

Geldanamycin-Induced Down-Regulation of ErbB2 from the Plasma Membrane Is Clathrin Dependent but Proteasomal Activity Independent

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Abstract

ErbB2, a member of the epidermal growth factor receptor family, is overexpressed in a number of human cancers. In contrast to the epidermal growth factor receptor, ErbB2 is normally endocytosis resistant. However, ErbB2 can be down-regulated by inhibitors of heat shock protein 90, such as geldanamycin. We now show that geldanamycin induces endocytosis and lysosomal degradation of full-length ErbB2. We further report that the endocytosis of ErbB2 is dynamin and clathrin dependent. When ErbB2 was retained at the plasma membrane due to knockdown of clathrin heavy chain, the intracellular part of ErbB2 was degraded in a proteasomal manner. However, our data strongly suggest that proteasomal activity is not required for geldanamycin-induced endocytosis of ErbB2 in SKBr3 cells. Interestingly, however, proteasomal inhibitors retarded degradation of ErbB2, and electron microscopy analysis strongly suggested that proteasomal activity is required to sort internalized ErbB2 to lysosomes. Because geldanamycin derivatives and inhibitors of proteasomal activity are both used in experimental cancer treatment, knowledge of molecular mechanisms involved in geldanamycin-induced down-regulation of ErbB2 is important for future design of cancer treatment. (Mol Cancer Res 2008;6(3):491–500)

Introduction

Receptor down-regulation plays important roles in terminating growth factor signaling. ErbB2 is a member of the epidermal growth factor (EGF) receptor (EGFR) family, but unlike the other members (EGFR, ErbB3 and ErbB4), ErbB2

has no soluble ligand (ref. 1; reviewed in ref. 2). Still, ErbB2 is the preferred dimerization partner for the other family members (refs. 3, 4; reviewed in ref. 5), and heterodimers containing ErbB2 are strong signal transducers (6, 7). In human breast cancer, ErbB2 is amplified and overexpressed in 20% to 30% of the cases and often associated with aggressive disease and poor prognosis (ref. 8; reviewed in ref. 9). Addressing mechanisms involved in down-regulation of ErbB2 is therefore important. ErbB2 has been reported to be internalization resistant (10–12) with a long half-life at the plasma membrane. ErbB2 has also been found to cluster in lipid microdomains, and it has been speculated that ErbB2 is endocytosis deficient by being excluded from clathrin-coated pits (11, 13, 14). Furthermore, ErbB2 has been shown to inhibit EGF-induced formation of clathrin-coated pits (15).

The benzoquinone ansamycin geldanamycin, which specifically binds and inactivates the ATP/ADP binding pocket of the molecular chaperone heat shock protein 90 (Hsp90; ref. 16), disrupts the stabilizing interaction of Hsp90 with ErbB2 (17). This results in degradation of ErbB2 and also degradation of other Hsp90 target proteins (reviewed in refs. 18, 19). The mechanisms whereby geldanamycin induces down-regulation of ErbB2 have been elusive. However, when binding of ADP/ATP to Hsp90 is inhibited, the ubiquitin ligase COOH terminus of heat shock cognate protein 70–interacting protein (CHIP) is recruited via heat shock protein 70 and induces rapid ubiquitination of ErbB2 (19–21). This ubiquitination has been proposed to induce proteasomal degradation of ErbB2 (22), but ubiquitination has also been described as an internalization signal both in yeast and in mammalian cells (reviewed in refs. 23, 24). A requirement for ubiquitination in down-regulation of ErbB2 on treatment with geldanamycin has thus far not been firmly established.

It was recently proposed that geldanamycin induced internalization of full-length ErbB2 from the plasma membrane in a proteasome-dependent manner and that full-length ErbB2 was degraded in lysosomes (25). This is in contrast to previous findings concluding that caspase-dependent cleavage of ErbB2 is required for internalization and degradation of ErbB2 (26–28). This is further in contrast to reported findings claiming that geldanamycin exclusively acts by increasing the lysosomal sorting and not by increasing endocytosis of ErbB2 as such (29).

Incubation of cells with geldanamycin has been shown to increase the lateral mobility of ErbB2 within the plasma membrane and to cause more frequent localization of ErbB2 to clathrin-coated pits (25). This could suggest that internalization

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Table 1. Anti-ErbB2 Antibodies Used

| Antigenic Epitope | Antibody Source | Abbreviation | Used for |
|----------------------|---------------------------------|--------------|--|
| Extracellular domain | BD Transduction clone 42 | e.c. | Western blotting |
| Extracellular domain | Zymed Laboratories clone TAB250 | e.c.* | Flow cytometry, confocal and electron microscopy |
| Extracellular domain | R&D Systems AF1129 | e.c.** | Western blotting |
| Intracellular domain | Calbiochem Ab-3 | i.c. | Western blotting, confocal and electron microscopy |
| Intracellular domain | Zymed Laboratories PADZ4881 | i.c.* | Western blotting, confocal microscopy |
| Intracellular domain | NeoMarkers Ab-8 | i.c.** | Western blotting, confocal microscopy |

from clathrin-coated pits is involved in geldanamycin-induced down-regulation of ErbB2, but as the proportion of ErbB2 found in clathrin-coated pits is very small, also clathrin-independent endocytic mechanisms could play roles in endocytosis. In the current study, we have studied geldanamycin-induced down-regulation of ErbB2 in an attempt to characterize molecular mechanisms responsible for geldanamycin-induced down-regulation of ErbB2. Our results show that ErbB2 detected in vesicles on exposure to geldanamycin originates from the plasma membrane. We have found that the geldanamycin-induced down-regulation of ErbB2 is dynamin and clathrin dependent. Surprisingly, in cells depleted of clathrin heavy chain (CHC), where geldanamycin-induced internalization was inhibited, we found that the intracellular part of ErbB2 was degraded by a mechanism independent of caspases but requiring proteasomal activity. However, our flow cytometry analysis data clearly showed that proteasomal activity was not required for endocytic down-regulation of ErbB2 from the plasma membrane in SKBr3 cells, whereas proteasomal activity promoted lysosomal sorting of endocytosed ErbB2.

The geldanamycin derivative 17-*N*-allylamino-17-demethoxygeldanamycin and the proteasomal inhibitor bortezomib (Velcade, PS341) are currently both used in clinical trials (recent reviews in refs. 30-33), and knowledge of how they affect potential oncoproteins such as ErbB2 at the molecular level is therefore important in the design of cancer treatment. It is especially important to characterize requirements for ubiquitination and/or proteasomal activity to avoid adverse effects on ErbB2 down-regulation and on growth inhibitory pathways.

