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Regulation of Proto-Oncogenic Dbl by Chaperone-Controlled, Ubiquitin-Mediated Degradation▽

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The *dbl* proto-oncogene product is a prototype of a growing family of guanine nucleotide exchange factors (GEFs) that stimulate the activation of small GTP-binding proteins from the Rho family. Mutations that result in the loss of proto-Dbl's amino terminus produce a variant with constitutive GEF activity and high oncogenic potential. Here, we show that proto-Dbl is a short-lived protein that is kept at low levels in cells by efficient ubiquitination and degradation. The cellular fate of proto-Dbl is regulated by interactions with the chaperones Hsc70 and Hsp90 and the protein-ubiquitin ligase CHIP, and these interactions are mediated by the spectrin domain of proto-Dbl. We show that CHIP is the E3 ligase responsible for ubiquitination and proteasomal degradation of proto-Dbl, while Hsp90 functions to stabilize the protein. Onco-Dbl, lacking the spectrin homology domain, cannot bind these regulators and therefore accumulates in cells at high levels, leading to persistent stimulation of its downstream signaling pathways.

The *dbl* oncogene product was the first identified mammalian guanine nucleotide exchange factor (GEF) for Rho family GTPases and serves as a prototype for a growing family of proteins comprising more than 70 members in humans (16, 56, 57). Dbl family members are defined by the presence of a Dbl homology (DH on RhoGEF) (28) domain adjacent to a Pleckstrin homology (PH) domain. Physiologically, Dbl-like proteins activate members of the Rho family, such as Cdc42, Rac1, and RhoA. These GTP-binding proteins function as binary switches that cycle between an inactive (GDP-bound) and an active (GTP-bound) conformation to control diverse signaling pathways (33). Guanine nucleotide exchange factors facilitate the exchange of GDP for GTP, thus stimulating the formation of the activated form of Rho GTPases, which then activate downstream effectors.

Disruptions in the integrity of Dbl-like genes are intimately associated with cancer and malignancy (9, 67, 74). Thus, many Dbl family members were originally identified as potent fibroblast-transforming genes (18, 21, 37, 46, 66), while others were found mutated in a variety of human pathologies (23, 31, 32, 41, 50). In most cases, elevated oncogenic activity was associated with mutations in a sequence adjacent to the DH/PH region, leading to deregulated GEF activity and persistent stimulation of the GTPase substrate. Oncogenic activation of the *dbl* proto-oncogene involves the loss of the amino-terminal half of the protein, giving rise to a constitutively active GEF with potent transforming potential (27). Overexpression of the *dbl* proto-oncogene can also lead to an increase in the gene's transforming activity but to a smaller extent than observed with the oncogenic variant (55). At present, the molecular mechanisms that underlie the high oncogenic activity of mutated GEFs from the Dbl family are not fully understood.

We recently demonstrated that the molecular chaperone Hsc70 selectively interacts with the full-length, proto-oncogenic form of Dbl protein (proto-Dbl), that Hsc70 negatively regulates the GEF activity of proto-Dbl, and that the interaction with Hsc70 is dramatically diminished in the case of the truncated, oncogenic form of Dbl (onco-Dbl) (38). In addition to their well-established activity in protein folding, Hsc70 and the related chaperone Hsp90 regulate the expression level of their client proteins by controlling the rate of their ubiquitination and proteasomal degradation (13, 44). Of particular importance in this pathway is the cochaperone CHIP (carboxyl terminus of Hsc70-interacting protein), an E3 ubiquitin protein ligase that binds to the chaperones Hsc70 and Hsp90 and attaches polyubiquitin chains to client proteins, thus targeting them to degradation by the proteasome (14). By mediating the "triage decision" between stabilization and degradation, Hsc70/Hsp90/CHIP regulates the activities of its client proteins and, in turn, controls fundamental aspects of cell growth and proliferation (13). In this capacity, chaperone complexes function as master regulators of several important oncogenic pathways, where they were shown to control steady-state levels of critical signaling molecules, such as ErbB2/HER-2 (69), Raf (58, 59), Akt (3, 4), p53 (17), and steroid hormone receptors (8, 61).

We report here that, in addition to binding Hsc70, proto-Dbl associates with Hsp90 and the ubiquitin ligase CHIP. We further show that these chaperones function to regulate the ubiquitination, the degradation rate, and the signaling activity of proto-Dbl. Importantly, we show that that the oncogenic variant of Dbl is not subject to this mode of regulation. Consequently, onco-Dbl "escapes" degradation and accumulates in cells at high levels, causing persistent activation of its substrate GTPases and cell transformation.

MATERIALS AND METHODS

Molecular constructs. The proto-Dbl (residues 1 to 925), onco-Dbl (residues 498 to 925), DH-PH-Dbl (residues 498 to 825), N-Dbl (residues 1 to 498), N-spectrin-Dbl (residues 224 to 417), and PH-Dbl (residues 711 to 808) fragments have been previously described (38). S-tagged proto- and onco-Dbl were

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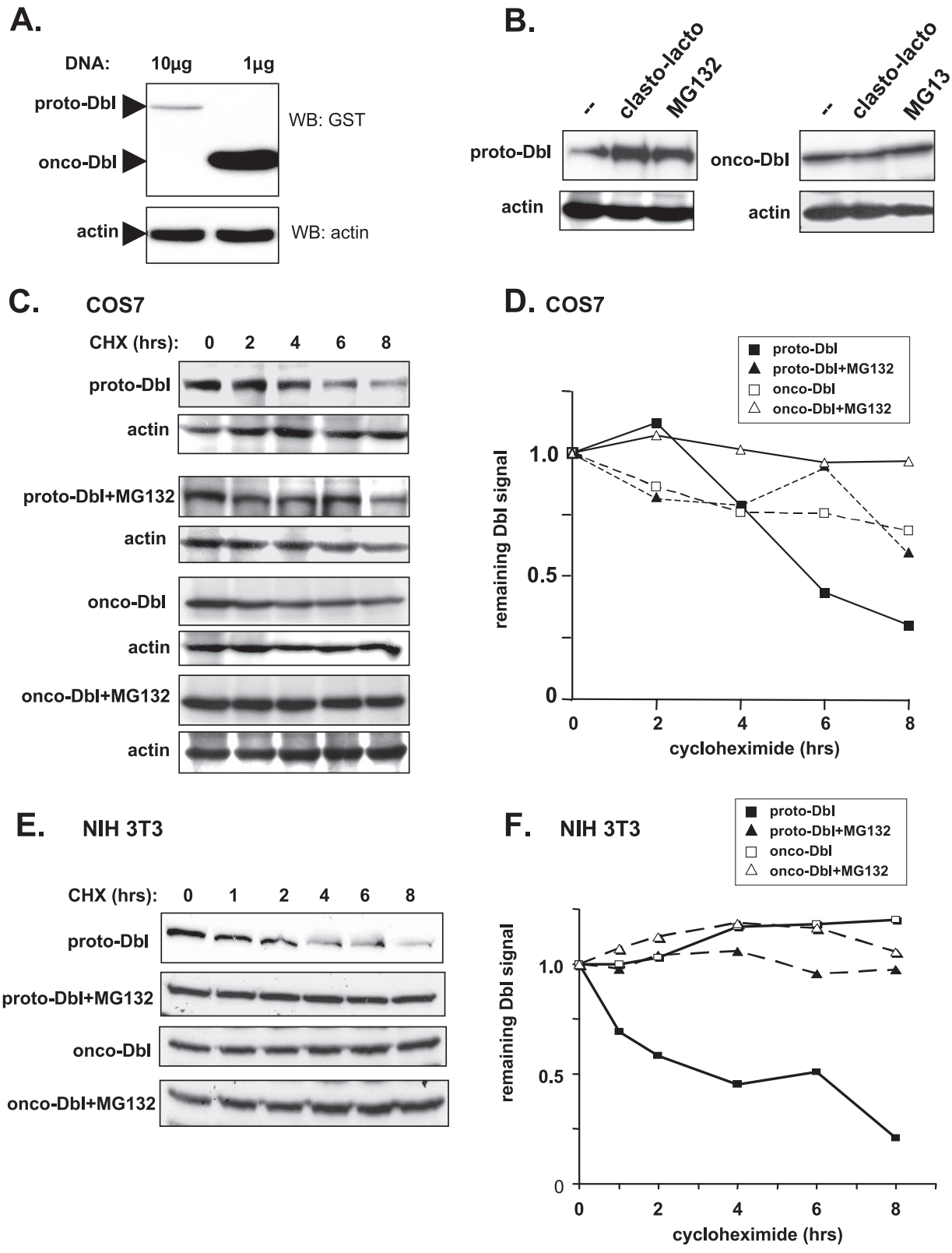

