Sensitivity of Mature ErbB2 to Geldanamycin Is Conferred by Its Kinase Domain and Is Mediated by the Chaperone Protein Hsp90*

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ErbB receptors are a family of ligand-activated tyrosine kinases that play a central role in proliferation, differentiation, and oncogenesis. ErbB2 is overexpressed in >25% of breast and ovarian cancers and is correlated with poor prognosis. Although ErbB2 and ErbB1 are highly homologous, they respond quite differently to geldanamycin (GA), an antibiotic that is a specific inhibitor of the chaperone protein Hsp90. Thus, although both mature and nascent ErbB2 proteins are down-regulated by GA, only nascent ErbB1 is sensitive to the drug. To reveal the underlying mechanism behind these divergent responses, we made a chimeric receptor (ErbB1/2) composed of the extracellular and transmembrane domains of ErbB1 and the intracellular domain of ErbB2. The ErbB1/2 protein is functional since its kinase activity was stimulated by epidermal growth factor. The sensitivity of ErbB1/2 to GA was similar to that of ErbB2 and unlike that of ErbB1, indicating that the intracellular domain of the chimera confers GA sensitivity. This finding also suggests that the GA sensitivity of mature ErbB2 depends on cytosolic Hsp90, rather than Grp94, a homolog of Hsp90 that is restricted to the lumen of the endoplasmic reticulum, although both chaperones bind to and are inhibited by GA. Lack of Grp94 involvement in mediating ErbB2 sensitivity to GA is further suggested by the fact that a GA derivative with low affinity for Grp94 efficiently depleted ErbB2 protein in treated cells. To localize the specific region of ErbB2 that confers GA sensitivity, we made truncated receptors with progressive deletions of the cytoplasmic domain and tested the GA sensitivity of these molecules. We found that ErbB2 constructs containing an intact kinase domain retained GA sensitivity, whereas those lacking the kinase domain (ErbB2/DK) lost responsiveness to GA completely. Hsp90 co-immunoprecipitated with all ErbB2 constructs that were sensitive to GA, but not with ErbB2/DK or ErbB1. Both tyrosine-phosphorylated and non-phosphorylated ErbB2 proteins were similarly sensitive to GA, as was a kinase-dead ErbB2 mutant. These data suggest that Hsp90 uniquely stabilizes ErbB2 via interaction with its kinase domain and that GA stimulates ErbB2 degradation secondary to disruption of ErbB2/Hsp90 association.

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‡ The abbreviations used are: EGF, epidermal growth factor; ECD, extracellular domain; ICD, intracellular domain; E3, ubiquitin-protein isopeptide ligase; GA, geldanamycin; ER, endoplasmic reticulum; Ab, antibody; PAGE, polyacrylamide gel electrophoresis; N-ethylcarboxamidoadenosine.
and nascent ErbB2 proteins are efficiently down-regulated by the drug (14, 17). Although association of a number of cytosolic kinases with Hsp90 is necessary to maintain their solubility, intracellular trafficking, and correct response to an activating signal (15), a requirement for chaperone association with transmembrane kinases is unclear. Likewise, although Grp94 is thought to be part of a chaperone cascade that participates in the maturation process undergone by transmembrane and secreted proteins as they pass through the ER, its direct requirement for or participation in ErbB maturation has not been demonstrated. Because a clinically tolerated GA derivative is currently in phase I trial, it is important to fully understand how it targets the ErbB2 protein for degradation and to uncover the biologic rationale for the divergent responses of ErbB1 and ErbB2 to this drug.

ErbB proteins are type I transmembrane proteins whose extracellular domain is glycosylated while in the ER; and thus, these proteins may come in contact with both Hsp90 and Grp94 during their synthesis and maturation. Therefore, the mechanism by which GA destabilizes mature ErbB2 (but not mature ErbB1) might be related to drug effects on ErbB interaction with either or both chaperone proteins. Although initial reports implicated GA interference with ErbB/Grp94 association as the drug’s primary mechanism of action with respect to ErbB2 (14, 16), at least one study has suggested an involvement of the protein’s kinase domain, which never contacts Grp94, in this process (18). If Grp94 association with ErbB2 at the plasma membrane were responsible for ErbB2 sensitivity to GA, the chaperone would have to remain associated with the extracellular domain of the kinase at the cell surface even though Grp94 is thought to be restricted to the ER. Conversely, if the kinase domain of ErbB2 determines the protein’s GA sensitivity, mature ErbB2 should associate with Hsp90.

