Available online at www.sciencedirect.com
ScienceDirect
Biochimica et Biophysica Acta xx (2006) xxx–xxx
Biochimica et Biophysica Acta BBA
www.elsevier.com/locate/bbamcr

Curcumin-induced degradation of ErbB2: A role for the E3 ubiquitin ligase CHIP and the Michael reaction acceptor activity of curcumin

Yunjin Jung [a,*], Wanping Xu [b], Heejung Kim [a], Namchul Ha [a], Len Neckers [b]

[a] *The Research Institute for Drug Development, College of Pharmacy, Pusan National University, Busan, 609-735, Korea*
[b] *Urologic Oncology Branch, National Cancer Institute, Bethesda, MD 20894, USA*

Received 1 August 2006; received in revised form 2 November 2006; accepted 4 November 2006

Abstract

We investigated the molecular mechanism underlying curcumin depletion of ErbB2 protein. Curcumin induced ErbB2 ubiquitination but pretreatment with proteasome inhibitors neither prevented curcumin depletion of ErbB2 protein nor further accumulated ubiquitinated ErbB2. Curcumin increased association of endogenous and ectopically expressed CHIP, a chaperone-dependent ubiquitin ligase, with ErbB2. In COS7 cells cotransfected with ErbB2 and various CHIP plasmids followed by curcumin treatment, CHIP-H260Q (a mutant lacking ubiquitin ligase activity) promoted less curcumin-induced ErbB2 ubiquitination than did wild type CHIP, and CHIP-K30A (a mutant incapable of binding Hsp90 and Hsp70) neither associated with ErbB2 nor promoted its ubiquitination. ErbB2 mutants lacking the kinase domain failed to associate with CHIP and were completely resistant to ubiquitination and depletion induced by curcumin. Finally, curcumin's Michael reaction acceptor functionality was required for both covalent association of curcumin with ErbB2 and curcumin-mediated ErbB2 depletion. These data suggest (1) that CHIP-dependent ErbB2 ubiquitination is implicated in curcumin-stimulated ErbB2 depletion, and (2) that covalent modification of ErbB2 by curcumin is the proximal signal which initiates this process.

Keywords: Curcumin; ErbB2; Michael reaction; E3 ubiquitin ligase; Molecular chaperones

1. Introduction

ErbB2 (HER2/neu) is a 185 kDa transmembrane tyrosine kinase that acts as a coreceptor for the other epithelial growth factor receptor (EGFR) family members, including EGF receptor (ErbB1), ErbB3, and ErbB4. Although no ligand for ErbB2 has been identified, ErbB2 heterodimerization with other EGFR family members prolongs receptor signaling, activation of multiple downstream pathways, and oncogenesis [1].

High expression of ErbB2 is frequently observed in many types of human cancers including breast, ovarian, gastric, lung, bladder, and kidney carcinomas. A growing body of evidence from clinical as well as laboratory data has revealed that overexpression of ErbB2 increases metastatic potential and resistance to anti-cancer agents, and is associated with a poor prognosis [2]. For this reason, therapeutic strategies that down-regulate the level of ErbB2 protein and/or its activity, including ErbB2 antibody [3], tyrosine kinase inhibitors [4], heat shock protein (Hsp) 90 inhibitors such as geldanamycin (GA) [5], or the polyphenolic natural product curcumin [6], have been investigated as potential treatments for ErbB2-overexpressing cancers.

Recently it was reported that the chaperone-dependent E3 ubiquitin ligase, carboxyl terminus of Hsc70-interacting protein (CHIP), is involved in GA-induced ErbB2 degradation [7]. GA fosters CHIP association with ErbB2 and subsequently induces ubiquitination and degradation of the kinase via the proteasome. These observations suggest that pharmacologic enhancement of the CHIP–ErbB2 interaction may be therapeutically useful for down-regulating ErbB2 activity.

Curcumin (1,7-bis (4-hydroxy-3-methoxyphenyl)-1,6-hepta-diene-3,5-dione) is the major yellow pigment extracted from turmeric derived from the rhizome of the plant *Curcuma longa*. It is known to have numerous biological activities including anti-inflammatory, antioxidant, and anti-proliferative properties

* Corresponding author. Tel.: +82 51 510 2527; fax: +82 51 513 6754.
E-mail address: jungy@pusan.ac.kr (Y. Jung).

0167-4889/$ - see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbamcr.2006.11.004

[8]. Ample evidence exists to support its use in cancer prevention for its anti-proliferative and anti-carcinogenic properties [9]. Recently, it was reported that curcumin down-regulates cellular ErbB2 signaling in breast cancer cells by depleting ErbB2 protein [6]. However, little is known regarding the molecular mechanism underlying ErbB2 depletion by curcumin. In this study, we show that curcumin-induced ErbB2 depletion involves CHIP, requires the kinase domain of ErbB2 and the Michael acceptor function of curcumin, but surprisingly is independent of the proteasome.

2. Materials and methods

2.1. Cells, antibodies and plasmids

SKBr3 and COS7 cells were purchased from American Type Culture Collection. Immunoprecipitating anti-ErbB2 antibodies (Ab-2, 5), which were premixed before use, were from Oncogene Science Inc. (Cambridge, MA), and Western blotting anti-ErbB2 antibodies (Ab-3 [clone 3B5] for the intracellular domain and AB-3 [clone L87] for the extracellular domain) were from Oncogene Science Inc. and NeoMarkers (Fremont, CA), respectively. Rabbit anti-ubiquitin polyclonal antibody was from Sigma (St. Louis, MO). Rat anti-Hsp90 monoclonal antibody (SPA-835) was from Stressgen Biotech Corp. (Victoria, BC, Canada), and goat anti-Hsp/Hsc70 polyclonal antibody was from Santa Cruz Biotechnology (Santa Cruz, CA). Plasmid constructs (wild type and mutant ErbB2 plasmids and wild type and mutant CHIP plasmids) were prepared as previously described [7,10]. Rabbit anti-CHIP polyclonal antibody and purified CHIP protein were a kind gift of Dr. C. Patterson (University of North Carolina, Chapel Hill, NC).

2.2. Drugs and chemicals

Curcumin (1,7-bis (4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione) was purchased from LKT (St. Paul, MN). PS-341 and geldanamycin were obtained from Millenium Pharmaceuticals (Cambridge, MA) and the National Cancer Institute (Rockville, MD), respectively. ALLnL was purchased from Sigma (St. Louis, MO). Radiolabeled curcumin ([^{3}H], specific activity=20 Ci/mmol) was purchased from Moravek Biochemicals Inc. (Brea, CA). Tetrahydrocurcumin (THC) was prepared by Pd/C-reduction of curcumin dissolved in ethanol in a pressure reactor (50 psi hydrogen). Formation of THC was verified by NMR and IR.

