COMPARATIVE ANALYSIS OF *HISTOPHILUS SOMNI* Ibpa WITH OTHER FIC ENZYMES REVEALS DIFFERENCES IN SUBSTRATE AND NUCLEOTIDE SPECIFICITIES

Seema Mattoo1*, Eric Durrant2, Mark J. Chen3, Junyu Xiao2, Cheri S. Lazar2, Gerard Manning3, Jack E. Dixon1,2, and Carolyn A. Worby2*

From Howard Hughes Medical Institute and Department of Pharmacology1, Departments of Pharmacology; Cellular and Molecular Medicine; and Chemistry and Biochemistry2, University of California, San Diego, La Jolla, California, 92093-0721; and Razavi Newman Center for Bioinformatics, Salk Institute, La Jolla, California, 920373

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Address correspondence to: Seema Mattoo, Ph.D. or Carolyn A. Worby, Ph.D., 9500 Gilman Drive, 0721, La Jolla, CA 92093-0721. Fax: (858) 822-5888

Email: smattoo@ucsd.edu or cworby@ucsd.edu

A new family of adenylyltransfases, defined by the presence of a Fic domain, was recently discovered to catalyze the addition of adenosine mono-phosphate (AMP) to Rho GTPases (Yarbrough et al., 2009, Science, 323:269; Worby et al., 2009, Mol. Cell, 34:93). This adenylylation event inactivates Rho GTPases by preventing them from binding to their downstream effectors. We reported that the Fic domain(s) of the protein Ibpa from the pathogenic bacterium *Histophilus somni* adenylylates mammalian RhoGTPases, RhoA, Rac1 and Cdc42, thereby inducing host cytoskeletal collapse, which allows *H. somni* to breach alveolar barriers and cause septicemia. The Ibpa-mediated adenylylation occurs on a functionally critical tyrosine in the switch 1 region of these GTPases. Here, we conduct a detailed characterization of Ibpa’s Fic2 domain and compare its activity to other known Fic adenylyltransferases, VopS from the bacterial pathogen *Vibrio parahaemolyticus* and the human protein HYPE. We also included the Fic domains of the secreted protein PfhB2, from the opportunistic pathogen *Pasteurella multocida*, in our analysis. PfhB2 shares a common domain architecture with Ibpa and contains two Fic domains. We demonstrate that the PfhB2 Fic domains also possess adenylyltransferase activity that targets the switch 1 tyrosine of Rho GTPases. Comparative kinetic and phylogenetic analyses of Ibpa-Fic2 with the Fic domains of PfhB2, VopS, and HYPE reveal important aspects of their specificities for Rho GTPases and nucleotide usage, and offer mechanistic insights for determining nucleotide and substrate specificities for these enzymes. Finally, we compare the evolutionary lineages of Fic proteins with those of other known adenylyltransfases.

INTRODUCTION

The bacterial pathogen *Histophilus somni* produces a large surface antigen called immunoglobulin-binding protein A (Ibpa) that is expressed on the cell surface via a two-partner secretion system (1). Ibpa contains filamentous hemagglutinin and coiled coil domains in the amino terminus along with two filamentation-induced by c-AMP (Fic) domains and a YopT-like cysteine protease domain in the carboxyl terminus. The *H. somni* Fic domains catalyze an adenylylation reaction where adenosine triphosphate (ATP) is hydrolyzed to add an adenosine monophosphate (AMP) to the tyrosine in the switch 1 region of Rho family guanosine triphosphatases (GTPases), RhoA, Rac, and Cdc42, thereby blocking their ability to bind to downstream effectors (2).

The covalent addition of AMP to proteins has been previously described. In the 1960s, bacterial glutamine synthetase was reported to be stably adenylylated on up to 12 tyrosine residues, with the degree of adenylylation controlling enzymatic activity (3). Transient adenylylation of the C-terminal glycine or the catalytic lysine also occurs during the activation of ubiquitin and ubiquitin-like proteins as well as during DNA and RNA ligation processes (4,5). These transient adenylylation events serve chiefly as priming reactions with the hydrolysis of the high energy phosphate bonds in ATP providing the necessary

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energy for the subsequent reactions. Fic proteins are not homologous to bacterial glutamine synthetase adenylyltransferase (GS-ATase), the polynucleotide ligases or the E1-like enzymes. Recently, another bacterial effector, *Legionella pneumophila* DrrA, was shown to adenylylate a Tyr residue in the switch 2 region of Rab1b (6). DrrA does not contain a Fic motif but instead bears structural similarity to bacterial GS-ATases.

We previously demonstrated that adenylylation of Rho GTPases by IbpA Fic domains requires the conserved histidine in the core motif of the Fic domain, HPFxGNGR, as mutating the histidine to alanine nearly eliminates adenylyltransferase (ATase) activity and prevents cytotoxicity (2). Adenylylated Rho GTPases have also been reported to retract the cytoskeleton in bovine alveolar type 2 (BAT2) cells, allowing *H. somni* to cross the alveolar barrier and cause septicemia (7).

