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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.

The *ErbB2* gene (also known as *Her2/neu*), a homolog of the rat *neu* gene, encodes a 185-kDa receptor-like glycoprotein that is a member of the ErbB family of receptor tyrosine kinases that also include the epidermal growth factor (EGF)[1] receptor (ErbB1) (1), ErbB3 (2), and ErbB4 (3). ErbB receptors are single transmembrane proteins with an extracellular domain (ECD) that bears two cysteine-rich clusters and is responsible for interaction with polypeptide ligands and an intracellular domain (ICD) that contains a tyrosine kinase motif and a long hydrophilic segment at the C-terminal end (4). Binding of the ECD with ligands causes hetero- and/or homodimerization of ErbB proteins, followed by stimulation of their intrinsic kinase activity, leading in turn to the phosphorylation of tyrosine residues in the C-terminal tail (5). In the case of EGF-stimulated ErbB1, the E3 ubiquitin ligase Cbl binds to the tyrosine-phosphorylated C terminus and mediates ubiquitination and down-regulation of the receptor (6, 7). Although no ligand has been found for ErbB2, the protein seems to be the preferred dimerization partner for the other ErbB receptors, perhaps because it is not normally a target of Cbl and may thus protect the other ErbB members with which it dimerizes from Cbl-mediated down-regulation (8).

The *ErbB2* gene was first isolated in the rat from chemically induced neuroblastomas based on its ability to transform NIH 3T3 cells (9, 10). It has been shown that overexpression of ErbB2 causes cell transformation and tumorigenesis (11), and recent *in vitro* experiments have shown that ErbB2 is required for induction of carcinoma cell invasion by other members of the ErbB family (12). Moreover, overexpression of ErbB2 in cells devoid of other ErbB proteins potentiates cell migration. These results suggest that ErbB2 expression is related to the malignancy of tumor cells. In fact, *ErbB2* is often amplified in various solid tumors, and the clinical implications of its overexpression in breast and ovarian cancers have been well described (5).

Geldanamycin (GA), a benzoquinone ansamycin antibiotic, is a specific inhibitor of the 90-kDa chaperone protein family, which includes the cytosolic heat shock protein Hsp90 (13) and the endoplasmic reticulum (ER)-localized glucose-regulated protein Grp94 (14, 15). GA has antitumor activity *in vivo* and has been shown to cause rapid depletion of the ErbB2 protein mediated by its ubiquitin-dependent proteasomal degradation (16). Although ErbB2 is highly homologous to ErbB1 in amino acid sequence, its sensitivity to GA is quite different. Whereas only newly synthesized ErbB1 is sensitive to GA, both mature

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[1] 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; NECA, *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 conclusively show 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. (LA1). Anti-ErbB2 immunoprecipitation antibody was from Oncogene Science Inc. (Ab-5), and Western blot antibodies were 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—pcDNA3-*ErbB1* and pcDNA3-*ErbB2* were described previously (19). Chimeric ErbB1/2 was made by joining three fragments. The first fragment was cut out of pcDNA3-*ErbB1* with *Acc*65I and *Bsm*I. 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′-CGCTTCCGGACGATGTGGCGCCTTCGCA-3′, which contains a silent mutation of T to C at position 2208 of *ErbB1* to make a *Bsp*EI cutting site. The third fragment was excised from pcDNA3-*ErbB2* using *Bsp*EI and *Xba*I. 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-*ErbB2* with *Hin*dIII/*Sph*I with another fragment amplified by polymerase chain reaction. The polymerase chain reaction was performed with the shared 5′-end primer 5′-GATGAGGAGGGCGCATGCCAGCCTT-3′ and the 3′-end primer 5′-GCGCTCGAGTTACTCAGAGGGCAGGGGTACTG-3′ for ErbB2/DT, 5′-GCGCTCGAGTTAGAAGAAGCCCTGCTGGGGTA-3′ for ErbB2/DHC, or 5′-GCGCTCGAGTTATCTCCGCATCGTGTACTTCC-3′ for ErbB2/DK. The products of polymerase chain reactions were digested with *Sph*I and *Xho*I 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′-ATTCCAGTGGCCATCGCAGTGTTGAGGGAAAA-3′.

