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# Activation of the Epidermal Growth Factor (EGF) Receptor Induces Formation of EGF Receptor- and Grb2-Containing Clathrin-Coated Pits

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**In HeLa cells depleted of adaptor protein 2 complex (AP2) by small interfering RNA (siRNA) to the μ2 or α subunit or by transient overexpression of an AP2 sequestering mutant of Eps15, endocytosis of the transferrin receptor (TfR) was strongly inhibited. However, epidermal growth factor (EGF)-induced endocytosis of the EGF receptor (EGFR) was inhibited only in cells where the α subunit had been knocked down. By immunoelectron microscopy, we found that in AP2-depleted cells, the number of clathrin-coated pits was strongly reduced. When such cells were incubated with EGF, new coated pits were formed. These contained EGF, EGFR, clathrin, and Grb2 but not the TfR. The induced coated pits contained the α subunit, but labeling density was reduced compared to control cells. Induction of clathrin-coated pits required EGFR kinase activity. Overexpression of Grb2 with inactivating point mutations in N- or C-terminal SH3 domains or in both SH3 domains inhibited EGF-induced formation of coated pits efficiently, even though Grb2 SH3 mutations did not block activation of mitogen-activated protein kinase (MAPK) or phosphatidylinositol 3-kinase (PI3K). Our data demonstrate that EGFR-induced signaling and Grb2 are essential for formation of clathrin-coated pits accommodating the EGFR, while activation of MAPK and PI3K is not required.**

Activation of the epidermal growth factor receptor (EGFR) initiates signal transduction important for gene expression. Additionally, ligand-binding results in localization of the EGFR to clathrin-coated pits, from where the EGFR is endocytosed at physiological concentrations of EGF (21, 42). It has previously been demonstrated that there are distinct saturable components involved in clathrin-mediated endocytosis (40) and that saturation of the endocytic pathway for the transferrin (Tf) receptor (TfR) does not affect the endocytosis of the EGFR (39). Consistently, our recent data demonstrated that even though EGFR and TfR to some extent colocalized in clathrin-coated pits, more EGFR was found in TfR-negative coated pits than in TfR-positive coated pits (36). This finding could potentially support the contention that clathrin-dependent endocytosis of the EGFR is independent of adaptor protein 2 (AP2), in contrast to clathrin-dependent endocytosis of the TfR (8, 22). The idea of specialized coated pits has been proposed by Cao et al. (7), and it is becoming increasingly clear that specific interactions between recruited coat subunits and cargo proteins direct the incorporation of cargo into budding vesicles and that adaptor molecules other than AP2 can interact with phosphatidylinositol (PI) (4,5), $P_2$ and clathrin (1). This highlights the idea of potential EGFR-specific adaptor molecules in clathrin-coated pits.

The demonstration that nerve growth factor (NGF) increased the number of coated pits at the plasma membrane of sympathetic neurons (9) and that NGF signaled through tyrosine kinase receptor A to increase clathrin at the plasma membrane and in this way enhanced clathrin-mediated membrane trafficking (2) could argue that NGF would induce formation of new clathrin-coated pits at the plasma membrane. It was also demonstrated that ligand-binding to the EGFR, the T cell receptor, and the B cell receptor through Src-induced phosphorylation of clathrin influenced the distribution of clathrin at the plasma membrane (10, 37, 41). Induced formation of coated pits at the plasma membrane was recently demonstrated in the case of viruses, where influenza viruses taking the clathrin pathway were demonstrated to enter cells via de novo formation of clathrin-coated pits at sites of virus binding (30).

We have recently demonstrated that overexpression of Grb2 with inactivating point mutations in both SH3 domains (d.n.Grb2), as well as overexpression of human Sprouty 2, inhibiting the enzymatic activity of Cbl, inhibited the EGF-induced transport of the EGFR to clathrin-coated pits. Furthermore, we demonstrated that ubiquitin binding proteins played a role in recruiting the activated EGFR to clathrin-coated pits. We thus concluded that a macromolecular complex containing Grb2 and Cbl as well as ubiquitin-ubiquitin-interacting motif interactions played a role in recruitment of activated EGFR to clathrin-coated pits (36). We have extended studies of localization of the EGFR to clathrin-coated pits, and we now demonstrate that upon efficient knock-down of AP2, endocytosis of the EGFR was partly inhibited, while endocytosis of the TfR was blocked. Upon sequestering of functional AP2 or small interfering RNA (siRNA)-mediated knock-down of AP2, EGF was found to induce formation of a large number of new clathrin-coated pits. Formation of these coated pits depended on the EGFR kinase activity and was blocked by d.n.Grb2, as well as by Grb2, with single point mutations in either of the SH3 domains. This confirmed the

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requirement for Grb2. The TfR was excluded from EGF-induced coated pits, even though the new coated pits still contained AP2, with a labeling density inside coated pits approximately half that in control cells. The new coated pits further contained Grb2 but not Eps15. Altogether, these data suggest that upon EGF-induced signal transduction, AP2 and clathrin form EGFR-containing coated pits. Our findings further suggest that the requirement for AP2 is mechanistically different in endocytosis of the TfR compared to in endocytosis of the EGFR.

## MATERIALS AND METHODS

**Materials.** Human recombinant EGF was from Bachem AG. $Na^{125}I$ and $^{125}I$-EGF were from Amersham Biosciences. Rhodamine (Rh)-conjugated EGF (Rh-EGF), Rh-conjugated Tf (Rh-Tf), Alexa 633-conjugated Tf (Alexa 633-Tf) and Alexa 488-conjugated EGF (Alexa 488-EGF) were from Molecular Probes, Inc. FuGene was from Roche Diagnostics. Other chemicals were from Sigma-Aldrich unless otherwise noted.

**Cell culture and treatment.** HeLa cells were grown in Dulbecco's modified Eagle's medium (3.7 g/liter sodium bicarbonate) (BioWittaker) containing 1× penicillin-streptomycin mixture (17-603; BioWittaker) and 2 mM L-glutamine (BioWittaker) and supplemented with 5% (vol/vol) fetal bovine serum (FBS) (PAA Innovations). Porcine aortic endothelial (PAE) cells (provided by Carl-Henrik Heldin, The Ludwig Institute, Uppsala, Sweden) were grown in Ham's F-12 (BioWhittaker) medium supplemented with 10% (vol/vol) FBS and 1× penicillin-streptomycin mixture. PAE.B2 cells (PAE cells stably transfected with human wild-type EGFR) (provided by Alexander Sorkin, University of Colorado Health Sciences Center, Denver, CO) were grown in Ham's F-12 medium supplemented with 10% (vol/vol) FBS, 1× penicillin-streptomycin mixture, and 400 μg/ml G418 sulfate (Invitrogen). The cells were plated at a density of 15,000 cells/cm$^2$ 48 h prior to experiments. For inhibition of EGFR kinase activity, Src kinase activity, PI 3-kinase (PI3K) activity, or MEK activity, cells were preincubated with PD153035 (100 nM) (Tocris Cookson, Inc.) for 1 h at 37°C, SU6656 (2 μM) (Calbiochem, Merck Biosciences) for 1 h at 37°C, wortmannin (500 nM) for 10 min at 37°C, or PD98059 (50 μM) (Tocris Cookson, Inc.) for 1 h at 37°C, respectively, in Eagle minimal essential medium (MEM) without $HCO_3^-$ (Gibco/ Invitrogen Corporation) before the experiments were carried out in the presence of the inhibitors.

**Plasmids and transient transfection of cells.** Enhancend green fluorescent protein (EGFP)-tagged Δ95/295-Eps15 (pEGFP-EH95, where EH95 refers to the construct Δ95/295-Eps15 lacking Eps15 homology [EH] domains 2 and 3) was provided by Alexandre Benmerah (Institut Cochin, Paris, France). Wild-type and kinase-negative (K721A) EGFR in the pRK5 vector was provided by Andrew Chantry (University of East Anglia, Norwich, United Kingdom). pEGFP-C2 was from BD Biosciences. Myc-tagged d.n.Grb2 (W36, 193K; nonfunctional N- and C-terminal SH3 domains) (pRK5Myc-d.n.Grb2) has been described previously (36). Plasmids encoding wild-type Grb2, N-SH3 Grb2 (W36K; nonfunctional N-terminal SH3 domain) and SH2 Grb2 (R86K; nonfunctional SH2 domain) were provided by Robin M. Scaife (University of Western Australia, Nedlands, Australia). Wild-type Grb2, N-SH3 Grb2, and SH2 Grb2 were amplified by PCR, and Myc fusion proteins of these Grb2 constructs were constructed by subcloning into a pRK5Myc vector provided by Alan Hall (UCL, London, United Kingdom). Myc-tagged C-SH3 Grb2 (G203R; nonfunctional C-terminal SH3 domain) was constructed by site-directed mutagenesis of Myc-tagged wild-type Grb2, using a QuikChange XL kit (Stratagene) according to the manufacturer's procedures. HeLa, PAE, and PAE.B2 cells were transiently transfected with plasmids 24 h prior to experiments using FuGene in accordance with procedures provided by the manufacturer. Transfection efficiency was approximately 30% for HeLa cells, 30% for PAE cells, and 15% for PAE.B2 cells, as estimated by counting fluorescing cells either directly (pEGFP-EH95) or upon immunocytochemical labeling either for the Myc tag (Grb2 constructs) using mouse anti-Myc antibody or for the EGFR using sheep anti-EGFR antibody. When HeLa cells were transfected with two different plasmids, approximately 25% of the cells were found to be cotransfected.

