Biochemical Comparisons of the \textit{Saccharomyces cerevisiae} Bem2 and Bem3 Proteins

DELINEATION OF A LIMIT Cdc42 GTPASE-ACTIVATING PROTEIN DOMAIN*

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The Bem2 and Bem3 proteins, which appear to play roles in the regulation of bud site formation in \textit{Saccharomyces cerevisiae}, show striking homology to a number of proteins that compose a family of GTPase-activating proteins (GAPs) for the rho-subgroup of ras-related GTP-binding proteins. These members include human platelet GAP for Cdc42Hs (the human homolog of a \textit{S. cerevisiae} GTP-binding protein that regulates bud site assembly), the break point cluster region protein, the brain protein chimerin, the 85-kDa regulatory subunit (p85) of the phosphatidylinositol 3-kinase, and the ras-GAP-binding protein (p180). A fusion protein composed of the glutathione S-transferase protein and the rho-GAP homology region of Bem3 (designated GST-Bem3) stimulates the GTPase activity of the wild-type Cdc42Hs protein (Cdc42HsGTP-Cdc42HsGDP), but has no stimulatory effect on a GTPase-defective mutant (Cdc42HsGTP-Cdc42HsGAP), whereas a GST-Bem2 fusion protein does not stimulate the GTPase activity of either form of Cdc42Hs. We have compared the ability of GST-Bem3 to serve as a GAP for Cdc42Hs relative to other members of the rho-GAP subfamily and found the following order of potency: human platelet Cdc42Hs GAP >> p180 >> Bem3 >> break point cluster region protein, whereas p85, like Bem2, shows no GAP activity or any ability to bind to the GTP-bound form of Cdc42Hs.

We have taken advantage of the functional specificity exhibited by Bem3 (\textit{versus} Bem2) in using Bem2/Bem3 chimeras, as well as different deletion mutant versions of the Bem3 protein, to delineate the limits of a functional Cdc42 GAP domain. The results of this study indicate that the carboxyl-terminal ~224 amino acids (which contain three regions of homology to the other members of the rho-GAP family) represent a "limit GAP." The first two appear to be important for binding to Cdc42Hs and for partial GAP activity.

The different members of the rho-subgroup of ras-related GTP-binding proteins appear to play "organizational roles" in different cytoskeleton-associated assembly processes (Hall, 1992; Ridley and Hall, 1992; Ridley et al., 1992). One example is the \textit{Saccharomyces cerevisiae} Cdc42 protein (designated Cdc42Sc), a cell division cycle protein that mediates the assembly of the bud site in yeast (Johnson and Pringle, 1990; Drubin, 1991). We have used the human homolog of the Cdc42Sc protein (designated Cdc42Hs) (Shinju et al., 1990) as a model to study the protein-protein interactions underlying the regulation of rho-subtype proteins. Although the role of Cdc42 in mammalian cells is currently unknown, significant progress has been made toward the identification of proteins that are capable of directly influencing the GTP binding and GTPase activities of the human Cdc42 protein. One of these, the dbl oncogene product, stimulates the dissociation of GDP from Cdc42Hs and thereby catalyzes the GDP-GTP exchange activity (Hart et al., 1991a). This putative exchange factor (or GTP dissociation stimulator) shares a region of homology (of 238 amino acids) with the \textit{S. cerevisiae} Cdc24 protein, which also has been implicated in bud site assembly in yeast (Sloat et al., 1981). A second Cdc42Hs regulatory protein effectively opposes the actions of the dbl oncogene product and inhibits GDP dissociation (Leonard et al., 1992). The available amino acid sequence for this regulatory protein indicates that it is very similar, if not identical, to a protein that earlier was shown to inhibit GDP dissociation from the rho- and rac-proteins and was designated rho-GDI1 (Fukumoto et al., 1990). The GDI protein also appears to be capable of triggering the release of Cdc42Hs from membranes (Leonard et al., 1992) and to inhibit the GTPase activity of Cdc42Hs (Hart et al., 1992).

