

Pertuzumab increases epidermal growth factor receptor down-regulation by counteracting epidermal growth factor receptor-ErbB2 heterodimerization

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Abstract

Epidermal growth factor receptor (EGFR) and ErbB2 readily form heterodimers when both are expressed in the same cell and the EGFR is activated by one of its ligands. Our data show that such heterodimers are constitutively formed also in a ligand-independent manner on overexpression of EGFR and ErbB2 in porcine aortic endothelial cells. Interestingly, cross-linking experiments showed that incubation with the antibody pertuzumab, which has been shown to bind the dimerization arm of ErbB2, resulted in dissolution of EGFR-ErbB2 heterodimers. Incubation with pertuzumab also increased the amount of EGF-induced EGFR homodimers, and under these conditions, endocytosis of radiolabeled EGF was increased. This increase was significant, although slightly more EGF was internalized in cells expressing EGFR only compared with pertuzumab-treated cells expressing both EGFR and ErbB2. By confocal microscopy analysis, more EGF was observed in endosomes on incubation with pertuzumab, and under similar conditions, immunoblotting experiments showed increased EGFR degradation on incubation with both EGF and pertuzumab. These results show that pertuzumab enhanced the endocytic down-regulation of EGFR by counteracting EGFR-ErbB2 heterodimerization. Our previous results showing that ErbB2 counteracts EGFR endocytosis can therefore be explained by tethering of EGFR to ErbB2 at the plasma membrane. [Mol Cancer Ther 2009;8(7):1885–92]

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Introduction

The human epidermal growth factor receptor (EGFR) family consists of four members (EGFR/ErbB1, ErbB2, ErbB3, and ErbB4). Additionally, at least 11 different ligands can interact with the ErbB proteins (1, 2). While EGFR and ErbB4 can form signaling homodimers, they also engage in heterodimerization, and ErbB2 and ErbB3 can only signal on heterodimerization (3, 4). ErbB2 is an orphan receptor, and no conventional ligands are known to bind ErbB2. However, because of its constitutively exposed dimerization arm, ErbB2 is the preferred dimerization partner, readily interacting with the other ligand-activated ErbB proteins (5). ErbB3 has a defective kinase domain (6), and ErbB3 can therefore also only signal on heterodimerization. ErbB2 is overexpressed in several human malignancies, and this overexpression is associated with poor clinical outcome (7). By heterodimerization, ErbB2 plays a dominant role in mediating the malignant phenotype (8–10). ErbB2-ErbB3 heterodimers are potent signaling units (11). This is explained by the many intracellular docking sites for phosphatidylinositol 3-kinase created by ErbB2-induced phosphorylation of the ErbB3 tail (12).

ErbB2 is normally endocytosis deficient (13, 14), and we have shown that ErbB2 negatively affects EGFR down-regulation (15). Ways of blocking ErbB2-induced heterodimerization do therefore potentially represent important treatment modalities. We have, in the current work, made use of stably transfected porcine aortic endothelial (PAE) cells overexpressing EGFR only or EGFR and ErbB2 together (see Supplementary Fig. S1).⁴ We have, by using these cells, investigated how the humanized version of the anti-ErbB2 monoclonal antibody 2C4 (pertuzumab) affects EGFR/ErbB2 dimerization and EGF-induced endocytic down-regulation of EGFR. Our present results show that pertuzumab efficiently counteracts EGFR-ErbB2 heterodimerization and that pertuzumab thereby efficiently enhances EGF-induced endocytosis of EGFR. This will, over time, expectedly reduce the amount of growth signaling EGFR/ErbB2 complexes at the plasma membrane.

Materials and Methods

Materials

Human recombinant EGF was from Bachem. Protein G-coupled magnetic beads were purchased from Invitrogen. Other chemicals were from Sigma-Aldrich unless otherwise noted. Mouse ¹²⁵I-EGF was from Perkin-Elmer Life and Analytical Sciences.

⁴ Supplementary material for this article is available at Molecular Cancer Therapeutics Online (<http://mct.aacrjournals.org/>).

Antibodies

Pertuzumab, a humanized monoclonal antibody directed to ErbB2, was a gift from Roche Diagnostics. Mouse anti-ErbB2 antibody (TAB250 to the extracellular part) and rabbit anti-ErbB2 antibody (clone PADZ4881 to the intracellular part) were from Invitrogen. Mouse anti-ErbB2 antibody (clone 42 to the extracellular part) was from BD Biosciences. Sheep anti-EGFR antibody was from Fitzgerald Industries International. Rabbit anti-early endosomal antigen 1 (EEA1) antibody was from Millipore. Rabbit anti-tubulin antibody was from Abcam. Peroxidase-conjugated donkey anti-rabbit and peroxidase-conjugated donkey anti-sheep antibodies were from Jackson Immuno Research Laboratories. Alexa 647-conjugated donkey anti-rabbit antibody was from Invitrogen.

