Geldanamycin Induces ErbB-2 Degradation by Proteolytic Fragmentation*

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Exposure of carcinoma cell lines to the antibiotic geldanamycin induces the degradation of ErbB-2, a co-receptor tyrosine kinase that is frequently overexpressed in certain tumors. Using ErbB-2 mutants expressed as chimeric receptors or green fluorescent protein fusion proteins, we report that the kinase domain of ErbB-2 is essential for geldanamycin-induced degradation. The kinase domain of the related epidermal growth factor receptor was not sensitive to this drug. The data further indicate mechanistic aspects of ErbB-2 degradation by geldanamycin. The data show that exposure to the drug induces at least one cleavage within the cytoplasmic domain of ErbB-2 producing a 135-kDa fragment and a 23-kDa fragment. The latter represents the carboxyl-terminal domain of ErbB-2, whereas the former represents the ectodomain and part of the cytoplasmic domain. Degradation of the carboxyl-terminal fragment is prevented by proteasome inhibitors, whereas degradation of the membrane-anchored 135-kDa ErbB-2 fragment is blocked by inhibitors of the endocytosis-dependent degradation pathway. Confocal microscopy studies confirm a geldanamycin-induced localization of ErbB-2 on intracellular vesicles.

ErbB-2 is a Type 1 transmembrane tyrosine kinase that functions as a co-receptor by forming dimers with other members of the ErbB receptor family (ErbB-1 (EGF1 receptor), ErbB-3, and ErbB-4; Refs. 1 and 2). Although ErbB-2 has a potential ligand-binding ectodomain, no direct ligand has yet been identified. In its role as a co-receptor, ErbB-2 enhances the signaling capacity of its dimerization partners. The association of ErbB-2 with these various receptors is, however, entirely ligand-dependent. In the absence of growth factor ErbB-2 is reported to interact with CD44, an adhesion receptor, in ovarian carcinoma cell lines (3) and with a large plasma membrane glycoprotein complex in microvilli of a mammary adenocarcinoma cell line (4). ErbB-2 has also been demonstrated to form ligand-dependent complexes with the IL-6 receptor component gp130 (5) and Trk A (6), the nerve growth factor receptor.

ErbB-2 was originally identified as the transforming oncogene neu in which a point mutation in the transmembrane domain is responsible for its oncogenic potential (7, 8). ErbB-2 also functions as an oncogene when overexpressed (9, 10) and in humans is frequently overexpressed in breast and ovarian tumors (11). ErbB-2 overexpression in breast cancer is associated with a poor prognosis (12), and hence it is a target for therapeutic reagents, including monoclonal antibodies and drugs (13). Frequently, antibodies that decrease the growth of ErbB-2-expressing tumors also reduce the level of ErbB-2 by a mechanism that is unclear. Hence, the transforming activity of ErbB-2 is related to structural changes or changes in its level of expression.

The benzoquinoid ansamycin antibiotics geldanamycin and herbimycin were first isolated from the culture broths of several actinomycete species (14, 15) and described as inhibitors of tyrosine kinase-dependent growth (16, 17). These compounds, particularly geldanamycin, have tumoural activity toward numerous tumor cell lines (18), including those that overexpress ErbB-2 (19). This action toward tumor cell lines is attributed to the capacity of geldanamycin to induce the degradation of several important signal transducers important in mitogenic pathways. These targets include protein kinases, such as Src, Raf, FAK, and ErbB-2, and other growth regulating proteins, such as p53 (20). The mechanism for the geldanamycin-induced degradation of these various molecules is centered on the Hsp90 family of chaperones, because Hsp90 is the major intracellular protein that binds geldanamycin (20, 21). Geldanamycin has been shown to dissociate Hsp90 from various proteins and thereby inhibit their function, such as the nuclear translocation of glucocorticoid receptors, or to induce their metabolic degradation, such as Src, Raf, and p53.

In the case of ErbB-2, association with Hsp90 has not been reported. However, it has been reported that the glucose-regulated chaperone GRP94, an Hsp90 family member that is localized to the lumen of the endoplasmic reticulum, does associate with ErbB-2 in a geldanamycin-sensitive manner (22). Geldanamycin-induced degradation of ErbB-2 is reported to involve, presumably as a consequence of the dissociation of GRP94, the polyubiquitination of ErbB-2 and its proteosomal degradation (23). On the basis of GRP94 localization, these studies would suggest an interaction with the ErbB-2 ectodomain in the lumen of the endoplasmic reticulum and drug-induced degradation during receptor biosynthesis. Others, however, have suggested that this interpretation does not account for the quantitative aspects of ErbB-2 degradation induced by geldanamycin (24).

