Evidence for a Novel Cdc42GAP Domain at the Carboxyl Terminus of BNIP-2

Received for publication, January 13, 2000, and in revised form, February 23, 2000

Boon Chuan Low, Kah Tong Seow, and Graeme R. Guy‡

From the Signal Transduction Laboratory, Institute of Molecular and Cell Biology, 30 Medical Dr., Singapore 117609, Republic of Singapore

We recently identified BNIP-2, a previously cloned Bcl-2- and E1B-associated protein, as a putative substrate of the FGF receptor tyrosine kinase and showed that it possesses GTPase-activating activity toward Cdc42 despite the lack of homology to previously described catalytic domains of GTPase-activating proteins (GAPs). BNIP-2 contains many arginine residues at the carboxyl terminus, which includes the region of homology to the noncatalytic domain of Cdc42GAP, termed BNIP-2 and Cdc42GAP homology (BCH) domain. Using BNIP-2 glutathione S-transferase recombinants, it was found that its BCH bound Cdc42, and contributed the GAP activity. This domain was predicted to fold into α-helical bundles similar to the topology of the catalytic GAP domain of Cdc42GAP. Alignment of exposed arginine residues in this domain helped to identify Arg-235 and Arg-238 as good candidates for catalysis. Arg-238 matched well to the arginine “finger” required for enhanced GTP hydrolysis in homodimerized Cdc42. Site-directed mutagenesis confirmed that an R235K or R238K mutation severely impaired the BNIP-2 GAP activity without affecting its binding to Cdc42. From deletion studies, a region adjacent to the arginine patch (288EYV290 on BNIP-2) and the Switch I and Rho family-specific “Insert” region on Cdc42 are involved in the binding. The results indicate that the BCH domain of BNIP-2 represents a novel GAP domain that employs an arginine patch motif similar to that of the Cdc42-homodimer.

Ras superfamily GTPase proteins act as molecular switches for signal transduction pathways to control cell growth, differentiation, and motility. The Ras superfamily consists of the Ras, Rho, Rab, and Arf families, which are classified according to their sequence similarities and functions (1, 2). These proteins cycle between two guanine-nucleotide bound states, the GTP-bound form, which is active, and the inactive GDP-bound form. Activation occurs as a result of a change in the conformation of discrete “switch regions” in these proteins that allow them to interact with their appropriate effector proteins. The GTP/GDP-regulated proteins possess a low intrinsic activity for hydrolyzing GTP to GDP, but for efficient physiological catalysis, they associate with other proteins, which can enhance their GTPase activity further. These proteins are termed GTPase-activating proteins (GAPs), and they have been recognized by conserved amino acid sequence motifs that are characteristic of each family (3).

The Rho subfamily of GAPs, which includes RhoA, RhoB, RhoC, RhoE, RhoG, Rac1, Rac2, Cdc42, and TC10, is involved in various aspects of cytoskeletal organization, cell polarity, and motility (4–6). For example, RhoA is involved in the regulation of stress fibers and focal adhesion formation (7, 8), Rac1 is involved in the formation of lamellipodia and membrane ruffling (9, 10), and Cdc42 is necessary for actin microspikes/filopodia to form (10, 11). Furthermore, all three can activate the Jun amino-terminal kinase, affect the transcription of certain target genes, and regulate the progression of the cell cycle (12, 13). Structural and biochemical studies show that all GAPs, although bearing no close overall sequence homology to each other, exert their effect either by contributing catalytic residues in-trans, by lowering the activation energy for GTP hydrolysis, or by stabilizing the conformation of the inherent GTPases. These mechanisms are employed by 120-kDa Ras-GAP and the 50-kDa RhoGAP/Cdc42GAP through a highly conserved arginine “finger” catalytic motif and by similar binding topology (3, 14–22). Recently, however, the crystal structure of rna1p, the Schizosaccharomyces pombe ortholog of the mammalian GAP of Ran, revealed a completely different folding pattern that nevertheless contributed to both the catalysis and the stabilization effect on Ran (23).

GAPs have been identified that act preferentially on members of the Rho subfamily. It is interesting to note that two different catalytic domains have been described for GAPs acting on Cdc42. The 50-kDa Cdc42GAP was shown in various crystallographic studies to possess the conventional arginine finger that interacted closely with the Switch I domain from Cdc42 to affect catalysis. The second type of catalytic domain, albeit of unproven physiological relevance, was identified when Cdc42 forms a homodimer. In this circumstance, one molecule can act as a GAP toward the other partner, with catalysis being mediated by a conserved arginine patch in the carboxyl terminus (24, 25).