Cell Culture and Transient Transfection—SKBR3 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 2 mM glutamine, 1 mM Hepes, and 100 units of penicillin and streptomycin. COS-7 cell culture medium contained 90% Dulbecco's modified Eagle's medium, 10% fetal calf serum, 2 mM glutamine, 1 mM Hepes, and 1 mM sodium pyruvate. For transient transfections, each plasmid, which was premixed with FuGene 6 (Roche Molecular Biochemicals), was added to cells at 50–70% confluency. Cells were continually cultured in the same medium for 24–48 h until lysis. For EGF stimulation, cells were first incubated with Opti-MEM (Life Technologies, Inc.) for 24 h; then EGF was added at 100 ng/ml; and incubations were continued at 37 °C for 10 min.

Immunoprecipitation and Western Blotting—Cells were washed once with cold phosphate-buffered saline (pH 7.0) and lysed by scraping in TMNSV buffer (50 mM Tris-HCl (pH 7.5), 20 mM Na_2MoO_4, 0.09% Nonidet P-40, 150 mM NaCl, and 1 mM sodium orthovanadate) supplemented with Complete™ proteinase inhibitors (Roche Molecular Biochemicals). Cell lysates were clarified by centrifugation at 14,000 rpm (4 °C) for 15 min, and protein concentration was determined by the BCA method (Pierce). For immunoprecipitation, 1 mg of lysate protein was incubated with 4 μg of mouse monoclonal antibodies at 4 °C for 2 h, followed by the addition of protein A-Sepharose beads (Amersham Pharmacia Biotech), which were precoated with rabbit anti-mouse IgG, and rotation at 4 °C overnight. The beads were washed five times with lysis buffer, resuspended in 1× SDS sample buffer (80 mM Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 100 mM dithiothreitol, and 0.0005% bromphenol blue), and boiled for 5 min. Immunoprecipitated proteins (or cell lysates mixed with 5× SDS sample buffer) were separated by 8% SDS-PAGE. Western blotting was performed as described previously (14).

Preparation and Use of Immobilized GA—GA was derivatized to Affi-Gel resin (Bio-Rad) as described (13). Briefly, GA was dissolved in chloroform and allowed to react with 1,6-hexanediamine for 2 h at room temperature. After aqueous extraction, the resulting 17-hexamethylenediamine-17-demethoxygeldanamycin was dried under vacuum, redissolved in dimethyl sulfoxide, and reacted with Affi-Gel 10 resin for 4 h at room temperature. After several washes, beads were stored in cell lysis buffer and used within 10 days for competition assays. For competition assays, increasing concentrations of soluble test drugs were incubated on ice with cell lysates for 30 min; 100 μl of a 50% suspension of GA resin was added; the volume was adjusted to 500 μl with lysis buffer; and samples were rotated at 4 °C for 2 h. The GA resin was washed four times with lysis buffer; 100 μl of 1× SDS sample buffer was added; and the samples were processed as described above. After SDS-PAGE and transfer to nitrocellulose, blots were probed for Hsp90, and band densities were obtained by densitometry using Adobe Photoshop and NIH Image software.

Analysis of GA and WX514 Binding to Grp94—250-μl binding reactions consisting of 5 μg of Grp94, 20 nM *N*-[^{3}H]ethylcarboxamidoadenosine (NECA), 50 mM Tris (pH 7.5), and various concentrations of competitor were incubated on ice for 1 h. Grp94 was then collected on polyethyleneimine-treated glass filters, washed twice with 4 ml of ice-cold 50 mM Tris (pH 7.5), dried, and counted in a scintillation counter to determine the amount of Grp94-bound [^{3}H]NECA (20). All assays were performed in triplicate and corrected for nonspecific ligand binding.