We have explored the question of how geldanamycin induces ErbB-2 degradation and show that the ErbB-2 kinase domain is essential for sensitivity of geldanamycin. Also we show that geldanamycin induces fragmentation of ErbB-2 within the carboxy-terminal region of the cytoplasmic domain and that the
resulting transmembrane fragment is degraded by a mechanism that involves the formation of intracellular vesicles.

**EXPERIMENTAL PROCEDURES**

**Materials**—Geldanamycin, N-acetyl-l-leucinyl-l-leucinyl-norleucil (ALLN), folimycin, proteasome inhibitor I, and 4-hydroxy-5-ido-3-nitrophenylacetyl-Leu-Leu-Valinal were purchased from Calbiochem. Batimatstat (BB94) was a generous gift of Dr. P. Dempsey (Vanderbilt University, Nashville, TN). TAPI-O was obtained from Peptide International. Other protease inhibitors, protein G or protein A, and ECL reagents were from Sigma. Monoclonal antibody (Ab5) against ErbB-2 extracellular domain and monoclonal antibody (Ab3) against carboxyl terminal of ErbB-2 were purchased from Oncogene Science. Monoclonal antibodies against EGFR extracellular domain (Ab13 and Ab14), monoclonal antibody (Ab2) against the ErbB-4, and monoclonal antibody (Abb) against ErbB-2 cytoplasmic domain were from NeoMarkers. Goat anti-mouse IgG labeled with Alexa 488 for confocal microscopy was from Molecular Probes. Human ErbB-2 cDNA and NIH 3T3 cell lines with overexpressed ErbB-2 or EGF receptor have been generous gifts from Dr. Pier Paolo Di Fiore (European Institute of Oncology, Milan, Italy) and have been previously described elsewhere (25). The mammalian expression vector pEGFP-C1 for heterologous fusion proteins to green fluorescent protein (GFP) and monoclonal antibody to GFP were obtained from CLONTECH.

**Cell Culture and Transfection**—Human mammary tumor-derived SKBr3 cells were grown in 5% CO₂ at 37 °C in McCoy medium with 10% fetal bovine serum, COS 7 cells were grown in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum, and all fibroblast cell lines were grown in Dulbecco’s modified Eagle’s medium containing 10% calf serum. Cells were grown to about 80% confluency then washed two times, and treated with indicated drugs in serum-free Dulbecco’s modified Eagle’s medium. For transfection and expression of GFP fusion protein, COS 7 cells were grown to ~70% confluency overnight and transfected with LipofectAMINE (Life Technologies, Inc.) according to manufacturer’s recommendations (10 μg of plasmid DNA mixed with 16 μl of LipofectAMINE were used per 60-mm tissue culture dish). The cells were grown for 48 h before assays.

**Construction of GFP Fusion Proteins**—The ErbB-2 kinase domain and cytoplasmic region cDNA fragments were generated by PCR with high fidelity VENT™ DNA polymerase (New England Biolabs). Following the numbering of Yamamoto et al. (26), the kinase domain fragment and cytoplasmic domain fragment correspond, respectively, to residues 715-1250 and 1256-1255 of ErbB-2. To prepare these two fragments, the following primers were synthesized: upstream primer with SacI restriction site 5′-GGG AGT ATC ATC AAA CGA GCT CGT AAG ATC-3′ (primer 1), downstream primers with SacI restriction site 5′-GGG ATC CTC ATC AAA CGA GCT CAG AAG ATC-3′ (primer 2), downstream primers with HindIII restriction site 5′-CAG ATG AGC GCG GGG-3′ (primer 3), and primer 2 as downstream primer. One round PCR with primers 5′-CTC GAT TCT GTC ACT GTA GGG-3′ (upstream primer) and 5′-TCC GGT GGC GGC CCG CCG GGC GGG-3′ (downstream primer). The cDNA fragment for ErbB-2 carboxyl terminus with primer 5′-CAT TGG CCA AGT CCT CTA GAC TCC AAC TTC-3′ (upstream primer). The cDNA fragment for ErbB-2 kinase domain was prepared as described above with primers 1 and 2. Both products were cut with XbaI and ligated. Then cDNA fragment and pEGFP vector were cloned into SacI and XhoI restriction sites. All the constructions described above were verified by sequencing in the regions that underwent genetic manipulations.