We have recently shown BNIP-2, a previously cloned Bcl-2 interacting protein, to be a putative substrate of the FGF receptor tyrosine kinase and to bind both Cdc42GAP and Cdc42 when not tyrosine-phosphorylated. Interestingly, BNIP-2 was also shown to possess a “GAP-like” activity toward Cdc42 (26). This protein contains no sequence homology to the canonical catalytic domain of a GAP, but it shares a highly conserved sequence with a region in the amino-terminal, noncatalytic half of Cdc42GAP (26, 27). We wished to determine the sequences in

* This work was supported by the Institute of Molecular and Cell Biology, National University of Singapore. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed. Tel.: 65-874-3737; Fax: 65-779-1117; E-mail: mcgg@imcb.nus.edu.sg.

1 The abbreviations used are: GAP, GTPase-activating protein; BCH, BNIP-2 and Cdc42GAP homology; BH, breakpoint cluster region homology; GST, glutathione S-transferase; GTPγS, guanosine 5′-3-O-(thio)triphosphate; HA, hemagglutinin; PAGE, polyacrylamide gel electrophoresis.
BNIP-2 that were responsible for the binding to Cdc42 and for the catalysis. To do this, we used a combination of site-directed mutagenesis and molecular modeling based on both the known biochemistry and structural topology of the catalytic Cdc42GAP domains. It enabled a hypothetical model to be constructed that formed the basis for the mutational studies. Using this approach, we have found that the BNIP-2 and Cdc42GAP homology (BCH) domain contains the GAP activity in BNIP-2, but not in Cdc42GAP. Within this novel domain, there are two critical arginine residues that are important for conferring the GAP activity. This arginine patch shares reasonable similarity to those residues that mediate the homodimerization-induced GAP activity seen with Cdc42 homodimers (24, 25). Other discrete regions likely to be important for the BNIP-2 and Cdc42 interaction were also identified.

MATERIALS AND METHODS

Plasmids—Full-length cDNA of BNIP-2 was cloned into a hemagglutinin (HA)-tagged expression vector, pXJ40 (Dr. E. Manser, Institute of Molecular and Cell Biology, Singapore), or into pGEX4T-1 vector for producing the GST recombinant protein as described previously (26). pGEX-Cdc42 and pGEX-Cdc42GAP (from Dr. A. Hall, University College, London, United Kingdom) were used in making GST fusion proteins or as templates to generate pXJ40/HA and pXJ40FLAG constructs. Point mutants of BNIP-2 or Cdc42 were generated by site-directed mutagenesis using the Quick-Change mutagenesis kit (Stratagene), whereas deletion mutants were generated by polymerase chain reaction using specific primers facilitated by restriction sites. All plasmids were purified using a Wizard Miniprep kit (Promega) or a Wizard Maxi/Mega-prep kit followed by ethanol reprecipitation for use in transfection experiments. Clones were confirmed correct by thermal-cycle sequencing using the SequiThermal EXCEL II DNA sequencing kit (Epicentre Technologies) or mapping analyses using restriction enzymes (New England Biolabs). Escherichia coli strain DH5α was used as host for propagation of the expression vectors. Reagents used were of analytical grade, and standard protocols for molecular manipulations and media preparations were referred from Ref. 53.

Cell Culture and Transfection—Human 293T cells were grown in RPMI 1640 medium supplemented with 10% (v/v) fetal bovine serum (HyClone), 2 mM l-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin (all from Sigma) and maintained at 37 °C in a 5% CO₂ atmosphere. Cells at 90% confluence in 100-mm plates were transfected for 1 h with 10 μg of indicated plasmid using Tfx-50 cationic lipids according to the manufacturer's instructions (Promega).

Precipitation Experiments and Western Blot Analyses—Cells were lysed in 1 ml of lysis buffer (50 mM HEPES, pH 7.4, 150 mM sodium chloride, 1.5 mM magnesium chloride, 5 mM EGTA, 10% (v/v) glycerol, 1% (v/v) Triton X-100, a mixture of proteases inhibitors (Roche Molecular Biochemicals), and 5 mM sodium orthovanadate). The lysates were directly analyzed as either whole cell lysates (25 μg) or their aliquots (500 μg) were used in affinity precipitation/pulldown experiments with GST-BNIP-2 (5 μg) or GST-Cdc42 (5 μg) that had been preloaded with guanosine 5'-O-3-thiotriphosphate (GTP·S) (Sigma) as described previously (26). Samples were run in SDS-PAGE gels and analyzed by Western blotting with HA antibody (Roche Molecular Biochemicals) or FLAG antibody (Sigma).

