The Inhibitory Effect of ErbB2 on Epidermal Growth Factor-induced Formation of Clathrin-coated Pits Correlates with Retention of Epidermal Growth Factor Receptor–ErbB2 Oligomeric Complexes at the Plasma Membrane

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By constructing stably transfected cells harboring the same amount of epidermal growth factor (EGF) receptor (EGFR), but with increasing overexpression of ErbB2, we have demonstrated that ErbB2 efficiently inhibits internalization of ligand-bound EGFR. Apparently, ErbB2 inhibits internalization of EGF-bound EGFR by constitutively driving EGFR–ErbB2 hetero/oligomerization. We have demonstrated that ErbB2 does not inhibit phosphorylation or ubiquitination of the EGFR. Our data further indicate that the endocytosis deficiency of ErbB2 and of EGFR–ErbB2 heterodimers/oligomers cannot be explained by anchoring of ErbB2 to PDZ-containing proteins such as Erbin. Instead, we demonstrate that in contrast to EGFR homodimers, which are capable of inducing new clathrin-coated pits in serum-starved cells upon incubation with EGF, clathrin-coated pits are not induced upon activation of EGFR–ErbB2 heterodimers/oligomers.

INTRODUCTION

Overexpression of epidermal growth factor (EGF) receptor (EGFR) and/or ErbB2 has been implicated in cancer development due to enhanced and altered growth factor signaling with ensuing effects on cell motility, cell anchoring, and cell transformation (Di Fiore *et al.*, 1987; Chazin *et al.*, 1992; Brandt *et al.*, 1999; Ignatoski *et al.*, 1999; Spencer *et al.*, 2000). EGF binds the EGFR, whereas ErbB2 has no known ligand. ErbB2 is still the main dimerization partner of all members of the EGFR family (Sliwkowski *et al.*, 1994; Yarden, 2001; Yarden and Sliwkowski, 2001). Dimerization is normally initiated by a conformational change in the extracellular domain of the EGFR upon ligand binding. This conformational change exposes a dimerization loop (a β hairpin), which can interact with a similarly exposed domain in another receptor (Garrett *et al.*, 2002; Ogiso *et al.*, 2002). However, this dimerization domain is constitutively exposed in ErbB2 (Schlessinger, 2002; Garrett *et al.*, 2003), and ErbB2 is therefore readily available for dimerization/oligomerization upon overexpression.

An important pathway inactivating receptors is endocytosis followed by lysosomal degradation (reviewed by Watanabe and Yarden, 2001). Whereas endocytosis and down-

regulation of the EGFR rapidly occurs upon ligand binding (Sorkin and Von Zastrow, 2002), endocytosis and down-regulation of ErbB2, ErbB3, and ErbB4 is inefficient (Wallasch *et al.*, 1995; Baulida *et al.*, 1996; Pinkas-Kramarski *et al.*, 1996). Defective endocytosis and enhanced recycling have been reported to characterize ErbB2-containing heterodimers (Lenferink *et al.*, 1998; Worthylake *et al.*, 1999). In a study using EGFR–ErbB2 chimeras, it was proposed that the cytoplasmic domain of ErbB2 either lacked an internalization motif or contained an inhibitory signal with respect to endocytosis from clathrin-coated pits (Sorkin *et al.*, 1993). Consistently, fractionation studies indicated that heterodimers containing ErbB2 did not reach endosomes (Wang *et al.*, 1999). Recently, Hommelgaard *et al.* (2004) reported that ErbB2 is retained at membrane protrusions and excluded from clathrin-coated pits. However, also recent studies have supported the contention that ErbB2 is endocytosed, but rapidly recycled, to the plasma membrane (Klapper *et al.*, 2000; Hendriks *et al.*, 2003; Austin *et al.*, 2004).

The fact that different conclusions have been reached on whether ErbB2 can be endocytosed or not could in part be explained by use of different model systems. All studies described have been performed by comparing results from different cell lines. We therefore set out to systematically investigate this issue by creating stably transfected cells where the expression of EGFR was constant in all the cell clones, but the expression of ErbB2 varied between the clones. From these studies, we now conclude that ErbB2 is not endocytosed and that in contrast to EGFR homodimers, EGFR–ErbB2 heterodimers are endocytosis resistant. We further demonstrate that the endocytosis resistance of ErbB2-containing heterodimers is associated with inefficient EGF-induced formation of clathrin-coated pits compared with when the EGFR is present in homodimers.

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Abbreviations used: PAE, porcine aortic endothelial.

MATERIALS AND METHODS

Materials

Human recombinant EGF was from Bachem (Bubendorf, Switzerland). Alexa Fluor 488-conjugated EGF (Alexa 488-EGF), rhodamine (Rh)-conjugated EGF (Rh-EGF), Rh-conjugated transferrin (Tf) (Rh-Tf), and Zeocin were from Invitrogen (Carlsbad, CA). Dako fluorescent mounting medium was from DakoCytomation Denmark A/S (Glostrup, Denmark). ^{125}I -EGF was from GE Healthcare (Little Chalfont, Buckinghamshire, United Kingdom). FuGENE 6 was from Roche Diagnostics (Mannheim, Germany). Other chemicals were from Sigma-Aldrich (St. Louis, MO) unless otherwise noted.