**Immunoprecipitation and Immunoblotting**—After indicated treatments, the cells were solubilized by scraping with rubber policeman into cold lysis buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2 mM EDTA, 0.5% NP-40, 1 mM diethylsulfoxide, 2 mM Pefabloc, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM Na3VO4). The lysates were then clarified by centrifugation (14,000 × g, 10 min). Receptors were immunoprecipitated with 1 μg of the indicated antibody immobilized on protein G or protein A by incubation for 1 h at 4 °C. Subsequently, the complexes were washed with lysis buffer three times and resuspended in Laemmli sample buffer for 7.5% SDS-PAGE. After electrophoresis, proteins were transferred to nitrocellulose membranes, and the membranes were blocked by incubation with 5% bovine serum albumin in PBS for 1 h at room temperature. The membranes were then incubated 1 h at room temperature with the indicated blotting antibody in TBSTw buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.05% Tween 20, 0.5% nonfat milk), washed three times in the same buffer, and incubated 1 h with horseradish peroxidase-conjugated mouse antibody or protein A. The membranes were then washed five times in TBSTw and visualized by ECL.

**RESULTS**

**Influence of ErbB-2 Kinase Domain on Geldanamycin-induced Degradation**—To measure the extent of ErbB-2 degradation in cells treated with geldanamycin, we have used, in parallel, antibodies that react with ectodomain or carboxy-terminal domain epitopes of ErbB-2. The results, as shown in Fig. 1A, demonstrate that immunoreactivity to both ectodomain and carboxyl-terminal domain antibodies is rapidly lost following exposure to geldanamycin. The cells employed in this experiment, SKBr3, overexpress ErbB-2, and quantitation of the data indicates that in these cells the half-life of ErbB-2 is approximately 2 h in the presence of geldanamycin. Others have reported a similar half-life for ErbB-2 under these conditions (19) and contrasts with the reported half-life of about 7–9 h for ErbB-2 under normal conditions (28, 29). This influence of geldanamycin on ErbB-2 metabolic stability is reasonably specific because no significant decrease in the structurally related receptors ErbB-1 or ErbB-4 was detected following geldanamycin treatment of A-431 cells or T47–17 cells, respectively (Fig. 1B).

To map the region of ErbB-2 that mediates geldanamycin sensitivity, we initially employed chimeric receptors in which the cytoplasmic domain of the EGF receptor is replaced by that of ErbB-2 (EFG/ErB-2(c2D)) or in which the carboxy-terminal domain of the EGF receptor is replaced by the corresponding region of ErbB-2 (EFG/ErB-2(c2)). These chimeric receptors are expressed in NIH 3T3 cells and have been described previously (25). The data in Fig. 2 show that following the addition of geldanamycin the chimeric receptor having the entire ErbB-2 cytoplasmic domain is sensitive to drug-induced degradation, whereas the chimeric receptor that contains only the carboxy-terminal domain of ErbB-2 is not influenced by the presence of geldanamycin. In this experiment the EFG/ErB-2(c2D) receptor has a half-life of approximately 3.9 h in the pres-
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exposed to geldanamycin (3 μM) for the indicated periods of time. The cells were then lysed, and Western blots (WB) were prepared from equal aliquots (50 μg) of each lysate using antibody to either the ErbB-2 cytoplasmic domain (CD) or ectodomain (ED). The blots in lanes 1–4 were evaluated by scanning densitometry to quantitate the amount of ErbB-2 present as a function of the time of drug exposure. B, A-431 cells that overexpress the EGF receptor (lanes 1–5) or NIH3T3 cells that overexpress a transfected human ErbB-4 receptor (lanes 5–8) were exposed to geldanamycin (3 μM) for the indicated periods of time. The cells were then lysed, and equal aliquots of each lysate (50 μg) were used for immunoprecipitation (IP) of the indicated receptor. Western blots were then performed as indicated, and bound antibody was visualized by ECL.