GAP Assay—The GAP activity toward Cdc42 was examined by determining the release of 32P, from the [γ-32P]GTP prebound to the molecule. GST-Cdc42 (5 μg) still conjugated to the Sepharose beads, were washed twice in Buffer A (50 mM HEPES, pH 7.4, 0.5 mM EDTA), and resuspended in final 10 μl of the same buffer with 5 μCi of [γ-32P]GTP (6,000 Ci/mmol; New England Nuclear) for 10 min at room temperature. The reaction was terminated by adding 25 mM magnesium chloride. Excess unincorporated radioactive GTP was removed by washing the beads five times in 1 ml of cold Buffer B (50 mM HEPES, pH 7.4, 150 mM sodium chloride, 1.5 mM magnesium chloride, 5 mM EGTA, 10% (v/v) glycerol, 1% (v/v) Triton X-100, a mixture of proteases inhibitors, and 5 mM sodium orthovanadate). The beads were finally resuspended in 10 μl of the same buffer. For GST-BNIP-2 assays using proteins expressed in bacteria, eluted recombinants of BNIP-2 (1 μg in 100 μl of Buffer B) were then added to the beads suspension and mixed well. The suspension was quickly centrifuged to collect the beads and incubated at room temperature for the times indicated, and aliquots of the supernatant (10 μl) were then taken for counting in a scintillation counter. For GAP assays involving 293T lysates, cells transfected with

RESULTS

BNIP-2 Binding to Cdc42—We previously demonstrated that BNIP-2 acts as a GAP protein toward Cdc42, despite its lack of obvious homology to the canonical GAP domain for Cdc42 (26). To gain an insight into the structural/functional relationship between BNIP-2 and Cdc42, we initially set out to identify which regions of BNIP-2 bind to Cdc42. Full-length and various deletion constructs of BNIP-2 were generated as GST recombinants as indicated (Fig. 1A). The full-length protein was arbitrarily dissected into various fragments designated as A, B, BCH, C1, C2, and C3. The C-series fragments encompassed the region of homology between BNIP-2 and Cdc42GAP (26, 27), which we termed BCH domain. The recombinants were expressed in E. coli, purified, and used to precipitate HA-Cdc42 expressed in 293T cells, as described under
“Materials and Methods.” The precipitated protein was separated by SDS-PAGE followed by Western blotting using HA antibody as the probe. Fig. 1B shows that the recombinant full-length BNIP-2 bound Cdc42 relatively strongly and that this interaction was mainly mediated via the C2 and C3 regions of BNIP-2. Some weaker binding could be seen with fragment A, but no interaction was detectable with the other regions of the molecule. To further demonstrate specificity of binding, two endogenous cellular proteins were assessed for their binding to the fragments of BNIP-2. It can be seen in Fig. 1B (Crk, Lyn, and GST) that neither the Crk adaptor protein nor the Lyn tyrosine kinase bound any of the BNIP-2 fragments. Similarly, no binding by these fragments could be seen when the phosphotyrosine phosphatase SHP-2 was overexpressed in these cells (data not shown). The blot was stripped and reprobed with anti-GST to reveal the integrity of these recombinants and to show equal loading. To further demonstrate that the BCH fragment of BNIP-2 could bind Cdc42, a precipitation experiment was performed. A GST recombinant of BNIP-2 BCH was generated and used to precipitate Cdc42 expressed in 293T cells. Their ability to co-precipitate was compared with the GST recombinants of full-length BNIP-2, Cdc42GAP, and GST beads alone. The results in Fig. 1C show that the BNIP-2 BCH binding to Cdc42 was equivalent to or greater than that by the full-length BNIP-2 and of Cdc42GAP.

Molecular Modeling of the BCH Domain—When BNIP-2 was first cloned and sequenced, it was found to have a domain that was conserved in only one other mammalian protein, an apparently noncatalytic sequence found on the amino-terminal half of Cdc42GAP (27). It could be assumed that BNIP-2 and Cdc42GAP might bind to a common substrate by means of their shared BCH domain. Indeed, multiple sequence alignment was recently presented that suggested that the BCH domain might be a phospholipid-binding domain similar to that of the Saccharomyces cerevisiae phosphatidylinositol transfer protein Sec14p (29). However, there is only a low degree of homology apparent with these two proteins. We demonstrated that the BCH domain mediates both hetero- and homophilic binding of the proteins containing this sequence, which would indicate that the BCH domain is likely to be a protein-protein interaction domain. Further alignment studies, however, indicated that the BCH domain was not confined to two known mammalian proteins but has been highly conserved throughout evolution, having been found also in two recently cloned but uncharacterized proteins from Arabidopsis thaliana (Fig. 2A).

