Regulation of the Dbl Proto-oncogene by Heat Shock Cognate Protein 70 (Hsc70)*

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The Dbl oncogene product is the defining member of a family of onco-proteins known as Dbl guanine nucleotide exchange factors (GEFs) that facilitate the activation of the small GTP-binding proteins Cdc42, Rac, and Rho. Oncogenic activation of proto-Dbl occurs through loss of the amino-terminal 497 residues, rendering the protein constitutively active. Because both onco- and proto-Dbl contain the structural elements required for GEF activity (i.e. the Dbl homology (DH) and pleckstrin homology (PH) domains), it is thought that the amino terminus of proto-Dbl somehow inhibits the biochemical activity of the protein. To better understand the molecular basis of this regulation, we set forth to identify cellular proteins that preferentially bind the proto-oncogenic form of Dbl. We identified the molecular chaperone heat shock cognate protein (Hsc70) as a binding partner that preferentially interacts with the proto-oncogenic form of Dbl. Dbl is complexed with Hsc70 in transfected cells, as well as in native mouse brain extracts. The interaction between Hsc70 and proto-Dbl is mediated by at least two regions in Dbl, the amino-terminal spectrin homology domain (residues 224–417) and the pleckstrin homology domain (residues 711–808). Overexpression of a dominant negative Hsc70 mutant leads to activation of proto-Dbl GEF activity, indicating that the chaperone negatively regulates proto-Dbl function in vivo. We propose that Hsc70 attenuates Dbl activity by maintaining an inactive conformation in which the amino terminus is “folded over” the catalytic DH-PH domain.

The Dbl oncogene was first identified in 1985 by transfection of fibroblasts with DNA from a human diffuse B-cell lymphoma (1). Since then, multiple other gene products have been described that bear strong sequence homology to the original Dbl protein (2). Operationally, Dbl family members have been defined as proteins that contain the tandem arrangement of a pleckstrin homology (PH) domain adjacent to a unique domain termed the Dbl homology (DH) domain. Many of these proteins possess high oncogenic potential and were found in gene transfer experiments to be potent fibroblast-transforming genes (3, 4). The physiological importance of Dbl family members is further underscored by their abundance in eukaryotic genomes (5). To date, most Dbl-family members have been documented to function as activators or guanine nucleotide exchange factors (GEFs) for Rho-like GTP-binding proteins (i.e. Cdc42, Rac, and Rho; reviewed in Refs. 2–4 and 6). Like all GTPases, Rho proteins function as binary molecular switches that are “on” in the GTP-bound state and “off” in the GDP-bound state. Deactivation is achieved by the intrinsic GTP hydrolytic capability of these proteins that is further stimulated by GTPase-activating proteins. GTPase activation, occurring in response to a variety of events (such as growth factor stimulation), is the reaction facilitated by GEFs that promotes dissociation of the bound GDP. GTP then re-binds to the GTPase, thus forming the active state of the molecule. Oncogenic activation of Dbl-like proto-oncogenes often occurs by a specific mutation or a chromosomal rearrangement event that leads to a truncation, resulting in unregulated GEF activity.

Current understanding of the chain of upstream events that lead to Dbl activation is extremely limited. One mode of regulation is provided by the PH domain, as it was shown to bind phosphoinositides (7) and because its deletion abolishes transformation by onco-Dbl (8). In addition, the fact that the amino terminus of proto-Dbl is lost during oncogenic activation suggests that this region exerts negative regulation on the DH-PH module. Indeed, Bi et al. demonstrated that co-expression of the amino terminus of Dbl leads to attenuation of onco-Dbl GEF activity, suggesting an auto-inhibitory regulatory mechanism (9). However, the molecular basis of this interaction is unknown, and it is unclear how proliferative signals affect it.

To better understand the molecular basis of Dbl regulation, we set forth to identify cellular proteins that preferentially bind the proto-oncogenic form of Dbl. Here we report the identification of the molecular chaperone heat shock cognate protein 70 (Hsc70) as a novel regulator of Dbl function.

EXPERIMENTAL PROCEDURES

Molecular Constructs—The proto-Dbl (residues 1–925), onco-Dbl (residues 498–925), DH-PH-Dbl (residues 498–825) (8), and T1-Dbl (residues 1–825) (9) genes were excised from pCMV6 or pZIP-Neo vectors with NcoI and HindIII and inserted into pCDNA3.1-Hygro (+) vector. To generate the pCDNA3.1-Hygro (+) vector, Hsc70 cDNA (kind gift of D. McKay, Stanford University) was amplified by PCR and inserted into the vector.

