Role of the pleckstrin homology domain in intersectin-L Dbl homology domain activation of Cdc42 and signaling

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Abstract

Intersectin-long (ITSN-L) contains the invariant Dbl homology (DH) and pleckstrin homology (PH) domain structure characteristic of the majority of Dbl family proteins. This strict domain topography suggests that the PH domain serves an essential, conserved function in the regulation of the intrinsic guanine nucleotide exchange activity of the DH domain. We evaluated the role of the PH domain in regulating the DH domain function of I TsN-L. Surprisingly, we found that the PH domain was dispensable for guanine nucleotide exchange activity on Cdc42 in vitro, yet the PH domain enhanced the ability of the DH domain to activate Cdc42 signaling in vivo. PH domains can interact with phosphoinositide substrates and products of phosphatidylinositol 3-kinase (PI3K). However, PI3K activation did not modulate I TsN-L DH domain function in vivo.

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1. Introduction

Members of the Dbl family of proteins function as guanine nucleotide exchange factors (GEFs) that activate Rho family GTPases (reviewed in Refs. [1,2]). Like Ras, Rho family GTPases function as regulated GDP/GTP switches, where Dbl family proteins catalyze guanine nucleotide exchange to promote formation of the active GTP-bound protein. Upon activation, Rho GTPases (with RhoA, Rac1, and Cdc42 being the best characterized) interact with a diverse spectrum of effectors to regulate actin cytoskeletal organization, gene expression, and cell cycle progression [3,4].

Mammalian Dbl family proteins now number over 50, with many identified initially as transforming proteins (e.g., Dbl, Vav, Ect2) [1,2]. All Dbl family proteins possess a catalytic Dbl homology (DH) domain that serves to accelerate the intrinsic GDP/GTP exchange activity of Rho family proteins. In addition to the DH domain, the vast majority of Dbl family proteins possess a pleckstrin homology (PH) domain. Although PH domains are found in other signaling proteins [5], the invariant positioning of a PH domain immediately COOH-terminal to the DH domain supports the notion that the PH domain is a critical mediator of DH domain function. In support of this possibility, mutation of the PH domain typically abolishes the transforming activity of Dbl family oncoproteins [1,2].

Current evidence attributes two possible functions to the PH domain of Dbl family proteins. First, the PH domain may promote Dbl protein association with the plasma membrane where Rho GTPase substrates reside. For example, the loss of transformation caused by mutation of the PH domain of Dbs and Lsc can be restored by adding a plasma membrane-targeting sequence [6,7]. Second, the PH

Abbreviations: DH, Dbl homology; PH, pleckstrin homology; PI3K, phosphatidylinositol 3-kinase; PtdIns(3,4,5)P3, phosphatidylinositide 3,4,5-triphosphate; I TsN-S, intersectin-short; I TsN-L, intersectin-long; GST, glutathione S-transferase; SRF, serum response factor

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domain may be critical for positive or negative modulation of the intrinsic catalytic activity of the DH domain. A comparison of the catalytic activity of the DH and DH–PH domains derived from several Dbl family proteins (e.g., Trio, Dbs) showed that the PH-domain-containing protein exhibited up to 100-fold greater exchange activity than that measured for the DH domain alone in vitro [8,9]. Conversely, the PH domain of other Dbl family proteins (Sos, Vav, and Dbl) may actually serve to negatively regulate DH domain activity, a function that is relieved by association with phosphoinositides. For example, there is evidence that products of agonist-stimulated phosphoinositide 3-kinases (PI3Ks), such as phosphatidylinositol 3,4,5-triphosphate (PtdIns(3,4,5)P3), can promote the in vivo activation of the DH domain of Vav and Sos through PH domain-dependent mechanisms [10,11]. Thus, PtdIns(3,4,5)P3 association with the PH domain is thought to facilitate the association of Dbl family proteins with the plasma membrane or to modulate DH domain catalytic activity or both. However, the critical structural determinants required for high-affinity binding to phosphoinositides are absent from the PH domains of Dbl family proteins [12,13]. Because PH domains significantly vary in phosphoinositide binding specificity and affinity [14,15], it is likely that the PH domains of Dbl family proteins will serve distinct and varied functions with regard to phosphoinositide interactions.

