The BNIP-2 and Cdc42GAP Homology Domain of BNIP-2 Mediates Its Homophilic Association and Heterophilic Interaction with Cdc42GAP*

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We recently showed that BNIP-2 is a putative substrate of the fibroblast growth factor receptor tyrosine kinase and it possesses GTPase-activating activity toward the small GTPase, Cdc42. The carboxyl terminus of BNIP-2 shares high homology to the non-catalytic domain of Cdc42GAP, termed BCH (for BNIP-2 and Cdc42GAP homology) domain. Despite the lack of obvious homology to any known catalytic domains of GTPase-activating proteins (GAPs), the BCH domain of BNIP-2 bound Cdc42 and stimulated the GTPase activity via a novel arginine-patch motif similar to that employed by one contributing partner in a Cdc42 homodimer. In contrast, the BCH domain of Cdc42GAP, although it can bind Cdc42, is catalytically inactive. This raises the possibility that these domains might have other roles in the cell. Using glutathione S-transferase recombinant proteins, immunoprecipitation studies, and yeast two-hybrid assays, it was found that BNIP-2 and Cdc42GAP could form homo and hetero complexes via their conserved BCH domains. Molecular modeling of the BNIP-2 BCH homodimer complex and subsequent deletion mutagenesis helped to identify the region 217RRKMP221 as the major BCH interaction site within BNIP-2. In comparison, deletion of either the arginine-patch 235RRLRK239 (necessary for GAP activity) or region 288EYV290 (a Cdc42 binding sequence) had no effect on BCH-BCH interaction. Extensive data base searches showed that the BCH domain is highly conserved across species. The results suggest that BCH domains of BNIP-2 and Cdc42GAP represent a novel protein-protein interaction domain that could potentially determine and/or modify the physiological roles of these molecules.

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two-hybrid analysis to demonstrate that while BNIP-2 and Cdc42GAP can individually bind to and enhance Cdc42 GTPase activity they could also bind to each other (2). Such an interaction between proteins that bind to and activate the same substrate provides a potential controlling mechanism with several layers of complexity. We are primarily interested in establishing which regions of BNIP-2 and Cdc42GAP are responsible for their homophilic and heterophilic interactions. To do this we have used a series of deletion studies and molecular modeling techniques that enabled a hypothetical model to be constructed and formed the basis for more detailed mutational studies. Using these approaches we found that the BCH domains of BNIP-2 and Cdc42GAP are responsible for their homophilic or heterophilic interactions. We further identified a discrete region within the BNIP-2 BCH domain that is responsible for the interactions involving BNIP-2. Having presented evidence to show that the BCH domain was involved in protein-protein interactions we searched various data bases to see what other proteins might contain this domain and to see what other domains they are associated with. The significance of this novel BCH domain-containing family is discussed.

MATERIALS AND METHODS

Plasmids—Full-length cDNA of BNIP-2 was cloned into a hemagglutinin (HA)-tagged or FLAG-tagged expression vector, pXJ40 (Dr. E. Manser, IMCB, Singapore), or into pGEX-1T-1 vector for producing the GST recombinant protein as described previously (3). 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 pXJ40HA and pXJ40FLAG constructs. SHP-2 mammalian expression construct was a gift from Dr. C. J. Pallen (IMCB, Singapore). Deletion mutants of BNIP-2 were generated by polymerase chain reaction using specific primers facilitated by restriction sites. All plasmids were purified using a Wizard miniprep kit (Promega) or Wizard Maxi/Mega-prep kit followed by ethanol re-precipitation 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 clones. Reagents used were of analytical grade and standard protocols for molecular manipulations and media preparations were as described in Ref. 30.

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 FxX-50™ cationic lipids according to the manufacturer’s instructions (Promega).

