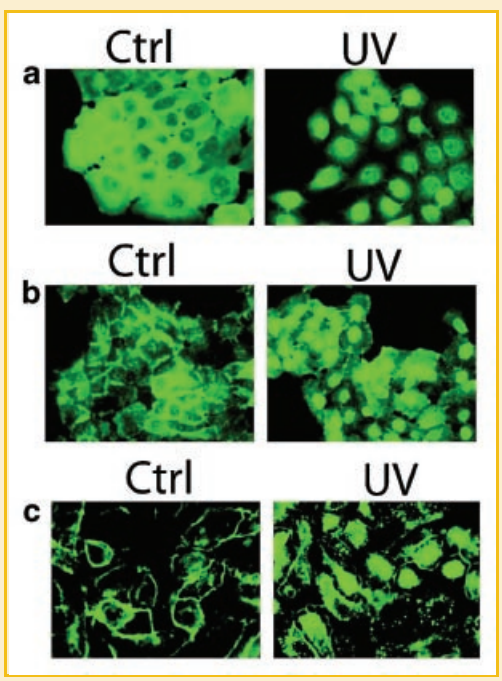

Fig. 2. EGFR nuclear translocation induced by UV irradiation in human keratinocyte HaCaT cells and primary keratinocytes. Human keratinocyte HaCaT cells (a,b) or primary human adult keratinocytes (c) were exposed to 40 mJ/cm^2 UV irradiation or sham irradiated (Ctrl). Cells were washed and fixed for immunofluorescence microscopy 30 min post-irradiation. EGFR antibodies used: (A), Santa Cruz (Cat#1005); (B), Transduction Laboratory (Cat #E12020); and (c), NeoMarker Ab-10 (Cat #MS-378). Data are representative of three independent experiments.

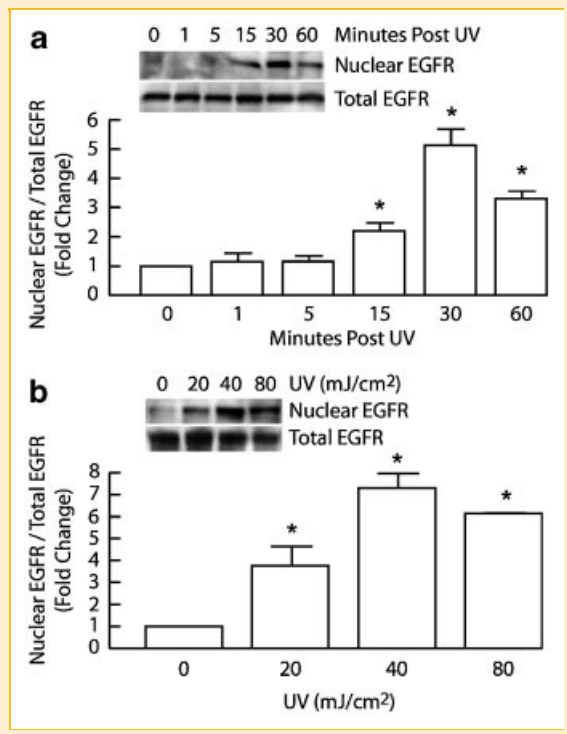

Fig. 3. Time and dose dependent induction of EGFR nuclear translocation by UV irradiation. (a) Human keratinocyte HaCaT cells were exposed to 40 mJ/cm^2 UV irradiation. Non-nuclear and nuclear fractions were prepared at the indicated times post-UV irradiation. (b) Human keratinocyte HaCaT cells were exposed to indicated doses of UV irradiation. Non-nuclear and nuclear fractions were prepared 30 min post-UV irradiation, separately normalized to equal protein content, and subjected to SDS–PAGE followed by Western blot probed with EGFR antibody. EGFR protein was analyzed by Western blot using EGFR antibody (Santa Cruz 1005) and quantified by STORM PhosphorImager. The amount of EGFR protein present in nuclear fraction was normalized with total EGFR protein and expressed as fold change relative to levels in sham-irradiated controls (Ctrl). Data are mean ± SEM of two independent experiments. $P < 0.05$.

fungal metabolite that binds exportin 1 in the nuclear export machinery and inhibits its function [Kudo et al., 1999]. Pretreatment of human keratinocyte HaCaT cells with 10 ng/ml LMB did not increase basal or UV irradiation-induced EGFR nuclear translocation, suggesting that UV irradiation-induced EGFR nuclear translocation does not involve nuclear export machinery (data not shown). In contrast, LMB treatment of human keratinocyte HaCaT cells caused significant nuclear accumulation of P65 NF-κB in the nucleus (data not shown), as previously described in HEK293, PC-3, and monkey arterial smooth muscle cells [Carlotti et al., 2000; Huang et al., 2000]. Nuclear accumulation of P65 for 10 mM LMB treatment serves as positive control, and indicates that the mechanism of EGFR nuclear trafficking differs from that of P65.

