Regulation of Proto-Dbl through the Ubox Domain of CHIP

Nora Muakkassa

**Summary**

The Dbl protein is a product of a proto-oncogene that functions as a guanine nucleotide exchange factor for Rho-family GTPases. It is responsible for activating the GTPases by facilitating the dissociation of GDP thus allowing for the binding of GTP. Intracellular levels of Dbl are regulated by ubiquitin-mediated proteolysis. CHIP is the E3 protein-ubiquitin ligase responsible for this ubiquitination. More specifically, the Ubox domain of CHIP is critical to this interaction. Oncogenic Dbl, which lacks its spectrin domain, cannot bind CHIP and therefore escapes degradation. This causes accumulation of the oncogene product in the cell, and leads to persistent activation of its downstream pathways.
Introduction

Dbi was first identified by isolating fibroblast-transforming DNA from a human diffuse B-cell lymphoma\(^7\). Since its discovery, >70 other proteins have been categorized as members of the Dbi family\(^3, 6, 19, 22\). Most of these proteins have been found to act as guanine nucleotide exchange factors (GEFs) that modulate the activity of Rho-like GTPases\(^3, 20\). These G-proteins are turned on by the exchange of GDP for GTP with the help of a GEF which catalyzes the dissociation of GDP. Once activated, GTPases stimulate a cascade of events affecting cytoskeletal structure and gene transcription among other cellular functions\(^11, 18\). Members of the Dbi family are identified as such by the presence of a Dbl-homology (DH) domain adjacent to a plekstrin homology (PH) domain\(^9, 10\). The DH domain is responsible for the GEF-activity, while the PH domain contributes to the intracellular localization of the protein\(^22\). Five-prime recombinations result in the loss of the amino-terminal half of proto-Dbl, producing a variant with elevated oncogenic potential, known as onco-Dbl\(^9\). This increased oncogenic potential is associated with increased GEF activity in vivo\(^9\). The molecular mechanisms that underlie the increased oncogenic potential exhibited by onco-Dbl are poorly understood.

One model for Dbl regulation is based on findings that proto-Dbl is a short-lived protein that is regulated by the ubiquitin-mediated proteolysis. Furthermore, onco-Dbl escapes this mode of negative regulation\(^8, 12, 21\). Recently, it was found that proto-Dbl is heavily ubiquitinated and accumulates upon proteasomal inhibition in vivo, and that this regulatory mechanism is attenuated in the case of onco-Dbl\(^12\). In addition, proto-Dbl was shown to interact with Hsc70\(^14\) and Hsp90\(^12\), molecular chaperones that aid in refolding misfolded proteins\(^4\). It is thought that these chaperones are involved in intracellular
‘triage decision’, determining whether a protein will be refolded or sent to the proteasome for degradation\textsuperscript{(4)}. Bi et al. proposed that an intramolecular interaction between the amino terminus of proto-Dbl and the PH domain maintains the protein’s basal, inhibited state in which the catalytic DH domain is not accessible to the substrate GTPase. Our objective was to determine whether other cellular factors are involved in regulating proto-Dbl’s activity, and to better understand the modes that regulate Dbl action.

The binding of proto-Dbl to both Hsc70 and Hsp90 suggests that these molecular chaperones play a role in regulating the stability of proto-Dbl\textsuperscript{(12, 14)}. Furthermore, a protein known as CHIP (carboxyl terminus of Hsc70-interacting protein) has been shown to interact with Hsc70 and therefore may also be important for the regulation of proto-Dbl\textsuperscript{(4)}. CHIP is a 35 kDa E3 ubiquitin-protein ligase that is responsible for attaching polyubiquitin chains to its substrates, thereby targeting them for proteasomal degradation\textsuperscript{(5)}. CHIP contains three tandem TPR (tetratricopeptide repeat) domains at the amino terminus that mediate binding to Hsc70\textsuperscript{(1)} and a Ubox domain encoding ubiquitin ligase activity\textsuperscript{(16)}. Here, we set to test the hypothesis that CHIP is the ligase that regulates proto-Dbl levels through ubiquitin-mediated proteolysis\textsuperscript{(12)}.