Because the BCH domain is found as a discrete homologous protein domain highly conserved in sequence throughout evolution, it is plausible that the BCH domain of BNIP-2 could form a functional and structural domain with GAP activity toward Cdc42. In order to perform mutational experiments to address this possibility, we need to know what residues are most likely to be involved in the catalysis and binding. As we currently lack a crystal structure of the complex, we decided to make some testable assumptions using molecular modeling techniques.

Most GAPs have well defined structure and substrate specificities, as exemplified by RasGAP, RanGAP, and RhoGAP acting only on Ras, Ran, and Rho respectively. To date, structures of two RhoGAP domains binding to Cdc42 have been identified, one for the catalytic GAP domain of Cdc42GAP (16, 19, 21) and the other one for the BH domain of the phosphoinositide 3-kinase p85α subunit (30). Although it binds to Cdc42, the BH domain fails to act as a functional GAP for Cdc42. We therefore set out to test whether the BCH domain, which also acts on Cdc42, would have a structure similar to these domains. To test this hypothesis, we predicted and modeled the tertiary structure of the BCH domain. First, we aligned the BCH domains of BNIP-2 and Cdc42GAP to the canonical GAP domain of Cdc42GAP and the BH domain of p85α. Both the GAP domain of Cdc42GAP and the BH domain of p85 α have similar structural folds (16, 19, 21, and 30). The alignment shows considerable conservation in regions corresponding to the secondary structural elements (α-helices) of the GAP domain or the p85-BH domain (Fig. 2B). We then used the structures of the Cdc42GAP “GAP” domain and the p85-BH domain as structural templates to model the BCH domain using the homology modeling techniques described under “Materials and Methods.” The predicted structure (Fig. 2C) was used as a basis for the design of site-directed mutagenesis and deletion experiments. Our assumption was that if the BCH domain folds into the predicted GAP-like structure and binds to Cdc42 in a manner similar to the catalytic GAP domain, we could use the model to facilitate the identification of BCH residues and regions involved in (a) GAP catalytic activity and (b) binding to Cdc42.

Identification of Candidate Residues Important for BNIP-2 GAP Activity—Based on the model above, we first sought to identify candidate residues that might be important in mediating BNIP-2 GAP activity. We assumed that these residues would fulfill the criteria as listed below with respect to the known structure and the biochemistry of the GAP domains of Cdc42GAP. The two GAP domains that have been described achieve catalysis either via an arginine finger (Cdc42GAP) or via a conserved arginine patch (found in Cdc42, Rac1, RhoG, and RhoC homodimers). Both arginine-containing sequences must be positioned on the outside of the three-dimensional structure of the protein. According to our model, there was no obvious region on BNIP-2 that corresponded to the reactive arginine finger Arg-305 of Cdc42GAP. We noted, however, a polybasic patch prominently displayed on the exterior of the protein model corresponding to a region containing Arg-235, Arg-236, and Arg-238 (as indicated in Fig. 2C). Somewhat surprisingly, this region matches a reactive arginine motif recently identified in the human Cdc42, Rac1, RhoC, and RhoG homodimers (Fig. 2D). Although not required for the formation of dimers, this polybasic patch in these members of the Rho subfamily was identified to be responsible for the GAP-like activity when homodimers were formed (24, 25). Of particular interest is that in this local alignment, the Arg-238 in BNIP-2 aligns to the critical arginine residue (Fig. 2D, arrow) that is indispensable for their GAP activities. It is notable that this arginine residue is absent from those members of the Rho family (and the BCH domain of Cdc42GAP) that lack the GAP activity.

We next mutated individual arginine residues to lysine to form R235K, R236K, and R238K and tested whether they contribute to the GAP-like catalytic activity of BNIP-2 on Cdc42. Each of these mutations was introduced into the full-length BNIP-2 and expressed in vitro in mammalian 293T cells. Lysates from control and transfected cells were then tested for their ability to catalyze the GTP-hydrolysis of GST-Cdc42 pre-loaded with [γ-32P]GTP, as described under “Materials and Methods.” Fig. 3A shows that the intrinsic GTPase activity of Cdc42 followed a linear time course over 30 min and showed minimal catalytic activity under the conditions studied. This activity was enhanced over 10-fold in the presence of wild-type BNIP-2. Interestingly, mutations of the prime candidate arginine, Arg-238, as well as Arg-235 markedly reduced the enhanced GAP activity toward Cdc42. In comparison, the R236K