Precipitation Experiments and Western Blot Analyses—Control cells or cells transfected with appropriate expression plasmids 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 protease inhibitors (Roche Molecular Biochemicals), and 5 mM sodium orthovanadate). The lysates that were directly analyzed either as whole cell lysates (25 μg) or aliquots (500 μg) were used in affinity precipitation/pull-down experiments with various GST fusion proteins (5 μg) or GST-Cdc42 (5 μg) that had been preloaded with GTPγS (Sigma) as described previously (2). 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 of Cdc42 was examined by determining the release of [32P] from the γ-[32P]GTP prebound to the molecule as described previously (25).

Yeast Two-hybrid Interaction Assays—Full-length BNIP-2 were fused downstream of the GAL4 DNA-binding domain in a pAS2 vector and tested for interaction with various proteins expressed from the construct fused with GAL4 DNA activation domain in the pACT vector according to the manufacturer’s instructions (CLONTECH). Positive yeast hosts were scored for the appearance of blue color of the yeast host in less than 8 h according to the protocol. In all experiments, however, the appearance of blue colonies was already apparent within 2 h indicating strong direct binding.

Molecular Modeling and Sequence Alignments—The methods for modeling the BNIP-2 BCH domain were as described previously (25, 31). One of the templates used in comparative modeling was the crystal structure of the breakpoint cluster region homology (BH) domain from the phosphatidylinositol 3-kinase p85α regulatory subunit (Protein Data Bank code: 1PBW). Crystals of the BH domain contained two monomers (A and B) which were present as a dimer in the asymmetric unit (32). The model of the BH homodimer was derived by superimposition of two BNIP-2 BH domains upon A and B monomers of BH domains at the level of the Cα carbon coordinates. The BH model was subjected to energy minimization in stages to refine the loops and side chains.

BNIP-2 and Cdc42GAP Interact via Their Respective BCH Domains—We have previously demonstrated that BNIP-2 could form a complex in vitro and in vivo with the 50-kDa Cdc42GAP, a GTPase-activating protein for the small GTPase, Cdc42 (2). To gain an insight into how this binding takes place we set out to perform reciprocal precipitation experiments with either full-length or various deletions of GST fusion proteins for BNIP-2 or Cdc42GAP. Each protein fragment represented various known domains or “domains” that were assumed on likely secondary structure evidence. The organization of the resultant protein fragments are depicted in Fig. 1A. Each protein was divided into three fragments, A-series fragments for BNIP-2 and B-series fragments for Cdc42GAP. The COOH-terminal of BNIP-2 (amino acids 167–314; fragment A3) and the region of Cdc42GAP encompassing amino acids 86–208 (fragment B2) share a high degree of homology with each other that we have previously termed the BCH (BNIP-2 and Cdc42GAP homology) domain (25). The Cdc42GAP-BCH domain is located proximal to its canonical catalytic GAP domain which is encompassed within fragment B3 (amino acids 208–440) while its NH2 terminus fragment B1 (amino acids 1–85) contains a small region of homology to part of the NH2 terminus (fragment A1) of BNIP-2 (amino acids 1–70; Ref. 2). The unique region within BNIP-2 that is flanked by A1 and the BNIP-2-BCH domain (fragment A3) is arbitrarily set as fragment A2 (amino acids 71–166).

These GST recombinants were expressed in E. coli, purified, and verified intact by standard Coomassie Blue staining prior to the precipitation experiments. Human 293T cells were transfected with an expression vector encoding Cdc42GAP tagged with a FLAG epitope and the lysates were subjected to precipitation using GST recombinants of BNIP-2 or Cdc42GAP as described in Fig. 1B. The precipitated proteins were separated by SDS-PAGE followed by Western blotting using FLAG antibody as described under “Materials and Methods.” The data in Fig. 1B (top panel) shows that the recombinant full-length BNIP-2 binds Cdc42GAP and this interaction was enhanced when the A3 region of BNIP-2 (BNIP2-BCH domain) was the binding partner. Some weaker binding could be seen with fragment A2 but no significant interaction was detectable with region A1 of BNIP-2. Interestingly, when full-length proteins are transfected into human 293T cells, we observed a reciprocal interaction using FLAG antibody. This reciprocal interaction is consistent with the data presented in this study.
Cdc42GAP was used in similar precipitation experiments, it was observed to bind very strongly to itself and this interaction was solely mediated by region B2, which is the BCH domain of Cdc42GAP. No binding was detectable with other regions.

