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A Novel Biochemical Pathway of ErbB2 Down-Regulation

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This proposal will examine a novel hypothesis that the HSP90/HSP70-associated co-chaperone CHIP functions as a selective ErbB2-directed ubiquitin ligase. This hypothesis is based on studies that while Cbl ubiquitin ligase negatively regulates ErbB1 (EGF receptor) by promoting its lysosomal traffic, lack of Cbl recruitment to ErbB2 upon heregulin stimulation promotes receptor recycling; however, ErbB2 can be targeted for ubiquitination via heterodimerization with ErbB1 or treatment with a therapeutic anti-ErbB2 antibody, highlighting the potential of ubiquitin ligase-mediated down-regulation of ErbB2 as a therapeutic strategy. We propose comprehensive strategies to test if CHIP functions as an ErbB2-selective ubiquitin ligase independent of Cbl, whether Cbl and CHIP can synergize to induce the ubiquitination and subsequent down-regulation of ErbB2 and reduction of cancer cell proliferation, and examine the mechanism of CHIP-mediated down-regulation of ErbB2. Validation of our hypothesis will be a major advance in ErbB receptor biology, and will provide a rationale to synergize anti-ErbB2 antibody therapy (e.g., with ansamycins or related drugs) of ErbB2-overexpressing breast cancer patients to increase the proportion of responders and to reduce toxicity.
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Introduction:

This application proposes to test a unique hypothesis that the HSP90/HSP70-associated co-chaperone CHIP (Carboxyl terminus of HSP70-interacting protein) functions as a selective ErbB2-directed ubiquitin ligase. This hypothesis is based on previous observations that inefficient Cbl-mediated ubiquitination of ErbB2 allows it to escape from a lysosomal fate. Importantly, a therapeutic anti-ErbB2 antibody was shown to induce the down-regulation of ErbB2 from the cell surface accompanied by an induced association with Cbl, highlighting the potential of ubiquitin ligase-mediated down-regulation of ErbB2 as a therapeutic strategy. Recent finding that geldanamycin, an ansamycin antibiotic with anti-tumor activity, induces ErbB2 down-regulation via ubiquitin modification has led to the possibility that a second ubiquitin ligase pathway distinct from Cbl can be recruited to ErbB2. Recent identification of CHIP as a HSP90/HSP70-associated ubiquitin ligase led to our hypothesis that this protein may target ErbB2 for ubiquitination and degradation, resulting in its functional downregulation. The proposed studies are designed to test this hypothesis in a comprehensive manner by assessing if CHIP indeed functions as an ErbB2-directed ubiquitin ligase, if CHIP is selective towards ErbB2 compared to ErbB1, and if CHIP-mediated ErbB2 ubiquitination is independent of Cbl. We will further assess if Cbl and CHIP can synergize to induce the ubiquitination and subsequent down-regulation of ErbB2, and if this can lead to synergistic reduction of cancer cell proliferation. Finally, we will examine the mechanism of CHIP-mediated down-regulation of ErbB2, focusing on the role of lysosomal targeting of ErbB2. Overall, our analyses will address a novel hypothesis that CHIP is an ErbB2-directed ubiquitin ligase that can be recruited to this receptor independently of Cbl ubiquitin ligase providing a rationale for combined recruitment of these two pathways to induce synergistic ErbB2 down-regulation. Validation of our hypothesis will represent a major advance in our understanding of ErbB receptor biology, and will provide rationale for the possibility of combining different agents (such as anti-ErbB2 and ansamycins or related drugs) to obtain a synergistic effect against ErbB2-overexpressing breast tumors. Such synergistic combinations could help increase the proportion of responders to anti-ErbB2 therapy and possibly help reduce toxicity. Given the significantly worse prognosis of patients with ErbB2-overexpressing tumors and their frequent hormone unresponsiveness, the proposed studies, if successful, could help ameliorate a major problem in breast cancer.

Body of Report:

To date, we have carried out studies proposed under Tasks 1-3 of the SOW. These findings have led to a successful test of our major hypothesis that CHIP functions an ErbB2-directed ubiquitin ligase, both in vitro and in vivo. We have further established that CHIP-mediated ubiquitination and degradation is selective for ErbB2 as compared to ErbB1 (EGF receptor). Finally, we have used Cbl-resistant ErbB2 mutant to show that CHIP-mediated ubiquitination and degradation of ErbB2 is Cbl-independent. These studies were carried out both in model cell line 293T as well as in mammary cell lines. The findings of our work have been published in the Journal of Biological Chemistry (Please see attached reprint: Zhou, P. et al. JBC, 2003).

Through these studies, we have also established the needed reagents and conditions to work on the remaining objectives. In this direction, we have already performed a series of
analyses (described below) that strongly support our hypothesis that CHIP-mediated ErbB2 down-regulation is mediated via lysosomal degradation. These studies have been carried out even though we are awaiting the transfer of the grant from Brigham and Women’s Hospital to Evanston Northwestern Healthcare Research Institute.

Anti-ErbB2 immunostaining of SKBR3 cells treated with GA was previously shown to induce the appearance of intracellular vesicular ErbB2 staining (Ref. #1). This finding, together with our work that ubiquitin modification of Erb receptors serves as a lysosomal sorting signal rather than a proteasome targeting signal, suggested that 17AAG-induced ErbB2 downregulation may proceed through lysosomal degradation. We therefore assessed the potential colocalization of ErbB2 and a lysosomal marker LAMP1 by immunofluorescence staining in SKBR3 cells treated with 17AAG for various time points. Consistent with previous results, 17AAG treatment of SKBR3 cells induced the surface ErbB2 to localize into intracellular vesicular structures. Double immunostaining for LAMP1 revealed these markers to colocalize (Fig. 1), consistent with our hypothesis that 17AAG-induced CHIP-mediated ubiquitinylation targets ErbB2 to lysosomes.

