We have previously shown that, although overexpression of mutant dynamin inhibits clathrin-dependent endocytosis and disrupts high affinity binding of epidermal growth factor (EGF) to the EGF receptor (EGFR), it does not inhibit ligand-induced translocation of the EGFR into clathrin-coated pits. In the present study, we demonstrate that, upon ligand binding and incubation at 37 °C, the EGFR was polyubiquitinated regardless of overexpression of mutant dynamin. In cells not overexpressing mutant dynamin, the EGFR was rapidly internalized and deubiquitinated. In cells being endocytosis-deficient, due to overexpression of mutant dynamin, however, the EGFR was upon prolonged chase first found in deeply invaginated coated pits, and then eventually moved out of the coated pits and back onto the smooth plasma membrane. Polyubiquitination occurred equally efficiently in cells with or without intact clathrin-dependent endocytosis, while the kinetics of ubiquitination and deubiquitination was somewhat different. We further found that the EGF-induced ubiquitination of Eps15 occurred both in the absence and presence of endocytosis with the same kinetics as polyubiquitination of the EGFR, but that the EGF-induced monoubiquitination of Eps15 was somewhat reduced upon overexpression of mutant dynamin. Our data show that EGF-induced polyubiquitination of the EGFR occurs at the plasma membrane.

Binding of epidermal growth factor (EGF)1 to its receptor at the plasma membrane results in activation and autophosphorylation of the EGF receptor (EGFR), as well as phosphorylation and activation of other molecules (1). Activation of EGFR thereby initiates signal transduction cascades important for cellular growth and differentiation. Furthermore, binding of EGF mediates translocation of the EGFR to clathrin-coated pits, internalization of the EGFR via clathrin-coated vesicles, transport through endosomes, and eventually degradation in lysosomes (2–4). Activation of the EGFR by ligand binding at 37 °C was also demonstrated to induce polyubiquitination of the EGFR (5). No EGF-stimulated EGFR ubiquitination was found in experiments using a mutant kinase-negative EGFR or in experiments where clathrin-dependent endocytosis was prevented by low temperature or K+ depletion (5). This suggested a functional coupling between clathrin-dependent endocytosis and ubiquitination of the EGFR.

Ubiquitination has been shown to be essential for endocytosis of the yeast α-factor receptor (6) and for the growth hormone receptor (GHR) (7). In cells with a temperature-sensitive defect in ubiquitin conjugation, neither growth hormone (GH)-dependent internalization nor GH-dependent degradation of the GHR was observed at the non-permissive temperature (7). Further studies have demonstrated that when the internalization of the GHR was inhibited, GHR ubiquitination was also inhibited (8). These results also imply a connection between ubiquitin conjugation and endocytosis.

It has so far been unclear whether ubiquitination occurs prior to or following the budding of clathrin-coated vesicles. In a recent study, Levkowitz et al. (9) proposed that polyubiquitination of the EGFR occurs in endosomes. This was, however, not directly demonstrated. Instead, the authors demonstrated increased EGFR polyubiquitination and increased EGFR down-regulation by the overexpression of c-Cbl in Chinese hamster ovary cells. They furthermore demonstrated complex formation between c-Cbl and the EGFR upon incubation with EGF in cells overexpressing both c-Cbl and EGFR. Additionally, they showed that c-Cbl and the EGFR colocalized in endosomes upon addition of EGF. These data strongly support the recently suggested role for c-Cbl in the process of EGFR polyubiquitination (10, 11), but do not clarify where in the cell ubiquitination occurs. It has been proposed by others that ubiquitination caused endocytosis of GHR by directing the GHR into clathrin-coated pits (8).

We have previously shown that EGF stimulation of the EGFR induced a rapid relocation of EGFR from the smooth plasma membrane into clathrin-coated pits in cells being endocytosis-deficient due to overexpression of the GTPase-deficient mutant K44A form of dynamin (12). Clathrin-coated pits do not bud in cells overexpressing this mutant form of dynamin (13), and we took advantage of this system to study the trafficking, tyrosine phosphorylation, and ubiquitination of the EGF-stimulated EGFR in the absence of clathrin-dependent endocytosis. We found that in cells overexpressing K44A dynamin the EGFR transiently localized to coated pits, but that hardly any EGF-EGFR complexes were internalized. Nevertheless, polyubiquitination occurred as efficiently in endocytosis-deficient cells as in cells where clathrin-dependent endocytosis took place in a normal fashion. Our data therefore demonstrate for the first time that ubiquitination of the EGFR occurs at the plasma membrane prior to endocytosis.