2.3. Cell culture and transient transfection

SKBr3 cells were cultured in Dulbecco's modified Eagle's medium (Biofluids, Rockville, MD), supplemented with 10% fetal bovine serum (Invitrogen, Frederick, MD), 2 mM glutamine, 1 mM HEPES, and 100 units of penicillin and streptomycin (Biofluids). COS7 cell culture medium contained 90% Dulbecco's modified Eagle's medium, 10% fetal bovine serum, 2 mM glutamine, 1 mM HEPES and 1 mM sodium pyruvate. For transient transfections, each plasmid was premixed with FuGene 6 (Roche Molecular Biochemicals, South San Francisco, CA) and added to cells at 50–70% confluency. Cells were continually cultured in the same medium for 24 h until appropriate treatment.

2.4. 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^{TM} 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, 0.5 mg–1 mg of lysates was incubated with 2 μg of mouse monoclonal antibodies at 4 °C for 2 h, followed by the addition of protein G-agarose beads (Invitrogen) and rotation at 4 °C overnight. The beads were washed five times with TMNSV 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 4–20% gradient gel electrophoresis (BioRad). Western blotting was performed as described previously [11].

2.5. Measurement of [^{3}H]-curcumin bound to ErbB2

After a 1 h incubation of ^{3}H-labeled curcumin with immunoprecipitated ErbB2 in the presence or absence of appropriate chemicals, immunobeads were washed thoroughly with buffer (50 mM Tris–HCl (pH 7.5), 1% Nonidet P-40, 150 mM NaCl), resuspended in 1× SDS sample buffer, and boiled for 5 min. Immunoprecipitated ErbB2 was separated by 4–20% gradient gel electrophoresis and gels were stained with GelCode (Pierce, Rockford, IL) for 30 min–1 h. Stained bands were excised and incubated for 24 h in NCS tissue solubilizer (GE Healthcare, Piscataway, NJ) to extract ErbB2 protein. Eluates were mixed with BSC-NA scintillation cocktail (GE Healthcare) and radioactivity was measured using an LS 6500 liquid scintillation counting system (Beckman Coulter).

3. Results

3.1. Curcumin induces ErbB2 ubiquitination but not proteasome-mediated degradation

Curcumin is reported to down-regulate ErbB2 protein [6]. To investigate the molecular mechanism underlying curcumin-induced ErbB2 depletion, we first examined whether curcumin affected the stability of ErbB2 protein. ErbB2-overexpressing SKBr3 cells were treated with cycloheximide, an inhibitor of protein synthesis, in the presence or absence of curcumin, and the level of ErbB2 protein was monitored at the indicated times. As shown in Fig. 1A, in the absence of new protein synthesis curcumin markedly reduced the level of pre-existing ErbB2 protein.

The Hsp90 inhibitor GA destabilizes ErbB2 protein via ubiquitination and subsequent proteasomal degradation [11]. To examine whether a similar process was involved in curcumin-induced destabilization of ErbB2, we treated SKBr3 cells with curcumin in the presence or absence of the proteasome inhibitor PS-341. For comparison, cells were treated with GA. ErbB2 protein was immunoprecipitated and its ubiquitination status was examined by Western blotting. Consistent with a previous report [11], GA rapidly induced proteasome inhibitor-enhanced ErbB2 ubiquitination (Fig. 1B), and its proteasome-mediated degradation (Fig. 1C). Interestingly, although curcumin also induced ErbB2 ubiquitination, its onset was slower than that caused by GA and was not further enhanced by concomitant proteasome inhibition (Fig. 1B), nor could curcumin-induced depletion of ErbB2 be rescued by proteasome inhibition (Fig. 1C). Lysosomal inhibitors failed to protect ErbB2 from curcumin-induced degradation (data not shown).

3.2. CHIP participates in curcumin-induced ErbB2 ubiquitination

CHIP plays a role in both GA-enhanced and steady-state ubiquitination of ErbB2 [7,12]. We wondered whether CHIP served as a ubiquitin ligase in the case of curcumin-induced

Please cite this article as: Y. Jung, et al., Curcumin-induced degradation of ErbB2: A role for the E3 ubiquitin ligase CHIP and the Michael reaction acceptor activity of curcumin, Biochim. Biophys. Acta (2006), doi:10.1016/j.bbamcr.2006.11.004

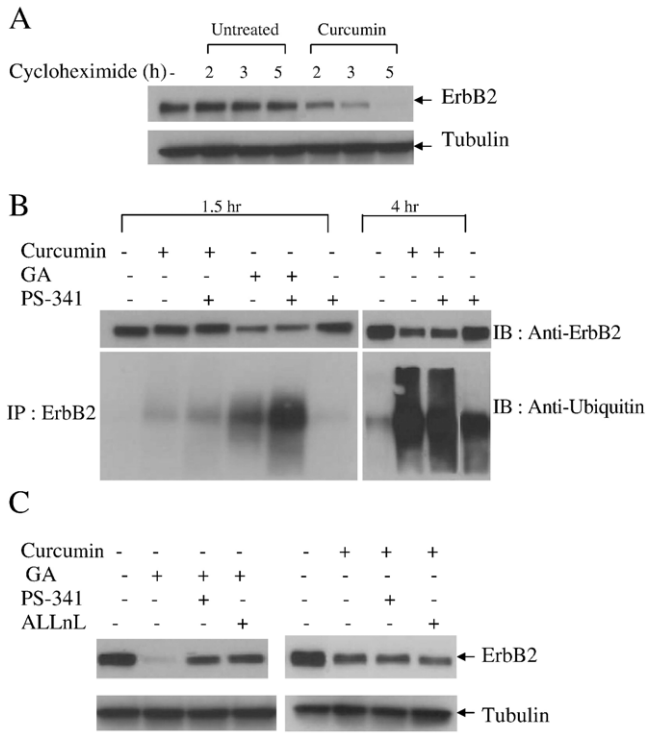

Fig. 1. Curcumin induces ubiquitination and degradation of ErbB2 in SKBr3 cells. (A) SKBr3 cells were either left untreated or were treated with curcumin (50 μM) for 1 h, followed by addition of cycloheximide (100 μM) for the indicated times. Cellular levels of ErbB2 protein were visualized from whole cell lysates (20 μg). The blot was reprobed for tubulin as a control for equal loading. (B) SKBr3 cells, either left untreated or treated with curcumin (50 μM) or GA (1 μM) for the indicated times in the presence or absence of the proteasome inhibitor PS-341 (1 μM), were lysed in TMNSV buffer. ErbB2 proteins were immunoprecipitated as described in Materials and methods, solubilized in SDS sample buffer, and separated by SDS-PAGE. Blots were probed with anti-ErbB2 or anti-ubiquitin antibodies. (C) SKBr3 cells were treated with curcumin or GA in the presence or absence of either PS-341 or ALLnL (100 μM) for 4 h. Cellular levels of ErbB2 protein were visualized as above. In the experiments shown in (B) and (C) cells were pre-treated with PS-341 for 30 min, and the inhibitor was then present continuously for the remainder of the experiment.

