UV induces tyrosine kinase-independent internalisation and endosome arrest of the EGF receptor

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Summary
We have compared the activation and trafficking of epidermal growth factor receptor (EGFR) induced by UV light and EGF. Tyrosine phosphorylation of EGFR was not detected in UV-exposed cells by immunoblotting of whole cell lysates or EGFR immunoprecipitates with antibodies specific for each of the five activated autophosphorylation sites of EGFR. In addition, EGFR of UV-irradiated cells did not demonstrate increased 32P-incorporation. However, UV-exposed cells demonstrated a gel mobility shift of EGFR, which was not abolished by alkaline phosphatase treatment. UV-exposure did not induce dimerisation of EGFR. Furthermore, UV induced internalisation of EGFR without polyubiquitination or degradation. UV-exposed EGFR was transferred to early endosomes and arrested in transferrin-accessible endosomes close to the cell surface. Whereas inhibition of the EGFR tyrosine kinase effectively inhibited tyrosine phosphorylation and internalisation of EGF-activated EGFR, internalisation of UV-exposed EGFR was unaffected. UV induced neither relocalisation of Shc and Grb2 nor activation of Raf, but activation of MEK and MAPK was observed.

Our work indicates that UV induces internalisation of EGFR independent of its phosphorylation or receptor tyrosine kinase activation, and altered EGFR trafficking compared with ligand-activated receptor. In addition, MAPK activation by UV does not appear to be mediated by EGFR activation.

Key words: UV, EGF receptor, Receptor internalisation, Intracellular trafficking

Introduction
The effects of short wavelength UV radiation on signal transduction have been intensively studied during the past decade. Exposure of mammalian cells to UV results in elevated expression of several growth-associated immediate-early genes, such as c-jun and c-fos (Buscher et al., 1988; Devary et al., 1991), through post-translational modification of several transcription factors, which include AP-1 family members and NF-kB (Stein et al., 1989; Devary et al., 1992; Devary et al., 1993). Involvement of growth factor receptors, Src and members of the Ras-Raf-MEK-MAPK signalling pathway in the mammalian UV response has been reported (Radler-Pohl et al., 1993; Sachsenmaier et al., 1994; Coffer et al., 1995; Rosette and Karin, 1996; Huang, R. P. et al., 1996). A signalling pathway resulting in activation of c-Jun N-terminal kinases (JNKs) has been described after UV-exposure (Derijard et al., 1994; Kyriakis et al., 1994). The mechanism of this UV-mediated activation is not fully understood. It has been suggested that the activation is not a response to DNA damage, but depends upon a signalling cascade starting at the plasma membrane (Devary et al., 1992; Sachsenmaier et al., 1994; Rosette and Karin, 1996).

The epidermal growth factor receptor (EGFR) is dimerised and becomes tyrosine phosphorylated on five tyrosine autophosphorylation sites in response to growth factors (Moghal and Sternberg, 1999). In their phosphorylated state the five cytosolic C-terminal autophosphorylation sites (Y992, Y1068, Y1086, Y1148 and Y1173) (Downward et al., 1984; Margolis et al., 1989; Walton et al., 1990) serve as binding sites for a large group of signal proteins, including Shc and Grb2, which initiate the Ras-Raf-MEK-MAPK signalling pathway (Carpenter, 2000; Moghal and Sternberg, 1999).

Increased EGFR tyrosine phosphorylation after UV-exposure has previously been reported in HeLa and A431 cells (Sachsenmaier et al., 1994), and HER14 and A431 cells (Coffer et al., 1995). Also, UV-induced EGFR complex formation with Shc and Grb2, which initiate the Ras-Raf-MEK-MAPK signalling pathway (Carpenter, 2000; Moghal and Sternberg, 1999). A signalling pathway resulting in activation of c-Jun N-terminal kinases (JNKs) has been described after UV-exposure (Derijard et al., 1994; Kyriakis et al., 1994). The mechanism of this UV-mediated activation is not fully understood. It has been suggested that the activation is not a response to DNA damage, but depends upon a signalling cascade starting at the plasma membrane (Devary et al., 1992; Sachsenmaier et al., 1994; Rosette and Karin, 1996).

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translational modifications of EGFR by direct physical perturbation of the plasma membrane, or by a conformational change caused by absorption of UV energy (Rosette and Karin, 1996; Huang, R. P. et al., 1996).