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Antibodies—Anti-HA (HA11), anti-Myc (9E10), and GST antibodies were from Covance Inc. Anti-Dbl antibody was purchased from Santa Cruz Biochemicals, and anti-Hsc70 antibodies (SIA 915 and SIA 820) were from StressGen Biotechnologies.

Identification of Hsc70 as a Proto-Dbl-binding Protein—Forty-eight hours post-transfection with pEBG-T1-Dbl or pEBG-DH-PH-Dbl, COS7 cells were lysed by rocking with 20 mM HEPES (pH 7.4), 1 mM EDTA, 150 mM NaCl, 1% Nonidet P-40, 20 mM sodium fluoride, 20 mM β-glycerophosphate, 1 mM activated sodium vanadate, 200 μM phenylmethylsulfonyl fluoride, and 10 μg/ml each leupeptin and aprotinin. Cleared lysates were incubated with glutathione agarose (Sigma) for 1.5 h at 4 °C. The beads were washed three times with lysis buffer, incubated with NIH 3T3 lysates (~30 mg protein) at 4 °C for 1.5 h, and washed three times. Bound proteins were resolved on SDS-PAGE and visualized with silver stain. A protein band at ~70 kDa that is selectively associated with the GST-T1-Dbl (but not with DH-PH-Dbl) was excised and sent for micro-sequencing analysis by tandem mass spectrometry at the Harvard Microchemistry Facility.

GTPase Activation Assays—To assess the level of activated (GTP-bound) Dbl substrates in cells, we utilized the “Rho binding assay” (10) as modified by Ren et al. (11). Briefly, COS7 cells were transfected with RhoA (HA-tagged in the pKH3 vector) together with the indicated Hsc70 (Myc- or HA-tagged in pcDNA3.1 vector) and Dbl (in pCMV6 vector) forty-eight hours post-transfection. The cells were serum-starved for 12 h and then lysed in 20 mM HEPES (pH 7.4), 150 mM NaCl, 1% Nonidet P-40, 10% glycerol, 10 mM MgCl₂, 1 mM EDTA, 0.2 mM phenylmethylsulfonylfluoride, and 10 μg/ml each leupeptin and aprotinin. Lysates were incubated with ~20 μg of the purified, GST-fused CRIB (Cdc42/Rac interactive binding) domain of rhoetin immobilized on glutathione agarose. After incubation at 4 °C for 2 h, the beads were washed and resolved on SDS-PAGE, and bound Rho protein was visualized by anti-HA immunoblotting.

Immunoprecipitations from Tissue—Frozen mouse brains were obtained from Pel-Freeze Inc. Thawed brains (1.4 g) were homogenized in an Eppendorf tube with minimal volume (1 ml) of 150 mM NaCl, 1 mM dithiothreitol, 1 mM EDTA, 150 mM NaCl, 25 mM Tris-HCl (pH 8.0), 0.25 mM phenylmethylsulfonylfluoride, and 5 μg/ml each leupeptin and aprotinin. Lysates were incubated with ~20 μg of the purified, GST-fused CRIB (Cdc42/Rac interactive binding) domain of rhoetin immobilized on glutathione agarose. After incubation at 4 °C for 2 h, the beads were washed and resolved on SDS-PAGE, and bound Rho protein was visualized by anti-HA immunoblotting.

Estimation of Complex Stoichiometry—COS7 cells were transfected with proto-Dbl cdNA (in pCMV vector), and lysates were prepared as described above. Anti-Dbl immunoprecipitates from three 100-mm plates were resolved on SDS-PAGE, and the amount of endogenous Hsc70 that associated with proto-Dbl was determined after staining.

RESULTS

Identification of Hsc70 as a Proto-Dbl-associated Protein—Our major goal in this study was to identify proteins that regulate the biological activity of proto-Dbl. Because the proto-oncogenic form of Dbl exhibits attentuated GEF activity (9) and diminished transformation potential (12) relative to the oncogenic form of Dbl, because little or no Hsc70 associates with the oncogenic form of Dbl, we tested whether a stable complex can be isolated from cells that express both proteins. We transfected GST-proto-Dbl or GST-onco-Dbl into COS7 cells that express Hsc70 and asked whether the proteins co-precipitate.