Furthermore, we used truncation mutants of CHIP to elucidate the importance of specific domains of CHIP in regulating proto-Dbl expression levels.
**Materials and Methods**

**Molecular Constructs.** The proto-Db1 (residues 1-925), onco-Db1 (residues 498-925) were previously described (14). S-tagged proto- and onco-Db1 were generated by PCR using primers containing in-frame amino terminal S-tag (Novagen), and ligated into the pCMV6 vector. PCEFL-GST-proto-Db1 and onco-Db1 were generated by ligation of the corresponding cDNAs into the pCEFL-GST vector, in frame with a 5’ GST tag (generous gift of Yi Zheng, Cincinnati Children's Hospital Medical Center, and Silvio Gutkind, National Institute of Dental and Craniofacial Research). 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. All constructs were verified by restriction mapping and sequencing at the Cornell BioResource 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 siRNA experiments.

**Antibodies.** Anti-Myc (9E10) and anti-GST antibodies were from Covance Inc. Anti-Db1 antibodies (sc-89 and sc-28582) were purchased from Santa Cruz Biochemicals. Anti-Hsc70 (SPA-815 and SPA-820) were from StressGen Biotechnologies. S-protein-HRP conjugates and S-protein cross-linked sepharose were from Novagen. Anti-Xpress tag
antibody was from Invitrogen. Alexa488-conjugated secondary antibodies were from Molecular Probes.

**Cell Culture.** Cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM). NIH3T3 cells were cultured with 10% bovine calf serum while COS7 cells were cultured with 10% fetal bovine serum. Cells were transfected with plasmid DNA using either FuGENE6 (Roche) or LipofectAmine2000 (Invitrogen).
Results

Proto-Dbl but not onco-Dbl interacts with CHIP. It was recently found that proto-Dbl is degraded by ubiquitin-mediated proteolysis\(^{(12)}\). Since steady-state expression levels of onco-Dbl are about 100-fold those of proto-Dbl, onco-Dbl appears to escape the mode of regulation that proto-Dbl is subject to\(^{(12)}\). Further support of this model is the observation that the half-life of proto-Dbl is significantly shorter than onco-Dbl\(^{(21)}\). As a ubiquitin-protein ligase, CHIP may play a role in this process\(^{(5)}\). To explore CHIP’s possible role in Dbl regulation, we transiently co-transfected COS7 cells with a plasmid encoding GST-tagged proto-Dbl, GST-tagged onco-Dbl or GST alone together with Myc-tagged CHIP. The cells were lysed forty-eight hours later and association between the GST proteins and CHIP were visualized by SDS-PAGE and western blotting (Figure 1). We found that CHIP interacts with proto-Dbl but not with onco-Dbl. This observation suggests that CHIP may be the ligase that attaches poly-ubiquitin chains to Dbl, thus ‘addressing’ it for degradation. Furthermore, since the spectrin domain is the only recognizable motif of proto-Dbl that is missing from onco-Dbl, this domain may mediate the interaction between proto-Dbl and CHIP.

Proto-Dbl but not onco-Dbl is ubiquitinated when CHIP is present. Data courtesy of Dr. Elena Kamynina\(^{(12)}\). The relative stability of onco-Dbl as compared to proto-Dbl suggests that proto-Dbl may be degraded at a faster rate. In order to determine if this difference is due to differential ubiquitination, COS7 cells were transiently co-transfected with either GST-tagged proto-Dbl or GST-tagged onco-Dbl together with Myc-tagged ubiquitin. The cells were treated with the proteasomal inhibitor MG132 and were lysed.
forty-eight hours after transfection. Cell lysates were incubated with glutathione sepharose beads and levels of ubiquitin and GST proteins were visualized by SDS-PAGE and Western blotting (Figure 2). We found that proto-Dbl, but not onco-Dbl, is heavily ubiquitinated in vivo. This suggests that proto-Dbl levels are regulated by ubiquitination and that CHIP may be the ubiquitin ligase that associates with proto-Dbl. Furthermore, this observation suggests that the spectrin domain, which is missing from onco-Dbl, may be responsible for Dbl’s association with CHIP.