FIG. 1. Proto-Dbl, but not onco-Dbl, is a short-lived protein that accumulates upon proteasomal inhibition. (A) Steady-state expression levels of onco- and proto-Dbl 48 h after transient transfection into COS7 cells. The expression level of proto-Dbl was lower despite using a 10-fold-larger amount of DNA. The indicated genes, in the pEBG vector, were transfected using FuGENE6 (10 μg proto-Dbl and 1 μg onco-Dbl per 100-mm plate). (B) Effect of proteasomal inhibition on the steady-state levels of Dbl proteins. Cells were treated with 25 μM MG132 or 10 μM *Clasto*-lactacystin β-lactone (or the DMSO control) for 6 h prior to lysis and anti-Dbl Western blotting (WB). Shown are lysates prepared from transiently transfected COS7 cells, expressing the GST-fused Dbl proteins in the pEBG vector. (C) COS7 cells transiently expressing proto- or

generated by PCR using primers containing an in-frame amino-terminal S tag (Novagen) and ligated into the pCMV6 vector. pCEFL–GST–proto-Dbl and onco-Dbl were generated by ligation of the corresponding cDNAs into the pCEFL-GST vector, in frame with a 5′ glutathione *S*-transferase (GST) tag (generous gift of Yi Zheng, Cincinnati Children's Hospital Medical Center, and Silvio Gutkind, National Institute of Dental and Craniofacial Research). Hemagglutinin (HA)-tagged proto- and onco-Dbl were generated by PCR with primers containing an in-frame, amino-terminal HA tag and ligated into pcDNA3.1-Hygro(+) plasmid. CHIP cDNAs (a generous gift from Cam Patterson, University of North Carolina) were ligated into pcDNA4.1 HisMax vector (Invitrogen) to generate Xpress-tagged CHIP constructs. CHIP(ΔUbox) contains a stop codon at residue 230. Myc-ubiquitin (in the pCW7 plasmid) was a generous gift from Ron Kopito (Stanford University). Human Hsp90 (generous gift from David Toft, University of Rochester) was ligated into pCDNA3.1-Hygro(+) vector (Invitrogen) with a 5′ HA tag. All constructs were verified by restriction mapping and sequencing at the Cornell BioResource Facility. Recombinant GST-fused proto-Dbl from Sf9 insect cells was prepared by the Cornell Cancer Protein Expression Facility. To disrupt the expression of endogenous CHIP, we ligated short DNA oligomer-containing BglII-HindIII overhangs, 19 bp of sense mouse CHIP cDNA starting at position 1242, a connecting loop, and the same CHIP sequence in the antisense orientation into the pSUPER vector (generous gift of Olivier Staub, University of Lausanne, Switzerland) which was then used for small interfering RNA experiments. Controls for this experiment were cells transfected with the empty pSUPER vector.

Materials. MG132 (Calbiochem) and *Clasto*-lactacystin β-lactone (Boston Biochem) were dissolved in dimethyl sulfoxide (DMSO) and stored at −20°C. Cycloheximide was obtained as a ready-to-use solution (Sigma-Aldrich) and stored at 4°C. 17-(Allylamino)-17-demethoxygeldanamycin (17-AAG; NSC 330507) was obtained from A.G. Scientific and from the Division of Cancer Treatment and Diagnosis, National Cancer Institute.

Antibodies. Anti-HA (HA.11), anti-Myc (9E10), and anti-GST antibodies were obtained from Covance Inc. Anti-Dbl antibodies (sc-89 and sc-28582) were purchased from Santa Cruz Biochemicals. Anti-Hsc70 (SPA-815 and SPA-820) and anti-Hsp90 (SPA-835) were obtained from StressGen Biotechnologies, and monoclonal anti-Hsp90 was obtained from Cell Signaling Inc. S-protein HRP conjugates and S-protein cross-linked Sepharose were from Novagen. Anti-HA fluorescein isothiocyanate conjugates were obtained from Covance. Anti-Xpress tag antibody was obtained from Invitrogen. Antivimentin clone 9 was obtained from Sigma. Alexa 488-conjugated secondary antibodies were obtained from Molecular Probes.

Cell culture, protein precipitation, and GTPase activation assays were carried out as described previously (38).

Focus formation assay. Subconfluent NIH 3T3 cells were transfected with proto- or onco-Dbl in the pCEFL-GST vector by using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. At 48 hours posttransfection, the cells were split into three 100-mm culture dishes. At 16 days posttransfection, cells were washed with phosphate-buffered saline, fixed with phosphate-buffered saline containing 10% formaldehyde, and stained with an 8% Giemsa stain. Prominent foci (>1 mm) were scored manually. The viability of Dbl-expressing cells was assessed by scoring the number of colonies in plates containing 700 μg/ml neomycin (G418, encoded on the pCEFL plasmid).

Ubiquitination assays. GST-tagged proto-Dbl (purified from baculovirus-infected Sf9 insect cells and immobilized on glutathione agarose) was incubated with His-tagged CHIP (affinity purified from COS7 cells) in 100 mM KCl, 20 mM Tris, 10 mM dithiothreitol, 1.5 mg/ml ubiquitin (Calbiochem), 0.15 mg/ml biotinylated ubiquitin (Affinity Bioreagents) or Myc-ubiquitin (Boston Biochem), 0.5 mg/ml ubiquitin-aldehyde (Boston Biochem), 25 μM MG132 (Sigma), 5 mM ATP-Mg (Boston Biochem), ATP regeneration solution (Boston Biochem), and E1 and E2 enzymes (UbcH5a; Boston Biochem). After 2 h at room temperature, the washed beads were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and visualized by immunoblotting.

Immunofluoresence confocal microscopy. COS7 cells were transfected with the indicated constructs using FuGENE6 (Roche) according to the manufacturer's instructions. Forty-eight hours after transfection, cells were fixed with 4% paraformaldehyde, permeabilized with 0.2% Triton X-100, stained with the indicated reagents, and viewed on a Leica TCS SP2 microscope at the Cornell BioResource Center. HA-tagged Dbl proteins were visualized using anti-HA antibody (purified monoclonal HA.11; Covance), followed by Alexa 488-conjugated goat anti-mouse secondary antibody (Molecular Probes).

RESULTS

Proto-Dbl, but not onco-Dbl, is a short-lived protein that is degraded by the proteasome. When transiently transfected into cultured cells, onco-Dbl expressed to much higher levels than its proto-oncogenic counterpart (ca. 100-fold) (Fig. 1A). This difference in steady-state expression levels persisted regardless of transfection procedure, cell line, promoter strength, the presence or absence of an epitope tag, or the amount of DNA used. One possible explanation for this phenomenon is that the proto-oncogenic Dbl degrades rapidly, while the oncogenic variant is more stable. We therefore set out to examine whether the cellular levels of proto-oncogenic Dbl are limited by ubiquitination and proteasomal degradation. Treatment of Dbl-expressing NIH 3T3 or COS7 cells with the specific proteasomal inhibitors *Clasto*-lactacystin β-lactone and MG132 (19, 42) caused a marked increase in the levels of proto-Dbl (Fig. 1B). In contrast, the steady-state expression levels of oncogenic Dbl were unaffected by the same treatment (Fig. 1B). These observations raise the possibility that the proto-oncogenic form of Dbl is subject to degradation by the proteasomal pathway, whereas onco-Dbl is resistant to degradation. To directly address this hypothesis, we measured the turnover rate of the two Dbl variants. COS7 cells transiently expressing proto- or onco-Dbl were treated with the protein synthesis inhibitor cycloheximide, and the kinetics of Dbl degradation were monitored by immunoblotting (Fig. 1C and D). Proto-Dbl degraded with a half-life ($t_{1/2}$) of approximately 4.5 h, and this process was significantly delayed upon proteasomal inhibition ($t_{1/2} \gg 8$ h). In contrast, the onco-Dbl protein was significantly more stable ($t_{1/2} \gg 8$ h), and its turnover rate was not affected by treatment with MG132. Similar results were obtained with NIH 3T3 cells that stably express proto- or onco-Dbl (Fig. 1E and F). We conclude that the expression levels of proto-Dbl in cells are kept at low levels by means of an efficient, proteasome-mediated degradation. The oncogenic form of Dbl avoids this fate and accumulates in cells at high levels. This striking difference in protein stability likely affects the biological activity of Dbl and, in turn, could be at the root of the difference in oncogenic potential exhibited by the two variants.