In this study, we demonstrate that a functional chimeric protein containing the ErbB1 extracellular and transmembrane domains and the ErbB2 intracellular domain displays GA sensitivity that is unlike that of ErbB1, but indistinguishable from that of ErbB2. Furthermore, we conclude that the GA sensitivity of the mature ErbB2 protein is conferred by its intracellular domain, secondary to GA-mediated disruption of Hsp90 association with this region. Lack of interaction of mature ErbB1 and certain C-terminal ErbB2 truncation mutants with Hsp90 is consistent with the relative insensitivity of these constructs to GA. Finally, a GA derivative with similar affinity for Hsp90, but with a 90-fold weaker affinity for Grp94, depletes ErbB2 protein from tumor cells at a concentration similar to that of GA. Taken together, these data confirm that the stability of the mature ErbB2 protein, in contrast to ErbB1, requires association of its kinase domain with Hsp90.

MATERIALS AND METHODS

Cells and Antibodies—SKBR3, A431, and COS-7 cells were purchased from American Type Culture Collection. Anti-ErbB1 immunoprecipitation antibody was purchased from Oncogene Science Inc. (Ab-1), and Western blot antibody was from Upstate Biotechnology, Inc. (LAI). Anti-ErbB2 immunoprecipitation antibody was from Oncogene Science Inc. (Ab-5), and Western blot antibody was from Oncogene Science Inc. (Ab-3 for the ICD) and Transduction Laboratories (clone 42 for the ECD). Rat anti-Hsp90 monoclonal antibody was from Stressgen Biotech Corp. (SPA-835), and goat anti-Hsp70 and anti-Hsc70 polyclonal antibodies were from Santa Cruz Biotechnology. Anti-phosphotyrosine monoclonal antibody was from Oncogene Science Inc. (Ab-2). Recombinant human EGF was purchased from Life Technologies, Inc.

Preparation of Plasmid Constructs—pcDNA-ErbB1 and pcDNA-Erb2 were described previously (19). Chimeric ErbB1/2 was made by joining three fragments. The first fragment was cut out of pcDNA3-ErbB1 with Acc65I and BsmI. The second fragment was amplified from ErbB1 by polymerase chain reaction using the 5′-end primer 5′-CCGGAGCCCAGGGACTGCGTCTCT-3′ and the 3′-end primer 5′-CGCTTCCGGAGCATGTTGGGGCCCGTCTC-3′, which contains a silent mutation of T to C at position 2208 of ErbB1 to make a BspEI cutting site. The third fragment was excised from pcDNA3-Erb2 using BspEI and XhoI. The three fragments were ligated and inserted into the pcDNA3 vector, and the resulting construct was verified by restriction enzyme mapping and sequencing. Truncated ErbB2 proteins were made by joining a fragment cut out of pcDNA3-Erb2 with HindIII/SphI with another fragment amplified by polymerase chain reaction. The polymerase chain reaction was performed with the shared 5′-end primer 5′-GATGAGGAGGCCGCGCATGC- CAGCCTT-3′ and the 3′-end primer 5′-GGCGGCTGATTCTACAGAG- GGAGGAGGTACTG-3′ for ErbB2/DT, 5′-GGCGTCGAGTTAGAAGAGAAG- GCCCTCTGGGTTA-3′ for ErbB2/DHC, or 5′-GGCGTCGAGTTATC- TCGCGATCTGTTACTC-3′ for ErbB2/DR. The products of polymerase chain reaction were digested with SpfI and XhoI and, together with the first fragment, were ligated into the pcDNA3 vector. Kinase-deficient ErbB2 (ErbB2/K753A) was made by mutating lysine 753 of wild-type ErbB2 to alanine using the GeneEditor in vitro site-directed mutagenesis system (Promega) with the ErbB2-specific primer 5′-ATTCAGTGGCAGCATCAGGTGAAAGGA-3′.
Sensitivity of Mature ErbB2 to GA Is Confirmed by Its ICD—
ErbB1 and ErbB2 proteins are differentially sensitive to GA. A431 and SKBR3 cells, which overexpress ErbB1, and SKBR3 cells, which overexpress ErbB2, were both exposed to GA for varying times, and ErbB proteins in whole cell extracts were detected by Western blotting (Fig. 1). Whereas the ErbB2 protein level declined by >80% within 4 h and was nearly undetectable by 6 h, the ErbB1 level did not decline noticeably until 16 h after drug exposure. ErbB1 responded similarly to cycloheximide (data not shown), suggesting that GA effects on ErbB1 were mediated solely by its ability to destabilize newly synthesized ErbB1 protein, as suggested by Sakagami et al. (17), whereas mature ErbB2 retained GA sensitivity (14).