**siRNA knockdown of μ2- and α-adaptin.** HeLa cells were treated twice with siRNA directed against μ2-adaptin (target sequence, AAGUGGAUGCCUUU CGGGUCA) or α-adaptin (target sequence, AAGAGCAUGUGCACGCUGG CCA) essentially as described by Motley et al. (22). Due to problems with detaching cells, the following changes were made to the protocol: $1 \times 10^6$ cells were seeded in a T75 flask the day before the first transfection. Four to six hours after each transfection, the transfection solution containing siRNA was replaced with ordinary growth medium without antibiotics. Twenty-four hours after the last transfection, the cells were plated for experiments at a density of 30,000 cells/cm$^2$. All siRNAs including negative scrambled control siRNA (#4613) were synthesized and annealed by Ambion. When immunofluorescence was performed on siRNA-treated cells, siRNA was conjugated to Cy3 using a Silencer siRNA Labeling Kit-Cy3 (Ambion) according to manufacturer's procedures. Transfection efficiency was approximately 100% as measured by immunofluorescence of Cy3-conjugated siRNA.

**Antibodies.** Mouse anti-α-adaptin antibody, rabbit anti-Grb2 antibody, rabbit anti-EGF antibody, and mouse anti-EGFR (sc-120) antibody were from Santa Cruz Biotechnology, Inc. Sheep anti-EGFR antibody was from Fitzgerald. Rabbit anti-clathrin light chain antibody was a gift from Frances Brodsky, University of California, San Francisco, Calif. Mouse anti-clathrin heavy chain, anti-β-adaptin, anti-μ2-adaptin, anti-Grb2, and anti-early endosome antigen 1 antibodies were from BD Biosciences. Mouse anti-Myc antibody was from the 9E10 hybridoma (11). Rabbit anti-Myc antibody and rabbit anti-GFP antibodies were from Abcam Ltd. Mouse anti-EGFR (Ab-3) antibody was from Neomarkers, and rabbit anti-human TfR antibody was from HybriDomus. Rabbit anti-phospho p44/p42 mitogen-activated protein kinase (MAPK) (Thr 202/Thr 204) and rabbit anti-phospho Akt (Ser 473) antibodies were from Cell Signaling Technology. Rabbit anti-Eps15 antibody was from BAbCO. Peroxidase-conjugated anti-mouse immunoglobulin G (IgG) and peroxidase-conjugated anti-rabbit IgG antibodies were from Jackson ImmunoResearch Laboratories, Inc. Rabbit anti-mouse IgG antibody was from Cappel, ICN Biomedicals.

**Immunocytochemistry and confocal microscopy.** HeLa cells were plated on 12-mm coverslips in 24-well microtiter plates and treated as described in the legends of the figures. The cells were either incubated with Rh-Tf (20 μg/ml), Alexa 633-Tf (20 μg/ml), Alexa 488-EGF (2.4 nM), or Rh-EGF (2.4 nM) in MEM without $HCO_3^-$ containing 0.1% (wt/vol) bovine serum albumin (BSA) for 15 min at 37°C; 2.4 nM EGF corresponds to 15 ng/ml EGF. The cells were then washed three times with ice-cold phosphate-buffered saline (PBS) and fixed in paraformaldehyde (4% [wt/vol] in Soerensen's phosphate buffer) for 20 min on ice. Cells were mounted using fluorescent mounting medium with 15 mM $NaN_3$ (Dako Corporation). The cells were examined using a TCS XP confocal microscope (Leica Microsystems).

**Immunoelectron microscopy (immuno-EM).** Cells treated as described in the figure legends were incubated with or without EGF in MEM without $HCO_3^-$ containing 0.1% (wt/vol) BSA. Subsequently, the cells were fixed using paraformaldehyde (4%, wt/vol) and glutaraldehyde (0.1%, wt/vol) in Soerensen's phosphate buffer and processed as described by Griffiths et al. (13). Immunocytochemical labeling of thawed cryosections was performed essentially as described by Griffiths et al. (14) using protein A gold (purchased from G. Posthuma, Utrecht, The Netherlands) or gold coated with donkey anti-mouse or donkey anti-rabbit IgG (Jackson ImmunoResearch Laboratories, Inc.). EGFR was labeled using a mixture of mouse anti-EGFR (sc-120) and mouse anti-EGFR (Ab-3); clathrin was detected using the rabbit anti-clathrin light chain antibody, while cells transfected with the different Grb2-constructs were detected using either the rabbit or the mouse anti-Myc antibody. Sections were examined using a Philips CM120 transmission electron microscope with a Megaview II TEM Soft Imaging System and a Philips Tecnai 12 transmission electron microscope with a Megaview III TEM Soft Imaging System. To estimate the number of coated pits per micrometer of plasma membrane, randomly oriented sections were scanned in a systematic random fashion. The length of the plasma membrane on randomly chosen cells was measured using a 500-nm lattice overlay to score intersections with the plasma membrane. No less than 300 μm of plasma membrane was measured in each of the three independent parallel experiments. To estimate the number of EGFR-positive coated pits and the distribution of the EGFR at the plasma membrane, no less than 50 coated pits and 100 gold particles were counted for each of the three independent labeling experiments. To measure the labeling density for α-adaptin in coated pits, the length of the coated membrane was measured using a 100-nm lattice overlay to score intersections with the coated membrane. The results represent the mean of at least three independent labeling experiments ± standard deviation (SD).

**Western blotting.** Cells in 12-well microtiter plates were treated as indicated in the figure legends before being subjected to Western blot analysis as previously described (33). The reactive proteins were detected using enhanced chemiluminescence (Amersham Biosciences).

**Internalization of $^{125}$I-Tf.** Iron-saturated human Tf was iodinated with $Na^{125}I$, as previously described (36). HeLa cells in 24-well microtiter plates were incubated with 70 ng/ml of $^{125}$I-Tf in MEM without $HCO_3^-$ with 0.1% (wt/vol) BSA at 37°C for the times indicated in the figures. In the control (time point 0), the cells were incubated with $^{125}$I-Tf on ice for 1 min. The cells were washed three

times with ice-cold PBS before being incubated with 3% Pronase E (wt/vol) in MEM without $HCO_3^-$ for 1 h on ice. The cell suspension was transferred to Eppendorf tubes and centrifuged at 6,000 × *g* for 5 min at 4°C. The supernatant fraction was removed and counted in a γ-counter (1470 Wallac WIZARD; Perkin Elmer, Inc.). The pellet fraction was washed in cold PBS before analysis by γ-counting. Internalized $^{125}$I-Tf was measured as the ratio of cpm in the pellet to the supernatant fraction.

**Internalization of $^{125}$I-EGF.** HeLa cells in 24-well microtiter plates were incubated with 1 ng/ml of $^{125}$I-EGF in MEM without $HCO_3^-$ with 0.1% (wt/vol) BSA at 37°C for the times indicated. In the control (time point 0), the cells were incubated with $^{125}$I-EGF on ice for 1 min. The cells were washed three times with ice-cold PBS before surface-bound $^{125}$I-EGF was removed by incubating the cells twice with 0.2 M acetate buffer (pH 2.8) containing 0.5 M NaCl for 10 min on ice. The radioactivity released from the cell surface was subsequently measured in a γ-counter. The cells were hydrolyzed with 1 M NaOH on ice for 60 min before the internalized $^{125}$I-EGF was measured in a γ-counter. Internalized $^{125}$I-EGF was measured as the ratio of internalized to surface-localized cpm.

## RESULTS

**EGF induces formation of new clathrin-coated pits.** Eps15 mutants lacking Eps15 homology (EH) domains but containing the AP2-binding sites are not targeted to the plasma membrane, and overexpression of such constructs results in blocked clathrin-dependent endocytosis of the TfR (3, 5). Such mutations in Eps15 result in constitutive association with AP2, and blunted clathrin-dependent endocytosis is therefore due to sequestration of AP2 in the cytoplasm (3). In order to study the effect of functional down-regulation of AP2 at the plasma membrane on ligand-induced clathrin-dependent endocytosis, we overexpressed Eps15 with deleted EH domains in HeLa cells and investigated the effect on early steps of EGF-induced endocytosis of the EGFR. Consistent with previous findings (3, 5), we observed that overexpression of GFP-tagged Δ95/295-Eps15 (EH95) caused redistribution of AP2 from the plasma membrane to the cytoplasm, as detected by immunofluorescence, using an antibody to the α-adaptin subunit of AP2 (data not shown). Upon overexpression of EH95 in HeLa cells, we further found that endocytosis of Tf was strongly inhibited, consistent with previous findings (3) (Fig. 1A and B). However, in contrast to what was previously reported (4), ligand-induced endocytosis of the EGFR was unaffected (Fig. 1C and D). This was demonstrated both by immunofluorescence using Rh-EGF (2.4 nM) (Fig. 1C) and by an internalization assay with low and nonsaturating concentrations of $^{125}$I-EGF (1 ng/ml) (21, 42) (Fig. 1D). The internalization assay was performed by continuous incubation at 37°C in order to avoid saturating coated pits by binding EGF to cells on ice for long time periods (16). Internalized EGF further localized to early endosome antigen 1-positive vesicles both in nontransfected cells and in cells overexpressing EH95 (data not shown).