Cell Culture and Treatment

PAE cells were from Carl-Henrik Heldin (The Ludwig Institute for Cancer Research), and stably transfected PAE cells expressing EGFR (PAE.EGFR) were a generous gift from Alexander Sorkin (University of Colorado, Health Sciences Centre). PAE.EGFR.ErbB2 cells were established by transfection of PAE.EGFR cells with the plasmid pcDNA3.1-ErbB2 as described previously (15). PAE.ErbB2 cells were established by transfection of PAE cells with the plasmid pcDNA3.1-ErbB2. Stably transfected PAE cells were grown in Ham's F-12 medium (Lonza) supplemented with 10% (v/v) fetal bovine serum (Sigma-Aldrich) and 0.5× penicillin-streptomycin mixture (Lonza). The established cell lines were grown under different antibiotic selection pressure depending on the vector encoding the ErbB protein [400 µg/mL G418 sulfate (Invitrogen) for PAE.EGFR cells, 400 µg/mL G418 sulfate and 30 µg/mL zeocin (Invitrogen) for PAE.EGFR.ErbB2 cells, and 30 µg/mL zeocin for PAE.ErbB2 cells]. SKBr3 cells were from ATCC and were grown in DMEM supplemented with 10% (v/v) fetal bovine serum, 0.5× penicillin-streptomycin mixture, and 2 mmol/L L-glutamine.

Cross-linking of EGFR and ErbB2

Before cross-linking and immunoprecipitation, the cells were incubated with or without pertuzumab (25 µg/mL) for 1 h on ice followed by incubation with or without EGF (60 ng/mL) for 30 min on ice. On incubation, the cells were washed three times with ice-cold PBS and subjected to cross-linking with the membrane nonpermeable cross-linking reagent bis(sulfosuccinimidyl)suberate (Thermo Fisher Scientific; 2 mmol/L in PBS) on ice for 30 min. In all experiments, a freshly prepared solution of bis(sulfosuccinimidyl)suberate was used. Subsequently, the reaction was stopped with 10 mmol/L Tris (pH 7.5), 0.9% NaCl, and 0.1 mol/L glycine for 15 min on ice. Whole-cell lysates were prepared with 1% SDS in PBS and immediately subjected to immunoprecipitation.

Immunoprecipitation

Cells were lysed in 1% SDS in PBS, boiled for 5 min, and chilled on ice before homogenization using QIAshredder columns (Qiagen). The lysates were added to protein G-coupled magnetic beads preincubated with EGFR antibody. Coupling of EGFR antibody to beads was done according manufacturer's instructions with incubation for 1 h at room temperature. After washing with 1× immunoprecipitation buffer

[2% (v/v) Triton X-100, 0.5% (w/v) sodium deoxycholate, 1% (w/v) bovine serum albumin, 2 mmol/L EDTA, 40 mmol/L NaF, 2 mmol/L phenylmethylsulfonyl fluoride, 4 mmol/L Na₃VO₄, 40 µg/mL leupeptin, 20 µg/mL aprotinin, and 2 mmol/L NEM] containing SDS (50% of 2× immunoprecipitation buffer + 50% of 1% SDS in PBS), the beads were resuspended in 2× immunoprecipitation buffer. Antibody-coupled beads and cell lysates were gently mixed for 1 h at 4°C with shaking. The beads were then washed in 1× immunoprecipitation buffer and eluted in 2× sample buffer [20 mmol/L Tris-HCl (pH 6.8), 10 mmol/L EDTA, 100 mmol/L NaF, 60 mmol/L sodium pyrophosphate, 4% SDS, 2% β-mercaptoethanol, 20% glycerol, and 0.006% bromophenol blue]. The samples were incubated at 96°C for 5 min and subjected to SDS-PAGE using 6% SDS-polyacrylamide gels for 90 min at 150 V.

Immunoblotting

On SDS-PAGE, the cell lysates were electrotransferred to nitrocellulose membranes (GE Healthcare Life Sciences). The membranes were incubated with primary and secondary antibodies at 4°C overnight or at room temperature for 1 h, and proteins were detected using SuperSignal West Dura Extended Duration Substrate (Thermo Fisher Scientific) and KODAK Image Station 4000R (Carestream Health).

Internalization of ¹²⁵I-EGF

The internalization of ¹²⁵I-EGF in PAE.EGFR, PAE.EGFR.ErbB2, and SKBr3 cells was measured essentially as described previously (16). Cells were incubated in MEM without bicarbonate (Invitrogen) 10 min before the experiment. At the end, cells were lysed in 1% SDS in PBS at room temperature. Internalized ¹²⁵I-EGF was estimated as the ratio of internalized counts/min to surface-localized counts/min.

Degradation of EGFR

PAE.EGFR and PAE.EGFR.ErbB2 cells were incubated with or without pertuzumab (25 µg/mL), EGF (60 ng/mL), and cycloheximide (25 µg/mL) for 4 h at 37°C. On incubation, degradation of EGFR was investigated as described (17). The lysates were subjected to SDS-PAGE using 10% gels for 1 h at 150 V, and immunoblotting was done as described.

Immunocytochemistry and Confocal Microscopy

PAE.EGFR and PAE.EGFR.ErbB2 cells grown in 60 mm plastic Petri dishes were incubated with or without pertuzumab (25 µg/mL) for 1 h at 37°C followed by incubation with 60 ng/mL Alexa 555-EGF (Invitrogen) for 15 min at 37°C. On incubation, immunostaining was done essentially as described (14). The specimens were mounted using DAKO fluorescence mounting medium and examined using confocal microscopy (Olympus FW1000, objective ×60).