Since ErbB1 and ErbB2 proteins respond differently to GA, we constructed a chimeric protein (ErbB1/2) consisting of the ECD and transmembrane domain of ErbB1 and the ICD of ErbB2 to determine whether the ECD or ICD of ErbB2 determines sensitivity of the mature protein to GA (Fig. 2A). We ascertained that the chimeric protein was functional by testing its responsiveness to EGF after transfection into COS-7 cells. As is evident from Fig. 2B, EGF efficiently stimulated the autophosphorylation of ErbB1/2, in contrast to its lack of effect on the autophosphorylation of transfected ErbB2 (Fig. 2B, upper panel). The EGF responsiveness of ErbB1/2 suggests that the protein attained a native conformation in the transfected cells. Next, we compared the GA sensitivity of ErbB1/2 with that of both ErbB1 and ErbB2 following transient transfection into COS-7 cells. Treatment of cells with 1 μg GA for 6 h almost totally depleted ErbB1/2 and ErbB2 proteins, whereas only a portion of ErbB1 was degraded by 6 h (Fig. 2B, lower panel). These data indicate that mature ErbB1/2 retains ErbB2-like GA sensitivity, thus implying that the ICD of ErbB2, rather than its ECD or transmembrane domain, mediates the responsiveness of the mature protein to GA.

Depletion of the ErbB2 Protein by GA Requires the Presence of Its Kinase Domain—The ICD of ErbB2 is 580 amino acids long and is composed of a 14-amino acid juxtamembrane segment, a 343-amino acid kinase domain, and a long hydrophilic C-terminal tail (Fig. 3A). The juxtamembrane segment and the kinase domain are highly conserved between ErbB1 and ErbB2, whereas the C-terminal tail is moderately homologous, albeit a short stretch (amino acids 1097–1123) within this region shows increased homology. To further localize the region of the ICD that mediates the GA response, we progressively truncated ErbB2 from its C terminus (Fig. 3A) and tested the GA sensitivity of these truncations in transiently transfected COS-7 cells. Our data show that deletion of the entire hydrophilic C-terminal tail, including the autophosphorylation sites, did not affect the GA sensitivity of ErbB2 since both ErbB2/DT (deletion of 132 amino acids at the C terminus) and ErbB2/DHC (deletion of the last 224 C-terminal amino acids) were effectively depleted by GA (Fig. 3B). However, when the C-terminal deletion included the kinase domain (ErbB2/DK), the truncated protein lost its GA sensitivity completely. Thus, in cells exposed to 1 μg GA for up to 31 h, the ErbB2/DK protein level remained identical to that in untreated cells (Fig. 3B), suggesting that neither mature nor nascent ErbB2/DK is sensitive to GA and indicating that the ErbB2 kinase domain is obligatory for GA-induced down-regulation of the protein.

ErbB2 Kinase Activity Is Not Required for GA Sensitivity—Since we have shown that the ErbB2 kinase domain confers sensitivity to GA, we next asked whether an active kinase is required for such sensitivity, as is apparent for Cbl-mediated down-regulation of ErbB1 (7). This is of particular interest considering that ErbB3, a member of the ErbB family containing an inactive kinase domain, is insensitive to GA (21). To answer this question, we created a kinase-deficient ErbB2 construct by mutating lysine 753 to alanine, and we tested its sensitivity to GA. ErbB2/K753A was not autophosphorylated on tyrosine in COS-7 cells, in contrast with wild-type ErbB2, confirming its lack of kinase activity (Fig. 4, lower panel). However, ErbB2/K753A was depleted from cells by treatment with GA to a degree comparable to wild-type ErbB2 (Fig. 4, upper panel), confirming that the functional activity of the kinase domain is not important for GA-induced down-regulation of ErbB2 and suggesting instead that a structural motif contained within this region plays a major role in conferring GA sensitivity.