2 B. C. Low, K. T. Seow, and Graeme R. Guy, manuscript in preparation.
FIG. 2.  Modeling of the BCH domain and the putative arginine finger.  A, sequence alignment of the BCH domains of BNIP-2 and Cdc42GAP with two putative proteins from A. thaliana containing BCH-like domains.  The GenBank accession numbers of Arab1 and Arab2 are AL031986 and AC009400, respectively.  B, sequence alignment of the BCH domains of BNIP-2 and Cdc42GAP with the catalytic GAP domain of phosphoinositide 3-kinase p85α subunit.  The conserved α-helices corresponding to the GAP domain of Cdc42GAP (19) are depicted as bars on top of the sequences.  GenBank accession numbers for p85, Cdc42GAP, and BNIP-2 are P27986, Z23024, and U15173, respectively.  C, predicted structure of BNIP-2 BCH.  The
mutant showed a GAP-like activity that was 50% of that of wild-type BNIP-2. We also generated corresponding mutants as full-length GST recombinants in bacteria to test their effects on GTP hydrolysis in vitro. Results similar to those in Fig. 3A were obtained, verifying that these BNIP-2 mutants had significantly lost the ability to enhance the GTP hydrolysis of Cdc42 (data not shown).

Although each of these point mutations showed varying inhibitory effects on the rates of GTP-hydrolysis, none was completely inactive toward Cdc42, consistent with the notion that multiple residues are involved in the GAP activity. To ensure that the loss in the GAP activity by these mutants was not simply due to a loss of binding to the partner protein or by misfolding of the recombinant protein, we used GST-Cdc42 to test the binding to these mutants from the cell lysates in precipitation experiments. Fig. 3B shows that each of the mutants was soluble and that they had equal levels of expression in 293T cells. When subjected to precipitation experiments, each of the point-mutants exhibited an apparently equal affinity to the GTP-loaded GST-Cdc42. This indicates that the overall structural integrity of the BNIP-2 point mutants was maintained and that these putative catalytic arginine residues were unlikely to be directly involved in binding.

To further confirm that the BCH domain of BNIP-2 itself can fold separately and act effectively as a GAP, various BCH GST recombinants with or without the above point mutations were generated, purified, and used in GAP assays (Fig. 4A). Consistent with the full-length studies involving either BNIP-2 expressed in vivo, or in vitro, the recombinant BCH domain of BNIP-2 alone could stimulate the rate of GTP hydrolysis of Cdc42 by 10-fold. Again, the GAP-like activity associated with the BNIP-2 BCH domain was severely impaired by the R238K and R235K mutants and less severely by the R236K mutant. In contrast, the homologous BCH domain of Cdc42GAP was completely inactive toward Cdc42, although it can also bind Cdc42 (data not shown). To ensure that the integrity of these bacterially expressed recombinants remained intact, they were tested for binding HA-Cdc42 in precipitation experiments (Fig. 4B). The results show that none of the binding to Cdc42 had been compromised and again demonstrate that these residues are important in the catalysis but not for the binding.

Binding Regions within BCH and Cdc42—Most effectors/ regulators of Cdc42 bind to the Switch I region (effector binding domain) and/or the Rho family-specific “Insert” region (31). We had previously generated Cdc42 recombinants with the aminoterminal half containing the Switch I and Switch II regions, and the carboxyl-terminal half of the Cdc42 containing the Insert region. We found that either half of the molecule could bind to BNIP-2, suggesting that there are multiple sites of binding on Cdc42 for BNIP-2 (data not shown). In order to
domain of BNIP-2 was deleted (lane 2). However, when the BNIP-2 ΔT mutant was used as the target (lane 3), there was nearly a 50% loss in its binding to the Cdc42 mutants lacking either the Switch I region or the Insert region. No effect was seen in the binding of this ΔT mutant to the wild-type Cdc42 or its Switch II deletion mutant. The apparent synergistic effect that deletions of region T have with Switch I or the Insert would be consistent with the notion that region T does not interact directly with either the Switch I or the Insert. A similar degree in the loss of binding would have been seen by either single or combined deletions of these constructs if region T was binding to either the Switch I or Insert. This means that the Switch I and the Insert region of Cdc42 would both contribute to the binding to two distinct but unknown regions on BNIP-2, whereas the region T (encompassing 288EYV290) on BNIP-2 would represent another site of interaction with other region(s) of Cdc42.

Contrary to the requirement for multiple regions of binding to Cdc42 by BNIP-2, as demonstrated above, deletion of the Switch I region alone in the GTPγS-bound form of Cdc42 could reduce its binding to Cdc42GAP by two-thirds, whereas deletions of the Switch II or the Insert region had no effect on the binding (Fig. 5B). The direct involvement of the Switch I but not the Insert region of Cdc42 in binding Cdc42GAP is consistent with the crystallographic structure of the complex (15). However, we were surprised that deletion of the Switch II region had minimal effects on Cdc42 binding to Cdc42GAP, when similar studies had shown it to be a likely interacting sequence (15).