Recycling and Degradation of ¹²⁵I-EGF

Recycling of EGF was analyzed essentially as described (18), loading cells with 50 ng/mL of EGF (5 ng/mL ¹²⁵I-EGF and 45 ng/mL unlabeled EGF) in MEM without bicarbonate and with 0.1% (w/v) bovine serum albumin for 20 min at 37°C. On chase at 37°C in MEM containing 0.1% bovine serum albumin, the supernatants were collected and transferred to microtubes. The EGF remaining at the cell surface was removed using a low pH glycine-buffered solution [0.1 mol/L glycine and 0.15 mol/L NaCl (pH 3.0)] and pooled with the respective supernatants from the chase.

Subsequently, a solution containing 50% trichloroacetic acid and 10% phosphotungstic acid was added to the supernatant fractions and ^{125}I -EGF was precipitated overnight at 4°C . All fractions, the trichloroacetic acid-phosphotungstic acid precipitated ^{125}I -EGF (^{125}I -EGF representing both non-internalized, surface-localized and recycled EGF) and the trichloroacetic acid-phosphotungstic acid soluble radioactivity (representing degraded ^{125}I -EGF) were measured by γ -counting. Internalized EGF was measured by γ -counting on lysis of the cells in 1% SDS in PBS at room temperature. Internalized, recycled, and degraded fractions were estimated as percentage of the total ^{125}I -EGF.

Results

ErbB2-EGFR Heterodimerization Is Counteracted by Pertuzumab in PAE Cells Stably Expressing EGFR and ErbB2

Our group has shown previously that ErbB2 inhibits EGF-induced endocytosis of EGFR (15). This is probably

due to heterodimerization of EGFR and ErbB2. To study the effect of EGF and pertuzumab on EGFR-ErbB2 heterodimerization, stably transfected PAE cells expressing either EGFR only (PAE.EGFR cells) or EGFR and ErbB2 (PAE.EGFR.ErbB2 cells) were used. To investigate the nature of EGFR dimers in the absence and presence of pertuzumab, a membrane-impermeable chemical cross-linker [bis(sulfosuccinimidyl)suberate] was added to the cells. The cross-linked and immunoprecipitated receptors were analyzed by immunoblotting with antibodies to EGFR and ErbB2. When doing cross-linking and immunoprecipitation experiments with cells expressing EGFR only, we observed EGFR homodimers on incubation with EGF (Fig. 1A, lane 7). In cells expressing both EGFR and ErbB2, only minimal amounts of EGFR appeared as dimers on cross-linking in nonstimulated cells (Fig. 1A, lane 1). By using an antibody to ErbB2, we could, on the other hand, in the same cells clearly detect ErbB2 in dimeric state (Fig. 1B, lane 1). On incubation with EGF and subsequent cross-linking, the