Involvement of the Chaperone Protein Hsp90 in GA-induced Down-regulation of ErbB2—The chaperone protein Hsp90 is the molecular target of GA in the cytosol (22–24). GA exerts its destabilizing effects on soluble kinases by altering their association with Hsp90-containing multiprotein complexes, thereby targeting them for proteolytic degradation (15). Although it has not been shown previously, we reasoned that, in analogy to the soluble kinases, ErbB2 must also interact with Hsp90. We first examined ErbB2/Hsp90 association in ErbB2-overexpressing SKBR3 cells. We immunoprecipitated ErbB2 from SKBR3 cells using an antibody recognizing the ErbB2 ECD, and coprecipitation of Hsp90 was examined by Western blotting. We found that Hsp90 coprecipitated with ErbB2 proteins from untreated SKBR3 cells (Fig. 5A). In contrast, when cells were treated with GA for 1 h, Hsp90 disappeared from ErbB2 immunoprecipitates, even though at this time we detected no change in the amount of ErbB2 protein immunoprecipitated. As seen with Hsp90-dependent soluble kinases (13), an increased association of Hsp70 with ErbB2 coincided with the loss of coprecipitated Hsp90 after GA treatment (Fig. 5A). GA did not affect the level of either chaperone protein measured in total cell lysate (data not shown).

We next examined whether endogenous Hsp90 associated with ErbB1, the ErbB1/2 chimeric protein, and ErbB2 in transiently transfected COS-7 cells. Although Hsp90 coprecipitated with wild-type ErbB2 and with the ErbB1/2 chimera, the chaperone was not found in ErbB1 immunoprecipitates, even though all three ErbB constructs were expressed to a similar degree (Fig. 5B). Thus, Hsp90 association (or its lack of) with these transfected proteins correlates with their sensitivity profiles for GA. EGF did not affect the binding of Hsp90 to the EGF-responsive ErbB1/2 chimera, suggesting that Hsp90 association is not regulated by receptor tyrosine phosphorylation (Fig. 5B) and supporting our earlier data (see Fig. 4) that the
tyrosine phosphorylation state of ErbB2 does not affect its GA sensitivity. Last, we examined endogenous Hsp90 association with the panel of C-terminal ErbB2 truncation mutants expressed in COS-7 cells, and we found that Hsp90 coprecipitated with all of the ErbB2 truncations, except ErbB2/DK (Fig. 5). Again, this correlates perfectly with the respective GA sensitivities of these truncated ErbB2 proteins and indicates the importance of Hsp90 association in modulating ErbB2 stability.

**Binding to Hsp90, but Not Grp94, Determines Benzoquinone Ansamycin Activity for ErbB2**—To determine whether GA binding to Grp94 may also contribute to drug-stimulated ErbB2 destabilization, we utilized a GA derivative, WX514, with 3-fold less affinity than GA for Hsp90, but with 90-fold weaker affinity for Grp94. Relative drug binding affinities for Hsp90 were determined by competition of soluble drugs with immobilized GA for Hsp90 binding with an IC50 = 0.3 μM, whereas the IC50 for WX514 was 1 μM. In contrast, the IC50 for GA competition with [3H]NECA binding to Grp94 was 1 μM, whereas that of WX514 was 90 μM. With these data in hand, we examined the ability of WX514 to deplete the mature ErbB2 protein from SKBR3 cells (Fig. 6). Corresponding data obtained with GA are shown for comparison. The concentration of WX514 that caused 50% ErbB2 depletion was 3.5 μM, whereas at 10 μM WX514, ErbB2 depletion was complete. These concentrations are 3- and 10-fold, respectively, the relative affinity of WX514 for Hsp90, but they are one-twenty-fifth and one-ninth the relative affinity of WX514 for Grp94. Thus, these data support the hypothesis that inhibition of Hsp90, but not Grp94, is responsible for ErbB2 destabilization.