As ubiquitinylated receptor tyrosine kinases are targeted to lysosomes by virtue of their recognition by the ESCRT-1 complex, we tested if alteration of the ESCRT-1 machinery function affects the 17AAG-induced ErbB2 downregulation. Therefore, we examined the effect of overexpressing WT or dominant-negative VPS4, the ESCRT-1 regulator (Ref. # 2), on 17AAG-induced ErbB2 downregulation in SKBr3 cells. Both WT VPS4 and its dominant negative mutant (K173Q) were localized in endosomal structures, as expected (Ref. # 2), when transfected as GFP-tagged proteins in SKBr3 cells (Fig. 2). Overexpression of VPS4 enhanced the 17AAG-induced ErbB2 degradation in transfected 293T cells, whereas the expression of VPS4-K173Q mutant partially blocked ErbB2 degradation and increased the basal level of ERBB2 (Fig. 3). Together, these studies support the possibility that 17AAG-induced ErbB2 ubiquitinylation targets the receptor into lysosomes for degradation.

**Key Research Accomplishments:**

- Demonstrated that CHIP functions as a ubiquitin ligase towards ErbB2.
- Established that CHIP-dependent ubiquitination and down-regulation are ErbB2-selective.
- Determined that CHIP-mediated ErbB2 down-regulation is Cbl-independent.
- Demonstrated ErbB2 colocalization with lysosomal marker upon 17AAG treatment of breast cancer cells.
- Initial demonstration of ErbB2 association with ESCRT-1 regulator VPS4.

**Reportable Outcomes:**

**Reagents:**
- Generated tagged CHIP expression constructs, sequence verified these and demonstrated their expression in model cells.
- Established mutants of CHIP and their ability to express expected CHIP proteins.
- Established the ability of various CHIP constructs to influence the ubiquitination and down-regulation analyses of ErbB2.
- Established the Cbl-resistant ErbB2 mutants
- Generated and characterized anti-CHIP antibodies.
- Generated GFP-tagged ESCRT-1 components

Publications:


Conclusions:

In conclusion, we have successfully completed the first three stated objectives of the project and have obtained evidence supporting the hypotheses to be tested in the remaining objectives. Through these studies, we have established that CHIP functions as a ubiquitin ligase towards ErbB2, that CHIP-dependent ubiquitination and down-regulation are ErbB2-selective, and that CHIP-mediated ErbB2 down-regulation is Cbl-independent. These studies establish a firm basis for our further investigations aimed at assessing the potential synergistic ubiquitination and degradation of ErbB2 by Cbl and CHIP ubiquitin ligases (Objective 4) and the mechanism of ErbB2 downregulation by CHIP and Cbl-mediated ubiquitination (objective 5). Accomplishment of our objectives will represent a major advance in understanding the mechanisms of ErbB2 downregulation and provide a basis for harnessing the ErbB2-directed downregulation processes for breast cancer therapy.

References:


Fig. 1. 17AAG-induced HER2 co-localization in LAMP1-positive intracellular vesicles. Sub-confluent SKBr3 cells on glass coverslips were treated with 17AAG (2 μM) for the indicated time points. Cells were washed in PBS, fixed in 4% paraformaldehyde in PBS, permeabilized with 0.1% Triton X-100/PBS and blocked in 2% FBS/PBS. The cells were stained with a monoclonal anti-ErbB2 antibody (Neu-Ab-5, Oncogene) diluted in blocking buffer followed by anti-mouse IgG-Alexa594 (Molecular Probes). The cells were subsequently stained with directly FITC-conjugated LAMP1 monoclonal antibody (clone H4A3, BD Biosciences), and subjected to imaging on a Nikon Confocal microscope. Note the predominant surface staining at time zero with intracellular vesicles appearing after 17AAG treatment; the latter colocalize partially (yellow) with LAMP1. ErbB2 signals are lost after 3 h due to degradation.
Fig. 2. Subcellular localization of WT GFP-VPS4 and its dominant-negative mutant (K173Q) to endosomal structures upon transfection in SKBr3 cells. Sub-confluent SKBr3 cells on glass coverslips were transfected with GFP-VPS4 wild type or K173Q mutant plasmids. After 24 hours the cells are fixed with 4%PFA/PBS and images were acquired under a Nikon fluorescence microscope. Typical endosomal structures are noted.

Fig. 3. Enhancement and inhibition of 17AAG-induced ErbB2 degradation by WT and mutant VPS4, respectively. 293T cells were cotransfected with ErbB2 and myc-tagged human VPS4 (WT) or its indicated mutants, treated with 17AAG (2 μM) for the indicated time points, and cell lysates (50 μg) subjected to anti-ErbB2 and anti-myc immunoblotting. Note increased loss of ErbB2 signals upon WT VPS4 transfection and inhibition of ErbB2 degradation upon K173Q transfection.
ErbB2 overexpression contributes to the evolution of a substantial group of human cancers and signifies a poor clinical prognosis. Thus, down-regulation of ErbB2 signaling has emerged as a new anti-cancer strategy. Ubiquitinylation, mediated by the Cbl family of ubiquitin ligases, has emerged as a physiological mechanism of ErbB receptor down-regulation, and this mechanism appears to contribute to ErbB2 down-regulation induced by therapeutic anti-ErbB2 antibodies. Hsp90 inhibitory ansamycin antibiotics such as geldanamycin (GA) induce rapid ubiquitinylation and down-regulation of ErbB2. However, the ubiquitin ligase(s) involved has not been identified. Here, we show that ErbB2 serves as an in vitro substrate for the Hsp70/Hsp90-associated U-box ubiquitin ligase CHIP. Overexpression of wild type CHIP, but not its U-box mutant H260Q, induced ubiquitinylination and reduction in both cell surface and total levels of ectopically expressed or endogenous ErbB2 in vivo, and this effect was additive with that of 17-allylamino-geldanamycin (17-AAG). The CHIP U-box mutant H260Q reduced 17-AAG-induced ErbB2 ubiquitinylation. Wild type ErbB2 and a mutant incapable of association with Cbl (ErbB2 Y1112F) were equally sensitive to CHIP and 17-AAG, implying that Cbl does not play a major role in geldanamycin-induced ErbB2 down-regulation. Both endogenous and ectopically expressed CHIP and ErbB2 communoprecipitated with each other, and this association was enhanced by 17-AAG. Notably, CHIP H260Q induced a dramatic elevation of ErbB2 association with Hsp70 and prevented the 17-AAG-induced dissociation of Hsp90. Our results demonstrate that ErbB2 is a target of CHIP ubiquitin ligase activity and suggest a role for CHIP E3 activity in controlling both the association of Hsp70/Hsp90 chaperones with ErbB2 and the down-regulation of ErbB2 induced by inhibitors of Hsp90.