Results

Geldanamycin Induces Down-Regulation of ErbB2 from the Plasma Membrane

We took advantage of flow cytometry and an antibody recognizing the extracellular part of ErbB2 (for overview of ErbB2 antibodies used, see Table 1) to study the kinetics of geldanamycin-induced down-regulation of overexpressed ErbB2 in SKBr3 cells. As shown in Fig. 1A, a small, but measurable, down-regulation of ErbB2 was observed on incubation with geldanamycin for 2 hours, and ErbB2 was further down-regulated with increasing incubation times. Geldanamycin-induced down-regulation of ErbB2 from the plasma membrane could potentially result from interference with transport of ErbB2 from the endoplasmic reticulum through the Golgi apparatus (34, 35). However, incubation of cells with cycloheximide did not reduce effect on the level of

ErbB2 at the plasma membrane (Supplementary Fig. S1), indicating that down-regulation is due to geldanamycin-induced endocytosis. This is consistent with previous findings (10, 25, 28, 29, 36). Furthermore, confocal microscopy analyses showed that ErbB2 localized to vesicles on incubation of cells with geldanamycin. Immunostaining with antibodies to the intracellular and extracellular parts of ErbB2 showed a vesicular pattern (Fig. 1B; Supplementary Fig. S2), and ErbB2 was found to colocalize with early endosome antigen 1 (Supplementary Fig. S3). To make sure that ErbB2 localizing to vesicles was derived from the plasma membrane, cells were incubated on ice with antibody recognizing the extracellular part of ErbB2 and subsequently chased at 37°C with or without geldanamycin for 2 hours. The cells were then fixed, permeabilized, and immunostained with fluorescing secondary antibody and examined by confocal microscopy. As illustrated in Fig. 1C, the anti-ErbB2 antibody showed a vesicular staining pattern, indicating that the complex of ErbB2 and anti-ErbB2 antibody was endocytosed. In cells not exposed to geldanamycin, only plasma membrane labeling was observed, showing that incubation with anti-ErbB2 antibody as such did not induce endocytosis of ErbB2. Although we cannot exclude the possibility that a small part of the ErbB2-positive vesicles found in geldanamycin-incubated cells could represent endoplasmic reticulum-derived membrane, our data clearly show that the majority of ErbB2-positive vesicles contain ErbB2 originating from the plasma membrane.

To confirm that the observed effect of geldanamycin on ErbB2 is general and not cell type specific, we also studied geldanamycin-induced down-regulation of ErbB2 in PAE cells stably transfected with EGFR and ErbB2 (ref. 15; Supplementary Fig. S4) and in HEP2 cells (Supplementary Fig. S5). Incubation with geldanamycin induced internalization and degradation of ErbB2 in both these cell lines, illustrating a general down-regulatory effect of geldanamycin on ErbB2.

Geldanamycin-Induced Endocytosis of ErbB2 Is Dynamin and Clathrin Dependent

To characterize the geldanamycin-induced endocytosis of ErbB2, we initially investigated its dynamin dependency because dynamin is reported to regulate several endocytic pathways (37). SKBr3 cells were transiently transfected with a plasmid encoding the GTPase-defective dominant-negative mutant K44A dynamin 24 hours before incubation with geldanamycin. On 4-hour incubation with geldanamycin, the cells were fixed and immunostained for ErbB2 and dynamin. Transfected cells could, due to overexpression, be detected with the anti-dynamin antibody, and as shown in Fig. 2, in cells

overexpressing K44A dynamin, geldanamycin did not induce endocytosis of ErbB2. This is consistent with the idea that geldanamycin-induced endocytosis of ErbB2 is dynamin dependent. To investigate to what extent clathrin is required for geldanamycin-induced endocytosis of ErbB2, we transfected cells with small interfering RNA (siRNA) targeting CHC (38). The knockdown of CHC on transfection with siRNA was efficient (Fig. 3A), and endocytosis of ^{125}I -transferrin (^{125}I -Tf) was efficiently inhibited (Fig. 3B). We further studied the effect of geldanamycin on the plasma membrane level of ErbB2 in cells transfected with or without siRNA to CHC before incubation with geldanamycin. As shown in Fig. 3C, endocytosis of ErbB2 was inhibited in cells depleted of CHC

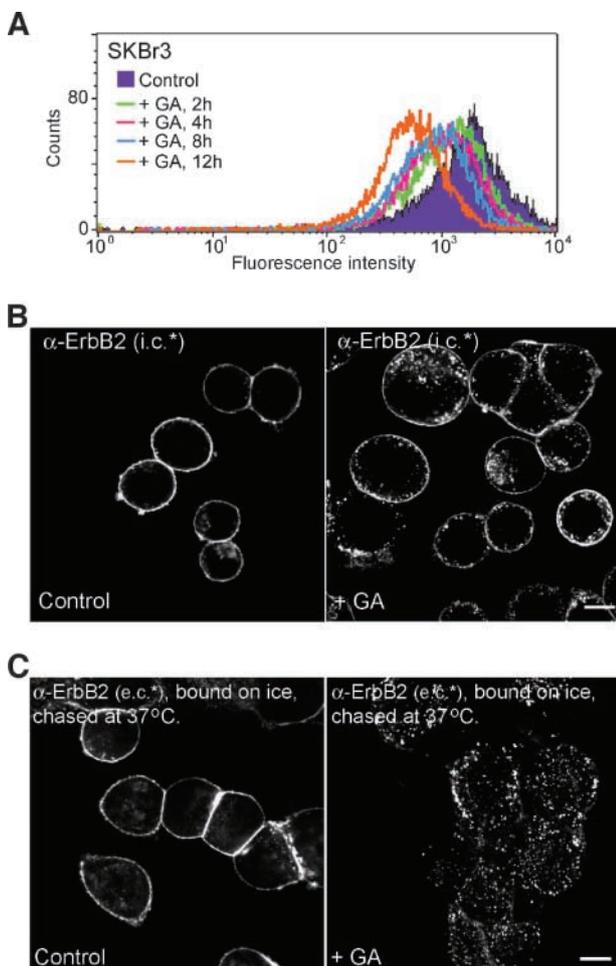


FIGURE 1. ErbB2 is down-regulated from the plasma membrane on incubation of cells with geldanamycin. **A.** SKBr3 cells were incubated with geldanamycin (GA; 3 $\mu\text{mol/L}$) for the indicated times. The cells were then fixed and immunostained with antibody e.c.* recognizing the extracellular part of ErbB2 followed by phycoerythrin-conjugated goat anti-mouse antibody and analyzed by flow cytometry. Representative of six experiments. **B.** SKBr3 cells on coverslips were incubated either with or without geldanamycin (3 $\mu\text{mol/L}$) for 2 h before being fixed and immunostained for ErbB2 (using the i.c.* anti-ErbB2 antibody) and analyzed by confocal microscopy. Bar, 10 μm . **C.** SKBr3 cells were incubated with the mouse anti-ErbB2 antibody e.c.* for 1 h on ice. The cells were then washed in cold PBS before being chased in MEM with or without geldanamycin (3 $\mu\text{mol/L}$) for 2 h at 37°C. Cells were fixed, permeabilized, and immunostained with Alexa 488-conjugated antimouse antibody and then analyzed by confocal microscopy. Bar, 10 μm .