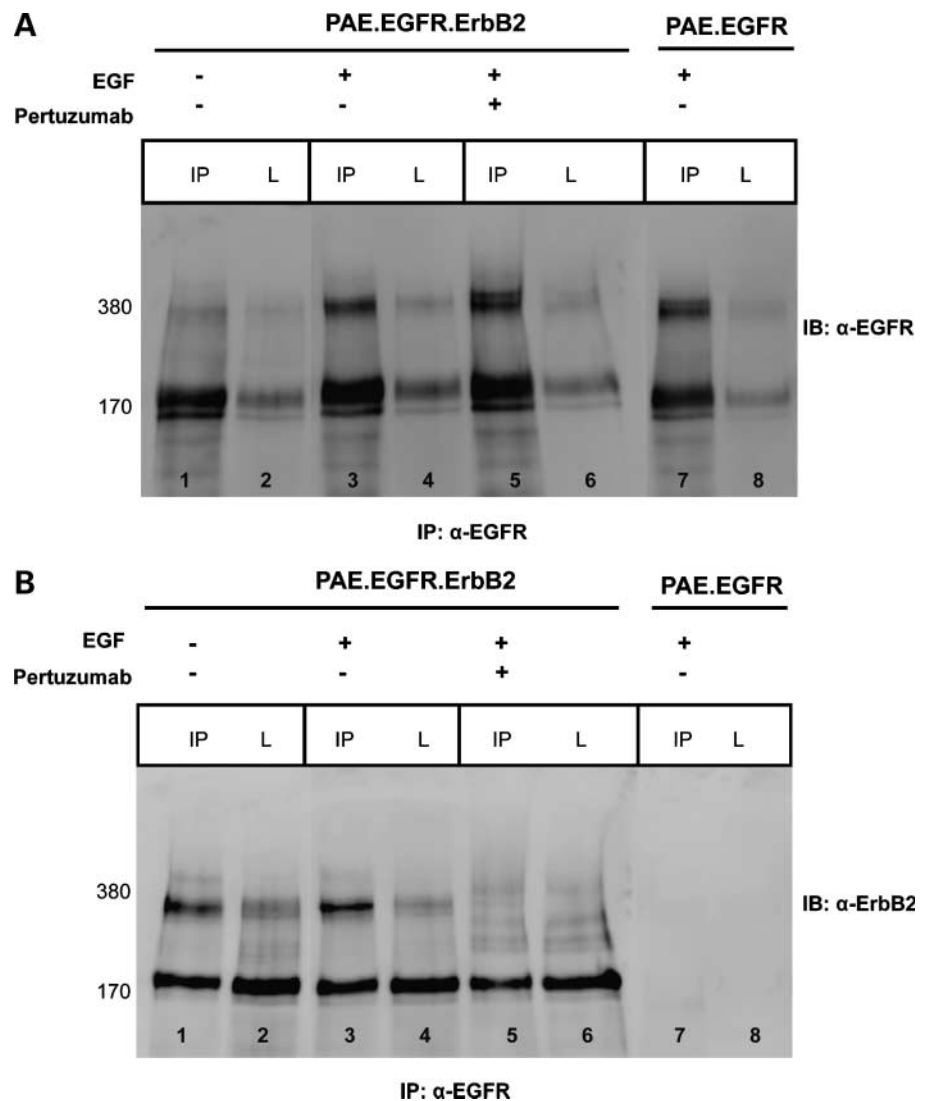


Figure 1. ErbB2 and EGFR form heterodimers in PAE cell lines, and heterodimerization is counteracted by pertuzumab. PAE.EGFR and PAE.EGFR.ErbB2 cells were incubated with or without pertuzumab (25 $\mu\text{g}/\text{mL}$) for 1 h on ice and subsequently with or without EGF (60 ng/mL) for 30 min on ice. Plasma membrane proteins were then cross-linked using 2 mmol/L bis(sulfosuccinimidyl)suberate in PBS. Cell lysates were subjected to immunoprecipitation using anti-EGFR antibody. The immunoprecipitated material was subjected to SDS-PAGE using 6% gels followed by immunoblotting with antibody to EGFR (**A**) or ErbB2 (**B**). The immunoblots show bands reactive to monomeric EGFR or ErbB2 at 170 kDa. Dimers are represented by bands at 380 kDa. **A**, lanes 3, 5, and 7, EGF-induced dimerization. **B**, lanes 1 and 3, ErbB2-containing dimers. Note that the ErbB2 reactivity disappears on incubation with pertuzumab and EGF (lane 5). Representative of one of six experiments.

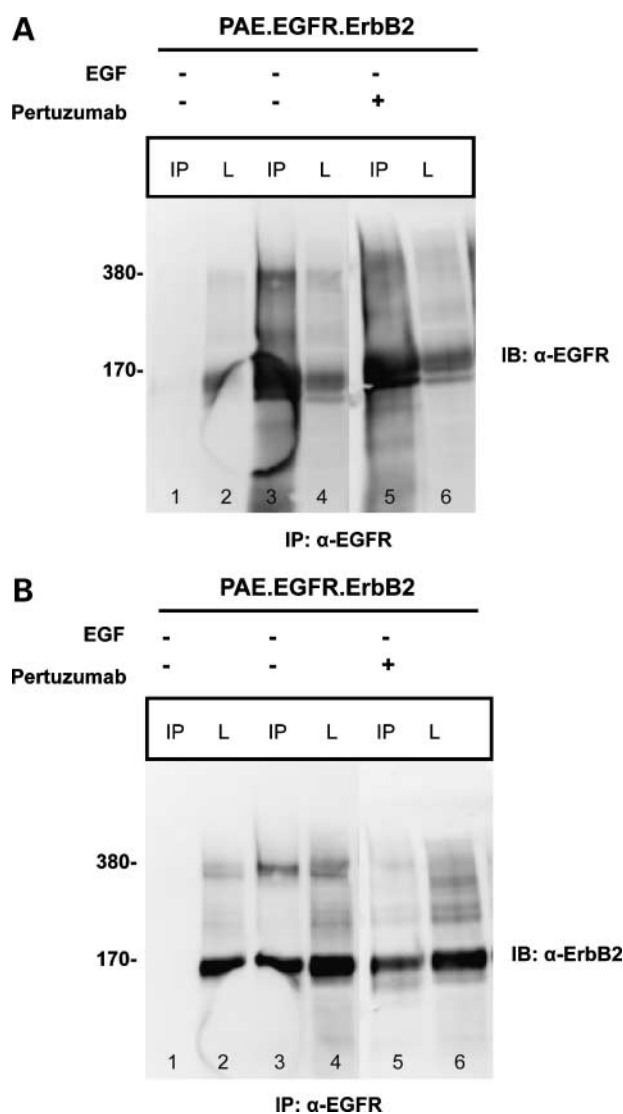


Figure 2. Pertuzumab efficiently disrupts EGFR-ErbB2 heterodimers. PAE.EGFR.ErbB2 cells were incubated with or without pertuzumab (25 μ g/mL) for 1 h on ice. Plasma membrane proteins were cross-linked using ice-cold 2 mmol/L bis(sulfosuccinimidyl)suberate in PBS. The cell lysates were subjected to immunoprecipitation using anti-EGFR antibody. The immunoprecipitated material was subjected to SDS-PAGE using 6% gels followed by immunoblotting with antibody to EGFR (**A**) or ErbB2 (**B**). The immunoblots display bands reactive to monomeric EGFR or ErbB2 at 170 kDa. Dimeric bands are represented by bands at 380 kDa. **A**, lanes 3 and 5, constitutive dimers. **B**, lane 3, ErbB2-containing dimers. The ErbB2 reactivity disappears on incubation with pertuzumab (lane 5). Lane 1, precipitated beads without antibody. Representative experiment.

amount of dimeric EGFR increased strongly (Fig. 1A, lane 3), whereas the ErbB2 reactivity of the dimeric bands showed no clear change (Fig. 1B, lane 3).