**Fig. 2. Construction of chimeric ErbB1/2 and comparison of its sensitivity to GA with that of ErbB1 and ErbB2.** A, shown is a schematic diagram showing the construction of ErbB1/2. ErbB1/2 is composed of the ECD and transmembrane domain of ErbB1 and the ICD of ErbB2, including its kinase domain and the hydrophilic C-terminal tail. B, COS-7 cells, transiently transfected with 1 μg of ErbB1, ErbB2, or ErbB1/2 plasmid DNA, were cultured in Opti-MEM for 24 h and then treated with recombinant human EGF (upper panel) or GA (lower panel) and lysed in TMNSV buffer. Lysates were separated by SDS-PAGE, transferred to nitrocellulose, and probed with antibodies directed against phosphotyrosine (upper panel) or against ErbB1 and ErbB2 (lower panel). PM, plasma membrane; CRD, cysteine-rich domain; IB, immunoblot.
Hsp90 forms a multi-chaperone complex that targets its client protein to the proteasome for degradation. The formation of both types of Hsp90-containing multi-chaperone complexes and their association with client proteins are labile and reversible and depend primarily upon intracellular ATP concentration. GA binds in the nucleotide pocket of Hsp90 with higher affinity than either ATP or ADP, but the conformation of GA-bound Hsp90 mimics that of the ADP-bound chaperone. Thus, GA promotes accumulation of the destabilizing Hsp90-containing multi-chaperone complex, leading to client protein degradation (for review, see Ref. 15).

Although a requirement for Hsp90 interaction has been demonstrated for several non-receptor tyrosine and serine/threonine kinases, including RIP, p60src, and p210bcr/abl, and for progesterone and glucocorticoid receptors, similar data showing dependence of mature transmembrane kinases on Hsp90 and the effects of GA on such client protein-chaperone complexes are lacking. Thus, although the rapid down-regulation of mature ErbB2 by GA is well established, a direct involvement of Hsp90 in this phenomenon has not been reported.

In this study, we have shown that endogenous Hsp90 and ErbB2 do indeed associate in ErbB2-overexpressing SKBR3 cells and in transiently transfected COS-7 cells. As is the case with non-receptor kinases, shortly after GA addition, the nature of the ErbB2-chaperone complex shifts from one that is destabilizing (containing Hsp90, lacking Hsp70) to one that is stabilizing (containing Hsp90, lacking Hsp70), and this is followed by rapid degradation of the kinase. Our data demonstrate that Hsp90 association is specific for ErbB2 and is not seen with ErbB1. Furthermore, the presence of the ErbB2 kinase domain is minimally required to observe Hsp90 interaction and GA sensitivity. The ErbB2-like GA sensitivity of an ErbB1/2 chimeric protein containing the ECD and transmembrane region of ErbB1 and the ICD of ErbB2 confirms the importance of the ICD in mediating the sensitivity of ErbB2 to GA. Interestingly, EGF-induced autophosphorylation of the ErbB1/2 chimera does not affect ErbB2/Hsp90 association. Thus, Hsp90 binding to the kinase domain of ErbB2 is not intrinsically inhibitory, as it is for v-Src (26). The stability of kinase domain-deleted ErbB2 (ErbB2/DK) in the presence of GA and our observation that WX514, an Hsp90-binding GA derivative, is able to deplete mature ErbB2 at concentrations far below its affinity for Grp94 both supply conclusive evidence that GA-induced destabilization of ErbB2 is secondary to disruption of Hsp90 association with the ErbB2 kinase domain.

Unlike endogenous ErbB1 expressed in A431 cells, on which GA has almost no effect within 6–8 h, ErbB1 expressed in...
transiently transfected cells is discernibly down-regulated in response to GA, although much less so than ErbB2. The apparently increased GA sensitivity of ErbB1 in COS-7 cells may be related to its higher basal level of autophosphorylation. Whereas in the absence of EGF, ErbB1 in A431 cells is not detectably phosphorylated (data not shown), transiently transfected ErbB1 in COS-7 cells is moderately autophosphorylated in the absence of ligand. EGF-stimulated ErbB1 phosphorylation results in recruitment of the E3 ubiquitin ligase c-Cbl, which mediates ubiquitination-dependent degradation of the