After growth factor binding to EGFR, the receptor is internalised through clathrin-coated pits, distributed to early endosomes and either recycled back to the cell surface, or transported to late endosomes and lysosomes for degradation. Whereas internalisation of EGFR induced by UV has been reported (Rosette and Karin, 1996), the trafficking of UV-exposed EGFR compared with ligand-activated EGFR has never been analysed in detail. The function of EGFR tyrosine kinase activity in receptor internalisation and routing is still not fully understood. It has been reported that the EGFR kinase activity is required for ligand-induced receptor internalisation (Wiley et al., 1991). However, the role of induced internalisation versus lysosomal targeting for EGFR downregulation is still unclear. Whereas Wiley and co-workers have suggested that receptor degradation is primarily regulated by endocytosis (Wiley et al., 1991), others have proposed that inhibition of receptor recycling is the mechanism that regulates receptor degradation (Felder et al., 1990). It has been suggested that EGFR autophosphorylation results in exposure of trafficking sequences directly (Cadena et al., 1994), or indirectly through formation of a receptor-substrate complex (Wiley et al., 1991; Chang et al., 1993). The aim of this study was to compare EGFr- and UV-induced EGFR activation and trafficking.

Materials and Methods

Materials

Receptor grade EGF from mouse submaxillary glands was purchased from Sigma Chemical Co. (St Louis, MO). Rabbit R42/pY1173 and R46/pY992 antisera, specific to tyrosine phosphorylated EGFR at position Y1173 and Y992, and without crossreactivity with non-phosphorylated receptor has been described elsewhere (Oksvold et al., 2000). Affinity purified rabbit-anti-EGFR phosphotyrosine 1068, 1086 and 1148 were kindly provided by A. J. Wong (Thomas Jefferson University, Philadelphia, PA). A sheep antibody to the intracellular domain of EGFR was obtained from Gibco BRL (Paisley, UK). Rabbit and mouse anti-She and mouse anti-EEA1 were purchased from Transduction Laboratories (Lexington, KY). A mouse anti-LAMP-1 (H4A3) was obtained from the Developmental Studies Hybridoma Bank, University of Iowa (Iowa City, IA). Rabbit anti-Cbl, -Grb2, and -Raf-1 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), and mouse anti-CD63 was obtained from Chemicon International (Temecula, CA). Rabbit anti-phospho-p44/42 MAP kinase (ERK1 and ERK2) was obtained from New England Biolabs (Beverly, MA), and rabbit anti-phospho-MEK and mouse anti-phosphotyrosine (4G10) were purchased from Upstate Biotechnology (Lake Placid, NY). All Cy2- and rhodamine Red-X-conjugated donkey antibodies to mouse, rabbit and sheep IgG were obtained from Molecular Probes (Eugene, OR). Peroxidase-conjugated donkey anti-sheep and mouse IgG were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA). Alexa Fluor 594 donkey anti-sheep IgG and FITC-conjugated human holo-transferrin from serum were purchased from Molecular Probes (Eugene, OR). Peroxidase-conjugated donkey anti-sheep and mouse IgG were obtained from Jackson ImmunoResearch. [32P]Orthophosphate (5 mCi/ml) was purchased from Amersham Pharmacia (Little Chalfont, UK). The EGFR kinase inhibitor PD153035 was obtained from Tocris Cookson (Bristol, UK). The MEK-inhibitor PD98059 was purchased from Calbiochem (La Jolla, CA). All other materials were purchased from Sigma Chemical Co. unless otherwise noted.

Materials

Cells

The human HeLa epidermal carcinoma cell line was obtained from American Tissue Type Collection (Rockville, MD). Cells were maintained in DMEM (BioWhittaker, Walkersville, MD) supplemented with 5% (v/v) fetal calf serum (BioWhittaker), 2 mM L-glutamine and 50-ng/ml gentamycin (Gibco BRL). Cells were grown to 80% confluency in petri dishes or flasks (Costar Corp., Cambridge, MA), and starved in serum-free medium overnight before the start of the experiment. UV exposure of cells was performed in a Vilber Lourmat BLX-254 (Marne La Vallée, France) with 10-200 J/m2 UV 254 nm. The medium was removed before UV irradiation, and replaced before further incubations. Tyrosine phosphorylation of EGFR was induced with EGF (5 nM in Hanks balanced salt solution, pH 7.4 (Gibco BRL)) on ice for 15 minutes. Cells were then washed in ice cold PBS, incubated in pre-warmed medium and chased at 37°C for different time intervals.

Western blot analysis

Cells were lysed in Tris lysis buffer, pH 7.4 (10 mM Tris-HCl, 10% (v/v) glycerol, 2% (w/v) SDS, 5 mM EDTA, 2.5 μg/ml aprotinin, 2.5 μg/ml leupeptin, 1 mM β-glycerophosphate, 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF) and 60 μM sodium orthovanadate). Further sample preparations were performed as described elsewhere (Oksvold et al., 2000). The nitrocellulose membranes were incubated overnight at 4°C with primary antibodies. Peroxidase-conjugated donkey anti-sheep or mouse IgG and goat anti-rabbit IgG were used for detection. All antibodies were diluted in 1% (w/v) nonfat dry milk in TBS containing 0.01% thimerosal. The filters were washed in TBS before detection by the enhanced chemiluminescence (ECL) method with Hyperfilm (Amersham Pharmacia).