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Espen Stang‡, Lene E. Johannessen§, Sigrun L. Knardal, and Inger Helene Madshus
From the Institute of Pathology, University of Oslo, National Hospital, 0207 Oslo, Norway

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‡ Supported by a research fellowship from the Norwegian Cancer Society. To whom all correspondence should be addressed. E-mail: espens@ulrik.uio.no.

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1 The abbreviations used are: EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; GH, growth hormone; GHR, growth hormone receptor; PBS, phosphate-buffered saline; PMSF, phenylmethylsulfonyl fluoride; SAB, sodium acetate buffer with 0.5 M NaCl; PAGE, polyacrylamide gel electrophoresis.
EXPERIMENTAL PROCEDURES

Materials—Human recombinant EGF was from Bachem Feinchemikalien AG (Budendorf, Switzerland). All reagents were from Sigma unless otherwise noted.

Cell Culture—HeLa cells transfected with K44A mutant dynamin (13) were a kind gift from Sandra L. Schmid ( Scripps Research Institute, La Jolla, CA). The cells were grown in Dulbecco’s modified Eagle’s medium (Gibco-BRL, Life Technologies, Inc., Paisley, United Kingdom), 200 ng/ml puromycin, 2 mM L-glutamine (BioWhittaker, Walkersville, MD) containing 400 μg/ml Geneticin (Life Technologies, Inc., Paisley, United Kingdom), 200 ng/ml porcine insulin, 2 mM L-glutamine (BioWhittaker, Walkersville, MD) containing 400 μg/ml Geneticin (Life Technologies, Inc., Paisley, United Kingdom), 200 ng/ml puromycin, 2 mM L-glutamine (BioWhittaker, Walkersville, MD), and 1% penicillin-streptomycin (70-756, BioWhittaker) supplemented with 10% (v/v) fetal bovine serum (BioWhittaker). Cells were seeded at a density of 15,000 cells/cm² and grown for 24 h at 37 °C in the presence (uninduced) or absence (induced) of 1 μg/ml doxycycline for 15 min on ice, washed with ice-cold phosphate-buffered saline (PBS), and chased in prewarmed EGF-free medium at 37 °C. At the end of the chase period, the cells were fixed, and immunoprecipitation was performed essentially as described by Strous et al. (7). The cells were lysed in boiling buffer, containing 1% (w/v) SDS in PBS. After 15 min at 100 °C, the lysates were sonicated and homogenized by QIAshredder columns (Qiagen GmbH, Hilden, Germany). Then protein A-agarose (Amersham Pharmacia Biotech) was incubated with sheep anti-EGF antibody for 1 h at room temperature. The complexed antibody was washed twice in the buffer used for immunoprecipitation, and immunoprecipitation was performed at 4 °C for 60 min. In the experiment demonstrating ubiquitination of the EGF, the immunoprecipitation was performed essentially as described by Strous et al. (7).

Antibodies—The following antibodies were used: sheep anti-EGF (Life Technologies, Inc., catalog no. 13287-016); rabbit anti-conjugated ubiquitin (Sigma); rabbit anti-Eps15 (C terminus) (Berkeley Antibody Company, Berkeley, CA); mouse anti-phosphotyrosine (Upstate Biotechnology, Inc., Lake Placid, NY); peroxidase-conjugated donkey anti-mouse IgG (Sigma); peroxidase-conjugated goat anti-rabbit IgG, peroxidase-conjugated donkey anti-sheep-IgG, and alkaline phosphatase-conjugated goat anti-rabbit antibodies (Jackson Immunoresearch Laboratories Inc., West Grove, PA); and phosphatase-conjugated anti-mouse antibodies (Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom).

Immunoelectron Microscopy—Cells were incubated with 10−8 M EGF for 15 min on ice, washed with ice-cold phosphate-buffered saline (PBS) (140 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 2 mM NaH2PO4, pH 7.4), and chased in prewarmed EGF-free medium at 37 °C. At the end of the chase period, the cells were washed with ice-cold PBS, fixed with 4% paraformaldehyde, 0.1% glutaraldehyde in Soerensen’s phosphate buffer, and processed as described (14). A thin section of the tissue was processed for immunoelectron microscopy. From each experiment micrographs from at least 10 randomly chosen cells were examined, and a minimum of 100 gold particles were counted. The percentage of distribution of gold particles at the plasma membrane were counted and sorted into two groups depending on whether the gold particle was associated with clathrin-coated pits or with smooth plasma membrane areas. Labeling along the tube connecting coated pits with the rest of the plasma membrane was interpreted as labeling of non-coated plasma membrane. From each experiment micrographs from at least 10 randomly chosen cells were examined, and a minimum of 100 gold particles were counted. The percentage of distribution of gold particles to coated pits was calculated as (no. of gold particles on coated plasma membrane)/total no. of gold particles at the plasma membrane × 100.