A third group of Cdc42Hs regulatory proteins are the GTPase-activating proteins (GAPs), which stimulate the GTP hydrolytic event. GAPs are especially interesting because they are likely to represent a diversity of structures and because these regulatory proteins may serve as target/effectors as well as negative regulators of Cdc42Hs and/or related GTP-binding proteins. A Cdc42Hs GAP was first identified and purified from human platelets (Hart et al., 1991b). The apparent molecular mass of this GAP (25 kDa) is similar to that of the rho-GAP (~30 kDa) that has been purified from the cytosol of spleen (Garrett et al., 1989) and bovine adrenal glands (Mori et al., 1991). Based on the available amino acid sequence from rho-GAP, a number of proteins (of varying size) that share sequence homology with rho-GAP were identified. One of these is the break point cluster region protein (bcr), the product of a gene that is rearranged in the Philadelphia chromosome and fused to the abl tyrosine kinase-encoding gene (Heisterkamp et al., 1985). The carboxyl-terminal third of bcr was shown to act as a GAP for the rac1 protein (Diekmann et al., 1991), and more recently, full-length bcr was reported to act as a GAP for Cdc42Hs (Hart et al., 1992). The brain protein chimerin (~30 kDa) also contains a rho-GAP homology region and was shown

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‡ The abbreviations used are: GDI, GDP dissociation inhibitor; GAPs, GTPase-activating proteins for low molecular mass GTP-binding proteins; bcr, break point cluster region protein; PAGE, polyacrylamide gel electrophoresis.
to serve as a GAP for rac1 (Diekmann et al., 1991). Two other proteins sharing homology with rho-GAP are the 85-kDa regulatory domain (p85) of the phosphatidylinositol 3-kinase and the ras-GAP-binding protein (p190) (Hall et al., 1992). The p190 protein was shown to have GAP activity for the rho-, rac-, and Cdc42Hs GTP-binding proteins (Settleman et al., 1992).

Recently, two S. cerevisiae gene products, designated Bem2 (~245 kDa) and Bem3 (~125 kDa), that are suspected to be involved in the regulation of bud site formation (presumably acting downstream from Cdc42Sc) have been identified. These two yeast proteins contain sequences (near their carboxyl-terminal ends) that bear striking similarity to rho-GAP, bcr, chimerin, and p190. In this study, we compare the abilities of Bem3, bcr, p190, and platelet Cdc42Hs GAP to couple functionally to the human Cdc42 protein and take advantage of the functional differences between the Bem2 and Bem3 proteins in utilizing Bem2/Bem3 chimeras in combination with various deletion mutant versions of the Bem3 protein to delineate a limit catalytic Cdc42 GAP domain.

EXPERIMENTAL PROCEDURES

Construction of Expression Plasmids for Bem2 and Bem3 Mutants—The Escherichia coli vector pGEX-KG (for the expression of glutathione S-transferase fusion proteins) was generated from pGEX-2T (Pharmacia LKB Biotechnology Inc.) by insertion of a polylinker into the EcoRI cloning site (Gaan and Dijon, 1991). The plasmids for expressing the Bem2 and Bem3 carboxyl-terminal polypeptides as glutathione S-transferase fusion proteins (GST-Bem2, GST-Bem2', and GST-Bem3) were constructed by digesting pPG/BEM3 and pPG/BEMZ3 with HincII and HindIII to make GST-Bem2 (which contains residues 1652-2167 of the Bem2 protein), Ban1 and HindIII to make GST-Bem3 (residues 751-1128 of the Bem3 protein). The segments of the BEM2 gene were further subcloned from pGEX-KG-BEM3 into HincII and HindIII to make GST-Bem2 (which contains residues 1652-2167 of the Bem2 protein), Ban1 and HindIII to make GST-Bem3 (residues 904-1128). The chimeras of the Bem2 and Bem3 proteins were made by ligating segments of the BEM3 gene to BEM2 using linkers that filled in any missing amino acid codons and make Bemafi (residues 904-1128). The chimeras of the Bem2 and Bem3 proteins were made by ligating segments of the BEM3 gene to BEM2 was ligated to the DraI-Hind11 fragment of BEM3 and continued on page...
extent of amino acid identity occurs in box 1 (i.e., the homology region nearest the amino-terminal end), where the Bem2 and Bem3 proteins share ~40% identity and where Bem3 is 44% identical to bcr, while Bem2 is 57% identical to bcr. The sequences within the middle region of homology (box 2) are ~30% identical among the different proteins, whereas the sequences composing box 3 (nearest the carboxyl-terminal end) are 20–25% identical among the different proteins.