ErbB2 is a member of the ErbB receptor tyrosine kinase family, which also includes the epidermal growth factor receptor (EGFR/ErbB1), ErbB3, and ErbB4. The members of this receptor tyrosine kinase family play key roles in normal cell proliferation, differentiation, survival, and migration and have emerged as important contributing factors in tumorigenesis (1). ErbB2 overexpression, found in nearly a third of breast cancer patients as well as in other cancers, is associated with poor responsiveness to conventional therapy and shorter relapse-free survival, validating ErbB2 as a molecular target for therapy through attenuation of ErbB2 signaling (2).

A crucial physiological mechanism of ErbB receptor signal attenuation involves the down-regulation of receptors from the cell surface, a process that includes initial ligand-induced endocytosis and subsequent sorting to the lysosome for degradation (3). Recent studies have focused on the role of ErbB receptor ubiquitinylation, mediated by the ubiquitin ligase Cbl, as a crucial mechanism to control ErbB receptor sorting to the lysosome. Upon EGF stimulation, EGFR associates with Cbl and undergoes ubiquitinylination, which facilitates its lysosomal sorting and eventual degradation (3–8). In contrast, ErbB2 activated by heregulin (through heterodimer formation with ErbB3/4) fails to interact with Cbl and is inefficiently ubiquitinylated (9–12). As a result, ErbB2 avoids the lysosomal fate and recycles to the cell surface instead. Furthermore, ErbB2 heterodimerized with other ErB family members also avoids the lysosomal fate and is preferentially recycled, accounting for its enhanced signaling potency (9–13). Importantly, the therapeutic anti-ErbB2 antibody Trastuzumab (Herceptin™) induces an ErbB2-Cbl interaction, which results in ErbB2 ubiquitinylation and degradation (14, 15). These findings highlight the potential use of ubiquitin ligase-mediated ErbB2 down-regulation as a therapeutic strategy in ErbB2-overexpressing cancers.

It has been demonstrated that treatment of breast and other cancer cell lines with ansamycin antibiotics such as geldanamycin (GA) depletes the cell surface ErbB2 via ubiquitinylation and subsequent degradation (16, 17). The effect of GA on ErbB2 is apparently not mediated by Cbl, since EGFR, a Cbl target, is GA-resistant. Furthermore, ErbB2 kinase activity is dispensable for GA action, whereas the kinase activity is required for Cbl-mediated EGFR or ErbB2 ubiquitinylation (16–21). These findings point to the existence of a novel ubiquitin ligase machinery that could be recruited to attenuate ErbB2 signaling. To date, however, the identity of such an ubiquitin ligase remains unknown.

Previous studies reveal that GA is a specific inhibitor of the

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‡Supported by National Institutes of Health Training Grant T32AR07530.

1 The abbreviations used are: EGFR, epidermal growth factor (EGF) receptor; CHIP, carboxyl terminus of Hsc70-interacting protein; GA, geldanamycin; 17-AAG, 17-allylamino-geldanamycin; GST, glutathione S-transferase; HRP, horseradish peroxidase; IP, immunoprecipitate; Ab, antibody; E1, ubiquitin-activating enzyme; E2 or Ubc, ubiquitin-conjugating enzyme; RIPA, radiolabeled precipitation assay buffer.
molecular chaperone Hsp90, suggesting that Hsp90 is required for mature ErbB2 stability (21). Indeed, mature ErbB2 is associated with Hsp90, and GA treatment leads to the disassociation of Hsp90 from ErbB2, with concomitant recruitment of Hsp70 (21). It is, therefore, likely that GA treatment leads to remodeling of the ErbB2-associated chaperone complex, resulting in the recruitment of a ubiquitin ligase for the ubiquitylation of ErbB2.

Recent studies identify a co-chaperone protein, CHIP (carboxyl terminus Hsc70-interacting protein) (Fig. 2A), whose three tetratricopeptide repeats bind to Hsp70/Hsc70 and Hsp90, whereas its carboxyl-terminal U-box domain associates with ubiquitin-conjugating enzymes (Ubc), thus satisfying the requirements of a chaperone-associated ubiquitin ligase (22–26). Importantly, CHIP has been demonstrated to negatively regulate Hsp70 and Hsp90 function and convert Hsp90 complexes from a chaperone function to one that promotes the ubiquitylation and degradation of client proteins, such as the glucocorticoid receptor (23), the cystic fibrosis transmembrane conductance regulator (24), and c-Raf kinase (26). Here we investigated if CHIP serves as an ErbB2-directed ubiquitin ligase. We demonstrate that CHIP associates with ErbB2 in vivo and that wild type CHIP, but not its U-box mutant, induces ErbB2 ubiquitylation and down-regulation. CHIP also enhances the effects of GA treatment, and the U-box mutant of CHIP inhibits GA-induced ErbB2 ubiquitylation. Together with results published by Neckers and co-workers while this paper was under review (27) our studies support the idea that CHIP ubiquitin ligase provides a novel mechanism to down-regulate ErbB2 via the ubiquitin pathway.