It was shown previously that in the Ras-RasGAP complex (32) the Gly-12 residue in the Switch I region of the GTPase contacts part of the GAP closely. Mutation of this residue to valine (G12V) significantly reduced the binding of RasGAP to Ras (32, 33). We hypothesized that the BCH domain of BNIP-2 would have a similar recognition profile. To test this hypothesis, HA-Cdc42 constructs were expressed in 293T cells either as the wild-type, G12V, or T17N point mutations, and each was subjected to precipitation experiments using GST recombinants of either the full-length BNIP-2 (FL) or the BCH domain of BNIP-2 as indicated. Bound proteins were separated by SDS-PAGE, blotted and probed with HA antibody. Aliquots of whole cell lysates were probed with HA antibody to determine the equal expression of the HA-tagged wild-type or mutant Cdc42.

delineate the sites of interaction further, small deletion mutants of the Switch I (amino acids 32–40; ΔSI), Switch II (amino acids 60–70; ΔSII), or the Insert region (amino acids 89–94; ΔIns) were generated. These deletion mutants were then used to test for binding to either the Switch I or the BH domain of BNIP-2 or the BH domain of BNIP-2 deletion mutant, ΔT, that is devoid of residues 288EYV290. This region is located on the outer surface and is potentially involved in binding Cdc42 as predicted from our model above (Fig. 2C). As a control, we deleted the 290RRLRK299 region (ΔR) in BNIP-2, assumed by modeling and experimental data (shown above) to be important for catalysis and not for binding.

To test the various interactions, the wild-type or deletion constructs of HA-BNIP-2 (ΔR or ΔT) were expressed in 293T cells, and the lysates were subjected to precipitation experiments with GTPγS-bound GST-Cdc42, either as the wild-type or with deletions in the Switch I, Switch II, or Insert region. The results in Fig. 5A show that each of the HA-BNIP-2 wild-type or mutant was expressed equally well in 293T cells (see WCL), and none of them bound the controls with GST beads alone. The binding by the wild-type BNIP-2 was not significantly affected by any single deletion on Cdc42 (Fig. 5A, compare results in lane 1). This confirmed our previous observation that there exist multiple sites of interaction on Cdc42 for BNIP-2. Similar results were seen when the proposed catalytic

DISCUSSION

We have identified several arginines as key residues within the BNIP-2 BCH domain that are responsible for the GAP activity of the protein. This region containing the arginines bears no similarity to the arginine motifs employed by the “cradle-fold” structural topology in the RasGAP or Cdc42GAP/RhoGAP catalytic structures (3, 14–22) or to the leucine repeat folds of RanGAP binding to Ran (23). It does, however, show a striking similarity to the polybasic carboxyl terminus of Cdc42. Although not required for the formation of dimers, this polybasic patch in Cdc42 was identified to be responsible for its
BCH Domain of BNIP-2 Confers GAP Activity

GAP-like activity when homodimers are formed (24, 25). Of particular interest is that in this local alignment, the Arg-238 in BNIP-2 matches the critical arginine residue that is indispensable for the homodimer-enhanced GAP activity. In those members of the Rho subfamily that fail to display GAP activity when homodimerized, i.e. RhoA and the yeast form of Cdc42 (25), this arginine is absent, as it is from the BCH domain of Cdc42GAP, which is also catalytically inactive (Fig. 2D). Although it lacks GAP activity, the BCH domain of Cdc42GAP can still bind to Cdc42 (data not shown).

Despite the varying degrees of the loss of GAP activity displayed by the R238K, R235K, and R236K mutants, BNIP-2 still retains its ability to bind Cdc42, indicating that these residues are not involved in the binding to Cdc42. The conserved arginine fingers in the GAP domains of Cdc42GAP (34) or RasGAP (18, 35) have all been shown to be important for catalysis and not for binding to their respective partners. In both Cdc42GAP and RasGAPs, catalytic enhancement is likely to be the result of stabilization of the conformation most complementary to the transition state and from ground state destabilization (3, 14–22, 34–37). It is most likely that the reactive “arginine patch” in BNIP-2 would confer one or more of the residues in-trans for the catalysis. In this regard, we observed that Arg-235 and Arg-238 are potent residues. The current work utilizes semi-quantitative measurements to demonstrate the involvement of such arginine fingers in the BCH domain of BNIP-2. The actual relative contribution from each arginine residue or combination of arginines 235, 236, and 238 awaits a more thorough kinetic determination.

