# A Cdc42 Mutant Specifically Activated by Intersectin†

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ABSTRACT: The Rho family GTPase Cdc42 functions as a molecular switch and controls many fundamental cellular processes such as cytoskeletal regulation, cell polarity, and vesicular trafficking. Guanine nucleotide exchange factors of the Dbl family activate Cdc42 and other Rho GTPases by catalyzing the removal of bound GDP, allowing for GTP loading, and subsequent effector recognition ultimately leading to downstream signaling events. Analysis of existing structural data reveals that the Dbl exchange factor intersectin engages a strictly conserved GTPase residue of Cdc42 (tyrosine 32) in a unique mode with respect to all other visualized exchange factor−Rho GTPase interfaces. To investigate this differential binding architecture, we analyzed the role of tyrosine 32 of Cdc42 in binding, and stimulation by Dbl family exchange factors. Deletion of the hydroxyl side chain of tyrosine 32 substantially increases the affinity of Cdc42 for intersectin, yet severely cripples interaction with Dbs, a normally potent exchange factor of Cdc42. Moreover, Cdc42(Y32F) is exclusively activated by intersectin, while virtually unresponsive to other Cdc42-activating exchange factors in vitro and in vivo. Further, the structural determinants unique to intersectin, which permit selective recognition and concomitant stimulation of Cdc42(Y32F), have been defined. Cdc42 and other individual Rho GTPases receive input stimulatory signals from a multitude of Dbl exchange factors, and therefore, Cdc42(Y32F) could act as a valuable reagent for understanding the specific influence of ITSN on Cdc42-mediated signaling phenomena.

The Ras-related GTP-binding protein, Cdc42, is activated by a variety of extracellular stimuli and has been implicated in a number of fundamentally important cellular processes, including the establishment of cell polarity and motility through influences on the actin cytoskeleton, control of mitogenic signaling pathways, and the regulation of intracellular trafficking and endocytosis (*1*−*4*). Cdc42 is a member of the Rho family of small GTPases, and cycles between a GTP-bound active and a GDP-bound inactive conformation, thereby regulating its ability to recognize downstream targets and/or effectors. Tight regulation of the GTP-binding/GTPase cycle of Rho GTPases is a necessary requirement for its cellular function, and an altered regulation of this cycle gives rise to cellular transformation and tumor formation in nude mice (*5*, *6*).

To orchestrate the tight regulation of the GTPase cycle, GTP-binding proteins are controlled by distinct types of regulatory proteins. Particularly, the exchange of GDP for GTP has been suggested to be the rate-limiting step in the GTPase cycle, and this process is directly governed by guanine nucleotide exchange factors (GEFs)[1] (*7*). GEFs catalyze the removal of bound GDP from a GTPase, thus permitting GTP loading, and subsequent downstream effector recognition. Specifically, misregulation of Rho family GTPases is associated with malignant cellular transformation, underscoring the importance of understanding how an exchange factor stimulates its GTPase substrate (*8*, *9*).

Cdc42 receives stimulatory input from a multitude of proteins, including integrins, receptor tyrosine kinases, and G protein-coupled receptors (*10*). These diverse stimuli modulate the enzymatic capacity of guanine nucleotide exchange factors, resulting in Rho GTPase activation. Dbl family proteins encompass the most well understood class of GEFs for Rho family GTPases (*11*). More than sixty human members of the Dbl family have been identified to date, each possessing the minimal catalytic unit for these GEFs: a Dbl (diffuse B-cell lymphoma) homology (DH) domain directly preceding a pleckstrin homology (PH) domain (*12*). Cdc42 is directly activated by numerous Dbl proteins, including Vav, Dbl, Dbs, WGEF, Fgd1, and the long isoform of intersectin (ITSN) (*13*−*16*). Each GEF responds to unique upstream signals to catalyze removal of GDP within Cdc42 at specific cellular locations. For instance, ITSN is a multidomain scaffold protein that binds several endocytic proteins at the location of clathrin-coated vesicles, and has been established as an adaptor between endocytosis

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[1] Abbreviations: GEF, guanine nucleotide exchange factor; Dbl, diffuse B cell lymphoma; DH, Dbl homology; PH, pleckstrin homology; ITSN, intersectin; GAP, GTPase activating protein; wt, wild type.

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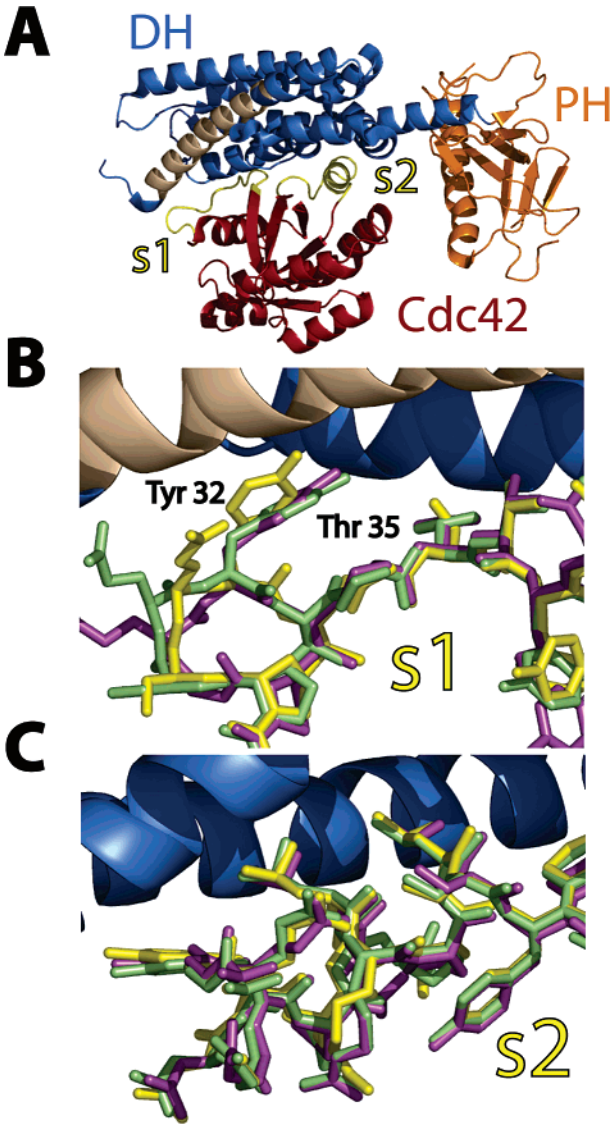