Bem3 Protein Serves as GAP for Human Homolog of Cdc42

The carboxyl-terminal portions of the Bem2 and Bem3 proteins have been expressed in E. coli as glutathione S-transferase fusion proteins using the pGEX-KG expression system. Two forms of the BEM2 gene cDNA (containing 600 base pairs and 1.7 kilobases) and one form of the BEM3 gene (containing 1.3 kilobases), which included all three boxes of the GAP homology region, were fused to the glutathione S-transferase gene under the control of the tac promoter. Induction by isopropyl-1-thio-β-D-galactopyranoside resulted in the appearance of a protein band of ~70 kDa for GST-Bem3 and protein bands of ~90 and 55 kDa for GST-Bem2 that are designated below (also see Fig. 2A) as GST-Bem2 and GST-Bem2', respectively. Fig. 2B shows that the recombinant GST-Bem3 protein is able to effectively stimulate the GTPase activity of the human Cdc42 protein to an extent that is comparable to the stimulation obtained with Cdc42Hs GAP purified from human platelets (Hart et al., 1991b) (also see below). Neither the GST-Bem2 protein nor GST-Bem2' showed any ability to stimulate the GTPase activity of Cdc42Hs, i.e., the activities measured in the presence of these proteins were essentially identical to the basal rate of GTP hydrolysis and were indistinguishable from the GTPase activity for Cdc42Hs measured in the presence of glutathione S-transferase alone. The GST-Bem3 and GST-Bem2 or GST-Bem2' proteins also showed no effect on the binding of [γ-32P]GTP to Cdc42Hs (relative to the binding of [γ-32P]GTP to Cdc42Hs in buffer alone), indicating that these glutathione S-transferase fusion proteins did not affect the GDP-GTP exchange activity of the GTP-binding protein.

One oncogenic form of H-ras has an amino acid alteration at position 12 (i.e., a valine residue is substituted for a glycine residue); this mutated ras-protein has an impaired (basal) GTPase activity that cannot be stimulated by ras-GAP (Trahey and McCormick, 1987). An analogous mutation in Cdc42Hs yields a similar result, i.e., a GTPase-defective protein that cannot be stimulated by platelet Cdc42Hs GAP (Hart et al., 1991b). As shown in Fig. 2C, the GST-Bem3 protein also is completely ineffective in stimulating the GTPase activity of the Cdc42Hs51Val protein.

Comparisons of Activities of Different GAPs for Cdc42Hs

The abilities of different proteins to stimulate the GTPase activity of the human Cdc42 protein were assessed using a quantitative assay that measures 32P, release (Brant et al., 1983). The results of these studies, presented in Fig. 3, illustrate that the different proteins that serve as GAPs for Cdc42Hs vary in specific (GAP) activity over an ~30–50-fold range. Human platelet Cdc42Hs GAP was the most potent stimulator of the GTPase activity, with half-maximal stimulation occurring at ~5 nM. The p190 protein was slightly less effective, with half-maximal stimulation occurring at ~10 nM. The GST-Bem3 protein was 10-fold less potent as a GAP with half-maximal effects occurring at ~100 nM, and Spodoptera frugiperda-expressed, full-length bcr was the least potent, with half-maximal stimulation occurring at >200 nM. It should be noted that although full-length bcr was capable of eliciting a measurable stimulation of the Cdc42Hs GTPase activity, assays performed with insect cell lysates expressing fragments of bcr typically showed a stronger ability to stimulate the GTPase activity, thereby suggesting that fragments of bcr (that contain the rho-GAP homology region) may have a higher specific activity than the full-length protein. Neither the GST-Bem2 protein nor an E. coli-expressed GST-P55 protein showed any ability to stimulate the GTPase activity of Cdc42Hs (even when the concentrations of these proteins exceeded 500 nM).