Other Fic domain-containing proteins, such as VopS (*Vibrio* outer protein S) from *Vibrio parahaemolyticus*, have been shown to adenylylate Rho GTPases on a nearby conserved threonine residue in the switch 1 region (8). IbpA and VopS do not share sequence similarities other than the conserved Fic core motif, but they do share significant structural similarity (8,9). We recently solved the structure of IbpA’s Fic2 domain in complex with Cdc42 (PDB: 3N3V) (9). This structure reveals that IbpA-Fic2 contains an NH2-terminal extension, which we refer to as an ‘arm segment’, that recognizes a three-dimensional epitope in the Rho-family proteins involving their switch 1 and switch 2 regions, resulting in tight substrate specificity. Consistent with our structure, the recently published VopS structure (PDB: 3LET) also contains an arm-like segment (8). Superimposing VopS onto IbpA-Fic2 suggests that this arm is in a perfect position to interact with the switch 2 region of Cdc42 (9). As mentioned above, VopS adenylylates a Thr rather than a Tyr residue in the switch 1 region. The significance of Tyr versus Thr adenylation is currently unclear, as both modifications serve to block downstream signaling by the GTPases (2,10). Another secreted Fic protein, AnkX from *L. pneumophila*, has been implicated in breakdown of the golgi network, though an ATase activity and substrate for it have not been identified (11). In addition, a single Fic domain-containing protein, HYPE (huntingtin yeast interacting protein E; also called FicD), exists in humans. HYPE and its *Drosophila* homolog have also been demonstrated to possess ATase activity (2,12). Several other Fic domain structures have been solved by structural genomics efforts (PDB 2F6S, 2G03, 3CUC, 3EQX, 2JK8, and 2VZA); all contain variations on the conserved core Fic domain but none contain the arm segment common to IbpA and VopS (9,13). Not unexpectedly, while many of these enzymes autoadenylylate, they fail to adenylylate Rho GTPases, making their cellular substrates of paramount interest (9,14).

Fic proteins are evolutionarily related to the toxin Doc (death on curing), a component of the toxin-antitoxin module encoded by the *phd/doc* operon in P1 bacteriophage (15). Doc is a ribosomal toxin that binds and inhibits the 30S ribosomal subunit in a manner similar to hygromycin (16). While an adenylyltransferase activity for Doc has not been demonstrated, the histidine of Doc’s Fic motif is important for its ability to function as a ribosomal toxin (15). Fic proteins bear significant sequence and structural similarity to P1 bacteriophage Doc. As such, Doc and Doc-like proteins have been recently classified as the FiDo (Fic/Doc) family (12).

Over 4300 proteins contain a Fic domain (http://www.ebi.ac.uk/interpro). Is the presence of a Fic domain sufficient to confer ATase activity? What other substrates do Fic proteins target? Finally, does the variability in the Fic motif sequence dictate substrate specificity, or perhaps specificity for nucleotide usage? In this manuscript, we have attempted to answer these questions while conducting a detailed enzymatic characterization of IbpA-Fic2. First, we demonstrate that another Fic protein, the *Pasteurella multocida* secreted virulence factor PfhB2 (*Pasteurella* filamentous hemagglutinin B2), also adenylylates Rho GTPases in a manner similar to IbpA. We, further, compare the ATase activity of the Fic domains of *H. somni* IbpA, *P. multocida* PfhB2, *V. parahaemolyticus* VopS, and human HYPE, and find that VopS displays the ability to effectively use a nucleotide other than ATP as a co-substrate. This finding greatly broadens our appreciation for the scale of post-translational modifications carried out by the Fic family of enzymes. Using mutant proteins with chimeric Fic motifs, we address the role of the Fic
motif sequence in determining substrate and nucleotide specificities, and conduct a phylogenetic analysis to understand the functional evolution of these proteins. Finally, we determine the catalytic parameters for the IbpA-Fic adenylylation of a constitutively active form of Cdc42 and compare our results to those determined for VopS.

**EXPERIMENTAL PROCEDURES**

**Cloning, Protein Expression and Purification**

Rho GTPase clones were obtained from the Missouri S&T cDNA Resource Center (www.cdna.org). GST-fusion, His-SUMO-fusion, and MBP-His-TEV-fusion proteins were expressed in *E. coli* BL21 RILP (Stratagene) in LB medium containing 100 µg/ml of ampicillin (pET-GSTx and pSJ8) or kanamycin (pSMT3) to a density of 0.6 A600. Protein expression was induced overnight at room temperature with 0.4 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Cells were lysed in 20 mM Hepes pH 7.4, 100 mM NaCl, 0.5 mM TCEP, and protease inhibitors by sonication and affinity purified using GST-Bind resin (Novagen) or Ni-NTA resin (Qiagen). The His6-SUMO tag and MBP-His-TEV tags were cleaved using recombinant ULP1 and TEV proteases, respectively. The affinity tags were then removed by passing the proteins over Ni2+-NTA resin a second time. Protein concentrations were measured using the Bradford method, purity was determined by SDS-PAGE, and proteins were stored at -80°C.

**In Vitro Adenylylation and Nucleotide Exchange Assays**

Approximately 5 µg of GST-IbpAFic1, GST-IbpAFic2, GST-PfhB2Fic1, GST-PfhB2Fic2, and GST-HYPE were incubated with 5 µg of GST-RhoA, Rac, or Cdc42 or their Gly, Thr, or Tyr mutants in 40 µl adenylylation reactions containing 25 mM Tris-HCl (pH 7.5), 3.0 mM MgCl2, 1 mM DTT, 0.5 mM EDTA, and 5 µCi α32P-ATP for 30-60 minutes at 30°C. Reactions were stopped with NuPAGE loading buffer (Invitrogen).