Antibodies

Sheep anti-EGFR antibody was from Fitzgerald Industries International (Concord, MA). Mouse anti-ErbB2 (Ab-8, intracellular domain), rabbit anti-ErbB2 (Ab-1, aa 1243–1255), and mouse anti-EGFR (Ab-3) antibodies were from NeoMarkers (Fremont, CA). Mouse anti-ErbB2 (extracellular domain), rabbit anti-ErbB2 (intracellular domain), mouse anti-hemagglutinin (HA) and mouse anti-Tf receptor (TfR) antibodies were from Zymed Laboratories (South San Francisco, CA). Rabbit anti-phospho EGFR (pY1086), rabbit anti-Myc, and rabbit anti-green fluorescent protein (GFP) antibodies were from Abcam (Cambridge, United Kingdom). Mouse anti-phospho EGFR (pY1173) antibody was from Upstate Biotechnology (Lake Placid, NY). Mouse anti-phospho EGFR (pY1068), rabbit anti-phospho EGFR (pY1045), and rabbit anti-p-Akt antibodies were from Cell Signaling Technology (Beverly, MA). Rabbit anti-EGF, mouse anti-EGFR (sc-120), mouse anti- α -adaptin, and rabbit anti-extracellular signal-regulated kinase (Erk) antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit anti-pErk antibody was from New England Biolabs (Beverly, MA). Phycoerythrin-conjugated goat anti-mouse, Cy2-conjugated donkey anti-rabbit, rhodamine red-X-conjugated donkey anti-rabbit, rhodamine red-X-conjugated donkey anti-mouse, Cy5-conjugated donkey anti-rabbit, peroxidase-conjugated donkey anti-mouse IgG, and peroxidase-conjugated donkey anti-sheep IgG antibodies were all from Jackson ImmunoResearch Laboratories (West Grove, PA). Alexa 488-conjugated goat anti-mouse antibody was from Invitrogen. Peroxidase-conjugated donkey anti-rabbit IgG was from Sigma-Aldrich. Rabbit anti-mouse IgG was from Cappel/ICN Biomedicals (Aurora, OH).

Plasmids

pcDNA3.1-ErbB2 was generated by PCR amplification of full-length ErbB2 from the pRK5-HER2-GFP (a gift from Andrew Chantry, University of East Anglia, Norwich, United Kingdom) using gene-specific primers 5'-AGA AGC TTC ACA CTG GCA CGT CCA GAC CCA G-3' and 5'-AGG CTA GCC GCA GTG AGC ACC ATG G-3' (Invitrogen) with restriction sites for *NheI* and *HindIII* included. The PCR product was directly cloned into the pCR-Blunt II-TOPO (Invitrogen). A positive clone was digested with *NheI* and *HindIII* and ligated into the respective sites of pcDNA3.1/Zeo (Invitrogen). pRK5-myc-ErbB2 Δ N was generated by PCR amplification of the DNA encoding the 200 amino acids at the C-terminal end of ErbB2 from pcDNA3.1-ErbB2. This was performed by using the gene-specific primers 5'-GGC GAA TTC CTA CAC TGG CAC GTC CAC ACC-3' and 5'-CCG GGA TCC GGT GGG GAC CTG ACA CTA GG-3' with restriction sites for *BamHI* and *EcoRI* included. The PCR product was cloned into the *BamHI* and *EcoRI* sites of pRK5-myc, which was provided by Alan Hall (University College, London, United Kingdom). The plasmid pcDNA3.1-ErbB2 Δ C was generated by subjecting pcDNA3.1-ErbB2 to mutagenesis changing Tyr-1248 of ErbB2 into a stop codon by using the QuikChange XL kit (Stratagene, La Jolla, CA). Primers were designed containing a mismatch in this codon of the ErbB2 DNA-sequence. The mismatch is underlined: 5'-CAC GTC CAG ACC CAG CTA CTC TGG GTT CTC TGC-3' and 5'-GCA GAG AAC CCA GAG TAG CTG GGT CTG CAG GTG-3'. The pMT123 plasmid encoding HA-ubiquitin $\times$ 8 was obtained from Dirk Bohmann (University of Rochester, Rochester, NY).

Cell Culture, Treatment, and Transfection

Stably transfected porcine aortic endothelial (PAE) cells expressing wild-type (wt) EGFR (PAE.B2) were obtained from Alexander Sorkin (University of Colorado Health Sciences Centre, Denver, CO). The cells were grown in Ham's F-12 (Cambrex Bio Science Copenhagen, Copenhagen, Denmark) supplemented with 10% (vol/vol) fetal bovine serum (FBS) (PAA Innovations, Linz, Austria), 0.5 $\times$ penicillin-streptomycin mixture (Cambrex Bio Science Copenhagen), and 400 $\mu\text{g}/\text{ml}$ G418 sulfate (Invitrogen). Clones of PAE.B2 cells (clones 1–4) stably expressing ErbB2 were established using FuGENE 6 transfection reagent, standard single-cell cloning procedures (Johansen *et al.*, 2001), and zeocin selection (30 $\mu\text{g}/\text{ml}$). Cells from PAE.B2 and clone 4 were transiently transfected with a pMT123 plasmid encoding HA-ubiquitin $\times$ 8, with pRK5-myc-ErbB2 Δ N or pcDNA3.1-ErbB2 Δ C using FuGENE 6. Transfected cells were analyzed 24 h upon transfection.

Immunoblotting

Cells were lysed in lysis buffer [10 mM Tris, pH 6.8, 5 mM EDTA, 50 mM NaF, 30 mM sodium pyrophosphate, 2% (wt/vol) SDS (Applichem, Darmstadt,

Germany), 1 mM phenylmethylsulfonyl fluoride (PMSF) (Fluka, Buchs, Switzerland), and 1 mM Na_3VO_4 (Stem Chemicals, Newburyport, MA)] for 10 min on ice. Sample buffer (4% (vol/vol) glycerol, 4% (vol/vol) β -mercaptoethanol, and 0.005% (wt/vol) bromophenol blue) was added before incubation at 95°C for 10 min. The lysates were subjected to SDS-PAGE before electrotransfer to nitrocellulose membranes (Hybond; GE Healthcare). The membranes were incubated with primary and secondary antibodies at room temperature for 1 h, and the reactive proteins were detected using enhanced chemoluminescence (GE Healthcare).