FIGURE 1: Comparison of the structural differences between RhoGEF−Rho GTPase complexes. (A) Ribbon representation of the DH domain (blue) and PH domain (orange) portion of ITSN in complex with nucleotide free Cdc42 (red) (PDB entry 1KI1). The α1 helix of the DH domain is colored wheat. The switch regions (s1 and s2) of Cdc42 are colored yellow. (B) Close-up of the ITSN−Cdc42 crystal structure displaying s1 of Cdc42 superimposed with the equivalent s1 regions of Rho GTPases from the Dbs−Cdc42 (PDB entry 1KZ7) (green) and Tiam1−Rac1 (PDB entry 1FOE) (purple) structures. (C) Close-up of the ITSN−Cdc42 crystal structure displaying s2 of Cdc42 superimposed with the s2 regions of Rho GTPases from the Dbs−Cdc42 and Tiam1−Rac1 structures. The coloring is as described for panel B.

and stimulation of actin polymerization mediated through activation of N-WASP by Cdc42 (*17*, *18*).

Atomic resolution structures of the catalytic portions (DH/PH domains) of several RhoGEFs bound to cognate nucleotide free Rho GTPases (Tiam1−Rac1, Dbs−Cdc42, Dbs−RhoA, ITSN−Cdc42, LARG−RhoA, and PDZ−RhoGEF−RhoA) (*19*−*23*) have been determined (Figure 1A), and afford a detailed examination of the mechanism of nucleotide exchange catalyzed by a Dbl protein. Each RhoGEF−Rho GTPase complex displays the all-helical DH domain utilizing conserved helical stretches to embrace the switch regions (regions within the nucleotide-binding pocket of GTP-binding proteins that undergo a conformational change upon association with either GTP or GDP) of the GTPase. RhoGEFs impart subtle changes in the nucleotide-binding cleft of the GTPase, ultimately resulting in the displacement of the bound magnesium cation, critical for stabilizing the negative charge stemming from the β- and γ-phosphates of the guanine nucleotide (*24*). The transient RhoGEF−nucleotide free Rho GTPase complex allows unhindered access to the guanine nucleotide binding pocket where excess GTP (relative to GDP) concentrations in the cellular environment drive subsequent GTP binding leading to remodeling of the switch regions resulting in dissociation of the GEF (*25*).

Comparison of the existing RhoGEF−Rho GTPase structural data reveals that upon GEF binding, the switch regions within each Rho GTPase adopt identical conformations that are important for expelling bound nucleotide (Figure 1B,C). Inspection of the Dbs−Cdc42, Tiam1−Rac1, Dbs−RhoA, and LARG−RhoA structures reveals a key interaction involving a highly conserved acidic residue (Glu639 of Dbs, Glu1047 of Tiam1, and Glu794 of LARG) within the α1 helix of the DH domain forming a hydrogen bond with the hydroxyl of a strictly conserved tyrosine within switch 1 of the GTPase (Tyr32 in Cdc42 and Rac1 and Tyr34 in RhoA) which constrains switch 1 in a position favorable for displacement of bound GDP. Notably, several studies have identified the importance of residue Tyr32 of Cdc42 and Rac1 in responsiveness toward GEF-mediated nucleotide exchange (*26*, *27*). However, unlike all other existing RhoGEF−Rho GTPase structures determined to date, within the ITSN−Cdc42 complex, the conserved Tyr32 of Cdc42 adopts a unique side chain configuration at the interface of the DH domain (Figure 1B). Specifically, the side chain of Tyr32 in the ITSN−Cdc42 complex is rotated ∼40° from its position relative to the Dbs−Cdc42 complex, and does not interact with the conserved acidic Glu1244 of ITSN. Instead, Tyr32 of Cdc42 contacts the aromatic ring of Tyr1241 of ITSN, and makes a hydrogen bond interaction with the backbone carbonyl of Gly1240 (Figure 2A). In an effort to understand the potential differential role of Tyr32 of Cdc42 in GEF-stimulated guanine nucleotide exchange by various Dbl proteins, we analyzed the effect of deletion of the hydroxyl of Tyr32 of Cdc42 using a range of biophysical and cellular-based techniques. These studies have identified a Cdc42 mutant that displays selective RhoGEF-mediated stimulation from ITSN over other similar Cdc42-activating Dbl family proteins.

## EXPERIMENTAL PROCEDURES

*Expression Constructs and Protein Purification*. Expression and purification of the DH/PH portions of ITSN and Dbs have been described previously (*20*, *21*). The coding region for amino acids 299−802 of human WGEF and amino acids 188−375 of mouse Vav1 were amplified by PCR and subcloned into pET21 (Novagen) to create hexahistidine−GEF fusion expression constructs. Both WGEF and Vav1 proteins were expressed and purified in a manner similar to that described for Dbs. The coding region for full-length Cdc42 was amplified by PCR and subcloned into pEGFP-C (Clonetech), generating a GFP−Cdc42 fusion construct. The above-mentioned DH/PH portion of murine Dbs and the DH/PH/C2 portion of human ITSN-L were amplified by PCR and subcloned into pCDNA (Invitrogen) to create hemagglutin (HA) and Myc fusions, respectively. All point muta-