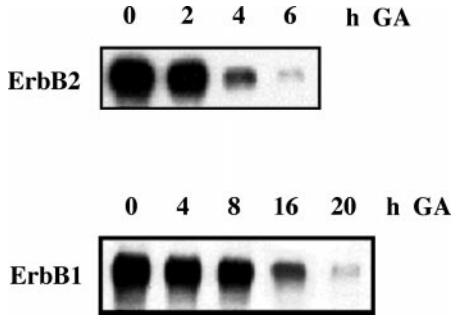

FIG. 1. **ErbB2, but not ErbB1, is sensitive to brief exposure to GA.** A431 and SKBR3 cells were treated with 3 μM GA for increasing times. Cell lysates were separated by SDS-PAGE and Western-blotted for either ErbB1 (A431 lysates) or ErbB2 (SKBR3 lysates). Equal loading of total proteins in each lane was confirmed by Ponceau red staining of the nitrocellulose membranes prior to blocking and antibody probing.

RESULTS

Sensitivity of Mature ErbB2 to GA Is Conferred by Its ICD—ErbB1 and ErbB2 proteins are differentially sensitive to GA. A431 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. 2*A*). 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. 2*B*, EGF efficiently stimulated the autophosphorylation of ErbB1/2, in contrast to its lack of effect on the autophosphorylation of transfected ErbB2 (Fig. 2*B*, *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 μM GA for 6 h almost totally depleted ErbB1/2 and ErbB2 proteins, whereas only a portion of ErbB1 was degraded by 6 h (Fig. 2*B*, *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. 3*A*). 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. 3*A*) 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. 3*B*). 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 μM GA for up to 31 h, the ErbB2/DK protein level remained identical to that in untreated cells (Fig. 3*B*), 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. 5*A*). 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. 5*A*). 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. 5*B*). 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. 5*B*) and supporting our earlier data (see Fig. 4) that the