Immunoprecipitation

Cells were lysed with immunoprecipitation (i.p.) buffer A (phosphate-buffered saline [PBS], pH 7.5, with 10 mM EDTA, 1% Triton X-100, 10 mM NaF, 1 mM PMSF, 1 mM Na_3VO_4 , 20 $\mu\text{g}/\text{ml}$ leupeptin, 10 $\mu\text{g}/\text{ml}$ aprotinin, and 1 mM *N*-ethylmaleimide [NEM]) and incubated with protein G- or protein A-coupled magnetic beads (DynaL Biotech, Oslo, Norway). The magnetic beads were precoupled with antibody to EGFR or ErbB2 in 50 mM Tris-HCl, pH 7, at room temperature for 1 h or at 4°C overnight. The beads were washed four times with i.p. buffer A before cell lysates were added. Beads and cell lysates were gently mixed for 1 h at room temperature or at 4°C overnight, before being washed four times with i.p. buffer A and once with 10% (vol/vol) PBS in H_2O . The immunoprecipitate was eluted in 2 $\times$ sample buffer [10 mM Tris-HCl, pH 6.8, 10 mM EDTA, 100 mM NaF, 60 mM sodium pyrophosphate, 4% (wt/vol) SDS, 2% (vol/vol) β -mercaptoethanol, 20% (vol/vol) glycerol, and 0.006% (wt/vol) bromophenol blue], incubated at 95°C for 5 min, and subjected to SDS-PAGE and immunoblotting. To investigate ubiquitination of EGFR, cells were lysed in SDS (1%) containing PBS, incubated at 100°C for 5 min, and chilled on ice before homogenization using a QIA-shredder column (QIAGEN, Valencia, CA). The lysates were added to protein G-coupled magnetic beads (DynaL Biotech) precoupled to EGFR (as described above). The beads were dissolved in 2 $\times$ i.p. buffer B (2% (vol/vol) Triton X-100, 0.5% (wt/vol) sodium deoxycholate, 1% (wt/vol) bovine serum albumin (BSA), 2 mM EDTA, 40 mM NaF, 2 mM PMSF, 4 mM Na_3VO_4 , 40 $\mu\text{g}/\text{ml}$ leupeptin, 20 $\mu\text{g}/\text{ml}$ aprotinin, and 2 mM NEM). Antibody-coupled magnetic beads and cell lysates were gently mixed for 1 h at 4°C. The beads were then washed in 1 $\times$ i.p. buffer B (50% 2 $\times$ i.p. buffer B + 50% SDS [1%] in PBS), eluted in 2 $\times$ sample buffer, and eventually subjected to SDS-PAGE and immunoblotting, as described above.

Immunocytochemistry and Confocal Microscopy

The cells were grown on MENZEL-GLÄSER 12-mm coverslips (Gerhard Menzel, Glasbearbeitungswerk, Braunschweig, Germany). After incubation with indicated compounds, the cells were washed in PBS and fixed in pre-heated (37°C) 4% (wt/vol) paraformaldehyde (PFA) (Riedel-de Haën, Seelze, Germany) in Soerensen's phosphate buffer for 5 min. Cells were then washed three times in PBS before anti-quenching in 50 mM NH_4Cl for 10 min at room temperature and washing twice in PBS. Fixed cells were permeabilized with 0.1% Triton X-100 in PBS and incubated with BSA [1% (wt/vol) in PBS] for 30 min before incubation with a primary antibody for 1 h. Coverslips were washed with PBS before subsequent incubation with a secondary antibody for 30 min before mounting, using Dako fluorescent mounting medium. The cells were examined using a confocal microscope (TCSXP; Leica, Wetzlar, Germany).

Internalization and Recycling of ^{125}I -EGF

Cells in 24-well microtiter plates were incubated with 1 ng/ml ^{125}I -EGF in minimal essential medium (MEM) without HCO_3^- with 0.1% (wt/vol) BSA at 37°C for the times indicated. In the control (0-min time point), ^{125}I -EGF was added and then immediately removed from the cells. The cells were washed three times with PBS. Surface-bound ^{125}I -EGF was removed by incubating the cells in MEM with 3 $\mu\text{g}/\text{ml}$ Pronase E for 1 h at 4°C. The ^{125}I -EGF in the supernatant fraction (representing surface-bound EGF) and the pelleted cells (representing internalized ^{125}I -EGF) was separated by centrifugation and subsequently measured in a gamma counter (Wallac 1470 Wizard; PerkinElmer Wallac, Turku, Finland). The ratio of internalized to surface localized cpm was plotted against time. Recycling of EGF was analyzed essentially as described previously (Babst *et al.*, 2000). Because clone 4 cells and PAE.B2 cells easily detach from plastic on ice, recycling of EGF was measured in cells in solution that had been trypsinized using 0.05% trypsin/EDTA solution (Cambrex Bio Science Copenhagen) and subsequently resuspended and incubated in MEM without HCO_3^- and with 0.1% BSA at 37°C for 30 min. The cells were pelleted by centrifugation at $410 \times g$ for 5 min before loading with 50 ng/ml ^{125}I -EGF in MEM without HCO_3^- and with 0.1% BSA for 20 min at 37°C. On loading, the surface-localized radioactivity was removed by a glycine-buffered solution, pH 3.0 (Babst *et al.*, 2000), followed by chase in MEM without HCO_3^- and with 0.1% BSA at 37°C. Then, the cells were washed once with the pH 3.0 buffer to remove recycled EGF at the cell surface. At the 0-min point, cells were incubated with ice-cold MEM without HCO_3^- and with 0.1% BSA for 2 min on ice before being washed once with the pH 3.0 buffer. The chase medium and the pH 3.0 wash buffer were combined in one fraction and analyzed for degraded and recycled EGF as

described previously (Skarpen *et al.*, 1998). The cpm in the cell pellet represents intracellularly localized EGF.

Flow Cytometry

Cells were harvested by trypsinization and washed twice in buffer A (PBS with 2% FBS and 2 mM EDTA) before being fixed in 4% PFA (wt/vol) in Soerensen's phosphate buffer. After fixation, the cells were washed twice and incubated with primary antibody diluted in buffer A for 30 min. The cells were washed twice before incubation for 30 min with secondary antibody (phycoerythrin-conjugated goat anti-mouse). The cells were washed twice, resuspended in buffer A and analyzed using a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA).