Results from the initial binding experiment strongly indicated that binding of BNIP-2 to Cdc42GAP and binding of Cdc42GAP to Cdc42GAP were both mediated by the BCH domains, thus implying that BNIP-2 would also bind in a similar homophilic manner via its BCH domain. To test this experimentally, BNIP-2 tagged with a HA epitope was expressed in human 293T cells and lysates were used in similar precipitation experiment to those described above. The precipitated proteins were separated by SDS-PAGE followed by Western blotting using HA antibody (Fig. 1B, middle panel). As predicted, GST-BNIP-2 could precipitate BNIP-2 (lane 5) expressed in the cells and its BCH domain essentially mediated this interaction. As demonstrated in Fig. 1B (top panel) recombinant full-length Cdc42GAP precipitated full-length BNIP-2 (lane 6) and this was primarily mediated by the BCH domain of Cdc42GAP (lane 7) and not by the other regions of the protein (lanes 8 and 9). The apparent homophilic and heterophilic interaction between BNIP-2 or Cdc42GAP that was mediated by the respective BCH domains was deemed specific because none of their GST fragments was seen to bind endogenous proteins such as the Crk adaptor protein or the Lyn tyrosine kinase (data not shown). Similarly, no binding by these fragments could be seen when the phosphotyrosine phosphatase SHP-2 was overexpressed in 293T cells and similar precipitation experiments were performed (Fig. 1B, bottom panel).

BNIP-2 Forms Homophilic Associations in Vivo—As we had previously demonstrated that BNIP-2 binds Cdc42GAP in vivo (2) we next set out to verify that BNIP-2 could form a homophilic complex in vivo, as suggested by the in vitro results shown in Fig. 1. To this end, BNIP-2 was constructed with two different epitope tags, one with the HA epitope and the other one with the FLAG epitope. Human 293T cells were transfected with or without expression vector encoding the described constructs, either alone or together. Equal amounts of lysates were then subjected to immunoprecipitation using either HA or FLAG antibodies and the precipitated proteins were separated by SDS-PAGE followed by Western blotting using the FLAG or HA antibodies as described under “Materials and Methods.” Fig. 2 shows that FLAG-BNIP-2 immunoprecipitated with FLAG antibody could bring down HA-BNIP-2 (top panel). The blot was stripped and reprobed with FLAG antibody to show equal amounts of immunoprecipitated protein (second panel) while similar Western blots of the whole cell lysates (fifth panel) demonstrate similar levels of expression for FLAG-tagged BNIP-2. In a reciprocal experiment, HA-BNIP-2 immu-
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Fig. 2. Homomeric binding of BNIP-2 in vivo. A, 293T cells were transfected with (+) or without (−) expression vectors for HA-BNIP-2 and FLAG-BNIP-2, separately or together as indicated. Lysates were immunoprecipitated (IP) with FLAG or HA antibodies and the associated proteins were separated on SDS-PAGE, Western blotted (WB), and probed with HA or FLAG antibodies to reveal the binding of targets. WCL, whole cell lysates.

noprecipitated with HA antibody revealed the presence of FLAG-BNIP-2 in the complex (third panel). Equal precipitation and expression of HA-tagged BNIP-2 was likewise shown (fourth and bottom panels, respectively).

BNIP-2 and Cdc42GAP Associate Directly in Vivo—All binding experiments described above used overexpression systems where both the binding partner proteins (or fragments) were present in great excess in order to minimize the likely involvement of any other endogenous proteins. To further verify the direct involvement of BCH domains in the formation of BNIP-2 and Cdc42GAP hetero- and homophilic complexes in vivo, the yeast two-hybrid system was employed (36). BNIP-2 was constructed in the pAS GAL4 DNA-binding domain and tested against a panel of Cdc42GAP targets, either as the full-length or as the separate domains: B1, B2, and B3 (as shown in Fig. 1A). These constructs were cloned downstream of the pACT GAL4 DNA-activation domain (Table I). Standard assays for the lacZ reporter system for positive interactions were performed, as described under “Materials and Methods.” The results show that BNIP-2 interacted directly and strongly with full-length Cdc42GAP and also with the fragment B2 (i.e. Cdc42GAP-BCH domain). As we had previously seen, BNIP-2 also interacted directly with Cdc42, where it acts as a GAP (25). Furthermore, BNIP-2 was again shown to associate with itself but did not bind to protein kinase C-λ, that was included as a negative control.