Proto-Dbl, but not onco-Dbl, is efficiently ubiquitinated. The rapid degradation of proto-Dbl and its sensitivity to proteasomal inhibition raise the possibility that the protein is selectively targeted for degradation by ubiquitination. To examine this possibility, we cotransfected GST-tagged proto- or onco-Dbl into COS7 cells together with Myc-tagged ubiquitin.

onco-Dbl were treated with 100 μg cycloheximide (CHX) and with 10 μM MG132 (or vehicle control) and lysed at the indicated times, and Dbl expression was evaluated by Western blotting. (D) Quantitation of the data shown in panel C. (E) NIH 3T3 cells stably expressing GST-tagged proto- or onco-Dbl were treated with cycloheximide and with 10 μM MG132 (or vehicle control) and lysed at the indicated times, and Dbl expression was evaluated by Western blotting. (F) Quantitation of the data shown in panel E. Forty micrograms of lysate protein was loaded onto each lane.

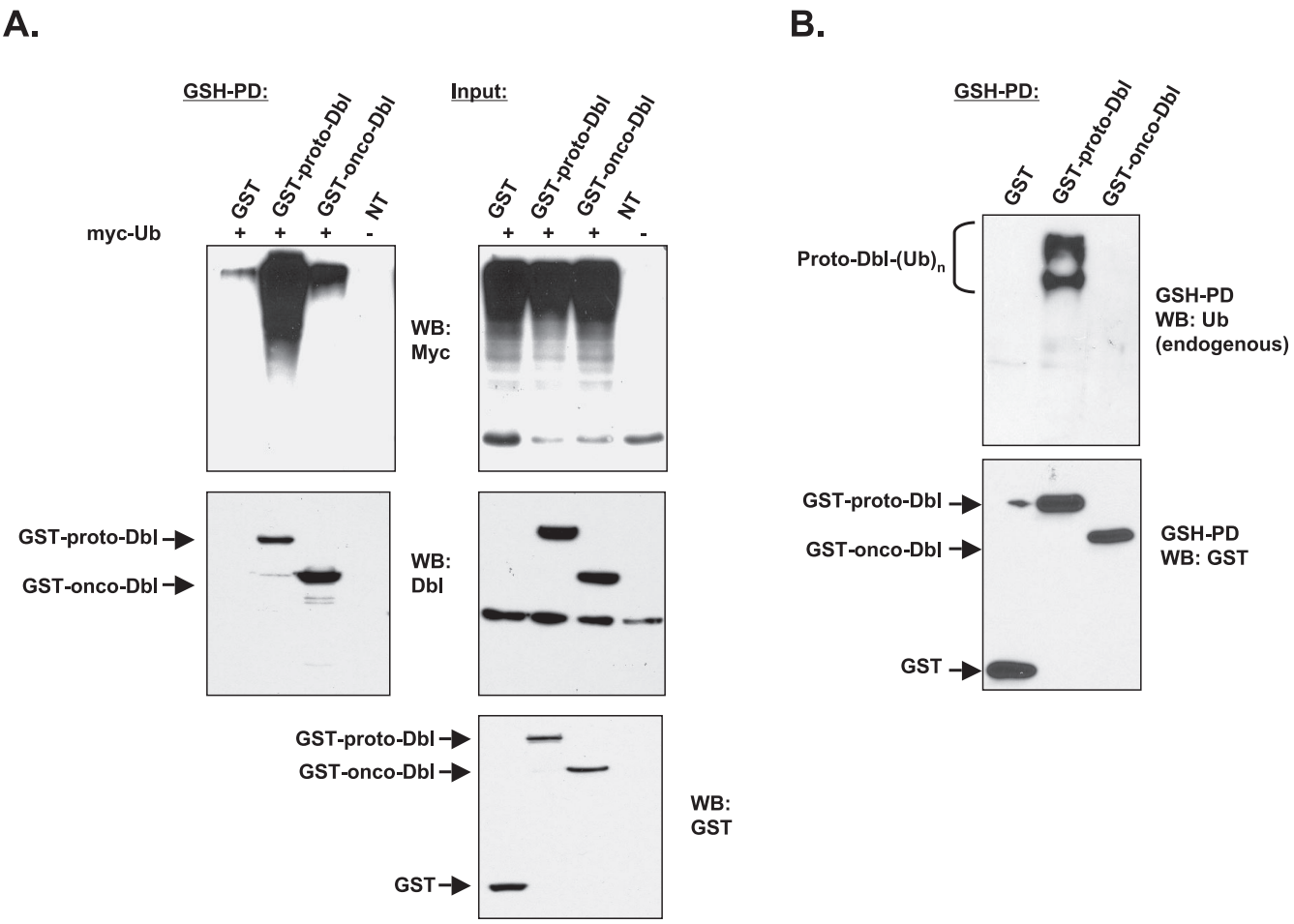

FIG. 2. Proto-Dbl, but not onco-Dbl, is heavily ubiquitinated in vivo. The indicated constructs (in the pEBG vector) were cotransfected into COS7 cells, with (A) or without (B) cDNA encoding Myc-tagged ubiquitin (Ub) (in the pCW7 vector). After 40 h, cells were treated with MG132 (25 μM, 6 h) to inhibit proteasomal degradation. Dbl complexes were precipitated from the lysates by glutathione affinity precipitation, and ubiquitination was evaluated by anti-Myc (A) or antiubiquitin (B) Western blotting (WB). NT, nontransfected; PD, pulldown.

Following cell lysis, glutathione agarose precipitates were resolved on SDS-PAGE and the ubiquitination level of the Dbl proteins was assessed by anti-Myc immunoblotting. We observed that proto-Dbl displays an intense "ladder" of high-molecular-weight ubiquitinated bands (Fig. 2). Importantly, the ubiquitination level of onco-Dbl was significantly weaker (ca. 50-fold). A similar pattern was observed for the modification of proto-Dbl with endogenous ubiquitin (Fig. 2B). We conclude that proto-Dbl is selectively ubiquitinated and that this modification triggers its rapid degradation, as established for many other proteins (reviewed in references 11 and 65).

The chaperones Hsc70 and Hsp90 and the protein-ubiquitin ligase CHIP form a complex in vivo. We recently demonstrated that proto-Dbl selectively associates with Hsc70 in both transfected cells and native brain extracts. Furthermore, we found that the binding of Hsc70 attenuates the GEF activity of Dbl (38). To identify other proteins that participate in this interaction, we performed differential affinity precipitations from cultured COS7 cells and observed a 90-kDa protein that associated with GST-proto-Dbl but not with GST-onco-Dbl. Microsequencing analysis revealed this protein to be the molecular chaperone Hsp90 (data not shown). To evaluate the role of Hsp90 in this complex, we transfected the GST-fused Dbl variants into COS7 cells and probed Dbl precipitates for the presence of cotransfected Hsp90. The interactions between Hsp90 and Dbl mirrored those previously observed with Hsc70 (38): Hsp90 strongly associated with proto-Dbl, while it did not exhibit any appreciable binding to the oncogenic form of Dbl, nor to the control GST "bait" (Fig. 3A). The specificity in interaction between proto-Dbl and Hsp90 was also observed when the endogenous Hsp90 protein was immunoprecipitated from proto-Dbl-expressing cells. Thus, only proto-Dbl associated with the endogenous Hsp90, whereas onco-Dbl did not (Fig. 3B).

The fate of most proteins that are regulated by chaperone-controlled ubiquitination and proteasomal degradation is dictated by the triage decision, a delicate interplay between molecular chaperones that regulate the stabilization of client proteins and ubiquitin-protein ligases that trigger protein degradation (13). It has been reported that Hsc70, in complex with Hsp90 and the ubiquitin ligase CHIP, can influence the fate of multiple cellular proteins (43). We thus examined whether Dbl proteins are present in a complex with Hsc70, Hsp90, and CHIP in vivo. We cotransfected GST-tagged proto- or onco-