ubiquitination of ErbB2. First, we investigated whether curcumin induced endogenous CHIP/ErbB2 association in SKBr3 cells. Cells were treated with curcumin for 1.5 h, ErbB2 protein was immunoprecipitated, and coimmunoprecipitated CHIP was examined by Western blotting. As shown in Fig. 2A, CHIP was barely detectable in the ErbB2 immunocomplex isolated from untreated cells, but curcumin treatment markedly increased CHIP coimmunoprecipitation. We next examined whether curcumin could enhance association of CHIP with ErbB2 protein *in vitro*. After immunoprecipitating ErbB2 from SKBr3 lysate, we treated the immunopellets with curcumin, and then washed and incubated the immunopellets in lysis buffer containing purified CHIP protein. After washing, we detected CHIP and ErbB2 in the immunopellets by Western blotting. As shown in Fig. 2B, CHIP level in the curcumin-treated immunopellet was markedly increased.

Since curcumin enhanced association of CHIP with ErbB2, we determined whether CHIP could act as a ubiquitin ligase mediating curcumin-induced ErbB2 ubiquitination. To do this, we compared the level of curcumin-induced ubiquitination of ErbB2 in the presence of wild type (CHIP-WT) or E3 ligase-deficient (CHIP-H260Q) CHIP protein. COS7 cells were transfected with ErbB2 and either CHIP-WT or CHIP-H260Q, and 24 h later the transfected cells were treated with curcumin for an additional 1.5 h. ErbB2 proteins were immunoprecipitated, and CHIP and ubiquitinated ErbB2 in the immunopellets were examined by Western blotting. As shown in Fig. 2C, curcumin induced both CHIP-WT association with and ubiquitination of ErbB2. That the curcumin-induced ErbB2 ubiquitination was CHIP-dependent is suggested by the finding that curcumin-induced ErbB2 ubiquitination was significantly reduced in COS7 cells transfected with ErbB2/CHIP-H260Q although, as previously reported [7,12], association of this inactive CHIP mutant with ErbB2 was much greater than that seen with CHIP-WT. If CHIP-dependent ErbB2 ubiquitination is related to curcumin-induced ErbB2 depletion, expression of CHIP-H260Q should at least partially protect ErbB2 from the effects of curcumin treatment. This is indeed what we observed (see Fig. 2D). When COS7 cells were transfected with CHIP-WT, ErbB2 depletion following curcumin (4 h exposure) was more pronounced than in cells not transfected with CHIP. Indeed, in the presence of over-expressed CHIP-WT protein, ErbB2 steady-state expression was reduced even in the absence of curcumin. However, in COS7 cells expressing ubiquitin ligase-deficient CHIP-H260Q protein, ErbB2 was partially protected from the effects of curcumin.

3.3. Involvement of molecular chaperones in curcumin-induced ErbB2 ubiquitination

Since CHIP is thought to depend on the molecular chaperones Hsp70 and/or Hsp90 to bring it into contact with its substrates [13], we examined whether a chaperone complex was required for CHIP-mediated ErbB2 ubiquitination induced by curcumin. ErbB2 and CHIP-WT or CHIP-K30A (a mutant incapable of binding to both Hsp90 and Hsp70) were transfected into COS7 cells and 24 h later the cells were treated with curcumin for an additional 1.5 h. ErbB2 proteins were immunoprecipitated and CHIP and ubiquitinated ErbB2 were examined by Western blotting. Consistent with our earlier data, curcumin enhanced CHIP-WT binding to and ubiquitination of ErbB2 (Fig. 3A; also see Fig. 2C). In contrast, co-transfection of CHIP-K30A prevented curcumin-induced ErbB2 ubiquitination (Fig. 3A). However, unlike the enzymatically inactive CHIP-H260Q, CHIP-K30A failed to associate with ErbB2, indicating that binding to a chaperone intermediate may be required for CHIP-dependent ErbB2 ubiquitination induced by curcumin. Alternatively, the data in Fig. 2B suggest that CHIP may interact directly with curcumin-modified ErbB2 (see also Fig. 5). Thus, we cannot rule out the possibility that the TPR domain mutation of CHIP-K30A, which prevents its interaction with Hsp90 and Hsp70, may also abrogate its ability to interact directly with ErbB2.

GA treatment remodels the ErbB2–chaperone complex, reducing association of Hsp90 while increasing the association of Hsp/Hsc70 [7]. We wondered whether a similar remodeling of the ErbB2–chaperone complex occurred following curcu-

Please cite this article as: Y. Jung, et al., Curcumin-induced degradation of ErbB2: A role for the E3 ubiquitin ligase CHIP and the Michael reaction acceptor activity of curcumin, Biochim. Biophys. Acta (2006), doi:10.1016/j.bbamcr.2006.11.004