Defining the Region of Interaction in the BNIP-2 BCH Domain—We were interested to further define the region within the BCH domain that was responsible for binding. The BCH domain of BNIP-2 was further arbitrarily subdivided into three fragments that were designated as C1, C2, and C3 (Fig. 3A). We performed precipitation experiments using GST recombinants corresponding to these subregions of the BNIP-2 BCH domain. Fragments A1 and A2 were the same as those used in the experiments shown in Fig. 1A. The C-series fragments had previously been used to help identify the region of BNIP-2 that binds Cdc42 (25). Human 293T cells were transfected with expression plasmids encoding HA-tagged BNIP-2, FLAG-tagged Cdc42GAP, HA-tagged Cdc42 (used as a comparison), or phosphotyrosine phosphatase SHP-2 (used as a negative control). The lysates were subjected to precipitation using equal amounts of various GST recombinants of BNIP-2 and the precipitated proteins were separated by SDS-PAGE, Western blotted (WB), and probed with HA antibody (top and third panels), FLAG antibody (second panel), or SHP-2 antibody (lower panel) to reveal the binding of targets. WCL, whole cell lysates; FL, full-length proteins.

Table I

| pACT constructs | pAS constructs |
|-----------------|----------------|
| pACT alone | BNIP-2 | pAS alone |
| Cdc42GAP (full-length) | Cdc42GAP | Cdc42GAP-B2 (BCH) |
| Cdc42GAP-B1 | Cdc42GAP-B3 | BNIP-2 |
| BNIP-2 | Cdc42 | PKK-λ |

Fig. 3. Discrete binding regions within BCH domains. A, defined are regions of BNIP-2 used in the production of GST recombinants in E. coli as described under “Materials and Methods.” B, GST recombinants from A were used to precipitate lysates of 293T cells transfected (77) with expression vectors for HA-BNIP-2, FLAG-Cdc42GAP, HA-Cdc42, or SHP-2. The associated proteins were separated on SDS-PAGE, Western blotted (WB), and probed with HA antibody (top and third panels), FLAG antibody (second panel), or SHP-2 antibody (lower panel) to reveal the binding of targets. WCL, whole cell lysates; FL, full-length proteins.
(top panel). Similarly, the C2 region in BNIP-2 was also predominantly involved in binding to Cdc42GAP (second panel). In contrast to the homophilic and heterophilic binding mediated by the respective BCH domains and consistent with our previous report, the C3 region of the BNIP-2 BCH domain was primarily involved in binding to Cdc42 (third panel) (25). None of the BNIP-2 fragments was shown to interact with SHP-2 that was overexpressed in the same system (bottom panel). These results indicate that the C2 region of BNIP-2 is the major binding site for BCH domain interactions.

Molecular Modeling of Binding Regions Within the BCH Domain of BNIP-2—We recently identified that the BCH domain of BNIP-2 confers GAP activity toward Cdc42 (25). Secondary structure predictions of the BCH domain suggest that it mainly consists of a helices (data not shown). This is consistent with that of the a-helical bundles of the catalytic GAP domain of Cdc42GAP (25). Since the majority of GAPs have specific substrates, we hypothesized that by having a common substrate (i.e. Cdc42), it may imply a common structural fold shared by the BCH domain of BNIP-2 and the other two Cdc42-binding proteins that are either active as a GAP (i.e. the RhoGAP domain of Cdc42GAP; Refs. 18 and 37) or catalytically inactive toward Cdc42 (the BH domain of the p85 regulatory subunit of phosphatidylinositol 3-kinase) (32). Molecular modeling of the BCH domain of BNIP-2 had helped us identify the arginine-patch 235RRLRK239 as a novel and essential arginine motif for catalysis, while another region adjacent to it, 288EYV290, was identified as a major binding site for Cdc42 (25). In this study, we used the same BCH model to test if it was possible to further delineate the interaction sites of homo- and heterodimerization observed in the in vitro and in vivo experiments shown above.