Nucleotide loading and GTPase activation assays were carried out as described previously (17) using untagged RhoA, Rac1, and Cdc42. RhoGDI binding and subsequent in vitro adenylylation reactions were carried out by transfecting HEK293A cells with HA-tagged RhoA, Rac1 or Cdc42. After recovering for 48h, cells were lysed in 600 µl 50 mM Tris-CI, pH 7.2, 1% (w/v) Triton X-100 500 mM NaCl, 0.2 mM PMSF and a protease inhibitor cocktail. HA-tagged GTPases were loaded with GDP or GMP-PNP (17). Nucleotide exchange was confirmed by assessing the ability of GDP or GMP-PNP bound GTPases to bind to GST-Rhotekin or GST-PAK (17), as assessed by Western blot using antibodies against RhoA, Rac1, and Cdc42 (BD Transduction Labs).

His6-SUMO-RhoGDI beads were added to the GDP or GMP-PNP loaded GTPases, and the samples rotated for 2h at 4°C. Beads were washed three times with 1ml lysis buffer and once with 1X adenylylation reaction buffer. The adenylylation reaction was carried out as described above in the presence of 10mM cold ATP. After 30 min, the beads were pelleted, and the supernatant and bead fractions saved. The bead fractions were then washed three times with 1ml of 50 mM Tris-CI, pH 7.2, 1% (w/v) Triton X-100, 150 mM NaCl, 10 mM MgCl2, 0.2 mM PMSF containing protease inhibitors. The HA-RhoGTPase-RhoGDI complex was eluted with wash buffer containing 300 mM imidazole. Laemmli loading buffer was added to the bead eluate and to the previously collected supernatant fraction, and the samples were separated on AnyKD gels (BioRad). The separated proteins were then transferred to nitrocellulose and subjected to autoradiography. Protein load was visualized by Ponceau S staining.

For nucleotide specificity assays, in vitro reactions were conducted as above with α32P-labeled ATP, GTP, CTP, UTP or dTTP (Perkin Elmer) containing 1mM of each respective cold dNTP. Adenylylation was visualized by autoradiography at various exposures.

**Kinetic Data Analysis**

The adenylylation of Cdc42Q61L by Fic2 was assayed using α32P-ATP (Perkin Elmer) and P81 Whatman filter paper. The reaction buffer consisted of 20 mM Hepes pH 7.4, 100 mM NaCl, 1 mg/ml BSA, 0.5 mM TCEP, 5 µCi α32P-ATP, and equal concentrations of ATP and MgCl2.
Reactions were performed at 25°C in triplicate for 120 seconds. The reaction was initiated with 0.56 nM Fic2, in a final volume of 25 µl, and stopped with an equal volume of STOP solution (0.1 M EDTA, 0.1M ATP). 25 µl of the stopped reaction was immediately pipetted onto P81 Whatman filter paper and dropped into a beaker containing 500 ml 0.4% phosphoric acid that was sitting on a rotating platform. The filters were washed 30 minutes with 0.4% phosphoric acid (500 ml per wash) for a total of four washes followed by a final wash of 95% ethyl alcohol. Afterwards, the filters were allowed to air dry before being placed in scintillation vials followed by counting in a Beckman LS 6000IC scintillation counter. To analyze the apparent kinetic values (K_m) of ATP using Fic2 with Cdc42-Q61L, the substrate concentration was held constant at 500 µM while varying the ATP concentration with equal molar MgCl_2, 0.1 to 10 mM. To determine the k_m for Cdc42-Q61L the ATP and MgCl_2 concentrations were held at 5 mM and substrate concentration varied from 0.10 to 2.8 mM. The kinetic values were fitted with the Michaelis-Menten equation (Equation 1) using GraphPad Prism 4.

\[ v = \frac{V_{\text{max}} [S]}{K_m + [S]} \]  

(Eq. 1)

V_{\text{max}} represents the maximum velocity, S is the substrate concentration, and K_m is the substrate concentration at half of the maximum velocity.

**RESULTS AND DISCUSSION:**

**Alignment of the Fic domains of IbpA with PhbB2, VopS and HYPE**

We characterized the enzymatic activity of IbpA’s Fic2 domain as it compares to other enzymatically active Fic domains, such as *H. somni* IbpA-Fic1, *V. parahemolyticus* VopS and human HYPE (Supplementary Figure 1A). We also included the Fic domains of *P. multocida* PhbB2 in this analysis. PhbB2’s Fic1 and Fic2 domains share 64% amino acid sequence identity with IbpA-Fic1 and –Fic2, respectively, but have not been shown to function as ATases. Supplementary Figure 1B shows an alignment of the Fic domains of IbpA (Fic1, aa2994-3358 and Fic2, aa3359-3781); PhbB2 (Fic1, aa2892-3191, and Fic2, aa3309-3609); VopS, aa30-388; and HYPE, aa181-458 and their predicted secondary structure as compared with the structure for IbpA-Fic2 (PDB 3N3U and 3N3V) (9). Despite their low sequence similarity, each of these Fic domains shares a common structural fold consisting of 7 alpha helices (α8-α14), with a surface exposed loop containing the Fic HxFxx(G/A)N(G/K)R motif. In addition, IbpA-Fic2 residues shown to be critical for binding to ATP (Supplementary Figure 1B, red arrows) and to the Switch 1 and Switch 2 regions of Rho GTPases (Supplementary Figure 1B, blue and black arrows, respectively) are conserved between IbpA and PhbB2 (9).