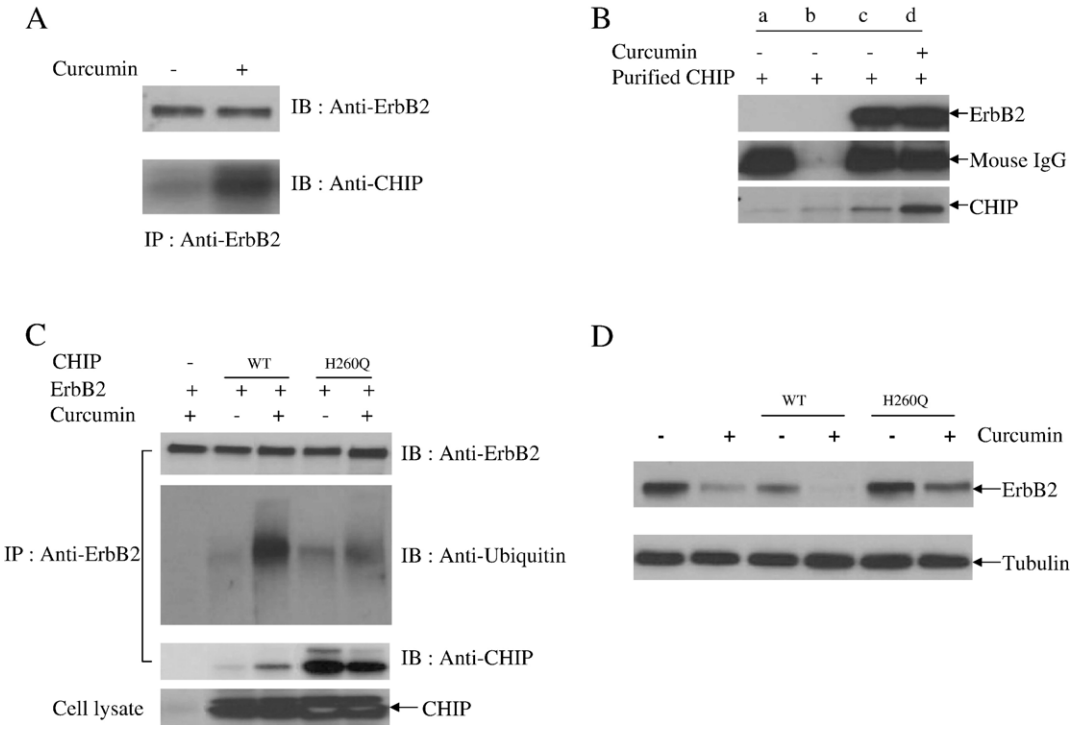

Fig. 2. CHIP participates in curcumin-induced ubiquitination of ErbB2. (A) SKBr3 cells, either left untreated or treated with curcumin for 1.5 h, were lysed in TMNSV buffer. ErbB2 protein in 0.5 mg cell lysate was immunoprecipitated (*IP*) using as described under Materials and methods. Immunoprecipitated proteins were solubilized in SDS sample buffer and separated by 4–20% SDS-PAGE. Blots were probed with anti-ErbB2 (for immunoprecipitation efficiency) or anti-CHIP antibodies. *IB*, immunoblot. (B) SKBr3 cells were lysed in TMNSV buffer. ErbB2 proteins were immunoprecipitated and the immunopellets were treated (d) or left untreated (c) with curcumin (50 μM) for 1 h, and then were washed and incubated in lysis buffer containing purified CHIP protein (100 ng/ml) for 1 h. The immunopellets were solubilized in SDS sample buffer and separated by 4–20% SDS-PAGE. Blots were probed with appropriate antibodies. Lanes a (beads+ErbB2 antibodies+CHIP) and b (beads+cell lysate+CHIP) represent controls. (C) COS7 cells were transfected with ErbB2 together with either wild type CHIP (CHIP-WT) or CHIP-H260Q (a U box mutant lacking ubiquitin ligase activity). After 24 h, cells were treated with or without curcumin for 1.5 h. ErbB2 proteins were immunoprecipitated (*IP*), solubilized in SDS sample buffer and separated by SDS-PAGE. Blots were probed with appropriate antibodies. (D) COS7 cells were transiently transfected with either ErbB2 alone or together with CHIP-WT or ubiquitin ligase-deficient CHIP-H260Q. The following day, cells were treated or not with curcumin for 4 h. ErbB2 levels were monitored by Western blot. Tubulin is shown as a loading control.

min. SKBr3 cells were treated with curcumin for 1.5 h and ErbB2 protein was immunoprecipitated. Coimmunoprecipitation of Hsp90 and Hsp/Hsc70 was examined by Western blotting. As shown in Fig. 3B, unlike GA curcumin recruited both Hsp90 and Hsp/Hsc70 to an ErbB2 immunocomplex. We suspected that Hsp90 could be recruited to curcumin-modified ErbB2 in a manner distinct from its normal association with the kinase. To investigate this possibility, we utilized ErbB2-5M in which the Hsp90 binding loop of ErbB2 has been replaced with the same loop in EGFR (to which Hsp90 does not bind) [10]. ErbB2-5M in addition to not binding Hsp90 is quite resistant to the effects of GA [10,14]. COS7 cells were cotransfected with CHIP-WT and either ErbB2-WT or ErbB2-5M, as previously described, and the cells were treated with curcumin for 1.5 h. ErbB2 proteins were immunoprecipitated and co-immunoprecipitated Hsp90, Hsp/Hsc70, and CHIP were examined by Western blotting. As shown in Fig. 3C, curcumin recruited a large amount of Hsp90 to ErbB2-WT but not to ErbB2-5M. These data demonstrate that Hsp90 associated with ErbB2 in the presence of curcumin bound to the same region of the kinase as does Hsp90 under steady-state conditions. In contrast, the amount of Hsp/Hsc70 recruited to ErbB2 by curcumin was modest but was not affected by ErbB2 mutation.

Since ErbB2-WT coprecipitated with CHIP to a greater extent than did ErbB2-5M upon curcumin treatment, and because curcumin induced a greater ubiquitination of ErbB2-WT compared to ErbB2-5M, we examined whether Hsp90 recruitment was necessary for curcumin-induced ErbB2 depletion. To do this, we compared the curcumin sensitivity of ErbB2-WT and ErbB2-5M in the presence of CHIP-WT. As shown in Fig. 3D, ErbB2-5M was depleted somewhat more slowly than was ErbB2-WT upon curcumin treatment, suggesting that although Hsp90 might provide a positive contribution to CHIP-dependent ErbB2 depletion, its presence is not absolutely necessary. Whether the small amount of Hsp/Hsc70 recruited by curcumin to ErbB2-5M mediates ErbB2 depletion in the absence of Hsp90, or whether modification of ErbB2 by curcumin (see below) recruits CHIP directly to the kinase remains to be conclusively determined.