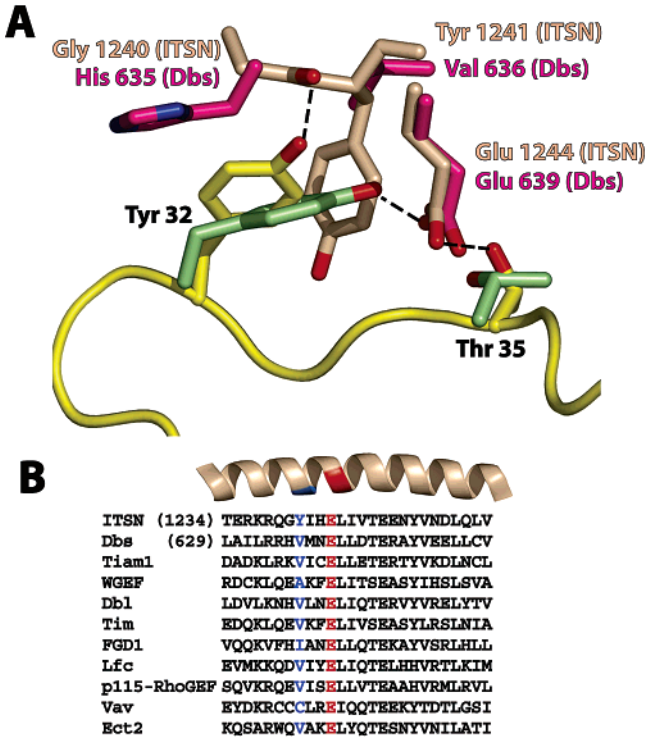


FIGURE 2: Unique binding mode of Tyr32 of Cdc42 within the ITSN−Cdc42 crystal structure. (A) Superposition of the GEF−GTPase interface of the ITSN−Cdc42 and Dbs−Cdc42 complexes. ITSN residues are colored wheat, and magenta for Dbs. Cdc42 from the ITSN−Cdc42 structure is colored yellow, and green for residues from the Dbs−Cdc42 structure. (B) Sequence alignment of the α1 helices of DH domains from a representative panel of Dbl family proteins. Key amino acid positions depicted in panel A are highlighted. The sequence alignment was constructed using ClustalX (*45*).

tions were generated using the Quick Change Site-Directed Mutagenesis Kit (Stratagene) and verified by nucleotide sequencing.

*Surface Plasmon Resonance.* The binding of Cdc42 to ITSN and Dbs was monitored using surface plasmon resonance (SPR) with a BIAcore3000 instrument (Biacore), at 25 °C, with a flow rate of 25 μL/min, using HBS-P buffer (Biacore) supplemented with 50 μM EDTA. Approximately 500 response units (RU) of ITSN-DH/PH was covalently immobilized to a CM5 sensor chip (Biacore) according to the manufacturer's protocol. For Dbs−DH/PH, approximately 500 RU of protein was captured on a NTA chip (Biacore) via the interaction of the hexahistidine tag of Dbs with the nickel-chelated surface. Injections of Cdc42 were performed with a KINJECT of 30 μL, with a dissociation time of 100 s. Between injections of GTPase, surfaces were regenerated with 1 mM GDP and 10 mM $MgCl_2$. Throughout the experiments, GEF sensor surface levels remained stable, even with the inclusion of EDTA in the running buffer over the NTA chip. All sensorgrams were normalized to the signal achieved due to binding a control "blank" flow cell surface. For kinetic evaluations, sensorgrams from each set of Cdc42 titrations (0.01, 0.05, 0.1, 0.25, 0.5, 1.0, and 5.0 μM) were aligned, and the normalized data were globally fit to a 1:1 Langmuir binding model (RI = 0), with BIAevaluation version 3.2 (Biacore). Thermodynamic and kinetic constants are the result of three independent sets of titrations.

*Guanine Nucleotide Exchange Assays.* The rate of guanine nucleotide loading into purified recombinant Cdc42 GTPase was analyzed using fluorescence spectroscopy with a PerkinElmer Life Sciences LS 50B spectrometer at 25 °C. Reaction mixtures were pre-equilibrated with 1 μM Cdc42−GDP, 100 μM *N*-methylanthraniloyl-GTP (Biomol), 20 mM Hepes (pH 8.0), 150 mM NaCl, 5 mM $MgCl_2$, and 1 mM DTT, prior to addition of 200 nM GEF. Nucleotide loading was measured by the tryptophan fluorescence signal ($\lambda_{ex}$ = 295 nm, $\lambda_{em}$ = 335 nm) upon addition of exchange factor as previously described (*24*). The rates of nucleotide loading ($k_{obs}$) were estimated from single-exponential decay best fits using GraphPad Prism. Fold activation values are reported as the ratio of the rate of catalyzed nucleotide loading divided by the rate of uncatalyzed (spontaneous) nucleotide loading.

*Cdc42 Activation Assay in Vivo Using the Limit Cdc42/Rac-Binding Domain from PAK.* The procedure for assaying the activation of Cdc42 has been previously described (*28*). HeLa cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS), and maintained in 5% $CO_2$ at 37 °C. For transfection, cells were cultured overnight to 80% confluency. The Effectene transfection procedures were performed according to the manufacturer's directions (Gibco-BRL). The cells were transiently transfected according to the manufacturer's protocol with the cDNA for either wild-type Cdc42 (0.5 μg) or Cdc42(Y32F) (0.5 μg) in pEGFP vector (Clontech) alone or in combination with myc-DH/PH/C2 of ITSN (0.5 μg) or HA-DH/PH of Dbs (0.5 μg) in pCDNA3 (Invitrogen). Cells were serum-starved for 24 h prior to lysis. Cell lysates were prepared by lysing cells at 4 °C and then rocking for 15 min with cold mammalian lysis buffer [20 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1% Triton X-100, 5 mM $CaCl_2$, and 5 mM $MgCl_2$] supplemented with the mammalian protease inhibitor cocktail (Sigma). The cell lysates were cleared by centrifugation at 12000*g* in a microcentrifuge at 4 °C, and the supernatants were transferred to a fresh tube before preclearing with glutathione−agarose (Amersham Pharmacia). Samples were next incubated for 1 h with 50 μL (1 μg/μL) of glutathione−agarose coupled GST−PBD (for p21-binding domain from Pak) that was expressed and purified from Bl21(DE3) cells. Beads were then precipitated by centrifugation, washed with mammalian lysis buffer supplemented with protease inhibitor cocktail, subjected to SDS−PAGE, and analyzed using Western blotting with antibody directed against GFP. Total lysate samples were subjected to SDS−PAGE, and membranes were sequentially blotted, probed, and washed using antibodies against myc (for myc−ITSN), HA (for HA−DBS), and GFP (for GFP−Cdc42). The presence of GST−PBD protein was visualized by SDS−PAGE, followed by Coomassie blue staining to ensure equivalent sample loading between each experiment.