Immunoelectron Microscopy (Immuno-EM)

Cells were fixed using PFA [4% (wt/vol)] and glutaraldehyde [0.1% (wt/vol)] in Soerensen's phosphate buffer and processed as described by Griffiths *et al.* (1984). Immunocytochemical labeling of thawed cryosections was performed essentially as described by Griffiths *et al.* (1983), using protein A-gold (purchased from G. Posthuma, Utrecht, The Netherlands) or gold coated with donkey anti-mouse IgG or donkey anti-rabbit IgG from Jackson ImmunoResearch Laboratories. Sections were examined using a Philips CM120 or Tecnai 12 transmission electron microscope equipped with a MegaView II or III TEM Soft Imaging System, respectively. To estimate the distribution (percentage) of EGFR at the plasma membrane and in endosomes in each experiment, at least 200 gold particles were counted for each labeling experiment. Identification of endosomes was based on morphology. To estimate the number of clathrin-coated pits at the plasma membrane, randomly oriented sections were scanned in a systematic random manner. 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. Identification of coated pits was based on morphology and labeling for α -adaptin, and the number of coated pits per micrometer of plasma membrane was calculated (Griffiths, 1993). The results represent the mean of three independent labeling experiments $\pm$ SD, and in each parallel 10 randomly chosen cells were quantified. The EGFR was detected with a mixture of mouse anti-EGFR (antibody-3) and mouse anti-EGFR (sc-120) antibodies. The ErbB2 was detected with mouse anti-ErbB2 (extracellular domain) antibody, and EGF was detected with a rabbit anti-EGF antibody.

RESULTS

ErbB2 Is Not Endocytosed, and Overexpression of ErbB2 Inhibits Down-Regulation of EGFR from the Plasma Membrane as Well as Degradation of EGFR

To investigate the effect of overexpressing ErbB2 on internalization of the EGFR, we created stably transfected PAE cells expressing the same amount of EGFR, but expressing increasing amounts of ErbB2. The expression of ErbB2 in several clones was investigated by flow cytometry and by Western blotting (our unpublished data), and four different clones were selected for further investigations. As demonstrated (Figure 1A) clones 1–4 expressed increasing levels of ErbB2 compared with the parent cell line PAE.B2. Subcellular localization of ErbB2, as well as internalization of Alexa 488-EGF ligated to the EGFR in EGFR homodimers and in EGFR–ErbB2 heterodimers/oligomers, was investigated by immunofluorescence and confocal microscopy. ErbB2 was consistently found at the plasma membrane and not in endosomes (Figure 1B). It should be noted that vesicular staining of ErbB2 was previously observed upon incubating ErbB2-overexpressing cells with geldanamycin (Longva *et al.*, 2005). The inability to find ErbB2 in endosomes can therefore not be explained by the antibodies used to recognize ErbB2. On incubation with Alexa 488-EGF (15 ng/ml) for 15 min at 37°C, fluorescing endosomes were observed in cells expressing low levels of ErbB2. However, in cells expressing higher levels of ErbB2, the punctuate staining representing endosomes was significantly reduced (Figure 1B). This indicated that ErbB2 is not endocytosed and further that overexpression of ErbB2 inhibits endocytosis of Alexa 488-EGF. To confirm that overexpression of ErbB2 inhibited initial steps of endocytosis, internalization of EGF was investigated by an internalization assay with low and nonsat-

urating concentrations of 125 I-EGF (1 ng/ml) (Lund *et al.*, 1990; Wiley *et al.*, 1998) (Figure 1C). The internalization assay was performed by continuous incubation at 37°C, as described (Huang *et al.*, 2004). As demonstrated, the rate of internalization was significantly inhibited in clone 4 cells compared with PAE.B2 cells.

Immuno-EM was further used to study EGF-induced internalization of the ligand-bound EGFR. PAE.B2 cells expressing different levels of ErbB2 (PAE.B2 and clones 3 and 4) were incubated with EGF (15 ng/ml) at 37°C for increasing time periods (Table 1). In all three cell clones, the EGFR localized mainly to the plasma membrane upon brief incubation with EGF (5 min). Prolonged incubation with EGF significantly reduced the fraction of EGFR localizing to the plasma membrane in PAE.B2 cells. In clone 3 cells, however, the fraction of EGFR at the plasma membrane was only slightly reduced, and in clone 4 cells, the EGFR was observed at the plasma membrane only, regardless of incubation with EGF. Comparable results were obtained when antibody to EGF instead of to the EGFR was used. Subcellular localization of EGF and ErbB2 upon incubation of the cells with EGF (15 ng/ml) at 37°C for 30 min is demonstrated in Figure 2. In PAE.B2 cells (Figure 2, A–C) labeling for EGF was upon incubation with EGF for 30 min found all along the endocytic pathway with a high amount of labeling in multivesicular bodies. In clones 3 cells (our unpublished data) and clone 4 cells, labeling for EGF (Figure 2, D–F) and ErbB2 (Figure 2F) was restricted to smooth parts of the plasma membrane and seemed to be excluded from clathrin-coated pits and endosomes. Quantitation of the labeling results is presented in Table 1. Together, these observations demonstrate that overexpression of ErbB2 efficiently inhibits EGF-induced endocytosis of the EGFR.

We additionally used flow cytometry to study the effect of overexpression of ErbB2 on down-regulation of EGFR from the plasma membrane (Figure 3A). On 5-h incubation with EGF (60 ng/ml) in the presence of cycloheximide, a significant decrease in the amount of EGFR at the plasma membrane was observed in PAE.B2 cells and in clones 1 and 2. However, no significant reduction of EGFR from the plasma membrane was observed in clones 3 and 4. We further studied the effect of ErbB2 on degradation of the EGFR, comparing the EGF-induced degradation in the parent cell PAE.B2 with the EGF-induced degradation of EGFR in clones 1–4. The cells were incubated with EGF (60 ng/ml) for 1–5 h at 37°C. When PAE.B2 cells were exposed to EGF, significant degradation of the EGFR was observed (Figure 3B). Incubation of clones 1 and 2 with EGF also resulted in degradation of the EGFR. However, in clones 3 and 4, no degradation could be detected by Western blotting using antibody to the EGFR. Together, these results demonstrate that increasing levels of ErbB2 increasingly inhibit EGF-induced internalization and down-regulation of the EGFR. Potentially, blunted degradation of EGFR could result from blocked endocytosis or from lack of lysosomal sorting due to rapid recycling. To investigate whether the effect of overexpressing ErbB2 on degradation of EGFR was due to lack of endocytosis or increased recycling, we first studied the subcellular localization of ErbB2 upon inhibiting recycling by incubating clone 4 cells with the ionophore monensin. As demonstrated in Figure 4A, the TfR was observed to accumulate perinuclearly. This demonstrated that monensin efficiently blocked recycling of the TfR. However, the subcellular distribution of ErbB2 was unaltered. This is consistent with a block in endocytosis of ErbB2 and does not support the hypothesis that ErbB2 is rapidly recycled upon constitutive endocytosis. The same result also was obtained upon