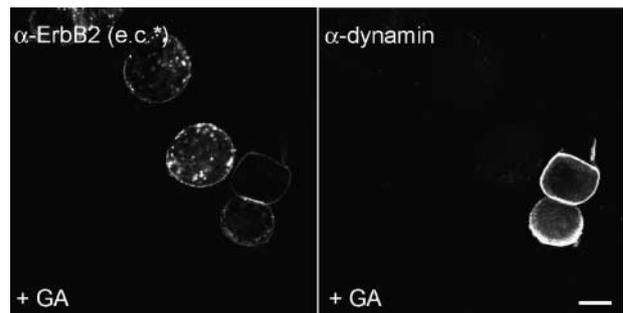


FIGURE 2. Geldanamycin-induced endocytosis of ErbB2 is dynamin dependent. SKBr3 cells were transiently transfected with plasmid encoding hemagglutinin-tagged K44A dynamin before incubation with geldanamycin (3 $\mu\text{mol/L}$) for 4 h. Then the cells were fixed and immunostained with rabbit anti-dynamin and mouse anti-ErbB2 (e.c.*) antibodies. The cells were examined by confocal microscopy. Note the lack of vesicular staining for ErbB2 in cells overexpressing K44A dynamin. Bar, 10 μm .

(bottom) when compared with control cells (top), indicating that clathrin-mediated endocytosis is the prime endocytic pathway for ErbB2 on incubation of cells with geldanamycin.

Geldanamycin-Induced Endocytosis of ErbB2 Is Growth Factor Receptor Binding Protein-2 Independent

It has been shown that clathrin-dependent endocytosis of the EGFR depends on the adaptor protein growth factor receptor binding protein 2 (Grb2; refs. 39, 40) and, additionally, that overexpression of Grb2 with point mutations inactivating both SH3 domains (d.n.Grbb2) inhibits EGF-induced formation of clathrin-coated pits and endocytosis of the EGFR (41, 42). To address whether Grb2 is also required in geldanamycin-induced endocytosis of ErbB2, cells were transfected with a plasmid encoding Myc-tagged d.n.Grbb2 24 hours before incubation with geldanamycin. On incubation with geldanamycin, the cells were fixed and immunostained for ErbB2. Transfected cells were identified with an anti-Myc antibody. We found that in cells overexpressing d.n.Grbb2, ErbB2-containing vesicles were formed. These vesicles stained with antibodies to intracellular parts of ErbB2³ and to extracellular parts of ErbB2 (Fig. 4), showing that intact ErbB2 is internalized in the absence of functional Grb2.

ErbB2 Can Be Cleaved by Proteasomes when Retained at the Plasma Membrane in Cells Depleted of CHC

Geldanamycin treatment has been reported to induce a caspase-dependent cleavage of ErbB2 at the plasma membrane (27), and proteasomal activity has been reported to be required for geldanamycin-induced endocytosis of ErbB2 (25). To study a possible geldanamycin-induced cleavage of ErbB2 at the plasma membrane, we used cells transfected with or without siRNA to CHC before incubation with geldanamycin. Although endocytosis of ErbB2 was clearly inhibited in cells transfected with siRNA to CHC, Western blotting with antibodies to the intracellular part of ErbB2 showed that the intracellular part of ErbB2 was degraded on incubation with geldanamycin

³ Our unpublished data.

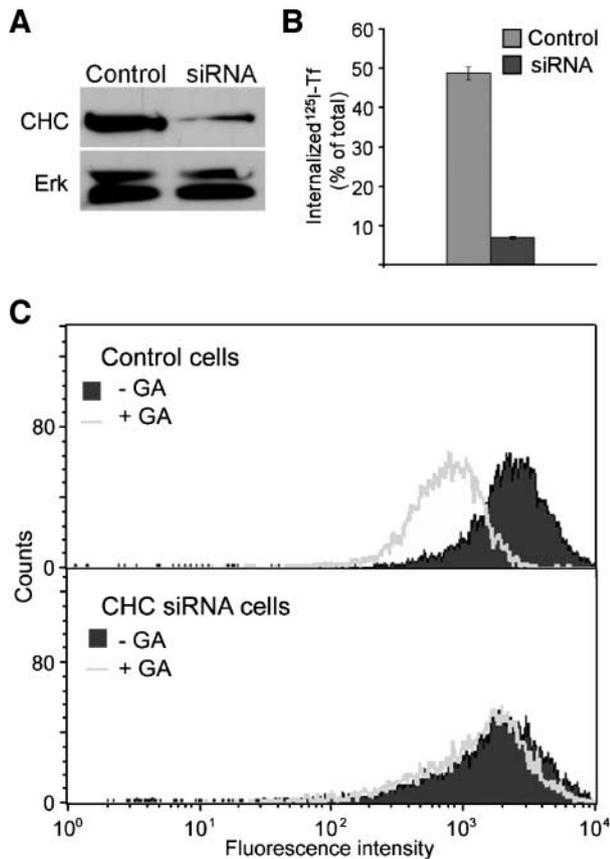


FIGURE 3. Knockdown of CHC inhibits geldanamycin-induced endocytosis of ErbB2. CHC was knocked down with siRNA as described in Materials and Methods. **A.** Lysates of SKBr3 cells transfected with or without siRNA to CHC were subjected to Western blotting with mouse anti-CHC and rabbit anti-extracellular signal-regulated kinase (*Erk*) antibodies (loading control). The reactive proteins were detected by enhanced chemiluminescence. **B.** SKBr3 cells, transfected without (control) or with siRNA to CHC, were incubated with ¹²⁵I-Tf (70 ng/mL) for 10 min at 37°C. Internalized ¹²⁵I-Tf is shown as percent of total cell-associated Tf (internalized + surface bound). Representative of four independent experiments with four parallels. Columns, mean; bars, SD. **C.** SKBr3 cells transfected without (control) or with siRNA to CHC were incubated with geldanamycin (3 μmol/L) for 12 h before being fixed and immunostained with mouse anti-ErbB2 antibody (e.c.*) followed by phycoerythrin-conjugated goat anti-mouse antibody. The level of ErbB2 at the plasma membrane was analyzed by flow cytometry. Representative of four experiments.

both in control cells and in CHC knockdown cells (Fig. 5A). However, when antibodies to the extracellular part of ErbB2 were used (Fig. 5B; Supplementary Fig. S6), we found that whereas the bands representing ErbB2 disappeared in control cells, the molecular weight of ErbB2 was reduced on incubation with geldanamycin in cells depleted of CHC. This suggested a geldanamycin-induced degradation or fragmentation of the intracellular part of ErbB2 in endocytosis-deficient cells. A small amount of the low molecular weight band was seen also in CHC-depleted cells not incubated with geldanamycin, indicating a minor geldanamycin-independent degradation of the intracellular part of ErbB2 when endocytosis was inhibited.