**Substrate specificity of Fic proteins against Rho GTPase substrates**

IbpA-Fic2 and HYPE have been shown in vitro to target the conserved Switch 1 Tyr32 of Cdc42 and Rac1, and Tyr34 of RhoA (2). In contrast, VopS has been demonstrated to target the conserved Switch 1 Thr35 of Cdc42 and Rac1, and...
Thr37 of RhoA (10). We, therefore, tested the ability of the Fic domains described in Supplementary Figure 1 to target Rho GTPases, RhoA, Rac1 and Cdc42 and their switch 1 mutants, to determine whether they targeted Rho GTPases and if so, which residue. IbpA-Fic1, IbpA-Fic2, PfhB2-Fic1, PfhB2-Fic2, and VopS were purified as GST fusions using glutathione sepharose. HYPE-Fic was purified as a His6-SUMO fusion. These purified Fic proteins were then incubated with GST-tagged and purified RhoA, Rac1 and Cdc42 in an in vitro adenylylation reaction. Results with Cdc42 as a substrate are shown (Figure 1A). As expected, IbpA-Fic1, IbpA-Fic2 and HYPE-Fic adenylylated wild type Cdc42 and its Thr35 to Ala mutant, but not the Tyr32 to Phe mutant. Likewise, VopS adenylylated the wild type and the Tyr32 to Phe mutant of Cdc42, but not the Thr35 to Ala mutant. Both Fic domains of PfhB2 displayed ATase activity against Cdc42 (Figure 1A and data not shown), specific to Tyr32. This is the first demonstration of ATase activity for PfhB2. Together, these data confirm that IbpA, PfhB2 and HYPE function as tyrosyl ATases, while VopS functions as a threonine specific ATase. Given that Tyr32 and Thr35 are in such close proximity, and that the arm segment of each of the Fic proteins likely serves as a docking site for Cdc42, it is plausible that these proteins would modify any available free hydroxyl group in Cdc42’s switch 1 region. Our data indicate that this is not the case. These data further indicate that Tyr32 and Thr35 are the only Cdc42 residues that are targeted for adenylylation by the Fic domains of IbpA, PfhB2, HYPE, and VopS. Similar results were obtained with RhoA and Rac1 (Supplementary Figures 2A and 2B, respectively).

**IbpA-Fic2 targets both the active and inactive forms of Rho GTPases**

To determine whether IbpA-Fic2 displays a substrate preference for the active (GTP-bound) or inactive (GDP-bound) form of the GTPases, we performed in vitro adenylylation reactions on bacterially expressed wild type untagged RhoA, Rac1 and Cdc42 loaded with either GDP or the non-hydrolyzable GTP analogue, GMP-PNP. To confirm the efficiency of nucleotide exchange, GDP or GMP-PNP loaded GTPases were tested for the ability to bind to downstream effectors, Rhotekin or PAK. Only the active (GMP-PNP bound) forms of RhoA, Rac and Cdc42 bound to Rhotekin and PAK, respectively, as determined by western blot analysis (Figure 1B). Further, IbpA-Fic2 was capable of adenylylating both the active and inactive forms of these GTPases, as determined by autoradiography (Figure 1B). This result is in agreement with our crystallographic data where IbpA-Fic2 co-crystallized with an adenylylated, GDP-bound form of Cdc42 (PDB: 3N3V) (9). This result contradicts previously reported observations where IbpA-Fic2 targeted only the active forms of the GTPases in HeLa cells following transfection with point mutants that lock the GTPases into constitutively active (G12/14V) or dominant negative (T17/19N) conformations (2,10). We reconcile this discrepancy by inferring that the inactivating point mutations used in the earlier assays alter the conformation of the GTPases, such that they may not be effectively recognized by the arm domain of IbpA-Fic2.