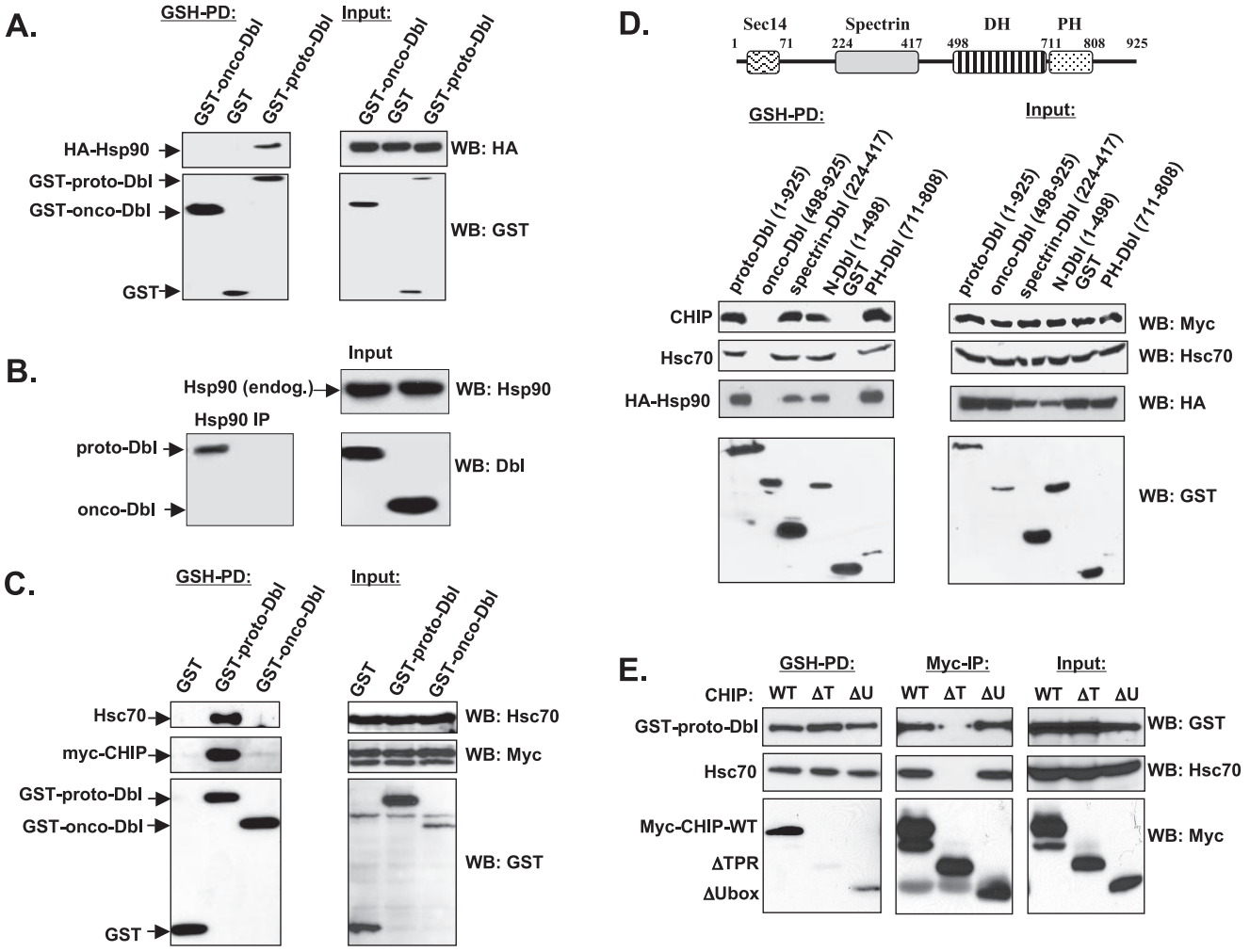

FIG. 3. Proto-Dbl, but not onco-Dbl, interacts with the chaperones Hsc70 and Hsp90 and the ubiquitin-ligase CHIP. (A) cDNAs encoding GST-tagged proto- or onco-Dbl (in the pEBG vector) or GST alone were transiently transfected into COS7 cells together with HA-tagged Hsp90. Forty-eight hours after transfection, cells were lysed and GST-tagged proteins isolated by glutathione-agarose precipitation (2 h at 4°C). After being washed, the proteins were analyzed by SDS-PAGE and Western blotting (WB) with the indicated antibodies. (B) Dbl constructs were transfected into COS7 cells, and endogenous (endog.) Hsp90 precipitates (αHsp90) were probed for the presence of Dbl proteins. (C) cDNAs encoding GST-tagged proto- or onco-Dbl (in the pEBG vector) were transiently transfected into COS7 cells together with the indicated Myc-tagged CHIP construct (in the pCDNA3 vector). Forty-eight hours after transfection, cells were lysed and Dbl complexes isolated by glutathione-agarose precipitation (4 h at 4°C). After being washed, the proteins were analyzed by SDS-PAGE and Western blotting with the indicated antibodies. (D) The spectrin domain of proto-Dbl mediates the association with Hsc70, Hsp90, and CHIP. The indicated Dbl constructs (in the pEBG vector) were transiently cotransfected into COS7 cells together with Hsp90 or CHIP. Dbl complexes were isolated by glutathione-agarose precipitation and associated proteins evaluated by immunoblotting. The top three panels show the presence of Myc-CHIP, endogenous Hsc70, and HA-Hsp90 in Dbl precipitates. The bottom panels show the presence of Dbl constructs ("baits") in the lysates (right) and in the affinity precipitates (left) of the Hsc70/CHIP transfection. Identical "bait" blots were obtained in the Hsp90 experiment. (E) cDNAs encoding GST-tagged proto-Dbl together with the indicated Myc-tagged CHIP constructs (in the pCDNA3 vector) were transiently transfected into COS7 cells. Forty-eight hours after transfection, cells were lysed and glutathione-Sepharose pulldowns (PD) were performed in parallel with the anti-Myc immunoprecipitations (IP). After being washed, the proteins were analyzed by SDS-PAGE and Western blotting with the indicated antibodies. WT, wild type.

Dbl (or control) constructs into COS7 cells together with cDNA encoding Myc-tagged CHIP. Dbl proteins were then affinity precipitated, resolved on SDS-PAGE, and probed for interacting CHIP by anti-Myc immunoblotting. As shown in Fig. 3C, proto-Dbl strongly associated with the ubiquitin ligase CHIP and with Hsc70. Importantly, this interaction is specific: we could not detect any CHIP immunoreactivity in precipitates prepared from cells that express either the oncogenic form of Dbl or the control vector (Fig. 3C). These observations raise the possibility that CHIP is the E3 ubiquitin ligase that mediates the ubiquitination of proto-Dbl. Based on these results, we conclude that in cells, proto-Dbl exists in a complex that contains Hsp90, the ubiquitin ligase CHIP, and Hsc70.

Next, we set out to delineate the regions in proto-Dbl that mediate the interactions with CHIP and its associated chaperones. Since the difference between onco-Dbl and its proto-oncogenic variant is the presence of an extended amino terminus in the latter, we examined the ability of isolated amino-

terminal fragments of proto-Dbl to interact with CHIP, Hsc70, and Hsp90 by using affinity precipitations and immunoblotting. We found that the isolated amino terminus of proto-Dbl (N-Dbl, residues 1 to 498) as well as the spectrin homology domain (residues 224 to 417) bound to Hsp90 and CHIP with an affinity similar to that observed with the full-length proto-oncogene (Fig. 3D). Neither onco-Dbl nor the control GST "bait" showed any appreciable binding to these molecules. Interestingly, the isolated PH domain of Dbl exhibited significant binding to Hsp90 and CHIP, although it did not bind these factors in the context of onco-Dbl (Fig. 3D). While the explanation for this apparent enigma is not evident, it is important to note that the exact same domain selectivity was reported regarding binding of Hsc70 to Dbl (38). In summary, Hsp90 and CHIP bind to proto-Dbl through the same protein modules that mediate binding to Hsc70, suggesting the existence of a multicomponent complex.

The CHIP protein has two functional domains: the tetratricopeptide repeat (TPR) domain, which mediates binding to chaperones, and the U-box domain, where the ubiquitin-protein ligase activity resides (34). The U-box domain also serves as a dimerization interface in CHIP homodimers (49, 72). To investigate the roles of these domains in the CHIP-chaperone-Dbl complex, we used truncated CHIP cDNAs in which either domain is deleted [i.e., the CHIP(ΔTPR) and CHIP(ΔUbox) constructs]. We observed that endogenous Hsc70 associated with proto-Dbl in cells expressing either CHIP construct (Fig. 3E), suggesting that CHIP does not function as a "bridge" between the GEF and the chaperones. The interaction between CHIP and Dbl, on the other hand, is dependent on the functional integrity of the ectopic CHIP, particularly on the TPR domain: no association between Dbl and CHIP was observed when the CHIP(ΔTPR) construct was overexpressed (Fig. 3E). In reverse experiments, where the ectopically expressed CHIP proteins were precipitated, we observed that neither Hsc70 nor proto-Dbl associated with CHIP(ΔTPR) (Fig. 3). Taken together, these observations suggest that interaction between proto-Dbl and CHIP is mediated by Hsc70/Hsp90 that binds CHIP through its TPR domain (72).