Intersectin (ITSN; also known as Dap-160, EHSH-1, and Ese-1) was originally identified as an adaptor protein that contains two Eps 15 homology (EH) domains followed by five Src homology 3 (SH3) domains [16,17]. Current evidence supports a function for ITSN in regulation of endocytosis and mitogenesis [18]. Subsequent studies have described a long isoform of ITSN (designated ITSN-L) that is created by alternative RNA splicing and is expressed primarily in the nervous system [19,20]. ITSN-L is distinct from the short form (ITSN-S) in that, in addition to the NH2-terminal EH and SH3 domains, it contains tandem DH and PH domains followed by a C2 domain at its COOH terminus. Thus, ITSN-L may function as a GEF for Rho family small GTPases and this activity may be regulated by PI3K activation.

We and others recently determined that the ITSN-L DH domain promotes GDP/GTP exchange on Cdc42, but not Rac1 or RhoA [21,22]. In addition, we also determined that the PH domain of ITSN-L binds to a broad spectrum of phosphoinositides, including PtdIns(3,4,5)P3, at low micromolar affinities in vitro [23]. However, phosphoinositide binding did not regulate the intrinsic catalytic activity of the DH domain. Finally, our structural analyses of the ITSN-L DH–PH domain showed that the PH domain is not in contact with the complexed Cdc42 substrate [24]. This contrasts with the PH domain of another Dbl family protein, Dbs, where the PH domain is in contact with bound Cdc42 or RhoA and is required for full DH domain catalytic activity in vitro [25]. The importance of the PH domain in ITSN DH domain function in vivo, and whether it is regulated by PI3K-stimulated phosphoinositides, has not been determined. In this study, we have analyzed the role of the PH domain in regulation of ITSN DH domain function and assessed the contribution of PI3K activation to its regulation. We found that the PH domain is dispensable for ITSN DH domain activity in vitro but not in vivo, and that PI3K is not a regulator of ITSN DH domain function in cells.

2. Materials and methods

2.1. Molecular constructs

A cDNA fragment corresponding to the DH domain of human ITSN-L (GenBank accession no. AF064244) was amplified using polymerase chain reaction (PCR). A 5′ DH primer (CTG CGG AAT TCC ATG GCA GAT ATG TTG ACC CCA ACT GAA) containing EcoRI and Neol sites and seven DH domain NH2-terminal residues was used in conjunction with a 3′ PH primer (CGC CTC GAG CTA CAG GCC TTC ACA CTG CAC GTG) containing an Xhol site and seven DH COOH-terminal residues to generate a 0.6-kb fragment. To subclone the DH–PH domain fragment, the same 5′ DH domain primer was used in conjunction with a 3′ PH primer (CGC CTC GAG CTA CAG GTA CGC TTT CTC GGC CTT) containing an Xhol site and seven PH domain COOH-terminal residues to generate a 1.1-kb fragment.

An NheI–BamHI cDNA cassette encoding the NH2-terminal myristoylation sequence from human lck (MGGC/GSSHPEDD) as well as c-Myc epitope and heptadistidine tags were ligated into NheI–BamHI-digested pcDNA3.1 to create a plasmid, referred to as pTag14, that contains NH2-terminal myristoylation signal and plasma membrane targeting sequences to direct localization to the plasma membrane [26]. ITSN DH and DH–PH PCR products were digested with EcoRI and Xhol before ligation in frame with appropriately digested pTag14 plasmid. Expression of ITSN DH and DH–PH domains was verified by immunoblotting with anti-Myc monoclonal antibody (clone 9E10; Roche Molecular Biochemicals).

2.2. Protein purification and guanine nucleotide exchange assays

cDNA fragments generated as described above were digested with Neol and Xhol and ligated into the appropriately digested bacterial expression vector, pET28a (Novagen). The bacterial expression constructs were transformed into the BL21 (DE3) strain of Escherichia coli and protein expression and purification were done as standard. A bacterially expressed glutathione S-transferase (GST) fusion protein containing sequence of human Cdc42 was purified as described previously [8]. Fluorescence spectroscopic
analysis of N-methylantraniloyl (mant)-GDP incorporation into GDP-loaded Cdc42 was carried out as described previously [27].