Immunoprecipitation

Whole cell lysates (2 mg protein/ml) from UV or EGF-exposed cells were prepared in IP buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 50 mM NaF, 10 mM β-glycerophosphate, 0.5% (v/v) NP-40, 1 mM EDTA, 1 mM orthovanadate, 1 mM DTT, 1 mM PMSF, 1 mM benzamidine, trypsin inhibitor (10 μg/ml), and aprotinin and leupeptin (2.5 μg/ml)). The lysates were passed four times through a 25G needle, and homogenised by 15 strokes in a dounce homogenizer. Sheep anti-EGFR or rabbit anti-Cbl (7 μg) were conjugated to 50 μl protein G-Sepharose (Pharmacia Biotech, Uppsala, Sweden) by incubation for 1 hour at room temperature. The anti-EGFR-protein G-Sepharose conjugate was incubated with 100 μl cell lyses for 2 hours on ice. The pellets were washed three times in IP-buffer, before resuspension in 100 μl Tris lysis buffer containing 2% β-mercaptoethanol and 0.002% bromophenol blue, and heated at 100°C for 5 minutes. Immunoprecipitates were separated by SDS-PAGE, and prepared for autoradiography or processed for immunoblotting as described above.

In vivo labelling of EGFR

Cells were washed with phosphate-free MEM, and incubated in the same buffer for 2 hours at 37°C, before incubation with [32P]Orthophosphate (0.2 mCi/ml) for 2 hours. Cells were exposed to UV (50 or 200 J/m2) or EGF (5 nM) and lysated directly, or incubated at 37°C for 10 minutes. The cells were rinsed in ice cold PBS, followed by lysisation in Tris-lysis buffer containing 2% β-mercaptoethanol and 0.002% bromophenol blue, and heated at 100°C for 5 minutes. Immunoprecipitated with SDS-PAGE, and prepared for autoradiography using a Molecular Imager FX (Bio-Rad, Hercules, CA).
Treatment with alkaline phosphatase
Immunoprecipitated EGFR from unstimulated, UV- or EGF-exposed cells were incubated with 50 diethanolamine (DEA) units of alkaline phosphatase from bovine intestinal mucosa in 150 μl DEA-buffer (1 M DEA, pH 9.8, containing 0.5 mM MgCl₂) at 37°C for 30 minutes.

Crosslinking of the EGFR
Cells were either exposed to UV at 37°C and incubated for 2 minutes, or rinsed in PBS and exposed to UV or EGF in HBSS on ice and further incubated for 15 minutes on ice. Cells were rinsed in PBS, and treated with 3 mM of the nonpermeable crosslinking agent bis(sulphosuccinimidyl) suberate (BS³) (Pierce, Rockford, IL) in PBS for 20 minutes on ice. The reaction was stopped by incubation in PBS with 250 mM glycine for 5 minutes on ice. The cells were washed in PBS with 250 mM glycine, lysed in Tris lysis buffer, and prepared for western immunoblotting as described elsewhere (Oksvold et al., 2000). The lysates were subject to SDS-PAGE and immunoblotting with an antibody to EGFR, and further prepared as described above.

Immunofluorescence staining and microscopy
Cells exposed to UV or EGF were fixed in 4% paraformaldehyde in PBS for 20 minutes at room temperature and prepared for immunocytochemistry as described elsewhere (Oksvold et al., 2000). Combinations of Cy2 and rhodamine Red-X-conjugated donkey antibodies monospecific for IgG of the appropriate species were used. The samples were mounted with coverslips using Dako fluorescent mounting kit (Dako, Carpinteria, CA). The cells were examined with a Leica TCS SP confocal microscope (Leica, Heidelberg, Germany) equipped with an Ar (488 nm) and two He/Ne (543 and 633 nm) lasers. A Plan apochromat 100x/1.4 oil objective was used. Images from multi-labeled cells were acquired sequentially.

Results
Effects of UV-exposure on EGFR phosphorylation
It has previously been reported that UV induced EGFR tyrosine phosphorylation in HeLa and A431 cells (Sachsenmaier et al., 1994) and A431 and HER14 cells (Coffer et al., 1995). To examine this effect, whole cell lysates and EGFR immunoprecipitates from HeLa cells exposed to UV were analysed by SDS-PAGE and immunoblotting. Antibodies specific for the five different EGFR autophosphorylation sites in their activated state (pY992, pY1068, pY1086, pY1148 and pY1173) were used. UV-induced tyrosine phosphorylation was not detected for any of the five EGFR autophosphorylation sites in whole cell lysates or in EGFR immunoprecipitates (Fig. 1A and B, respectively). By contrast, all five EGFR autophosphorylation sites were strongly phosphorylated in cells incubated with 5 nM EGF (Fig. 1A,B). Immunoblotting analysis with the anti-phosphotyrosine antibodies 4G10 and PY20 revealed similar results (Fig. 1A, pTyr, and data not shown, respectively). Anti-EGFR antibodies were used to show that the levels of EGFR were similar in the different cell lysates and immunoprecipitates (Fig. 1A,B, EGFR).