Physiological consequences of the interactions between proto-Dbl and the chaperone complex. (i) CHIP mediates the degradation arm of the triage decision. The observation that proto-Dbl selectively and specifically associates with CHIP in intact cells raises the possibility that CHIP is the ligase that mediates proto-Dbl's ubiquitination and its subsequent rapid degradation by the proteasome. Indeed, we observed that overexpression of CHIP caused a dose-dependent decrease in the steady-state expression level of proto-oncogenic Dbl (Fig. 4A). This effect required the ubiquitin ligase activity of CHIP, since overexpression of the catalytically inactive CHIP(ΔUbox) did not affect the expression levels of proto-Dbl. Interestingly, the CHIP(ΔTPR) construct, unable to bind the chaperone Hsc70/Hsp90, caused a marked, dose-dependent increase in the level of proto-Dbl (Fig. 4A). A possible explanation for this is that CHIP(ΔTPR) acts in a dominant-negative fashion by sequestering the endogenous CHIP through U-box-mediated dimerization (49, 72). That CHIP modulates Dbl stability is also supported by the observation that overexpression of CHIP in cycloheximide-treated cells shortened proto-Dbl's half-life from 4 h to 2 h (Fig. 4C). Importantly, overexpression of CHIP did not affect the expression level of the oncogenic form of Dbl (Fig. 4B). To conclusively implicate CHIP as a Dbl-ubiquitin ligase, we employed small interfering RNA that specifically targets endogenous CHIP. Expression of the interfering RNA in the pSUPER vector (7) in NIH 3T3 cells expressing proto-Dbl resulted in a ca. 90% reduction in the endogenous expression levels of CHIP (Fig. 4D). Importantly, the decrease in CHIP levels was accompanied by a marked (>3-fold) increase in the level of the proto-Dbl protein but not of the unrelated c-Jun protein (Fig. 4D). Taken together, these observations indicate that the E3 ubiquitin ligase CHIP is an important regulator of Dbl expression levels.

(ii) Hsp90 regulates the stabilizing arm of the triage decision. The observations that proto-Dbl is ubiquitinated, degraded, and kept at low cellular concentrations point at the possibility that the activity of this protein is regulated by the so-called triage decision, a delicate balance between opposing pathways that facilitate ubiquitination/degradation reactions and pathways that promote stabilization/refolding of the client protein (13, 43, 47). In light of the known role of Hsp90 as a facilitator of protein stabilization during this process (70, 73) and the selective association between proto-Dbl and Hsp90 (Fig. 3B), we set out to examine whether Hsp90 function impacts the cellular fate of proto-Dbl. Overexpression of Hsp90 typically favors the stabilization of its client proteins, whereas pharmacologic inhibition of Hsp90 shifts the triage process toward degradation, leading to a reduction in the levels and biological activity of the client protein. To investigate the role of Hsp90 in Dbl function, we utilized the ansamycin antibiotic 17-AAG, which binds with high affinity and specificity to the ATP binding pocket of Hsp90 (48, 71). We cotransfected COS7 or NIH 3T3 cells with cDNAs encoding proto-Dbl and Myc-tagged ubiquitin and examined the effect of 17-AAG treatment on proto-Dbl status. 17-AAG caused a pronounced increase in the ubiquitination of proto-Dbl. In addition, treatment with 17-AAG caused a marked increase in the association between proto-Dbl and CHIP, suggesting that CHIP mediates the observed increase in Dbl ubiquitination (Fig. 5A). Similar results were observed when modification of proto-Dbl by endogenous ubiquitin was assessed: 17-AAG caused a pronounced increase in the ubiquitination of proto-Dbl that was further enhanced by overexpression of CHIP (Fig. 5B).

We also tested whether CHIP can ubiquitinate proto-Dbl in a cell-free system. We incubated recombinant proto-Dbl (purified from baculovirus-infected Sf9 insect cells) with purified ubiquitin and CHIP that was immunoprecipitated from COS7 cells. In the presence of other components required for the ubiquitination reaction (the E1 and E2 enzymes and an ATP-regenerating system), we observed efficient, CHIP-dependent ubiquitination of proto-Dbl (Fig. 5C).

Since protein ubiquitination often precedes degradation, we anticipated that inhibition of Hsp90 would also cause a reduction in the steady-state levels of the proto-Dbl protein. Indeed, treatment with 17-AAG caused a rapid decline in the cellular levels of proto-Dbl, down to ~20% of the pretreatment level within 4 h (Fig. 6A). Furthermore, cotreatment with the proteasomal inhibitor MG132 completely abolished the effect of 17-AAG, indicating that the proteasome is responsible for the 17-AAG-induced degradation of proto-Dbl (Fig. 6A). These observations indicate that Hsp90 stabilizes proto-Dbl, serving

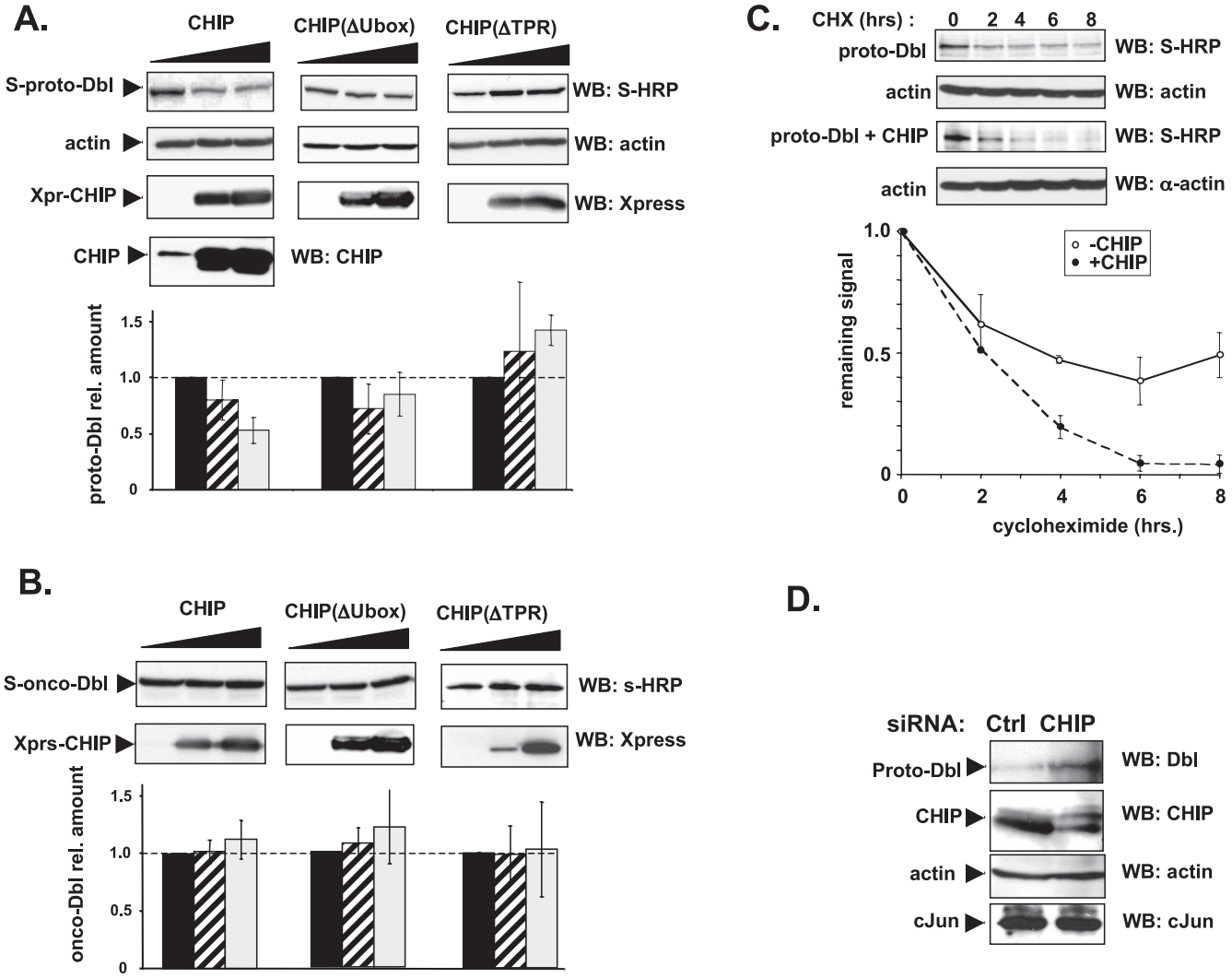

FIG. 4. CHIP regulates the steady-state levels of proto-Dbl and shortens its half-life. (A) Effect of CHIP expression on steady-state levels of proto-Dbl. Proto-Dbl (S tagged in the pCMV6 vector) was cotransfected with increasing amounts (0, 0.2, and 1.0 μg per 32-mm dish) of the indicated CHIP cDNA (Xpress tagged in the pCDNA4.1 vector), and protein levels were examined after 48 h by Western blotting (WB) with the indicated antibodies. The intensity of the Dbl signal was averaged from three to five independent experiments. (B) CHIP expression does not affect the steady-state levels of onco-Dbl (experimental details as described for panel A). (C) CHIP accelerated the turnover rate of proto-Dbl. COS7 cells were transiently transfected with the indicated constructs and treated with cycloheximide (CHX) for the indicated amount of time before lysis and Western blotting with anti-Dbl antibodies. Shown are the averages for three independent experiments. (D) Small interfering RNA (siRNA) directed against endogenous CHIP (in the pSUPER vector) was transfected into NIH 3T3 cells stably expressing proto-Dbl. Transfection with an empty pSUPER vector was used as a control (Ctrl). Protein levels of CHIP and proto-Dbl were evaluated by immunoblotting with the indicated antibodies. cJun was used as a specificity control. "Xpr" and "Xprs," Xpress; rel., relative.