INHIBITION OF INTRINSIC TYROSINE KINASE ACTIVITY REDUCES UV IRRADIATION-INDUCED EGFR NUCLEAR TRANSLOCATION

EGFR activation by UV irradiation and ligands results from increased tyrosine phosphorylation, which is catalyzed by intrinsic tyrosine kinase activity. Therefore, we next investigated the role of EGFR tyrosine kinase activity on EGFR nuclear translocation. EGFR tyrosine kinase inhibitor PD169540 [Fry et al., 1998] inhibited UV irradiation-induced EGFR tyrosine phosphorylation 80%, in human keratinocyte HaCaT cells [Xu et al., 2006b]. PD169540 inhibited EGFR nuclear translocation induced by UV irradiation 50% (Fig. 4), indicating involvement of EGFR tyrosine kinase activity.

PHOSPHORYLATION OF EGFR TYROSINES 1148 AND 1173 ARE REQUIRED FOR NUCLEAR TRANSLOCATION

To investigate the role of individual tyrosine residues in the carboxyl-terminus of EGFR in UV irradiation-induced EGFR nuclear translocation, tyrosine residues at position 1068, 1148, and 1173 were mutated to phenylalanine, which cannot be phosphorylated. Wild-type or mutated EGFR were expressed in Chinese Hamster Ovary (CHO) cells, which lack endogenous EGFR. Expression of wild-type EGFR tended to be lower than the three mutant EGFR proteins, which were expressed at similar levels. UV irradiation stimulated nuclear translocation of expressed wild-type EGFR in CHO cells similar to that observed in keratinocytes (Fig. 5). EGFR containing mutation of either tyrosine 1148 or tyrosine 1173 did not translocate to the nucleus in response to UV irradiation (Fig. 5). In contrast, mutation of tyrosine 1068 had no effect on UV irradiation-induced EGFR nuclear translocation (Fig. 5).

We have previously reported that receptor type protein tyrosine phosphatase-kappa (RPTP-κ) co-localizes with EGFR on the surface of human keratinocytes and specifically dephosphorylates EGFR tyrosines 1068, 1173 [Xu et al., 2005], and 1148 [Xu et al., unpublished work]. RPTP-κ reduces basal, ligand-stimulated, and UV irradiation-induced tyrosine phosphorylation, thereby inhibiting down-stream signal transduction. Therefore, we over-expressed RPTP-κ to further investigate the role of EGFR tyrosine phosphorylation on UV irradiation-induced nuclear translocation in human keratinocytes. Over-expression of RPTP-κ significantly reduced UV

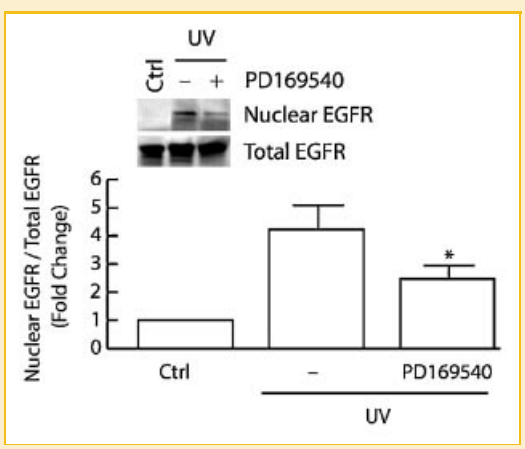

Fig. 4. Inhibition of UV irradiation-induced EGFR nuclear translocation by EGFR tyrosine kinase inhibitor PD169540. Keratinocyte HaCaT cells were treated with vehicle or EGFR tyrosine kinase inhibitor PD169540 (200 nM) for 2 h and then sham-irradiated (Ctrl) or exposed to 40 mJ/cm^2 UV irradiation. Non-nuclear and nuclear fractions were prepared 30 min post-UV irradiation, separately normalized to equal protein content, and analyzed by Western blot using EGFR antibody (Santa Cruz 1005). Quantification was performed using STORM PhosphorImager. The relative amount of EGFR protein present in nuclear fraction was normalized with total EGFR protein and expressed as fold change relative to levels in sham-irradiated, untreated controls (Ctrl). Data are mean ± SEM, N = 3, $^*P < 0.05$ compared to UV-irradiated sample without PD169540 (Ctrl).