To verify the specificity and selectivity of the interaction between Dbl and Hsc70, we asked whether a stable complex can be isolated from cells that express both proteins. We transfected GST-proto-Dbl or GST-onco-Dbl into COS7 cells that express Hsc70 and asked whether the proteins co-precipitate. As can be seen in Fig. 1A (left lane), when proto-Dbl and Hsc70 are co-expressed in cells the chaperone is associated with precipitated proto-Dbl. The interaction is specific, as no Hsc70 is found in control (GST alone) precipitates. More importantly, Hsc70 selectively binds to the proto-oncogenic form of Dbl, because little or no Hsc70 associates with the oncogenic form of Dbl (Fig. 1A, center lane). In a reverse experiment, proto-Dbl was found to co-precipitate with ectopically expressed Hsc70 (Fig. 1B, middle lane), whereas onco-Dbl did not (Fig. 1B, left lane). To better characterize the interaction between Dbl and Hsc70, we evaluated relative amounts of the two proteins in complexes immunoprecipitated from transfected cells. We found that the complex contains the two proteins at a mole ratio of ~1:1 (data not shown). This observation suggests that in this context Hsc70 functions as an “escort” protein and not as a catalytic component. The selectivity and specificity of this interaction suggest the possibility that Hsc70 may function as a cellular regulator of Dbl activity.

Hsc70 and Proto-Dbl Associate in Vivo—Our next goal was to determine whether an Hsc70-Dbl complex exists in native tissue where the two proteins are expressed endogenously. Because expression of proto-Dbl is limited to tissues of neuroectodermal origin (4), we used mouse brain as source material for these studies. As can be seen in Figs. 2, A and B (left lanes), both proto-Dbl and Hsc70 proteins are present in the soluble fraction of mouse brain. When the extract is immunoprecipitated with a specific anti-Hsc70 antibody, proto-Dbl can be found in the precipitate (Fig. 2A, center lane). Similarly, when an anti-Dbl antibody is used for immunoprecipitation, Hsc70 co-precipitates with Dbl (Fig. 2B, center lane). Thus, we conclude that the proto-oncogenic form of Dbl exists in a stable complex with the Hsc70 chaperone in native brain extracts. To test whether the Hsc70/Dbl interaction is direct, we tried to reconstitute the complex using purified components. We could not detect significant binding using in vitro translated Hsc70 and insect cell-expressed proto-Dbl, suggesting that interaction between Hsc70 and Dbl involves additional cellular components. It should be noted in this regard that most Hsc70-substrate complexes contain a number of additional proteins that regulate the chaperone function (e.g. see Refs. 17, 24, 25). Also, truncation of the carboxyl terminus of Hsc70 is often required for its association with substrates in vitro (26).

Spectrin Repeats and the Pleckstrin Homology Domain of Proto-Dbl Mediate the Interaction with Hsc70—We next set out to identify the domain(s) in Dbl that mediates its association with Hsc70. As Hsc70 preferentially associates with the proto-native state and assist in their proper folding (13). Unlike the stress-induced (and highly homologous) Hsp70, Hsc70 is constitutively and ubiquitously expressed in virtually all cell types. Hsc70 activity is regulated by an ATPase cycle that governs substrate binding, release, and conformational changes. In addition to its “conventional” role in assisting in the proper folding of nascent and misfolded peptides, Hsc70 has recently been implicated in multiple other cellular functions. Thus, Hsc70 appears to regulate the activity of a number of proteins, including transcription factors (14, 15) and nuclear receptors (16–18). Hsc70 binding can also direct a substrate to proteosomal degradation (19, 20). Finally, Hsc70 was demonstrated to function as a clathrin-uncoating ATPase, a key regulator of receptor-mediated endocytosis (21–23).
oncogenic form of Dbl and because the proto- and onco-versions of Dbl differ only by the amino terminus of proto-Dbl, we hypothesized that the primary Hsc70 binding site resides in residues 1–498. Indeed, the isolated amino terminus of proto-Dbl (N-Dbl, residues 1–498) associates tightly with Hsc70 (Fig. 4A). We also tested the ability of two amino-terminal truncations, ΔN1-Dbl (residues 71–925) and ΔN2-Dbl (residues 349–925), to associate with Hsc70. We found that whereas deletion of residues 1–70 did not affect the interaction, removal of residues 1–348 from proto-Dbl amino terminus abolished the association between Dbl and Hsc70 (Fig. 4B). Thus, we conclude that an important interaction domain between Dbl and Hsc70 resides between residues 71–348 of the proto-oncogene. Taking into account the primary structure of that region (Fig. 3), we hypothesized that the interaction is mediated by the spectrin homology domain of Dbl (residues 224–417). As shown in Fig. 4C, we find that the isolated spectrin domain exhibits robust association with Hsc70. We conclude that the amino-terminal spectrin domain of proto-Dbl serves as a major binding “handle” for the complex with Hsc70. Interestingly, a con-
struct encoding the PH domain (residues 711–808; Fig. 3) also exhibited tight association with Hsc70 (Fig. 4). This observation was surprising, because onco-Dbl (which contains the PH domain) displays only weak affinity to Hsc70 (Figs. 1 and 4). We do not know the basis for the different affinities of Hsc70 toward the PH domain when the latter is in isolation versus