Deletion Mutations of Bem3 and Generation of Bem2/Bem3 Chimeras: Identification of Limit Cdc42Hs GAP Domain—To delineate the domain necessary for the Cdc42Hs GAP activity, we have constructed a series of Bem3 deletion mutants and Bem2/Bem3 chimeras (Fig. 4A). Each of these mutants was expressed as glutathione S-transferase fusion proteins and was affinity-purified and visualized by SDS-PAGE (Fig. 4B). We often have observed proteolytic fragments when expressing the different Bem3 proteins and Bem2/Bem3 chimeras in E. coli;
however, adjustments were made in the amount of the recombinant parent proteins added to the GTPase assay mixtures so that all assays were performed using an identical amount of the parent Bem3 mutants or Bem2/Bem3 chimeras. We found that the N\_H\_2 terminus of Bem3 could be truncated as far as residue 904 (i.e. Bem3\_2 in Fig. 4A) and still retain GAP activity. However, when the deletion was extended to residue 981 (Bem3\_1) in the first GAP homology box (also see Fig. 1), no stimulation of the Cdc42Hs GTPase activity was detected. The truncation of the third homology box (residues 751–1067) (Bem3\_3) rendered the Cdc42Hs GTPase activity was detected. The truncation of the first GAP homology box of Bema and the middle and carboxyl-terminal third GAP homology boxes of Bem3; the only difference between these two chimeras was that Bem2\_1/Bem3\_2 has a serine residue (position 952) from Bem3 at

**FIG. 3. Stimulation of Cdc42Hs GTPase by rho-GAP-related proteins.** GTPase activities at the indicated concentrations of human platelet GAP ( ), bcr ( ), p190 ( ), GST-Bem3 ( ), GST-p85 ( ), and GST-Bem2 ( ) were measured in 20 mm Tris (pH 7.5), 5 mm MgCl\_2, 1 mm dithiothreitol, 100 mm NaCl, and 0.5 mg/ml bovine serum albumin for 5 min at 22 °C by the charcoal absorbance method (Brandt et al., 1983). Prebinding of [γ-32P]GTP-bound Cdc42Hs was as described (Hart et al., 1991b), and the final concentration of GTP-bound Cdc42Hs was 100 nM.

This suggestion is reinforced from the results of experiments with different Bem2/Bem3 chimeras. Four different Bem2/Bem3 chimeras have been examined (Fig. 4A). Bem2\_2/Bem3, joins residues 1652–2065 from the amino-terminal portion of Bem2 to residues 1023–1128 of Bem3, i.e. this chimera contains the first two GAP homology boxes of Bem2 and the carboxyl-terminal box (box 3) of Bem3. Bem3\_1/Bem2, is composed of the first two GAP homology boxes of Bem3 (residues 904–1022) and the third homology box of Bem 2 (residues 2066–2167). Bem2\_1/Bem3\_2 and Bem2\_1/Bem3\_2 were essentially identical, containing the first (amino-terminal) GAP homology box of Bem2 and the middle and carboxyl-terminal third GAP homology boxes of Bem3; the only difference between these two chimeras was that Bem2\_1/Bem3\_2 has a serine residue (position 952) from Bem3 at

