Interactions between Conserved Domains within Homodimers in the BIG1, BIG2, and GBF1 Arf Guanine Nucleotide Exchange Factors*

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Small GTP-binding proteins of the Arf (ADP-ribosylation factor) family are major regulators of membrane traffic in the exocytic and endocytic pathways (reviewed in Ref. 1). They are activated by the exchange of GDP for GTP, which is stimulated by guanine nucleotide exchange factors (ArfGEFs)4 carrying a catalytic Sec7 domain (reviewed in Refs. 2 and 3). Evidence is accumulating that ArfGEFs integrate upstream signals that define the conditions of Arf activation. First, ArfGEFs localize to specific trafficking organelles (4–9), which allows them to specify which subcellular site requires Arf activity. Second, binding partners involved in cell signaling, such as protein kinase A, FK506-binding protein 13, and the AKAP-interacting protein AMY-1, have been identified for the large Golgi-localized ArfGEFs (10–12). Finally, ArfGEFs may play a role in membrane recruitment of Arf effectors, such as coats, thus assembling downstream components of Arf signaling pathways prior to Arf activation (5, 13).

An essential issue is to decipher how ArfGEFs implement these functions and coordinate them with their biochemical GDP/GTP exchange activity. To address this question, we chose to focus on the large ArfGEFs, since (i) they are the only ArfGEFs found in all eukaryotes, and (ii) their multidomain architecture may allow them to recapitulate the largest number of ArfGEF functions within a single polypeptide (14, 15). Large ArfGEFs comprise two groups, which we refer to as the GBF and BIG groups after their names in mammals. Both function in maintaining organelle integrity and membrane traffic at the Golgi or at endosomes (reviewed in Ref. 1). The best studied representatives are yeast Gee1p/Gee2p, Arabidopsis thaliana GNOM and mammalian GBF1 for the GBF group, and yeast Sec7p and mammalian BIG1/BIG2 for the BIG group (reviewed in Refs. 1 and 2). We predicted earlier from a bioinformatics analysis that the GBF and BIG groups share a common architecture, suggesting that both ArfGEF groups follow a common scenario for their activation of Arf (15). The predicted organization comprises two noncatalytic domains (dimerization and cyclophilin-binding (DCB) and homology upstream of Sec7 (HUS)) in the N terminus of the Sec7 domain and three in its C terminus (HDS1, HDS2, and HDS3). The N-terminal DCB domain (Fig. 1A) is the only domain to which a molecular func-

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4 The abbreviations used are: ArfGEF, guanine nucleotide exchange factor of Arf protein; GBF, Golgi-associated brefeldin A-resistant guanine nucleotide exchange factor; BIG, brefeldin A-inhibited guanine nucleotide exchange factor; DCB, dimerization/cyclophilin binding; HUS, homology upstream of Sec7; BD, binding domain; AD, activation domain; GFP, green fluorescent protein; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.
tion has been assigned. It was originally identified in plant GNOM, where it was shown to be capable of dimerization in yeast two-hybrid and in vitro pull-down assays (16). Little is known about the other domains, except for an almost invariant 5-residue motif in the HUS domain, the HUS box (15, 17) (Fig. 1B), which is essential for aspects of Golgi traffic in yeast (18).

Here we combine biochemical, yeast two-hybrid, and cellular assays to analyze the domain architecture and interdomain interactions of the GBF and BIG groups of large ArfGEFs. Our data demonstrate the existence of two distinct interactions involving the DCB domain of the mammalian large ArfGEFs: homodimerization via a DCB/DCB interaction (generalizing previous results from plants) and a novel DCB/HUS interaction depending on the HUS box. We propose that the DCB/DCB and DCB/HUS interactions define a common structure in all members of the BIG and GBF groups of ArfGEFs, which probably also exists in the related eukaryotic Mon2p family.

**EXPERIMENTAL PROCEDURES**

Expression of Recombinant DCBBIG1, DCBBIG2, and DCB-HUS-Sec7BIG1—The DCB domain of human BIG1 (DCBBIG1, residues 2–224) was introduced into pET28a (Novagen) modified to remove the thrombin cleavage site and to include alternative restriction sites (KpnI and AgeI). The E221K mutation was introduced into DCBBIG1 by PCR using the QuickChange site-directed mutagenesis kit (Stratagene). Both wild type and mutant DCBBIG1 were expressed in the Rosetta (DE3)pLysS Escherichia coli strain (Merck KGaA), DCBBIG1 was purified on a Ni2+-nitrilotriacetic acid affinity column (GE Healthcare) followed by precipitation in ammonium sulfate to 70% saturation and gel filtration on a Superdex 75 column (GE Healthcare). The DCB domain of human BIG2 (DCBBIG2, residues 2–224) was cloned, expressed, and purified as described for DCBBIG1.