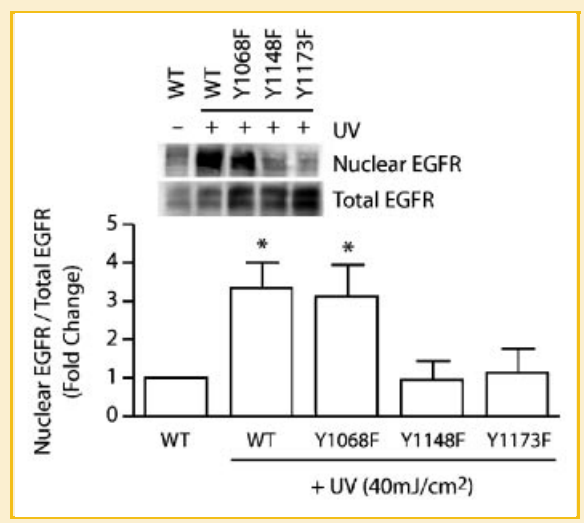

Fig. 5. Phosphorylation of tyrosines Y1148F and Y1173F, but not Y1068F, is required for UV irradiation-induced EGFR nuclear translocation. CHO cells were transfected with wild-type (WT) EGFR or mutant forms of EGFR harboring single tyrosine to phenylalanine mutation (Y1068F, Y1148F, or Y1173F). One day after transfection, CHO cells were sham-irradiated or exposed to 40 mJ/cm^2 UV irradiation. Nuclear translocation of EGFR was analyzed by subcellular fractionation and Western blot using EGFR antibody (Santa Cruz 1005). Quantification was performed using STORM PhosphorImager. The relative amount of EGFR protein present in nuclear fraction was normalized with total EGFR protein and expressed as fold change. Sham irradiated, WT EGFR-expressing CHO cells were used as control (WT without UV). Data are mean ± SEM, N = 3, $^*P < 0.05$ compared to WT without UV irradiation.

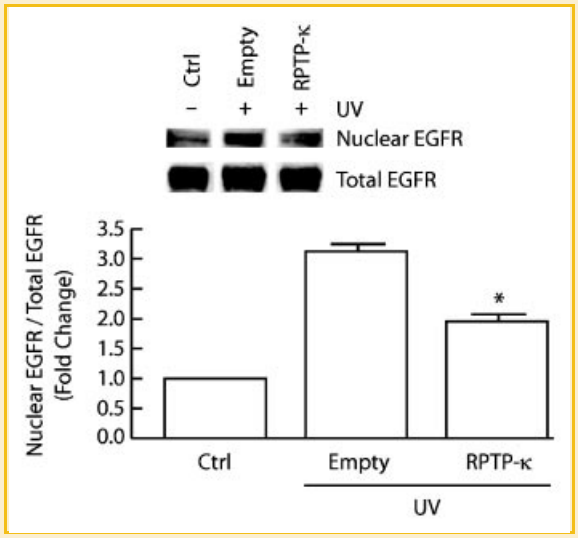

Fig. 6. Over-expression of RPTP-κ inhibits UV irradiation-induced EGFR nuclear translocation in human keratinocytes. Human primary keratinocytes were infected with either empty or RPTP-κ expressing adenovirus. Cells were subjected to sham irradiation (Ctrl) or 40 mJ/cm^2 UV irradiation 24 h after infection. Non-nuclear and nuclear fractions were prepared 30 min post-UV irradiation, separately normalized to equal protein content, and analyzed by Western blot using EGFR antibody (Santa Cruz 1005). Quantification was performed using STORM PhosphorImager. The relative amount of EGFR protein present in nuclear fraction was normalized with total EGFR protein and expressed as fold change relative to levels in sham-irradiated, untreated controls (Ctrl). Data are mean ± SEM, N = 3, $^*P < 0.05$ compared to empty virus UV irradiated.