3.4. ErbB2 kinase domain is required for curcumin-induced, CHIP-dependent ubiquitination and degradation

The ErbB2 kinase domain is required for GA-induced and CHIP-dependent ErbB2 ubiquitination and degradation [7]. We examined whether curcumin required a similar motif to induce

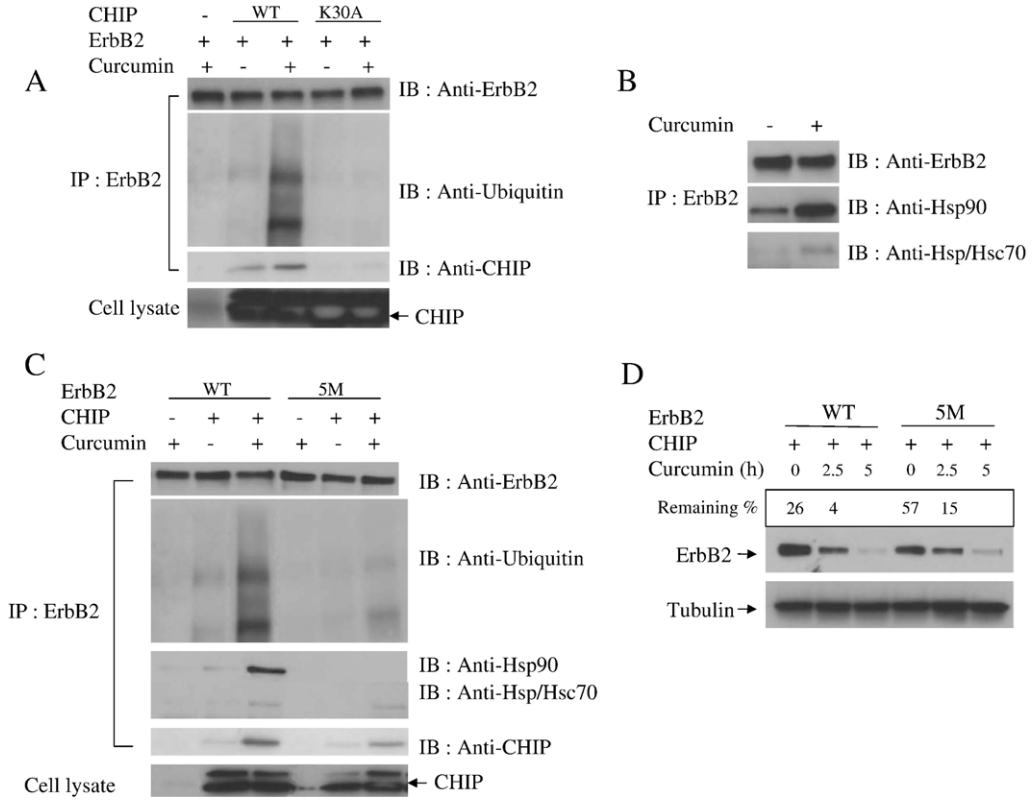

Fig. 3. Curcumin recruited-Hsp90 positively regulates CHIP-dependent ErbB2 ubiquitination. (A) SKBr3 cells, either left untreated or treated with curcumin for 1.5 h, were lysed in TMNSV buffer. ErbB2 protein in 0.5 mg cell lysate was immunoprecipitated (*IP*), solubilized in SDS sample buffer, and separated by 4–20% SDS-PAGE. Blots were probed with anti-ErbB2 (for immunoprecipitation efficiency), anti-Hsp90 or anti-Hsp/Hsc70 antibody. *IB*, immunoblot. (B) COS7 cells were transfected with ErbB2 together with either CHIP-WT or CHIP-K30A (a CHIP mutant incapable binding to Hsp90 or Hsp70). After 24 h, cells were treated with or without curcumin for 1.5 h. 1 mg of cell lysate was immunoprecipitated (IP) using anti-ErbB2 antibodies, solubilized in SDS sample buffer, and separated by SDS-PAGE. Blots were probed with anti-ErbB2, anti-ubiquitin or anti-CHIP antibody. (C) COS7 cells were transfected with ErbB2 (WT) or ErbB2-5M (5 M, an ErbB2 mutant incapable of binding to Hsp90) together with CHIP for 24 h, followed by treatment with or without curcumin for 1.5 h. Immunoprecipitation and immunoblotting were performed as in "B". Blots were probed with anti-ErbB2 (for immunoprecipitation efficiency), anti-CHIP, anti-ubiquitin, anti-Hsp90 or anti-Hsp/Hsc70 antibody. (D) COS7 cells were cotransfected with CHIP and either ErbB2 (WT) or ErbB2-5M (5 M) for 24 h, followed by treatment with or without curcumin for the indicated times. Cell lysates (40 μg) were mixed with 5× SDS sample buffer and separated by 4–20% SDS-PAGE. Blots were probed with anti-ErbB2 antibody. Percent remaining was determined by densitometric analysis of the blots.

ErbB2 ubiquitination and depletion. COS7 cells were cotransfected with CHIP-WT and various ErbB2 truncation constructs as shown in Fig. 4A. After 24 h, cells were treated with curcumin for 3 h and ErbB2 proteins were immunoprecipitated as described above. Ubiquitinated ErbB2 and CHIP in the immunocomplex were examined by Western blotting. As shown in Fig. 4B, curcumin induced CHIP association with and ubiquitination of only full-length ErbB2 and ErbB2-DHC (deletion of 224 amino acids C-terminal to the kinase domain), but not of ErbB2/Δ750–971 (an ErbB2 construct with an internal deletion of the kinase domain) or ErbB2/DK (deletion of most of the intracellular domain). We next examined whether curcumin-induced ErbB2 ubiquitination via CHIP association correlated with curcumin sensitivity of the different ErbB2 constructs. COS7 cells were cotransfected with CHIP-WT and the various ErbB2 constructs as described and were treated with curcumin for 5 h. ErbB2 protein was monitored by Western blotting. As shown in Fig. 4C, while full-length ErbB2 and ErbB2-DHC (including the kinase domain) were depleted by curcumin, ErbB2-DK and ErbB2/Δ750–971 proteins (not including the kinase domain) were resistant.

3.5. The Michael reaction acceptor functionality of curcumin is required for ErbB2 depletion

Since we had reason to believe that curcumin may directly modify ErbB2, we explored whether curcumin's Michael reaction acceptor functionality was necessary. Curcumin contains two Michael reaction (nucleophile) acceptors. We reduced curcumin using Pd/C under 50 psi hydrogen to obtain tetrahydrocurcumin, which lacks Michael reaction acceptor activity, and we compared the ability of tetrahydrocurcumin with that of curcumin to deplete ErBb2 in SKBr3 cells. For comparison, the structures of curcumin and tetrahydrocurcumin are shown in Fig. 5A. Tetrahydrocurcumin did not deplete ErbB2 at concentrations up to 100 μM, indicating that a Michael reaction acceptor in curcumin is required for its ability to promote ErbB2 depletion. We wondered whether the effect of this chemical functionality on ErbB2 depletion was general. To examine this question, we treated SKBr3 cells with several structurally distinct compounds which have Michael reaction acceptor functionality (Fig. 5B) and we monitored their effect on ErbB2 level.