To model the BCH homodimer complex, the protein structure data base was searched for a template that would mimic the configuration of protein-protein interactions of the BCH domain. The BH domain of the p85 regulatory subunit of phosphatidylinositol 3-kinase was known from crystallographic studies to consist of two monomers in the asymmetric unit and related by a 2-fold non-crystallographic symmetry axis (32). The dimerization interface is hydrophobic with the side chain of Met176 from one monomer inserting into a small, exposed pocket formed by three hydrophobic residues located in the AB loop of the BH domain. Recently, using both in vitro and in vivo experiments, the BH domain was shown to be involved in the protein dimerization interface of p85 (38). Since one of the structural templates used in the comparative modeling of BCH domain was the BH domain, we set out to determine if the BCH domain of BNIP-2 could form a dimer in a similar structural configuration as the BH domain.

Two previously modeled BCH domains of BNIP-2 were superimposed onto the monomer A and B of the BH dimer structure. The resultant BCH dimer was then energy minimized. The BCH homodimer model assumes similar 2-fold symmetry in agreement to that observed with the BH homodimer (Fig. 4). Based on the model of the BCH dimer, it was deduced that a small amount of residues 217RRKMP221 could potentially contribute to the dimer interaction. These residues are part of the C2 region that was described earlier as the major binding fragment of the BNIP-2 BCH domain (see Fig. 3). Furthermore, this region is part of the BCH structure loop region that is homologous to the AB loop found in the BH domain (32). The model also predicts that the previously identified arginine-patch 235RRLRK239 (necessary for GAP activity) and the region 288EYV290 (necessary for binding to Cdc42) are not part of the dimerization interface and are seemingly useful as internal controls to test the binding specificity (Fig. 4).