**FIG. 4. Deletion and chimera analysis of Bem3 GAP domain.** A, diagrammatic representation of deletion and chimeric mutants and their activities. The three GAP homology regions are depicted by large-square checkered boxes for Bem3 and by small-square checkered boxes for Bem2. The Bem3 sequences between the first and second homology boxes and between the second and third boxes are depicted by rightward-slanted lined boxes. The corresponding sequences for Bem2 are depicted by leftward-slanted lined boxes. GAP activities were compared by assaying for 5 min at 22 °C using 1 μg of glutathione S-transferase fusion protein and 1 μg of the E. coli-expressed recombinant Cdc42Hs protein. B, analysis of the purified mutant proteins. The E. coli-expressed GST-Bem3 or GST-Bem2/Bem3 proteins were purified by glutathione-agarose affinity chromatography, and 1–2 μg of each protein were subjected to SDS-PAGE (10% acrylamide).

the joining point (i.e. instead of the proline residue that is normally present in Bem2; see Fig. 1). Bem2\_2/Bem3\_1, Bem2\_2/Bem3\_2, and Bem2\_2/Bem3\_2 were all completely inactive as GAPs, and none of these chimeras were able to compete with
GST-Bem3 for the GTP-bound form of Cdc42Hs. However, the chimera Bem3K/Bem3S was able to retain some GAP activity (i.e. ~30% of the activity measured with GST-Bem3). These results suggest that the region responsible for GAP activity for Cdc42 primarily exists within the first two GAP homology boxes.

DISCUSSION

In S. cerevisiae, bud site formation appears to be regulated by the rho-subtype GTP-binding protein Cdc42Sc as well as by Cdc24 and the Bem1/Bem3 proteins, which in turn may interact with or regulate Cdc42Sc (Adams et al., 1990; Johnson and Pringle, 1990; Drubin, 1991). Given that mammalian cells do not bud, it is interesting that the primary sequence of Cdc42 has been strongly conserved from yeast to humans. One approach that we have taken toward determining the role that Cdc42 plays in mammalian cells has been to identify and characterize mammalian proteins that regulate the GTP binding/GTPase cycle of this ras-like protein. Recently, we have shown that the DBl oncogene product (which shares homology with the S. cerevisiae CDC24 gene product) catalyzes the GDP-GTP exchange activity of the human Cdc42 protein (Cdc42Hs) (Hart et al., 1991a). This suggests a possible involvement of Cdc42Hs in pathways that regulate cell growth. Further support for this possibility comes from the findings that both bcr and the ras-GAP-binding protein (p190) are GAPs for Cdc42Hs. In addition to bcr and p190, a 25-kDa protein from human platelet membranes will serve as a GAP for Cdc42Hs (Hart et al., 1991b). It seems likely that platelet Cdc42Hs GAP will be similar, if not identical, to a 29-kDa GAP for the rho-GTP-binding protein that was first identified in spleen cytosol (Garre et al., 1989).

The identification and biochemical characterization of Cdc42Hs GAPs could provide important clues as to the function of Cdc42Hs since GAPs may represent physiological targets for this GTP-binding protein. However, an important question concerns the physiological significance of the ability of these different proteins (bcr, p190, and the 25-kDa platelet protein) to serve as GAPs for Cdc42Hs in vitro, i.e. are the in vitro activities representative of interactions between Cdc42 and the different GAPs that occur in vivo, or do they represent “cross-reactivities” that are not reflective of in vivo interactions? This consideration makes the discovery that the Bem3 gene product serves as a GAP for Cdc42 especially important since there are strong indications that Cdc42 and Bem3 interact within a common biological pathway in yeast.

Direct comparisons of the Cdc42Hs GAP activities for the platelet 25-kDa protein, bcr, p190, and Bem3 show that the specific GAP activities of these different proteins vary by as much as 30–50-fold. Human platelet GAP was the most potent stimulator of the Cdc42Hs GTPase activity, followed by the p190 protein, the yeast Bem3 protein, and then full-length bcr. The simplest explanation for these differences is that the different GAPs bind to the GTP-bound form of Cdc42Hs with different affinities. This is consistent with the finding that neither the GST-Bem2 nor GST-p85 protein shows any capability of binding to the GTP-bound form of Cdc42Hs based on their inability to compete with any of the GAPs. In the future, we hope to develop fluorescence spectroscopic read-outs to monitor Cdc42-GAP interactions in order to obtain direct determinations of the relative affinities of the different GAPs for the GTP-bound (and GDP-bound) forms of Cdc42Hs.