*Microscopy.* Subconfluent HeLa cells were transfected with GFP−Cdc42 and GEF constructs exactly as described above for the Cdc42 activation assays. HeLa cells were plated on glass coverslips for 24 h post-transfection and allowed to grow for an additional 12 h in DMEM supplemented with 10% FBS. Cells were then serum-starved for 12 h and subsequently fixed in 4% (w/v) paraformaldehyde in PBS for 10 min, washed with PBS, and incubated in 10% FBS with PBS for 20 min. Antibodies directed against myc (9E10; Babco) and HA (Covance) epitopes were prepared as 1:500 dilutions into 0.1% saponin in 10% FBS with PBS and incubated on coverslips for 1 h. Coverslips were then washed

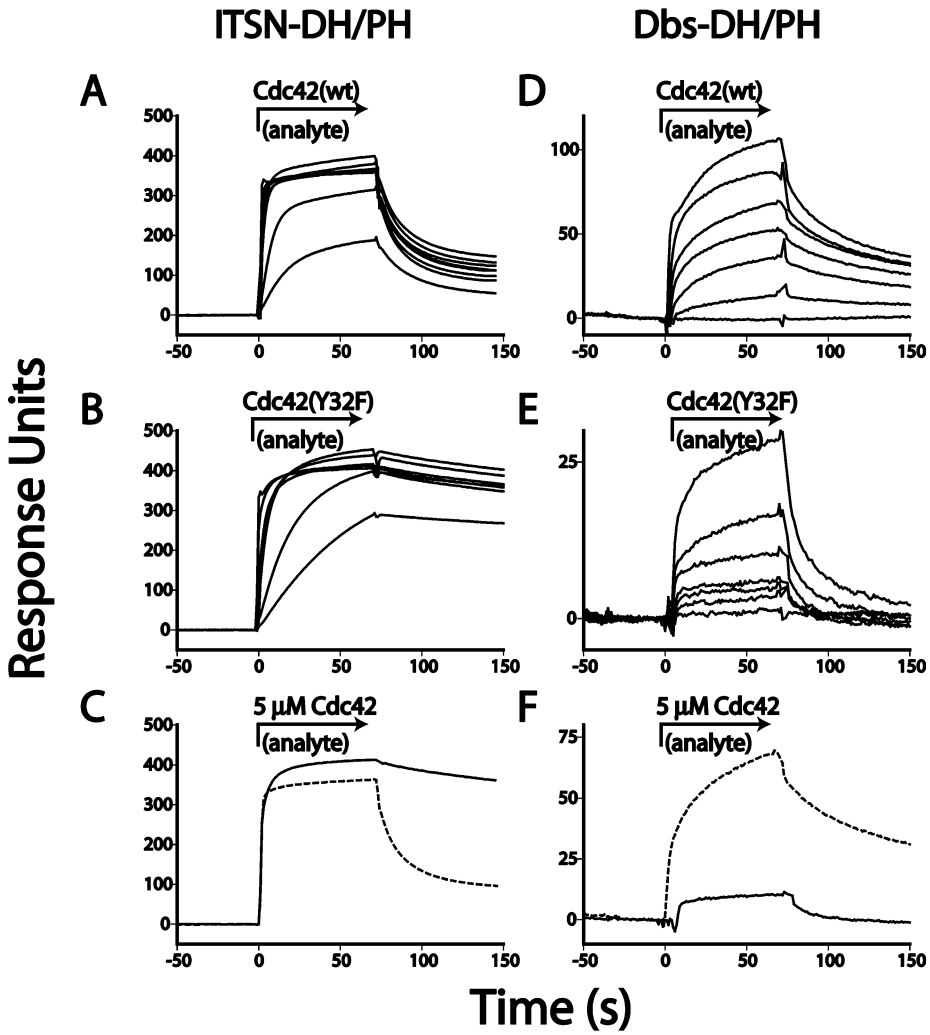


FIGURE 3: Differential binding to Cdc42(Y32F) by ITSN and Dbs. Cdc42(wt) and Cdc42(Y32F) protein analytes were injected at time zero for 72 s over surfaces of immobilized ITSN−DH/PH (A−C) or Dbs−DH/PH (D−F) proteins, followed by injection of running buffer to promote dissociation monitored using a Biacore 3000 instrument as described in Experimental Procedures. Panels A, B, D, and E display sensorgrams from titration sets of Cdc42 concentrations (0.01−5 $\mu$M) over the immobilized ITSN−DH/PH or Dbs−DH/PH surfaces. Panels C and F display comparisons of sensorgrams of individual injections of both Cdc42(wt) (dashed line) and Cdc42(Y32F) (solid line), each at 5 $\mu$M, over indicated GEF surfaces.

with 10% FBS with PBS three times for 5 min each. Texas Red-conjugated phalloidin and Donkey Cy5-conjugated AffiniPure anti-mouse Igs (Jackson ImmunoResearch Laboratories) were prepared as 1:1000 dilutions into 0.1% saponin in 10% FBS with PBS and incubated on coverslips for 1 h. Coverslips were then washed with 10% FBS with PBS for 5 min each with one final rinse in PBS. Coverslips were mounted by inverting them onto 20 $\mu$L of Fluoromount-G (Southern Biotech) and sealed. Slides were examined using an inverted confocal laser scanning microscope (model Leica TCS SP2) equipped with a 63 $\times$ 1.5 oil immersion objective. GFP, Texas Red, and Cy5 fluorescence was visualized using excitation filters at 488, 596, and 633 nm, respectively, in sequential scanning mode.

## RESULTS

Comparison of Cdc42 in complex with either the DH/PH portion of Dbs or ITSN reveals minimal conformational changes within the GTPase with the exception of a significant deviation occurring within switch 1 at residue Tyr32 of Cdc42. Importantly, several RhoGEFs (Dbs, Tiam1, and LARG) each make ~2.6 Å hydrogen bonds with their respective GTPase position equivalent to Tyr32 of Cdc42 (Tyr32 of Rac1 and Tyr34 of RhoA) using an invariant glutamate reside within the α1 helix of the DH domain (Figure 2). Notably, the interface of the DH domain of ITSN and switch 1 of Cdc42 shows Tyr32 of Cdc42 making significant $\pi$ orbital stacking with the side chain of Tyr1241 of ITSN, as well as making a polar contact with the main chain carbonyl of Gly1240. In turn, the conserved Glu1244 of ITSN is positioned in the spatial location equivalent to Glu639 of Dbs, yet does not contact Tyr32 of Cdc42, but rather makes a hydrogen bond with the polar side chain of Thr35 of Cdc42. To test the significance of these structural differences between key GEF and GTPase interface positions, and rule out incorrect model building of the ITSN−Cdc42 structure, we analyzed functional abilities of several Cdc42 and GEF interface mutants.