Immunoprecipitation was done using beads coated with antibody to EGFR. However, because also monomeric ErbB2 appeared to be precipitated by this antibody, this method did not allow us to differentiate between homodimerization and heterodimerization. Whether the presence of monomeric ErbB2 was due to cross-reactivity of antio-

dies used for precipitation, or whether the monomeric ErbB2 band represents heterodimers dissolved on precipitation, is unclear. The strong EGF-induced increase in EGFR-positive dimeric bands does, however, suggest formation of EGFR homodimers in addition to EGFR-ErbB2 heterodimers.

Most importantly, however, on incubation with pertuzumab and EGF in the PAE.EGFR.ErbB2 cells, dimeric ErbB2 was no longer detectable (Fig. 1B, lane 5), whereas antibodies to EGFR showed a strong reactivity to the dimeric band (Fig. 1A, lane 5). This indicated that incubation with pertuzumab counteracted EGFR-ErbB2 heterodimerization and that ErbB2 mainly existed as monomers on this incubation.

To investigate whether pertuzumab in its own capacity would dissolve heterodimers or whether EGF facilitated this dissolution by driving EGFR homodimerization, we incubated PAE.EGFR.ErbB2 cells with pertuzumab only on ice for 60 min. Importantly, and as shown in Fig. 2A and B, the preexisting EGFR-ErbB2 heterodimers were more or less completely dissociated by this incubation (compare lanes 3 and 5 in Fig. 1B).

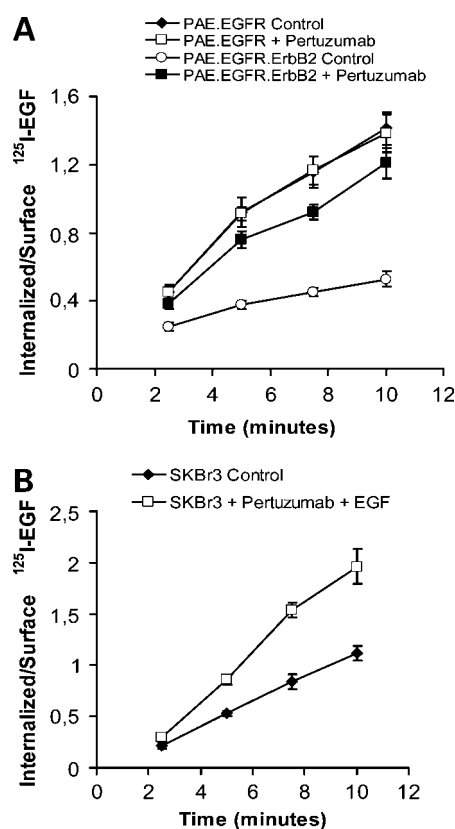


Figure 3. Pertuzumab enhances EGF-induced internalization of EGFR in cells expressing EGFR and ErbB2. PAE cells stably expressing EGFR alone or EGFR and ErbB2 (**A**) and SKBr3 cells (**B**) were incubated with or without pertuzumab (25 μ g/mL) for 1 h at 37°C and subsequently with 1 ng/mL 125 I-EGF at 37°C for the times indicated. The ratio of internalized to surface-localized 125 I-EGF was plotted as a function of time. Mean \pm SE of three independent experiments with four parallels.

Because IgG molecules have two antigen-binding sites, incubation with pertuzumab could potentially induce formation of ErbB2 homodimers. Whether functional ErbB2 homodimers were formed on incubation with pertuzumab in PAE.ErbB2 cells expressing ErbB2 only was difficult to address, because immunoblotting with antibody to ErbB2 showed a large number of reactive bands on cross-linking (data not shown). Many of these bands probably represented various ErbB2-pertuzumab complexes. Analysis of potential ErbB2 homodimerization thus awaits further investigation and will probably require proteomics studies.

Pertuzumab and EGF Induce Internalization of EGFR in Cells Expressing EGFR and ErbB2

ErbB2 has been shown to have a negative effect on endocytosis of EGFR. To investigate whether pertuzumab

has an effect on EGFR endocytosis, we used PAE.EGFR and PAE.EGFR.ErbB2 cells. The cells were preincubated with or without pertuzumab (25 $\mu\text{g}/\text{mL}$) for 1 h at 37°C. Then, internalization of EGF was investigated by incubating the cells with 1 ng/mL ^{125}I -EGF at 37°C for different incubation times. Comparing endocytosis in pertuzumab-treated cells with endocytosis in control cells, we observed that, in cells expressing EGFR only, pertuzumab did not interfere with EGFR endocytosis. In cells expressing EGFR and ErbB2, however, endocytosis of EGFR was strongly increased on incubation with pertuzumab and EGF (Fig. 3A). The same was observed when SKBr3 cells overexpressing ErbB2 were preincubated with pertuzumab (Fig. 3B). This shows that pertuzumab, by dissolving EGFR-ErbB2 heterodimers, enables