To verify that UV-exposure did not induce an increased tyrosine phosphorylation of the EGFR, we examined the total phosphorylation of EGFR by in vivo labelling with 32P orthophosphate. Immunoprecipitated EGFR from UV-irradiated cells was analysed by SDS-PAGE and autoradiography. No increased phosphorylation was detected in the EGFR immunoprecipitates from cells exposed to 50-200 J/m² UV and homogenised immediately, or after 10 minutes incubation (Fig. 2A). By contrast, EGFR immunoprecipitated from EGF-stimulated cells showed increased phosphorylation.

Western immunoblotting analysis of the 32P-labelled EGFR immunoprecipitates from UV- and EGF-treated cells with anti-EGFR confirmed similar levels of EGFR in the different immunoprecipitates (Fig. 2B).

Also, two other stress factors and their effects on EGFR tyrosine phosphorylation were examined. Both hydrogen peroxide and heat shock induced tyrosine phosphorylation of the EGFR, as revealed by western immunoblotting of whole cell lysates (data not shown).

The results from HeLa cells were reproduced in A431 and PC12 cells, and primary cultures of hepatocytes from Sprague-

![Fig. 1](image-url) UV-exposed cells did not show increased EGFR tyrosine phosphorylation. Tyrosine phosphorylation was analysed in whole cell lysates (A) and EGFR immunoprecipitates (B) subject to SDS-PAGE and western immunoblotting with rabbit anti-pY992, pY1068, pY1086, pY1148 and pY1173. Cells were either left untreated or exposed to the indicated UV-doses and chased for the indicated time intervals (minutes). The whole cell lysates (A) were also analysed for tyrosine phosphorylation with a general anti-phosphotyrosine antibody (4G10; pTyr). The total levels of EGFR, determined with anti-EGFR antibodies, are shown (bottom line). Cells stimulated with 5 nM EGF for 5 minutes were used as a positive control. C and E denote control and EGF-stimulated cells, respectively, and I denotes J/m². The molecular weight markers presented at the left indicate 220 kDa.
Dawley rats (data not shown). In addition, EGF dose-response studies showed that 0.05 nM EGF induced EGFR tyrosine phosphorylation detectable by immunoblotting (data not shown).

EGFR from UV-exposed cells is not dimerised and displays an electrophoretic mobility shift associated with receptor internalisation without degradation

To examine whether UV-exposure induced EGFR dimerisation, we used the chemical crosslinking agent BS3 to preserve dimerised receptors (Sorkin and Carpenter, 1991). UV- or EGF-treated cells were incubated with BS3 before lysis of the cells. The cell lysates were analysed by SDS-PAGE and western immunoblotting with antibody to EGFR. In cells stimulated with 5 nM EGF for 5 minutes were used as positive controls. C and E denote control and EGF-stimulated cells, respectively. The molecular weight markers presented at the left indicate 220 kDa.

UV-exposed cells did not reveal increased phosphate incorporation in the EGF receptor. In vivo labelling of cells were performed by preincubation of cells with phosphate-free medium (MEM) for 2 hours, followed by incubation with $[^{32}P]$ orthophosphate in DMEM for 2 hours. Cells were then left untreated or exposed to UV or EGF, and incubated for the indicated time intervals. Cell lysates were subject to immunoprecipitation with an anti-EGFR-protein G-sepharose conjugate. The EGFR immunoprecipitates were separated by SDS-PAGE, and identified by (A) autoradiography and (B) western immunoblotting with anti-EGFR. Cells stimulated with 5 nM EGFR for 5 minutes were used as positive controls. C and E denote control and EGF-stimulated cells, respectively. The molecular weight markers presented at the left indicate 220 kDa.

Fig. 2. UV-exposed cells did not reveal increased phosphate incorporation in the EGF receptor. In vivo labelling of cells were performed by preincubation of cells with phosphate-free medium (MEM) for 2 hours, followed by incubation with $[^{32}P]$ orthophosphate in DMEM for 2 hours. Cells were then left untreated or exposed to UV or EGF, and incubated for the indicated time intervals. Cell lysates were subject to immunoprecipitation with an anti-EGFR-protein G-sepharose conjugate. The EGFR immunoprecipitates were separated by SDS-PAGE, and identified by (A) autoradiography and (B) western immunoblotting with anti-EGFR. Cells stimulated with 5 nM EGFR for 5 minutes were used as positive controls. C and E denote control and EGF-stimulated cells, respectively. The molecular weight markers presented at the left indicate 220 kDa.