Fig. 2. Sensitivity of GFP-ErbB2 fusion proteins to geldanamycin-induced degradation. A, constructs encoding GFP, GFP-ErbB2(ED), or GFP-ErbB2(CT) were expressed in COS 7 cells and tested for metabolic stability following incubation for 6 h in the absence (−) or presence (+) of geldanamycin (3 μM). Following the incubations, the cells were lysed, equivalent aliquots (30 μg) of each lysate were electrophoresed, and fusion proteins were detected by Western blotting with anti-GFP. B, constructs encoding GFP fusions with the ErbB-2 kinase domain (GFP-ErbB2(ED); lanes 1–4) or the EGF receptor kinase domain (GFP-EGFR(ED); lanes 5–8) were expressed in COS 7 cells. The cells were treated with geldanamycin (3 μM) for the indicated times prior to cell lysis. Equal aliquots (30 μg) of each lysate were then electrophoresed, and Western blotting with anti-GFP was used to detect fusion proteins. C, scanning densitometry of the data in B was used to quantitate the level of each fusion protein. Bound antibody was detected in each panel by ECL. Closed bars represent GFP-ErbB2(ED), and open bars represent GFP-EGFR(ED).

Fig. 3. Sensitivity of GFP-ErbB2 fusion proteins to geldanamycin-induced degradation. A, constructs encoding GFP, GFP-ErbB2(ED), or GFP-ErbB2(CT) were expressed in COS 7 cells and tested for metabolic stability following incubation for 6 h in the absence (−) or presence (+) of geldanamycin (3 μM). Following the incubations, the cells were lysed, equivalent aliquots (30 μg) of each lysate were electrophoresed, and fusion proteins were detected by Western blotting with anti-GFP. B, constructs encoding GFP fusions with the ErbB-2 kinase domain (GFP-ErbB2(ED); lanes 1–4) or the EGF receptor kinase domain (GFP-EGFR(ED); lanes 5–8) were expressed in COS 7 cells. The cells were treated with geldanamycin (3 μM) for the indicated times prior to cell lysis. Equal aliquots (30 μg) of each lysate were then electrophoresed, and Western blotting with anti-GFP was used to detect fusion proteins. C, scanning densitometry of the data in B was used to quantitate the level of each fusion protein. Bound antibody was detected in each panel by ECL. Closed bars represent GFP-ErbB2(ED), and open bars represent GFP-EGFR(ED).

The results in Fig. 3 show that the GFP fusion protein containing the ErbB-2 kinase domain (GFP-ErbB2(ED)) was rapidly degraded in the presence of geldanamycin, whereas there was no degradation of a fusion protein containing the EGF receptor kinase domain (GFP-EGFR(ED)). However, the GFP fusion protein containing the ErbB-2 kinase domain (GFP-ErbB2(ED); lanes 1–4) was rapidly degraded in the presence of geldanamycin, whereas there was no degradation of a fusion protein containing the EGF receptor kinase domain (GFP-EGFR(ED); lanes 5–8). The results in Fig. 3B are quantitated in Fig. 3C and show the increased sensitivity of the ErbB-2 kinase domain to geldanamycin compared with the EGF receptor kinase domain.

In this system, the lack of sensitivity to geldanamycin is, on
The cells were then treated with (+) or without (−) geldanamycin (3 μM) for 6 h. Lysates were prepared, and equal aliquots (30 μg) were electrophoresed on 10% SDS-PAGE gels. Subsequently, Western blotting with anti-GFP was used to detect the fusion protein bands, which were visualized by ECL. B, a construct encoding a fusion protein of GFP with the ErbB-2 kinase domain and the EGF receptor carboxyl terminus (GFP-ErbB2KD-EGFRCT) was expressed in COS 7 cells. The cells were then treated with (+) and without (−) geldanamycin (3 μM) for 6 h, and lysates were prepared. Equal aliquots (30 μg) of each lysate were electrophoresed and blotted with antibody to GFP. Bands were visualized by ECL.

The basis of previous data as expected for fusion proteins containing the ErbB-2 carboxyl-terminal domain or the EGF receptor kinase domain. That the fusion protein containing only the ErbB-2 kinase domain is rapidly degraded in the presence of geldanamycin indicates that the kinase domain is sufficient to mediate its degradation. However, the lack of degradation of the fusion protein containing the entire ErbB-2 cytoplasmic domain was unexpected and is in apparent discordance with the geldanamycin-induced degradation of the EGF receptor/ErbB-2 chimeric receptor, which contains the entire ErbB-2 cytoplasmic domain (Fig. 2, lanes 1–4).