**IbpA-Fic2 is active against the RhoGTPase-RhoGDI complex**

Small GTPases cycle between an inactive, GDP-bound and active, GTP-bound state. Three families of proteins regulate this switching of molecular states, namely, GEFs (GTPase exchange factors), GAPs (GTPase activating proteins), and GDI (GDP disassociation inhibitors) (18). GEFs act as GDP-dissociation stimulators catalyzing the exchange of GDP for GTP, thus activating RhoGTPases. The GAPs enhance the intrinsic ability of the GTP-binding proteins to hydrolyze GTP to GDP, thus inactivating Rho GTPases. Finally, GDIs extract Rho family GTPases from the membrane and inhibit the exchange of GDP for GTP as well as the hydrolysis of GTP. Since crystallographic data indicates that IbpA-Fic2 locks Cdc42 in a conformation that resembles its RhoGDI-bound state (9), we sought to determine whether RhoGTPase:RhoGDI complexes could be adenylylated by IbpA-Fic2. Specifically, HA-tagged RhoA, Rac1 or Cdc42 exchanged with either GDP or GMP-PNP were bound to an Ni2+-NTA agarose column containing bacterially purified His6-SUMO-tagged RhoGDI. This column with the RhoGTPase:RhoGDI complex was then subjected to Fic-mediated adenylylation. Following the adenylylation reaction, the
supernatant and eluate of the bead (agarose) fractions were separated by SDS-PAGE and analyzed by autoradiography and Ponceau S staining. Figure 1C shows the results with the GDP-loaded Cdc42-RhoGDI complex. The autoradiograph indicates that IbpA-Fic2 is able to adenylylate Cdc42 in a complex with RhoGDI and does not effectively disassociate the complex, as determined by the lack of 32P signal in the supernatant lane. Similar results were obtained for GDP-loaded RhoA and Rac1, as well as with GMP-PNP loaded RhoA, Rac1, and Cdc42 bound to RhoGDI (Supplementary Figure 2C and data not shown).

Kinetics of IbpA-Fic2 activity on Cdc42

Since IbpA-Fic2 efficiently targets activated Cdc42, we performed kinetic analyses on IbpA-Fic2's ATase activity using a Cdc42-Q61L point mutant that mimics Cdc42 in its active/GTP-bound form (19). This mutant displays greater stability than Cdc42-G12V at the high protein concentrations required for the kinetic analyses (8).

IbpA-Fic2 hydrolyzes ATP to AMP and PPi while it catalyzes the addition of AMP to the invariant Tyr in the switch 1 region of the Rho GTPases. We, therefore, surveyed several representatives from each sub-family to determine the variety of GTPases that can be targets of Fic-mediated adenylylation. An alignment of the Switch 1 region of these GTPases indicates several highly conserved amino acid residues, including an invariant threonine corresponding to Thr35 of Cdc42 that is adenylylated by VopS (Supplementary Figure 3). Additionally, the Tyr32 of Cdc42 that is adenylylated by IbpA, PfhB2 and HYPE is conserved in several Rho and Ras proteins (Supplementary Figure 3). We cloned and purified GST-tagged versions of additional Rho (RhoB, RhoC, RhoG, and TC10), Ras (H-Ras, RheB, RheS, Rap1a, Ral1A, and Rit1), Rab (Rab1a, Rab1b, Rab4a, Rab4b, Rab5a, and Rab11a), Arf (Arfl and Arl1), and Ran (Ran) subfamily members and tested them in vitro as substrates for adenylylation by IbpA-Fic1, IbpA-Fic2, PfhB-Fic1, PfhB-Fic2, VopS, or HYPE-Fic (Figure 3). Surprisingly, only Rho family members RhoB, RhoC, RhoG, and TC10 were efficiently adenylylated by any of the Fic enzymes, despite high Switch 1 sequence conservation and presence of the invariant Thr35/37 (Figure 3A and Supplementary Figure 3). Interestingly, while most Rho family members, including RhoG (Figure 3B), were efficiently adenylylated by the various enzymes except HYPE-Fic, TC10 was efficiently adenylylated only by the Fic domains of IbpA and PfhB2, and was only weakly modified by VopS (Figure 3C). Further, HYPE-Fic did not modify TC10, supporting our hypothesis and previous observations that Rho GTPases are not the physiological target(s) of HYPE (Figure 3C and (2)). RhoB, RhoC, RhoG, and TC10 belongs to the Rho subfamily, which includes RhoA, Rac1 and Cdc42, and displays a structural similarity to these GTPases. We, therefore, infer that the Fic domains of IbpA, PfhB2, and VopS preferentially...
target only the Rho subfamily of GTPases for adenylylation.

**Nucleotide specificity of Fic ATases**

We next surveyed the ability of the Fic domains of IbpA, PfhB2, VopS, and HYPE to utilize nucleotides other than ATP while modifying Rho GTPases. Results using the active form of Cdc42 (Cdc42-Q61L) are shown (Figure 4). Cdc42-Q61L was incubated with Fic enzymes in identical *in vitro* reactions using equal amounts of $\alpha^{32}$P-labeled ATP, GTP, CTP, UTP or dTTP. ATP was the preferred nucleotide source for all the Fic domains tested (Figure 4A). As expected, HYPE-Fic displayed weakest activity and required longer exposure for detection by autoradiography (Figure 4B). Surprisingly, VopS displayed an equal preference for utilizing ATP or GTP, indicating that VopS may also function as a guanylyltransferase. Additionally, the Fic domains of IbpA, PfhB2 and HYPE displayed a moderate level of activity with CTP, while VopS preferred UTP over CTP. Longer exposures indicated that all the Fic enzymes tested could utilize any of the nucleotides to some extent (data not shown).

Point mutations in the IbpA-Fic2’s Fic motif did not alter its affinity for nucleotides. IbpA-Fic2-P3718G or IbpA-Fic2-E3721D mutations, which mimic the corresponding residues in the Fic motif of VopS, did not confer specificity for GTP (Figure 4C). Thus, the Fic motif sequence alone does not dictate nucleotide specificity.