Because both proteasomal activity and caspases have been reported to be involved in geldanamycin-induced ErbB2 down-regulation (25, 27), we examined whether inhibitors of

proteasomes or caspases affected degradation of the intracellular domain of ErbB2 in CHC knockdown cells. As shown in Fig. 6, it is clear that preincubation of cells with the proteasomal inhibitor lactacystin efficiently inhibited degradation of the intracellular part of ErbB2, in contrast to the caspase inhibitor z-VAD-fmk, which had a minor effect only. The finding that the caspase inhibitor did not block degradation of ErbB2 is consistent with the notion that geldanamycin-induced degradation of the intracellular part of ErbB2 in cells depleted of CHC depended on proteasomal activity. However, whereas this did not exclude the possibility that caspase activity could be required for geldanamycin-induced internalization of ErbB2, we also studied the effect of z-VAD-fmk on down-regulation of ErbB2 from the plasma membrane by flow cytometry. Inhibition of caspases had no effect on geldanamycin-induced down-regulation of ErbB2 from the plasma membrane (Supplementary Fig. S7), showing that caspase activity is not required for initial internalization steps.

The observed lactacystin-inhibitable geldanamycin-induced degradation of the intracellular part of ErbB2 in cells depleted of CHC was confirmed by immunoelectron microscopy (Fig. 7). In cells transfected with siRNA to CHC, but not incubated with geldanamycin, antibodies both to the extracellular and intracellular parts of ErbB2 showed strong labeling at the plasma membrane (Fig. 7, left). When CHC-depleted cells were incubated with geldanamycin for 6 hours, labeling with antibody to the extracellular part of ErbB2 was almost as intense as in control cells, whereas labeling for the intracellular part was strongly reduced (Fig. 7, middle). However, if the cells were incubated with both geldanamycin and lactacystin, the labeling was similar in control cells using antibodies to both the intracellular and extracellular parts of ErbB2 (Fig. 7, right). Quantification of the labeling densities obtained with the two antibodies at the plasma membrane of 10 cells from each specimen confirmed these observations (Table 2).

Proteasomal Cleavage of ErbB2 Is Involved in Lysosomal Sorting, but not in Internalization of ErbB2 from the Plasma Membrane

As shown in Fig. 5, geldanamycin-induced degradation of ErbB2 can be monitored by reduced reactivity in Western blots

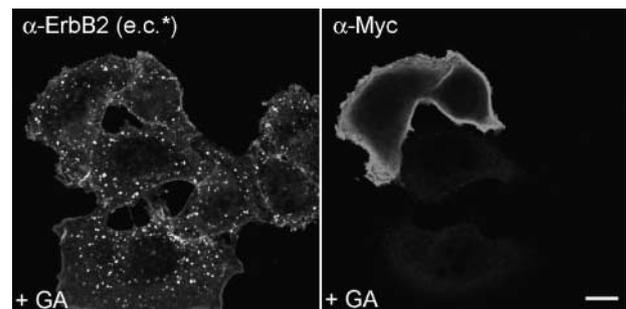


FIGURE 4. Geldanamycin-induced endocytosis of ErbB2 happens independently of Grb2. SKBr3 cells were transiently transfected with a plasmid encoding Myc-tagged d.n.GrB2 before incubation with geldanamycin (3 μmol/L) for 4 h. Then the cells were fixed and immunostained with rabbit anti-Myc and mouse anti-ErbB2 (e.c.*) antibodies. The cells were examined by confocal microscopy, and vesicular staining for ErbB2 is shown in cells overexpressing d.n.GrB2. Bar, 10 μm.

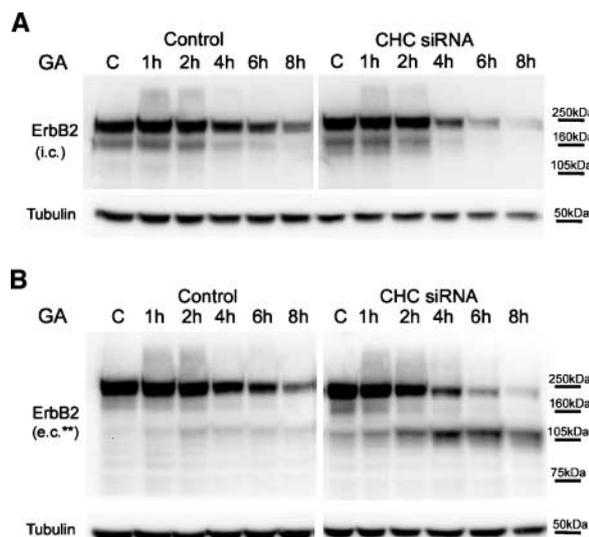


FIGURE 5. The intracellular part of ErbB2 can be degraded when ErbB2 is retained at the plasma membrane in cells depleted of CHC. SKBr3 cells treated without (control) or with CHC siRNA were incubated with geldanamycin (3 $\mu\text{mol/L}$) for increasing time periods before being lysed and subjected to Western blotting with antibody i.c. to the intracellular part of ErbB2 (**A**) or antibody e.c.** to the extracellular part of ErbB2 (**B**). Note the geldanamycin-induced shift in molecular weight observed with the anti-ErbB2 antibody recognizing the extracellular part of ErbB2. Mouse anti-tubulin antibody was used as loading control. C, cells not incubated with geldanamycin.

with antibodies to the extracellular or intracellular domains on 4 to 8 hours of incubation. Furthermore, lactacystin was found to inhibit geldanamycin-induced degradation of ErbB2 (see control column in Fig. 6). This could potentially be compatible with the notion that proteasomal degradation is required for geldanamycin-induced endocytosis and down-regulation of ErbB2 (25). To investigate whether proteasomal activity was in fact required for geldanamycin-induced endocytosis of ErbB2, cells were preincubated with or without lactacystin for 1 hour before incubation with geldanamycin, and the amount of ErbB2 at the plasma membrane was assayed by flow cytometry. Surprisingly, we found that the level of ErbB2 at the plasma membrane was reduced also in cells preincubated with lactacystin (Fig. 8A and B). This suggested that although proteasomes can cleave ErbB2 at the plasma membrane in SKBr3 cells, proteasomal activity is not essential for initial steps of internalization.

We further studied the endocytosis of ErbB2 in SKBr3 cells incubated with lactacystin before geldanamycin using confocal microscopy and antibodies to NH₂-terminal or COOH-terminal parts of ErbB2. ErbB2 was detected in early endosome antigen 1-positive vesicles (Supplementary Fig. S8). On 6-hour exposure to lactacystin and geldanamycin, ErbB2-positive vesicles accumulated (Supplementary Fig. S8, *bottom*), indicating that the intracellular transport of ErbB2 was inhibited when proteasomal activity was blocked. We used immunoelectron microscopy to study the transport of ErbB2 in more detail. Cells preincubated with or without lactacystin before incubation with geldanamycin for 6 hours were prepared for immunoelectron microscopy and labeled with antibodies to both extracellular and intracellular epitopes of ErbB2. In cells incubated with

geldanamycin only, both antibodies showed strong labeling for ErbB2 on the internal membranes of multivesicular bodies (Fig. 9, *top*). In cells incubated with both lactacystin and geldanamycin, however, both antibodies labeled intracellular compartments that morphologically resembled early endosomes (Fig. 9, *middle and bottom*). These compartments, which often appeared clustered in groups, contained few internal vesicles, but frequently had tubular protrusions. Only a limited number of the ErbB2-positive compartments in these cells were multivesicular bodies, and the labeling for ErbB2 was restricted to the outer limiting membranes. Quantification of the labeling on endosome-like compartments within 20 randomly chosen cell profiles from each specimen confirmed these observations (Table 3).