To analyze the significance of the side chain hydroxyl of Tyr32 of Cdc42 observed making differential interactions with various RhoGEFs, we evaluated both the wild type (wt) and Cdc42(Y32F) for affinity for the DH/PH portions of both ITSN and Dbs using surface plasmon resonance (Figure 3 and Table 1). As expected, the Cdc42(wt) protein displays

Table 1: Kinetic and Equilibrium Binding Data for the GEF−Cdc42 Interactions[a]

| | Cdc42(wt) | | | Cdc42(Y32F) | | |
|---|---|---|---|---|---|---|
| GEF | $k_{on}$ ($M^{-1}$ $s^{-1}$) | $k_{off}$ ($s^{-1}$) | $K_D$ (nM) | $k_{on}$ ($M^{-1}$ $s^{-1}$) | $k_{off}$ ($s^{-1}$) | $K_D$ (nM) |
| ITSN | $6.6 \times 10^5$ | $2.2 \times 10^{-2}$ | 33 | $3.7 \times 10^5$ | $6.0 \times 10^{-4}$ | 1.6 |
| Dbs | $9.5 \times 10^3$ | $1.4 \times 10^{-3}$ | 147 | $2.5 \times 10^3$ | $5.2 \times 10^{-3}$ | 2100 |

[a] Kinetic rates and equilibrium binding constants derived from the representative data displayed in Figure 3 were estimated from best fit global fitting analysis using BIAevaluation software, and are the average of three independent trials.

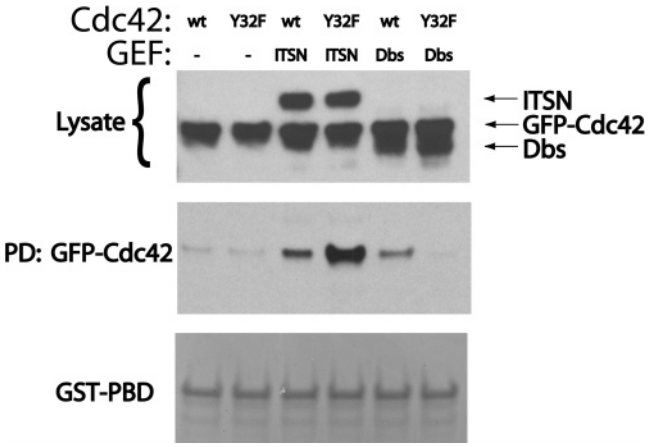


FIGURE 4: In vivo selective activation of Cdc42(Y32F) by ITSN. HeLa cells were transiently cotransfected with the indicated plasmids encoding GFP-tagged Cdc42(wt) or Cdc42(Y32F) in pEGFP, and either the DH/PH/C2 portion of ITSN or the DH/PH portion of Dbs in pCDNA or no GEF. Cells were serum-starved for 24 h, and the relative amount of activated (GTP-loaded) Cdc42 was assessed by Western blot analysis for its ability to recognize and therefore coprecipitate with the GST−PBD protein as presented in the PD (GFP−Cdc42 row). Total expression of GFP−Cdc42, myc−ITSN, and HA−Dbs proteins was visualized by Western blot analysis. GST−PBD protein was visualized by Coomassie staining.

significant affinity for immobilized recombinant ITSN−DH/PH and Dbs−DH/PH protein surfaces. In the presence of EDTA, known to promote GEF−GTPase interactions by destabilizing bound GDP via chelation of $Mg^{2+}$, the affinity constants for these measured interactions are 33 and 140 nM for ITSN and Dbs, respectively. In contrast, Cdc42(Y32F) binds the ITSN−DH/PH surface with an increased affinity constant ($K_D$ = 1.6 nm) relative to that of Cdc42(wt), yet binds the Dbs−DH/PH surface with a 15-fold weakened affinity ($K_D$ = 2100 nm) relative to that of Cdc42(wt). Additionally, real-time binding measurements taken using surface plasmon resonance demonstrate that, relative to Cdc42(wt), Cdc42(Y32F) displays ~20-fold enhanced binding affinity for ITSN exclusively in the form of a decreased dissociation rate constant (Table 1). Therefore, removal of the hydroxyl moiety of Tyr32 of Cdc42 increases the binding affinity for ITSN, with this improved affinity stemming entirely from slower dissociation kinetics, yet diminishes the affinity for Dbs.

The capacity of RhoGEFs to activate Cdc42(Y32F) was next assessed in vivo. Serum-starved HeLa cells were transfected with either GFP−Cdc42(wt) or GFP−Cdc42-(Y32F), and either constructs containing the catalytic portion of ITSN, Dbs, or no GEF construct (Figure 4). The amount of activated (GTP-loaded) Cdc42 within the cells was assessed for its ability to recognize the Cdc42/Rac1 binding region of p21-activated kinase (Pak), a known effector of Cdc42, and therefore coprecipitate with the glutathione-bound, recombinant GST-fused Cdc42/Rac1 binding region of Pak. As expected, single transfection with either Cdc42-(wt) or Cdc42(Y32F) produces negligible amounts of activated GTPase, as displayed in the PD (GFP−Cdc42 row). Importantly, cotransfection of Cdc42(Y32F) and ITSN yields the largest proportion of precipitated GTPase, relative to the other Cdc42(wt)/ITSN, Cdc42(Y32F)/Dbs, and Cdc42(wt)/Dbs cotransfections. These data strongly suggest that Cdc42-(Y32F) is potently activated by ITSN, and not Dbs.