to protect it from degradation by the proteasome. In support of this notion, treatment with 17-AAG also caused a pronounced enhancement of proto-Dbl's turnover rate in cycloheximide-treated cells, reducing its half-life from 4 h to 2 h. Furthermore, ectopic overexpression of Hsp90 lead to a substantial stabilization of proto-Dbl, prolonging its half-life to $\gg$4 h (Fig. 6A). Under identical conditions, treatment with 17-AAG did not affect the status of onco-Dbl (data not shown).

To evaluate these findings in a physiologically relevant context, we examined the effect of 17-AAG treatment on the GEF activity of proto-Dbl, i.e., its ability to facilitate nucleotide exchange on the substrate GTPase RhoA. We cotransfected COS7 cells with cDNAs encoding Dbl and RhoA and subjected them to serum starvation. The activated (GTP-bound) form of RhoA was then selectively precipitated from cell lysates by using an immobilized GTPase binding domain of the effector rhotekin (1, 53, 62). Expression of either onco- or proto-Dbl caused a pronounced increase in the fractional pool of RhoA that is in the GTP-bound state (Fig. 6B), indicating that both Dbl constructs are fully functional. Treatment with 17-AAG caused a marked reduction in the expression levels of proto-Dbl and completely abolished its ability to activate RhoA. In contrast, treatment with 17-AAG did not affect the expression level or the GEF activity of the truncated, oncogenic form of Dbl (Fig. 6B). We also examined the effect of 17-AAG treatment on the ability of Dbl-expressing cells to

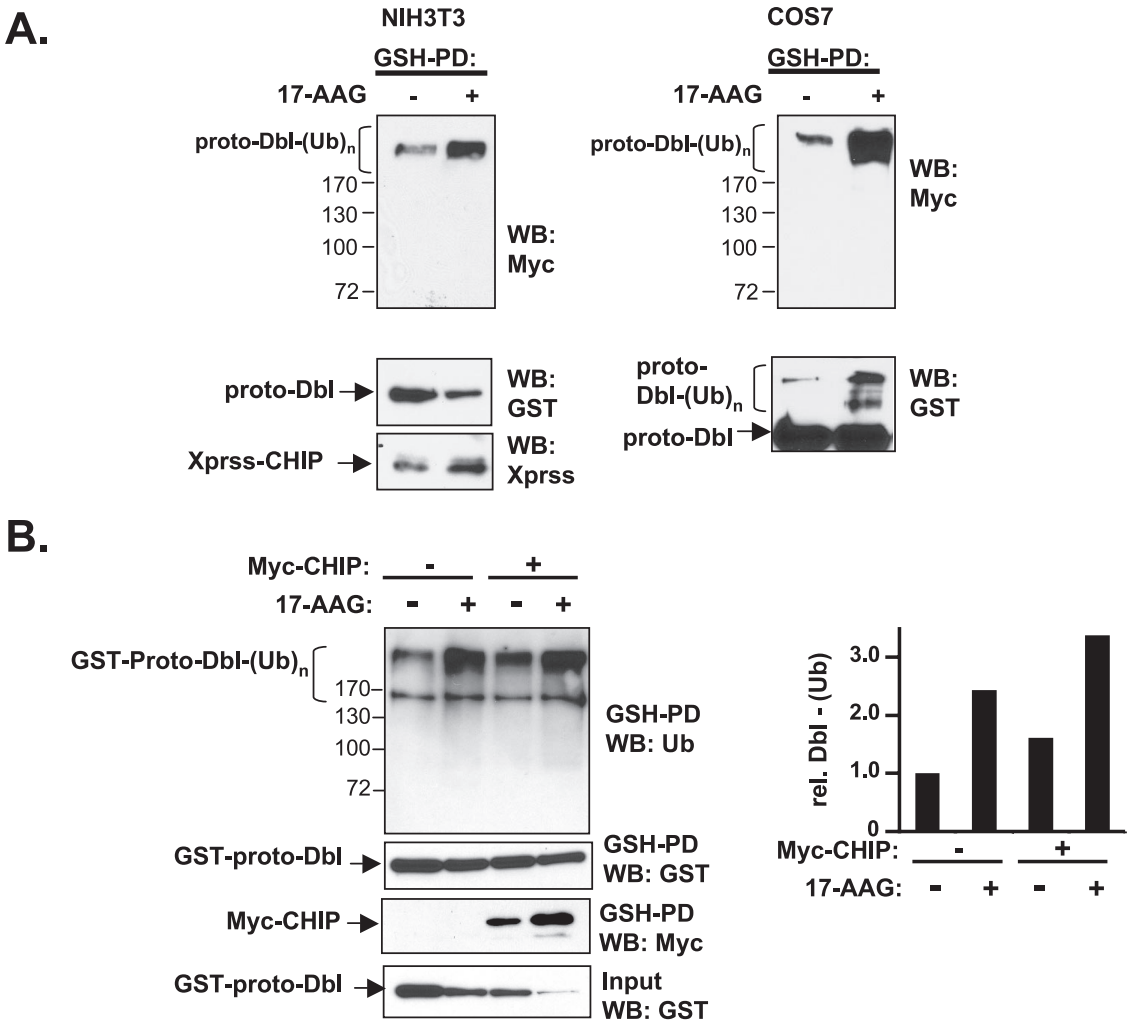

FIG. 5. Hsc70/Hsp90/CHIP complex is involved in proto-Dbl ubiquitination. (A) Treatment with 17-AAG decreases steady-state levels of proto-Dbl and increases its ubiquitination and association with CHIP. NIH 3T3 cells stably expressing proto-Dbl (left panels) or COS7 cells transiently expressing proto-Dbl (right panels) were cotransfected with cDNAs encoding Myc-ubiquitin and, where indicated, CHIP (Xpress tagged in the pCDNA4.1 vector). 17-AAG (or the DMSO vehicle control) was added at 3 μM for 2 h prior to lysis in lysis buffer containing 25 μM MG132. Proto-Dbl ubiquitination and association with CHIP were assessed in glutathione affinity precipitates. (B) COS7 cells were transiently transfected with GST-tagged proto-Dbl and Myc-CHIP (where indicated). Cells were treated and lysed and proto-Dbl complexes precipitated as described for panel A. The ubiquitination level of Dbl was evaluated by probing precipitates for the presence of endogenous ubiquitin (Ub) (top panel). The proto-Dbl ubiquitination signal was quantitated by densitometry and normalized. (C) GST-tagged proto-Dbl (purified from baculovirus-infected Sf9 insect cells and immobilized on glutathione agarose) was incubated with His-tagged CHIP (affinity purified from COS7 cells) in 100 mM KCl, 20 mM Tris, 10 mM dithiothreitol, 1.5 mg/ml ubiquitin (Calbiochem), 0.15 mg/ml Myc-ubiquitin (Boston Biochem), 0.5 mg/ml ubiquitin-aldehyde (Boston Biochem), 25 μM MG132 (Sigma), 5 mM ATP-Mg (Boston Biochem), ATP regeneration solution (Boston Biochem), and E1 and E2 enzymes (UbcH5a; Boston Biochem). After 2 h at room temperature, the beads were washed with radioimmunopreciptation assay buffer, resolved by SDS-PAGE, and visualized by immunoblotting with anti-Myc and anti-GST antibodies. Xprss, Xpress; PD, pulldown; WB, Western blotting; rel., relative.

avoid contact inhibition and to form foci in confluent NIH 3T3 cells. Inhibition of Hsp90 with 17-AAG resulted in a marked reduction in the number of foci formed by proto-Dbl (Fig. 6C). Onco-Dbl, on the other hand, exhibited significantly more potent fibroblast-transforming activity (55), which was not sensitive to 17-AAG treatment. The inhibitory effects of 17-AAG did not stem from global effects on cell growth, as the drug did not impact the viability of Dbl-transformed cells (Fig. 6D).