By using immuno-EM and stereological methods, we quantified the number of coated pits per micrometer of plasma membrane in HeLa cells with or without overexpression of EH95. As demonstrated in Fig. 1E, the number of coated pits in cells not incubated with EGF was reduced to approximately 15% in cells overexpressing EH95 compared to control cells. However, upon incubation with EGF on ice, the number of coated pits in cells overexpressing EH95 increased to approximately 70% of the control. Labeling, using antibody to clathrin light chain, confirmed that EGF-induced coated pits contained clathrin (data not shown). This clearly demonstrated that upon incubation with EGF, new clathrin-coated pits were formed in cells depleted of functional AP2.

Immuno-EM was further used to study the localization of EGF/EGFR and TfR to coated pits at the plasma membrane. In nontransfected cells incubated with EGF on ice (Fig. 2A and D), both EGF and the TfR could be found in coated pits. Upon overexpression of EH95 and incubation of cells with EGF on ice, EGF and EGFR were found in coated pits (Fig. 2B and C, respectively). While less than 2% of the coated pits in control cells not incubated with EGF were EGFR positive (Table 1), incubation with EGF resulted in a more than 10-fold increase in the number of EGFR-positive coated pits both in control cells and in cells overexpressing EH95. The plasma membrane distribution of the EGFR changed correspondingly. No TfR was found in coated pits in cells overexpressing EH95 (Fig. 2E and F), and no endocytosis of Tf was observed even when Tf was added to the cells together with EGF at 37°C (results not shown). These results are consistent with the notion that the TfR is unable to internalize through the clathrin-coated pits used by the EGFR upon removal of AP2 from the plasma membrane and could potentially confirm the contention that the EGFR does not depend on AP2 for internalization from clathrin-coated pits (8, 22).

In addition to functional depletion of AP2 by overexpression of EH95, we depleted AP2 by using siRNA to the μ2 and α subunit of AP2, as previously described (22). Figure 3A demonstrates down-regulation of the μ2 and α subunits of AP2 in cells transfected with siRNA to knock down either μ2 or α. Partial down-regulation of the α and β subunits of AP2, but not of the clathrin heavy chain, was observed upon treatment with siRNA to the μ2 subunit. Similarly, partial down-regulation of the μ2 and β subunits of AP2 was observed with siRNA to the α subunit. This is consistent with the findings of Motley et al. (22). It should be noted that the α subunit was most efficiently down-regulated by siRNA to the α subunit (Fig. 3A). Scrambled negative control siRNA had no effect on the expression of μ2 or α (data not shown). We confirmed that uptake of $^{125}$I-Tf was fully blocked, while the uptake of $^{125}$I-EGF (1 ng/ml) at 37°C was not affected by siRNA to the μ2 subunit (Fig. 3B and C, respectively). However, upon knockdown of the α subunit, $^{125}$I-Tf uptake was completely inhibited, and the uptake of $^{125}$I-EGF (1 ng/ml) at 37°C was significantly inhibited (Fig. 3B and C). By immuno-EM, the number of coated pits found in μ2 and α siRNA-treated cells not incubated with EGF was strongly reduced compared to control cells. However, the number of coated pits increased approximately 10 times upon incubation of the siRNA-treated cells with EGF (Fig. 3D). Slightly more coated pits were induced in cells that had been incubated with siRNA to the μ2 subunit than in cells that had been incubated with siRNA to the α subunit (Fig. 3D). The labeling for EGFR within EGF-induced coated pits in cells treated with siRNA to the μ2 subunit was comparable to control cells incubated with EGF (compare Table 1 and Table 2). However, in agreement with the results on endocytosis of $^{125}$I-EGF, the EGF-induced increase in localization of EGFR to coated pits was less pronounced in cells that had been incubated with siRNA to the α subunit.

To make sure that the internalization of EGF in cells where AP2 had been knocked down was not a result of observing internalization in a pool of cells in which knock-down of AP2

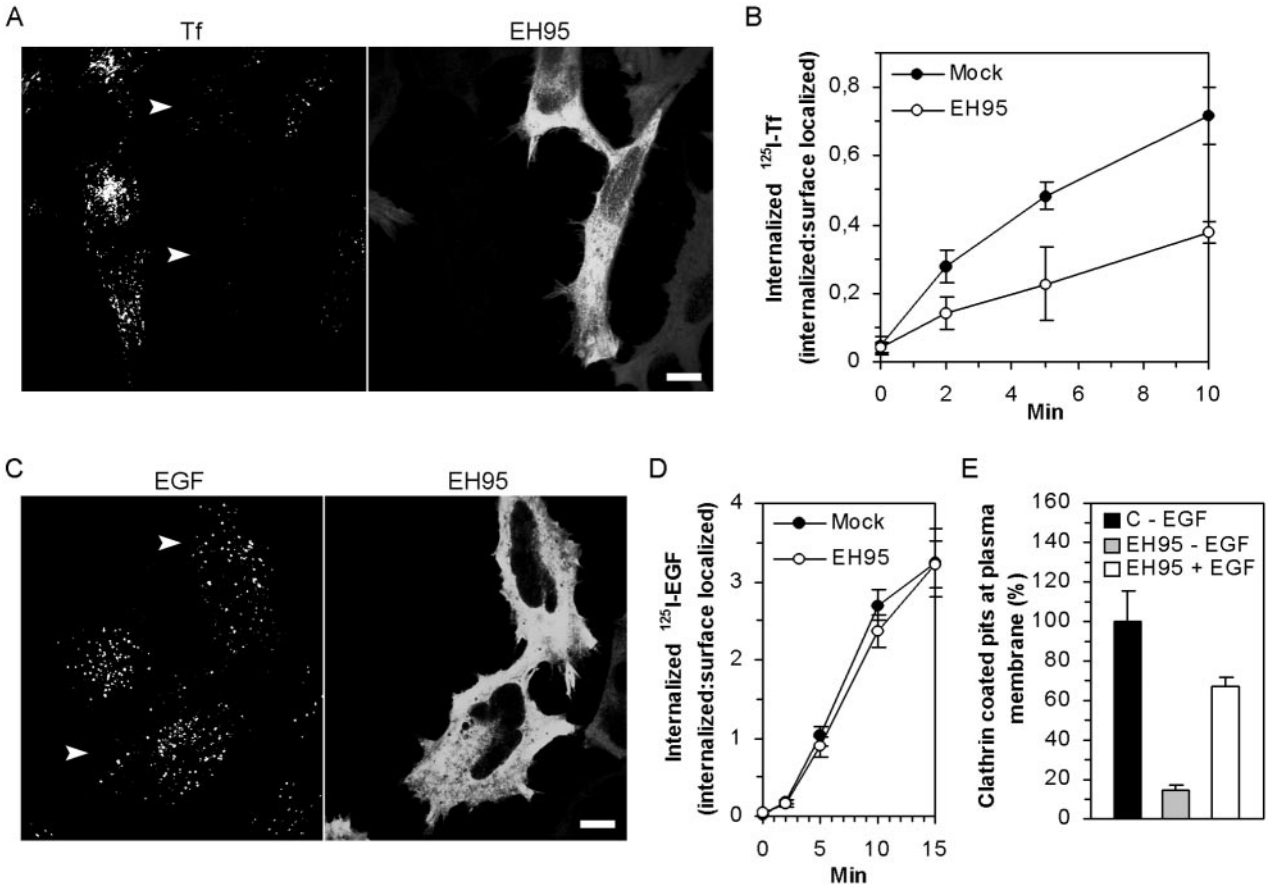


FIG. 1. Effect of overexpression of Eps15 with EH domain deletions (EH95) on internalization of Tf and EGF and on the number of clathrin-coated pits. HeLa cells were transfected with or without the plasmid pEGFP-EH95 expressing GFP-tagged EH95. (A) Internalization of Rh-Tf (left panel). Arrowheads indicate cells overexpressing GFP-EH95 (overexpression demonstrated in right panel). (B) Internalization of $^{125}$I-Tf. Internalized $^{125}$I-Tf is demonstrated as cpm of internalized $^{125}$I-Tf divided by cpm of surface-localized $^{125}$I-Tf. (C) Internalization of Rh-EGF (2.4 nM) (left panel). Arrowheads indicate cells overexpressing GFP-EH95 (overexpression demonstrated in right panel). (D) Internalization of $^{125}$I-EGF (1 ng/ml). Internalized $^{125}$I-EGF demonstrated as cpm of internalized $^{125}$I-EGF divided by cpm of surface-localized $^{125}$I-EGF. (E) Stereological analysis of the number of coated pits at the plasma membrane. Cells incubated for 1 h on ice with or without EGF (60 ng/ml) were prepared for immuno-EM and labeled for GFP. C, nontransfected cells. The results are presented as the percentage of the number of coated pits found in nontransfected cells not incubated with EGF. Mock, cells transfected with empty vector. Scale bar, 10 μm. Each data set in panels B and D was derived from three independent experiments ± SD. Each data set in panel E was derived from three independent labeling experiments ± SD.

was insufficient, we fluorescently labeled the siRNA to the μ2 subunit. Then confocal microscopy was performed on Alexa 488-EGF- and Alexa 633-Tf-treated cells. As shown in Fig. 4, fluorescent siRNA was evenly distributed in all cells. Furthermore, endocytosis of Tf was observed to be blunted, while EGF was seen to be endocytosed in all the cells.