Please cite this article as: Y. Jung, et al., Curcumin-induced degradation of ErbB2: A role for the E3 ubiquitin ligase CHIP and the Michael reaction acceptor activity of curcumin, Biochim. Biophys. Acta (2006), doi:10.1016/j.bbamcr.2006.11.004

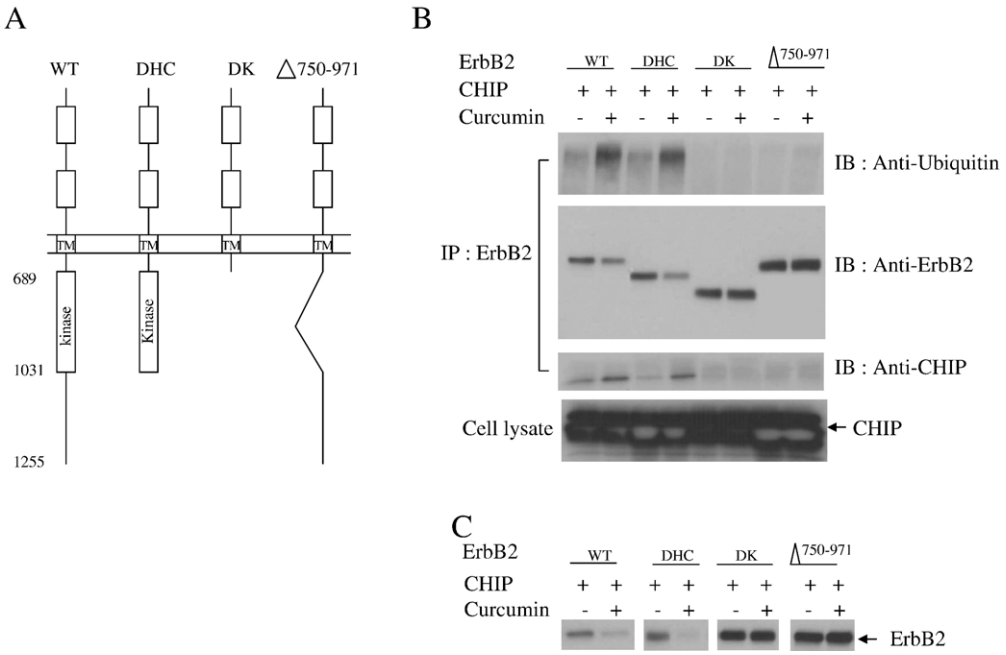

Fig. 4. ErbB2 kinase domain is required for curcumin-induced CHIP-dependent ubiquitination of ErbB2. (A) Schematic description of ErbB2 constructs used in the experiments. (B) COS7 cells in 10 cm dishes were transfected with CHIP, plus full-length, truncated or internal-deleted ErbB2 constructs. Twenty-four hours after transfection, cells, either left untreated or treated with curcumin for 3 h, were lysed with TMNSV buffer. For immunoprecipitation (*IP*), 1 mg of cell lysate was incubated with anti-ErbB2 antibodies, and the immunocomplex was precipitated with protein-G-agarose beads. Immunoprecipitated proteins were solubilized in SDS sample buffer and separated by SDS-PAGE. Blots were probed with anti-ErbB2 (AB-3, Neomarkers), anti-ubiquitin or anti-CHIP antibody. (C) Cell lysates (40 μg) obtained from the transfected COS7 cells treated with or without curcumin for 5 h were mixed with 5× SDS sample buffer and separated by 4–20% SDS-PAGE. Blots were probed with anti-ErbB2 antibody (AB-3, Neomarkers).

All compounds were able to deplete ErbB2 to some degree (Fig. 5B).

Since covalent modification of a protein involves reaction between nucleophile(s) on the protein and nucleophile acceptors [15], we speculated that curcumin may exert its effects on ErbB2, at least in part, by covalent association with the kinase. To test this hypothesis, we incubated [^{3}H] curcumin with ErbB2 that had been immunoprecipitated from SKBr3 cells. After 1 h incubation with labeled curcumin, the immunoprecipitated ErbB2 was separated by SDS-PAGE and the gel was stained. Bands corresponding to ErbB2 were excised and extracted for 24 h followed by measurement of radioactivity, as described in Methods. A control gel slice was prepared by cutting out an amount of gel matching the ErbB2 band (at approximately the same location) obtained after electrophoresis of a sample prepared from incubating [^{3}H] curcumin with beads obtained from the immunoprecipitation process without anti-ErbB2 antibodies. To test whether Michael reaction acceptor functionality was required for covalent association, we performed the same experiment after pre-treating the immunoprecipitated ErbB2 with either excess non-radioactive curcumin (50 μM) or tetrahydrocurcumin (100 μM). As shown in Fig. 5C, while incubation of ErbB2 with labeled curcumin increased ErbB2-associated radioactivity more than 3-fold, pretreatment with unlabeled curcumin, but not with tetrahydrocurcumin, markedly reduced the ErbB2-associated radioactivity. These data indicate that curcumin covalently binds to ErbB2 through a Michael reaction acceptor functionality.

Finally, we wished to explore the relevance of covalent association of curcumin with ErbB2 to curcumin's ability to deplete ErbB2. Since the kinase domain of ErbB2 was required for curcumin depletion, we examined whether curcumin bound to the kinase domain. [^{3}H] curcumin was incubated with either ErbB2-WT or ErbB2/Δ750–971 immunoprecipitated from COS7 cells. The radioactivity associated with ErbB2 was determined as described above. As shown in Fig. 5D, while incubation of ErbB2 with labeled curcumin resulted in an approximately 2-fold increase in signal compared to control, incubation of ErbB2/Δ750–971 with labeled curcumin did not produce a radioactive signal above background, supporting the hypothesis that the kinase domain of ErbB2 contains the binding site for curcumin. In contrast, using [^{3}H] 17AAG we could detect no covalent association with either WT or mutant ErbB2 proteins (data not shown).

4. Discussion

Our data demonstrate that CHIP mediates curcumin-induced ubiquitination of ErbB2 and that the kinase domain of ErbB2 is required for curcumin-induced ErbB2 ubiquitination and degradation. Moreover, curcumin's Michael reaction acceptor functionality appears to be the pharmacophore responsible for curcumin's ability to promote ErbB2 degradation.