Taken together, our observations indicate that proto-Dbl

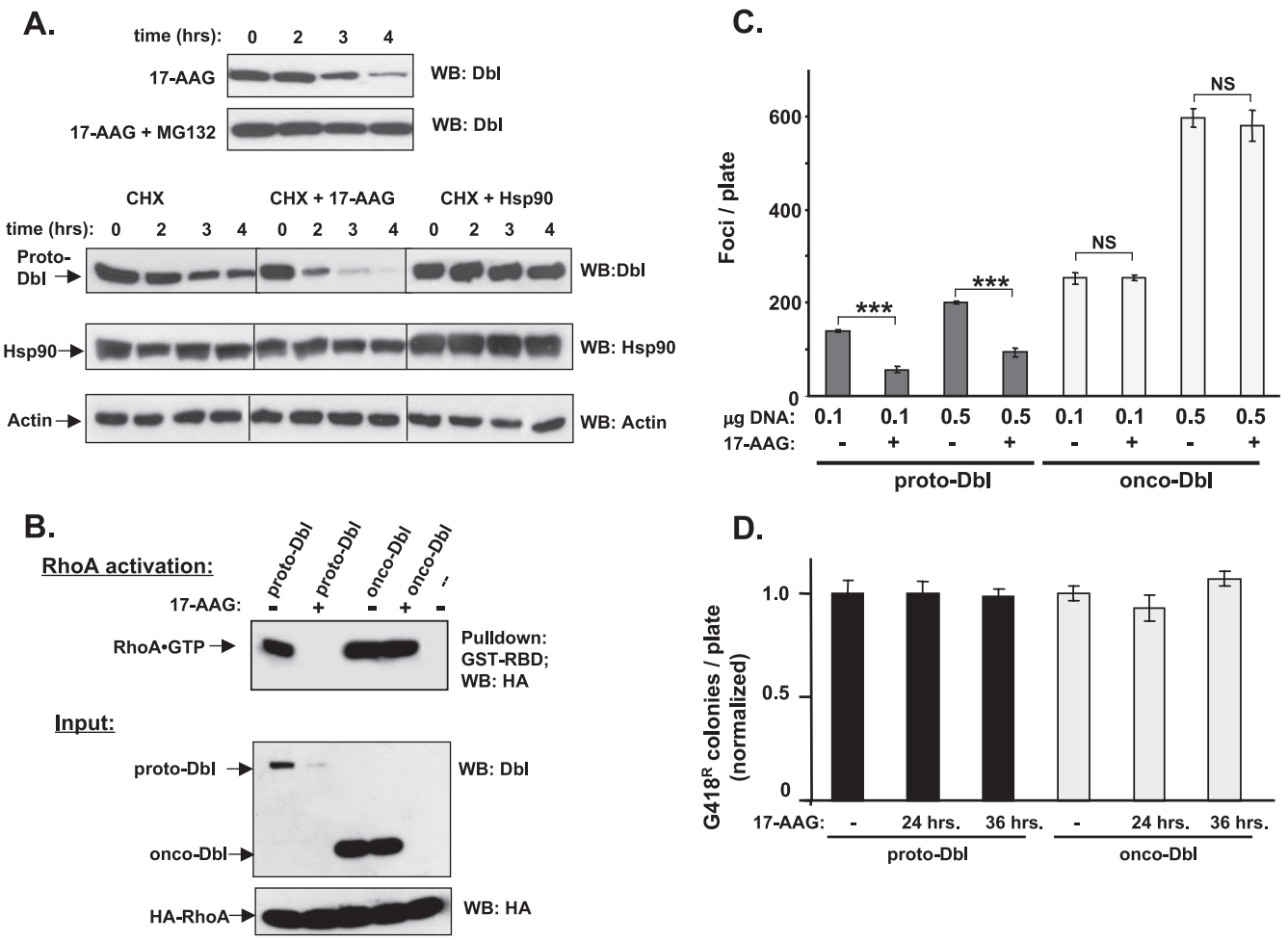

FIG. 6. Pharmacological inhibition of Hsp90 attenuates the level and activity of proto-Dbl. (A) Treatment with 17-AAG decreases steady-state levels of proto-Dbl. In the top two panels, COS7 cells were transiently transfected with proto-Dbl in the pCMV6 vector. The cells were treated with 1 μM 17-AAG for the indicated time periods; where indicated, cells were pretreated with 25 μM MG132 starting 4 h prior to 17-AAG treatment. At 48 hours posttransfection, the cells were lysed and lysates subjected to SDS-PAGE, followed by immunoblotting with anti-Dbl. In the bottom three panels, COS7 cells were transiently transfected with proto-Dbl (GST tagged in the pEBG vector) and, where indicated, with Hsp90 (HA tagged in the pCDNA3.1-Hygro(+) vector). The cells were treated with 50 ng/ml cycloheximide (CHX) for the indicated duration and, where indicated, with 1 μM 17-AAG for the indicated duration. At 48 hours posttransfection, Dbl expression was evaluated in cell lysates by anti-Dbl immunoblotting. (B) Inhibition of Hsp90 attenuates the GEF activity of proto-Dbl. COS7 cells were transiently transfected with RhoA (HA tagged in the pKH3 vector) and either proto-Dbl or onco-Dbl (in the pCMV6 vector). After serum deprivation for 16 h, the cells were treated with 2 μM 17-AAG (or the DMSO vehicle control) for 2 h. In the upper panel, activated (GTP-bound) RhoA was precipitated using the immobilized Rho-binding domain (RBD) of rhotekin as described in Materials and Methods. In the bottom panels, protein expression levels were assayed in lysates by immunoblotting using the indicated antibodies. (C) Inhibition of Hsp90 attenuates the transforming activity of proto-Dbl. The formation of morphologically transformed foci was assayed 16 days after transfection of NIH 3T3 cells with proto-Dbl or onco-Dbl (in the pCEFL-GST vector). 17-AAG (or the DMSO vehicle control) was added at 0.5 μM for the last 36 h of the experiment. Values represent average numbers of foci (± standard deviations) as determined in triplicate plates. Where indicated by asterisks, differences between 17-AAG and vehicle control treatment groups were found to be highly significant ($P = 0.001$) by the Student t test. (D) Treatment with 17-AAG does not affect the viability of Dbl-transformed cells. Viability was evaluated by quantitating cell proliferation in the presence of the antibiotic neomycin (the resistance for which is encoded on the pCEFL-GST vector). WB, Western blotting.

undergoes efficient ubiquitination and degradation that limit its cellular levels and that this process is mediated by the protein-ubiquitin ligase CHIP. Hsp90, on the other hand, serves to stabilize proto-Dbl and protect it from proteasomal degradation. These two opposing processes regulate the cellular levels of proto-Dbl. Onco-Dbl, in contrast, is not regulated by the chaperone-CHIP machinery and, consequently, accumulates in cells at high levels.

Effect of Hsp90 inhibition on the intracellular localization of Dbl proteins. Disruption of Hsp90 function has been reported to cause misfolding of its client substrates, leading in many cases to their sequestration to specific cellular compartments prior to degradation (71). We therefore set out to examine the effect of inhibiting Hsp90 activity on the subcellular localization of Dbl proteins. Confocal fluorescence microscopy of COS7 cells transiently transfected with proto-Dbl revealed two primary patterns: in most cells, proto-Dbl exhibited diffuse cytosolic distribution with pronounced perinuclear staining (Fig. 7A). In cells that express high levels of the protein, proto-Dbl concentrated in a distinct round, juxtanuclear spot

A.
proto-Dbl
proto-Dbl +17AAG
onco-Dbl
onco-Dbl+17AAG
B.
proto-Dbl
vimentin
merge
γ-tubulin
C.
% Dbl cells with punctate structures
30
20
10
0
17-AAG: - +

FIG. 7. Pharmacological inhibition of Hsp90 alters the intracellular localization pattern of proto-Dbl but not of onco-Dbl. (A) COS7 cells were transiently transfected with proto- or onco-Dbl (HA tagged in the pCDNA3.1 vector) and prepared for microscopy as detailed in Materials and Methods. Proto-Dbl was visualized with anti-HA antibody, followed by Alexa 488-conjugated anti-mouse secondary antibody (green). Where indicated, 17-AAG was added (3 μM, 2 h) prior to fixing and immunostaining. White arrows point to proto-Dbl-positive aggresomes. (B) Colocalization of proto-Dbl with markers of the aggresome. The indicated antibodies were used to evaluate the intracellular distribution of vimentin and γ-tubulin. Arrowheads indicate vimentin "cages" around the aggresome, and arrows point to the microtubule-organizing center. (C) Total proto-Dbl-transfected cells (about 900 cells per condition, representing six to eight independent slides) were counted, and the percentage of cells exhibiting punctate structures larger than 1 μm was charted. Bar = 20 μm.