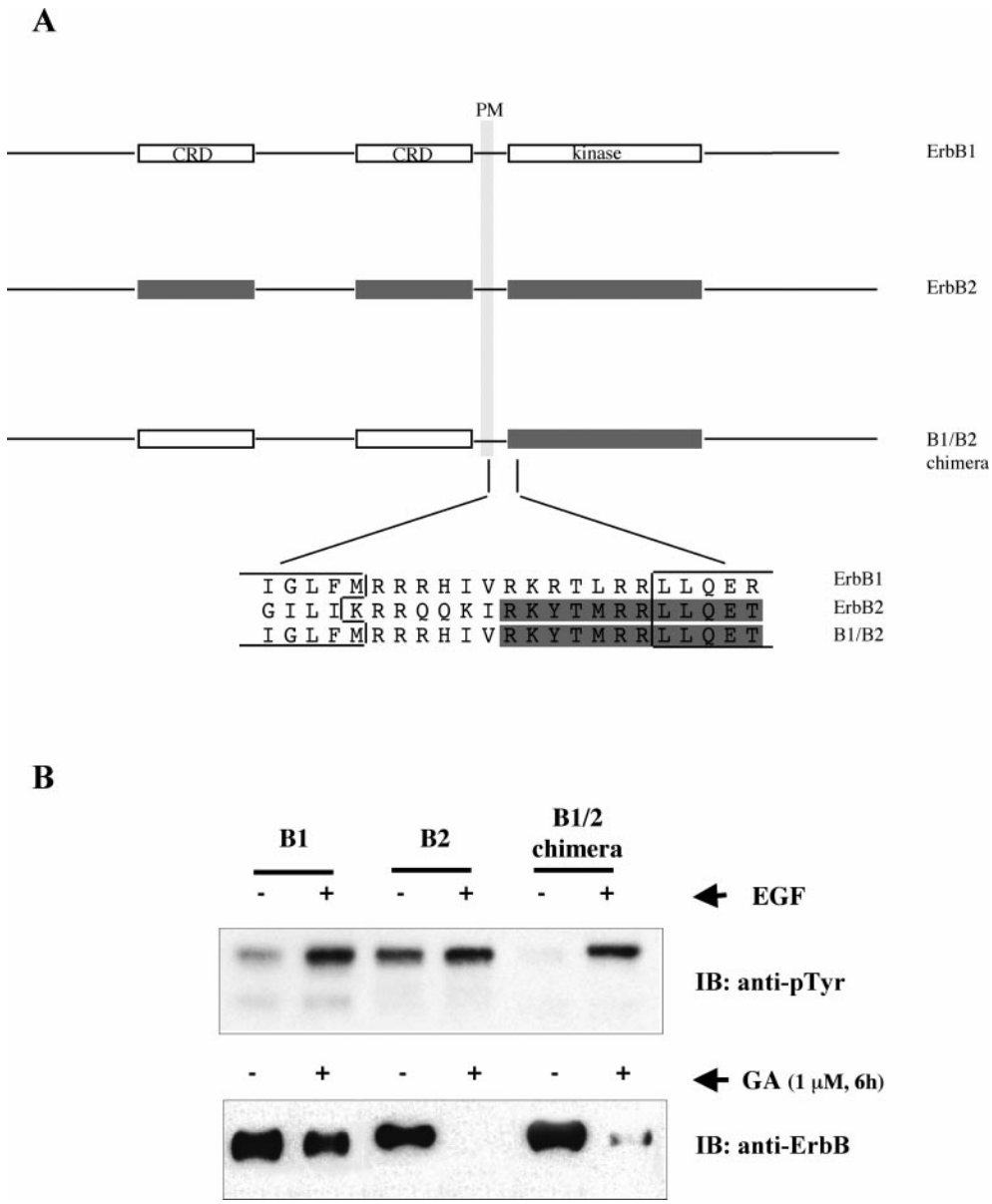

FIG. 2. **Construction of chimeric ErbB1/2 and comparison of its sensitivity to GA with that of ErbB1 and ErbB2.** *A*, shown is a schematic diagram showing the construction of ErbB1/2. ErbB1/2 is composed of the ECD and transmembrane domain of ErbB1 and the ICD of ErbB2, including its kinase domain 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.

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*C*). 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 Hsp90 binding to immobilized GA, as described previously (13). Relative binding affinities for Grp94 were determined by drug competition with the adenosine nucleotide analog [^{3}H]NECA binding to soluble Grp94, as NECA and GA share the same binding site on Grp94 (20). As shown in Fig. 6 (*A* and *B*), soluble GA competed with immobilized GA for Hsp90 binding with an IC_{50} ~0.3 μM, whereas the IC_{50} for WX514 was ~1 μM. In contrast, the IC_{50} for GA competition with [^{3}H]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*C*). 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.

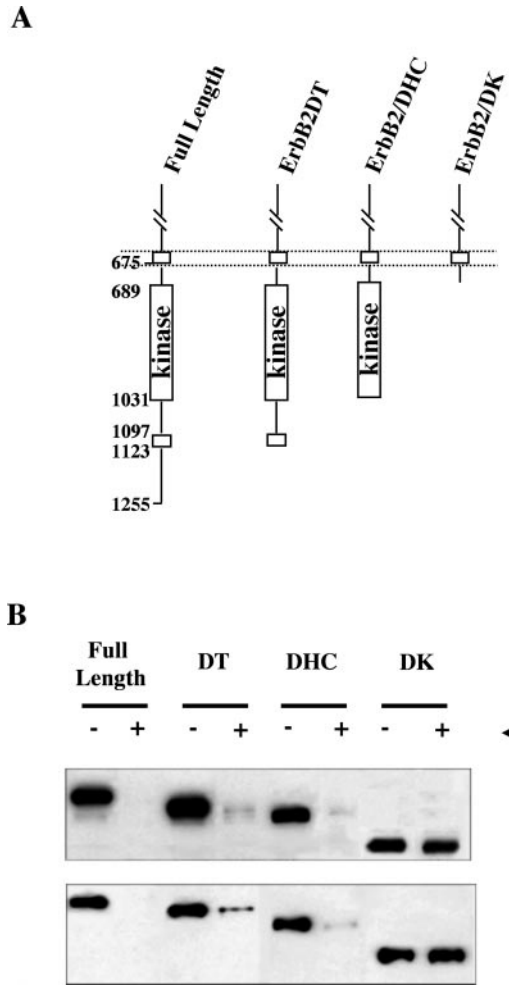

FIG. 3. **Sensitivity of truncated ErbB2 proteins to GA.** Truncated ErbB2 proteins were made by introducing a stop codon at the locations indicated. COS-7 cells (6-well plate) were transfected with 1 μg of plasmid DNA for each construct. 16 h after transfection, cells were treated with or without 1 μM GA for 6 or 31 h and lysed in TMNSV buffer. ErbB2 protein levels were detected by Western blotting using a monoclonal antibody against the ErbB2 ECD (clone 42).