These latter results suggested that perhaps within the context of cytosolic GFP fusion proteins, but not in the transmembrane ErbB-2 molecule, the ErbB-2 carboxyl terminus had a protective effect on the sensitivity of the kinase domain to degradation induced by geldanamycin. To test this possibility, we constructed a series of GFP fusion proteins containing the ErbB-2 kinase and carboxyl-terminal domains with progressive deletions of the ErbB-2 carboxyl-terminal domain. The geldanamycin sensitivity of these constructs, when expressed in COS 7 cells, is shown in Fig. 4. Deletion of the carboxyl-terminal 19 residues (Δ1236–1255, lanes 3 and 4) or 59 residues (Δ1196–1255, lanes 5, 6) did not increase sensitivity of the fusion proteins to degradation in the presence of geldanamycin. However, increased sensitivity to geldanamycin-induced degradation was observed when deletions of 105 or more residues were made in the ErbB-2 carboxyl-terminal domain (lanes 7–14). Hence, loss of the amino-terminal half of this carboxyl-terminal domain significantly increases the sensitivity of the ErbB-2 kinase domain to geldanamycin.

In this series of fusion proteins we also determined whether the carboxyl-terminal domains of the EGF receptor would abrogate sensitivity of the ErbB-2 kinase domain to geldanamycin. Hence, we prepared a fusion protein construct to encode the kinase domain of ErbB-2 and carboxyl-terminal domain of the EGF receptor. When this molecule was expressed in COS 7 cells, the results (Fig. 4B) showed complete sensitivity to geldanamycin-induced degradation in contrast to the fusion protein containing the carboxyl-terminal sequences of ErbB-2 (lanes 1 and 2).

**Detection of Geldanamycin-induced ErbB-2 Fragments**—The above results, which indicate a role of the ErbB-2 carboxyl-terminal domain in determining the sensitivity of ErbB-2 kinase domain to geldanamycin-induced degradation, suggested that perhaps the carboxyl-terminal region of the transmembrane receptor might be cleaved following the addition of geldanamycin and that this event may be necessary for subsequent degradation of the ErbB-2 molecule, perhaps in a manner that required localization at the plasma membrane and not in the cytosol. Therefore, we re-examined the geldanamycin-induced degradation of the chimeric EGF receptor/ErbB-2KD molecule, whose degradation in the presence of geldanamycin was shown in Fig. 2 (lanes 1–4). If the carboxyl terminus is, in fact, cleaved prior to degradation, then the antibodies used in the experiment shown in Fig. 2 would not detect the remaining membrane-localized fragment because they are to an epitope in the ErbB-2 carboxyl terminus. Hence, we tested the geldanamycin-induced degradation of this molecule using an antibody to the EGF receptor ectodomain. As shown in Fig. 5, this antibody detected both the native 185-kDa form of the chimeric receptor plus a geldanamycin-induced fragment of approximately 135 kDa (lanes 2 and 3). As shown in lane 5, the formation of this fragment in cells exposed to geldanamycin was blocked by the presence of the protease inhibitor ALLN and to a lesser extent by lactacystin (data not shown).

We next attempted to confirm that geldanamycin induced a carboxyl-terminal cleavage in the native ErbB-2 molecule as well as the chimeric receptor. To test this we used SKBr3 cells and an antibody to an epitope in the ErbB-2 ectodomain. As shown in Fig. 6A (lane 2) incubation of these cells with geldanamycin for 6 h resulted in the loss of the native ErbB-2 molecule, and no fragment was detected. This result is similar to that reported in Fig. 1A (lanes 5–8). We reasoned that perhaps in these cells the fragment was metabolically unstable and might be detectable if the geldanamycin treatment were performed at a low temperature to reduce metabolic degradation. In this part of the experiment (lanes 3 and 4), geldanamycin was added to the cells for 1 h at 37 °C, and then the cells were cooled to 4 °C and incubated for an additional 5 h. Under these conditions an
ErbB-2 fragment of 135 kDa was detectable, and in the presence of ALLN this fragment was not detected. These data indicated that endogenous ErbB-2 is cleaved within the cytoplasmic domain in geldanamycin-treated cells.