In some of the most efficient phosphoryl-transferring enzymes, such as adenylate kinase and uridylylate kinase (38, 39), several arginine residues are located in the active sites and they catalyze the reaction by stabilizing developing negative charges in the transition state. Although the involvement of multiple neighboring polybasic residues had not been tested in the similar arginine patches in Cdc42 and other Rho family members, it is tempting to speculate that these tandem arginine residues in BNIP-2 would provide a positive-charge interface to stabilize the negative charges that develop during the transition state of GTP hydrolysis in Cdc42. In Cdc42GAP, the Arg-305 within the classical GAP domain has been identified both structurally and biochemically as the key catalytic residue in promoting GTP hydrolysis in Cdc42. It does not account, however, for the full GAP activity. Recently, its adjacent arginine residue, Arg-306, was identified to be necessary to further augment its GAP activity (40). Similarly, the p120-RasGAP and the other RasGAP, neurofibromin NF1, both require at least the input of Arg-789 and Arg-903 (p120-RasGAP (17, 41)) or Arg-1276 and Arg-1391 (for NF1 (42)) for optimal catalysis. Therefore, it appears that although the BCH domain of BNIP-2 shows no clear sequence homology to any of these molecules, it too could utilize the same means to facilitate GTP hydrolysis by Cdc42.

Recently, evidence has begun accumulating that demonstrates that various GTPases are the preferred eukaryotic substrates of diverse bacterial toxins and exoenzymes (43–45). Several of these bacterial products have been shown to interfere with Rho family GTPase activity by means of chemical modifications on important residues (46, 47) or increasing the lifetime of the active GTP-bound form of the Rho proteins (48). Recently, the amino-terminal domain of Pseudomonas aeruginosa exoenzyme S was shown to activate the GTPase activity of Rho, Rac, and Cdc42 (45). It was interesting to note that the catalytic domain on this protein had no strong sequence homology to any canonical Rho-GAPs, thus providing evidence that dissimilar primary sequences are capable of performing a similar catalytic function when folded in the appropriate conformation.

Based on modeling, we attempted to identify the candidate regions for interaction between BNIP-2 and Cdc42. We observed that at least three sites on each protein are involved in interprotein binding. We showed that the region in BNIP-2 together with the Switch I or Insert region of Cdc42 synergistically caused a 50% loss in binding, suggesting that they include at least three of these binding sites. These data differ from those derived from studies on the “classical” GAP domain of Cdc42GAP, in which deletion of the Switch I region on Cdc42 alone was sufficient to markedly reduce the binding to this domain.

More than 10 direct interacting partners of Cdc42 have been identified to date (Ref. 26 and references therein). It remains a major issue as to how the incoming signals determine the specificity of functional coupling between this molecule and its effectors/regulators. Recently, Li et al. (49) utilized a mutational and chimeric approach and mapped unique regions on Cdc42 responsible for binding to three different target proteins. They showed that the Switch I and the immediate neighboring region on Cdc42 contain all the necessary determinants for PAK1 binding, whereas WASP and IQGAP1 did not bind the same sequence. Two distant located regions on Cdc42, residues 155–184 and residues 83–120, constitute the WASP- and IQGAP1-binding regions, respectively. Furthermore, it was shown that the Rho family-unique Insert region of Cdc42 is dispensable for PAK1 and WASP binding but is required for high affinity binding by IQGAP1. Recently, solution structures for the complex of Cdc42 binding to ACK (50) and Cdc42 binding to WASP (51) have highlighted the involvement of unique regions within Cdc42 and the respective effectors in mediating the specificity in the binding. Our results show that the binding of BNIP-2 to Cdc42 was mainly mediated by the Switch I and the Insert region. It will be interesting to test how BNIP-2 affects the binding of other effectors to Cdc42 and to what extent this would affect cellular functions. We have recently shown that BNIP-2, when tyrosine-phosphorylated, failed to bind Cdc42 (26). This phosphorylation could conceivably act as an additional switch to regulate the formation/dissociation of the complex.

Based on our model, the BCH domain of BNIP-2 was predicted to display folding similar to that of the classical GAP domain of Cdc42GAP. Nevertheless, the refined structure is likely to be different from that of the predicted structure. As noted previously, BNIP-2 binds equally well to each form of Cdc42, whereas Cdc42GAP recognizes only the GDP or GTP-bound form of Cdc42 (26, 52). This difference in binding selectivity is further supported by our observations that a deletion in the Switch I region of Cdc42 caused severe loss of binding to Cdc42GAP but not to BNIP-2, suggesting that this region alone is not sufficient to confer tight binding to BNIP-2.