Fig. 5. Association of Hsp90 with ErbB proteins detected by immunoprecipitation. A, SKBR3 cells, treated for 1 h with or without GA (1 μM), were lysed in TMNSV buffer. ErbB2 proteins were immunoprecipitated (IP) by first incubating the clarified cell lysate with monoclonal antibody Ab-5, followed by the addition of protein A-Sepharose beads coated with rabbit anti-mouse IgG. Immunoprecipitated proteins were solubilized in SDS sample buffer and separated by SDS-PAGE. Blots were probed with anti-Hsp90, anti-Hsp70/Hsc70, or anti-ErbB2 antibodies. B, COS-7 cells, cultured in 200 × 15-mm plates, were transfected with 10 μg of ErbB1, ErbB2, or ErbB1/2 plasmid DNA. 24 h after transfection, cells were treated with 100 ng/ml EGF for 10 min at 37 °C and lysed in TMNSV buffer. For immunoprecipitation, anti-ErbB2 antibody (Ab-5) was used for ErbB2-transfected cell lysate, and anti-ErbB1 antibody (Ab-1) for ErbB1- and ErbB1/2-transfected cell lysate. Membranes were Western-blotted for Hsp90 (upper panel) or with a mixture of anti-ErbB1 and anti-ErbB2 antibodies (lower panel). C, COS-7 cells were transfected with 10 μg of full-length or truncated ErbB2 DNA. Cell lysis, immunoprecipitation (with anti-ErbB2 antibody only), and Western blotting were performed as described above. IB, immunoblot.

Fig. 6. Depletion of the ErbB2 protein correlates with drug binding to Hsp90 but not to Grp94. A, increasing concentrations of either geldanamycin or WX514 were added to cell lysates and allowed to incubate for 20 min at 4 °C prior to the addition of immobilized GA. Hsp90 bound to immobilized GA was analyzed as described under “Materials and Methods.” Data are expressed as a percentage of Hsp90 bound to immobilized GA in the absence of competitor. B, increasing concentrations of geldanamycin or WX514 were incubated together with purified Grp94 and [3H]NECA on ice for 1 h. The amount of [3H]NECA bound to Grp94 was determined by filtration as described under “Materials and Methods.” C, SKBR3 cells were incubated for 6 h with increasing concentrations of GA or WX514. Cells were lysed, and the ErbB2 content of total lysates was determined as described in the legend to Fig. 1. Ponceau red staining was used to confirm equal loading of all samples.

Geldanamycin Targets ErbB2 Kinase Domain/Hsp90 Interaction
phosphorylated receptor (7, 27). When added to the enhanced instability of newly synthesized ErbB1 in the presence of GA, this phenomenon may contribute to the decrease in the ErbB1 steady-state level observed in transfected, drug-treated cells.

Although ErbB2 is not normally efficiently ubiquitinated by c-Bl, this ubiquitin ligase has been implicated in mediating antibody-induced ErbB2 down-regulation (28). However, c-Bl-mediated down-regulation requires tyrosine phosphorylation of C-terminal ErbB2 residues, whereas GA-mediated down-regulation of ErbB2 requires no motifs C-terminal to the kinase domain. In addition, neither ErbB2 phosphorylation nor kinase activity is required for GA sensitivity. Thus, an ErbB1/2 chimeric protein unstimulated by EGF is sensitive to GA, as are autophosphorylated ErbB2 and a kinase-dead ErbB2 point mutant. Although GA-induced ErbB2 polyubiquitination is observed prior to degradation of the protein, the ubiquitin ligase responsible for mediating the GA effect remains elusive. Recently, a novel GA-inducible ubiquitinating activity was identified in cell lysates, and characterization of this activity is in progress.

Our data definitively show that Hsp90 interacts with the kinase domain of ErbB2 and that GA-stimulated destabilization of ErbB2 is preceded by disruption of ErbB2/Hsp90 association, yet it remains unclear why ErbB2 stability, and not the kinase activity of ErbB2 is fixed in the plasma membrane, and this fixation following ligand stimulation (15). However, the location of mature ErbB2 is fixed in the plasma membrane, and this may have simultaneously acquired dependence on Hsp90 for its stability. The growth and survival advantage conferred by ErbB2 would certainly favor its ultimate evolutionary selection.

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