Labeling for Eps15 was performed using rabbit anti-Eps15 antibodies followed by protein A-coated colloidal gold (purchased from G. Posthuma and J. Slot, Utrecht, Holland).

Brefeldin A Experiments—HeLa cells were preincubated with brefeldin A (17, 18) (5 μg/ml) on ice for 15 min before the cells were washed with ice-cold PBS, and chased in prewarmed EGF-free medium at 37 °C. At the end of the chase period, the cells were washed three times with ice-cold PBS to remove unbound ligand. The cells were then chased in minimal essential medium without HCO3− and with 0.1% (w/v) bovine serum albumin at 37 °C for the indicated time period. The medium was collected, and the 125I-EGF was precipitated using 5% (w/v) trichloroacetic acid and 1% (w/v) metaphosphoric acid. The precipitate was dissolved with 1M NaOH, and the radioactivity was measured in a γ-counter. Cells were washed three times with PBS, treated with 0.2 mM sodium acetate buffer (pH 4.5 or 7.4) containing 0.5 mM NaCl (SAB pH 4.5/SAB pH 7.4) on ice for 10 min, and washed once with the same buffer. Then 125I-EGF was precipitated from the cells with trichloroacetic acid and 1% (w/v) phosphotungstic acid. Finally, the precipitate was dissolved in 1% NaOH, and the radioactivity was measured. Counts/min from cells treated with SAB pH 4.5 represent internalized 125I-EGF, while counts/min from cells treated with SAB pH 7.5 represent both internalized and surface-localized 125I-EGF.

RESULTS

Approximately 50% of Initially EGF-bound EGF Localize to Coated Pits in a Transient Manner—Overexpression of K44A dynamin has been shown to inhibit clathrin-mediated endocytosis (13). We have recently shown that although the overexpression of K44A dynamin disrupts high affinity binding of EGF to the EGFR, the EGFR is efficiently recruited to coated pits in a transient manner (12). Due to overexpression of K44A dynamin, coated pits were often found at the end of long tubular plasma membrane invaginations, and sometimes gathered in groups. In our previous study we only examined cells chased for 2 min at 37 °C. As coated pits do not bud and form coated vesicles in cells overexpressing K44A dynamin, we wanted to examine the trafficking of the EGF upon extended chase periods at 37 °C. EGF (10−8 M) was added to cells on ice for 15 min, the cells were washed free of unbound ligand and chased for increasing time periods at 37 °C. At the end of the chase period, the cells were fixed, sectioned, and immunocytochemically labeled using antibodies as described above before incubation with alkaline phosphatase-conjugated anti-mouse antibodies or alkaline phosphatase-conjugated anti-rabbit antibodies for 2 h at room temperature. The immunobinding was detected by the enhanced chemiluminescence method (Amersham Pharmacia Biotech), and the chemiluminescence was measured by a phosphorimager (STORM840, Molecular Dynamics, Sunnyvale, CA). Due to dispersion of the EGF caused by ubiquitination, both the main band and the smear above the main band were quantified. When membranes were incubated with anti-ubiquitin antibody, 3% (w/v) gelatin in Tris-buffered saline containing 1% (w/v) Tween 20 was used as blocking reagent, and the immunobinding was detected by enhanced chemiluminescence using an ECL Plus kit (Amersham Pharmacia Biotech).

Immunoprecipitation—When immunoprecipitating the EGF to determine the EGF phosphorylation, the cells were lysed in immunoprecipitation buffer (PBS with 10 mM EDTA, 1% (w/v) Triton X-100, 10 mM NaF, 200 units/ml aprotinin, 1 mM PMSF, and 1 mM Na3VO4). Then protein G-agarose (Amersham Pharmacia Biotech) was incubated with sheep anti-EGF antibody for 1 h at room temperature. The complexed antibody was washed twice in the buffer used for immunoprecipitation, and immunoprecipitation was performed at 4 °C for 60 min. In the experiment demonstrating ubiquitination of the EGF, the immunoprecipitation was performed essentially as described by Strous et al. (7). The cells were lysed in boiling buffer, containing 1% (w/v) SDS in PBS. After 15 min at 100 °C, the lysates were sonicated and homogenized by QIAshredder columns (Qiagen GmbH, Hilden, Germany). Then protein A-agarose and protein G-agarose (Amersham Pharmacia Biotech) were incubated with rabbit anti-ubiquitin antibody and sheep anti-EGF antibody, respectively, for 1 h at room temperature. The complexed antibody was washed twice in the immunoprecipitation buffer, consisting of 1% (w/v) Triton X-100, 0.5% (w/v) SDS, 0.25% (w/v) sodium deoxycholate, 0.5% (w/v) bovine serum albumin, 1 mM EDTA, 1 mM PMSF, 2 mM Na3VO4, 20 mM NaF, 10 μg/ml leupeptin, and 200 units/ml aprotinin in PBS, and immunoprecipitation was performed at 4 °C for 60 min. The immunoprecipitate was washed and subjected to SDS-PAGE and Western blotting, as described above.