Figure 1. Overexpression of ErbB2 inhibits endocytosis of EGF. (A) Flow cytometry analysis of ErbB2, using mouse anti-ErbB2 antibody (extracellular domain), in stably transfected cells expressing the same amount of EGFR, but different amounts of ErbB2. Flow cytometry was used to generate histograms of expression of ErbB2 in different clonal populations. PAE.B2 is the parental cell line, and clones 1–4 are generated by stable transfection with a plasmid encoding ErbB2 (see *Materials and Methods*). (B) PAE.B2 cells as well as clones 1–4 were incubated with Alexa 488-EGF (15 ng/ml) for 15 min at 37°C before fixation and immunocytochemical labeling with mouse anti-ErbB2 antibody (extracellular domain). Confocal microscopy was used to localize ErbB2 (left) and Alexa 488-EGF (right). Bar, 10 μ m. (C) Cells from clone 4 and PAE.B2 cells were incubated with 1 ng/ml 125 I-EGF for 0, 4, and 8 min, and the ratio of surface-bound/internalized 125 I-EGF was calculated and plotted against time.

incubation of the cells with EGF and monensin, demonstrating that ErbB2 was not endocytosed and recycled in the presence of EGF (our unpublished data). We further investigated the constitutive recycling of the EGFR, again by incubating PAE.B2 cells and clone 4 cells with monensin in the absence of EGF. As demonstrated in Figure 4B, the EGFR slightly accumulated perinuclearly in PAE.B2 cells, whereas almost no change in subcellular localization could be observed in clone 4 cells. This argues that there is less constitutive endocytosis and recycling of the EGFR in cells overexpressing ErbB2 than in cells not expressing ErbB2. To investigate whether ErbB2 affected the rate of recycling of activated EGFR, the rate of recycling of EGFR-bound 125 I-EGF (50 ng/ml) was measured in PAE.B2 cells and in clone 4 cells essentially as described previously (Babst *et al.*, 2000).

As demonstrated (Figure 5), the rate of recycling was similar whether or not ErbB2 was overexpressed. This finding further supports the conclusion that ErbB2 is not significantly internalized and recycled.

Ligand-Independent Heterodimerization/Oligomerization of the EGFR and ErbB2 Correlates with the Level of ErbB2 Expression

Heterodimerization of EGFR and ErbB2 was studied in clones 1–4. Immunoprecipitation of EGFR and ErbB2 was performed using cells that had been incubated in the absence or presence of EGF (60 ng/ml) for 2 min at 37°C. The cells were lysed, and the cell lysate was immunoprecipitated with antibodies to EGFR or to ErbB2. The immunoprecipi-

Table 1. ErbB2 inhibits EGF-induced endocytosis of the EGFR

	Incubation time (min) with EGF		
	5	15	30
PAE.B2	97	83	53
Clone 3	100	94	82
Clone 4	100	100	95
PAE.B2	3	17	47
Clone 3	0	6	18
Clone 4	0	0	5

PAE.B2 cells and cells from clones 3 and 4 were incubated with EGF (15 ng/ml) for 5, 15, and 30 min at 37°C and prepared for immuno-EM. Thawed cryosections were labeled using anti-EGFR antibodies, and the labeling distribution (plasma membrane or endosomes) was quantified. Top three rows, localization of EGFR to the plasma membrane. Bottom three rows, localization of EGFR in early and late endosomes. In each labeling experiment the total number of gold particles localizing to the plasma membrane, including clathrin-coated pits, and to endosomes, was counted, and the distribution (percentage) was calculated. Approximately 200 gold particles were counted in each experiment.

tated material was then analyzed by Western blotting, using antibodies to ErbB2 and EGFR, respectively. Immunoprecipitation of the EGFR coprecipitated increasing amounts of ErbB2 from clones 1–4. Correspondingly, immunoprecipitation of ErbB2 coprecipitated increasing amounts of EGFR (Figure 6). It should be noted that the heterodimerization/oligomerization seemed to be independent of EGF. Our data thus suggest that increasing the expression of ErbB2 results in ligand-independent heterodimerization/oligomerization of EGFR and ErbB2.

Overexpression of ErbB2 Does Not Inhibit EGF-induced Phosphorylation and Ubiquitination of the EGFR

To examine whether the level of ErbB2 qualitatively or quantitatively affected activation of the EGFR, we performed Western blotting using antibodies recognizing phosphorylated tyrosines (pY1045, pY1068, pY1086, and pY1173) in the EGFR tail. This experiment (Figure 7A) demonstrated that the activation of EGFR was equally efficient and that tyrosines 1045, 1068, 1086, and 1173 were as strongly phosphorylated in clone 4 cells as in PAE.B2 cells harboring EGFR homodimers only. We further studied the effect of heterodimerization/oligomerization on EGF-induced ubiquitination of the EGFR. The cells were first transiently transfected with a plasmid encoding HA-ubiquitin. Then, the cells were incubated with or without EGF (60 ng/ml, 37°C), and the EGFR was immunoprecipitated under denaturing conditions. The precipitated material was subjected to Western blotting using an antibody to HA. As demonstrated in Figure 7B, the same extent of ubiquitination of the EGFR was observed in PAE.B2 cells and in clone 4 cells. These data demonstrate that the EGFR was phosphorylated similarly in EGFR homodimers and in EGFR–ErbB2 heterodimers/oligomers and that the EGFR was ubiquitinated regardless of overexpression of ErbB2.