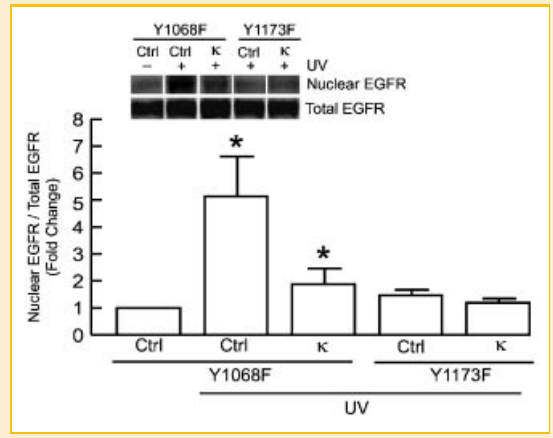

Fig. 7. Over-expression of RPTP-κ differently alters UV irradiation-induced nuclear localization of EGFR Y1068F and Y1173F mutants. CHO cells were co-transfected with protein tyrosine phosphatase RPTP-κ or RPTP-ξ (negative control, Ctrl) and EGFR mutant Y1068F or Y1173F. One day after transfection, CHO cells were sham-irradiated or exposed to 40 mJ/cm^2 UV irradiation. Nuclear translocation of EGFR was analyzed by subcellular fractionation and Western blot using EGFR antibody (Santa Cruz 1005). Quantification was performed using STORM PhosphorImager. The relative amount of EGFR protein present in nuclear fraction was normalized with total EGFR protein and expressed as fold change. Data are mean ± SEM, N = 4, $^*P < 0.05$.

irradiation-induced nuclear EGFR translocation (Fig. 6). Furthermore, expression of RPTP-κ significantly reduced UV irradiation-induced nuclear translocation of tyrosine 1068 mutant, and did not alter translocation of tyrosine 1173 mutant, in CHO cells (Fig. 7). These data confirm the importance of phosphorylation on tyrosine residues 1148 and 1173 for UV irradiation-induced EGFR nuclear translocation.

DISCUSSION

Activation of EGFR function in response to either ligand or UV irradiation results from increased phosphorylation of specific tyrosine residues in the cytoplasmic domain of EGFR. Although the mechanism by which these two stimuli increase tyrosine phosphorylation differs [Jorissen et al., 2003; Xu et al., 2006a], both ligand and UV irradiation stimulate EGFR nuclear translocation. These data suggest that increased tyrosine phosphorylation, independent of mechanism responsible for phosphorylation, triggers nuclear translocation. This conclusion is supported by our findings that inhibition of intrinsic receptor kinase activity, mutation of specific tyrosine residues, or over-expression of EGFR tyrosine phosphatase (RPTP-κ), substantially reduced nuclear translocation.

Tyrosine residues 1148 and 1173, but not 1068, were required for UV irradiation-induced EGFR nuclear translocation. The proximity of these two residues suggests that this region of EGFR is critical for nuclear translocation. Phospho-tyrosines serve as docking sites for proteins that transmit down-stream signal transduction. It is interesting that both phospho-tyrosines 1148 and 1173 bind the adaptor protein Shc [Zwick et al., 1999]. In contrast, tyrosine 1068, which was not found to be critical for UV irradiation-induced EGFR nuclear translocation, binds the adaptor protein Grb2 [Rojas et al., 1996]. These data suggest that Shc may be involved in the mechanism of shuttling EGFR into the nucleus. However, redundancy of protein-binding to specific phospho-tyrosine residues in EGFR makes this interpretation tentative. Interestingly, RPTP-κ, which preferentially dephosphorylates tyrosines 1068, 1148, and 1173, effectively, blocked UV irradiation-induced EGFR nuclear localization. These data provide further support for an important role of phospho-tyrosine 1148 and 1173 in UV irradiation-induced EGFR nuclear translocation.