2.3. Cell culture and transient expression reporter gene assays

NIH 3T3 cells were seeded onto six-well culture dishes (1.25 × 10^5 cells/well) in growth medium (DMEM supplemented with 10% bovine calf serum), and transient transfections were performed by calcium phosphate co-precipitation followed by glycerol shock as previously described [28]. Twenty-four hours after transfection, cells were starved of serum for 16 h in DMEM supplemented with 0.5% calf serum to reduce the level of serum-stimulated activation of serum response factor (SRF). Lysates of transiently transfected cells were analyzed using enhanced chemiluminescence reagents using Monolight 1010 luminometer (Analytical Luminescence) as described previously [29]. The (SREm)2 reporter plasmid contains a luciferase gene whose expression is under control of an SRF-responsive promoter as has been previously described [30].

2.4. Subcellular fractionation analyses

NIH 3T3 cells (5 × 10^5 cells per 100-mm dish) were transiently transfected with 1 μg of pTag14 empty vector or pTag14 constructs encoding ITSN DH and DH–PH domains. Twenty-four hours after transfection, cells were starved of serum in DMEM supplemented with 0.5% calf serum for 16–18 h, washed with ice-cold phosphate-buffered saline, and swelled in HYPO buffer (2 M Tris, pH 7.4, 1 M MgCl_2) supplemented with 5 μg/ml leupeptin, 5 μg/ml aprotinin, 5 μg/ml pepstatin A, and 1 ng/ml phenylmethyl-sulfonyl fluoride. After 10-min incubation on ice, lysates were homogenized and 1 M NaCl was added to adjust the ionic strength to 0.15 M. Lysates (500 μl) were set aside for the total fraction (T) and the remaining 1-ml volume was centrifuged at 100,000 g for 30 min, and resuspended in 50 ml (T) or 100 ml (S and P) of electrophoresis sample buffer. Equal volumes of each fraction were resolved by SDS-PAGE and protein expression was determined following transfer to Immobilon-P membranes (Millipore) by immunoblotting with anti-Myc epitope (clone 9E10; Roche Molecular Biochemicals), anti-RhoGDI rabbit polyclonal (sc-360; Santa Cruz Biotechnology), or an anti-pan Ras (OP-40; Oncogene Research Products) mouse monoclonal antibody.

2.5. Pull-down assay for GTPase activation

ITSN-L-mediated activation of cellular Cdc42 was measured using a GST:Pak-RBD fusion protein for affinity purification of GTP-bound Cdc42 as previously described [22]. Pak-RBD is the fragment of Pak1 that binds preferentially to GTP-bound Cdc42 and Rac1. Subconfluent NIH 3T3 cells were transfected with expression plasmids encoding full-length or the DH–PH domain fragment of ITSN-L, then starved for 24–48 h in DMEM supplemented with 0.5% calf serum and lysed in 25 mM HEPES (pH 7.5), 150 mM NaCl, 1% NP-40, 0.25% Na-deoxycholate, 10% glycerol, 10 mM MgCl_2, supplemented with 1 mM PMSF. Lysates (250–400 μg) were then incubated with GST:PAK-RBD immobilized on glutathione beads for 30 min at 4 °C. The beads were then washed twice in cold lysis buffer and processed for SDS-PAGE followed by Western blot analyses with an anti-Cdc42 monoclonal antibody (C70820; Transduction Labs).

3. Results and discussion

3.1. The PH domain is dispensable for DH domain activity in vitro

We recently showed that the ITSN-L DH–PH is a Cdc42-specific GEF in vitro and in vivo [22]. However, the precise contribution of the PH domain to DH domain function has not been determined. Therefore, we first compared the activity of the isolated DH domain fragment of ITSN-L (residues 1229–1449) to the activity of the bacterially expressed fragment of ITSN-L that contains the DH domain and its COOH-terminal PH domain (residues 1229–1586, designated ITSN DH–PH) (Fig. 1). For that purpose, we employed fluorescence spectroscopic analyses to determine the ability of ITSN DH and ITSN DH–PH to catalyze the incorporation of mant-GDP into bacterially expressed GDP-preloaded proteins. We found that the DH–PH polypeptide and the DH domain fragment displayed nearly identical exchange activities toward Cdc42 (Fig. 2), but not toward Rac1 or RhoA (data not shown). These results suggest that the PH domain is dispensable for DH domain activation, and differ from previous observations where the DH–PH domains of Trio and Dbs exhibited greatly enhanced catalytic activities in vitro compared to their DH domain counterparts [8,9]. Finally, the DH domain alone retained specificity for Cdc42 and did not show activity toward RhoA or Rac1 in vitro (data not shown). Thus, the presence of the PH domain does not alter GEF exchange activity or specificity in vitro.