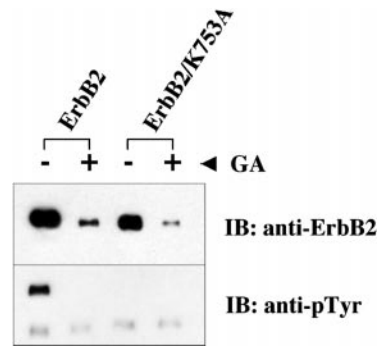

FIG. 4. **Sensitivity of kinase-deficient ErbB2 to GA.** Kinase-deficient ErbB2 was made by mutating lysine 753 in wild-type ErbB2 to alanine. ErbB2/K753A (1 μg) was transiently transfected into COS-7 cells, which were then treated with 1 μM GA for 6 h and lysed in TMNSV buffer. The kinase activity of the ErbB2 mutant was monitored by its autophosphorylation using anti-phosphotyrosine antibody, and its sensitivity to GA was detected by Western blotting as described in the legend to Fig. 3*B*. *IB*, immunoblot.

DISCUSSION

ErbB1 and ErbB2 are highly homologous type I transmembrane receptor tyrosine kinases whose overexpression in various solid tumors correlates with poor clinical prognosis. Mature ErbB1 and ErbB2 proteins are differentially sensitive to GA. Although in their nascent form, both ErbB proteins are destabilized by this drug, only mature ErbB2 retains GA sensitivity. The reason for this discrepancy is not known. GA binds specifically to the cytosolic chaperone protein Hsp90(13) and to its ER-restricted homolog Grp94(14). Although GA binding to Grp94 probably inhibits its chaperone activity in the ER (20), the nature of Grp94 function is still not well understood, although along with other chaperones, it is thought to participate in the maturation of transmembrane and secreted proteins (25). The function of Hsp90 is somewhat clearer. It forms two multiprotein complexes, each composed of a set of distinct co-chaperone proteins. These Hsp90-containing multi-chaperone complexes in turn associate with a distinct set of client proteins (*e.g.* many soluble kinases and steroid receptors). The nature of the particular multi-chaperone complex in which Hsp90 participates is determined by its conformation, which is regulated by occupation of an amino-terminal nucleotide-binding pocket by either ATP or ADP. When ATP-bound, Hsp90 forms a multi-chaperone complex that stabilizes its client protein and permits its activation. In contrast, when ADP-bound, 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, $p60^{v\text{-}src}$, and $p210^{bcr/abl}$, 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 stabilizing (containing Hsp90, lacking Hsp70) to one that is destabilizing (reduced Hsp90, elevated 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