125I-EGF-Cell Interaction Experiments—125I-EGF (10−8 M; Amersham Pharmacia Biotech) was added to the cells grown in minimal essential medium (Life Technologies, Inc.) without HCO3−. After 15 min at 4 °C, the cells were washed three times with ice-cold PBS to remove unbound ligand. The cells were then chased in minimal essential medium without HCO3− and with 0.1% (w/v) bovine serum albumin at 37 °C for the indicated time periods. The medium was collected, and the 125I-EGF was precipitated using 5% (w/v) trichloroacetic acid and 1% (w/v) phosphotungstic acid as described (22). Both the trichloroacetic acid-precipitable and the trichloroacetic acid-soluble radioactivity were measured in a γ-counter. Cells were washed three times with PBS, treated with 0.2 mM sodium acetate buffer (pH 4.5 or 7.4) containing 0.5 mM NaCl (SAB pH 4.5/SAB pH 7.4) on ice for 10 min, and washed once with the same buffer. Then 125I-EGF was precipitated from the cells with trichloroacetic acid and 1% (w/v) phosphotungstic acid. Finally, the precipitate was dissolved in 1% NaOH, and the radioactivity was measured. Counts/min from cells treated with SAB pH 4.5 represent internalized 125I-EGF, while counts/min from cells treated with SAB pH 7.5 represent both internalized and surface-localized 125I-EGF.
EGFR Ubiquitination Occurs at the Plasma Membrane

TABLE I

EGF-induced redistribution of EGFR to coated pits in cells overexpressing mutant dynamin

The distribution of the EGFR to coated versus non-coated areas of the plasma membrane in cells overexpressing mutant (K44A) dynamin was quantified as described under “Experimental Procedures.” The distribution of EGFR was determined in cells after binding of EGF on ice and chase at 37 °C in EGF-free medium (Pulse-chase) and in cells exposed to brefeldin A prior to, as well as during, a pulse-chase incubation (Brefeldin A). The table shows the percentage of total plasma membrane-associated EGFR labeling, that under each experimental condition localized to coated membrane areas. The control column shows the percentage amount of EGFR localized to coated pits in cells prior to incubation with EGF. The 0, 2, 5, 10, 15, 30 and 60 min columns indicate the percentage amount of EGFR in coated pits upon these times of incubation at 37 °C. Labeling along the tube connecting the coated pits with the uninvaginated plasma membrane was interpreted as labeling of non-coated plasma membrane. Results are presented as the mean ± S.E. from a minimum of three independent experiments.

|                    | Control | 0 min | 2 min | 5 min | 10 min | 15 min | 30 min | 60 min |
|--------------------|---------|-------|-------|-------|--------|--------|--------|--------|
| Pulse-chase        | 13 ± 2  | 11 ± 2| 34 ± 3| 40 ± 4| 49 ± 2 | 47 ± 3 | 30 ± 3 | 19 ± 1 |
| Brefeldin A        | 12 ± 2  | 11 ± 2| 11 ± 3| 24 ± 2| 41 ± 3 | 40 ± 1 | 12 ± 1 | 10 ± 3 |

ies recognizing the EGFR. To estimate the EGF-induced redistribution of the EGFR, we quantified the amount of EGFR localizing to coated pits compared with the total amount of EGFR found at the plasma membrane.

Immunocytochemical labeling showed that in cells not exposed to EGF, as well as in cells fixed immediately after binding of EGF on ice, the EGFR mainly localized outside coated pits (Table I and Fig. 1A). However, upon chasing at 37 °C, the EGFR was seen to relocalize rapidly from non-coated, uninvaginated plasma membrane areas into areas where the plasma membrane had a clear cytoplasmic coat (Fig. 1B). At early time points (2–5 min) (Table I and Fig. 1B), several of these coated areas were flat or only slightly invaginated. After a longer chase period (10–15 min) (Table I and Fig. 1C), however, most of the coat-associated labeling was seen in coated pits connected to the plasma membrane by long tubular necks. After 10–15 min of chase, almost 50% of the EGFRs were seen in coated pits (Table I). The labeling was usually found associated with the coated, bulb-shaped blind end of the tubules, and it should be noted that almost no labeling was found along the tubular neck. Upon further chase, however, the EGFR started to appear along the tube while the bulb-shaped end remained coated and the number of EGFR associated with coated areas decreased (Table I and Fig. 1, D and E). Upon 60 min of chase at 37 °C, the distribution of plasma membrane-associated EGFR was almost as in cells not exposed to EGF (Table I).