MATERIALS AND METHODS

Cell Lines and Reagents—The human breast cancer cell line SKBR-3 and the embryonic kidney epithelial cell line 293T were obtained from ATCC (Manassas, VA). 17-allylamino-geldanamycin (17-AAG, NSC 330507, provided by the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment and Diagnosis of the NCI, National Institutes of Health, Bethesda, MD) and MG-132 (Calbiochem) were dissolved in Me₂SO at 1 mM and 50 mM, respectively. Cycloheximide (Calbiochem) was dissolved in ethanol at 100 mM.

Antibodies—The rabbit polyclonal antibody (Ab) neu C-18 (anti-ErbB2) and mouse monoclonal antibodies W27 (anti-Hsp70), F-8 (anti-Hsp90), and D-10 (anti-β tubulin) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), monoclonal Ab c-neu Ab-5 (anti-ErbB2) was from Calbiochem, and monoclonal Abs P4G7 (anti-ubiquitin) was from Covance Research Products Inc. (Denver, PA). Monoclonal Ab 9E10 (anti-Myc) (28) was purified in-house. Purified humanized anti-ErbB2 antibody Herceptin™ was provided by Genentech Inc. (South San Francisco, CA). Rabbit polyclonal anti-CHIP antibody was supplied by Covance Research Products Inc. against a peptide corresponding to human CHIP residues 251–268, and its reactivity was confirmed by immunoprecipitation and immunoblotting against transfected Myc-tagged CHIP. Horseradish peroxidase (HRP)-conjugated protein A and rabbit anti-mouse IgG reagents were from Zymed Laboratories Inc. (South San Francisco, CA).

Plasmids—A pcDNA3 expression construct encoding the Myc epitope-tagged full-length CHIP protein (amino acids 1–303) was generated from a human CHIP IMAGE clone (ID 3847704, ATCC, Manassas, VA) by PCR (primer sequences are available upon request). The pGEX4T-2 bacterial expression constructs encoding the full-length or ΔU-box (amino acids 1–189) CHIP proteins fused to the carboxyl terminus of glutathione S-transferase (GST) were also generated by the PCR. The pcDNA3 constructs encoding ErbB2 expression constructs in pcDNA3 was a kind gift of Dr. Kermit Carraway III (University of California, Davis, CA). The QuikChange™ mutagenesis system (Invitrogen) was used to generate point mutants of ErbB2 (Y1112F) and CHIP (H260Q) using mutant primers (sequences available upon request). All constructs were sequence-verified. The pGEX4T-2 constructs expressing GST-Chip-N (Cbl residues 1–303) and GST-Chip (full-length) fusion protein have been described previously (29, 30).

Transient Transfection—Cell Lysis, Immunoprecipitation, and Immunoblotting—SKBR-3 or HEK 293T cells were plated in 100-mm dishes and transfected with the indicated expression plasmids using the Fu

**Figure 1.** Ubiquitylation and down-regulation of endogenous as well as ectopically expressed ErbB2 by 17-AAG. Endogenous ErbB2-expressing SKBR-3 cells or ErbB2-transfected 293T cells were treated with vehicle (−) or 100 nM (for SKBR-3 cells) or 5 μM (for 293T cells) 17-AAG (+) with (+) or without (−) 50 μM MG-132 for 4 h. MG-132 was added to the cells 1 h before and continued during the 17-AAG treatment. One-mg aliquots of lysate were subjected to immunoprecipitation (IP) with an anti-ErbB2 antibody, washed with RIPA buffer, resolved by SDS-PAGE, and immunoblotted (IB) with anti-ubiquitin antibody (top panel). The membrane was stripped and reprobed with the anti-ErbB2 antibody (bottom panel). 25-μg aliquots of whole cell lysate were immunoblotted directly with the anti-ErbB2 antibody to visualize the levels of endogenous or transfected ErbB2 protein (bottom panel). ErbB2-Ub indicates ubiquitylated ErbB2.

**Table 1.** Ubiquitinylated and down-regulation of ErbB2 by CHIP

<table>
<thead>
<tr>
<th>Cell line:</th>
<th>SKBR-3</th>
<th>ErbB2-transfected 293T</th>
</tr>
</thead>
<tbody>
<tr>
<td>MG-132:</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>17-AAG:</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>IP:</td>
<td>anti-ErbB2</td>
<td>anti-ErbB2</td>
</tr>
<tr>
<td>IB:</td>
<td>anti-ubiquitin</td>
<td>anti-ubiquitin</td>
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</tbody>
</table>

**Table 2.** Down-regulation of ErbB2 by CHIP

<table>
<thead>
<tr>
<th>Lysate:</th>
<th>SKBR-3</th>
<th>ErbB2-transfected 293T</th>
</tr>
</thead>
<tbody>
<tr>
<td>IP:</td>
<td>anti-ErbB2</td>
<td>anti-ErbB2</td>
</tr>
<tr>
<td>IB:</td>
<td>anti-ErbB2</td>
<td>anti-ErbB2</td>
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**Table 3.** Ubiquitylation of ErbB2 by CHIP

<table>
<thead>
<tr>
<th>Lane:</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
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<tbody>
<tr>
<td>17-AAG:</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>IP: anti-ErbB2</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>IB: anti-ErbB2</td>
<td>+</td>
<td>+</td>
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</table>
ErbB2 Degradation by CHIP

RESULTS

Ubiquitinylation and Down-regulation of Both Endogenous and Ectopically Expressed ErbB2 by 17-AAG—Ansamycin antibiotics, such as GA, specifically bind to Hsp90 and inhibit its ATPase activity (18). Exposure of cells to GA induces the dissociation of Hsp90 from ErbB2 and other Hsp90-associated signaling proteins, resulting in their degradation (19–21). A more potent GA derivative, 17-AAG, is undergoing clinical assessment as a potential anti-cancer agent (31). We examined whether 17-AAG, like its parent compound, induces the down-regulation of ErbB2 via the ubiquitin pathway. As anticipated (19–21), 17-AAG treatment led to a significant loss of ErbB2 in both SKBR-3 cells (endogenous ErbB2) and 293T cells (exogenous ErbB2) (Fig. 1). Treatment of cells with the proteasome inhibitor MG-132 prevented 17-AAG-induced loss of ErbB2 (Fig. 1, middle and bottom panels). Furthermore, 17-AAG treatment induced the ubiquitinylation of ErbB2, as revealed by anti-ubiquitin immunoblotting of ErbB2 IPs, and MG-132 treatment markedly enhanced the ubiquitinylated signal (Fig. 1, top left panels, compare lane 1 with lane 2 and lane 3 with lane 4; top right panel, compare lane 1 with lane 2 and lane 3 with lane 4). Thus, 17-AAG, like GA (19–21), induces ErbB2 down-regulation via the ubiquitin pathway.