To investigate whether EGF-induced coated pits were formed only at high concentrations of ligand, HeLa cells, where AP2 had been knocked down by siRNA to μ2, were incubated without EGF or with 1 and 60 ng/ml EGF on ice. Interestingly, similar amounts of coated pits were induced both at high and low concentrations of EGF (Fig. 5A). Results showed that 20% ± 1.2% of coated pits were EGFR positive on incubation with 1 ng/ml EGF and 30% ± 0.5% of coated pits were EGFR positive on incubation with 60 ng/ml EGF. To investigate whether EGF-induced coated pits were found only in cells depleted of AP2, we incubated serum-starved cells harboring normal levels of AP2 with or without EGF (60 ng/ml). Initially, such experiments were performed on ice, since coated pits do not bud at 4°C, and a potential increase in new coated pits could be more readily observable. As demonstrated in Fig. 5B, a clear increase in the number of clathrin-coated pits was observed when serum-starved HeLa cells were incubated with EGF on ice for 1 h. This experiment was further performed with incubation with or without EGF at 37°C for 3 min. Also in this case, we observed formation of new coated pits in serum-starved EGF-treated cells not depleted of AP2 (Fig. 5C). The results describing formation of coated pits in cells containing normal amounts of AP2 are consistent with recently published results by Puri et al. (28). Quantification of the EGFR labeling (Table 3) confirmed that the EGFR was recruited into coated pits both on ice and at 37°C. We thus conclude that EGF induces formation of new EGFR-positive coated pits at high and low concentrations of ligand on ice as well as at 37°C and in AP2-depleted cells as well as in cells with normal amounts of AP2.

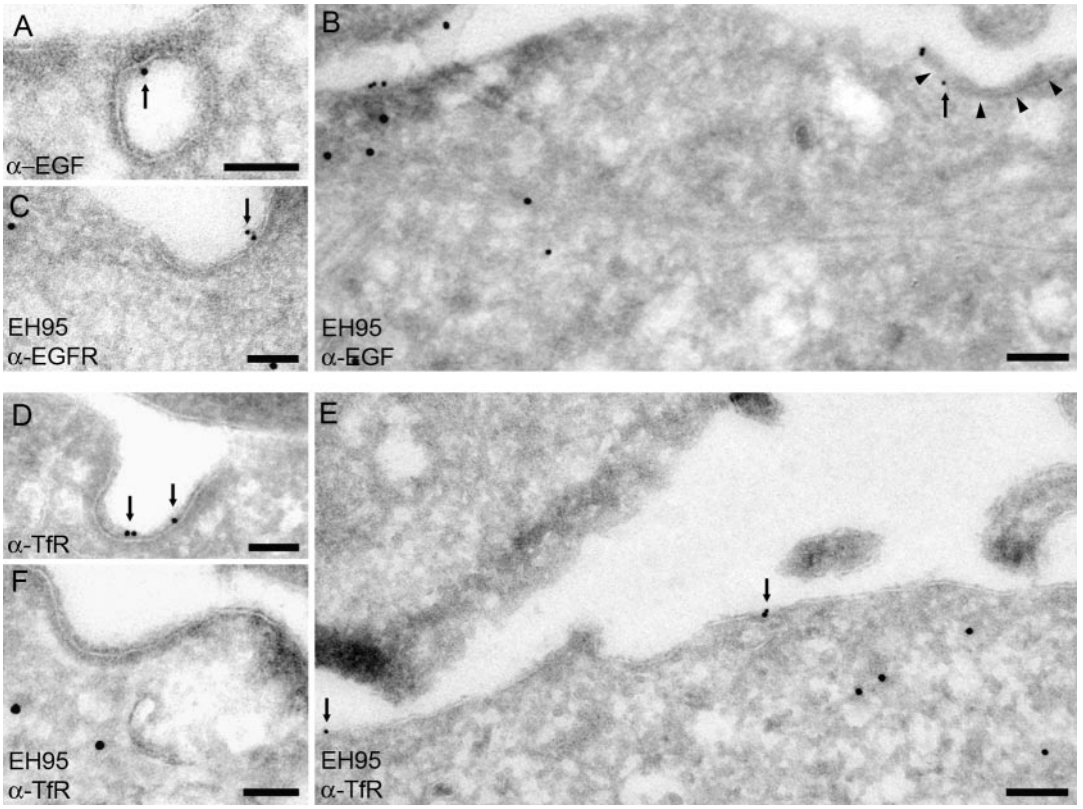


FIG. 2. EGF and EGFR, but not the TfR, localized to coated pits upon incubation of cells overexpressing EH95 with EGF. (A) Localization of EGF in nontransfected HeLa cells incubated for 1 h on ice with EGF (60 ng/ml). Labeling for EGF (arrow) indicates localization of EGF in a coated pit in HeLa cells not overexpressing EH95. (B and C) EGF and EGFR localized to coated pits (outlined by arrowheads in panel B) in HeLa cells overexpressing EH95 upon incubation with EGF (60 ng/ml) for 1 h on ice. Double labeling for EGF (B) or EGFR (C) (small particles, arrows) and GFP (large particles) demonstrates the localization of EGF and EGFR in coated pits. (D) The TfR localized to coated pits in nontransfected HeLa cells incubated with EGF (60 ng/ml) for 1 h on ice. Arrows indicate labeling for the TfR. (E and F) Double labeling for TfR (small particles, arrows) and GFP (large particles) demonstrate that the TfR was restricted to smooth plasma membrane (E) and not localized in coated pits (F) in cells overexpressing EH95 upon incubation with EGF (60 ng/ml) for 1 h on ice. Bar, 100 nm.

It should be noted that when cells where μ2-adaptin had been knocked down by siRNA were briefly incubated with EGF at 37°C, EGF was found both in invaginated coated pits (data not shown), in coated vesicles (Fig. 6A), and in early endosomes (Fig. 6B). This demonstrates that new EGF-induced coated pits in AP2-depleted cells are fully functional. As in cells overexpressing EH95, the TfR was not detected in EGF-induced coated pits upon siRNA-mediated knockdown of the μ2 or α subunit of AP2, and Tf was not endocytosed even when added together with EGF (data not shown). The size of the EGF-induced coated pits was the same as in control cells and showed normal labeling for clathrin (data not shown). However, compared to control cells, the labeling for α-adaptin inside coated pits was reduced in density in cells overexpressing EH95 and in cells treated with siRNA to the μ2 or α subunit of AP2 (Fig. 7). Quantification showed that in cells overexpressing EH95, the labeling density was reduced by approximately 50%, and in cells treated with siRNA to the μ2 or α subunits of AP2, the reduction of labeling for α-adaptin inside coated pits was almost 80% (results not shown).

TABLE 1. EGF-induced recruitment of the EGFR to coated pits in cells transfected with EH95[a]

| Cell culture | EGFR-positive coated pits (%) | EGFR in coated pits (%) |
|---|---|---|
| Control without EGF | 1.3 ± 0.9 | 0.7 ± 0.5 |
| Control with EGF | 23.0 ± 2.2 | 9.2 ± 2.7 |
| EH95 with EGF | 26.7 ± 1.2 | 13.6 ± 1.4 |

[a] By immuno-EM, the number of coated pits and the plasma membrane distribution of the EGFR were quantified in nontransfected (control) HeLa cells and in HeLa cells transfected with EH95 and incubated with or without EGF (60 ng/ml) for 1 h on ice. The number of EGFR-positive coated pits is presented as the percentage of the total number of coated pits observed. The EGFR localized to coated pits is presented as the percentage of the total labeling for EGFR found at the plasma membrane. Each data set was derived from three independent labeling experiments ± SD.

**EGFR kinase activity controls formation of EGFR-positive clathrin-coated pits.** As new coated pits were formed upon adding EGF, we investigated whether the EGFR kinase activity was required. Inhibition of the EGFR kinase activity by PD153035 (12) led to full inhibition of EGF internalization in cells transfected with or without μ2 siRNA, as measured by the uptake of $^{125}$I-EGF (1 ng/ml) (Fig. 8A). However, the Src kinase inhibitor SU6656, earlier demonstrated to be specific for Src kinases (6), the MEK inhibitor PD98059, and the PI3K inhibitor wortmannin did not affect internalization of the EGFR (Fig. 8A). Our findings that Src kinase activity, in con-