Although the antibodies used recognize the intracellular part of the EGFR, labeling was in some cases found on the extracellular side of the plasma membrane (Fig. 1). Due to the size of the primary antibody recognizing the EGFR and the secondary antibody coating the colloidal gold, the observed distance from the gold particle to the antigen may be more than the width of a membrane (16). The specificity of the anti-EGFR antibody was confirmed by Western blotting and was further reflected by the EGF-induced change in labeling distribution. The colloidal gold contained a small amount of doublets (see Fig. 1D), but when quantifying, eventual clusters were counted as one gold particle.

Labeling of sections showed that independent of EGF binding and chase at 37 °C, some EGFR localized to the Golgi apparatus (data not shown). This labeling most likely represents newly synthesized EGFR. As including newly synthesized EGFR appearing at the plasma membrane during the chase period could complicate the study of ligand-induced trafficking of the EGFR, we treated cells with brefeldin A before addition of EGF as well as during the chase period. Brefeldin A prevents the transport of newly synthesized proteins from the endoplasmic reticulum to the plasma membrane by redistributing the cis and medial stacks of the Golgi apparatus back into the endoplasmic reticulum (17, 18). Quantification of labeling on sections from brefeldin A-treated cells showed basically the same EGF-induced EGFR redistribution and transient localization to coated pits as found in cells not exposed to brefeldin A (see Table I). We conclude that, in cells deficient in endocytosis due to overexpression of K44A dynamin, EGFR moves to coated pits upon binding of EGF. The localization to clathrin-coated pits is transient, and the EGFR seems to lose its association with coat components and move back onto smooth, uninvaginated parts of the plasma membrane.

Internalization of EGF-EGFR Complexes Is Efficiently Inhibited in Cells Overexpressing Mutant Dynamin—To study the ligand-dependent redistribution of EGFR quantitatively, both with and without overexpression of mutant dynamin, we incubated cells with 10−8 M 125I-EGF on ice for 15 min before washing away unbound ligand and chasing the cells at 37 °C. At different time points, the fate of the initially bound 125I-EGF was analyzed as described under “Experimental Procedures.” The results show that the majority of the initially bound 125I-EGF in cells overexpressing K44A dynamin rapidly dissociated from the EGFR. Of the 125I-EGF that remained cell-associated, only a small amount was endocytosed, and eventually degraded (Fig. 2A). This is consistent with the findings of Vieira et al. (23). The inhibition of EGFR endocytosis was further confirmed by immunocytochemical labeling for the EGFR. Quantification of labeling on sections from cells exposed to brefeldin A showed that the EGFR labeling density at the plasma membrane remained unchanged for up to 60 min of chase at 37 °C (data not shown). In cells grown with tetracycline (not overexpressing mutant dynamin), 125I-EGF was rapidly endocytosed, with most ligand internalized upon 10 min of chase at 37 °C (Fig. 2B).

Autophosphorylated EGFR Is Dephosphorylated upon Entry into Coated Pits and Re phosphorylated upon Endocytosis—Tyrosine-phosphorylated and dimerized EGFR have previously been shown to exist in endosomes (24). In order to correlate the tyrosine phosphorylation of the EGFR with its cellular localization, we incubated cells with 10−8 M EGF on ice for 15 min before washing away unbound ligand and chasing the cells at 37 °C. Incubation with EGF on ice caused tyrosine phosphorylation of the EGFR both in noninduced cells and in cells overexpressing K44A dynamin (Fig. 3A). However, the EGFR was less efficiently tyrosine-phosphorylated in cells overexpressing K44A dynamin, compared with cells not expressing mutant dynamin (Refs. 12 and 23; Fig. 3B). It should be noted that, upon overexpression of mutant dynamin, more EGFR were found at the plasma membrane (12). We have demonstrated the specific tyrosine phosphorylation of the EGFR (Fig. 3B) by normalizing the intensity of the phosphotyrosine signal with respect to the EGFR signal both in cells with and without overexpression of mutant dynamin. Chase at 37 °C caused a rapid dephosphorylation of the EGFR in both induced and noninduced cells. Once dephosphorylated, the EGFR in cells overexpressing K44A dynamin remained dephosphorylated. The kinetics of the dephosphorylation coincided with both entry of EGFR into coated pits and dissociation of ligand. In cells not over-
expressing mutant dynamin, however, some of the EGFRs were transiently rephosphorylated upon prolonged chase at 37 °C (Fig. 3, A and B; 8, 10, and 12 min). When considering the kinetics of trafficking and phosphorylation, the rephosphorylation observed in cells not overexpressing mutant dynamin seemed to occur upon the EGFR reaching endosomes.