The steady-state activation of Cdc42(Y32F) was next examined by looking at actin polymerization events in serum-starved HeLa cells (Figure 5). As in the Pak binding experiments described above, cells were cotransfected with GFP−Cdc42(wt) or GFP−Cdc42(Y32F), and constructs containing the DH/PH portion of ITSN or Db, and evaluated for GFP−Cdc42 expression by GFP signal (green), GEF expression by staining with Cy5 directed against the myc or HA epitopes (blue), and fillipodial formation by staining with Texas Red-conjugated phalloidin (red). Each transfection produces a GFP fluorescent signal suggesting successful expression of GFP−Cdc42; however, only the Cdc42(Y32F)/ITSN cotransfection results in detectable levels of actin fillipodia, a hallmark of Cdc42 signaling (arrows in Figure 5). While these experiments utilize constructs that express truncated portions of ITSN (DH/PH/C2) and Dbs (DH/PH), and not the full-length multidomain proteins, these data are consistent with a model of ITSN-specific stimulation of Cdc42(Y32F) compared to Dbs.

As highlighted in Figure 2, ITSN and Dbs contact Tyr32 of Cdc42 utilizing distinct DH domain positions. Indeed, compared to all existing RhoGEF−Rho GTPase structures, only ITSN utilizes an aromatic side chain residue (Tyr1241) to embrace Tyr32 of Cdc42. Inspection of a multiple-sequence alignment of various DH domains from within Dbl proteins reveals that Tyr1241 of ITSN is unique in that all other GEFs possess small nonpolar side chains at this equivalent position, which cannot form a $\pi$ orbital stacking interaction. Therefore, Tyr1241 of ITSN was evaluated as a primary determinant for specific binding and stimulation of Cdc42(Y32F).

Using a well-established in vitro fluorescent guanine nucleotide exchange assay (*24*), the rates of GTP loading within Cdc42(wt) and Cdc42(Y32F) in response to GEF were measured (Figure 6). As previously reported (*29*), the DH/PH portions of ITSN and Dbs both stimulate Cdc42(wt) exchange activity potently with stimulation values of 48- and 52-fold, respectively, compared to spontaneous (uncatalyzed) rates of nucleotide exchange (Table 2). Significantly, ITSN retains the ability to activate Cdc42(Y32F) with a rate enhancement of 42-fold, while Dbs is severely crippled toward Cdc42(Y32F) (rate enhancement of 2.9-fold). Importantly, mutation of Tyr1241 of ITSN to mimic the equivalent amino acid in Dbs (Val636) alters ITSN, making

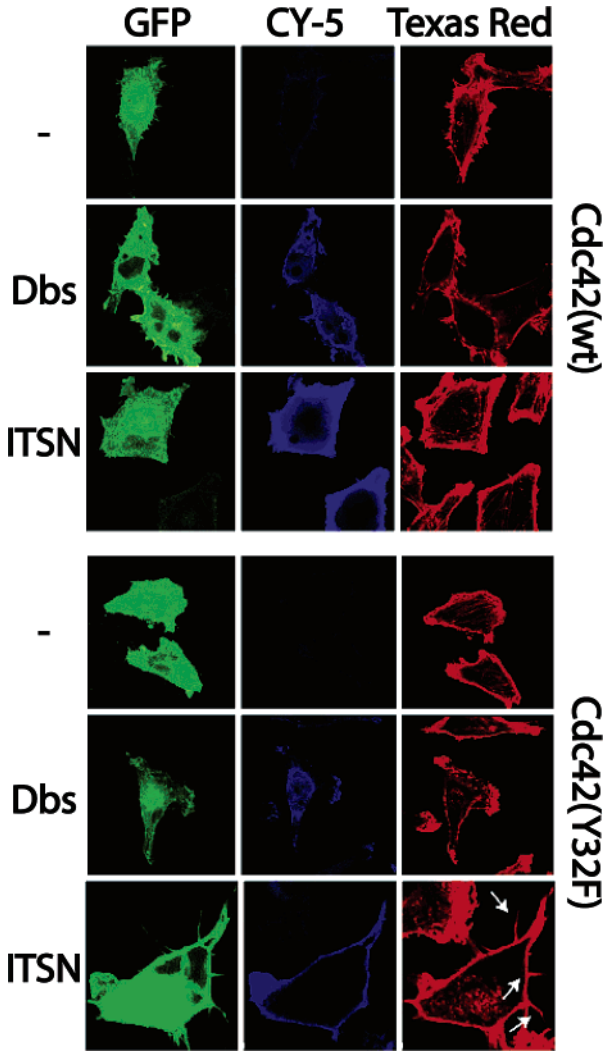


FIGURE 5: Cdc42(Y32F)-induced fillipodia formation is selectively activated by ITSN and not Dbs. Fixed HeLa cells were visualized for GFP expression (left column, green), Cy5 staining for RhoGEF expression (center column, blue), and Texas Red-conjugated phalloidin staining for actin (right column, red), after cotransfection with the indicated GFP−Cdc42 construct, and either no GEF (−), Dbs (HA−Dbs), or ITSN (myc−ITSN) as described in Experimental Procedures.

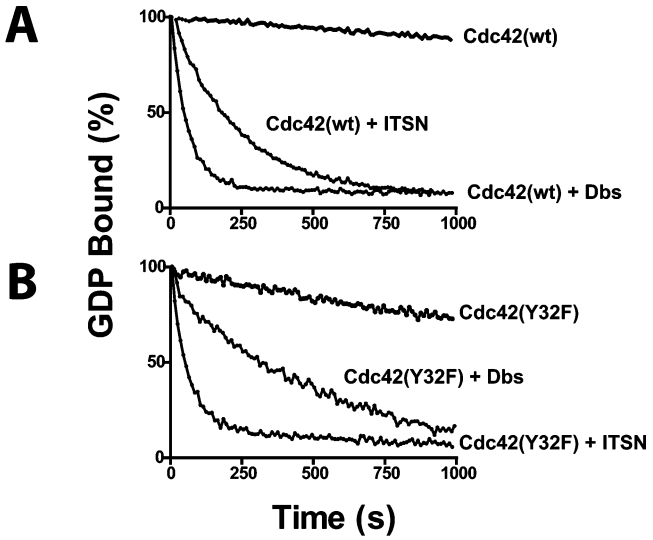


FIGURE 6: Specific stimulation of nucleotide exchange activity within Cdc42(Y32F) by ITSN. Representative data displaying tryptophan fluorescence changes due to *N*-methylanthraniloyl-labeled GTP loading within Cdc42(wt) (A) and Cdc42(Y32F) (B), upon addition of guanine nucleotide exchange factor as described in Experimental Procedures. Estimated rates of GTP loading and corresponding rate enhancement values are presented for the complete set of performed nucleotide exchange experiments in Table 2.