We further investigated individual ErbB2-overexpressing cells by immunofluorescence and confocal microscopy. Cells from clone 4 were incubated with Rh-EGF for 15 min at 37°C. Then, the cells were fixed and immunostained using antibodies to ErbB2 as well as to pY1068 in the EGFR. To investigate whether the tyrosine mainly responsible for re-

Figure 2. Endocytosis of EGF happens in the absence of ErbB2, but not in the presence of overexpressed ErbB2. PAE.B2 cells (A–C) and clone 4 cells (D–F) were incubated with EGF (15 ng/ml) for 30 min at 37°C, processed for immuno-EM, and singly labeled against EGF (A–E) or doubly labeled against EGF (large gold) and ErbB2 (small gold) (F). In PAE.B2 cells, EGF localized to clathrin-coated pits (A), to clathrin-coated vesicles (B), and to multivesicular bodies (C). (A) Arrowheads indicate the neck region of the clathrin-coated pit before scission. (B) The arrow indicates EGF in a clathrin-coated vesicle. In clone 4 cells (D–F), EGF (D–E) and ErbB2 (F, small gold particles) localized to the plasma membrane. (D) Arrowheads indicate a shallow clathrin-coated pit. CV, clathrin-coated vesicle; CP, clathrin-coated pit; MVB, multivesicular body). Bar, 100 nm.

cruiting Grb2 as well as phospholipase C (PLC)γ (pTyr1068) was equally phosphorylated in homodimers and heterodimers, we used cells from clone 4 with a high passage number. On multiple passages, some cells had lost ErbB2 (Figure 7C). In cells expressing EGFR and overexpressing ErbB2, Rh-EGF was not internalized; however, the EGFR still showed labeling with an antibody recognizing pTyr1068 (Figure 7C).

Overexpression of the C-Terminal Part of ErbB2 Does Not Induce Endocytosis of Full-Length ErbB2

It has been demonstrated that the C-terminal part of ErbB2 interacts with PDZ domain-containing proteins such as

Figure 3. Endocytic down-regulation of EGFR is reduced as a result of overexpression of ErbB2. (A) Flow cytometry was performed to detect down-regulation of EGFR from the plasma membrane upon incubation with EGF (60 ng/ml) for 5 h at 37°C. Cycloheximide (CHX; 25 μ g/ml) was included in the medium, and control cells were incubated with CHX only. The cells were then fixed and immunostained with mouse anti-EGFR antibody. (B) Cells were incubated with EGF (60 ng/ml) at 37°C in the presence of CHX and lysed upon the indicated incubation periods (hours). The lysates were subjected to Western blotting with sheep anti-EGFR antibody and rabbit anti-Erk antibody (loading control).

Figure 4. ErbB2 and EGFR are not constitutively endocytosed and recycled in cells overexpressing ErbB2. (A) Cells from clone 4 were incubated with or without 10 μ M monensin at 37°C for 60 min. Then, the cells were fixed and immunochemically labeled with mouse anti-TfR antibody and rabbit anti-ErbB2 antibody (intracellular domain) before confocal microscopy. (B) PAE.B2 cells and clone 4 cells were incubated with or without monensin as described in A. The cells were fixed and immunocytochemically labeled with sheep anti-EGFR antibody before confocal microscopy. Bar, 10 μ M.

Lin-7 and Erbin, and such interactions have been proposed as partly responsible for slowing down endocytosis of ErbB2 (Borg *et al.*, 2000; Jaulin-Bastard *et al.*, 2001; Birrane *et al.*, 2003; Shelly *et al.*, 2003). To investigate whether anchoring of the C-terminal part of ErbB2 was responsible for the lack of endocytosis observed, we cloned and overexpressed the C-terminal part of ErbB2 encompassing the 200 very C-terminal amino acids (ErbB2 Δ N). This Myc-tagged part of ErbB2 was overexpressed in PAE cells with EGFR and ErbB2 (clone 4) upon transient transfection. As demonstrated in Figure 8, B and D, there was no vesicular staining of ErbB2 in cells overexpressing the Erbin-binding part of ErbB2 when cells

Figure 5. Overexpression of ErbB2 does not affect the rate of EGFR recycling. Recycling of EGF in PAE.B2 cells and clone 4 cells was analyzed as described in *Materials and Methods*. Cells were loaded with 50 ng/ml 125 I-EGF for 20 min before stripping of surface localized 125 I-EGF and subsequent chase at 37°C in medium without EGF. 125 I-EGF (cpm intracellularly, degraded, and recycled) was measured, and percent recycled 125 I-EGF was plotted against time. The mean of two representative experiments $\pm$ SD is demonstrated.

had been incubated in the absence (B) or presence (D) of EGF. This experiment therefore demonstrates that overexpressing the C-terminal part of ErbB2 does not induce endocytosis of ErbB2. To ensure that the entire C-terminal fragment was overexpressed, we analyzed the transfected cells by Western blotting, using an antibody to the C-terminal part of ErbB2 (Ab-1). As demonstrated in Figure 8E, a band of ~20 kDa was recognized by the anti-ErbB2 antibody. It should be noted that only ~20% of the cells were transfected, and the amount of the fragment relative to full-length ErbB2 in the transfected cells is therefore underestimated. The fact that the C-terminal part of ErbB2 is indeed overexpressed without facilitating endocytosis of ErbB2, strongly suggests that upon overexpression, the C-terminal part of ErbB2 cannot compete out a potential anchoring of ErbB2 to a scaffolding protein. This suggests that the inhibited endocytosis of ErbB2 must be explained by other mechanisms.

Figure 6. Increasing numbers of EGFR-ErbB2 heterodimers/oligomers are constitutively formed upon increasing overexpression of ErbB2. Clones with different levels of ErbB2 were incubated with or without EGF (60 ng/ml) for 2 min at 37°C before lysis and immunoprecipitation with sheep anti-EGFR or rabbit anti-ErbB2 (Ab-1) antibodies. The immunoprecipitates were finally subjected to Western blotting using mouse anti-ErbB2 (Ab-8) and sheep anti-EGFR antibodies.

Mutant ErbB2 Lacking the 8 C-Terminal Amino Acids Is Endocytosis Deficient

The interpretation that overexpressing the C-terminal fragment of ErbB2 does not compete out an anchoring interaction that could normally explain the endocytosis deficiency of ErbB2 relies on correct folding of the overexpressed ErbB2 fragment. We therefore additionally constructed an ErbB2 mutant encoding a protein lacking the C-terminal amino acids that interact with PDZ domain proteins (ErbB2ΔC). This truncated ErbB2 was efficiently overexpressed upon transient transfection of PAE.B2 cells harboring the EGFR only (Figure 9, A and C). By Western blotting experiments, we found that the antibody recognizing the 12 very C-terminal amino acids of ErbB2 (Ab-1) did, as expected, not recognize ErbB2ΔC (Figure 9C), in contrast to the anti-ErbB2 antibody Ab-8. Overexpression of the truncated ErbB2 inhibited endocytosis of fluorescing EGF, as did wild-type ErbB2 (compare Figure 9B with Figure 1B). This strengthens the interpretation that ErbB2 is not endocytosis deficient due to anchoring of the tail.