Because it has been shown that geldanamycin and proteasomal inhibitors induce formation of large perinuclear vacuoles derived from the endoplasmic reticulum (34), we confirmed that ErbB2 observed in vesicles can originate from the plasma membrane by incubating cells with anti-ErbB2 antibody on ice for 1 hour before chase in MEM with or without lactacystin and geldanamycin. As shown in Fig. 10, vesicles containing anti-ErbB2 antibody were detected also on incubation of cells with lactacystin and geldanamycin. Although this does not prove that all ErbB2-containing vesicles represent endosomes, it shows that a high proportion of ErbB2 found in vesicles originated from the plasma membrane.

Discussion

The EGFR is efficiently down-regulated from the plasma membrane by clathrin-dependent endocytosis and degraded in lysosomes on ligand binding. However, ErbB2 seems to be internalization resistant (10-12). ErbB2 differs from the EGFR in being unable to bind ligand and in being an Hsp90 client protein. Hsp90 stabilizes ErbB2 by binding to the kinase domain of ErbB2. When cells are incubated with the ansamycin

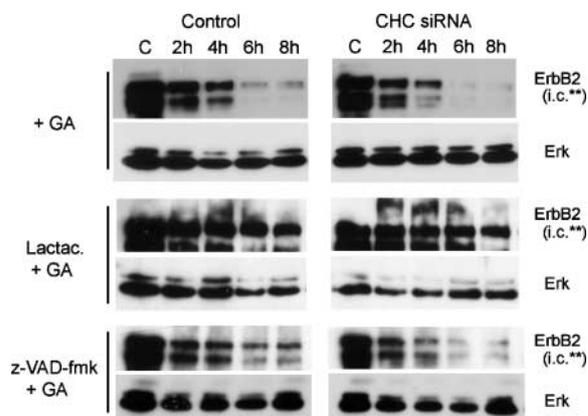


FIGURE 6. Degradation of ErbB2 retained at the plasma membrane due to depletion of CHC is dependent on proteasomal activity, but independent of caspases. SKBr3 cells transfected without (control) or with siRNA against CHC were preincubated with lactacystin (10 $\mu\text{mol/L}$) or z-VAD-fmk (50 $\mu\text{mol/L}$) for 1 h before incubation with geldanamycin (3 $\mu\text{mol/L}$) for increasing time periods. The inhibitors were present during the entire incubation. The cells were lysed and subjected to Western blotting with antibody i.c.** against the intracellular part of ErbB2 or with rabbit anti-extracellular signal-regulated kinase antibody (loading control).

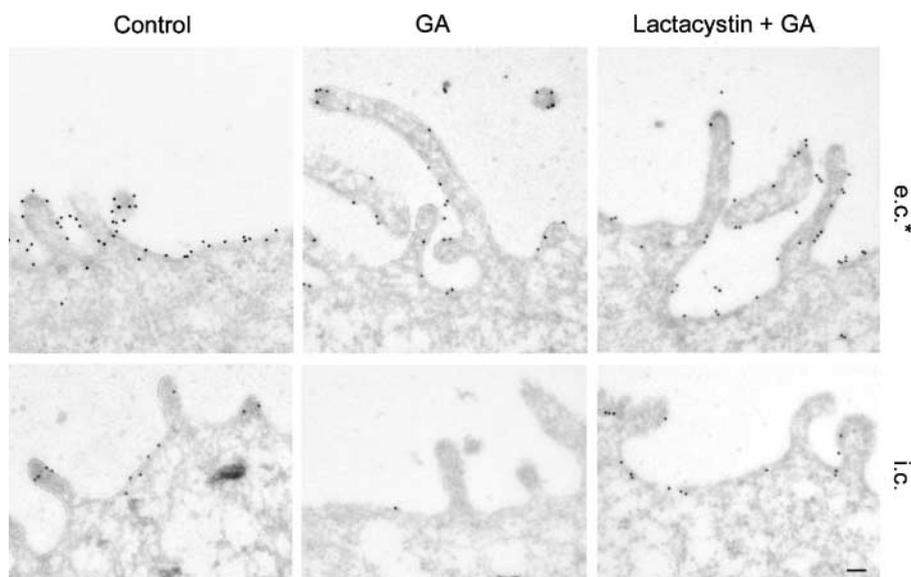


FIGURE 7. Geldanamycin induces a proteasome-dependent degradation of the intracellular part of ErbB2 in endocytosis-deficient cells. SKBr3 cells transfected with siRNA against CHC were incubated without geldanamycin (control; *left*) or with geldanamycin (3 $\mu\text{mol/L}$) alone for 6 h (*middle*), or preincubated with lactacystin for 1 h followed by a 6-h incubation with lactacystin and geldanamycin (*right*). The specimens were prepared for immunoelectron microscopy, and the sections were labeled with antibody e.c.* against the extracellular part of ErbB2 (*top*) or antibody i.c. against the intracellular part of ErbB2 (*bottom*). Bar, 100 nm.

derivative geldanamycin, the binding of ADP/ATP to Hsp90 is inhibited and the ubiquitin ligase CHIP is recruited to the ErbB2-chaperone complex (reviewed in refs. 19, 43). This results in ubiquitination, endocytosis, and degradation of ErbB2 (20, 21). In agreement with previous findings (25), we report that ErbB2-containing endosomes are formed on exposure to geldanamycin and that ErbB2 in these vesicles is derived from the plasma membrane. Austin et al. (29) reported that ErbB2 is constitutively internalized and recycled back to the plasma membrane in nonstimulated cells, and that geldanamycin enhanced the degradative sorting in endosomes instead of increasing initial endocytosis from the plasma membrane. However, in agreement with the results reported by Lerdrup et al. (25), our present results argue that initial endocytosis of ErbB2 is induced by geldanamycin-mediated inhibition of Hsp90. As a consequence of more ErbB2 delivered to endosomes, more ErbB2 is subsequently degraded in lysosomes. Whether geldanamycin additionally increases translocation of ErbB2 from early to late endosomes, as suggested by Austin et al. (29), could not be investigated because we could not detect ErbB2 in endosomes in cells not incubated with geldanamycin.