Finally, we compared the ability of IbpA-Fic2 and VopS to target switch 1 Tyr32 and Thr35 mutants of Cdc42 using different nucleotides (Figure 4D). As seen with ATP, IbpA-Fic2 modified the Cdc42-T35A mutant but not Cdc42-Y32F, irrespective of the nucleotide source. In contrast, VopS was impaired in its ability to add CMP, UMP and dTMP to both the Cdc42-T35A and Cdc42-Y32F mutants. These results indicate that coordination of nucleotides within the Fic enzymatic pocket depends not just on the Fic enzyme but also on the substrate. We reason that mutations in the switch 1 Tyr32 induce minor conformational changes in Cdc42, which affect the ability of VopS to target Thr35. In contrast, mutations in Thr35 are tolerated by IbpA-Fic2. This synergistic role of the enzyme-nucleotide-substrate complex may be what allows efficient adenylylation by Fic enzymes despite having high Km values for ATP and Cdc42.

**Phylogenetic analysis of IbpA’s Fic domains**

Of the 4300 known Fic proteins that constitute the FiDo family, only IbpA-Fic2 and VopS have previously been enzymatically characterized (2,8,12). In addition, the Enzyme Commission (EC) lists several distinct classes of enzymes as ATP-dependent ATases. These include glutamine synthetase ATases (EC # 2.7.7.42), UBA4 E1-ligase activating ATases (EC #2.7.7.B4), phenylalanine ATases (EC #2.7.7.54) that target amino acids, polynucleotide ATases (EC #s 2.7.7.19 and 2.7.7.25), DNA and RNA specific ligases (EC # 6.5.1), aminoglycoside ATases (EC #2.7.7.47), and sugar specific ATases (EC #s 2.7.7.27, 2.7.7.35, and 2.7.7.36) (Figure 5). All these ATases have evolved to target proteins, nucleotides, and sugars, and are involved in diverse biological processes, illustrating the importance of adenylylation in cellular signaling. We reasoned that a phylogenetic analysis of IbpA’s Fic domains with these other classes of ATP-dependent ATases might help understand the nature of the FiDo family. We generated a phylogenetic tree for each of the ATase families using the adenylyltransferase domain of index proteins shown in Supplementary Table 1. ATases appear to have evolved early in life, and have been reinvented several times to generate 4 dominant clades (Figure 5, yellow, orange, red and chocolate ellipses).

Like each of the ATase families analyzed, the FiDo family appears to have evolved independently from other clades of ATases (Figure 5). It can be further classified into three main groups – a) Doc, b) HYPE and c) Fic (Figure 5, red ellipse). The Doc group contains proteins with a Fic motif resembling bacteriophage P1 Doc’s HIFNDANKR sequence. These are typically small proteins (125-150 amino acids), lacking any other protein domain, and are often part of a toxin-antitoxin complex (21). The Doc group proteins are members of a larger group of proteins classified as the PSK (post-segregational cell killing) system, which allows plasmids to maintain themselves within their bacterial host (21). The HYPE group
consists of a single Fic locus found in each animal genome, and has potential orthologs in other eukaryotic lineages as well as in bacteria and archaea. These have a core motif of HPFxxGNGR, and all animal members contain a signal peptide and a TPR domain. We speculate that HYPE’s TPR domain is involved in substrate recognition. Many prokaryotic HYPE proteins are associated with helix-turn-helix domains, which may be involved in transcriptional activation. Finally, the Fic group includes E. coli Fic and its bacterial homologs. These proteins are generally longer than Doc proteins, but are not associated with a PSK system (21). They also do not display a conserved operon architecture, suggesting that they may have evolved to carry out different functions.

Within the Fic family, IbpA’s Fic domains branch closely with those of PfB2, as expected from their nearly 64% amino acid sequence identity (Figure 5, red ellipse). Interestingly, IbpA, PfB2, and VopS form a distinct clade that branches away from E. coli Fic, suggesting that these enzymes may have evolved to target proteins from their mammalian hosts. A similar divergence is observed for the L. pneumophila virulence factor AnKX, which has a non-canonical Fic motif resembling that of P1 bacteriophage Doc (Figure 5, red ellipse). Recently, L. pneumophila DrrA was shown to adenylylate a tyrosine residue in the switch 2 region of Rab1b, by an enzymatic mechanism similar to E. coli GS-ATase (6). The GS-ATase phylogeny indicates that DrrA diverged away from E. coli GS-ATase but retained the ability to utilize ATP to adenylylate a mammalian substrate, possibly due to its intimate association with the human host (Figure 5, orange ellipse). Further, DrrA also guanylylates Rab1b (6). Unlike Fic proteins, adenylylation of Rab1b by DrrA constitutively activates the GTPase (6). It would be interesting to determine whether DrrA and AnKX use adenylylation as a mechanism to counter each other and finely tune Legionella’s ability to modulate vesicular trafficking.

Finally, a comparison of the Fic phylogeny with DNA/RNA ligases reveals that while many ATP/ADP/NAD+ specific ligases cluster together, those that are exclusively NAD+ specific branch separately from the rest of the family members (Figure 5, yellow ellipse). It remains to be determined whether a nucleotide or NAD+ specific branch exists within the FiDo family.