Identification of a BCH-interacting Motif in BNIP-2—Based on the above model, deletion mutants corresponding to 217RRKMP221 (a-M), the arginine-patch 235RRLRK239 (a-R), and the region 288EYV290 (a-T) were introduced into BNIP-2 tagged with the HA epitope and expressed in 293T cells for use in binding studies. To test for various interactions, lysates containing equal amounts of HA-BNIP-2 wild-type or mutants were subjected to precipitation experiments using equal amounts of GST heads alone or GST recombinants of the wild-type BNIP-2-BCH, Cdc42GAP-BCH, or Cdc42 preloaded with GTP-S. The precipitated proteins were separated by SDS-PAGE followed by Western blotting using HA antibody as described under "Materials and Methods." The results from Fig. 5A (top panel) show that the recombinant BNIP-2 BCH domain bound full-length BNIP-2 strongly and this interaction was severely impaired by the deletion at region M, whereas deletions at regions R and T had no noticeable effect. Similar results were seen when the GST recombinant of Cdc42GAP-BCH was used as the precipitating agent (second panel). However, deletion of region M, similar to deletions of region R and
Fig. 5. BCH-interacting motif in BNIP-2. A, 293T cells were transfected with expression vectors for HA-BNIP-2 either as the wild type (WT), deletion mutants Δ-M, Δ-R, or Δ-T as described under "Materials and Methods." Lysates were precipitated using GST-BNIP2-BCH, (top panel), GST-Cdc42GAP-BCH (second panel), GST-Cdc42 preloaded with GTPγS (third panel), or just GST beads alone (fourth panel) as described under "Materials and Methods." Associated proteins were separated by SDS-PAGE, Western blotted (WB), and probed with HA antibody. Aliquots of the whole cell lysates (WCL) were also analyzed for equal expression of the WT or deletion mutants of HA-BNIP-2 proteins by HA Western analysis (bottom panel). B, 293T cells were transfected with plasmids expressing HA-BNIP-2 wild-type or Δ-M mutant, or FLAG-Cdc42GAP, either alone or together, as indicated in the figure. The Western blots (bottom panels) show that the cells expressed correct and intact proteins from the indicated combination of plasmids. Aliquots of these lysates were then used for GAP assays with GST-Cdc42 preloaded with radioactive GTP as described under "Materials and Methods." The activity was expressed as the fold increase over the control using the vector alone. Results are mean ± S.D. of three replicate determinations.
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T, had no effect on the binding of BNIP-2 with Cdc42 (third panel). Deletion of the region T of BNIP-2 was recently identified to cause loss of binding to Cdc42 only when the Switch I or Insert region of Cdc42 was also deleted. No effect was seen if only a single deletion of region T was introduced (25). As further controls, none of the HA-BNIP-2 forms bound to the GST beads alone (fourth panel). In all the experiments described above, each of the BNIP-2, wild-type, or deletion mutants were expressed equally well and confirmed intact in 293T cells as seen when their whole cell lysates were probed with anti-HA (bottom panel). In reciprocal precipitation experiments, the deletion of region M was introduced into a GST recombinant of BNIP-2-BCH and tested for binding to BNIP-2 or Cdc42GAP expressed in the cells. In all cases, and consistent with the results above, this deletion severely impaired complex formation (data not shown). These results lend credibility to our predictive model as they strongly indicate that the region 217RRKMP221 of BNIP-2 is a prime binding site within the BCH domain that it is involved in homophilic or heterophilic interactions. We also performed a preliminary mutation experiment with a similarly located sequence in the Cdc42GAP-BCH domain and results indicated that it was not involved in mediating the BCH domain-dependent homo- or heterophilic binding. We are interested in determining the sequence(s) on Cdc42GAP-BCH that mediates such interaction, which will involve further molecular modeling of the domain itself and more extensive mutational studies.

We had earlier reported that BNIP-2 and Cdc42GAP antagonized each others GAP activity (2). We were therefore interested to see what effects the Δ-M mutant of BNIP-2 had on the GAP activity of either BNIP-2 or Cdc42GAP, either singly or in combination. 293T cells were transfected with plasmids expressing HA-BNIP-2 wild-type or Δ-M mutant, or FLAG-Cdc42GAP, either alone or together, as indicated in Fig. 5B. Equal aliquots of these lysates were then used for GAP assays with GST-Cdc42 preloaded with radioactive GTP as described under “Materials and Methods.” BNIP-2 and Cdc42GAP each conferred a 4- and 10-fold increase in the GTP hydrolysis by Cdc42, respectively, but there was no additive effect when both GAPs were present. However, if deletion of region M was introduced, there was a further increase in the activity of both GAPs. Furthermore, when BNIP-2 Δ-M was present together with Cdc42GAP an additive GAP effect was observed. The Western blots (bottom panels) show that the cells expressed correct and intact proteins from the indicated combination of plasmids. These results demonstrate that the BCH-mediated homophilic binding of BNIP-2 (via the region-M) influences the GAP activity of BNIP-2 or the cumulative GAP activity when in combination with Cdc42GAP. Since currently we have not established the site(s) in the Cdc42GAP-BCH domain that is involved in its homophilic and heterophilic interaction, it remains to be seen whether deletion of such binding regions in Cdc42GAP could lead to an increase in its GAP activity as was seen for BNIP-2.

The BCH Domain Is Highly Conserved Throughout Evolution—In light of our data that indicated that the BCH domain of BNIP-2 or Cdc42GAP was a novel protein-protein interaction sequence, we were intrigued to know how many other proteins contained this sequence, if the sequence was conserved throughout evolution, and if resultant analyses would give us some clues as to other potential physiological functions of proteins that harbor this domain. For such an analysis we used PSI-BLAST to search the NCBI non-redundant protein data bases using the BCH domains of either BNIP-2 or Cdc42GAP as described under “Materials and Methods.” Similar sets of proteins were reproducibly identified (Fig. 6A). The BCH domain is evolutionarily conserved with representatives from S. cerevisiae, P. falciparum, A. thaliana, C. elegans, and H. sapiens. Such conservation over a prolonged time period would indicate an important physiological function for this novel domain.