A construct spanning the DCB, HUS, and Sec7 domains of human BIG1 (DCB-HUS-Sec7BIG1, residues 2–888) was introduced into pFastBac HTA vector (Invitrogen) using EcoRI and KpnI restriction sites. SF21 cells infected by baculoviruses harboring this construct were used to express DCB-HUS-Sec7BIG1. The recombinant protein was purified on a Ni2+-nitrilotriacetic acid affinity column (GE Healthcare) followed by a desalting column and a gel filtration Superdex 200 column (GE Healthcare). Limited proteolysis was performed with 10 units of thrombin (Amersham Biosciences) per mg of protein at room temperature overnight. DCB-HUS-Sec7BIG1 and its proteolysis products were analyzed by SDS-PAGE and Western blot.

Biophysical Assays—Sedimentation velocity was measured at 40,000 × g for 24 h and analyzed with the SVEDBERG software (available on the World Wide Web). Sedimentation equilibrium experiments were carried out at 10,000, 15,000, and 20,000 × g for 46 h and analyzed with the Origin software (Beckman Coulter). CD scans were recorded between 185 and 260 nm. Thermal denaturations were carried out at the temperature range of 5–95 °C at a rate of 2 °C/min. Secondary structure composition was estimated with the CDDSTR software (19). The effect of protein concentration on its thermal denaturation was measured with 0.3, 0.75, and 3 μM DCBBIG1 and analyzed at 222 nm, a wavelength minimum that is characteristic of α-helices.

**TABLE 1**

| Liposome composition in all cases of the fluorescent lipid nitrobenzoxadizoldihexadecanoyl-phosphatidylethanolamine was added as a tracer. PC, phosphatidylcholine; PG, phosphatidylglycerol; PIP2, phosphatidylinositol 4,5-bisphosphate. | 1 | 2 | 3 | 4 |
|---|---|---|---|---|
| Soybean PC | 95 | 65 | 92.5 | 62.5 |
| Cholesterol | 5 | 5 | 5 | 5 |
| Egg PG | 0 | 30 | 0 | 30 |
| Brain PIP2 | 0 | 0 | 2.5 | 2.5 |

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**Yeast Two-hybrid Assays**—Plasmids expressing human BIG1 and BIG2 and Cricetulus griseus GBF1 are gifts from P. Melançon (University of Alberta, Canada), the plasmid expressing coxsackievirus 3A is a gift from F. van Kuppevelt (Radboud University Nijmegen Medical Centre, The Netherlands). All yeast two-hybrid constructs were cloned in the pASΔ and pACT2 vectors to create fusions with the Gal4-DNA binding domain (BD) and Gal4 transcription activation domain (AD), respectively. The Y190 (MATa, gal4-542, gal80-538, his3, trp1-901, ade2-101, ura3-52, leu2-3, 112,URA3::GAL1-LacZ, Lys2::GAL1-HIS3c), and AH109 (MATa, trp1-901, leu2-3, 112, ura3-52, his3-200 gal4Δ, gal80Δ, LYS2::GAL1-UAS::GAL1-TATA-HIS3, GAL2::GAL2-UAS::GAL2-TATA-ADE2, URA3::MEL1-UAS::MEL1-TATA-lacZ) yeast strains were transformed with the different recombinant plasmids using the lithium acetate method (20). Y190 transformants autotrophic for tryptophan and leucine were assayed for β-galactosidase activity using the filter technique (21). AH109 transformants were tested for expression of HIS3 and ADE2 reporter genes. Stable expression of each clone in pACT2 and pASΔ was confirmed by Western blot using the Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) Gal4-AD and Gal4-BD monoclonal antibodies, respectively. All experiments were performed at least three times.

Biochemical Assays—Liposome binding experiments were performed with liposomes prepared as described in Ref. 22 (Table 1). DCBBIG1 (1 μM) was incubated at room temperature in 50 mM Heps, pH 7.2, and 120 mM potassium acetate with sucrose-loaded vesicles (final lipid concentration, 1 mM) in small polycarbonate tubes. The samples were centrifuged at 360,000 × g for 20 min, and the supernatants and the pellets were analyzed by SDS-PAGE with Sypro-orange staining. The Sec7 domain of BIG1 was used as a negative control. Exchange reaction assays were performed by tryptophan fluorescence kinetics using Δ17Arf1 as described in Ref. 23. The effect of DCBBIG1 on the exchange rate of Sec7BIG1 was analyzed by comparing the results of experiments done in the absence or presence of DCBBIG1 (10 μM).