Activated EGFR-ErbB2 Heterodimers Do Not Induce Formation of Clathrin-coated Pits, in Contrast to EGFR Homodimers

We have demonstrated that binding of EGF to the EGFR induces formation of new clathrin-coated pits (Johannessen, Pedersen, Pedersen, Madhus, and Stang, unpublished data). To investigate whether the endocytosis deficiency of ErbB2 could be explained by inability of EGFR-ErbB2 heterodimers/oligomers to induce formation of coated pits, we incubated PAE.B2 cells and cells from clone 4 with EGF for 3 min at 37°C upon serum starvation. By immuno-EM, we quantified the number of clathrin-coated pits per micrometer of plasma membrane in cells with or without overexpression of ErbB2 and with or without incubation with EGF. As demonstrated in Figure 10, the number of clathrin-coated pits per micrometer of plasma membrane was increased more than twofold, when EGF was added to serum-starved PAE.B2 cells. However, when clone 4 cells were incubated in the same way, the number of clathrin-coated pits per micrometer of plasma membrane did not increase. Together, these findings argue that activated heterodimers/oligomers of EGFR and ErbB2 do not, in contrast to EGFR homodimers, induce formation of clathrin-coated pits. The reason that heterodimers, in contrast to homodimers, do not induce new clathrin-coated pits can either be due to altered downstream signaling or to inefficient recruitment of an essential protein. As demonstrated in Figure 11, we found that activation of mitogen-activated protein kinase (MAPK) (measured by using an antibody to pErk), as well as activation of Akt (measured by using an antibody to pAkt as readout of phosphoinositide-3 kinase [PI3K]) was similar when EGF was added to PAE.B2 cells and to clone 4 cells. This is consistent with our unpublished findings (Johannessen, Pedersen, Pedersen, Madhus, and Stang, unpublished data) that neither MAPK nor PI3K seems to be required for EGF-induced coated pit formation.

DISCUSSION

ErbB2 is frequently overexpressed in epithelial cancers such as breast and ovarian carcinomas, and this is associated with poor prognosis and treatment resistance (Slamon *et al.*, 1987). ErbB2 is initially overexpressed due to gene amplification, but ErbB2 also has a very long half-life due to lack of lysosomal degradation (Yarden, 2001). Wang *et al.* (1999)

Figure 7. Phosphorylation and ubiquitination of the EGFR happens regardless of overexpression of ErbB2. (A) Cells from PAE.B2 and clone 4 were incubated with or without EGF (60 ng/ml) for 2 min at 37°C. The cells were lysed and subjected to Western blotting using antibodies recognizing phosphorylated EGFR (rabbit anti-phospho EGFR [pY1045], mouse anti-phospho EGFR [pY1068], rabbit anti-phospho EGFR [pY1086], or mouse anti-phospho EGFR [pY1173]) or a rabbit anti-Erk antibody (loading control). (B) Cells from PAE.B2 and clone 4 were transfected with HA-ubiquitin. The cells were incubated with or without EGF (60 ng/ml) for 2 min at 37°C before lysis and immunoprecipitation with sheep anti-EGFR antibody. The immunoprecipitate was subjected to Western blotting using mouse anti-HA or sheep anti-EGFR antibodies. (C) Cells from clone 4 were incubated with Rh-EGF (15 ng/ml) for 15 min at 37°C. On fixation, the cells were immunocytochemically labeled with rabbit anti-ErbB2 (intracellular domain) and mouse anti-phospho EGFR (pY1068) antibodies before confocal microscopy. Asterisk (*) indicates ErbB2 overexpressing cell. Bar, 10 μ m.

demonstrated that in the four breast cancer cell lines MDA453, SKBr3, BT474, and BT20, the EGFR–ErbB2 heterodimerization levels were positively correlated with the ratio of ErbB2/EGFR expression levels and negatively correlated with endocytosis of the EGFR and that microinjection of an ErbB2 expression plasmid into BT20 cells significantly inhibited EGF-stimulated EGFR endocytosis. However, the question of whether ErbB2 is in fact endocytosis deficient is still debated. It was recently published that ErbB2 is constitutively endocytosed but rapidly recycled (Austin *et al.*, 2004). We recently reported that ErbB2 is endocytosis deficient (Longva *et al.*, 2005), and Hommelgaard *et al.* (2004) reported that ErbB2 is being retained on cellular protrusions and cannot be observed to enter clathrin-coated pits.

Because comparing nonisogenic cell lines obviously has inherent problems, we have now generated isogenic cell lines that express the same level of EGFR but that increasingly overexpress ErbB2. PAE cells originally lacking all members of the EGFR family were initially stably transfected with cDNA encoding the EGFR. These PAE.B2 cells

(Jiang *et al.*, 2003) were then stably transfected with cDNA encoding ErbB2, and clones expressing different amounts of ErbB2 were selected and expanded. On analysis of these cells, we again conclude that ErbB2 is endocytosis deficient. We further found that increasing overexpression of ErbB2 inhibited endocytosis of the EGFR as well as down-regulation of the EGFR upon incubation of the cells with EGF. Consistent with the findings of Wang *et al.* (1999), we found that heterodimers/oligomers of ErbB2 and EGFR are constitutively formed upon overexpression of ErbB2 and that such oligomers are not internalized. By immuno-EM analysis of the cell lines expressing the most ErbB2 (clones 3 and 4), we observed both ErbB2 and the EGFR at cellular protrusions (our unpublished data), as did Hommelgaard *et al.* (2004), and we found virtually no EGFR in clathrin-coated pits or in endosomes upon incubation of cells with EGF. This is consistent with a lack of endocytosis. We have further demonstrated that the inability to find ErbB2 in endosomes by immunofluorescence microscopy and by immuno-EM is not the result of rapid recycling upon endocytosis, because incubation of the cells with monensin, resulting in inhibited