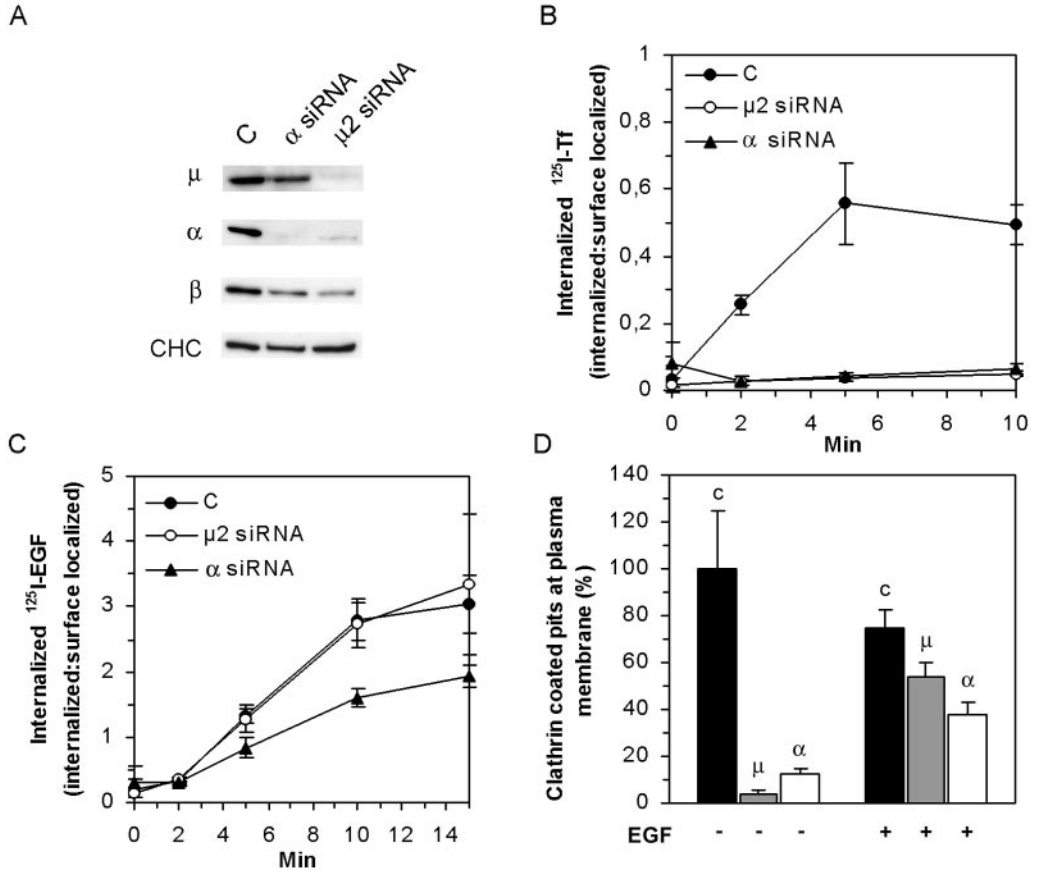


FIG. 3. Effect of down-regulation of the μ2 or the α subunit of AP2 on internalization of Tf and EGF and on the number of coated pits. HeLa cells were transfected with siRNA directed against μ2-adaptin or α-adaptin. C, control cells (transfection reagent w/o siRNA). (A) Down-regulation of AP2 subunits in siRNA-transfected cells. Cell homogenates were subjected to Western blotting using antibodies to α-adaptin, β-adaptin, μ2-adaptin, or clathrin heavy chain (CHC). (B) Internalization of $^{125}$I-Tf in siRNA-transfected cells. Internalized $^{125}$I-Tf was measured as described in the legend to Fig. 1B. (C) Internalization of $^{125}$I-EGF (1 ng/ml) in siRNA-transfected cells. Internalized $^{125}$I-EGF was measured as described in the legend to Fig. 1D. (D) EGF induced formation of coated pits. siRNA-transfected cells were incubated for 1 h on ice with or without EGF (60 ng/ml). By immuno-EM, the coated pits were counted and presented as the percentage of the number of coated pits found in control cells without EGF. Each data set in panels B and C was derived from three independent experiments ± SD. Each data set in panel D was derived from three independent labeling experiments ± SD.

trast to the EGFR kinase activity, is not important for endocytosis of the EGFR are in agreement with the findings of Sorkina et al. (35), who showed that a specific inhibitor of the EGFR kinase blunted endocytosis of EGFR localized outside clathrin-coated pits, but not of EGFR already localized in coated pits (35). Our results argue that activation of Src, MAPK, and PI3K is not important for EGF-induced endocytosis of the EGFR, while the intrinsic kinase activity of the EGFR is required for formation of coated pits.

TABLE 2. EGF-induced recruitment of the EGFR to coated pits in cells transfected with siRNA to the μ2 or α subunit of AP2[a]

| Cell transfection | EGFR-positive coated pits (%) | EGFR in coated pits (%) |
|---|---|---|
| μ2 siRNA with EGF | 30.0 ± 0.5 | 7.8 ± 1.5 |
| α siRNA with EGF | 17.0 ± 2.5 | 4.7 ± 1.2 |

[a] By immuno-EM, the number of coated pits and the plasma membrane distribution of the EGFR were quantified in nontransfected (control; see Table 1) HeLa cells and in HeLa cells transfected with siRNA to the μ2 or α subunit of AP2 upon incubation with EGF (60 ng/ml) for 1h on ice. The number of EGFR-positive coated pits is presented as the percentage of the total number of coated pits observed. The EGFR localized to coated pits is presented as the percentage of the total labeling for EGFR found at the plasma membrane. Each data set was derived from three independent labelling experiments ± SD.

By stereological methods, the effect of PD153035 on the number of coated pits at the plasma membrane in EGF-treated cells transfected with μ2 siRNA was measured (Fig. 8B). Upon inhibition of the EGFR kinase, EGF-induced formation of clathrin coated pits was inhibited, demonstrating that formation of EGF-induced clathrin-coated pits depended on the intrinsic EGFR kinase. Since PD153035 could potentially inhibit other receptors in the EGFR family, we investigated whether kinase-defective EGFR could promote formation of clathrin coated pits upon addition of EGF. PAE cells not harboring members of the EGFR family were transiently transfected with EH95 to functionally deplete the cells of AP2 and additionally transfected with wild-type EGFR or with kinase dead (K721A) EGFR. Then the cells were incubated with or without EGF (15 ng/ml) for 3 min at 37°C. Immuno-EM and stereology were then used to estimate the relative number of coated pits. As demonstrated in Fig. 8C, overexpression of

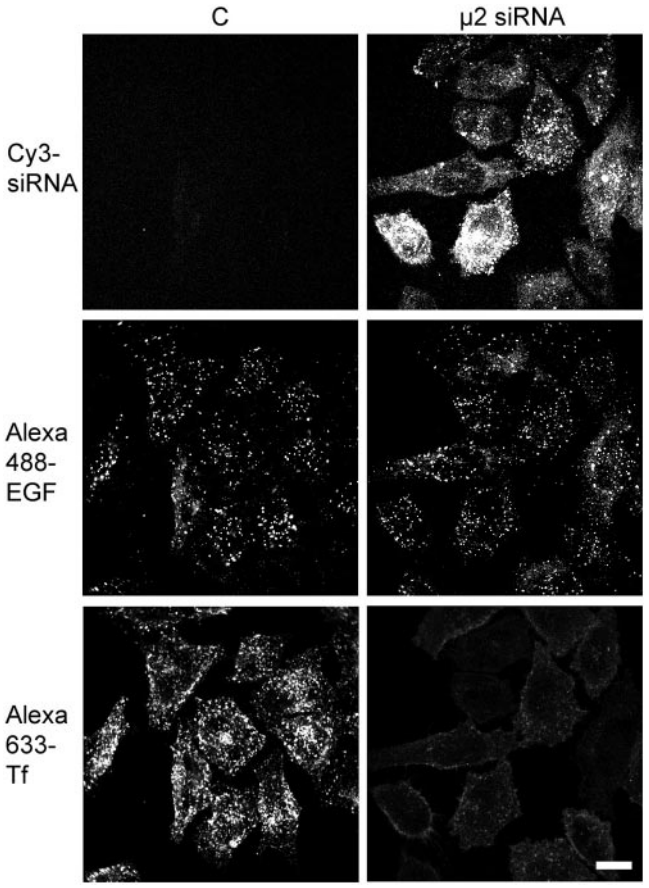


FIG. 4. siRNA directed against μ2 was evenly distributed in all cells, and endocytosis of Tf, but not of EGFR, was inhibited in all siRNA-treated cells. HeLa cells were transfected with Cy3-conjugated siRNA directed against μ2-adaptin. C, control cells (transfection reagent without siRNA). Internalization of Alexa 488-EGF (2.4 nM) and Alexa 633-Tf was assayed upon incubation for 15 min at 37°C. Bar, 10 μm.

TABLE 3. EGF-induced recruitment of the EGFR into coated pits in serum-starved cells[a]

| Culture conditions | EGFR-positive coated pits (%) | EGFR in coated pits (%) |
|---|---|---|
| On ice | | |
| Without EGF | 2.0 ± 1.6 | 0.7 ± 0.6 |
| With EGF | 33.7 ± 4.5 | 13 ± 2.2 |
| At 37°C | | |
| Without EGF | 2.3 ± 0.5 | 1.1 ± 0.5 |
| With EGF | 24.0 ± 4.2 | 16.7 ± 2.5 |

[a] By immuno-EM, the number of coated pits and the plasma membrane distribution of the EGFR were quantified in serum-starved HeLa cells incubated with or without EGF (60 ng/ml) for either 1h on ice or 3 min at 37°C. The number of EGFR-positive coated pits is presented as the percentage of the total number of coated pits observed. The EGFR localized to coated pits is presented as the percentage of the total labeling for EGFR found at the plasma membrane. Each data set was derived from three independent labeling experiments ± SD.

EH95 down-regulated the number of coated pits to approximately 50% in PAE cells, and incubation with EGF induced new clathrin-coated pits only in cells expressing wild-type EGFR.