To appreciate the domain organization of proteins containing the BCH domain, PFAM analyses were used and the results are displayed in Fig. 6B. There are two types of distribution for the BCH domain. The human proteins BNIP-2 and CAB07531.1 and putative proteins from A. thaliana, AAF02821 and CAA20045, exemplify the first distribution type. These proteins have BCH domains consistently located at the COOH-terminal. Individual two-sequence BLAST searches between each one of them with the full-length BNIP-2 showed no other regions of homology between these molecules and that all proteins lack the canonical GAP domain. This indicates that these A. thaliana proteins are not plant homologs of human BNIP-2 or Cdc42GAP, but rather represent distinct proteins containing conserved BCH domains. The second type of BCH distribution has representatives from yeast, plasmodium, and worm to human. These proteins have the classical RhoGAP catalytic GAP domain that is consistently found distal to the BCH domain. Interestingly, the spacing between these two domains is relatively well conserved, implying that they have co-evolved and could be under some form of co-structural or co-functional constraints.

DISCUSSION

The present study examined the interaction between BNIP-2 and Cdc42GAP by using in vitro and in vivo binding experiments and demonstrated that their homologous BCH domains primarily mediate both homophilic and heterophilic interaction between the proteins. Deletion studies aided by computer modeling allowed us to further define a unique region at 217RRKMP221 of BNIP-2 as the major determinant in the complex formation. This region is distinct from two other regions of BNIP-2 we had recently identified; the arginine-patch 235RRHK239 and the region 286ETY290 both of which are important for GAP activity of BNIP-2 and its binding to Cdc42, respectively (25). Since all these regions lie within the BCH domain it raises the interesting question as to how the homophilic and heterophilic interaction of BNIP-2 and/or Cdc42GAP (either in their BCH forms or as their full-length entities) would influence their GAP activity toward Cdc42. Our previous studies had shown that the presence of both BNIP-2 and Cdc42GAP led to a decreased GAP activity toward Cdc42 in comparison to when either protein was present alone (2). Such experiments suggest that the presence of BNIP-2 could
antagonize the GAP activity of Cdc42GAP and vice versa. We have now demonstrated that their binding via BCH domains is at least partly responsible for this inhibitory effect. The BCH-mediated binding of BNIP-2 (specifically via the region-M) negatively regulates the GAP activity of BNIP-2 as well as the Cdc42GAP. We are now trying to establish the site(s) in the Cdc42GAP-BCH domain that is involved in its homophilic and heterophilic interaction, and it remains to be seen whether deletion of such a binding region(s) in Cdc42GAP could lead to an increase in its GAP activity as was seen for BNIP-2.

In the case of Cdc42GAP, it was intriguing to see that this molecule harbors two Cdc42-binding domains. In addition to the canonical GAP domain at the carboxyl terminus, we recently identified that the BCH domain of Cdc42GAP can also bind Cdc42 but lacks catalytic activity as it is devoid of the arginine-patch motif found in the BNIP-2 BCH domain. The question arises as to what is the role of the BCH domain of Cdc42GAP? Potentially the Cdc42GAP-BCH domain can act as another binding interface for Cdc42, perhaps by interacting with other regions of the GTPase. Our current findings that Cdc42GAP is also capable of homophilic binding and/or heterophilic interactions with BNIP-2, via the same BCH domain, has added another layer of complexity to the potential regulation of both GAP proteins.

The notion that both the BCH and GAP domains in Cdc42GAP and in other members of the RhoGAP subfamily are vital for their possible activation and function is supported by our observations that the spacing between the two domains is well conserved (Fig. 6B). Nevertheless, the BCH and GAP domains of the various RhoGAPs in tandem are not a universal corollary of RhoGAP catalysis as they appear in only a subset of RhoGAP family proteins. It remains to be seen if there are any unique biochemical or cellular locational peculiarities for this subclass of RhoGAP when compared with those without the proximal BCH domains. Currently there is scant information pertaining to this class of RhoGAP proteins. Similarly, all proteins containing a type-1 BCH distribution, i.e. with their BCH domains at the carboxyl end, have no known functions. Work is currently underway in our laboratory to characterize proteins with each type of BCH domain distribution.