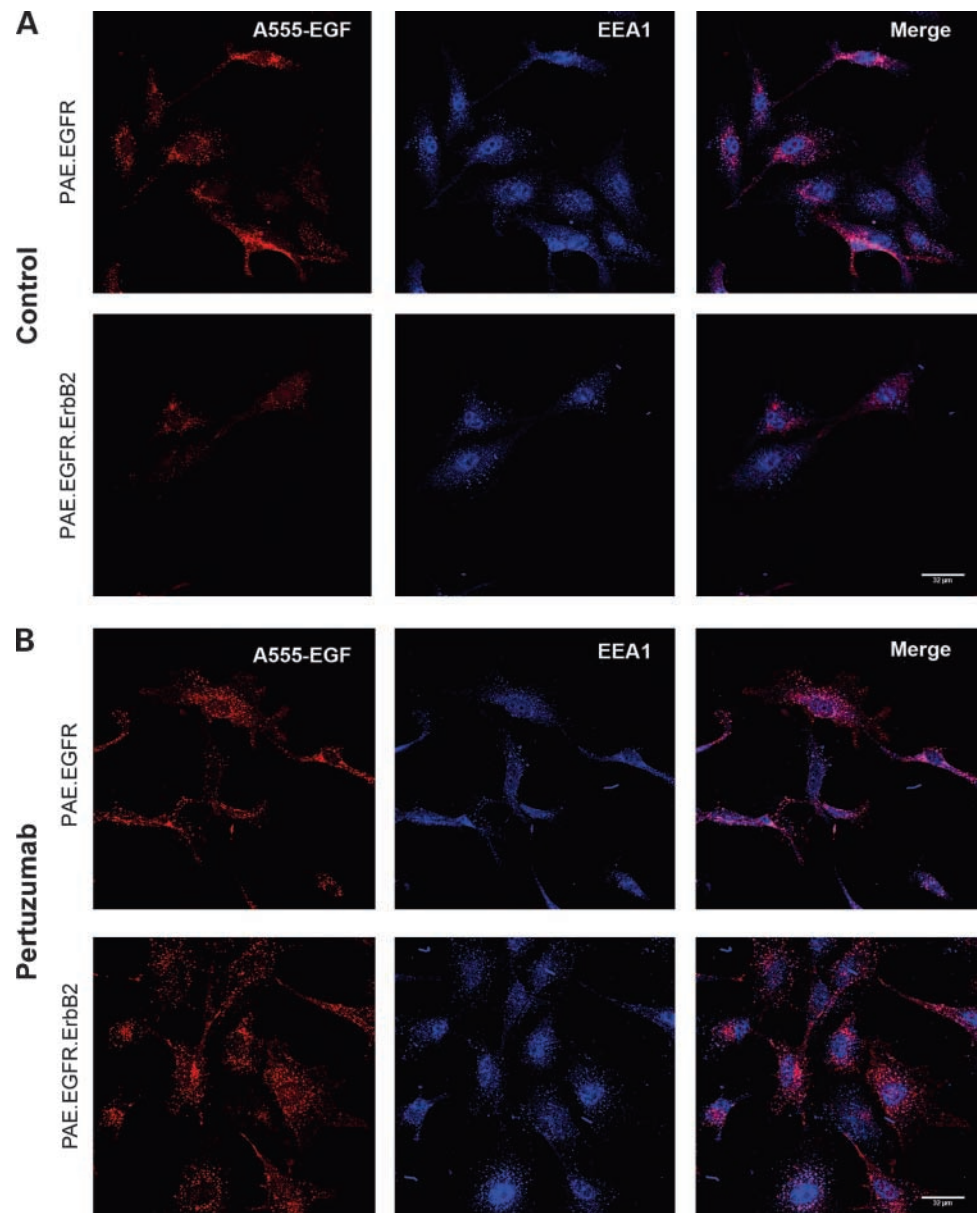


Figure 4. Pertuzumab does not affect EGF internalization in PAE cells expressing EGFR only but rescues EGF internalization in PAE cells expressing EGFR and ErbB2. **A**, PAE.EGFR and PAE.EGFR.ErbB2 cells were incubated with Alexa 555-EGF (60 ng/mL) for 15 min at 37°C. The cells were fixed and immunostained using rabbit anti-EEA1 antibody followed by Alexa 647-conjugated donkey anti-rabbit antibody. **B**, PAE.EGFR and PAE.EGFR.ErbB2 cells were incubated with pertuzumab (25 $\mu\text{g}/\text{mL}$) for 1 h at 37°C followed by Alexa 555-EGF (60 ng/mL) for 15 min at 37°C and subsequently fixed and stained as described in **A**. Endocytosed EGF is observed to colocalize with EEA1 in early endosomes (pink dots). Representative experiment. Bar, 32 μm .