**Functional importance of Grb2 in EGF-induced formation of coated pits.** We have recently demonstrated that Grb2 is recruited to the rim of EGFR-positive coated pits (36). This could imply that the EGF-induced formation of clathrin-coated pits directly results from recruitment of Grb2 to the activated EGFR. This would be consistent with the recent results by Jiang et al. (18), demonstrating that Grb2 is required for endocytosis of the EGFR. We therefore investigated the effect of d.n.Grb2 (inactivating mutations in both SH3 domains), incapable of binding proline-rich sequences, on EGF-induced formation of coated pits. As demonstrated in Fig. 8D, EGF was found not to increase the number of coated pits when cells were cotransfected with d.n.Grb2 and EH95. As the two SH3 domains of Grb2 are responsible for recruitment of different signaling molecules, we further explored the effect of overexpression of the N-SH3 domain or the C-SH3 domain individually mutated within the context of full-length Grb2. As

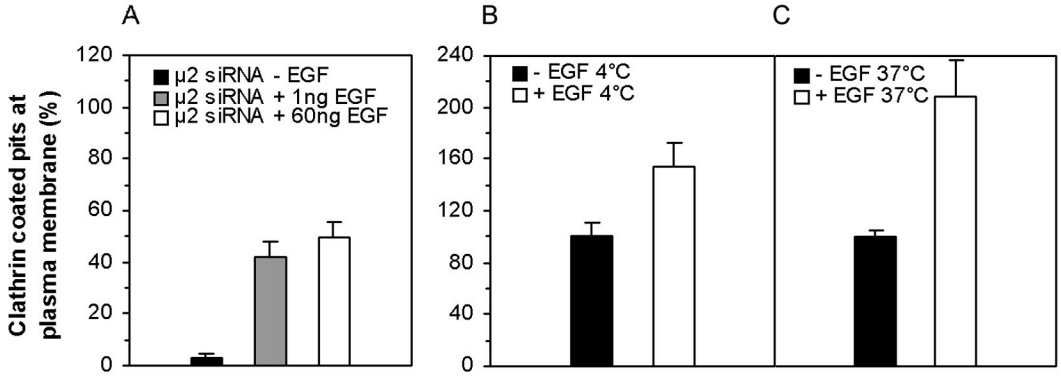


FIG. 5. Concentration dependency and temperature dependency of EGF-induced formation of coated pits. (A) HeLa cells were transfected with siRNA directed against μ2-adaptin and incubated with or without EGF (1 or 60 ng/ml) for 1 h on ice. The cells were then processed for electron microscopy. The data are presented as the percentage of the number of coated pits found at the plasma membrane in nontransfected HeLa cells incubated without EGF for 1 h on ice. (B and C) HeLa cells not depleted of AP2 were serum starved for 24 h at 37°C, followed by incubation with EGF (60 ng/ml) for 1 h on ice (B) or 3 min at 37°C (C) and subsequently processed for immuno-EM. Data in panel B are presented as the percentage of the number of coated pits found in serum-starved HeLa cells incubated without EGF for 1 h on ice. Data in panel C are presented as the percentage of the number of coated pits found in serum-starved HeLa cells incubated without EGF at 37°C. The data represent the mean of a minimum of three independent labeling experiments ± SD.

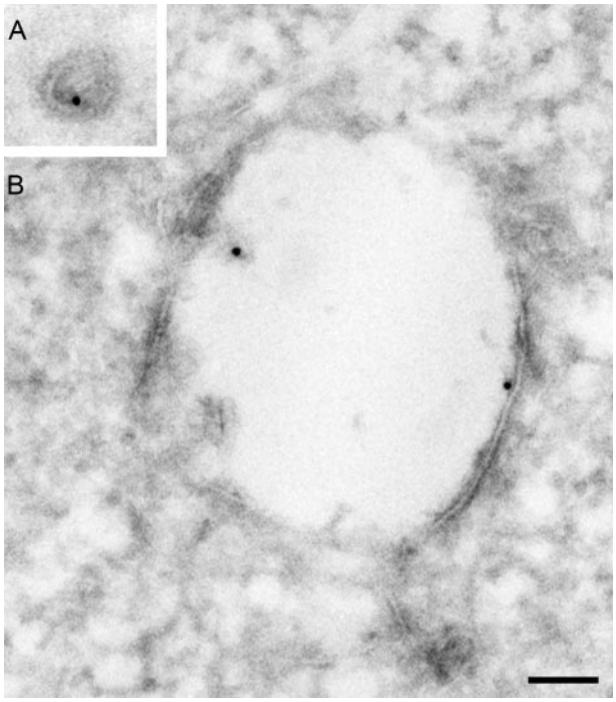


FIG. 6. The EGFR was efficiently internalized through new EGF-induced coated pits. HeLa cells were transfected with siRNA directed against μ2-adaptin and incubated with EGF (60 ng/ml) for 5 min at 37°C, processed for immuno-EM, and labeled against EGF. EGF was found localized to clathrin-coated vesicles (A) and to endosomal compartments (B). Bar, 100 nm.

demonstrated, Grb2 mutated in the N-SH3 domain inhibited EGF-induced formation of coated pits almost as efficiently as did the d.n.Grb2 and more efficiently than did Grb2 mutated in the C-SH3 domain (Fig. 8D). It should be noted that even upon overexpression of Grb2 with an inactivating point mutation in the SH2 domain and upon overexpression of wild-type Grb2, the EGF-induced formation of coated pits was inhibited (Fig. 8D). The inhibiting effect of the SH2 mutant and of wild-type Grb2 can probably be explained by binding of proline-rich proteins to the functional SH3 domains, thereby sequestering such proteins away from the activated EGFR-Grb2 complex.

It has been reported that the Ras guanine nucleotide exchange factor Sos mainly interacts with the N-terminal SH3 domain of Grb2 (31, 32, 38), while Gab1 interacts with the C-terminal SH3 domain of Grb2 (20, 26). Accordingly, activation of MAPK in most cells requires a functional N-SH3 domain (43), while activation of PI3K usually requires recruitment of p85 to Gab1, and consequently a functional C-SH3 domain. To correlate the blocked recruitment of Sos and Gab1 to activation of MAPK and PI3K, we investigated the effect of overexpressing Grb2 with inactivating single and double point mutations on activation of MAPK and on activation of PI3K (assayed indirectly as phosphorylation of Akt). To increase the fraction of cells expressing the Grb2 mutants, the cells were cotransfected with pEGFP and subsequently subjected to fluorescence-based cell sorting. As demonstrated in Fig. 9, we found that Grb2 with mutations in N- or C-terminal SH3 domains or with mutations in both SH3 domains did not blunt activation of MAPK or of PI3K, arguing that redundant pathways exist. As reported by Huang and Sorkin, it should further be noted that extracellular signal-regulated kinase 1 and 2 (ERK1/2) activity was inhibited only by 50% in cells depleted of Grb2 and also fully rescued by Grb2-yellow fluorescent protein in HeLa cells. As discussed (17), the significant activation of ERK1/2 in cells with very low MEK1/2 activity can probably be explained by the substantial molar excess of MEK1/2 over ERK1/2 in the cells and a 1,000-fold increase of ERK kinase activity by MEK1/2, allowing dramatic signal amplification at this step of the ERK activation cascade. We thus conclude that the inhibitory effect on EGF-induced formation of coated pits by point mutations in either the N-SH3 or the C-SH3 of Grb2 cannot be explained by blunted MAPK or PI3K activity. The contention that PI3K is not involved in coated pit formation is consistent with our immuno-EM experiments demonstrating EGF-induced formation of coated pits in Wortmannin-treated HeLa cells transfected with siRNA to μ2 (data not shown). Since the ubiquitin ligase Cbl has been demonstrated to be required for endocytosis of the EGFR (19, 36), we investigated recruitment of Cbl to the Grb2 variants described above. Consistent with previously described findings (18), we found that both SH3 domains were required to efficiently recruit Cbl to Grb2 (data not shown).

Immuno-EM of μ2 siRNA-treated cells demonstrated that Grb2 localized to EGF-induced coated pits. In fact, quantitative immuno-EM analysis demonstrated that compared to control cells, where approximately 25% of coated pits were positive for Grb2, more than 50% of EGF-induced coated pits were positive for Grb2 (Fig. 10). Considering labeling efficiency, this could be interpreted to mean that most coated pits induced by EGF in cells depleted of μ2-adaptin contain Grb2. This supports the notion that recruitment of Grb2 to the EGFR is essential for endocytosis of the EGFR, consistent with the findings of Jiang et al. (18), demonstrating that EGFRs lacking Tyr 1068/1086 are endocytosis deficient due to blocked direct binding of Grb2 to the EGFR. Interestingly, when investigating labeling of coated pits with respect to Eps15, we found that in contrast to control cells, where approximately 70% of coated pits were found to be positive, only 5 to 10% of EGF-induced coated pits were positive for Eps15 (Fig. 10). This is in accordance with the reported findings that the endocytosis of EGF was not affected in cells where Eps15 had been knocked down (16).