Co-immunoprecipitation Assays—COS7 cells in 10-cm culture dishes were cotransfected with the plasmids pHA-GBF1 expressing human HA-GBF1 and either Venus-GBF1, Venus-GBF1A889, or YFP-GBF1-C expressing, respectively, human GBF1 and GBF1 deleted of the first 297 (ΔDCB) and 710 amino acids (ΔDCB-HUS).5 After 20 h of expression, cells were washed two times with 5 ml of cold phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 19 mM NaH2PO4, 1.8 mM KH2PO4)

5 T. K. Niu and C. L. Jackson, unpublished data.
and then disrupted in 0.5 ml of cold lysis buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA, 1% Nonidet P-40). After centrifugation at 4 °C, soluble cellular extracts were pre-cleared with 20 μl of protein G-Sepharose 4 Fast Flow (GE Healthcare) at 4 °C for 30 min. Supernatants were incubated with 3 μg of anti-GFP antibodies (Roche Applied Science) for 1.5 h at 4 °C. Then 30 μl of protein G-Sepharose 4 Fast Flow was added, and the mixtures were incubated at 4 °C for 1.5 h. The resin was washed two times with 1 ml of W100 buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA), followed by two washes with 1 ml of phosphate-buffered saline. Proteins were then eluted by incubation with 60 μl of SDS-PAGE sample buffer for 5 min at 95 °C. Eluted proteins were separated on a 6% SDS-polyacrylamide gel and analyzed by Western immunoblotting using anti-HA antibodies as primary antibody (Sigma).

Plasmid Shuffle Assays in Yeast—The geo1-ΔDCB construct (coding for residues 233–1408 of Gea1p) was obtained by introducing a gap in the pAP22 (CEN, TRP1, GEA1) (gift of A. Peyroche, Commissariat à l’Énergie Atomique, Saclay, France). The PCR product extended to 1825 and 154 base pairs past the 5′ and 3′ ends of the gap, respectively. The mutated fragment and gapped plasmid were used to transform CJY52-10-2 yeast cells that contained the pAP23 plasmid (CEN, URA3, GEA1) (24). Transformants were plated onto synthetic medium plates lacking tryptophan. Trp+ clones were grown at 30 °C in minimal medium (YNB) containing 0.67% yeast nitrogen base without amino acids (BD), supplemented with appropriate nutrients and with 2% glucose. 5-Fluoroorotic acid monohydrate (Toronto Research Chemicals) was added to a final concentration of 0.1% to counterselect the URA3-containing cells. The presence of protein product of the geo1-ΔDCB allele was controlled by Western immunoblotting as follows. Total yeast cell protein extracts were prepared using the NaNH/trichloroacetic acid lysis technique (25). Proteins were separated by SDS-PAGE in 10% Tricine gels and were analyzed by immunoblotting. The primary antibody was either a polyclonal anti-Gea1p antibody or a monoclonal anti-Vat2p antibody directed against a subunit of the vacuolar ATPase (Invitrogen).

RESULTS

Mammalian BIG and GFB ArfGEFs Have an N-terminal Dimerization DCB Domain—Using a bioinformatics approach, we predicted previously that the DCB domain, originally identified as a dimerization domain in the GFB group member GNOM (16), is also present at the N terminus of large ArfGEFs from the BIG group, where it should form an all-helical domain (15). In order to address this question experimentally, we expressed in E. coli the N terminus of human BIG1, encompassing the helical subdomain of highest sequence homology and the more variable N-terminal subdomain (Fig. 1A), and purified it to homogeneity. The recombinant protein behaved as a 2 × 26-kDa dimer (Fig. 2A), a molecular mass that was confirmed by analytical ultracentrifugation equilibrium sedimentation (54.3 kDa). A similar construct from human BIG2 was also expressed and purified to homogeneity and, as for BIG1, eluted as a dimer on a gel filtration column (Fig. 2A). Deconvolution of CD spectra for both proteins was consistent with a mostly helical secondary structure. We thus conclude that the N-terminal domain of human BIGs qualifies as the bona fide homolog of the DCB domain of the GFB group member GNOM and refer to it as DCB-BIG hereafter.