It has previously been reported that the amount of ErbB2 found in coated pits increased on incubation of cells with geldanamycin compared with control cells, suggesting that internalization occurred clathrin dependently (25). We now directly show that the geldanamycin-induced endocytosis of ErbB2 is clathrin dependent because siRNA-mediated knock-down of CHC efficiently blocked down-regulation of ErbB2 from the plasma membrane. It is unclear whether incubation of cells with geldanamycin exposes an otherwise cryptic internalization signal in ErbB2. It should be noted that consistent with the findings of Lerdrup et al. (25), but in contrast to the findings of Tikhomirov and Carpenter (26-28), we were unable to detect ErbB2 fragments on incubation of cells with geldanamycin. Potentially, this could be a technical problem because small fragments tend to be unstable. However, in agreement with Lerdrup et al., we found that inhibition of caspases had no effect on geldanamycin-induced down-regulation of ErbB2,

arguing that caspase-mediated cleavage is not required for endocytosis of ErbB2. Geldanamycin reportedly induces ubiquitination of ErbB2 (20-22), and ubiquitination has been shown to play a role in regulated endocytosis (reviewed in ref. 44). Polyubiquitination could potentially also act by promoting proteasomal degradation of ErbB2. When cells were depleted of CHC, we found that the intracellular part of ErbB2 was degraded in a proteasome-dependent manner. Whether such degradation plays a role under more physiological conditions is, however, unclear. It should be noted that geldanamycin-induced degradation of the intracellular part of ErbB2 seemed to be more pronounced in CHC-knockdown cells compared with control cells. The reason for this is unclear.

Different results have been reported with respect to geldanamycin-induced cleavage of ErbB2 before endocytosis (25, 26, 28, 45). In agreement with the original findings of Lerdrup et al. (25), we found intact ErbB2 in endosomes when using antibodies recognizing both the extracellular and intracellular parts of ErbB2. Lerdrup et al., however, more recently reported that incubation with geldanamycin induced cleavage of ErbB2 at the plasma membrane and that cleaved

Table 2. The Intracellular Part of ErbB2 Is Degraded by Proteasomal Activity in Clathrin-Depleted Cells

| | Labeling Intensity (% of Control) | |
|----------------------------|-----------------------------------|------|
| Antibody | e.c.* | i.c. |
| Geldanamycin | 78.2 | 9.1 |
| Lactacystin + geldanamycin | 85.6 | 90.8 |

NOTE: SKBr3 cells transfected with siRNA to CHC were preincubated with or without lactacystin (10 $\mu\text{mol/L}$) for 1 h before geldanamycin (3 $\mu\text{mol/L}$) was added for 6 h. The cells were then prepared for immunoelectron microscopy and labeled with antibody e.c.* recognizing the extracellular part of ErbB2 or antibody i.c. recognizing the intracellular part of ErbB2. The labeling for ErbB2 at the plasma membrane was quantified as gold particles per unit length of plasma membrane of 10 cell profiles for each experimental condition. The results are presented as percentage of the labeling density found for each antibody in control cells transfected with siRNA to CHC, but not incubated with lactacystin or geldanamycin.

ErbB2 was endocytosed more efficiently compared with intact ErbB2. The efficient labeling found in endosomes when using antibodies to the intracellular part of ErbB2, however, strongly suggests that ErbB2 is in general not cleaved before endocytosis. However, this does not rule out the possibility that one ErbB2 molecule in a dimeric or oligomeric complex could be cleaved. Whereas Lerdrup et al. reported that proteasomal activity was required at the plasma membrane for geldanamycin-mediated endocytosis (25, 45), we found by flow cytometry analysis that geldanamycin-induced endocytosis of ErbB2 was equally efficient in the presence and absence of lactacystin. It is difficult to explain this apparent contradiction because the same cells and the same concentration of lactacystin were used. However, differences in experimental design could potentially explain this difference. The conclusion that proteasomal activity is required for endocytosis was based on immunofluorescence and quantitative colocalization measurements of ErbB2 at the plasma membrane and of total cellular ErbB2, followed by estimations of internalized ErbB2 in a limited number of cells (25). In contrast, our conclusion that proteasomal activity is not required for endocytosis of ErbB2 is based on flow cytometry analysis of nonpermeabilized cells using an antibody to the extracellular part of ErbB2, allowing thousands of randomly selected cells to be monitored. As we then limit the measurements to changes occurring at the plasma membrane and do not have to take into consideration proteasome-dependent differences in degradation of total ErbB2, we believe that the different conclusions can, at least in part, be explained by different methods. We strongly feel that flow cytometry analysis is the most sensitive and best suited method in this case.

In line with the results of Lerdrup et al. (25), we also report that the overall geldanamycin-induced degradation of ErbB2 depends on proteasomal activity. However, whereas Lerdrup et al. conclude that the rate-limiting step is down-regulation of ErbB2 from the plasma membrane, we find that lactacystin inhibits transport of ErbB2 from the limiting membrane to inner vesicles of multivesicular bodies. As intact ErbB2 is found on the inner vesicles of multivesicular bodies (our current results; ref. 25), ErbB2 itself does not seem to be the proteasomal target. Our immunoelectron microscopy experiments showed that whereas geldanamycin eventually induced transport of ErbB2 to inner vesicles of multivesicular bodies in the absence of lactacystin, ErbB2 was found localizing to intracellular compartments that morphologically resembled early endosomes when cells were incubated with both lactacystin and geldanamycin. These compartments contained few internal vesicles, and the labeling for ErbB2 was restricted to the outer limiting membrane. This effect of lactacystin on sorting of endocytosed ErbB2 is similar to what was observed when the effect of lactacystin on lysosomal sorting of the EGFR was investigated (46). The inhibiting effect of lactacystin on transport of endocytosed EGFR and ErbB2 to inner vesicles of multivesicular bodies and eventually to lysosomes is further consistent with recent results showing that in yeast, ubiquitination and proteasomal activity regulates a late-stage, membrane fusion event (47). It was reported that ubiquitinated proteins accumulated on vacuolar membranes. The most prominent of these ubiquitinated proteins was Ypt7. Vacuolar

Ypt7 and vacuolar proteasomes were found to be interdependent, and ubiquitinated Ypt7 became a proteasomal substrate during fusion. Indeed, the authors concluded that fusion cannot proceed without degradation of ubiquitinated Ypt7 (47). This is consistent with earlier unexplained findings reporting that proteasomal cleavage was required to sort the EGFR to inner vesicles of multivesicular bodies, although the EGFR itself was not a proteasomal target (46).

Materials and Methods

Materials

Na¹²⁵I was from Amersham Biosciences. Fugene was from Roche Molecular Biochemicals, and geldanamycin and lactacystin were from Sigma-Aldrich or Tocris Bioscience. Other chemicals were from Sigma-Aldrich unless otherwise noted.