Although curcumin induced ErbB2 ubiquitination, we found that ubiquitinated ErbB2 did not further increase in the presence of proteasome inhibitors. Furthermore, consistent with a

Please cite this article as: Y. Jung, et al., Curcumin-induced degradation of ErbB2: A role for the E3 ubiquitin ligase CHIP and the Michael reaction acceptor activity of curcumin, Biochim. Biophys. Acta (2006), doi:10.1016/j.bbamcr.2006.11.004

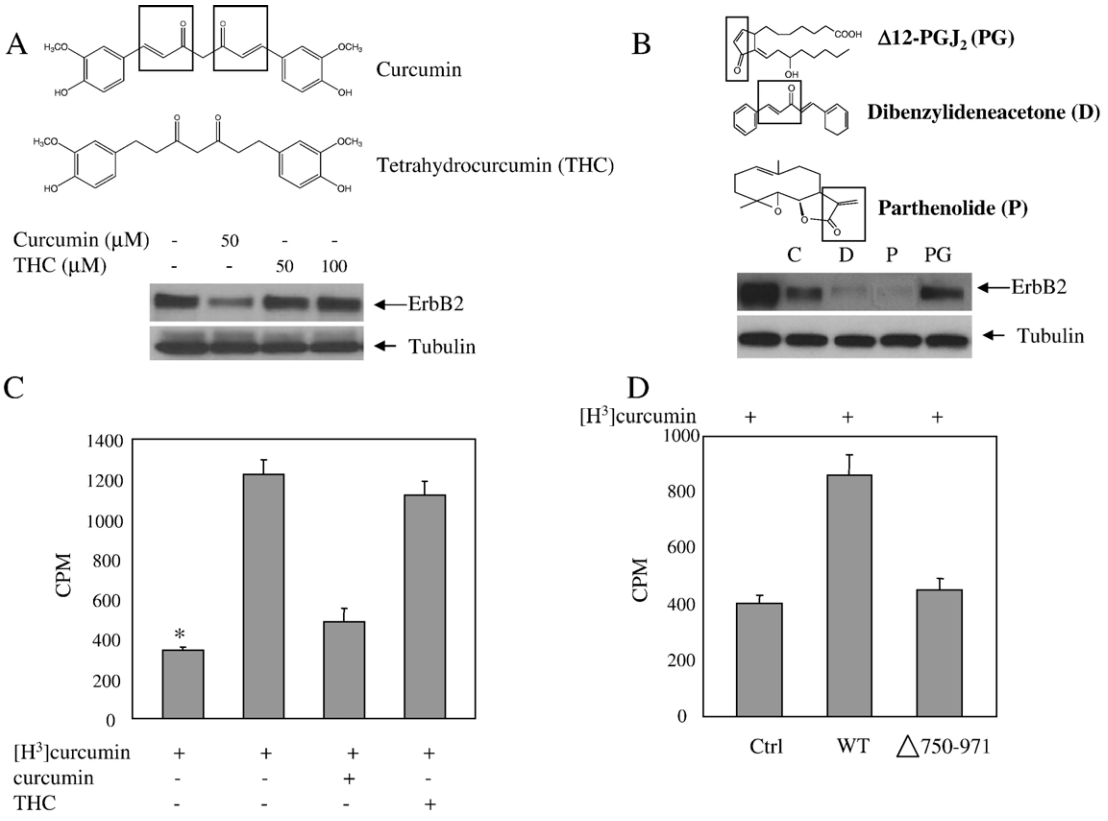

Fig. 5. The Michael reaction acceptor functionality of curcumin is required for curcumin-mediated ErbB2 depletion. (A) Upper panel, chemical structures of tetrahydrocurcumin (THC) and curcumin; the functional group in the boxes indicates Michael reaction acceptor. Lower panel, SKBr3 cells were treated with THC (50, 100 μM) or curcumin (50 μM) for 4 h and lysed to obtain whole cell lysates. Western blotting was performed to monitor ErbB2 levels. The membrane was reprobed with tubulin to verify equivalent loading. (B) Upper panel, chemical structures of ρ12-PGJ$_2$ (PG), Dibenzylideneacetone (D), and Parthenolide (P); the functional group in the boxes indicates Michael reaction acceptor. Lower panel, SKBr3 cells were treated with curcumin (C, 50 μM), PG (50 μM), D (20 μM) or P (20 μM) for 4 h and lysed to obtain whole cell lysates. Western blotting was performed to monitor ErbB2 levels. The membrane was reprobed with tubulin to verify equivalent loading. (C) After a 1-h incubation of ^{3}H-labeled curcumin with ErbB2 immunoprecipitated from SKBr3 cells, immunobeads were washed thoroughly with buffer (50 mM Tris–HCl, pH 7.5, 1% Nonidet P-40, 150 mM NaCl), resuspended in 1× SDS sample buffer and boiled for 5 min. Immunoprecipitated ErbB2 was separated by 4–20% SDS-PAGE and the gel was stained for 30 min. The stained band was cut out and incubated (24 h) in NCS tissue solubilizer to extract ErbB2 protein. The eluates were mixed with BSC-NA scintillation cocktail and radioactivity in the eluates was measured using a liquid scintillation counter. *: control (see text). The same experiment was done after 1 h pretreatment with excess unlabeled THC or curcumin. (D) The same experiment as in C was performed using either WT ErbB2 or kinase domain-deleted ErbB2 (Δ750–971) that had been immunoprecipitated from COS7 cells. Ctrl: control (prepared as in C).

previous report [16], proteasome inhibitors did not prevent curcumin-induced ErbB2 depletion. These data suggest that ubiquitinated ErbB2 accumulating in response to curcumin is not degraded via a proteasomal pathway. A recent report demonstrating that curcumin inhibits proteasomal activity by up to 90% [17] supplies a possible explanation for this phenomenon. The proteolytic pathway mediating curcumin-induced ErbB2 depletion remains under investigation. Recognition of ubiquitinated substrates by non-proteasomal proteolytic pathways has been described [18–20]. Lysosomal inhibitors failed to protect ErbB2 from curcumin-induced degradation (data not shown).

Participation of the ubiquitin ligase CHIP in curcumin-induced ErbB2 ubiquitination is supported by our data showing that (1) curcumin enhanced endogenous CHIP association with ErbB2 in SKBr3 cells, and in COS-7 cells transiently co-transfected with ErbB2 and CHIP plasmids; (2) curcumin increased association of CHIP with immunoprecipitated ErbB2 *in vitro*; (3) curcumin induced less ErbB2 ubiquitination in COS7 cells transiently transfected with ubiquitin ligase-deficient CHIP-H260Q; (4) transfection with CHIP-H260Q partially protected ErbB2 from curcumin-induced depletion; (5) CHIP-K30A, a mutant incapable of binding to both Hsp90 and Hsp70, did not support curcumin-induced ErbB2 ubiquitination.