The lack of a requirement for the PH domain for the full catalytic function of the DH domain of ITSN-L is consistent with our determination of the three-dimensional structure of the ITSN DH–PH domain complexed with Cdc42, where the PH domain does not contribute to the Cdc42 binding interface [24]. This contrasts with the role of the PH domain in Dbs, which does form contacts with the bound Cdc42 or RhoA [25]. The contrasting functional and structural relationship of the PH domains with the DH domains of ITSN-L...
and Dbs emphasizes that PH domains can exhibit very distinct functions in different Dbl family proteins, and can serve positive, negative, or in the case of ITSN-L, no role in regulating the intrinsic catalytic activity of the DH domain.

3.2. The PH domain enhances ITSN-L function in vivo

Our exchange analyses in vitro indicated that the PH domain did not serve to enhance the catalytic activity of the DH domain (Fig. 2). However, it remains possible that the PH domain may still contribute to DH domain function in vivo. One approach for these analyses would be to utilize full-length ITSN-L. However, because there is a diversity of other protein–protein and protein–lipid interaction motifs in sequences flanking the DH–PH domain, we were concerned that any activities we observed could not be solely ascribed to the PH domain. For example, a recent study found that SH3A, B, and D domains of ITSN interact and inhibit a Rac1 GAP (GTPase activating protein called CdGAP), resulting in positive regulation of Rac1 activity [32], which may indirectly affect Cdc42 activation via the DH–PH domains. Therefore, we focused on the DH–PH fragment and compared its activity to that of the isolated ITSN DH domain in vivo. Our initial analyses with expression vectors encoding these fragments did not result in significant GEF activity in vivo. Although the proteins were expressed adequately, their association with membranes was weak. Moreover, we observed no differences in the localization patterns of the DH fragment compared to the DH–PH protein (data not shown). We reasoned that the lack of downstream activity was most likely due to improper membrane-targeting activity of the PH domain, which would facilitate the DH domain interaction with its membrane-bound GTPase substrates. Therefore, we decided to generate membrane-targeted versions of ITSN DH and ITSN DH–PH for use in these analyses.

We and others have used peptide sequences that signal for co-translational modification of proteins by the myristate fatty acid to target proteins to the plasma membrane [33]. Therefore, we generated expression vectors that encode NH2-terminal myristoylation signal sequence-tagged versions of ITSN DH and DH–PH. We first verified that the resulting peptides were indeed associated with membranes in vivo. NIH 3T3 cells transiently transfected with expression vectors encoding these myristoylation signal sequence-tagged peptides as well as full-length ITSN-L were subjected to fractionation by high-speed centrifugation into crude cytosolic (S100) or membrane-containing particulate (P100) fractions. Western blot analyses showed that ITSN DH and DH–PH domains were predominantly found in the P100 fraction, indicating that the myristoylation signal sequence did promote consistent association with membranes (Fig. 3A).

To investigate the contribution of the PH domain to ITSN-L DH domain function in vivo, we compared the ability of the DH and DH–PH domains to activate transcription from a luciferase reporter gene expressed from a promoter that is responsive to SRF activation. Hill et al. [34] have previously shown that Rho GTPases are activators of the SRF and we determined that SRF activation is also stimulated by Dbl family proteins [35]. When expressed in NIH 3T3 cells with the SRF reporter construct (and the β-
galactosidase transfection control), ITSN DH stimulated SRF activity 4-fold relative to the control empty vector (Fig. 3B). This level of SRF activity was comparable to that observed with the mutationally activated Cdc42(Q61L) (data not shown), indicating that membrane targeting of the DH domain stimulates its catalytic activity. By comparison, the ITSN DH–PH domains caused SRF activation that was elevated about 11-fold relative to the control vector. The DH–PH fragment was also more potent in promoting filopodia formation in Swiss 3T3 cells when compared to the DH domain alone (data not shown). This difference in activity was not due to lower levels of expression of the
isolated DH domain compared to the DH–PH fragment because Western blot analyses showed that the DH was consistently expressed at approximately 2- to 3-fold higher levels than the DH–PH fragment (Fig. 3C). Together, these results indicate that the presence of the PH domain potentiated DH domain activity in vivo. It remains possible, however, that the PH domain may not enhance all of ITSN functions. Such a possibility has been indicated in studies of the Dbl family GEFs Lfc and Lbc, where the PH domain was shown to be required for transformation, but not c-Jun activation or stress fiber formation, respectively [35,36]. Nevertheless, our data suggest that whereas it is not essential for full DH domain catalytic function in vitro, the PH domain appears to facilitate DH-domain-mediated signaling events in vivo. Moreover, the Cdc42-binding domain of the Cdc42 effector WASP (WASP-RBD) specifically blocked ITSN DH and ITSN DH–PH function (Fig. 3D)—but not Rac1(61L)-mediated SRF activation (data not shown)—suggesting that ITSN DH or DH–PH variants transmit their signals essentially through a Cdc42-dependent mechanism.