Antibodies

Mouse anti-ErbB2 antibody (TAB250 to the extracellular part) and rabbit anti-ErbB2 antibody (clone PADZ4881 to the intracellular part) were from Zymed Laboratories. Mouse anti-ErbB2 antibody (Ab-8 to the intracellular part) was from

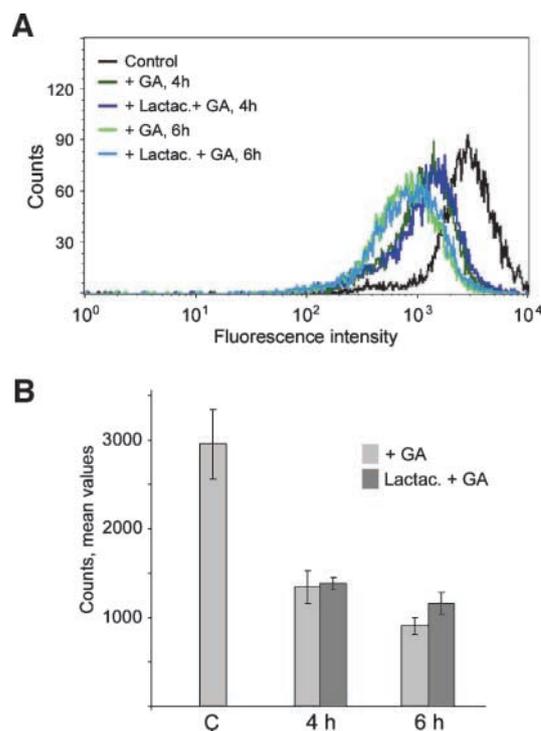


FIGURE 8. Proteasomal activity is not required for geldanamycin-induced endocytosis of ErbB2. **A.** The amount of ErbB2 at the plasma membrane was measured by flow cytometry in SKBr3 cells preincubated with or without lactacystin (10 μ mol/L) for 1 h before being incubated with geldanamycin (3 μ mol/L) for 4 or 6 h. The cells were fixed and immunostained with mouse anti-ErbB2 antibody (e.c.*) followed by phycoerythrin-conjugated goat anti-mouse antibody. Representative flow diagrams with one sample from each condition are illustrated. **B.** Statistical analysis of the flow experiments shown in **A.** Columns, mean values of ErbB2-positive peaks as measured by flow cytometry. Light columns, incubation with geldanamycin only; dark columns, preincubation with lactacystin before geldanamycin. The data set is generated from one representative experiment with six parallels. Columns, mean; bars, SD.

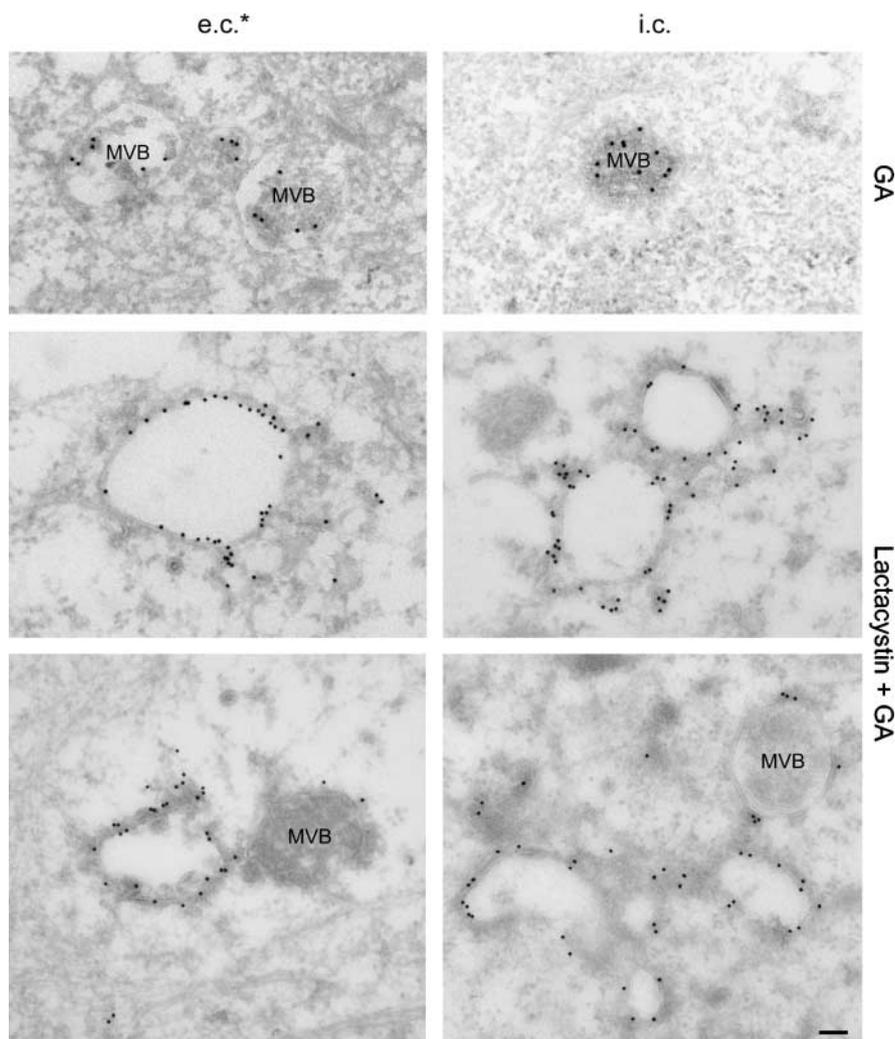


FIGURE 9. Inhibition of proteasomal activity blocks translocation of ErbB2 to internal vesicles of endosomes. SKBr3 cells were incubated with geldanamycin ($3 \mu\text{mol/L}$) alone for 6 h (top) or preincubated with lactacystin for 1 h, followed by a 6-hour incubation with lactacystin and geldanamycin ($3 \mu\text{mol/L}$; middle and bottom). The specimens were prepared for immunoelectron microscopy, and the sections were labeled with antibody e.c.* against the extracellular part or antibody i.c. against the intracellular part of ErbB2. MVB, multivesicular body. Bar, 100 nm.

NeoMarkers, and mouse anti-ErbB2 antibody (Ab-3 to the intracellular part) was from Calbiochem, MERCK KGaA. Mouse anti-early endosome antigen 1, mouse anti-ErbB2 antibody (clone 42 to the extracellular part), and mouse anti-CHC antibody were from BD Transduction. Goat anti-ErbB2 antibody (AF1129 to extracellular part of ErbB2) was from R&D Systems. Rabbit anti-dynamin antibody was a gift from Sandra Schmid (The Scripps Research Institute, La Jolla, CA), and rabbit anti-early endosome antigen 1 and mouse anti-dynamin antibody (Hudy 1) were from Upstate Biotechnology. Mouse anti-tubulin antibody was from Sigma-Aldrich and human anti-early endosome antigen 1 antibody (48) was a gift from Harald Stenmark (Centre for Cancer Biomedicine, Oslo, Norway). Rabbit anti-extracellular signal-regulated kinase antibody was from Santa Cruz Biotechnology and rabbit anti-Myc antibody was from Abcam. Peroxidase-conjugated donkey anti-mouse, peroxidase-conjugated donkey anti-rabbit, peroxidase-conjugated donkey anti-goat, Cy5-conjugated goat anti-mouse, Cy5-conjugated donkey anti-rabbit, Rhodamine Red-X-conjugated donkey anti-rabbit, Cy5-conjugated donkey anti-human, and phycoerythrin-conjugated goat anti-mouse

antibodies were from Jackson ImmunoResearch Laboratories. Alexa 488-conjugated goat anti-mouse antibody was from Molecular Probes, Invitrogen, and rabbit anti-mouse IgG antibody was from Cappel, ICN Biomedicals. For overview of the abbreviations and the use of various anti-ErbB2 antibodies, see Table 1.