UV-irradiation did not induce dimerisation of the EGFR. Cells were left untreated, or exposed to 50 J/m² UV (lanes 1,2), 200 J/m² UV (lanes 3,4), or EGF (10 nM on ice for 15 minutes). The UV-exposed cells were either irradiated at 37°C and incubated for 2 minutes at the same temperature (lanes 1,3), or irradiated on ice and further incubated on ice for 15 minutes (lanes 2,4). All samples were incubated with the crosslinker BS3 on ice for 20 minutes. Cell lysates were subject to SDS-PAGE and western immunoblotting with anti-EGFR. The band with molecular mass around 340 kDa representing dimeric EGFR is indicated (arrow). C and E denote control and EGF-stimulated cells, respectively. The molecular weight marker presented at the left indicates 220 kDa.

Fig. 3. UV-irradiation did not induce dimerisation of the EGFR. Cells were left untreated, or exposed to 50 J/m² UV (lanes 1,2), 200 J/m² UV (lanes 3,4), or EGF (10 nM on ice for 15 minutes). The UV-exposed cells were either irradiated at 37°C and incubated for 2 minutes at the same temperature (lanes 1,3), or irradiated on ice and further incubated on ice for 15 minutes (lanes 2,4). All samples were incubated with the crosslinker BS3 on ice for 20 minutes. Cell lysates were subject to SDS-PAGE and western immunoblotting with anti-EGFR. The band with molecular mass around 340 kDa representing dimeric EGFR is indicated (arrow). C and E denote control and EGF-stimulated cells, respectively. The molecular weight marker presented at the left indicates 220 kDa.

UV-induced EGFR mobility shift was not an immediate event since it was not observed less than 1 minute after UV-treatment. The EGFR gel mobility shift was seen 5 minutes after irradiation, and was present even 2 hours after exposure (Fig. 4A). In addition, while degradation of the EGFR was observed in EGF-stimulated cells incubated for 2 hours, EGFR-degradation was not detected in UV-exposed cells (Fig. 4A).

To exclude the possibility that the UV-induced electrophoretic mobility shift of EGFR was due to phosphorylation not detectable by immunoblotting and ³²P-labelling methods, we treated UV- or EGF-exposed EGFR with alkaline phosphatase. Incubations of EGFR-immunoprecipitates from EGF-stimulated cells with alkaline phosphatase effectively dephosphorylated the receptor (Fig. 4B, pY1173). However, the UV-induced EGFR mobility shift in SDS-PAGE was still present after treatment with alkaline phosphatase (Fig. 4B, EGFR).

Polyubiquitination, in addition to other functions, is a signal for degradation by the 26S proteasome (Dubiel and Gordon, 1999). Since polyubiquitination leads to electrophoretic mobility shift of targeted proteins, we compared EGFR polyubiquitination in UV- and EGF-exposed cells. Whole cell lysates from EGF- and UV-treated cells incubated for the indicated time intervals were analysed by western immunoblotting with anti-EGFR. In western immunoblots, polyubiquitination can be detected by gel retardation observed as smears, as previously shown in HeLa cells (Stang et al., 2000). EGF-stimulated EGFR appeared polyubiquitinated from 1-10 minutes after incubation at 37°C (Fig. 5A). In comparison, no ubiquitination was observed in cells exposed to 200 J/m² UV (Fig. 5A). It has previously been shown
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that EGFR polyubiquitination is dependent of tyrosine phosphorylation (Levkowitz et al., 1998).

To further compare EGFR ubiquitination in UV- and EGF-exposed cells, we studied the product of the proto-oncogene c-Cbl, which binds activated protein tyrosine kinases such as EGFR, and regulates their polyubiquitination (Waterman et al., 1999). Increased tyrosine phosphorylation of Cbl was not observed in Cbl-immunoprecipitates from cells exposed to UV (Fig. 5B). In addition, complex formation between EGFR and Cbl was not observed (Fig. 5B). Cbl-immunoprecipitates from EGF-treated cells showed increased Cbl tyrosine phosphorylation, and complex formation between tyrosine phosphorylated EGFR and Cbl (Fig. 5B).

It has previously been described that, similar to EGF, UV induces internalisation of the EGFR (Rosette and Karin, 1996). We therefore studied the effects of UV on EGFR distribution. Whereas EGFR in unstimulated cells are located in the plasma membrane, we have previously described that the activated receptor was located in EEA1-positive early endosomes 10 minutes after EGF stimulation (Oksvold et al., 2000). In unstimulated cells EGFR was located in the plasma membrane, as seen with immunofluorescence confocal microscopy (Fig. 6A). We did not observe internalisation of EGFR in cells exposed to 10 J/m² UV and incubated for 10 minutes (data...
not shown). When irradiated with 50 J/m², some EGFR internalisation was seen (data not shown). After exposure to 200 J/m², an extensive receptor internalisation comparable with that seen after EGF-stimulation was observed (Fig. 6B). The internalised EGFR was located to early endosomes, as shown by co-localisation with EEA1 (Fig. 6B-D).