In order to assess a possible dimer/monomer equilibrium, we analyzed the CD thermal denaturation spectra of DCB-BIG1 (Fig. 2B). The model providing the best fit for the data was a two-state transition between an all-helical structure and a random coil denatured state, without formation of a monomeric intermediate. This and a denaturation temperature (69 °C) independent of the protein concentration and higher than the average for proteins (around 55 °C) suggest that the dimer is stable with a dissociation constant below the concentration used in the experiment (100 nM). Analytical ultracentrifugation confirmed the predominance of a dimeric species and the absence of monomer.

To determine whether dimerization is a general feature of DCB-like domains of the large ArfGEFs, we tested mammalian GBF1 and BIG2 DCB/DCB interactions using the yeast two-hybrid system. We observed a strong interaction between DCB domains of hamster GBF1 (98% identity with human GBF1) and between human BIG2 DCB domains, indicating that the DCB domain mediates dimerization in all mammalian large ArfGEFs (Fig. 3, sectors 1 and 12). We next looked for residues that contribute to the DCB/DCB interaction. Two highly conserved residues, Lys91 and Glu130 in DCB-BIG1, are found in both large ArfGEF groups (Fig. 1A). Mutation of either residue to alanine in DCB-BIG1 abolished the interaction between mutant and wild-type DCB-BIG1 in the two-hybrid system (Fig. 3, sectors 2 and 11). Thus, these residues are either part of the dimer interface or induce an abnormal structure in this interface. We then analyzed a mutation found in the C terminus of the DCB domain of human BIG2 (Fig. 1A), which has been associated with a congenital disease, autosomal recessive periventricular heterotopias with microencephaly (26). DCB-BIG1 carrying the equivalent mutation, E221K, was expressed in E. coli with a solubility similar to that of wild-type DCB-BIG1. Analytical ultracentrifugation experiments showed that DCB-BIG1E221K forms a dimer (data not shown). Thus, functions of DCB-BIG2 other than dimerization are affected by this mutation in the autosomal recessive periventricular heterotopia with microencephaly disorder.

A Novel Interaction between the DCB and HUS Domains in Large ArfGEFs—To determine whether interactions exist between the different domains of the large ArfGEFs, we carried out an extensive yeast two-hybrid analysis of domain-domain interactions for mammalian GBF1 and BIG1. All five noncata- lytic domains in addition to the catalytic Sec7 domain were considered. For GBF1 (Table 2A) and BIG1 (Table 2B), only one interaction was detected in addition to the DCB/DCB interaction described above. In both cases, a strong interaction between the DCB and HUS domains of each ArfGEF was observed (Fig. 3, sectors 3 and 4). This interaction was independent of the variable DCB-HUS linker added on either the DCB or HUS side (Table 2A). A strong interaction was also found between the DCB and HUS domains of human BIG2 (Fig. 3, sector 5).

The HUS box is an almost invariant N(Y/F)DC(D/N) motif, which is predicted to lie between two α-helices (Fig. 1B). Mutations
tion of the central aspartate to alanine in this motif abolished the DCB/HUS interaction in both GBF1 and BIG1 (Fig. 3, sectors 18 and 19). Thus, the HUS box supports the DCB/HUS interaction, which is the first molecular function to be associated with this motif. We then analyzed whether mutations that impair the DCB/DCB interaction (see above) also affect the DCB/HUS interaction. The E130A mutation in GBF1, but not the K91A mutation, abolished the DCB/HUS interaction (Fig. 3, sectors 16 and 17). These results indicate that residue Lys91 is involved only in the DCB/DCB interaction, whereas the Glu130 residue is involved in both interactions.

Next, we used the yeast two-hybrid system to analyze DCB/DCB and DCB/HUS interactions within GBF1 constructs spanning more than one domain. We first analyzed the ability of the DCB domain alone to interact with larger ArfGEF fragments. DCBGBF1 interacted with DCB-HUSGBF1, DCB-HUS-Sec7GBF1, and full-length GBF1 (Fig. 3, sectors 7, 8, and 10). Although these yeast two-hybrid interactions appeared to be weaker than...
the interaction between two DCB domains alone, they strongly suggest that the DCB/DCB interaction occurs also in the context of the full-length GBF1 protein. We then analyzed the DCB/HUS interaction in the context of multiple domains. A strong interaction was found between the DCB domain and a HUS-Sec7 construct (Fig. 3, sector 6). HUSGBF1 also interacted with DCB-HUS-Sec7GBF1 and full-length GBF1 although more weakly than with DCBGBF1 alone (Fig. 3, sectors 14 and 15). In support for the dimerization of the N-terminal region taking place in larger constructs, recombinant DCB-HUS-Sec7BIG1 eluted on a gel filtration column with a molecular weight consistent with a dimer (Fig. 2A).