**FIG. 4.** Spectrin and PH domains of proto-Dbl mediate association with Hsc70. A, COS7 cells were transiently transfected with the indicated constructs in the pEBG vector. Endogenous Hsc70 was immunoprecipitated (IP) from cell lysates using an anti-Hsc70 antibody (αHsc70), and associated Dbl proteins were visualized by anti-GST immunoblotting. Asterisk denotes a nonspecific band. B, the indicated Dbl constructs were immunoprecipitated from transfected cells, and association with endogenous Hsc70 was assessed by anti-Hsc70 immunoblotting. C, COS7 cells were transiently transfected with the indicated Dbl constructs in the pEBG vector. GST-Dbl proteins were precipitated with glutathione-agarose, and associated endogenous Hsc70 was visualized with an anti-Hsc70 antibody. WB, Western blot.

**FIG. 5.** Hsc70 modulates the nucleotide exchange activity of Dbl in vivo. A, HA-tagged RhoA (in the pKH3 vector) and proto-Dbl (in the pCMV6 vector) were transfected into COS7 cells with either wild-type (wt) or K71M mutant Hsc70. After a 12-h starvation, lysates were blotted for the expressed proteins (upper two sections). The remaining lysates were subjected to GST-Rho-binding domain precipitations followed by anti-HA immunoblotting to visualize activated (GTP-bound) Rho (bottom section). B, a control experiment is shown in which the amino-terminal truncated (oncogenic) version of Dbl was used.

We do not know the basis for the different affinities of Hsc70 toward the PH domain when the latter is in isolation versus
when it is in the context of onco-Dbl. It is possible that this difference arises from different intracellular localization of the protein, a property that is known to be greatly affected by the PH domain (8). Interestingly, Bi et al. demonstrated binding between an amino-terminal fragment (residues 286–482) and the PH domain of Dbl previously (9). These authors suggested that this intramolecular association leads to masking of the catalytic (DH) domain, thus inhibiting the GEF activity of Dbl. The data presented here suggest that this interaction is indirect and is mediated by Hsc70.

**Hsc70 Modulates the Nucleotide Exchange Activity of Proto-Dbl in Vivo**—We hypothesized that if the Hsc70-Dbl interaction is physiologically significant, then interfering with Hsc70 activity should influence the biochemical activity of proto-Dbl in cells. To address this question, we made use of a known loss-of-function mutant of Hsc70 in which lysine 71 in the ATP binding pocket is substituted by a methionine (K71M). This mutation was shown to impair Hsc70 function by inhibiting its ability to hydrolyze ATP (26, 27). Importantly, ecytoplastic expression of the K71M construct in cultured cells was shown to interfere with endogenous Hsc70 function in a dominant negative fashion (28–30). To test the impact of Hsc70 on Dbl function, we co-transfected proto-Dbl and Hsc70 (wild-type or K71M mutant) into COS7 cells and measured the GEF activity of Dbl in vivo (see “Experimental Procedures”). As can be seen in Fig. 5, overexpression of wild-type Hsc70 leads to a marked reduction in cellular levels of GTP-bound RhoA, confirming that, in vivo, Hsc70 functions as a negative regulator of Dbl activity (Fig. 5A, compare lanes 1 and 2). Furthermore, overexpression of the dominant negative Hsc70(K71M) mutant leads to a significant increase in proto-Dbl activity (Fig. 5A, compare lanes 3 and 4). The lack of effect on RhoA-GTP levels in the absence of proto-Dbl expression (Fig. 5A, lanes 5 and 7) suggests that Hsc70 specifically impacts RhoA by attenuating proto-Dbl activity. To address the possibility that the chaperone modulates RhoGTP levels through other mechanisms (i.e. by stimulating an endogenous GTPase-activating protein), we assessed the effect of Hsc70 overexpression on the GEF activity of a Dbl construct that is defective in association with the chaperone. We observed that onco-Dbl, which is severely compromised in chaperone binding (Figs. 1 and 2), is not affected by overexpression of either wild-type Hsc70 or the K71M mutant (Fig. 5B). Taken together, our observations demonstrate that Hsc70 functions as a selective and specific negative regulator of proto-Dbl activity in vivo.