Figure 8. Overexpression of the C-terminal fragment of ErbB2 does not induce internalization of ErbB2. (A–D) Cells from clone 4 were transiently transfected with a plasmid encoding the Myc-tagged 200 C-terminal amino acids of ErbB2 (ErbB2ΔN). Cells were incubated without EGF (A and B) or with EGF (15 ng/ml) (C and D) for 15 min at 37°C, fixed, and immunocytochemically labeled with rabbit anti-Myc (A and C) or mouse anti-ErbB2 (extracellular domain) (B and D) antibodies (asterisk [*] indicates transfected cells). Bar, 10 μ m. (E) Cells from clone 4 were transiently transfected with ErbB2ΔN and subsequently subjected to Western blotting with two different antibodies to ErbB2 (lanes 1 and 2: antibody to aa 1243–1255 of ErbB2 [Ab-1] and lane 3: antibody to the intracellular domain of ErbB2 [Ab-8]) as well as antibody to Erk (loading control).

recycling and accumulation of the TfR in endosomes, did not cause redistribution of ErbB2 from the plasma membrane to endosomes (Longva *et al.*, 2005; this study).

As will be described in more detail elsewhere, we have recently discovered that the EGFR is in fact able to induce formation of new clathrin-coated pits (Johannessen, Pedersen, Pedersen, Madhus, and Stang, unpublished data). Such clathrin-coated pits were found to be induced when HeLa cells, where preexisting clathrin-coated pits had been removed by knocking down the α or μ subunits of activator protein-2 (AP2) by RNA interference, were subsequently incubated with EGF. EGF-induced formation of new clathrin-coated pits could further be observed when cells functionally depleted of AP2 by overexpression of a mutant of Eps15 lacking EH domains (EH95) (Benmerah *et al.*, 1999) were incubated with EGF (Johannessen, Pedersen, Pedersen,

Figure 9. ErbB2 lacking the eight very C-terminal amino acids is not endocytosed. PAE.B2 cells were transiently transfected with a plasmid encoding a truncated ErbB2 with a stop codon replacing Tyr-1248 (ErbB2ΔC). (A and B) Transfected cells were incubated with Alexa 488-EGF (15 ng/ml) for 15 min at 37°C and immunocytochemically labeled with mouse anti-ErbB2 antibody (extracellular domain). (C) PAE.B2 cells transiently transfected with ErbB2ΔC were lysed and subjected to Western blotting with antibodies to ErbB2 (lanes 1 and 2: antibody to aa 1243–1255 of ErbB2 [Ab-1]; lane 3, antibody to the intracellular domain of ErbB2 [Ab-8]) and to Erk (loading control).

Madhus, and Stang, unpublished data). Also, in EGF-treated HeLa and PAE.B2 cells with normal amounts of AP2, we were able to see EGF-induced formation of clathrin-coated pits upon serum starvation. We now report that the EGF-induced formation of clathrin-coated pits was indeed

Figure 10. EGFR–ErbB2 heterodimers do not induce formation of clathrin-coated pits, in contrast to EGFR homodimers. PAE.B2 and cells from clone 4 were serum-starved for 24 h before incubation with or without EGF (15 ng/ml) for 3 min at 37°C. Quantitative immuno-EM analysis of the number of clathrin-coated pits at the plasma membrane was then performed as described in *Materials and Methods*. The results are presented as percentage of the number of coated pits found in serum-starved cells not incubated with EGF. Each point is derived from three different labeling experiments $\pm$ SD.

Figure 11. EGFR–ErbB2 heterodimers activate Akt (readout of PI3 K) and Erk as efficiently as do EGFR homodimers. PAE.B2 cells and cells from clone 4 were incubated with or without EGF (60 ng/ml) for 15 min at 37°C. The cells were lysed, and the lysate was subjected to Western blotting using antibodies to pAkt, pErk, and Erk (loading control).

counteracted by overexpression of ErbB2. We currently have no explanation for this. Overexpression of dominant negative Grb2, incapable of interacting with proline rich domains, inhibited induction of clathrin-coated pits (Johannesen *et al.*, unpublished data), highlighting the importance of the major Grb2 binding sites of the EGFR in EGF-induced formation of coated pits. We therefore investigated whether Grb2 binding sites in the EGFR were phosphorylated in heterodimers. Our results demonstrate that Tyr1068 as well as Tyr1086 was efficiently phosphorylated regardless of overexpression of ErbB2. Additionally, the docking site for Cbl (pTyr1045) was efficiently phosphorylated in cells overexpressing ErbB2. This agrees with the finding that the EGFR was equally efficiently ubiquitinated whether ErbB2 was overexpressed or not. The EGFR in heterodimers/oligomers is therefore in principle able to interact with Grb2, Cbl, and PLC γ . However, there is the possibility that proper binding of Grb2 to the EGFR is compromised upon heterodimerization/oligomerization of EGFR with ErbB2.

It has been reported that interactions of ErbB2 with PDZ domain-containing proteins such as Lin-7 and Erbin, could partly be responsible for slowing down endocytosis of ErbB2 (Borg *et al.*, 2000; Jaulin-Bastard *et al.*, 2001; Birrane *et al.*, 2003; Shelly *et al.*, 2003). However, we have investigated this possibility by two different approaches. First, we overexpressed the C-terminal part of ErbB2 in ErbB2-overexpressing cells also expressing the EGFR to compete out a potential endocytosis-inhibiting interaction between ErbB2 and, for example, Erbin. However, this did not induce endocytosis of ErbB2. We then transiently overexpressed a mutant ErbB2 lacking the C-terminal part, reported to be involved in the interaction with PDZ-domain proteins. However, this mutant of ErbB2 was as inefficiently internalized as was wild-type ErbB2. We thus conclude that even though ErbB2 can interact with proteins such as Erbin and Lin7 (Borg *et al.*, 2000; Jaulin-Bastard *et al.*, 2001; Birrane *et al.*, 2003; Shelly *et al.*, 2003), such interactions do not explain the endocytosis resistance of ErbB2. Rather, we conclude that ErbB2 in fact inhibits EGF-induced formation of clathrin-coated pits when oligomerizing with the EGFR. This argues that the ability of the EGFR to induce clathrin-coated pits is physiologically

important and advances the understanding of the strong oncogenic effect of ErbB2.

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