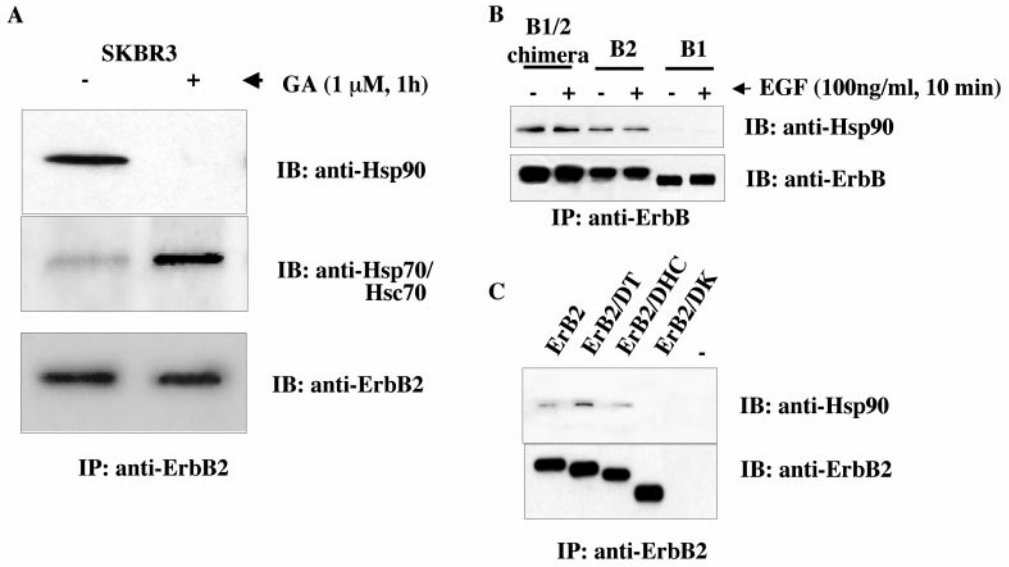

FIG. 5. **Association of Hsp90 with ErbB proteins detected by immunoprecipitation.** *A*, SKBR3 cells, treated for 1 h with or without GA (1 μM), were lysed in TMNSV buffer. ErbB2 proteins were immunoprecipitated (*IP*) by first incubating the clarified cell lysate with monoclonal antibody Ab-5, followed 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.

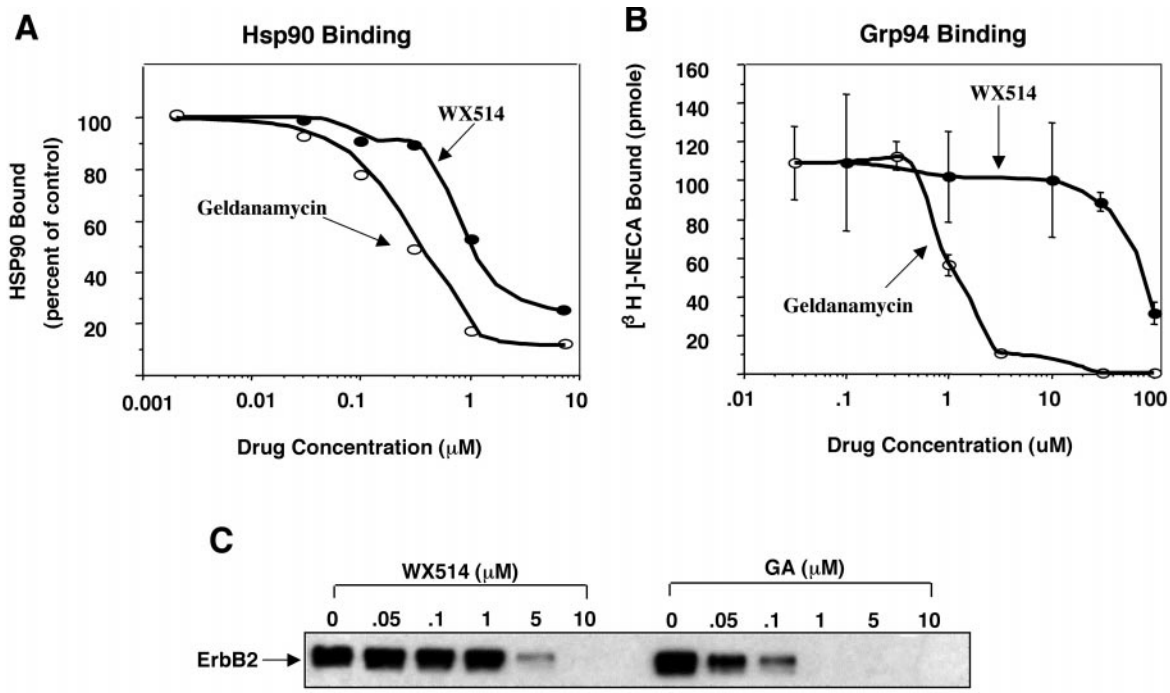

FIG. 6. **Depletion of the ErbB2 protein correlates with drug binding to Hsp90 but not to Grp94.** *A*, increasing concentrations of either geldanamycin or WX514 were added to cell lysates and allowed to incubate for 30 min at 4 °C prior to the addition of immobilized GA. Hsp90 bound to immobilized GA 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 [^{3}H]NECA on ice for 1 h. The amount of [^{3}H]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.