**Mechanism of Dbl Regulation by Hsc70**—In the studies described above, we identified two regions in proto-Dbl that mediate binding to Hsc70, the amino-terminal spectrin homology domain (residues 224–417) and the PH domain (residues 711–808). Because the Hsc70 (K71M) mutant activates proto-Dbl (Fig. 5), we wondered whether its binding interactions with Dbl are perturbed. We find that the Hsc70(K71M) mutant is indeed severely compromised in its ability to bind the amino terminus of Dbl (Fig. 6A), whereas its association with the PH domain is not affected (data not shown). These data support a mechanism in which Hsc70 mediates the inhibitory interaction between the amino terminus of Dbl and its PH domain (Fig. 7). Because the Hsc70(K71M) mutant is unable to bind the amino-terminal spectrin domain (Fig. 6A) and, thus, cannot maintain the inhibited conformation of Dbl, its overexpression results in GEF activation (Fig. 5). To test this hypothesis, we separately expressed the amino terminus (N-Dbl, residues 1–498) and the carboxyl terminus (either onco-Dbl, residues 498–925, or the isolated PH domain, residues 711–808) of Dbl. In agreement with an earlier report (9), both onco-Dbl and the isolated PH domain associate with N-Dbl (Fig. 6, B and C, leftmost lanes).

Strikingly, overexpression of Hsc70(K71M) disrupts the interaction between N-Dbl and either the PH domain (Fig. 6B) or the entire carboxyl terminus encoded on onco-Dbl (Fig. 6C). Importantly, the inhibitory activity of Hsc70(K71M) is dose-dependent (Figs. 6, B and C) and specific, because wild-type Hsc70 had no such effect (data not shown). Taken together, these data provide support for an intermolecular inhibition model whereby the association of Hsc70 with the amino-terminal and PH domain of Dbl maintains the inactive conformation of Dbl, thereby negatively regulating its GEF activity (Fig. 7).

**DISCUSSION**

The critical roles that GTP-binding proteins play in intracellular signaling pathways have been firmly established in the past decade. Specifically, members of the Rho GTPase family (Cdc42, Rac, and Rho) were shown to regulate multiple cellular activities through distinct and well-characterized downstream
signaling pathways (reviewed in Ref. 31). It is well established that the regulation of Rho-family GTPases is highly relevant to cancer, as activated alleles of these proteins were shown to promote transformation of cultured fibroblasts and to be tumorigenic in immunocompromised mice (32–40). In addition, the physiological activators of Rho-family proteins, i.e., GEFs from the Dbl family, comprise one of the largest families of cellular proto-oncogenes (2–4, 41).

Whereas a wealth of information has been uncovered regarding signal flow downstream of Dbl-family GEFs, very little is known about the upstream events that regulate their activity. Although mechanisms involving intramolecular inhibition were proposed for a number of Dbl homologues (42–44), limited experimental attention was given to this issue. In the case of Vav1, the proto-oncogene is auto-inhibited by an intramolecular association between an amino-terminal helix and the DH-PH module (45). Activation leads to detachment of the amino terminus from the DH-PH domain, whereupon Rac can interact with the catalytic module of the GEF and undergo nucleotide exchange. Along similar lines, Bi et al. have suggested that proto-Dbl exists in an auto-inhibited basal state in which the amino terminus and the DH-PH catalytic core interact (9). However, the mechanism regulating Dbl is likely to be more complex, because the “auto-inhibited” mode cannot be reconstituted in vitro (i.e., addition of purified amino terminus does not affect the GEF activity of onco-Dbl) (9).

To gain further insights into Dbl activation mechanism, we investigated whether other cellular proteins participate in the regulation of proto-Dbl. Using differential affinity precipitations, we identified Hsc70 as a novel Dbl-associated protein. We demonstrate here that the association with Hsc70 is specific and, importantly, highly selective to the proto-oncogenic form of Dbl (Fig. 1). Furthermore, an Hsc70/proto-Dbl complex is found in native brain extracts where both proteins are endogenously expressed (Fig. 2).

Fig. 7 summarizes our understanding of the role that Hsc70 plays in the regulation of Dbl. In quiescent cells, Hsc70 is complexed to proto-Dbl (likely together with other cellular factors) and maintains the catalytically inactive state. Receptor signaling leads to a conformational change in the chaperone and, consequently, to stimulation of the GEF activity of proto-Dbl.

The interaction between Hsc70 and Dbl appears to require additional cellular factors. This indeed is the case in multiple instances where Hsc70 functions as part of a multi-compo-
nent complex that regulates different cellular activities (17, 19, 20, 46, 47). Our future efforts will be directed toward unveiling the identity of other proteins that participate in the Hsc70-Dbl complex and toward gaining a better understanding of the mechanism by which this complex communicates with surface receptors.

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