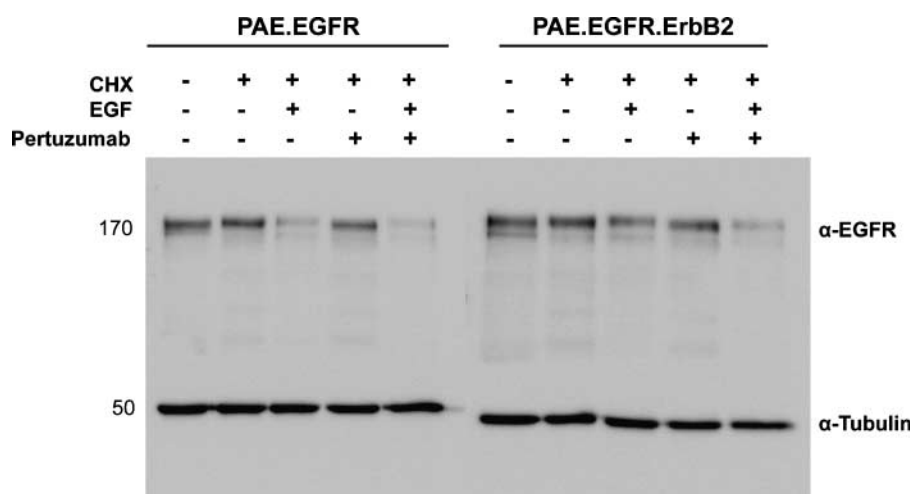


Figure 5. Pertuzumab increases EGF-induced degradation of EGFR in PAE.EGFR.ErbB2 cells. PAE.EGFR and PAE.EGFR.ErbB2 cells were incubated with pertuzumab (25 $\mu\text{g}/\text{mL}$) and EGF (60 ng/mL) for 4 h in the presence of cycloheximide (CHX; 25 $\mu\text{g}/\text{mL}$). Cells were lysed and subjected to SDS-PAGE and immunoblotting with anti-EGFR antibody. Tubulin was used as loading control. Incubation with pertuzumab increased the EGF-induced degradation of EGFR in PAE.EGFR.ErbB2 cells, whereas degradation of EGFR was not affected by pertuzumab in PAE.EGFR cells. Bands at 170 kDa represent EGFR and bands at 50 kDa represent tubulin.

EGF-induced formation of EGFR homodimers capable of being endocytosed.

We further studied the effect of pertuzumab on endocytosis of EGF by confocal microscopy of PAE.EGFR and PAE.EGFR.ErbB2 cells. PAE.EGFR and PAE.EGFR.ErbB2 cells were incubated with or without pertuzumab before incubation with Alexa 555-EGF (60 ng/mL) for 15 min at 37°C. The cells were subsequently fixed and immunostained for EEA1. In PAE.EGFR cells not incubated with pertuzumab, EGF was readily detected in EEA1-positive vesicles. In PAE.EGFR.ErbB2 cells, however, only a small fraction of EEA1-positive endosomes contained EGF (Fig. 4A). However, on preincubation of the cells with pertuzumab, the number of EEA1-positive endosomes containing endocytosed EGF strongly increased in PAE.EGFR.ErbB2 cells (Fig. 4B). This further supports the notion that pertuzumab increases EGF-induced EGFR homodimerization and internalization by counteracting heterodimerization of EGFR.

Pertuzumab Enhances Degradation of EGFR in Cells Expressing EGFR and ErbB2

As overexpression of ErbB2 was found to inhibit EGF-induced endocytosis and degradation of the EGFR (15), we studied whether the EGFR homodimers formed in PAE.EGFR.ErbB2 cells on incubation with pertuzumab and EGF were degraded on endocytosis. After incubation for 4 h with EGF (60 ng/mL) and cycloheximide (25 $\mu\text{g}/\text{mL}$), degradation of EGFR was hardly detectable by immunoblotting in PAE.EGFR.ErbB2 cells (Fig. 5). This is in contrast to PAE.EGFR cells, where degradation was readily observed. However, when PAE.EGFR.ErbB2 cells were incubated with pertuzumab in addition to EGF, degradation could readily be observed. It should, however, be noted that EGFR was degraded to a smaller extent than in PAE.EGFR cells.

To investigate whether the reduced degradation of EGFR in EGF- and pertuzumab-treated PAE.EGFR.ErbB2 cells resulted from reduced endocytosis or from increased recycling of internalized EGF-EGFR complexes, the fate of

internalized radiolabeled EGF was investigated as described in Materials and Methods. Clearly, the recycling and degradation of radiolabeled internalized EGF was not affected by incubation with pertuzumab (Fig. 6). This is consistent with our current and previous (15) findings that ErbB2 was not internalized and therefore incapable of affecting intracellular sorting of internalized EGFR. The differences in degradation of EGFR when comparing PAE.EGFR and PAE.EGFR.ErbB2 cells in the presence and absence of pertuzumab are therefore explained by different EGFR endocytosis efficiency.