The biological function of nuclear EGFR remains unclear. Early observations suggested that ligand-induced DNA synthesis required EGFR translocation from the cell surface [Wakshull and Wharton, 1985]. Holt et al. [1994] reported that EGFR translocates into the nucleus, where it associates with chromatin and directly phosphorylates other nuclear proteins. Lin et al. demonstrated that nuclear EGFR is capable of binding to AT-rich consensus DNA sequences and trans-activating the cyclin D1 promoter [Lin et al., 2001]. In addition, nuclear EGFR, which possess tyrosine kinase activity, can phosphorylate and consequently stabilize chromatin-bound proliferating cell nuclear antigen (PCNA), contributing to prolonged cell proliferation [Wang et al., 2006]. These observations raise the possibility that increased localization of EGFR in the nucleus participates in cell cycle regulation. Interestingly, accumulation of nuclear EGFR has been associated with poor prognosis in patients with breast [Lo et al., 2005] and oropharyngeal cancer [Psyrri et al., 2005]. It has been reported that following ligand stimulation, EGFR

translocates to the mitochondria, where it binds cytochrome c oxidase subunit II to positively regulate survival pathways [Boerner et al., 2004]. However, we did not observe mitochrondrial localization of EGFR following UV irradiation in the human keratinocytes.

Ionizing radiation has been shown to induce EGFR nuclear translocation and consequently stimulate DNA repair enzyme DNA-dependent protein kinase (DNA-PK) activity [Ditterman et al., 2005]. Blocking of EGFR nuclear translocation increased radio-sensitivity of irradiated cells. In the same study, the authors showed that inhibition of nuclear export by leptomycin B increased basal nuclear EGFR, but had no effect on ionizing irradiation-induced EGFR nuclear translocation, while radical scavengers such as N-acetyl cysteine inhibited EGFR nuclear translocation. However, whether N-acetyl cysteine inhibited EGFR tyrosine phosphorylation was not investigated.

A putative nuclear localization sequence (NLS) in the cytoplasmic domain of the EGFR (residue 645–657) has been described [Holt et al., 1995]. Fusion of EGFR NLS to β-galactosidase directs the fusion protein to the nucleus [Lin et al., 2001]. Mutation of EGFR NLS impairs interaction between EGFR and importins, while EGF treatment increases the interaction [Lo et al., 2006], implying that EGFR NLS may be buried in non-phosphorylated EGFR. Although we found that UV irradiation-induced EGFR nuclear translocation did not involve nuclear export machinery in human keratinocytes, our data demonstrate that tyrosine phosphorylation of EGFR is required for EGFR nuclear translocation. It is tempting to speculate that tyrosine phosphorylation results in functional exposure of the nuclear targeting sequence, which directs EGFR to the nucleus utilizing conventional nuclear importing machinery. Analyses of the effects of mutating the putative nuclear localization sequence on UV irradiation-induced EGFR nuclear translocation would likely be informative. It has recently been reported that after ligand stimulation, EGFR is trafficked to endoplasmic reticulum (ER), where it associates with Sec61 translocon complex and is retro-translocated from ER to cytoplasm before it is translocated into the cell nucleus [Liao and Carpenter, 2007].

The amount of EGFR translocated to the nucleus in response to UV irradiation was approximately 2% of total cellular EGFR, in HaCaT keratinocytes. While this amount is relatively low, it is likely sufficient to have functional impact by catalyzing phosphorylation of specific nuclear substrates. Interestingly, engagement of only 5% of cell surface EGFR by ligand has been shown to elicit full biological response including inositol phosphate production, release of $Ca2+$ from intracellular stores, induction of c-fos gene expression, and cell morphological change in A431 cells [Defize et al., 1989].

Several other receptor protein tyrosine kinases (RPTKs) such as ErbB2 [Xie and Hung, 1994], ErbB4 [Srinivasan et al., 2000; Ni et al., 2001], growth hormone receptor (GR) [Lobie et al., 1994], nerve growth factor (NGF) [Rakowicz-Szulczynska et al., 1986], platelet-derived growth factor receptor (PDGFR) [Rakowicz-Szulczynska et al., 1986], fibroblast growth factor receptor (FGFR) [Maher, 1996; Stachowiak et al., 1996], and insulin receptor (IR) [Vigneri et al., 1978] have been reported to be present in the nucleus. Interestingly, among them, PDGFR, FGFR, and IR are known to be activated by UV irradiation [Sachsenmaier et al., 1994; Coffer et al., 1995; Huang et al., 1996; Knebel et al., 1996]. Whether UV irradiation stimulates nuclear translocation of these RPTKs, in addition to EGFR, remains to be determined.

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