A number of protease inhibitors were tested without success for their capacity to prevent degradation of the 135-kDa ErbB-2 fragment produced in response to geldanamycin. However, the cathepsin B inhibitor CA074-Me (30 μM) did stabilize the level of the ErbB-2 fragment in geldanamycin-treated cells (Fig. 6B). Because cathepsin B is mainly localized in late endosomes (31), we tested compounds that interfere in the acidification and/or processing of endosomes. As shown in Fig. 6C (lanes 1–5), chloroquine, monensin, and folimycin each significantly increased accumulation of the 135-kDa ErbB-2 fragment in geldanamycin-treated cells. In the absence of geldanamycin, none of these compounds revealed major immunoreactive bands other than the native ErbB-2. Detection of this accumulated 135-kDa fragment was not possible when an antibody to the ErbB-2 carboxyl-terminal domain was employed (lanes 6–10), supporting the conclusion that this fragment is produced by a cleavage at the carboxyl terminus of ErbB-2.

If ErbB-2 is cleaved at the carboxyl terminus such that an antibody epitope is lost from the native molecule, an antibody to a carboxyl-terminal epitope may be able to detect the released fragment if it is sufficiently metabolically stable. That such a carboxyl-terminal ErbB-2 fragment can be detected in lysates from geldanamycin-treated cells blotted with an antibody to the ErbB-2 carboxyl terminus is shown in Fig. 7. As demonstrated in Fig. 7A, the presence of protease inhibitors ALLN or proteasome inhibitor I reveals the presence of a 23-kDa ErbB-2 carboxyl-terminal fragment produced during geldanamycin exposure of SKBr3 cells. The data in Fig. 7B show the influence of incubation time in geldanamycin on the accumulation of this fragment in SKBr3 cells. The fragment is readily detected within 1 h following the addition of geldanamycin. The previously described 135-kDa ErbB-2 fragment is also readily detected in the same period of time (data not shown).

Geldanamycin-induced Intracellular ErbB-2 Containing Vesicles—The capacity of folimycin, chloroquine, or monensin to increase accumulation of the 135-kDa ErbB-2 fragment suggests that this fragment is normally degraded by a mechanism that involves endocytic vesicles. Therefore, we used confocal microscopy to determine whether ErbB-2 is internalized during geldanamycin treatment. Following drug or vehicle exposure for 6 h, the cells were fixed and permeabilized prior to incubation with antibody to ErbB-2 and fluorescein-conjugated secondary antibody. Shown in Fig. 8 are SKBr3 cells with endogenous ErbB-2 (Fig. 8, A and B), NIH 3T3 cells stably expressing transfected ErbB-2 (Fig. 8, C and D), and COS 7 cells transiently expressing a GFP fusion protein with the ErbB-2 kinase domain (Fig. 8E). In the absence of geldanamycin, the transmembrane form of ErbB-2 is clearly expressed at the cell surface in SKBr3 (Fig. 8A) and NIH 3T3 cells (Fig. 8C), whereas the GFP fusion protein is located in the cytosol of COS 7 cells (Fig. 8E). In each case the distribution is changed dramatically following geldanamycin incubation, such that immunoreactivity is concentrated in intracellular vesicles, which...
mimic the appearance of lysosomes (Fig. 8, B, D, and F). The vesicles could be detected within 2 h of geldanamycin consistent with the time course of ErbB-2 degradation. The presence of either ALLN or folimycin decreased the redistribution of ErbB-2 immunoreactivity observed in the presence of geldanamycin (data not shown). Also, the GFP fusion protein with the entire ErbB-2 cytoplasmic domain, which we have previously shown is metabolically stable in the presence of geldanamycin (Fig. 3A, lanes 5 and 6), was not redistributed in the presence of the drug (data not shown).

**DISCUSSION**

In this manuscript we report several novel aspects of the mechanism by which geldanamycin induces the degradation of ErbB-2. Using antibodies to both ectodomain and carboxyl-terminal epitopes, we detect two fragments of ErbB-2 produced following geldanamycin incubation. One fragment of 135 kDa represents the ErbB-2 ectodomain plus the transmembrane domain and part of the kinase cytoplasmic domain. A second fragment of approximately 23 kDa is also detected under these conditions and represents the carboxyl-terminal domain of ErbB-2. These fragments are detectable within 30–60 min of geldanamycin addition to cells and continue to be detected for several hours. These two fragments do not, however, account for the entire mass of the native ErbB-2 molecule. In previous studies of geldanamycin-induced ErbB-2 degradation, fragments of this molecule have not been detected. This is likely due to two factors. The first is the absence of appropriate inhibitors and the second is the use of different antibodies for sequential precipitation (cytoplasmic domain epitope) and blotting (ectodomain epitope).