Polyubiquitination of the EGFR Occurs at the Plasma Membrane—When Western blotting with the anti-EGFR antibody, we found that the mobility of the EGFR was shifted as a function of ligand binding and chase at 37 °C, and by overexposing the film, a smear was clearly visible (Fig. 4A). As the Western blotting was performed subsequent to SDS-PAGE, this indicated that the decrease in mobility was caused by a covalent modification of the EGFR. It has previously been reported that the EGFR can be polyubiquitinated (5). We therefore investigated whether the decreased mobility of the protein, reactive to both anti-phosphotyrosine antibody (data not shown) and anti-EGFR antibody, was due to polyubiquitination by immunoprecipitating with an antibody to ubiquitin and Western blotting the immunoprecipitate, using an antibody to the EGFR. As demonstrated in Fig. 4B, the slowly migrating EGFR was indeed ubiquitinated. We further immunoprecipitated EGFR and Western-blotted the immunoprecipitate employing an antibody to conjugated ubiquitin. By this procedure, bands with the same apparent molecular weight were visualized (data not shown). As demonstrated in Fig. 4, the EGFR in cells not overexpressing mutant dynamin was clearly polyubiquitinated upon 2, 5, and 10 min of chase at 37 °C. However, in cells that were endocytosis-deficient due to overexpression of mutant dynamin, there was a clear polyubiquitination of the EGFR even upon 15 min of chase. In cells not overexpressing mutant dynamin, a large fraction of the EGFR was observed to be endocytosed upon 10 min of chase at 37 °C (Ref. 12 and Fig. 2). As demonstrated in Fig. 4, at this time point, the EGFR had started to become deubiquitinated. In conclusion, a transient polyubiquitination of the EGFR was observed upon ligand binding at 4 °C, followed by chase at 37 °C, and the transient polyubiquitination was observed regardless of EGFR endocytosis, demonstrating that the polyubiquitination of the EGFR happened at the plasma membrane. The polyubiquitination of the EGFR furthermore appeared to precede endocytosis of the EGFR (compare Figs. 2 and 4).

EGF Causes a Transient Ubiquitination of Eps15—Activation of EGFR has previously been shown to induce phosphorylation as well as monoubiquitination of the coated pit localized protein Eps15 (25–27). Following incubation of cells with EGF on ice and chase at 37 °C, we found a rapidly induced shift in Eps15 mobility upon SDS-PAGE and Western blotting with antibody to Eps15 (Fig. 5). EGF induced the same shift in mobility both in cells with and without overexpression of mutant dynamin. However, more Eps15 was shifted in cells that did not overexpress mutant dynamin. The shift in Eps15 mobility has been shown previously to represent monoubiquitination of Eps15 (27). It should be noted that, in cells overexpressing mutant dynamin, Eps15 was slightly ubiquitinated even in the absence of EGF (compare controls with and without tet, Fig. 5). While the shift in Eps15 mobility was reversed upon 10 min of chase at 37 °C in cells without overexpression of mutant dynamin, Eps15 remained ubiquitinated for 30 min in cells with deficient clathrin-dependent endocytosis due to overexpression of mutant dynamin (Fig. 5). This demonstrated that ubiquitination of Eps15 is also transient and followed the same kinetics as ubiquitination and coated pit localization of the EGFR. To investigate whether ubiquitination affected the localization of Eps15, we labeled the sections with antibody to Eps15. The labeling for Eps15 was restricted to the rim of the coated area even on coated pits attached to the K44A dynamin-induced tubules both in the absence of EGF and upon binding of EGF followed by 30 min of chase at 37 °C (Fig. 6).
FIG. 2. Distribution of 125I-EGF in HeLa cells with (A) or without (B) overexpression of mutant (K44A) dynamin. 125I-EGF (10 ng) was added to the cells on ice. After binding on ice for 15 min, the cells were washed and chased at 37 °C for the time periods indicated. The cells were treated with low pH buffer to release EGFR-bound surface-localized 125I-EGF. The cells were analyzed for surface-bound (○) and intracellular (●) 125I-EGF, and the medium was analyzed for released 125I-EGF (△) and degraded 125I-EGF (▽) as described under “Experimental Procedures.” Each point in panel A represents the mean of three independent experiments with S.E. indicated, while in panel B one representative experiment is shown.