## DISCUSSION

In the present work we have addressed the problem of how the EGFR enters clathrin-coated pits. Our current data do not confirm the findings that endocytosis of the EGFR happens independently of AP2 (8, 22) but are consistent with the report of Huang et al. (16), demonstrating that by efficient knockdown of AP2, the endocytosis of the EGFR was inhibited. It should be noted that the endocytosis of the TfR was readily blocked, while reduced endocytosis of EGF was more difficult to effect. Only by knocking down the α subunit of AP2 could we clearly observe inhibition of EGF/EGFR endocytosis in HeLa cells. By overexpressing Eps15 with a deletion of EH domains (EH95), thereby sequestering AP2 in the cytosol, we

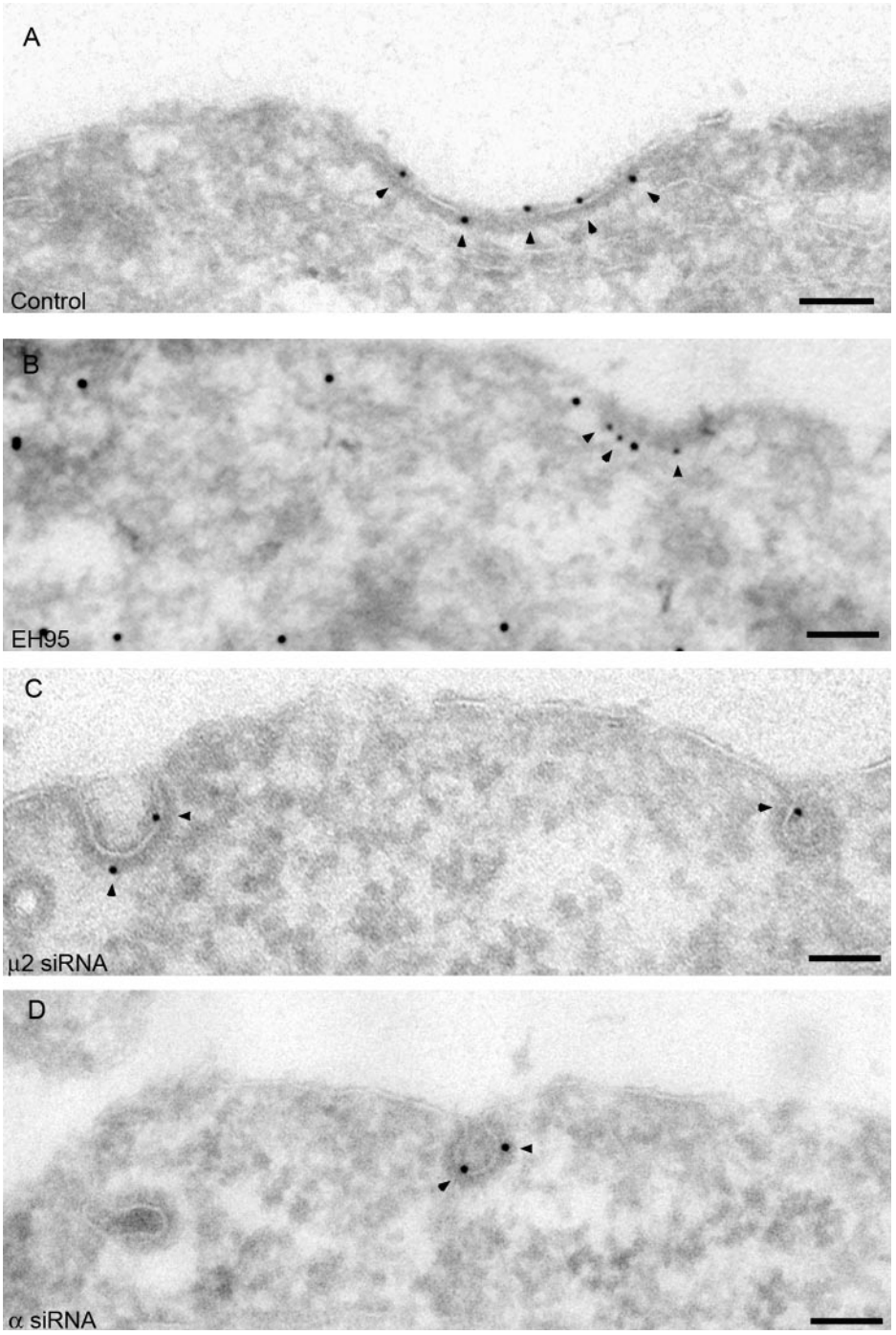


FIG. 7. α-Adaptin was present in EGF-induced coated pits, but the level of α-adaptin in these coated pits was reduced. HeLa cells were mock transfected, transfected with EH95, or transfected with siRNA directed against μ2- or α-adaptin, followed by incubation with EGF (60 ng/ml) for 1 h on ice and processed for immuno-EM and either singly labeled for α-adaptin (A, C, and D) or doubly labeled for α-adaptin (small particles) and GFP (large particles) (B). Coated pits labeled for α-adaptin (arrowheads) in mock-transfected cells (A), in cells transfected with EH95 (B), in cells transfected with siRNA directed against μ2-adaptin (C), and in cells transfected with siRNA directed against α-adaptin (D). Bar, 100 nm.

found that endocytosis of the TfR but not endocytosis of the EGFR was inhibited. This finding is inconsistent with the findings of Benmerah et al., describing inhibitory effects on both endocytosis of EGF and Tf upon overexpressing Eps15 with deleted EH domains (4). It is likely that overexpression of Eps15 sequesters AP2 with different efficiency in different cell lines. This is consistent with our unpublished findings that knocking down the μ2 subunit inhibited endocytosis of the EGFR in A431 cells but not in HeLa cells. Our finding that more efficient knockdown of AP2 is required to block endocytosis of the EGFR than of the TfR argues that AP2 plays a different role in clathrin-mediated endocytosis of the EGFR

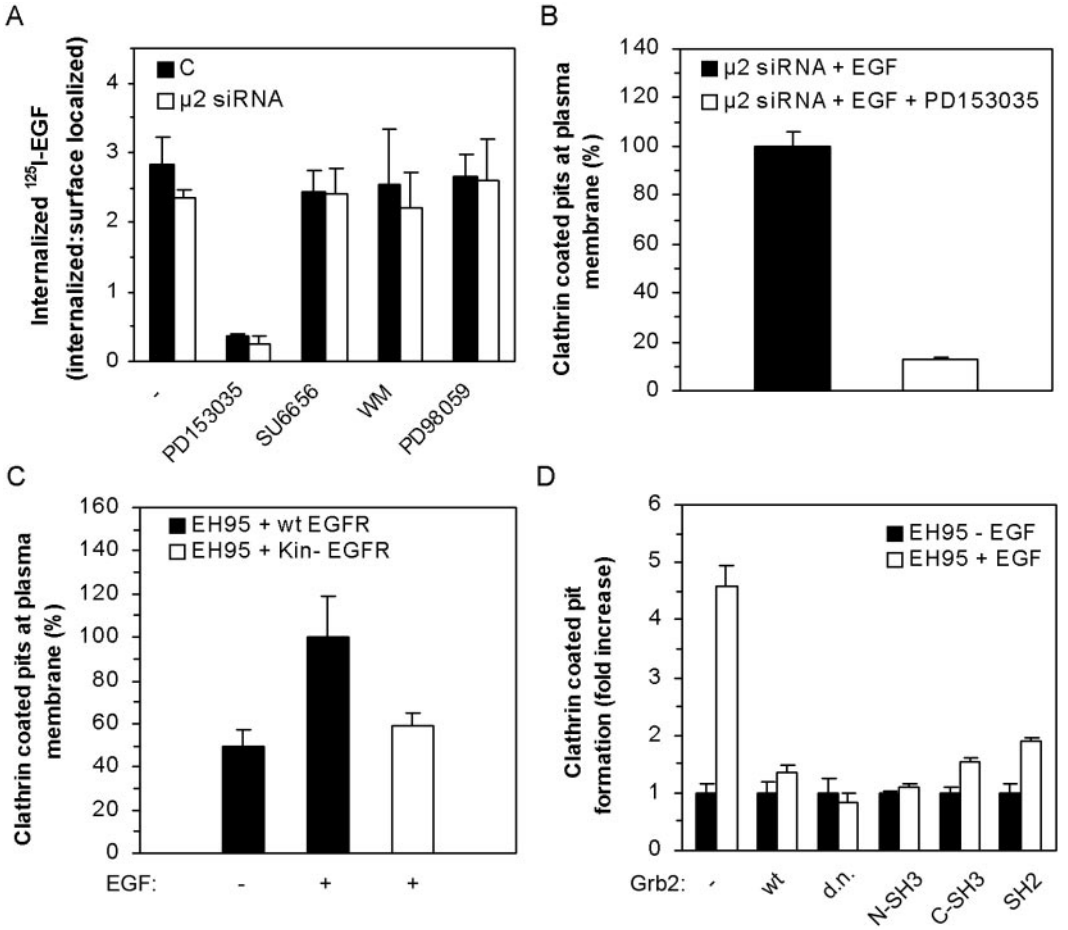


FIG. 8. Impact of EGFR kinase activity and intact Grb2 on internalization of EGF and on formation of coated pits. (A and B) HeLa cells were transfected with or without siRNA directed against μ2-adaptin. In panel A, the cells were incubated with or without PD153035 (100 nM), PD98059 (50 μM), wortmannin (WM) (500 nM), or SU6656 (2 μM). (A) Internalized $^{125}$I-EGF was assayed upon incubation for 15 min as described in the legend of Fig. 1D. C, control cells (transfection reagent without siRNA). One representative experiment with four parallels ± SD is demonstrated. In panel B, EM was used to determine the relative number of coated pits in cells transfected with siRNA to μ2 and untreated or treated with PD153035 (100 nM) prior to and during incubation with EGF (60 ng/ml) for 1 h on ice. The number of EGF-induced coated pits in AP2-depleted cells incubated with PD153035 is presented as the percentage of the number of coated pits found in AP2-depleted cells incubated with EGF only. (C) PAE cells were cotransfected with EH95 and wild-type (wt) EGFR or with EH95 and kinase dead (Kin−) EGFR (K721A EGFR), and the number of coated pits upon incubation with or without EGF (15 ng/ml) for 3 min at 37°C was determined. The data are presented as the percentage of the number of coated pits found in cells cotransfected with EH95 and wild-type EGFR and incubated with EGF. (D) HeLa cells were transfected with EH95 only (−) or cotransfected with EH95 and wild-type (wt) Grb2, EH95 and Grb2 W36K, G203R (d.n.), EH95 and Grb2 W36K (N-SH3), EH95 and Grb2 G203R (C-SH3), or EH95 and Grb2 R86K (SH2). By immuno-EM, the coated pits were counted in cells incubated with or without EGF (60 ng/ml) for 1 h on ice. The data are presented separately for each Grb2 variant as the increase upon incubation with EGF (60 ng/ml). The data represent the mean of three independent labeling experiments ± SD.