To date, all structural studies, and their functional inferences, pertaining to Cdc42GAP and Cdc42 are based on bimolecular complexes between Cdc42 or Rho with the catalytic GAP domain. No studies have been made with either the BCH domain or full-length Cdc42GAP. Structural determinations involving the BCH domain, or better still the whole protein, would give a more complete understanding of the molecular mechanism involved in the regulation of this protein and its interaction with other proteins.

The occurrence of at least a dozen distinct proteins with highly conserved BCH domains across so many species suggests that this domain should play a significant role(s) in some biological processes. With more genomes being sequenced, it is anticipated that the number of proteins harboring similar domains would increase. Although we have shown that the BCH domains of BNIP-2 and Cdc42GAP represent a novel protein-protein interaction domain it remains to be seen if all other BCH domains in other proteins are also involved in mediating protein-protein interaction. If they do, interaction among some of these BCH-containing molecules would confer functional diversity for various biological processes. It is worth noting that in the model of the BNIP-2 BCH domain, the region involved in mediating its binding is not conserved in all other “family members” including one of its binding partners, the Cdc42GAP-BCH domain. This non-homology of actual binding region within a conserved structure might provide a mechanism for the regulation of target specificity.

Recently, part of the BCH domains of BNIP-2 and Cdc42GAP were deemed, after multiple rounds of iteration in PSI-BLAST analysis, to share a limited homology to the Sec14p-like domain, previously known to mediate the exchange of phosphatidylinositol and phosphatidylcholine in S. cerevisiae (28). The implications of this observation are manifold. One possibility is that in vivo the BCH domain actually targets to some specific phospholipid moiety and this would direct associated catalytic domains into favorable locations near their substrates. A second possibility is, in addition to mediating protein-protein interaction, BCH domains might bind lipids such that their interaction could be modified and thus regulated, either directly on the protein-binding site or in an allosteric fashion. Regulation of protein-protein interaction by lipids has recently been reported for the intramolecular interaction between the lipid-binding pleckstrin homology and the catalytic Dbl homology domains of Vav or Sos1, two guanine nucleotide exchange factors (39). It was shown that phosphatidylinositol 3-kinase substrate promotes the binding of these two domains and blocks Rac binding to the Dbl homology domain, whereas products of phosphatidylinositol 3-kinase disrupt such interaction and allows Rac binding for activation. A third possibility is that BCH domains are purely protein-protein interaction domains that have diverged sufficiently from Sec14p lipid-binding domains to have evolved a separate function.

Our preliminary data on indirect immunofluorescence of BNIP-2 does not show any unique membrane localization of the protein in cells. Neither plasma or organelle membranes appeared to be stained; instead we observed a punctate pattern of distribution more likely linked to a cytoskeletal distribution (data not shown). This observation apparently rules out the notion of at least the BNIP-2 BCH domain being a membrane lipid-targeting device. Current work is aimed at addressing the detailed intracellular localization of BNIP-2.

In conclusion, our present work has shown that BCH domains of BNIP-2 and Cdc42GAP define a novel class of protein-protein interaction domain that includes various uncharacterized proteins. It may represent another example of proteins that form dimers as a functional necessity such as: various receptor tyrosine kinases (40), STAT transcription factors (41), c-Raf (42, 43), and various members of the Bcl family (44, 45). Our work also highlights the fact that although several structural studies have used the catalytic domain of Cdc42GAP to define a precise interaction with Cdc42 (and Rho) (18, 46) there is still much to be understood about how this protein is targeted and activated and indeed what its actual physiological role is. A better understanding of the structure and functional roles of the BCH domains of BNIP-2, Cdc42GAP, and of other proteins will answer these questions.

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