To exclude the possibility that the observed UV-induced internalisation of EGFR was due to a general increased inward membrane flow, we compared the liquid phase endocytosis of horseradish peroxidase (HRP) in unstimulated and UV-exposed cells. We observed a decrease in the internalisation of HRP in UV-exposed cells compared with that in unstimulated cells (data not shown).

We have earlier reported that EGFR was relocated to CD63 and LAMP-1-positive late endosomes in EGF-stimulated HeLa cells incubated for 20 minutes (Oksvold et al., 2001). EGFR was not detected in CD63-positive compartments of UV-exposed cells incubated for the same time interval (Fig. 7A-C). UV-exposed cells incubated for 20-120 minutes showed no co-localisation of EGFR and the early (EEA1) and late (CD63 and LAMP-1) endosome markers (data not shown). To determine whether the compartments containing internalised EGFR 60-120 minutes after UV-exposure were part of the endocytic pathway, we studied the accessibility of these compartments for transferrin. Transferrin bound to the transferrin receptor is transported from the cell surface to early endosomes, from where it recycles back to the cell surface (Lok and Loh, 1998). In UV-irradiated cells chased for 60 minutes and co-incubated with FITC-labeled transferrin (50 μg/ml) the last 20 minutes, EGFR was located in transferrin-positive vesicles (Fig. 7D-F). Aggregates of compartments containing EGFR and transferrin were observed close to the cell surface (Fig. 7D-F, arrow). These aggregates were observed in the majority of the cells studied (data not shown). In EGF-stimulated cells incubated for 60-120 minutes, little or no EGFR was located in vesicles (data not shown). This finding was in agreement with our previous result, which showed EGF-induced degradation of EGFR, but little or no degradation in UV-exposed cells (Fig. 4A).

It has earlier been suggested that the reported UV-induced activation of EGFR is due to production of ROS (Huang, R. P. et al., 1996). We therefore studied the effects of ROS scavengers on the UV-induced EGFR gel mobility shift and internalisation. Treatment with the ROS scavenger NAC (10 mM) inhibited H₂O₂-induced tyrosine phosphorylation of the receptor, observed by western blotting with anti-pY1173 (data not shown). However, incubation with NAC for 15 minutes to 12 hours before exposure to UV showed minor effects on both the EGFR gel mobility shift and receptor internalisation (data not shown). These results were confirmed by use of two other known ROS scavengers, α-tocopherol and L-ascorbic acid (data not shown).

UV-induced EGFR internalisation is independent of receptor tyrosine kinase activity

It has previously been reported that EGFR kinase activity is required for ligand-induced sequestration of receptors into coated pits (Lamaze and Schmid, 1995) and for EGFR internalisation (Wiley et al., 1991). In order to test whether UV-induced EGFR internalisation was dependent on receptor tyrosine kinase activity, we used the specific EGFR tyrosine kinase inhibitor PD153035 (Kunkel et al., 1996) prior to UV exposure. Incubation with 100 nM PD153035 for 2 hours effectively inhibited EGF-induced EGFR tyrosine phosphorylation (Fig. 8, upper panel). The EGF-induced internalisation of the receptor was also inhibited in cells incubated with PD153035, as shown by immunofluorescence confocal microscopy (Fig. 8A-C). In UV-exposed cells, inhibition of the EGFR tyrosine kinase with PD153035 had no effect on the internalisation of EGFR (Fig. 8D). Our result showed that UV-induced EGFR internalisation was independent of the receptor tyrosine kinase activity.

EGF-EGFR complexes are internalised by clathrin-coated pit-mediated endocytosis. To examine whether UV-irradiated EGFR follows the same pathway, we used the cholesterol-extracting drug β-cyclodextrin to inhibit clathrin-coated pit-mediated endocytosis. Recently it was reported that β-cyclodextrin treatment strongly inhibited endocytosis of transferrin and EGF in HEp-2 and other cell lines (Rodal et al., 1999). Incubation with 15 mM β-cyclodextrin revealed strong
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Inhibition of both EGF-mediated EGFR internalisation, and UV-induced EGFR internalisation (data not shown). We also studied the effects of β-cyclodextrin on the UV-induced EGFR mobility-shift in SDS-PAGE. The UV-induced electrophoretic retardation of EGFR was similar with and without β-cyclodextrin, suggesting that the receptor mobility shift was not dependent on internalisation (data not shown).