The Co-chaperone Protein CHIP Functions as a Ubiquitin Ligase toward ErbB2 in Vitro—Given the linkage of GA-induced ErbB2 degradation to inhibition of Hsp90 (21) and the recent observations that the Hsp70/Hsp90 co-chaperone CHIP functions as a ubiquitin ligase toward several Hsp70/Hsp90 client proteins (22–26, 32–36), we asked if CHIP functions as a ubiquitin ligase toward ErbB2, using an in vitro ubiquitination assay with purified GST-CHIP fusion proteins (Fig. 2A). Purified CHIP could readily auto-ubiquitinylate itself in vitro in the presence of purified E1, E2, ubiquitin, and ATP, whereas a CHIP mutant lacking the U-box domain was completely inactive (Fig. 2B), and the point mutation of an invariant histidine in the U-box domain, corresponding to a conserved RING finger domain histidine residue crucial for E2 binding, greatly reduced the auto-ubiquitinylation activity (32–34).

To assess if recombinant CHIP could function as a ubiquitin ligase toward ErbB2, recombinant ErbB2 immunopurified from SKBR-3 cells was incubated with purified CHIP fusion proteins, ubiquitin, and ATP in a rabbit reticulocyte lysate. Relatively little in vitro ErbB2 ubiquitination was observed in the absence of CHIP or ErbB2 (Fig. 2C). Inclusion of recombinant wild type mutant (residues 1–189); H260Q, U-box point mutant. NS indicates nonspecific signals. C, in vitro ErbB2 ubiquitination by recombinant CHIP but not its U-box mutants. Anti-ErbB2 IPs from 500-μg aliquots of SKBR-3 cell lysate were collected using protein G-Sepharose beads and washed with RIPA buffer. The bead-bound ErbB2 or anti-ErbB2 antibody was subjected to in vitro ubiquitination in rabbit reticulocyte lysate (RRL, a source of E1 and E2) and other components as indicated in A. The beads were further processed as in A to detect the ubiquitinated ErbB2 (ErbB2-Ib). D, CHIP mediates more efficient ErbB2 ubiquitination than Cbl. In vitro ErbB2 ubiquitination and detection was carried out as described in C.

Fig. 2. Recombinant CHIP functions as a ubiquitin ligase toward ErbB2 in vitro. A, schematic representation of the domain structure of CHIP. The numbers above correspond to amino acid residues in human CHIP. The positions of introduced mutations are indicated by arrows. TPR, tetratricopeptide repeat. B, anti-ubiquitinyl-
CHIP in the reaction resulted in a marked increase in the level of ubiquitylated ErbB2 (Fig. 2C, lane 5). In contrast, little or no increase in ErbB2 ubiquitylation was observed with the CHIP U-box domain mutants A-U-box or H260Q (Fig. 2C, lanes 6 and 7). The relative specificity of CHIP-induced ErbB2 ubiquitylation was shown by the relatively modest ubiquitylation of ErbB2 by GST-Cbl (Fig. 2D), whereas GST-Cbl efficiently induced the ubiquitylation of EGFR (data not shown). These in vitro studies showed that CHIP could function as a ubiquitin ligase toward ErbB2, suggesting the possibility that CHIP may regulate ErbB2 turnover in vivo.

**Overexpression of CHIP Promotes the Down-regulation of ErbB2**—To assess the effect of CHIP on ErbB2 turnover in vivo, we cotransfected ErbB2 and Myc-tagged CHIP into 293T cells and examined the overall levels of ErbB2 with or without 17-AAG treatment. Coexpression of CHIP led to a slight reduction in the level of ErbB2 in the absence of 17-AAG treatment (Fig. 3A, lane 3). Furthermore, CHIP overexpression modestly augmented the reduction in ErbB2 protein levels induced by 17-AAG treatment (Fig. 3A, lane 4).

Previous analyses have identified the role of Cbl, a RING finger domain ubiquitin ligase, in ErbB2 down-regulation induced by anti-ErbB2 antibodies or by EGF-induced heterodimerization with EGFR; the phosphorylated Tyr-1112 in ErbB2 serves as the Cbl-binding site in the context of antibody-mediated ErbB2 down-regulation (14, 15). Notably, the ability of 17-AAG and/or CHIP to induce the loss of ErbB2 protein was unaltered by the Y1112F mutation (Fig. 3A, lanes 7 and 8). That the Y1112F mutation indeed abrogated the ability of ErbB2 to interact with Cbl was demonstrated by a pull-down assay using lysates of ErbB2-transfected 293T cells stimulated with the humanized anti-ErbB2 antibody Herceptin™ and the GST-Cbl-N fusion protein, which incorporates the tyrosine kinase binding domain of Cbl (29). Wild type ErbB2 bound to GST-Cbl-N and Herceptin™ treatment enhanced this interaction (Fig. 3B, lane 6); in contrast, the ErbB2 Y1112F mutant failed to bind to GST-Cbl-N with or without Herceptin™ treatment (Fig. 3B, lane 8). Taken together, these data indicate that CHIP enhances ErbB2 degradation in a Cbl-independent manner.