**CHIP regulates the steady-state levels of proto-Dbl.** The selective interaction between proto-Dbl (but not onco-Dbl) and CHIP suggests that CHIP may be the ligase responsible for attaching a poly-ubiquitin chain to proto-Dbl, thus targeting it for degradation. In order to test this hypothesis, we transfected NIH3T3 cells with small interfering RNA (siRNA) specific for endogenous CHIP. As shown in Figure 3, transfection with the CHIP siRNA (but not with control siRNA) decreases the levels of endogenous CHIP in the cells by 51%. Furthermore, we observed that concomitant with the reduction in CHIP protein levels, the levels of proto-Dbl increased by 58%. At the same time, actin levels in the cell were not changed, suggesting that the effect of CHIP on Db1 levels is specific. The inverse relationship between the steady-state levels of CHIP and those of Dbl suggests that CHIP is involved in the degradation of proto-Dbl. It is likely that CHIP is the ligase that ubiquitinates proto-Dbl, probably by catalyzing its ubiquitination and proteasomal degradation.
CHIP regulates steady-levels of proto-Dbl but not onco-Dbl in a dose-dependent manner. The results from the siRNA experiment suggest that CHIP is a ubiquitin ligase that regulates proto-Dbl levels. Since onco-Dbl does not associate with CHIP (Figure 1), we hypothesized that the over-expression of CHIP would not change the expression levels of onco-Dbl. In order to specifically address this hypothesis, we asked whether the expression levels of onco-Dbl or proto-Dbl are altered by overexpression of CHIP. We co-transfected COS7 cells with s-tagged proto-Dbl or s-tagged onco-Dbl, together with increasing amounts of Xpress-tagged CHIP cDNA. The cells were lysed forty-eight hours after the transfection and protein levels were visualized by SDS-PAGE and Western blotting (Figure 4). We observed that as the expression levels of CHIP increased, expression levels of proto-Dbl decreased. In contrast, the expression levels of onco-Dbl were not affected by over-expression of CHIP. These observations indicate that CHIP is the ubiquitin ligase that regulates proto-Dbl levels and that onco-Dbl escapes this regulation.

Interaction between CHIP and proto-Dbl is mediated by CHIP’s Ubox domain. We then explored the domain(s) of CHIP that are important for regulation of proto-Dbl levels. Toward this end, we utilized truncation mutants that lack either the ubiquitin ligase domain (the ΔUbox construct in which a stop codon was inserted at residue 230\(^{(12)}\)), or the chaperone interaction domain (the ΔTPR construct, which had residues 32-145 deleted\(^{(4)(15)}\)). We found that proto-Dbl levels were not significantly affected by expression of the CHIP(ΔUbox) reagent, indicating that the ubiquitin ligase domain is critical for regulating Dbl levels.
CHIP(ΔTPR) mutant has a dominant negative effect on the regulation of proto-Dbl.

Interestingly, we found that upon expression of the CHIP(ΔTPR) construct, proto-Dbl levels increased in a dose-responsive manner (Figure 4A). Such dominant-negative behavior could be explained based on known existence of CHIP homo-dimers\(^{(17)}\). We hypothesize that each molecule in the dimer must bind to Hsc70 for its activity. Thus CHIP(ΔTPR), which cannot bind Hsc70, may dimerize with the endogenous CHIP, thereby sequestering it and inhibiting its ligase activity.
Discussion

The molecular mechanisms by which the regulation of the guanine nucleotide exchange factor Dbl is regulated are incompletely understood. Here, we propose a novel regulatory mechanism involving the regulation of proto-Dbl steady-state levels through ubiquitin-mediated proteasomal degradation. We identified CHIP as a ligase that facilitates ubiquitination of proto-Dbl, thus targeting it for proteasomal degradation. Importantly, we show that onco-Dbl escapes this mode of regulation, probably due to lacking the spectrin domain that mediates binding between the GEF and the molecular chaperones Hsc70 and Hsp90.