Based on the characteristics of these fragments, at least two mechanisms can be proposed to account for the generation of these fragments (Fig. 9). In model I, an endoproteolytic cleavage within the carboxyl-terminal domain could directly generate the observed 23-kDa fragment plus a 160-kDa fragment representing the rest of the ErbB-2 molecule. Subsequent cleavage of this latter fragment within the kinase domain could generate the 135-kDa fragment plus a small fragment of approximately 27 kDa that is undetectable with available immunologic reagents. Alternatively, it can be proposed that fragments are produced in the order predicted in model II. In this case an endoproteolytic event within the kinase domain would directly generate the observed 135-kDa transmembrane fragment plus a fragment of about 50 kDa. Subsequent cleavage of this latter fragment would produce the observed 23-kDa carboxyl-terminal domain fragment.

In either model intermediate fragments have not been detected (the 160-kDa fragment in model I or the 50-kDa fragment in model II), and this may be due to the rapidity with which the second cleavage occurs. We prefer the scheme depicted in model I for the following reasons. Our data show that expression of the ErbB-2 cytoplasmic domain as a cystolic GFP fusion protein is not sensitive to geldanamycin-induced degradation, whereas a GFP fusion with the ErbB-2 kinase domain without the carboxyl-terminal domain is sensitive to geldanamycin-induced degradation. Also, the transmembrane ErbB-2 receptor is sensitive to degradation induced by geldanamycin. This suggests that proteolytic cleavage of the carboxyl-terminal domain may be restricted topologically within the cell to an area near the cytoplasmic face of the plasma membrane and not available to mediate cleavage of the cystolic GFP fusion protein containing the entire ErbB-2 cytoplasmic domain. The exact protease(s) that generate these ErbB-2 fragments in response to geldanamycin have not been identified.

The metabolic stability of ErbB-2 in cells is likely complex, and ectodomain cleavage by metalloprotease activity has been
reported (32–35). In these experiments we have not observed ectodomain fragmentation, which produces fragments of different sizes than those we have detected in geldanamycin-treated cells.

The data in this manuscript indicate that complex series of proteolytic events are involved in the degradation of Erb-B-2. This would include the proteosome as well as cathepsin B, an endosomal protease. Minnaugh et al. (23) reported that lactacystin (10 μM), a proteosome inhibitor, blocked the geldanamycin-induced loss of the 185-kDa native form of Erb-B-2. We find that lactacystin (10–40 μM) only partially prevents cleavage of the native Erb-B-2 molecule and that ALLN is more effective in this regard. ALLN, like lactacystin, is a proteosome inhibitor, but it also inhibits other proteases such as calpain and cathepsin B and L. However, various calpain inhibitors (calpain inhibitor III, calpain inhibitor V, and calpastatin) do not block geldanamycin-induced fragmentation of Erb-B-2 in our system.

In our experiments geldanamycin induces the formation of intracellular vesicles containing Erb-B-2. Previously the accumulation of such vesicles was only reported after a prolonged 22-h incubation in the presence of geldanamycin and was attributed to the relocalization of newly synthesized Erb-B-2 molecules (22). We observed the much more rapid formation of intracellular Erb-B-2-containing vesicles, within 2 h of geldanamycin exposure. These vesicles also form when cells expressing the cytosolic GFP fusion protein with the Erb-B-2 kinase domain are treated with geldanamycin. Also, the formation of these vesicles containing Erb-B-2 is blocked by agents that interfere in the processing and acidification of endosomes, such as chloroquine, folinycin, and monensin. Hence, we conclude that these vesicles containing Erb-B-2 are derived from the plasma membrane and that endosomal proteases, such as cathepsin B, participate in the degradation of internalized Erb-B-2. The manner in which these vesicles are actually formed is, however, not clear. Inhibitors of cathepsin B, such as lactacystin, ALLN, and lactacystin (10–40 μM), a proteosome inhibitor, blocked the geldanamycin-induced loss of the 185-kDa native form of Erb-B-2. We find that lactacystin (10–40 μM) only partially prevents cleavage of the native Erb-B-2 molecule and that ALLN is more effective in this regard. ALLN, like lactacystin, is a proteosome inhibitor, but it also inhibits other proteases such as calpain and cathepsin B and L. However, various calpain inhibitors (calpain inhibitor III, calpain inhibitor V, and calpastatin) do not block geldanamycin-induced fragmentation of Erb-B-2 in our system.

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