Table 2: Rate Enhancement of GTP Loading Provided by RhoGEFs within Cdc42(wt) and Cdc42(Y32F)[a]

| | Cdc42(wt) | | Cdc42(Y32F) | |
|---|---|---|---|---|
| GEF | $k_{obs}$ ($\times 10^{-4}$ s$^{-1}$) | *x*-fold stimulation | $k_{obs}$ ($\times 10^{-4}$ s$^{-1}$) | *x*-fold stimulation |
| no GEF | 1.6 ± 1.2 | − | 6.2 ± 1.3 | − |
| ITSN | 77 ± 22 | 48 | 260 ± 120 | 42 |
| ITSN(Y1241V) | 40 ± 14 | 25 | 40 ± 18 | 6.5 |
| Dbs | 83 ± 36 | 52 | 18 ± 10 | 2.9 |
| Dbs(V636Y) | 69 ± 42 | 43 | 26 ± 15 | 4.2 |
| WGEF | 19 ± 5 | 12 | 12 ± 7 | 1.9 |
| Vav1 | 43.2 ± 12 | 27 | 22.9 ± 8 | 3.7 |

[a] Kinetic rates ($k_{obs}$) were estimated from single-exponential decay fits of GTP loading measurements within Cdc42 GTPases in the presence or absence of the indicated GEF as highlighted in Figure 6. *x*-fold stimulation values represent the ratio of the rates of catalyzed to uncatalyzed nucleotide exchange.

it behave like Dbs with retained activity on Cdc42(wt) (rate enhancement of 25-fold), yet a severely diminished capacity for Cdc42(Y32F) (rate enhancement of 6.5-fold). Conversely, replacement of Val636 of Dbs with a tyrosine side chain does not modify the activity of Dbs toward Cdc42(wt) or Cdc42(Y32F). Additionally, we assessed WGEF and Vav1, Cdc42-activating Dbl proteins that possess alanine and cysteine residues, respectively, at the position equivalent to Tyr1241 of ITSN for catalytic nucleotide exchange ability toward Cdc42(wt) and Cdc42(Y32F). As expected, a fragment of recombinant WGEF encompassing the DH/PH/SH3 domains, and the DH domain of Vav1, both stimulate nucleotide exchange within Cdc42(wt), but are dramatically less active toward Cdc42(Y32F). Together, these combined data strongly suggest that Cdc42(Y32F) displays specific responsiveness toward ITSN over other Cdc42-activating RhoGEFs: Dbs, WGEF, and Vav1. Additionally, structural and biochemical evidence implicates the phenyl group of Tyr1241 of ITSN as the chief determinant for this selective interaction, and corresponding enzymatic activity toward Cdc42(Y32F).

## DISCUSSION

This study has employed structure-based analysis of the interfaces between multiple RhoGEF−Rho GTPase complexes to characterize a mutant Cdc42 protein capable of solely responding to a single Dbl family exchange factor in vitro and in a cellular context. Specifically, the hydroxyl of Tyr32 of Cdc42 has differential effects on DH domain recognition of Cdc42. If ITSN is excluded, all other structurally examined Dbl proteins make a functionally important hydrogen bond with the equivalent hydroxyl of Tyr32 of Cdc42 using an invariant glutamate residue within the α1 helix of DH domains (Figure 2). In contrast, the ITSN−Cdc42 interface presents a unique architecture. As the hydroxyl of Tyr32 of Cdc42 is 4.5 Å from the side chain carboxylate of Glu1244 of ITSN, Thr35 of Cdc42 adopts a

unique rotamer with respect to all other RhoGEF−Rho GTPase complexes to stabilize Glu1244 of ITSN with a hydrogen bond. The unique positioning of Tyr32 of Cdc42 when in complex with ITSN is maintained by apparent significant partial $\pi$ orbital stacking with Tyr1241 of ITSN, and a polar backbone contact with the carbonyl of Gly1240.

The mechanistic effect of deletion of the hydroxyl of Tyr32 of Cdc42 is twofold. First, Cdc42(Y32F) cannot foster a polar contact with the conserved DH domain residue equivalent to Glu1244 of ITSN, thus severely impairing most GEFs (except ITSN) from stabilizing switch 1 of the GTPase in the critical conformation for destabilization of bound GDP and $Mg^{2+}$. With respect to this claim, our data show that Dbs, WGEF, and Vav1 have significantly impaired nucleotide exchange capacity for Cdc42(Y32F). In contrast, ITSN retains potent catalytic function toward Cdc42(Y32F). Second, the nonpolar Y32F side chain of Cdc42 can now more readily stack with the aromatic Tyr1241 of ITSN to form a tight complex. This notion is supported by the dramatic increase in the affinity of ITSN for Cdc42(Y32F) [~20-fold relative to that for Cdc42(wt)] due exclusively to the slower dissociation kinetics of the GEF−GTPase complex. Unlike ITSN, Dbs displays an ~14-fold loss in affinity for the mutant Cdc42 protein, with this reduction in affinity observed in both association and dissociation kinetics.

A survey of Dbl proteins with exchange capacity for Cdc42 reveals that only ITSN possesses an aromatic residue at the position equivalent to Tyr1241 to presumably effectively restrain Tyr32 of Cdc42 away from contacting the conserved glutamate residue within the $\alpha$1 helix of DH domains of Dbl proteins (Figure 2). Our data demonstrate the importance of the aromatic side chain of Tyr1241 of ITSN in selective nucleotide exchange activity toward Cdc42(Y32F), as ITSN(Y1241V) behaves like Dbs, WGEF, and Vav1 displaying potent exchange activity toward Cdc42(wt), but severely crippled catalytic function toward Cdc42(Y32F). However, Dbs could not be straightforwardly converted to activate Cdc42(Y32F) by the opposite V636Y mutation, suggesting that other determinants of Dbs preclude Tyr32 of Cdc42 from embracing the V636Y position of Dbs. It is very likely that the bulky side chain of His635 of Dbs would sterically hinder the approach of Tyr32 of Cdc42 toward Dbs(V636Y), given that the equivalent DH domain position within ITSN lacks any side chain substituent (Gly1240). Nonetheless, our data clearly define Tyr1241 of ITSN as the chief determinant for selective recognition of Cdc42(Y32F).