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

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-Cbl, this ubiquitin ligase has been implicated in mediating antibody-induced ErbB2 down-regulation (28). However, c-Cbl-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,[2] 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 that of ErbB1, requires chaperone binding. Hsp90 association with several non-receptor kinases is necessary to maintain their solubility and to permit their correct intracellular trafficking following ligand stimulation (15). However, the location of mature ErbB2 is fixed in the plasma membrane, and this kinase has no known ligand. Although ErbB2 is the only ligandless ErbB family member (5), it is the preferred partner in ErbB heterodimers (19, 29). ErbB2 is recruited to an ErbB heterodimer in response to ligand binding to its ErbB partner (5). We therefore currently favor the hypothesis that mature ErbB2 requires Hsp90 association with its kinase domain to maintain the conformation necessary to heterodimerize with other ligand-activated ErbB proteins. In fact, a motif within the C-terminal region of the ErbB2 kinase domain has been suggested to play a role in dimerization (30). Studies to investigate the possible involvement of this motif in Hsp90 binding to ErbB2 are in progress.

How did ErbB2 acquire its unique dependence on Hsp90? *ErbB2* is likely to have arisen from *ErbB1* through a gene duplication event since invertebrates contain only an ErbB1-like ligand-activated protein (30). Analysis of the ErbB2 amino acid sequence reveals that it contains an insert not found in the other ErbB proteins, and this insert occurs near a residue in ErbB1 shown to be in close proximity to bound EGF (31). It has been speculated that this altered sequence in the ErbB2 ECD may prevent it from binding ligand (30). The appearance of such a protein must have surely been considered a mutational event by the vertebrate organism in which it arose. Rutherford and Lindquist (32) recently proposed that Hsp90 binding to mutated proteins may stabilize them while masking their phenotypic expression, thus allowing accumulation of multiple silent mutations during evolution and providing the organism with a greater diversity of responses when faced with unexpected environmental stress. If *ErbB2* evolved by mutation from *ErbB1* to become a ligandless heterodimerization partner, it 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.

[2] I. Alroy and Y. Yarden, unpublished observations.