Effects of UV on signal proteins downstream of EGFR

To examine the effects of UV-exposure on intracellular signal transduction downstream of EGFR, we studied the effects of UV on the activation of the signalling proteins Shc, Grb2, Raf, MEK and MAPK. The adaptor proteins Shc and Grb2 bind to the activated tyrosine autophosphorylation sites on the EGFR after growth factor activation, and initiates the Ras-Raf-MEK-MAPK signalling cascade. In unstimulated cells, Shc and Grb2 were redistributed to vesicles after UV exposure. Cells were either left untreated (A and B), stimulated with 5 nM EGF for 10 minutes (C and D), or exposed to 200 J/m² UV (E and F). Immunofluorescence confocal microscopy with anti-Shc (A, C, E) and anti-Grb2 (B, D, F) was used to study the distribution of the adaptor proteins. In unstimulated cells, Shc and Grb2 showed a diffuse cytosolic distribution (A and B, respectively). In cells stimulated with EGF for 10 minutes, Shc and Grb2 were redistributed to vesicles (C and D, respectively). In cells exposed to 200 J/m² UV and incubated for 10 minutes, no redistribution of Shc and Grb2 was observed (E and F, respectively). Bar, 10 μM.
Discussion

Cellular stress including UV-exposure induces cellular responses through activation of several cytoplasmic kinases. The involvement of plasma membrane receptors in this signalling pathway has been investigated by several groups. Previous reports of UV-induced EGFR tyrosine phosphorylation are not conclusive with respect to the level of receptor phosphorylation and phosphorylation kinetics. Sachsenmaier and co-workers reported a low and short-lived EGFR tyrosine phosphorylation, compared with EGF-induced phosphorylation (Sachsenmaier et al., 1994). When A431 and HeLa cells were pretreated with high doses of the protein phosphatase inhibitor vanadate, tyrosine phosphorylation was detectable 0.2 minutes after UV-exposure, and was not detectable at later time intervals. In HC11 cells, a similar, low UV-induced EGF receptor tyrosine phosphorylation was detected 5 minutes after irradiation, and not after longer incubation intervals (Huang, R. P. et al., 1996).

By contrast, Coffer and co-workers found EGFR tyrosine phosphorylation levels similar or even stronger than that observed after incubations with relatively high doses of EGF (Coffer et al., 1995). In this work, the receptor tyrosine phosphorylation was still present 1 hour after UV-exposure. In all these studies, UV-induced EGFR tyrosine phosphorylation was demonstrated in cells exposed to 30-200 J/m² UVC by western blotting of EGFR immunoprecipitates using anti-phosphotyrosine antibodies. However, dose-response studies with UV have not been performed or were not shown. In our attempt to analyse the reported UV-induced EGFR tyrosine phosphorylation, we were unable to detect increased receptor tyrosine phosphorylation in whole cell lysates or EGFR immunoprecipitates using antibodies specific to the five different activated EGFR autophosphorylation sites. In addition, we used two different anti-phosphotyrosine antibodies, including the PY20 antibody that was earlier used to report UV-induced phosphorylation. We have no explanation...
for the discrepancy between our work and the previously reported UV-induced EGFR tyrosine phosphorylation. It cannot be explained by differences in antibody sensitivity, since the antibodies to the EGFR autophosphorylation sites appeared to be more sensitive than the general anti-phosphotyrosine antibody in western immunoblotting analyses (data not shown). We also failed to detect UV-induced EGFR tyrosine phosphorylation in A431 and PC12 cells, and primary cultures of rat hepatocytes. Therefore, the discrepancy appeared not to be due to cell-type-specific factors. One explanation could be that other EGFR family members are activated by UV, although dimer formations were not detected by the crosslinking assay.

We applied 10-200 J/m² UVC 254 nm, similar to that used in previous studies reporting UV-induced EGFR tyrosine phosphorylation. Our finding that UV-exposure did not induce EGFR tyrosine phosphorylation was supported by a set of further observations: (1) UV did not induce increased incorporation of $[^{32}\text{P}]$; (2) treatment of UV-exposed EGFR immunoprecipitates with alkaline phosphatase did not eliminate the UV-induced mobility shift in SDS-PAGE; (3) UV-exposure did not induce EGFR tyrosine kinase-dependent polyubiquitination of EGFR; (4) Inhibition of the EGFR tyrosine kinase revealed no effect on the UV-induced receptor gel mobility shift and internalisation of EGFR; (5) UV-exposure did not induce redistribution of Shc and Grb2 to vesicles containing EGFR, indicating lack of specific receptor phosphotyrosine-containing Shc and Grb2 binding sites.

It has previously been suggested that direct absorption of UV energy induces an EGFR conformational change (Rosette and Karin, 1996; Huang, R. P. et al., 1996). We found by dose-response studies that there was a correlation between increasing UV-doses and induced EGFR gel mobility shift. This observation suggested either a UV-induced receptor conformational change, or a post-translational modification of EGFR, directly or via other signal proteins. Although a conformation alteration is not necessarily detectable by SDS electrophoresis, the fact that the UV-induced mobility shift for EGFR was intact under reduced conditions by western immunoblotting analysis indicated the involvement of an unknown post-translational modification. Huang and co-workers have recently suggested that ROS are involved in the UV-mediated activation of EGFR (Huang, R. P. et al., 1996). We were not able to inhibit the UV-induced EGFR gel mobility-shift and receptor internalisation by treatment with ROS scavengers. We therefore find it unlikely that ROS have an important role in the UV-induced modification and internalisation of EGFR.