It has been shown that some members of the Dbl family, such as Dbl and Vav, display increased transforming potential when their amino terminus is deleted. This enhanced oncogenic potential was initially attributed to an auto-inhibitory function of Dbl’s amino terminus. Bi et al. suggested that through an intramolecular mechanism, Dbl’s amino terminus binds to its PH domain thereby limiting access of the GTPase to the catalytic DH domain of the GEF. Recent evidence suggests that this mechanism is more complex and involves several other factors. We observed that proto-Dbl, but not onco-Dbl, is heavily ubiquitinated suggesting that a ubiquitin ligase may play a role in the regulation of Dbl. Here, we identified this ubiquitin ligase as CHIP. We show that CHIP associates with proto-Dbl, but not onco-Dbl. Additionally, we observed that as CHIP levels increase, proto-Dbl levels decrease. Conversely, as endogenous CHIP levels decrease, the levels of proto-Dbl increase. Furthermore, we explored which domain(s) of CHIP is/are important for its function. Previous data provides as evidence that the Ubox domain is responsible for ligation of ubiquitin to the substrate protein. Our data
supports this conclusion, as we found that the steady-state levels of proto-Dbl levels did not change when the ligase-defective mutant CHIP(ΔUbox) was over-expressed. We also found that proto-Dbl levels increased upon over-expression of CHIP(ΔTPR), raising the possibility that this mutant somehow inhibits the activity of endogenous CHIP. This study describes a novel mechanism that regulates an oncogenic GEF and offers the possibility that other oncogenic proteins are similarly regulated.
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References


Figure Legends

Figure 1. Proto-Dbl but not onco-Dbl interacts with CHIP. Proto-Dbl (9 µg) and onco-Dbl (0.1 µg) in the pEBG vector were co-transfected with Myc-tagged CHIP (2 µg) using 10cm plates of COS7 cells with Fugene6 (Roche). The cells were lysed forty-eight hours later. The expression of the lysates was visualized by western blotting using anti-GST antibodies and anti-Myc antibodies (B). GST-tagged proteins were precipitated from the lysates using glutathione-agarose precipitation (5 hours, 4°C) and visualized by western blotting with anti-GST and anti-myc antibodies (A).

Figure 2. Proto-Dbl but not onco-Dbl is ubiquitinated in vivo. (Data courtesy of Dr. Elena Kamynina). Proto-Dbl and onco-Dbl (in pEBG vector) were co-transfected with Myc-tagged ubiquitin (in pCW7 vector). In order to prevent proteasomal degradation, the cells were treated with MG132 (25 µM for six hours) forty hours post-transfection. The cells were lysed and the lysates were visualized by running a western blot and incubating with anti-Dbl and anti-Myc antibodies. GST-tagged proteins were precipitated from the lysates using glutathione-agarose precipitation and ubiquitination was evaluated by western blotting with anti-Myc antibodies.

Figure 3. Expression of proto-Dbl increases with decreasing levels of endogenous CHIP. NIH3T3 cells were transfected with siRNA specific for endogenous CHIP (pSUPER vector) using LipfectAmine2000 (Invitrogen). They were lysed and the expression of Dbl and CHIP were visualized using anti-Dbl and anti-CHIP antibodies. As a control, the membrane was stripped and incubated with anti-actin antibodies.
Figure 4. Proto-Dbl levels *in vivo* decrease with increasing levels of wild-type CHIP, remain constant with increasing levels of CHIP(ΔUbox) and increase with increasing levels of CHIP(ΔTPR) while onco-Dbl levels remain unaffected. (A) COS7 cells were transfected with s-tagged proto-Dbl (in pCMV6 vector) and either Xpress-tagged wild-type CHIP, CHIP(ΔTPR) or CHIP(ΔUbox) (in pcDNA4.1 HisMax vector). Each CHIP construct was transfected in increasing amounts (0, 0.2 and 1.0 μg cDNA per 32mm plate). The cells were lysed forty-eight hours post-transfection and the expression of proto-Dbl and CHIP were visualized by western blotting. Proto-Dbl levels were quantified by measuring the intensity of the bands. These values were averaged from 3-5 independent experiments and were used to produce the graph shown. (B) Onco-Dbl levels were not affected by varying levels of CHIP or by using mutants of CHIP (experimental details described in panel A).
Figure 1

GSH-PD:
- GST
- GST-proto-DbI
- GST-onco-DbI
- GST
- CHIP

Input:
- GST
- GST-proto-DbI
- GST-onco-DbI
- WB: GST
- WB: myc
Figure 2
Figure 3
Figure 4