The identification of the BCH domain as a binding domain for Cdc42 in BNIP-2 and Cdc42GAP has further implications. Whereas the BCH domain of BNIP-2 acts as a GAP, the corresponding region in Cdc42GAP does not, partly because of its lack of Arg-236 and Arg-238 (see Fig. 2D). This raises an interesting issue as to what role this region may play in the physiological function of Cdc42GAP. Most previous biochemical and functional studies involving the interaction of various GAPs with their binding partners were performed using the minimal GAP domains, and any potential regulation via the non-GAP domains would have been overlooked. We have evidence that suggests that the BCH domain is also involved in homophilic and heterophilic interactions involving BNIP-2 and Cdc42GAP. The discrete regions that mediate these interac-
tions are different from those involved in the binding to Cdc42. It therefore seems likely that, because of this binding specificity, this domain will have some regulatory function.

In conclusion, our present work has shown that the BCH domain of BNIP-2 define a new catalytic GAP domain that contains an arginine patch similar to that found in certain members of the Rho subfamily when they form homodimers. Although the actual mechanism of GAP catalysis by either BNIP-2 or Cdc42 has yet to be determined by x-ray or NMR structural analyses, current evidence suggests that strategically placed arginine residues may represent GAP domains in different primary sequences and tertiary structures. This notion is supported by the recent identification of the arginine finger of rna1p/RanGAP present in a novel folding structure that is mediated by leucine-rich repeats (23).

Acknowledgments—We thank Dr. Catherine Pallen, Dr. Permeen Yusoff, and Jormay Lim for constructive criticism and help in reading the manuscript. We are also grateful to Dr. Alan Hall for the generous donation of materials.

REFERENCES

1. Lamarche, N., and Hall, A. (1994) Trends Genet. 10, 436–440
2. Kjoller, L., and Hall, A. (1999) Exp. Cell Res. 253, 166–179
3. Scheffzek, K., Ahmadian, M. R., Wittinghofer, A. (1998) Trends Biochem. Sci. 23, 257–262
4. Chant, J., and Stowers, L. (1995) Cell 81, 1–4
5. Hall, A. (1998) Science 279, 509–514
6. Hall, A. (1999) Br. J. Cancer 80, Suppl 1, 25–27
7. Chardin, P., Boquet, P., Mosale, P., Popoff, M. R., Rubin, E. J., and Gill, D. M. (1989) EMBO J. 8, 1087–1092
8. Paterson, H. F., Self, A. J., Garrett, M. D., Just, I., Aktories, K., and Hall, A. (1990) J. Cell Biol. 111, 1001–1009
9. Waterman-Storer, C. M., Worthylake, R. A., Liu, B. P., Burridge, K., Salmon, E. D. (1999) Nat. Cell Biol. 1, 45–50
10. Kozma, R., Ahmed, S., Best, A., and Lim, L. (1995) Mol. Cell. Biol. 15, 1842–1952
11. Nobes, C. D., and Hall (1995) Cell 81, 53–62
12. Minden, A., Lin, A., Clarett, F. X., Abo, A., and Karin, M. (1995) Cell 81, 1147–57
13. Aspenstrom, P. (1999) Curr. Opin. Cell Biol. 11, 95–102
14. Sprang, S. R. (1997) Curr. Opin. Struct. Biol. 7, 849–856
15. Rittinger, K., Walker, P. A., Eccleston, J. F., Nurnahmed, K., Owen, D., Lau, E., Gamblin, S. J., and Smerdon, S. J. (1997) Nature 388, 693–697
16. Nasso, N., Hoffman, G. R., Manor, D., Claridy, J. C., Cerione, R. A. (1998) Nat. Struct. Biol. 5, 1047–1052
17. Scheffzek, K., Ahmadian, M. R., Kabsch, W., Wissmuller, L., Lautwein, A., Schmitz, F., and Wittinghofer, A. (1997) Science, 277, 333–388
18. Ahmadian, M. R., Stege, P., Scheffzek, K., and Wittinghofer, A. (1997) Nat. Struct. Biol. 4, 686–689
19. Barrett, T., Xiao, B., Dodson, E. J., Dods, G., Lushbrook, S. B., Nurnahmed, K., Gamblin, S. J., Musacchio, A., Smerdon, S. J., and Eccleston, J. F. (1997) Nature 385, 458–461
20. Baz, A. (1998) Nature 392, 447–448
21. Rittinger, K., Taylor, W. R., Smerdon, S. J., and Gamblin, S. J. (1998) Nature 392, 448–449