Given the in vitro ubiquitin ligase activity of CHIP (Fig. 2, B and C) (26, 32, 33, 35) and its ability to induce the loss of ErbB2 treated with plasmids encoding wild type ErbB2 or ErbB2 Y1112F together with a plasmid encoding Myc-tagged CHIP (+) or with control vector (−). After 36 h of transfection, the cells were treated with 3 μM 17-AAG (+) or MeSO control (−) for 2 h before cell lysis with lysis buffer. 25-μg aliquots of whole cell lysate were resolved by SDS-PAGE and sequentially immunoblotted (IB) with anti-ErbB2, anti-Myc (CHIP), and anti-tubulin antibodies. 25-μg aliquots of lysates of ErbB2-transfected 293T cells were stimulated with 50 μg/ml of glutathione-Sepharose-bound GST-Cbl-N fusion proteins. The bead-bound ErbB2 was detected by anti-ErbB2 immunoblotting. C, CHIP-induced ubiquitylation of ErbB2 ubiquitylation in 293T cells. 293T cells were transfected with plasmids encoding ErbB2 with (+) or without (−) CHIP. The cells were treated with 50 μM proteasome inhibitor MG-132 for 4 h before lysis. Anti-ErbB2 IPs from 1.5-μg aliquots of lysate were serially immunoblotted with anti-ubiquitin (top panel) and anti-ErbB2 (middle panel) antibodies. 25-μg aliquots of lysate were directly immunoblotted with anti-Myc antibody to visualize the transfected CHIP protein (bottom panel). D, inhibition of 17-AAG-induced ErbB2 ubiquitylation by CHIP H260Q. The SKBR-3 cells were transfected with plasmids encoding wild type CHIP or CHIP H260Q or with vector alone (−). After 36 h of transfection, the cells were pretreated with 50 μM MG-132 (+) or with MeSO (−). Anti-ErbB2 IPs from 1-μg aliquots of lysate were serially immunoblotted with anti-ubiquitin or anti-ErbB2 antibodies. 25-μg aliquots of lysate were directly immunoblotted with anti-Myc antibody to visualize the transfected CHIP (bottom panel).
Fig. 4. CHIP-induced down-regulation of cell surface ErbB2. A, vector- or CHIP-transfected SKBR-3 cells were surface-labeled with biotin and treated with 100 nM 17-AAG or Me₂SO control for the indicated time points before lysis. Anti-ErbB2 IPs from 100-μg aliquots of lysate protein were immunoblotted (IB) with streptavidin-HRP to visualize the surface-labeled ErbB2 (top panel) followed by anti-ErbB2 immunoblotting to visualize the total immunoprecipitated ErbB2 (second panel). 25-μg aliquots of lysate were directly resolved by SDS-PAGE and immunoblotted with anti-Myc antibody to detect CHIP expression (bottom panel) (representative of two independent experiments). The bands in the top panel of A were quantified by densitometry using the Scion Images software. The data was expressed as a percentage of the signals obtained with untreated cells (bottom graph). B, overexpression of CHIP facilitates ErbB2 degradation even after new protein synthesis is blocked with cycloheximide (CHX) treatment. 293T cells were cotransfected with ErbB2 and/or CHIP as described in Fig. 3. The cells were treated with vehicle or 100 μM cycloheximide for 2 h before the addition of 3 μM 17-AAG. 25-μg aliquots of cell lysate were immunoblotted with the indicated antibodies. ErbB2 signals in the top panel were quantified by densitometry and plotted as a percentage of signals in vehicle-treated cells. The results are representative of three independent experiments.

In vivo (Fig. 3A), we asked if CHIP promotes ErbB2 ubiquitinylation in vivo. For this purpose, 293T cells transfected with ErbB2 with or without CHIP were incubated with the proteasome inhibitor MG-132, and the lysates of these cells were subjected to anti-ErbB2 IP followed by anti-ubiquitin immunoblotting. Indeed, CHIP overexpression was associated with an increase in the ubiquitinylation of ErbB2 (Fig. 3C). That CHIP-dependent enhancement of ErbB2 ubiquitinylation reflected the E3 activity of CHIP was shown by the inability of the U-box point mutant H260Q to enhance ErbB2 ubiquitinylation in transfected SKBR-3 cells (Fig. 3D). Furthermore, expression of the H260Q mutant (Fig. 3D, lane 6 versus lane 2), but not wild type CHIP (lane 4 versus 2), inhibited the 17-AAG-induced ubiquitinylation of ErbB2, suggesting a role for endogenous
 CHIP (and/or CHIP-related E3s) in 17-AAG-induced ErbB2 ubiquitylation.

CHIP-induced Down-regulation of Cell Surface ErbB2—Given that GA targets the Hsp90-associated mature cell surface ErbB2 for degradation, it was important to assess if CHIP overexpression specifically down-regulates this pool of ErbB2, particularly since previous studies reported CHIP as a ubiquitin ligase toward misfolded Hsp70/Hsp90 client proteins in the endoplasmic reticulum (24, 35). Therefore, we carried out cell surface biotinylation of vector- or CHIP-transfected SKBR-3 cells, which express endogenous ErbB2, and monitored the levels of biotin-labeled cell surface ErbB2 by streptavidin blotting of anti-ErbB2 IPs and densitometry (Fig. 4A). These analyses showed an enhancement of the 17-AAG-induced loss of surface ErbB2 in CHIP-overexpressing SKBR-3 cells as compared with the vector-transfected cells (Fig. 4A). The reduction in cell surface (biotin-labeled) ErbB2 closely paralleled the reduction in total ErbB2 (Fig. 4A, compare streptavidin versus anti-ErbB2 blots). Although the effect of CHIP was modest, it was reproducible in additional experiments (data not shown). CHIP-dependent enhancement of 17-AAG-induced loss of ErbB2 was also observed when the synthesis of new ErbB2 was inhibited with cycloheximide treatment (Fig. 4B), consistent with CHIP-induced loss of mature cell surface ErbB2. Although we could not exclude the possibility that CHIP also targets newly synthesized ErbB2, the combined results clearly show that CHIP can facilitate the 17-AAG-induced loss of cell surface ErbB2.