It is interesting that we have not yet identified any protein with Cdc42Hs GAP homology that will bind to the GTP-bound Cdc42Hs species, but not stimulate its GTPase activity. However, recently, we have demonstrated that a 28-kDa protein from brain that acts to inhibit the dissociation of GDP from Cdc42Hs (as well as from the rho- and rac-GTP-binding proteins (Ueda et al., 1990; Hirao et al., 1992)) and triggers the dissociation of Cdc42Hs from plasma membranes also elicits a strong inhibition of both the intrinsic and the GAP-stimulated GTPase activities of Cdc42Hs (Hart et al., 1992). The ability of GDI to inhibit the GAP-stimulated GTPase activity (which has been demonstrated both for 25-kDa human platelet GAP and full-length bcr) appears to be the outcome of a competition between GDI and GAPs for the GTP-bound Cdc42Hs protein. Thus far, we have not found any significant sequence similarity between GDI and GAPs that might point to a structural motif that was important for the recognition of the GTP-bound Cdc42Hs species. This may be due to the differences in the specific modes of interaction of GDI and GAPs with Cdc42Hs. Specifically, GDI will only bind to the isoprenylated form of the GTP-binding protein, whereas none of the Cdc42Hs GAPs appear to discriminate between prenylated and non-prenylated forms of the GTP-binding protein.

We have taken advantage of the fact that the Bem3 protein is a GAP for Cdc42Hs, whereas the structurally related protein Bem2 is not, in the use of deletion analysis and chimeric approaches to obtain information regarding what constitutes a limit Cdc42Hs GAP domain. Comparisons of the sequences of the different members of the rho-subtype GAP family indicate that the general region of “GAP homology” can be subdivided into three subdomains (designated as homology boxes) of ~25–30 amino acids each. It was the limited sequence that was initially obtained from the box 3 homology region of spleen rho-GAP (Diekmann et al., 1991) that led to the identification of bcr and chimerin as members of the rho-subtype GAP family. The results of our studies indicate that all three homology boxes are essential for full GAP activity. In fact, it is necessary that an ~30–40-amino acid segment extend both from the amino-terminal end of the GAP homology region (i.e. from box 1) and from the carboxyl-terminal end (box 3); otherwise no GAP activity (or ability to bind to the GTP-bound Cdc42Hs species) is observed. Presumably, the requirement of these overhanging sequences is for the proper folding of the complete GAP domain since these sequences are not conserved among the different members of the GAP family.

Of the various Bem2/Bem3 chimeras that we have examined, only the chimera that contains the first two homology boxes from Bem3 and the third homology box from Bem2 is a functional GAP. Interestingly, despite the fact that box 3 was instrumental in the identification of various members of the GAP family, a Bem2/Bem3 chimera that contains the third homology box of Bem3 is inactive as a GAP for Cdc42Hs. These results suggest that the first two homology boxes may be the most important for the effective (functional) coupling of GAP to the rho-subtype protein. When comparing the first two homology boxes for the different proteins that serve as GAPs for Cdc42Hs versus the first two homology boxes of Bem2, which is not a GAP, one of the most obvious differences is that all of the Cdc42Hs GAPs contain a serine at the second position from the carboxyl-terminal residue of box 1, whereas Bem2 has a proline at this position. Nonetheless, the single change of a proline to a serine at this position does not result in Cdc42Hs GAP activity. Thus, it seems likely that like the situation for the ras-GAP family (Marshall et al., 1988), a small sequence of amino acids will not be sufficient to bind to the GTP-bound Cdc42Hs species and to catalyse its GTPase activity, but that the three homology boxes provide a specific tertiary conformation that forms the proper binding (and catalytic) pocket.

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