Discussion

Overexpression of ErbB2 seriously affects disease outcome in cancer. This is, in many cases, explained by the preactivated conformation of ErbB2 irrespective of ligand binding. The exposed dimerization arm readily interacts with the dimerization arm of EGFR, ErbB3, and ErbB4 on ligand-mediated activation of these proteins, and ErbB2 inhibits down-regulation of EGFR (15). ErbB2 is endocytosis deficient (13, 14), and tethering to ErbB2 could therefore explain the endocytosis deficiency of EGFR. To investigate this more closely, we took advantage of the monoclonal antibody pertuzumab (2C4), which specifically binds the ErbB2 dimerization arm (19). Our cross-linking experiments show that, in cells expressing both EGFR and ErbB2, incubation with pertuzumab and EGF induced formation of EGFR homodimers, whereas the amount of ErbB2-containing dimers was reduced compared with cells incubated with EGF only. This indicates that the equilibrium was strongly shifted, and the dimers observed were mostly EGFR homodimers. In fact, our experiments also showed that the amount of EGFR-ErbB2 heterodimers was strongly decreased on incubation with pertuzumab only. This showed that pertuzumab in fact disrupted preexisting heterodimers. This conclusion is mainly based on loss of ErbB2 immunoreactivity in cross-linked ErbB dimers and is consistent with previous findings (20).

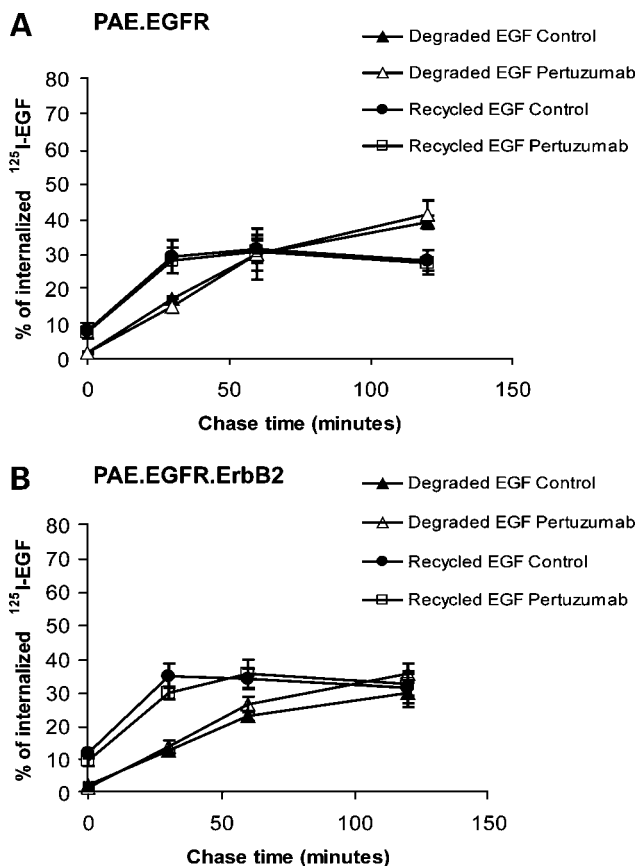


Figure 6. Pertuzumab does not affect recycling or degradation of internalized EGF. PAE.EGFR cells (**A**) and PAE.EGFR.ErbB2 cells (**B**) were incubated with or without pertuzumab (25 μ g/mL) for 1 h at 37°C and subsequently with 5 ng/mL 125 I-EGF and 45 ng/mL unlabeled EGF for 20 min at 37°C followed by chase in EGF-free medium at 37°C for the times indicated. Analysis of degraded and recycled EGF was done as described in Materials and Methods. Mean \pm SE of three different experiments with four parallels.

To investigate whether the EGFR homodimers formed were capable of being endocytosed, we studied the internalization of radiolabeled EGF in cells expressing EGFR and ErbB2 in the absence and presence of pertuzumab. There was a clear increase in uptake of 125 I-EGF in the presence, but not in the absence, of pertuzumab. This is consistent with the observation of significantly more EGFR homodimers induced by pertuzumab and argues that only EGFR homodimers are capable of being internalized. This is again consistent with the idea that EGFR complexed to ErbB2 cannot be internalized (15).

Normally, the EGFR is degraded in lysosomes on ligand binding and homodimerization (21). We therefore investigated the degradation of EGFR on incubation with EGF with or without pertuzumab in PAE cells expressing EGFR only compared with PAE cells expressing both EGFR and ErbB2. In cells expressing EGFR only, degradation was readily observed in the presence of EGF only. In cells expressing EGFR and ErbB2, degradation of EGFR was somewhat reduced on incubation with EGF and per-

tuzumab. This could be explained by the observed reduction of internalized EGF in PAE.EGFR.ErbB2 cells incubated with pertuzumab and EGF compared with internalization of EGF in PAE.EGFR cells. Experiments investigating degradation and recycling of radiolabeled EGF showed that internalized ligand was routed similarly in cells with or without expression of ErbB2.

In conclusion, pertuzumab could be a very useful agent in treatment of cancers with overexpressed EGFR and ErbB2. Patients could be injected with pertuzumab repeatedly, and this would eventually result in down-regulation of ErbB proteins. Also, the block in heterodimer formation will affect proliferative signal transduction. Additionally, like trastuzumab, pertuzumab could potentially modulate immune responses, for example, by recruitment of natural killer cells (22).

Disclosure of Potential Conflicts of Interest

Max Hasmann is a Roche employee.

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