Our finding that EGFR was internalised without phoshorylation and independent of receptor tyrosine kinase activity was surprising. The UV-induced EGFR gel mobility shift may serve as a signal for internalisation, directly or indirectly through other signal mediators. It has been reported earlier that EGF binding induces a conformational change in the external domain of its receptor (Greenfield et al., 1989). Opresko and co-workers have suggested that a conformational change in the EGFR is important for ligand-induced internalisation of the receptor (Opresko et al., 1995). It is possible that an EGFR modification induced by UV resembles the receptor modification seen after ligand binding. This was supported by our finding that treatment of EGFR immunoprecipitates from EGF-stimulated cells with alkaline phosphatase eliminated tyrosine phosphorylation, but not the EGFR mobility shift. In addition, inhibition of clathrin-coated pit-mediated endocytosis with β-cyclodextrin showed no inhibitory effect on the EGFR mobility shift in SDS-PAGE. This supports our hypothesis that the UV-induced EGFR modification observed as a receptor gel mobility shift, in some way mimics an EGF-mediated internalisation signal.

Whereas polyubiquitination did not seem to be involved in UV-induced EGFR internalisation, there is a possibility that monoubiquitination played a role. We failed to detect monoubiquitination by immuno-coprecipitation studies. However, it is generally difficult to detect monoubiquitin, and for that reason we cannot exclude the possible involvement of monoubiquitination. The findings that a single ubiquitin can serve as a signal for sorting to the degradative pathway (Urbanowski and Piper, 2001), do not support a role for monoubiquitination in UV-induced internalisation of EGFR.

We have shown that UV-exposed EGFR was internalised independently of receptor dimerisation and its receptor tyrosine kinase activity. The involvement of the EGFR tyrosine kinase for receptor internalisation and intracellular sorting has been intensively investigated in the past years. Whereas receptor kinase activity has been shown to be required for ligand-activated internalisation of EGFR (Chen et al., 1989; Lund et al., 1990; Wiley et al., 1991; Lamaze and Schmid, 1995), its role in intracellular receptor trafficking is still unclear. One hypothesis is that endosomal sorting is controlled by tyrosine kinase activity, and that the EGFR tyrosine kinase is necessary for trafficking to lysosomes for degradation (Honegger et al., 1987; Honegger et al., 1990; Felder et al., 1990; Felder et al., 1992; Futter et al., 1993). Felder and co-workers found that the distribution to internal vesicles of multivesicular bodies is inhibited in EGFR with a mutated tyrosine kinase, suggesting that the receptor tyrosine kinase is important for trafficking to lysosomes for degradation (Felder et al., 1990). Others have found that tyrosine kinase activity is not crucial for lysosomal targeting (Sorkin et al., 1991; Helin and Beguinot, 1991; Sorkin and Waters, 1993; Wiley et al., 1991; Lamaze et al., 1993; French et al., 1994; Herbst et al., 1994; Opresko et al., 1995). These studies support the model originally introduced by Linderman and Lauffenburger in which selective sorting occurs by endosomal retention (Linderman and Lauffenburger, 1988). Recently, a leucine-based lysosomal sorting signal was found in the EGFR (Kil et al., 1999).

Whereas UV-exposed EGFR internalisation was similar to ligand-mediated internalisation, the receptor trafficking in UV-irradiated cells was altered. We did not observe degradation of EGFR in UV-exposed cells. The receptor was not transported from early endosomes to late endosomes and lysosomes, but instead redistributed to compartments accessible to transferrin, where it was arrested. We presume that these compartments were recycling vesicles. We have two possible explanations for the altered receptor trafficking in UV-exposed cells: (1) lysosomal targeting of EGFR is dependent on an active receptor tyrosine kinase, or intact tyrosine phosphorylated autophosphorylations sites; (2) the endocytic transport machinery is in some way abrogated by UV-irradiation.

It has been suggested that UV-induced activation of MEK and MAPK mimics the ligand-induced signalling pathway that
initiates from EGFR (Huang, R. P. et al., 1996). Our results reveal that the UV-mediated activation of MEK and MAPK occurs independently of tyrosine phosphorylated EGFR and receptor binding of Shc and Grb2. Other studies yield support to our findings. Migliaccio and co-workers reported UV-induced gel mobility shift for p66 Shc, without tyrosine phosphorylation (Migliaccio et al., 1999). Furthermore, it has been shown that UV-induced AP-1 activation does not require EGFR (Huang, C. et al., 1996).

In conclusion, our work demonstrated that the UV-induced internalisation of EGFR was independent of its receptor tyrosine kinase. The trafficking of UV-exposed EGFR was altered compared with ligand-activated receptor, and the internalised receptor was not directed to lysosomes for degradation. In addition, the UV-induced activation of MEK and MAPK was independent of an active EGFR tyrosine kinase.

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