(Fig. 7A). The localization pattern of proto-Dbl in these spots is highly reminiscent of aggresomes, juxtanuclear bodies formed by the transport of protein aggregates along microtubule tracks to the microtubule-organizing center (35, 40, 45). It is thought that aggresome formation is a response of cells to an "overload" of cell folding and/or proteasomal degradation machinery (35, 40). When in juxtanuclear spots, the localization pattern of proto-Dbl shows extensive overlap with known markers of the aggresomes (Fig. 7B): the microtubule organizing center (visualized by anti-γ-tubulin staining) and the intermediate filament vimentin, known to form a structural "cage" around the aggresome (20, 35, 40). Pharmacologic inhibition of Hsp90 lead to a dramatic change in the intracellular localization pattern of proto-Dbl, with two specific morphological characteristics: (i) the proto-Dbl protein redistributed to discrete spherical particles distributed throughout the cell, and (ii) proto-Dbl displayed juxtanuclear spot localization in more cells (Fig. 7C). Oncogenic Dbl displayed a diffuse cytoplasmic distribution pattern in most cells, with some colocalization with cortical actin and the plasma membrane. The localization pattern of onco-Dbl did not change upon treatment with 17-AAG (Fig. 7A, bottom panels). Hence, we conclude that Hsp90 protects proto-Dbl from degradation and from concentrating in intracellular aggregates.

DISCUSSION

Despite the critical roles of Dbl family GEFs in mitogenic signaling, the mechanisms that regulate their activity are incompletely understood. Especially enigmatic are the mechanisms that maintain GEF activity "in check" prior to, and following, cell stimulation. Here, we describe the identification and characterization of a novel regulatory mechanism for the guanine nucleotide exchange factor Dbl. This pathway includes the molecular chaperones Hsc70 and Hsp90 and the U-box ubiquitin-protein ligase CHIP. We show that through interactions with these regulators, the proto-oncogenic form of Dbl is efficiently ubiquitinated and rapidly degraded, resulting in low steady-state expression levels of the protein. Importantly, we show that the oncogenic form of Dbl "escapes" regulation by the Hsc70/Hsp90/CHIP complex.

For many Dbl family members, mutations outside the catalytic DH/PH domain cause constitutive activity and elevated oncogenic potential. In the case of Dbl and its homologs Vav, Ect2, Tiam1, Asef, and Net1, oncogenic activation results from the deletion of sequences in the amino terminus of the proto-oncogene (10, 36, 39, 46, 54, 63). Thus, it appears that regions in the amino terminus have an inhibitory function that attenuates the GEF's catalytic activity. In support of this notion, it has been reported that coexpression of the isolated amino terminus of Dbl inhibits the GEF activity of oncogenic Dbl, suggesting an intramolecular, autoinhibitory mode of regulation (5). However, the molecular basis of this regulation is likely to be more complex, since the autoinhibited state could not be reconstituted in vitro with purified components (5). Our findings indicate that the amino terminus of proto-Dbl attenuates the protein's GEF activity by binding the regulators of ubiquitination and degradation.

It was previously reported that the oncogenic form of the Dbl protein degrades at a lower rate than its proto-oncogenic

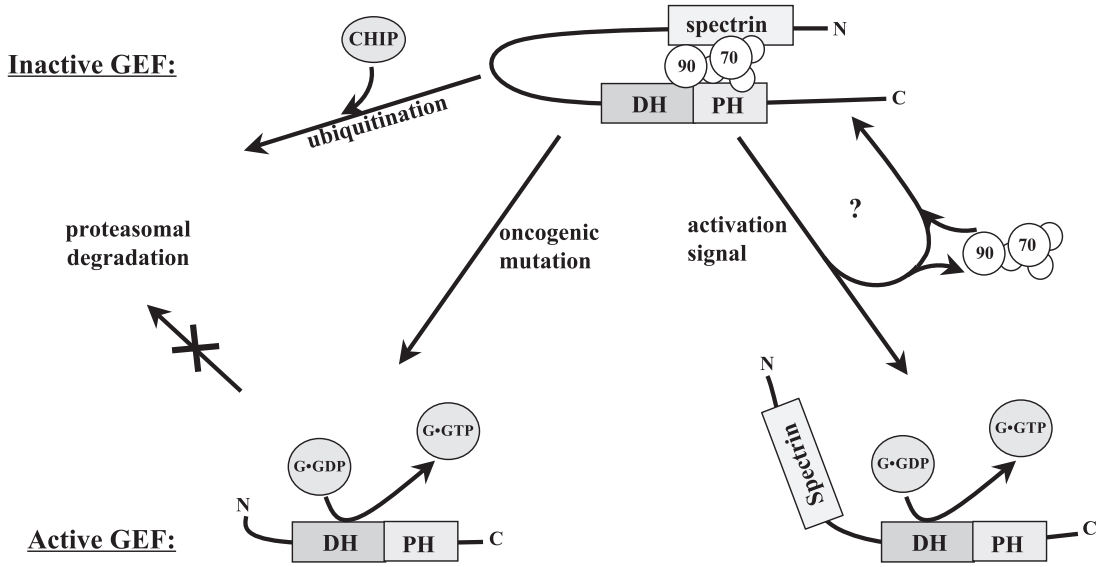

FIG. 8. A schematic model of the regulation of Dbl proteins by ubiquitination and proteasomal degradation. See Discussion for details.

counterpart (24, 54, 64). The basis for this difference, however, remained unknown. We show here that, through its amino-terminal spectrin domain, proto-Dbl binds the molecular chaperones Hsc70 and Hsp90 and the ubiquitin ligase CHIP and that these molecules regulate the levels, the localization, and the activity of proto-Dbl. These interactions render proto-Dbl a short-lived, aggregation-prone protein, the fate of which is dictated by the triage decision, stabilization versus degradation. While Hsp90 dictates stabilization of proto-Dbl, CHIP directs the protein to ubiquitination and subsequent degradation (Fig. 8). Hence, these interactions maintain the steady-state expression of proto-Dbl at low levels and comprise an efficient mechanism for keeping the protein's activity in check. Oncogenic Dbl, lacking the spectrin domain association interface, cannot bind the mediators of the triage decision. The result of the oncogenic truncation mutation, therefore, is that onco-Dbl escapes degradation and accumulates in cells at high levels. This increase in expression levels leads to persistent activation of the substrate GTPases and their downstream pathways, ultimately causing cell transformation. In that respect, onco-Dbl resembles many other growth regulatory molecules that transform cells by virtue of their elevated protein levels.

The role that ubiquitin-mediated degradation plays in the response of proto-Dbl- expressing cells to external stimuli is presently unknown. In view of the present observations, it is tempting to speculate that the activation of proto-Dbl by cell surface receptors will influence its association with the (co) chaperones and thus modulate its rate of degradation (Fig. 8). Verification of this point awaits further experimental examination.

The interactions that regulate the ubiquitination and degradation of proto-Dbl are different from those that control the fate of other growth-promoting molecules that are subject to the triage decision. For example, in the case of p53, Akt, B-Raf, the growth factor receptor ErbB2/Neu, and v-Src, inhibition of Hsp90 results in selective degradation of the mutated, oncogenic variant of the protein (3, 4, 6, 12, 25, 29, 60, 68). Consequently, Hsp90 inhibitors are presently used to treat malignancies in which the mutated, oncogenic protein is expressed (2, 22, 26, 52). In the case of Dbl, the situation is reversed: only the proto-oncogenic form of the protein is subject to downregulation, while the constitutively active form evades this fate. These observations suggest that malignancies in which truncated versions of Dbl-related GEFs are expressed will be resistant to treatment with inhibitors of Hsp90 function.

The molecular chaperones Hsc70 and Hsp90 have been shown to modulate the intracellular levels and activities of a number of signaling molecules, including the GEFs FGD1 (30), CNRasGEF (51), and Vps9p (15). The present study describes a new Hsp90/Hsc70 client protein, the guanine nucleotide exchange factor proto-Dbl, suggesting the possibility that other proteins that bear structural and functional homology may be similarly regulated.

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