In contrast to an earlier observation that the Hsp90 inhibitor GA promotes CHIP-dependent ErbB2 ubiquitination concomitant with Hsp90 dissociation from the kinase [7], our data demonstrate that curcumin-induced CHIP association with ErbB2 and subsequent ErbB2 ubiquitination occur together with enhanced recruitment of Hsp90 to ErbB2. Thus, curcumin-induced CHIP association with and subsequent ubiquitination of ErbB2 were markedly reduced in COS7 cells transiently transfected with CHIP-WT and ErbB2-5M, an ErbB2 mutant that does not bind Hsp90 [10]. Nonetheless, in agreement with an earlier report [14], ErbB2-5M remains sensitive to curcumin, suggesting that Hsp90 recruitment to ErbB2 may enhance but is not essential for curcumin-induced degradation. However, our data demonstrate that truncated or internally deleted ErbB2

Please cite this article as: Y. Jung, et al., Curcumin-induced degradation of ErbB2: A role for the E3 ubiquitin ligase CHIP and the Michael reaction acceptor activity of curcumin, Biochim. Biophys. Acta (2006), doi:10.1016/j.bbamcr.2006.11.004

mutants lacking a kinase domain fail to associate with CHIP and are neither ubiquitinated nor degraded in response to curcumin.

Lastly, our results demonstrate that the Michael reaction acceptor functionality of curcumin is required both for ErbB2 depletion and for covalent association of curcumin with ErbB2. This is in contrast to the Hsp90 inhibitor 17AAG, for which no evidence exists of direct modification of ErbB2. We found that the ErbB2 kinase domain, required for ErbB2 sensitivity to curcumin, is a potential site for covalent association of curcumin. Since thiols are more nucleophilic than amines in an aqueous biological environment, we think it most likely that thiols (especially ones surrounded by acidic or basic amino acids) are the predominant targets of curcumin-mediated covalent modification. This prediction is currently being tested. Since structurally different compounds with the same Michael reaction acceptor functionality were also able to deplete ErbB2, our data suggest that one might be able to design an effective ErbB2 targeting drug by chemical modification(s) of a lead compound to modulate its Michael reaction acceptor activity and affinity for ErbB2. Considering its relative safety, curcumin could be used as such a lead compound.

Acknowledgements

This work was supported in part by Korea Research Foundation Grant KRF-2004-002-E00199 awarded to Y. J.

References

[1] M.A. Olayioye, R.M. Neve, H.A. Lane, N.E. Hynes, The ErbB signaling network: receptor heterodimerization in development and cancer, EMBO J. 19 (2000) 3159–3167.
[2] N.G. Anderson, T. Ahmad, ErbB receptor tyrosine kinase inhibitors as therapeutic agents, Front. Biosci. 7 (2002) d1926–d1940.
[3] J. Baselga, J. Albanell, M.A. Molina, J. Arribas, Mechanism of action of trastuzumab and scientific update, Semin. Oncol. 28 (2001) 4–11.
[4] D.W. Fry, Mechanism of action of erbB tyrosine kinase inhibitors, Exp. Cell Res. 284 (2003) 131–139.
[5] L. Neckers, Hsp90 inhibitors as novel cancer chemotherapeutic agents, Trends Mol. Med. 8 (2002) S55–S61.
[6] R.L. Hong, W.H. Spohn, M.C. Hung, Curcumin inhibits tyrosine kinase activity of p185neu and also depletes p185neu, Clin. Cancer Res. 5 (1999) 1884–1891.
[7] W. Xu, M. Marcu, X. Yuan, E. Mimnaugh, C. Patterson, L. Neckers, Chaperone-dependent E3 ubiquitin ligase CHIP mediates a degradative pathway for c-ErbB2/Neu, Proc. Natl. Acad. Sci. U. S. A. 99 (2002) 12847–12852.
[8] B. Joe, M. Vijaykumar, B.R. Lokesh, Biological properties of curcumin-cellular and molecular mechanisms of action, Crit. Rev. Food Sci. Nutr. 44 (2004) 97–111.
[9] B.B. Aggarwal, A. Kumar, A.C. Bharti, Anticancer potential of curcumin: preclinical and clinical studies, Anticancer Res. 23 (2003) 363–398.
[10] W. Xu, X. Yuan, Z. Xiang, E. Mimnaugh, M. Marcu, L. Neckers, Surface charge and hydrophobicity determine ErbB2 binding to the Hsp90 chaperone complex, Nat. Struct. Mol. Biol. 12 (2005) 120–126.
[11] W. Xu, E. Mimnaugh, M.F. Rosser, C. Nicchitta, M. Marcu, Y. Yarden, L. Neckers, Sensitivity of mature Erbb2 to geldanamycin is conferred by its kinase domain and is mediated by the chaperone protein Hsp90, J. Biol. Chem. 276 (2001) 3702–3708.
[12] P. Zhou, N. Fernandes, I.L. Dodge, A.L. Reddi, N. Rao, H. Safran, T.A. DiPetrillo, D.E. Wazer, V. Band, H. Band, ErbB2 degradation mediated by the co-chaperone protein CHIP, J. Biol. Chem. 278 (2003) 13829–13837.
[13] H. McDonough, C. Patterson, CHIP: a link between the chaperone and proteasome systems, Cell Stress Chaperones 8 (2003) 303–308.
[14] O. Tikhomirov, G. Carpenter, Identification of ErbB-2 kinase domain motifs required for geldanamycin-induced degradation, Cancer Res. 63 (2003) 39–43.
[15] R. Uy, F. Wold, Posttranslational covalent modification of proteins, Science 198 (1977) 890–896.
[16] O. Tikhomirov, G. Carpenter, Caspase-dependent cleavage of ErbB-2 by geldanamycin and staurosporin, J. Biol. Chem. 276 (2001) 33675–33680.
[17] N.R. Jana, P. Dikshit, A. Goswami, N. Nukina, Inhibition of proteasomal function by curcumin induces apoptosis through mitochondrial pathway, J. Biol. Chem. 279 (2004) 11680–11685.
[18] W. Liao, L. Chan, Tunicamycin induces ubiquitination and degradation of apolipoprotein B in HepG2 cells. Biochem. J. 353 (2001) 493–501.
[19] A. d'Azzo, A. Bongiovanni, T. Nastasi, E3 ubiquitin ligases as regulators of membrane protein trafficking and degradation. Traffic 6 (2005) 429–441.
[20] S. Urbe, Ubiquitin and endocytic protein sorting, Essays Biochem. 41 (2005) 81–98.

Please cite this article as: Y. Jung, et al., Curcumin-induced degradation of ErbB2: A role for the E3 ubiquitin ligase CHIP and the Michael reaction acceptor activity of curcumin, Biochim. Biophys. Acta (2006), doi:10.1016/j.bbamcr.2006.11.004