CONCLUSION

By conducting a detailed analysis of IbpA-Fic2’s enzymatic, kinetic, and phylogenetic properties, we have gained important insights into factors that determine substrate specificity for Fic adenylyltransferases. We show that while most of the Fic enzymes tested displayed higher specificity for ATP as a nucleotide source, VopS displayed an equal affinity for GTP. Thus, Fic proteins have the potential to carry out post-translational modifications beyond adenylylation alone. We further demonstrate that nucleotide specificity can be dictated by the enzyme-nucleotide-substrate complex formed during the chemical reaction. Additionally, IbpA’s Fic domains can target RhoGTPases both in their active (GTP-bound) as well as their inactive (GDP-bound and RhoGDI-bound) states, thus preventing their downstream signaling function. It must be noted that unlike IbpA’s Fic domains which target Tyr32/Tyr34 of Rac1/Cdc42/RhoA, VopS adenylylates Thr35/Thr37 of Rac1/Cdc42/RhoA. Thr35 of Rac1 has been shown to play a critical role in binding to the regulatory arm of RhoGDI (22). It, thus, remains to be determined whether VopS functions differently than IbpA in its ability to adenylylate RhoGTPases in complex with RhoGDI. We also determined that Pasteurella PfB2 displayed ATase activity similar to the Fic domains of IbpA. Finally, we compared IbpA-Fic2’s kinetic properties to those of VopS and find that the two enzymes display similar affinities for ATP and Cdc42, despite targeting different residues on the Cdc42 substrate. The comparative phylogenetic analysis of Fic proteins with other known nucleotidyltransferases provides a perspective for the chemical diversity observed within the Fic family.

Fic proteins have been implicated in processes as diverse as bacterial pathogenesis, cell division, protein translation, eukaryotic cell signaling, and cellular trafficking. Chemical characterization of these enzymes is essential for providing a unifying, conserved catalytic mechanism to explain these otherwise disparate biological processes.
ACKNOWLEDGEMENTS

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REFERENCES

FIGURE LEGENDS

Figure 1: Substrate specificity of IbpA-Fic2.  
A) The Fic domains of IbpA, PfH2 and HYPE target Tyr32 of Cdc42, while VopS targets Thr35. Bacterially expressed GST-tagged IbpA-Fic2, IbpA-Fic1, PfH2-Fic2, VopS or HYPE-Fic were incubated with wild type (W), Y32F (Y) or T35A (T) versions of Cdc42 expressed as GST fusion proteins in bacteria in an in vitro adenylylation assay using α-32P-ATP. Samples were separated on SDS-PAGE and visualized by autoradiography (top panel) and Coomassie staining (bottom panel). The position of Cdc42 on the gel is indicated by arrows. The Fic domains of IbpA, PfH2 and HYPE adenylylate wild type Cdc42 and Cdc42-T35A but not Cdc42-Y32F, indicating their specificity for the switch 1 tyrosine. In contrast, VopS fails to adenylylate only Cdc42-T35A, indicating its specificity for the switch 1 threonine.  
B) IbpA-Fic2 targets both the active and inactive forms of Rho GTPases. Bacterially expressed untagged Cdc42, Rac and RhoA loaded with GDP or GMP-PNP (as described in Materials and Methods) were incubated with IbpA-Fic2 in an in vitro adenylylation assay. The protein load was visualized by Coomassie staining and the amount of adenylylation by autoradiography. The nucleotide status of the GTPases was confirmed prior to adenylylation by incubation with GST-Pak (Cdc42 and Rac) or GST-Rhotekin (RhoA) followed by separation on SDS-PAGE and Western analysis using antibodies directed against the individual GTPases.  
C) IbpA-Fic2 is active against the Cdc42-RhoGDI complex. HA-tagged Cdc42 was expressed in HEK293A cells. Bacterially expressed His6-SUMO-RhoGDI bound to nickel agarose beads was incubated with the HEK239A cell extract treated for GDP loading of RhoGTPases (as described in Materials and Methods) to allow Cdc42-RhoGDI complex formation. After washing, the beads were subjected to the in vitro adenylylation reaction in the presence or absence of GST-tagged IbpA-Fic2. The supernatant and bead eluate were separated on SDS-PAGE and visualized by autoradiography. The protein load was monitored by Ponceau S staining.  

Figure 2: Apparent steady-state kinetic measurements for ATP and constitutively active Cdc42.  
A) Initial-velocity measurements for ATP were obtained using a constant concentration of Cdc421-179Q61L of 500 µM while varying the ATP concentrations from 100 – 10,000 µM.  
B) Initial velocity measurements for Cdc42 were obtained at 5 mM ATP while varying the concentration of Cdc421-179Q61L between 100-2800 µM. Assays were preformed in triplicate with IbpA-Fic2 at 0.56 nM. The line represents the fit of this data using the Michaelis-Menten equation (Materials and Methods).  