CHIP Associates with ErbB2—To further explore the role of CHIP in ErbB2 turnover, we examined its in vivo association with ErbB2 with or without 17-AAG treatment. Because such an association was predicted to occur via Hsp70/Hsp90 chaperone proteins, we first demonstrated the coimmunoprecipitation of the endogenous Hsp70 and Hsp90 proteins with Myc-tagged CHIP in the lysates of CHIP-transfected 293 T cells (Fig. 5A). Next we used reciprocal IP/Western blotting to show that ErbB2 and CHIP proteins associate in 293 T cells cotransfected with both proteins (Fig. 5B). The level of ErbB2/CHIP coimmunoprecipitation increased when cells were treated with 17-AAG (Fig. 5B, top and third panels, lane 5 versus 6). The 17-AAG-induced CHIP-ErbB2 association correlated with the increased association of ErbB2 with the Hsp70 chaperone (see Fig. 6). Finally, we used the SKBR-3 cells, which express moderate levels of CHIP (compared with a number of other cell lines; data not shown), to demonstrate that both endogenous ErbB2 and CHIP also coimmunoprecipitate (Fig. 5C), indicating their association under more physiological conditions. Importantly, the level of the endogenous CHIP-ErbB2 association also increased substantially upon 17-AAG treatment (Fig. 5C).

Increased ErbB2 Interaction with the CHIP H260Q Mutant—In view of the reduced 17-AAG-induced ErbB2 ubiquitylation by CHIP expression plasmid (+) or vector (−), and lysates were prepared 36 h after transfection. One-mg aliquots of lysate were subjected to anti-Myc IPs and serially immunoblotted (IB) with anti-Hsp70, anti-Hsp90, and anti-Myc antibodies. B, association of transfected ErbB2 and CHIP proteins and increased association upon 17-AAG treatment. 293 T cells were transfected with ErbB2 and/or CHIP as indicated. Cells were then treated with 50 μM MG-132 for 1 h followed by a 2-h incubation in the presence of 3 μM 17-AAG (+) or MeSO (−). Anti-ErbB2 or anti-Myc IPs from 1-mg aliquots of lysate were serially immunoblotted with anti-ErbB2 and anti-Myc (CHIP) antibodies. C, association of endogenous ErbB2 and CHIP. SKBR-3 cells were treated with either MeSO vehicle (−) or MG-132 (+) for 1 h, and 100 nM 17-AAG (+) or MeSO (−) was then added for 1 h. Anti-ErbB2 IPs from 1-mg aliquots of lysate protein were then serially immunoblotted with anti CHIP (top panel) and anti-ErbB2 (second panel) antibodies. 50-μg aliquots of lysate were directly immunoblotted with anti CHIP (third panel) and anti-ErbB2 (bottom panel) antibodies.
ErbB2 Degradation by CHIP

**FIG. 6.** CHIP H260Q-induced enhancement of ErbB2-Hsp70 association and reduction of 17-AAG-induced Hsp90 dissociation from ErbB2. 293T cells were transfected with ErbB2 (+) and/or Myc-tagged wild type (WT) CHIP or its H260Q mutant, as indicated. 36 h post-transfection, the cells were pretreated with 50 μM MG-132 for 1 h followed by another 2-h incubation in the presence of 3 μM 17-AAG (+) or MeSO (−). Anti-Myc or anti-ErbB2 IPs from 1-mg aliquots of lysate were serially immunoblotted (IB) with the indicated antibodies (in the order of top panel to bottom panel).

ubiquitin ligase machinery is recruited to ErbB2 upon GA treatment. Given the recent studies that ansamycins inhibit the ATPase activity of Hsp90 and that ErbB2 constitutively associates with Hsp90, we examined the possibility that the Hsp70/Hsp90-associated co-chaperone CHIP may function as an ErbB2-directed ubiquitin ligase. Here we provide evidence that CHIP functions as a ubiquitin ligase toward ErbB2. Although our results provide an independent confirmation for similar data published while this paper was under review (27), the present study provides several additional insights that are discussed below.

In keeping with a role for CHIP in ErbB2 ubiquitinylation, both our results and the Xu et al. (27) study establish that ErbB2 functions as a substrate for CHIP-mediated ubiquitinylation in vitro (Fig. 2, C and D), an activity that requires the U-box domain, previously shown to be essential for CHIP ubiquitin ligase activity (26, 32, 33, 35). Furthermore, overexpression of intact CHIP, but not its U-box domain mutant, led to enhanced ubiquitinylation and degradation of ErbB2 in human cells (Fig. 3, C and D). The ability of the CHIP H260Q mutant to decrease 17-AAG-induced ErbB2 ubiquitinylation (Fig. 3D) is suggestive of a dominant negative effect on endogenous CHIP, although further studies are needed to clarify this possibility. The effects of CHIP on ErbB2 are reminiscent of those observed with cystic fibrosis transmembrane conductance regulator (24), another transmembrane receptor. However, the present study establishes that, in contrast to CHIP-induced ubiquitinylation of misfolded cystic fibrosis transmembrane conductance regulator in the ER (24), CHIP enhanced the loss of mature cell surface ErbB2 (Fig. 4, A and B). The sensitivity of mature cell surface ErbB2 to CHIP is consistent with the association of Hsp90 with mature ErbB2 and the requirement of this association for ErbB2 stability, as demonstrated by the sensitivity of ErbB2 to GA (21).

Our study also shows that CHIP overexpression enhances the ubiquitinylation and down-regulation of both wild type ErbB2 and a mutant (Y1112F) that is incapable of interacting with Cbl (Fig. 3B). Thus, our results define CHIP as a novel ErbB2-directed ubiquitin ligase distinct from the previously reported Cbl ubiquitin ligase.
of CHIP may be to facilitate the remodeling of chaperone complexes, but it is not clear at present whether CHIP-induced ErbB2 down-regulation in the absence of GA is mediated via the Hsp90-CHIP or the Hsp70-CHIP complexes, because both complexes were observed in CHIP-transfected cells, and both Hsp90 and Hsp70 coimmunoprecipitated with ErbB2.