It is at present unclear why the DH domain of ITSN utilizes a distinct binding architecture to stabilize the nucleotide free state of Cdc42. Previous work by several groups has clearly defined how Dbl proteins exploit nonconserved surface-exposed positions within the vicinity of the switch regions of Rho GTPases to discriminate between individual Rho GTPase substrates (*21*, *26*, *30*). In contrast, Tyr32 of Cdc42 is an invariant Rho GTPase position critical for GEF-stimulated nucleotide exchange activity. Additionally, the SopE virulence factor from *Salmonella enterica* functions as a GEF toward Cdc42 during the infection of mammalian host cells (*31*). While the catalytic portion of SopE does not resemble the tertiary structure of a DH domain, SopE targets a similar set of Cdc42 residues including a hydrogen bond contact with Tyr32 of Cdc42 by the acidic Asp124 of the virulence factor (*32*). Functional testing verified that deletion of the side chain of Asp124 of SopE renders the GEF unable to activate Cdc42 in vitro and in vivo. Therefore, RhoGEFs with distinct three-dimensional architectures from divergent organisms both make critical polar interactions with Tyr32 of Cdc42 for proficient nucleotide exchange. While other mammalian classes of RhoGEFs with nucleotide exchange capacity for Cdc42 have been identified, like Zizimin (*33*), it is tempting to speculate that these RhoGEFs also utilize the hydroxyl of Tyr32 of Cdc42 to effectively activate Cdc42, and that Cdc42(Y32F) is truly responsive to only ITSN.

Several mutations within nucleotide binding elements of the Ras superfamily and heterotrimeric GTPases have been identified which influence the nucleotide state, and hence the signaling capacity of GTPases. The G12V and Q61L mutations of Ras, and the equivalent individual mutations within many other GTPase proteins, both produce a constitutively active oncogenic phenotype (*34*). Both G12V and Q61L severely destabilize formation of the transition state of GTP hydrolysis, yet do not destroy binding to effector proteins (*35*, *36*). Additionally, Ras(D119N) displays reduced nucleotide affinity, and a dominant-negative phenotype in vivo (*37*). Further, the F28L mutation within Cdc42 [and also Rac1(F28L) and RhoA(F30L)] destroys a conserved key interaction with the nucleotide base to create a "fast-cycling" phenotype where the mutant GTPases spontaneously load GTP at rates normally achieved only with GEF participation (*9*). In contrast, as demonstrated through both in vitro and in vivo experimental formats, Cdc42(Y32F) represents a unique class of GTPase mutants which denies functional access to normally potent Cdc42-activating Dbl proteins, with the exception of ITSN.

A plethora of atomic resolution structures of various GTPases in different stages of the GTPase cycle implicates the conserved Tyr32 position within switch 1 of Cdc42 as possessing a functionally important role in regulation of nucleotide binding (*38*, *39*). Specifically, structures of the Rap2A GTPase bound to GDP, GTP, and a stable GTP analogue (GTP$\gamma$S) reveal that Tyr32 undergoes a transition from solvent-exposed to buried upon alternating between the GDP and GTP conformations (*40*). Interestingly, Tyr32 is observed making contacts with the $\gamma$-phosphate of GTP only in the Rap2A GTP-bound structure, but this interaction has been suggested to be dispensable for GTP loading. Our findings suggest that deletion of the phenol hydroxyl group of Tyr32 of Cdc42 results in ~4-fold greater intrinsic GTP loading, and activated Cdc42(Y32F) retains the ability to recognize p21-activated kinase in vivo. Additionally, a previous study characterized Cdc42(Y32F) as being severely impaired in its ability to hydrolyze GTP in response to Cdc42GAP (*41*). Importantly, this inability to hydrolyze GTP was not due to a decreased affinity of Cdc42(Y32F) for Cdc42GAP. Currently, it is not clear whether Cdc42(Y32F) can effectively engage all known Cdc42 effector proteins. The equivalent Y32F mutation in H-Ras does not affect binding to Raf-1 or the *Saccharomyces cerevisiae* isoforms of adenylyl cyclase, but activation of Raf-1 is weakened (*42*). Overall, although Tyr32 of Cdc42 has an intimate role in stabilizing switch 1 of the GTPase when in complex with both GEFs and GAPs, the subtle Cdc42(Y32F) mutant retains a functional ability to respond to ITSN, load GTP, and

recognize various downstream effector molecules with crippled ability to undergo GAP-stimulated GTP hydrolysis.

Our results support the differential interfacial architectures observed between the ITSN−Cdc42 structures and other published DH domain−Rho GTPase complexes. This analysis has spawned the functional characterization of an ITSN-specific Cdc42 mutant. As the Rho family GTPase member Cdc42 receives multiple input stimulatory signals, we envision Cdc42(Y32F) as a tool for dissecting the specific contribution of ITSN to Cdc42-induced cellular events. Currently, efforts to understand the consequences of specific Rho GTPase signaling pathways are limited; however, recent work has described the design of a small molecule inhibitor of Rac function (*43*), and specificity mutants of Dbs that cripple exchange activity toward RhoA, but not Cdc42 (*44*). Given the large number of identified Dbl family RhoGEFs (more than 60), with only 22 recognized Rho family GTPase substrates, it is paramount to understand the individual consequences of various GEFs acting on a single GTPase.

Rho GTPases control many important aspects of cellular function, including cell migration and gene expression, and misregulation of these small GTPases has been linked with several aspects of cancer development. A detailed understanding of the interactions between GTPase regulators and cognate Rho GTPases could provide powerful insight aimed at manipulating the signaling potency of a Rho GTPase. Specifically, controlling the ability of Dbl proteins to stimulate the release of bound GDP from within a Rho GTPase and therefore initiate downstream signaling pathways would allow dissection of the contributions made by various GEFs to individual Rho GTPase cellular function. With respect to this objective, we have demonstrated the functional characterization of a subtle Cdc42 mutant which displays specific responsiveness to Dbl family member ITSN.

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