Cell Culture and Treatment

The mammary carcinoma cell line SKBr3 was from American Type Culture Collection and was grown in DMEM (BioWhittaker) containing penicillin-streptomycin mixture ($0.5\times$; BioWhittaker), L-glutamine (2 mmol/L ; BioWhittaker), and fetal bovine serum ($15\% \text{ v/v}$). The HEP2 cells were grown in DMEM with penicillin-streptomycin mixture ($0.5\times$) and L-glutamine (2 mmol/L ; both from BioWhittaker) and fetal bovine serum ($5\% \text{ v/v}$; PAA Innovations) was routinely used. The PAE.EGFR.ErbB2 cells were made and grown as described (15). During experiments, cells were incubated with the indicated compounds either in MEM (Life Technologies) with 0.1% bovine serum albumin at 37°C or in DMEM if the experiment lasted for more than 5 hours.

Plasmids and Transient Transfection

pcDNA3.1-HA-Dynamin1 (encoding K44A dynamin, lacking GTPase activity) was kindly provided by Sandra Schmid. Myc-tagged d.n.Grb2 (W36, 193K; nonfunctional NH₂-terminal and COOH-terminal SH3-domains; pRK5Myc-d.n.Grb2) was previously described (41). SKBr3 cells were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer's recommendations. Transfected cells were analyzed ~24 hours on transfection, and transfected cells were detected with antibodies to the proteins of interest or to their tags.

Flow Cytometry

Flow cytometry analysis was done essentially as described (10, 15). For all flow cytometry experiments, a minimum of 10,000 cells were counted. Nonstained cells and cells only incubated with secondary antibody were always included as controls.

Immunoblotting

Western blot analysis was done as previously described (49). The reactive proteins were detected by enhanced chemiluminescence (Amersham Biosciences and Pierce).

Immunocytochemistry and Confocal Microscopy

Cells were grown on 12-mm coverslips (Menzel-Gläser) in 24-well microtiter plates, and immunostaining was done as described (10). The coverslips were mounted with DAKO fluorescent mounting medium (DAKO), and the cells were examined under a Leica TSC XP confocal microscope (Leica Microsystem AG).

Immunoelectron Microscopy

SKBr3 cells, treated as described in legends to figures, were prepared for immunoelectron microscopy as described (41). The sections were labeled with antibody e.c.* to the extracellular part of ErbB2 or antibody i.c. to the intracellular part of ErbB2, followed by rabbit anti-mouse IgG antibody and finally

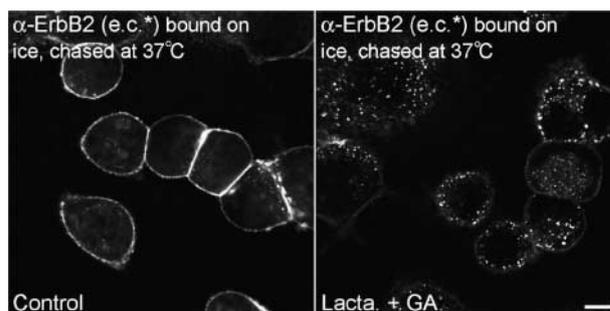


FIGURE 10. ErbB2-positive vesicles detected on incubation with lactacystin and geldanamycin originate from the plasma membrane. SKBr3 cells on coverslips were incubated with mouse anti-ErbB2 antibody (e.c.*) in MEM for 1 h on ice. Then the cells were washed with PBS before being chased with MEM with or without geldanamycin (3 μmol/L) for 2 h at 37°C. On incubation, the cells were fixed, permeabilized, and immunostained with Alexa 488-conjugated goat anti-mouse antibody and analyzed by confocal microscopy. Bar, 10 μm.

protein A gold (purchased from G. Posthuma). The sections were examined with a Philips CM 120 electron microscope.

CHC Knockdown

SKBr3 cells were transfected with siRNA to knock down CHC using Lipofectamine 2000 according to the manufacturer's recommendations (Invitrogen). Briefly, SKBr3 cells were transfected twice with siRNA with a 72-hour interval. The target sequence used was GCAAUGAGCUGUUUGAAGA (38). siRNA was synthesized and annealed by Ambion, Inc. Control cells were incubated with Lipofectamine 2000 only.

Internalization of ¹²⁵I-Tf

Iron-saturated human Tf was iodinated with Na¹²⁵I, as described (41). SKBr3 cells in 24-well microtiter plates were incubated with 70 ng/mL of ¹²⁵I-Tf as described (42). Internalized ¹²⁵I-Tf was measured as percent of total cell-associated ¹²⁵I-Tf (supernatant and pellet fraction).

Table 3. Distribution of ErbB2 on Endosomes in Cells Treated with Geldanamycin Only or with Geldanamycin and Lactacystin

| Experimental Condition | | Distribution of Anti-ErbB2 Labeling (%) | | | |
|----------------------------|----------|---|------|-------------------|----------------|
| | | A | | B | |
| Additive | Antibody | Early Endosome | MVB | Limiting Membrane | Inner Vesicles |
| Geldanamycin | e.c.* | 28.6 | 71.4 | 21.6 | 78.4 |
| | i.c. | 19.1 | 80.9 | 7.8 | 91.2 |
| Lactacystin + geldanamycin | e.c.* | 55.9 | 44.1 | 61.1 | 38.9 |
| | i.c. | 56.4 | 43.6 | 70.8 | 28.2 |

NOTE: SKBr3 cells were preincubated with or without lactacystin (10 μmol/L) for 1 h before geldanamycin (3 μmol/L) was added for 6 h. The cells were then prepared for immunoelectron microscopy and labeled with antibodies e.c.* or i.c. directed against the extracellular or intracellular part of ErbB2, respectively. For each experimental condition, the labeling of ErbB2-positive endosomal compartments within 20 cell profiles was quantified with respect to both type of endosome and localization of ErbB2 within the endosome. A: The numbers of ErbB2-positive early endosome-like compartments and ErbB2-positive multivesicular bodies were counted and are presented as percentage of the total number of ErbB2-positive endosomes under each condition. B: The distribution of the labeling was quantified with respect to localization to the limiting membrane of endosomes or to vesicles within the endosomal lumen. The number of gold particles showing different localization is presented as percentage of the total number of gold particles.

Abbreviation: MVB, multivesicular body.

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Molecular Cancer Research

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