DISCUSSION

We have demonstrated that, both in HeLa cells where endocytosis of the EGFR is inhibited due to overexpression of the K44A mutant dynamin and in HeLa cells with intact EGFR endocytosis, the EGFR became polyubiquitinated when the cells were chased at 37 °C following binding of EGF at 4 °C. At 4 °C the EGFR was maximally autophosphorylated, and during chase at 37 °C the EGFR rapidly became dephosphorylated. Ubiquitination was initiated by EGF binding, but was delayed with respect to EGFR autophosphorylation. The lag could indicate that ubiquitination follows phosphorylation of an EGFR substrate or, alternatively, that docking of a molecule onto the EGFR with ensuing complex formation is required for ubiquitination to occur. Interestingly, we found that the EGFR substrate Eps15 was ubiquitinated with the same kinetics as was the EGFR. In yeast, genetic interaction between the Eps15 homologue, Pan1p, and the ubiquitin-protein ligase Rsp5p has been demonstrated, and it was suggested that Pan1p could act as a connector bringing Rsp5p close to potential ubiquitination substrates (28). There is therefore the possibility that Eps15 plays a similar role in mammalian cells, and via an ubiquitin-protein ligase induces ubiquitination of membrane proteins like the EGFR. It was recently shown that c-Cbl may act as an ubiquitin-protein ligase (29) and that c-Cbl, synergistically with the ubiquitin-conjugating enzyme UbcH7, promoted ligand-induced ubiquitination of the EGFR (9–11). c-Cbl has previously been shown to become tyrosine-phosphorylated almost immediately upon EGF stimulation (30), and stimulation with colony-stimulating factor-1 caused a transient phosphorylation, membrane targeting, and ubiquitination of c-Cbl (31). c-Cbl was found to be associated with Crk upon stimulation with EGF (30). Crk has an Src homology 3 domain, which specifically binds to a proline-rich motif in Eps15 (32), further suggesting a role for Eps15 in induction of ubiquitination.

It was recently proposed that polyubiquitination of the EGFR occurs in endosomes (9). However, this hypothesis has not been confirmed, and, up to now, it has been unclear whether ubiquitination occurs before or after internalization of the EGFR. In the recent paper by Levkowitz et al. (9), the authors claimed that they were unable to detect ubiquitinated EGFR on the cell surface by using biotin labeling. However, due to the many technical difficulties in performing such experiments, a negative result does not necessarily mean that ubiquitination of the EGFR does not occur at the plasma membrane. Our present experiments demonstrated that EGF-EGFR complexes were not internalized in cells overexpressing mutant dynamin. However, EGF-stimulated EGFR were clearly polyubiquitinated. Our present results therefore show that polyubiquitination of the EGFR occurs at the plasma membrane. We cannot completely rule out the possibility that the EGFR can also be ubiquitinated in endosomes. However, the magnitude of the EGFR ubiquitination was comparable in cells with or without endocytosis. Furthermore, in cells not overexpressing mutant K44A dynamin, the ubiquitination appeared to precede endocytosis, and we observed no additional waves of ubiquitination during the 60-min chase following ligand binding. Interestingly, our unpublished data show that c-Cbl, believed to be instrumental in the ubiquitination of the EGFR (9–11), was maximally tyrosine-phosphorylated by EGF on ice regardless of overexpression of mutant dynamin.

In yeast, polyubiquitination serves as an internalization signal for the α-factor receptor and the α-factor receptor (6, 33). In mammalian cells, several tyrosine kinase or kinase-linked receptors are ubiquitinated upon ligand binding (reviewed in Ref. 34). Binding of GH to the GHR induces dimerization, activation and increased ubiquitination of the receptor. Using cells with a temperature-sensitive defect in ubiquitination status of EGFRs localizing to coated pits. Therefore it is required in order to conclude as to the ubiquitination.
vents clathrin-dependent receptor-mediated endocytosis, it does not inhibit the recruitment of receptors into coated pits. Both the constitutive transport of Tfn-R and the ligand-induced transport of EGFR into coated pits appear to occur as in normal cells (12, 13). In the present study, we demonstrate that, in HeLa cells that are endocytosis-deficient due to overexpression of K44A dynamin, the EGF-induced localization of EGFR to clathrin-coated pits was transient. Whether binding of EGF causes the EGFR to move into coated pits before or after assembly of clathrin has been debated (36–38). In cells overexpressing K44A dynamin, invaginated clathrin-coated pits accumulate at the expense of flat clathrin-coated membrane (13).