than in endocytosis of the TfR. It has been established that μ2 directly interacts with the cytoplasmic tail of membrane-bound receptors marked for internalization by tyrosine-based sorting signals (24). Phosphorylation of μ2 has further been demonstrated to increase the binding affinity of AP2 for tyrosine-based internalization motifs (29), and phosphorylation of μ2 has been demonstrated to be required for sequestration of TfR into coated pits (25). Our results suggest that interaction between μ2 and the EGFR is not important for recruitment of the EGFR to clathrin-coated pits. Instead, we suggest that AP2 plays a more catalytic role in endocytosis of the EGFR by assembling required adaptor and effector proteins around AP2 appendage domains, which are protein interaction hubs in the formation of clathrin-coated pits (27). This is consistent with the reported findings that an EGFR deficient in high-affinity binding to AP2 (Y974F) was endocytosed as efficiently as was wild-type EGFR when expressed at low levels and that this endocytosis was inhibited upon $K^+$-depletion of the cells (34). Furthermore, overexpression of a doubly mutated μ2 (D176A/W421A), which was fully incorporated into AP2 and was incapable of interacting with tyrosine-based internalization motifs, resulted in impaired endocytosis of the TfR, while endocytosis of the EGFR was unaffected (23).

Importantly, we found that overexpressing Eps15 with a deletion of EH domains (EH95), as well as knocking down the α or μ2 subunit of AP2, strongly reduced the number of clathrin-coated pits in the absence of EGF. However, when cells functionally depleted of AP2 were incubated with EGF, the

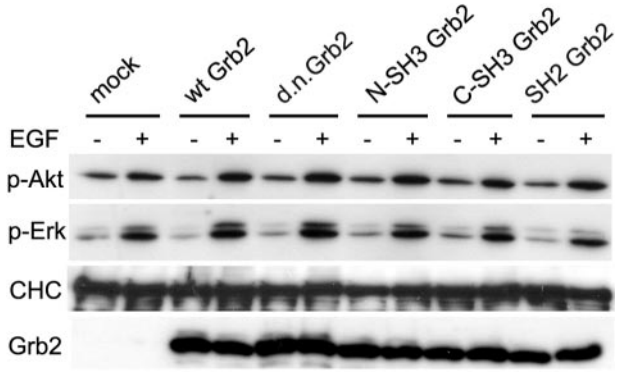


FIG. 9. Effect of Grb2 mutations on EGF-induced phosphorylation of MAPK (p-Erk) and phosphorylation of Akt (p-Akt). HeLa cells were transfected with pEGFP-C2 and with wild-type Grb2, Grb2 W36K, G203R (d.n.), Grb2 W36K (N-SH3), Grb2 G203R (C-SH3), or Grb2 R86K (SH2). The cells were trypsinized and sorted based on fluorescing GFP, using a FACSDIVa cell sorter (BD Biosciences) in order to increase the fraction of cells transfected with Grb2. Approximately 70% of the GFP-positive cells also contained tagged Grb2. The transfected cells (approximately $3 \times 10^5$ for each specimen) were resuspended and incubated with or without EGF (60 ng/ml) for 5 min at 37°C before being lysed and subjected to Western blotting with antibody to p-Akt, antibody to p-Erk, antibody to clathrin heavy chain (CHC), or antibody to Grb2, demonstrating overexpression of Grb2 in transfected cells.

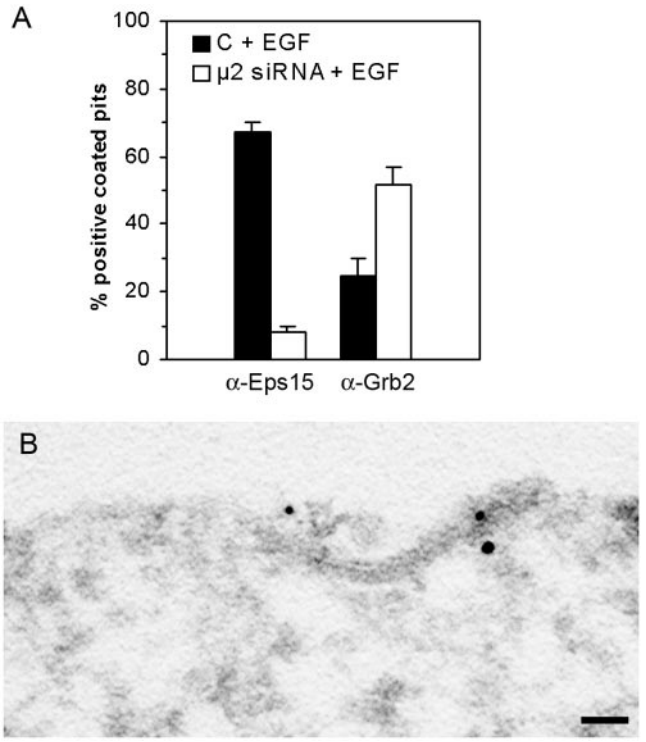


FIG. 10. EGF-induced coated pits are Grb2 positive but contain only trace amounts of Eps15. HeLa cells transfected with or without siRNA directed against μ2-adaptin were incubated with EGF (60 ng/ml) for 1 h on ice. C, transfection reagent only. (A) By immuno-EM, the coated pits labeling for either Eps15 or Grb2 were counted and presented as the percentage of the total number of coated pits observed. Each data set is derived from at least three independent labeling experiments ± SD. (B) Localization of Grb2 (large particles) and EGF (small particles) in EGF-induced coated pits in cells treated with siRNA directed against μ2-adaptin. Bar, 100 nm.

number of clathrin-coated pits increased dramatically. While coated pits were hardly detectable in cells overexpressing EH95 or lacking the α or μ2 subunit of AP2 in the absence of EGF, the number of coated pits in EGF-treated cells increased to approximately 70% of the number of coated pits in control cells. This, together with the finding that lack of EGFR kinase activity completely inhibited coated pit formation, demonstrates that clathrin assembly is induced upon activating the EGFR kinase.

Our findings thus argue that EGF-induced activation of the EGFR induces recruitment or rearrangement of AP2 and clathrin through signaling downstream of the activated EGFR. The formation of coated pits was blunted by overexpression of Grb2 with point mutations in both SH3 domains and thus incapable of recruiting proteins with proline-rich domains. Also, single point mutations in either SH3 domain inhibited EGF-induced formation of coated pits. Grb2 with an inactivating point mutation in N-SH3 was slightly more inhibitory than Grb2 with an inactivating point mutation in C-SH3. This argues that recruitment of signaling proteins to both SH3 domains is important. Our findings that none of the single SH3 point mutations separately blunted activation of MAPK or PI3K argue that neither MAPK nor PI3K is essential for formation of clathrin-coated pits upon EGFR activation. Consistent with previous findings (18), we found that the ubiquitin ligase Cbl was not efficiently recruited to Grb2 unless both SH3 domains were intact. As the ubiquitin ligase activity of Cbl has been demonstrated to be critical for clathrin-dependent endocytosis of the EGFR (17, 36), our data are compatible with the model that EGF-induced formation of coated pits only happens upon recruitment of the Grb2-Cbl complex to the EGFR and subsequent activation of Cbl's catalytic activity. The potential importance of ubiquitin–ubiquitin-interacting motif interactions in the process of coated pit assembly will be addressed more closely in future studies.

By comparing antibody labeling of coated pits in EGF-treated control cells with EGF-treated AP2-depleted cells, we found a significant increase in the proportion of Grb2-positive coated pits in AP2-depleted EGF-treated cells. This strongly suggests that Grb2 has an important function in EGF-induced formation of coated pits. Grb2 was consistently found to localize at the rim of the coat, as previously reported (36). New coated pits induced upon adding EGF to cells treated with siRNA to the μ2 or α subunit of AP2 did not contain TfR, even though α-adaptin was present in the coats and μ2 was detectable upon Western blotting in both cases. This could be explained by either too little μ2 at the plasma membrane or a lack of phosphorylation of the μ2 subunit of AP2. This could potentially mean that even though AP2 is localized to the plasma membrane upon activation of the EGFR, kinases responsible for phosphorylation of μ2 are not recruited to EGF-induced coated pits upon knock-down of the μ2 or α subunit of AP2. This should be addressed in future studies. On incubation with EGF, the proportion of EGFR-positive coated pits was similar whether or not the coated pits were preexisting or induced in AP2-depleted cells.

Normally, there will be AP2-positive coated pits at the

plasma membrane available for entry of the EGFR. However, there is reason to believe that formation of new coated pits is important in controlling endocytosis of the EGFR both under physiological and pathological conditions. Consistently, our present data demonstrate that also in serum-starved HeLa cells harboring normal levels of AP2 new coated pits are induced upon incubation with EGF. We have further demonstrated that cells strongly overexpressing ErbB2 do not significantly induce new coated pits upon incubation with EGF, in contrast to isogenic cells lacking ErbB2. The lack of EGF-induced formation of coated pits in cells overexpressing ErbB2 was found to coincide with a strongly reduced rate of endocytosis and down-regulation of the EGFR, while the endocytosis of the TfR was not affected by overexpression of ErbB2 (15).

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