The role of CHIP in GA-induced ErbB2 ubiquitinylation and degradation is supported by a number of findings presented here and the results of the Xu et al. (27) study. First, 17-AAG, a potent GA derivative (as well as GA, data not shown), induced a substantial increase in ErbB2-CHIP association (Fig. 5, B and C). Notably, this enhancement correlated with an increased association of Hsp70 with ErbB2 and concomitant Hsp90 dissociation (Fig. 6), consistent with a primarily Hsp70-mediated CHIP-ErbB2 association (21). Second, the overexpression of CHIP in the context of 17-AAG treatment led to an additive loss of ErbB2 (Figs. 3 and 4). Finally, the overexpression of a CHIP point mutant (H260Q), which exhibits reduced ubiquitin ligase activity in vitro (Fig. 2D), reduced the level of ErbB2 ubiquitinylation induced by 17-AAG. However, a substantial level of 17-AAG-induced degradation of ErbB2 was still observed when the CHIP H260Q mutant was expressed (Fig. 3D). The inability of this mutant to fully block the 17-AAG-induced ErbB2 degradation may be due to several factors. First, only a proportion of cells was likely to be transfected under the experimental conditions used. Second, the mutant construct employed, H260Q, has residual ubiquitin ligase activity (Fig. 2B), which may itself lead to ErbB2 degradation. We were unable to use a CHIP U-box-deleted mutant, which exhibits no in vitro ubiquitin ligase activity (Fig. 2B), for these studies because it showed markedly reduced association with ErbB2 (data not shown), potentially confounding the results. Third, CHIP may not be the sole ubiquitin ligase recruited to ErbB2 upon Hsp90 inhibition. This possibility is supported by the results of Xu et al. (27) with CHIP−/− fibroblasts, which showed no reduction in GA-induced ErbB2 degradation (27). Finally, CHIP may exert a more complex effect on ErbB2 regulation, as Hsp70 itself has been shown to be a CHIP substrate (33), and this modification may have regulatory effects on Hsp70-mediated folding and/or degradation of client proteins. In this regard, it is noteworthy that previous studies have shown that CHIP inhibits the protein folding function of Hsp70 and competes for binding of co-chaperones such as p23 (23). Furthermore, CHIP has been shown to decrease the ATPase activity of Hsp70 and inhibit its chaperone function in vitro (22). Further studies of CHIP should help clarify these issues.

Rather unexpectedly, and distinct from the results of Xu et al. (27), we observed a dramatically increased association of the CHIP H260Q mutant with ErbB2 under basal conditions; 17-AAG treatment further enhanced this association (Fig. 6). Notably, whereas the level of association of CHIP H260Q with Hsp70 was unaffected compared with wild type CHIP, dramatically more Hsp70 was associated with ErbB2 both under basal conditions and upon 17-AAG treatment. However, this mutant did not show a higher level of association with Hsp90, although it reduced the GA-induced Hsp90 dissociation from ErbB2 compared with that seen in untransfected and wild type CHIP-transfected cells. Thus, the mutant CHIP clearly alters the nature of the chaperone-substrate complex. These findings strongly suggest that the ubiquitin ligase activity of CHIP plays a critical role in controlling the chaperone association/dissociation cycle with client proteins. It is likely that one function of CHIP may be to facilitate the remodeling of chaperone complexes. Further studies will be needed to directly address these issues and to ascertain if the observed effects are specific for ErbB2 or are observed with other CHIP substrates as well. The apparent dichotomous effects of CHIP-H260Q on ErbB2 association with Hsp70 versus Hsp90 may simply reflect the higher basal level of Hsp90-ErbB2 association compared with Hsp70-ErbB2 association. Alternatively, these results may reflect a differential functional impact of CHIP-mediated ubiquitinylation (of substrates or chaperone proteins themselves) on Hsp70 versus Hsp90 association with client proteins. In this regard, it is well documented that Hsp90 and Hsp70 require distinct co-chaperone for association with client proteins and often engage distinct sets of client proteins (38–41).

Recently CHIP was shown to interact, albeit poorly, with the anti-apoptotic protein Bag-1, which possesses a ubiquitin-like domain capable of associating with the 26 S proteosome and additional sequences that interact with Hsp70 (26). These findings have led to a model that a ternary complex of the substrate (c-Raf in the reported study) with chaperone and ubiquitin ligase (CHIP) may be directly targeted to the proteosome for efficient degradation of the target proteins (26). In preliminary experiments we did not observe an enhancement of CHIP-induced ErbB2 degradation when Bag-1 was coexpressed (data not shown). Further studies will be needed to fully assess the potential role (or lack thereof) of the Bag-1 co-chaperone in CHIP-dependent ErbB2 degradation.

Our results together with those of Xu et al. (27) lead us to conclude that CHIP is a novel ErbB2-directed E3 ligase. The independence of this pathway from the previously characterized Cbl ubiquitin ligase pathway and the recruitment of these pathways by distinct pharmacologic agents (ansaamycins versus anti-ErbB2 antibodies) raise the possibility of combinatorial manipulations of both pathways for a more effective downregulation of ErbB2 and better anti-cancer therapy. Notably, a recent report demonstrated that kinase inhibitors of ErbB receptors also induce ubiquitin-dependent receptor degradation and Hsp70 recruitment, and such treatment additively downregulates the ErbB receptor levels when combined with anti-receptor antibodies (42). These findings provide added impetus to delineate the various ErbB2-directed ubiquitin ligase pathways to devise combinatorial strategies for treatment of ErbB2-overexpressing cancers.

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ErbB2 Degradation by CHIP