**FIG. 3.** EGF-dependent EGFR phosphotyrosine content in HeLa cells with (○) or without (●) overexpression of mutant (K44A) dynamin. EGF (10^{-8} M) was added to the cells on ice. After binding on ice for 15 min, the cells were washed and chased at 37 °C in EGF-free medium for the time periods indicated. A, the EGFR was isolated by immunoprecipitation and analyzed by Western blotting using an antibody to phosphotyrosine (α-pTyr) or to the EGFR (α-EGFR). B, the intensity of tyrosine-phosphorylated EGFR and of the EGFR was quantified as described under “Experimental Procedures.” The intensity of the phosphorylated EGFR was normalized with respect to the intensity of the EGFR, and the specific tyrosine phosphorylation of EGFR is therefore demonstrated. The data represent the mean of seven independent experiments, except the values at 8 and 12 min, which represent the means of three and four independent measurements, respectively.

**FIG. 4.** Time-dependent polyubiquitination of the EGFR in HeLa cells without or with overexpression of mutant (K44A) dynamin. EGF (10^{-8} M) was added to the cells on ice. After binding on ice for 15 min, the cells were washed and chased at 37 °C for the times indicated. The cells were then lysed as described under “Experimental Procedures.” A, the cell lysates were subjected to SDS-PAGE and Western blotting with an antibody to the EGFR, as described under “Experimental Procedures.” In order to demonstrate the mobility shift of the EGFR, the film was overexposed. B, the cell lysates were subjected to immunoprecipitation using an antibody to conjugated ubiquitin as described under “Experimental Procedures.” The immunoprecipitate was then subjected to SDS-PAGE and Western blotting with an anti-EGFR antibody.
FIG. 5. Time-dependent ubiquitination of Eps15 in HeLa cells without or with overexpression of mutant (K44A) dynamin. EGF (10⁻⁸ M) was added to the cells on ice. After binding on ice for 15 min, the cells were washed, chased at 37 °C for the times indicated, and lysed as described under “Experimental Procedures.” The lysates were subjected to SDS-PAGE and Western blotting, using an antibody to Eps15, as described under “Experimental Procedures.”

This is probably due to a limited cytoplasmic pool of coat components, and a consequence of this could be that new coated pits are prevented from forming. However, we observed coated pits at all stages of invagination, indicating that at least some new assembly of coated pits occurs. After short chase periods, the EGFR localized to flat, or only slightly invaginated, coated areas, but not to fully invaginated coated pits, or along the membrane tubes connecting these pits with the plasma membrane. This suggests that activated EGFR move into growing clathrin-coated pits. It was shown recently that EGF-induced activation of EGFR stimulated Src kinase activation with ensuing phosphorylation and redistribution of clathrin (39). EGF-induced recruitment of clathrin to the plasma membrane is consistent with the suggestion that the EGFR moves into growing coated pits and may explain why only 50% of EGFR were recruited to coated pits in endocytosis-deficient cells. This could be due to lack of clathrin and other molecules important for recruiting the EGFR into coated pits, possibly because of accumulation in preexisting coated pits.

In cells overexpressing K44A dynamin, the dephosphorylation of the EGFR correlated in time with dissociation of EGF. However, we also observed dephosphorylation in cells that did not overexpress mutant dynamin, where the EGFR was somewhat more firmly associated with the EGFR via high affinity binding. In cells exhibiting normal endocytosis, the dephosphorylation was, however, transient. The EGFR appeared to be dephosphorylated when localized to coated pits, but some receptors were dephosphorylated upon endocytosis. As dephosphorylation of EGFR in endosomes could be the result of both homo- and heterodimerization, the efficiency of endocytosis of other members of the ErbB family of proteins is expected to affect the specificity of signaling from endosomes. This could explain how EGFR localization to the plasma membrane or to endosomes results in qualitative and quantitative differences of signal transduction, as recently described (19, 40). Consistent with this notion, EGFR phosphorylation has been shown to be qualitatively different in endosomes, as compared with at the plasma membrane (41). Also consistent with our findings is the reportedly decreased EGF-induced tyrosine phosphorylation of the EGFR in cells deficient in endocytosis due to overexpression of K44A dynamin (23).

In conclusion, we have demonstrated that the EGFR was transiently localized to coated pits in endocytosis-deficient cells, where EGF-EGFR complexes were not internalized. Furthermore, we have shown that endocytosed, ligand-bound EGFR became dephosphorylated in endosomes, and that independently of endocytosis, the EGFR was polyubiquitinated at the plasma membrane in a transient manner.

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Polyubiquitination of the Epidermal Growth Factor Receptor Occurs at the Plasma Membrane upon Ligand-induced Activation

Espen Stang, Lene E. Johannessen, Sigrun L. Knardal and Inger Helene Madshus

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