REFERENCES

1. Ullrich, A., Coussens, L., Hayflick, J. S., Dull, T. J., Gray, A., Tam, A. W., Lee, J., Yarden, Y., Libermann, T. A., Schlessinger, J., Downward, J., Mayes, E. L. V., Whittle, N., Waterfield, M. D., and Seeburg, P. H. (1984) *Nature* **309,** 418–425
2. Kraus, M. H., Issing, W., Miki, T., Popescu, N. C., and Aaronson, S. A. (1989) *Proc. Natl. Acad. Sci. U. S. A.* **86,** 9193–9197
3. Plowman, G. D., Culouscou, J. M., Whitney, G. S., Green, J. M., Carlton, G. W., Foy, L., Neubauer, M. G., and Shoyab, M. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90,** 1746–1750
4. O'Rourke, D. M., Zhang, X., and Greene, M. I. (1997) *Proc. Assoc. Am. Physicians* **109,** 209–219
5. Klapper, L. N., Kirschbaum, M. H., Sela, M., and Yarden, Y. (2000) *Adv. Cancer Res.* **77,** 25–79
6. Levkowitz, G., Waterman, H., Ettenberg, S. A., Katz, M., Tsygankov, A. Y., Alroy, I., Lavi, S., Iwai, K., Reiss, Y., Ciechanover, A., Lipkowitz, S., and Yarden, Y. (1999) *Mol. Cell* **4,** 1029–1040
7. Levkowitz, G., Waterman, H., Zamir, E., Kam, Z., Oved, S., Langdon, W. Y., Beguinot, L., Geiger, B., and Yarden, Y. (1998) *Genes Dev.* **12,** 3663–3674
8. Muthuswamy, S. K., Gilman, M., and Brugge, J. S. (1999) *Mol. Cell. Biol.* **19,** 6845–6857
9. Schechter, A. L., Stern, D. F., Vaidyanathan, L., Decker, S. J., Drebin, J. A., Greene, M. I., and Weinberg, R. A. (1984) *Nature* **312,** 513–516
10. Bargmann, C. I., Hung, M. C., and Weinberg, R. A. (1986) *Nature* **319,** 226–230
11. Hudziak, R. M., Schlessinger, J., and Ullrich, A. (1987) *Proc. Natl. Acad. Sci. U. S. A.* **84,** 7159–7163
12. Spencer, K. S., Graus-Porta, D., Leng, J., Hynes, N. E., and Klemke, R. L. (2000) *J. Cell Biol.* **148,** 385–397
13. Whitesell, L., Mimnaugh, E. G., De Costa, B., Myers, C. E., and Neckers, L. M. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91,** 8324–8328
14. Chavany, C., Mimnaugh, E., Miller, P., Bitton, R., Nguyen, P., Trepel, J., Whitesell, L., Schnur, R., Moyer, J., and Neckers, L. (1996) *J. Biol. Chem.* **271,** 4974–4977
15. Neckers, L., Schulte, T. W., and Mimnaugh, E. (1999) *Invest. New Drugs* **17,** 361–373
16. Mimnaugh, E. G., Chavany, C., and Neckers, L. (1996) *J. Biol. Chem.* **271,** 22796–22801
17. Sakagami, M., Morrison, P., and Welch, W. J. (1999) *Cell Stress Chaperones* **4,** 19–28
18. Tikhomirov, O., and Carpenter, G. (2000) *J. Biol. Chem.* **275,** 26625–26631
19. Tzahar, E., Waterman, H., Chen, X., Levkowitz, G., Karunagaran, D., Lavi, S., Ratzkin, B. J., and Yarden, Y. (1996) *Mol. Cell. Biol.* **16,** 5276–5287
20. Rosser, M. F., and Nicchitta, C. V. (2000) *J. Biol. Chem.* **275,** 22798–22805
21. Zheng, F. F., Kuduk, S. D., Chiosis, G., Munster, P. N., Sepp-Lorenzino, L., Danishefsky, S. J., and Rosen, N. (2000) *Cancer Res.* **60,** 2090–2094
22. Grenert, J. P., Sullivan, W. P., Fadden, P., Haystead, T. A. J., Clark, J., Mimnaugh, E., Krutzsch, H., Ochel, H. J., Schulte, T. W., Sausville, E., Neckers, L. M., and Toft, D. O. (1997) *J. Biol. Chem.* **272,** 23843–23850
23. Prodromou, C., Roe, S. M., O'Brien, R., Ladbury, J. E., Piper, P. W., and Pearl, L. H. (1997) *Cell* **90,** 65–75
24. Stebbins, C. E., Russo, A. A., Schneider, C., Rosen, N., Hartl, F. U., and Pavletich, N. P. (1997) *Cell* **89,** 239–250
25. Kuznetsov, G., Chen, L. B., and Nigam, S. K. (1994) *J. Biol. Chem.* **269,** 22990–22995
26. Mimnaugh, E. G., Worland, P. J., Whitesell, L., and Neckers, L. M. (1995) *J. Biol. Chem.* **270,** 28654–28659
27. Joazeiro, C. A., Wing, S. S., Huang, H., Leverson, J. D., Hunter, T., and Liu, Y. C. (1999) *Science* **286,** 309–312
28. Klapper, L. N., Waterman, H., Sela, M., and Yarden, Y. (2000) *Cancer Res.* **60,** 3384–3388
29. Klapper, L. N., Glathe, S., Vaisman, N., Hynes, N. E., Andrews, G. C., Sela, M., and Yarden, Y. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96,** 4995–5000
30. Stein, R. A., and Staros, J. V. (2000) *J. Mol. Evol.* **50,** 397–412
31. Woltjer, R. L., Lukas, T. J., and Staros, J. V. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89,** 7801–7805
32. Rutherford, S. L., and Lindquist, S. (1998) *Nature* **396,** 336–342