Figure 3: Fic domains of IbpA, PfH2, and VopS preferentially target the Rho subfamily of GTPases for adenylylation.  
A) Survey of Ras family Rho GTPases as substrates for Fic-mediated adenylylation. The indicated GST-tagged Rho GTPases were bacterially expressed and purified, and incubated with purified IbpA-Fic2, in an in vitro adenylylation reaction. Samples were separated on SDS-PAGE and visualized by autoradiography (top panel) and Coomassie staining (bottom panel). The position of IbpA-Fic2 on the gel is indicated by an arrow. IbpA-Fic2 adenylylated only the Rho family members, RhoB, RhoC, RhoG, and TC10.  
B) Ability of Fic enzymes to adenylylate RhoG. GST-tagged and purified IbpA-Fic1, IbpA-Fic2, PfH2-Fic1, PfH2-Fic2, VopS, and HYPE-Fic were incubated with bacterially expressed and purified GST-RhoG in an in vitro adenylylation reaction. Samples separated by SDS-PAGE were visualized by autoradiography (top panel) and Coomassie staining (bottom panel). The position of RhoG on the gel is indicated by an arrow. The Fic domains of IbpA, PfH2, and VopS efficiently adenylylate RhoG, while the Fic domain of HYPE displays a weaker adenylylation activity.
C) Ability of Fic enzymes to adenylylate TC10. GST-tagged and purified IbpA-Fic1, IbpA-Fic2, PfhB2-Fic1, PfhB2-Fic2, VopS, and HYPE-Fic were incubated with bacterially expressed and purified GST-TC10 in an in vitro adenylylation reaction. Samples separated by SDS-PAGE were visualized by autoradiography (top panel) and Coomassie staining (bottom panel). The position of TC10 on the gel is indicated by an arrow. The Fic domains of IbpA and PfhB2 can efficiently adenylylate TC10, while VopS shows minimal activity towards it. HYPE did not adenylylate TC10 in vitro.

Figure 4: Nucleotide specificity of IbpA-Fic2.
A) GST-tagged and purified IbpA-Fic1, IbpA-Fic2, PfhB2-Fic1, PfhB2-Fic2, and VopS, and His6-SUMO-tagged HYPE-Fic were incubated with Cdc421-179Q61L in an in vitro reaction using α-32P-ATP, -GTP, -CTP, -UTP, or –dTTP. Samples separated by SDS-PAGE were visualized by autoradiography (top panel) and Coomassie staining (bottom panel). The ability of the indicated Fic enzymes to utilize different nucleotides for post-translationally modifying Cdc42 is shown. All the panels were given equal exposure times for autoradiography. Dotted line represents a break in the gels.
B) Reactions with His6-SUMO-tagged HYPE-Fic displayed in Figure 4A were re-run on SDS-PAGE and visualized by longer exposures for autoradiography (upper panel) and Coomassie staining (bottom panel). HYPE-Fic efficiently uses ATP, and CTP to a lesser degree, to modify Cdc42.
C) Point mutations in the IbpA-Fic2’s Fic motif did not alter its affinity for nucleotides. GST-tagged and purified Pro3718 to Gly (IbpA_Fic2-P/G) and Glu3271 to Asp (IbpA_Fic2-E/D) mutants of IbpA-Fic2, as well as wild type IbpA-Fic2 and VopS were incubated with Cdc42-Q61L using α32P-ATP and –GTP in an in vitro reaction. Samples were separated on SDS-PAGE and visualized by autoradiography (top panel) and Coomassie staining (bottom panel). Conversion of IbpA-Fic2’s Fic motif sequence to match the corresponding residues in the Fic motif of VopS did not confer specificity for nucleotides.
D) Comparison of IbpA-Fic2 and VopS to target switch 1 Tyr32 and Thr35 mutants of Cdc42 using different nucleotides. GST-tagged IbpA-Fic2 and VopS were incubated with wild type (W), Y32F (Y) or T35A (T) versions of Cdc42 expressed as GST fusion proteins in bacteria in an in vitro assay using α32P-ATP, -GTP, -CTP, -UTP, or –dTTP. Samples were assessed by autoradiography (top panel) with exposure times adjusted for optimal visualization, and by Coomassie staining (lower panel). Mutation of T35A in Cdc42 did not alter IbpA-Fic2’s ability to target the Switch 1 Tyr32 for modification. In contrast, the Y32F mutation in Cdc42 severely impaired VopS in modifying Thr35 using the different nucleotide sources.

Figure 5: Phylogenetic comparison of Fic enzymes with other classes of adenylyltransferases. A phylogenetic tree was generated using the Neighbor Joining method for each of the ATase families using the adenylyltransferase domain of index proteins shown in Table 1. The four families of ATases are shown – DNA/RNA ligases (in yellow) with a bracket indicating NAD+ specific enzymes; the glutamine synthetase adenylyltransferase (GS-ATase) family (in orange); the E1 ubiquitin ligase family (in chocolate brown); and the FiDo family (in red) with brackets indicating the HYPE, Doc, and Fic subgroups.
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Figure 1

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→ Adenylylated Cdc42
→ Cdc42

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→ HA-Cdc42
→ His-Sumo-RhoGDI
→ HA-Cdc42
Figure 2

A: Kinetics for ATP

B: Kinetics for Cdc42
Figure 3

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Figure 4
Figure 5

DNA/RNA ligase family

E1-like family

GS-ATase-like family

FiDo family

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Comparative analysis of Histophilus somni IbpA with other FIC enzymes reveals differences in substrate and nucleotide specificities
Seema Mattoo, Eric Durrant, Mark J. Chen, Junyu Xiao, Cheri S. Lazar, Gerard Manning, Jack E. Dixon and Carolyn A. Worby

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