3.3. PI3K activation does not stimulate ITSN-L DH domain function in vivo

Our phosphoinositide binding analyses argued that products of PI3K may not be sufficient to activate the catalytic activity of ITSN in vitro [23]. However, it remains significantly possible that association of PtdIns(3,4,5)P3 with the PH domain may modulate ITSN-L DH domain activity in vivo as has been shown for Vav, Sos1, and Tiam1 Dbl family proteins [10,11,37]. To address this possibility, we determined if co-expression of a constitutively activated variant of PI3K (p110-CAAX; a membrane-targeted variant of the p110 catalytic subunit of PI3K) could potentiate ITSN DH–PH signaling activity. As shown in Fig. 4A, expression of either p110-CAAX or ITSN DH–PH alone caused a 5-fold stimulation of SRF activity. Surprisingly, co-expression of p110-CAAX with ITSN DH–PH caused about 2-fold enhancement in SRF activation, suggesting an additive effect of ITSN DH–PH and PI3K. This finding indicates that PI3K activation does not modulate ITSN-L DH domain catalytic activity in vivo. To directly evaluate this lack of synergism, we determined the effect of p110-CAAX co-expression on ITSN DH–PH-mediated stimulation of the formation of active GTP-bound Cdc42. For these analyses, we used a GST fusion protein containing the Cdc42 binding domain of the Cdc42 effector PAK (designated GST:PAK RBD) in a pull-down assay for Cdc42-GTP. Expression of ITSN DH–PH alone promoted the formation of Cdc42-GTP (Fig. 4B). Interestingly, co-expression of the activated PI3K subunit with ITSN DH–PH failed to cause further increase in Cdc42-GTP levels. Thus, PI3K activation does not cooperate with ITSN DH–PH in promoting increased downstream signaling.

The role of PI3K activation in regulating DH domain function is best characterized for Vav. Han et al. [11] previously determined that substrates of PI3K inhibited the catalytic activity of Vav, whereas products of PI3K stimulated this activity in vitro. Recently, they determined that substrates of PI3K promoted the interaction of the PH domain with the DH domain and prevented Rho GTPase binding to the DH domain, whereas products of PI3K disrupted this interaction and permitted GTPase binding [38]. Thus, PtdIns(3,4,5)P3 association with the PH domain...
is thought to modulate DH domain catalytic activity. However, because the critical structural determinants required for high-affinity binding to phosphoinositides are absent from the PH domains of Dbl family proteins [12,13], we questioned whether this mechanism of regulation will be extended to other Dbl family members. Our recent in vitro analyses, where we found that the catalytic activity of ITSN DH–PH was not stimulated by phosphoinositide interactions, argued against such a role [23]. Similar negative results were also seen with the DH–PH fragments of Tiam1 and Dbs. However, because these analyses were done under in vitro conditions, in the present study, we evaluated whether PI3K activation was involved in PH domain regulation of DH domain function in vivo. We found only an additive stimulation of ITSN-L DH domain-mediated signaling and formation of Cdc42-GTP in vivo. Thus, we suggest that PI3K is not a significant regulator of DH domain function for ITSN-L in cells.

In summary, our observations with the PH domain of ITSN-L highlight the diverse functions that this domain may serve in regulating the function of adjacent DH domains. Thus, while the majority of PH domains are invariantly positioned COOH-terminal to all DH domains, their roles in regulation of DH domain function are not invariant and may range from a positive to negative role in activation of Rho GTPases. Finally, although earlier studies supported an important role for PI3K activation in regulation of Dbl GTPases. Finally, although earlier studies supported an important role for PI3K activation in regulation of Dbl family protein function, the results from this study and others argue against such a role for at least a growing subset of Dbl family proteins.

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