No interactions between the noncatalytic domains other than the DCB/DCB and DCB/HUS interactions were identified with the yeast two-hybrid assay. We also failed to detect an interaction of the noncatalytic domains with the Arf1 substrate (data not shown). In addition, we did not observe any DCB/DCB or DCB/HUS cross-interactions between BIG1, BIG2, and GBF1 in the yeast two-hybrid system (data not shown).

**Functions of DCB/DCB and DCB/HUS Interactions in Arf-GEF Dimers**—The DCB domain has features of a dimeric helical bundle, which is a frequent arrangement in cytosolic proteins involved in membrane recruitment, such as the mem-

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**TABLE 2**

| Summary of domain/domain interactions in mammalian ArfGEFs analyzed with the yeast two-hybrid assay |
|---------------------------------------------------|
| Strong interactions are indicated in dark gray, and weaker interactions are shown in gray. Baits (BD) and preys (AD) are indicated in rows and columns, respectively. |

|        | DCC | DCBBIG1 | DCBBIG2 | DCB-HUS-BIG1 | DCB-HUS-BIG2 | GBF1 (full-length) | DCBGBF1   | DCBBIG1   | GBF1   | DCBGBF1   | DCBBIG1   | GBF1   |
|--------|-----|---------|---------|--------------|--------------|-------------------|-------------|-----------|-------|------------|-----------|-------|
| 1      |     |         |         |              |              | +                 | +           | +         | +     | +          | +         |       |
| 2      |     |         |         |              |              | -                 | -           | +         | +     | +          | +         |       |
| 3      |     |         |         |              |              | -                 | -           | +         | +     | +          | +         |       |
| 4      |     |         |         |              |              | -                 | -           | +         | +     | +          | +         |       |
| 5      |     |         |         |              |              | -                 | -           | +         | +     | +          | +         |       |
| 6      |     |         |         |              |              | -                 | -           | +         | +     | +          | +         |       |
| 7      |     |         |         |              |              | -                 | -           | +         | +     | +          | +         |       |
| 8      |     |         |         |              |              | -                 | -           | +         | +     | +          | +         |       |
| 9      |     |         |         |              |              | -                 | -           | +         | +     | +          | +         |       |
| 10     |     |         |         |              |              | -                 | -           | +         | +     | +          | +         |       |
| 11     |     |         |         |              |              | -                 | -           | +         | +     | +          | +         |       |
| 12     |     |         |         |              |              | -                 | -           | +         | +     | +          | +         |       |
| 13     |     |         |         |              |              | -                 | -           | +         | +     | +          | +         |       |
| 14     |     |         |         |              |              | -                 | -           | +         | +     | +          | +         |       |
| 15     |     |         |         |              |              | -                 | -           | +         | +     | +          | +         |       |
| 16     |     |         |         |              |              | -                 | -           | +         | +     | +          | +         |       |
| 17     |     |         |         |              |              | -                 | -           | +         | +     | +          | +         |       |
| 18     |     |         |         |              |              | -                 | -           | +         | +     | +          | +         |       |
| 19     |     |         |         |              |              | -                 | -           | +         | +     | +          | +         |       |
| 20     |     |         |         |              |              | -                 | -           | +         | +     | +          | +         |       |
| 21     |     |         |         |              |              | -                 | -           | +         | +     | +          | +         |       |
| 22     |     |         |         |              |              | -                 | -           | +         | +     | +          | +         |       |
| 23     |     |         |         |              |              | -                 | -           | +         | +     | +          | +         |       |

**FIGURE 2.** Biochemical characterization of recombinant proteins. A, elution profiles of purified DCBBIG1, DCBBIG2, and DCB-HUS-Sec7BIG1 on a gel filtration column. The elution volumes of calibration proteins are indicated. B, circular dichroism spectra of DCBBIG1 (6 μM) as a function of temperature. CD spectra are taken every 5 °C. The isodichroic point lies at 200.5 nm (thin arrow), showing that unfolding occurs as a two-state helix-coil transition.

**FIGURE 3.** Yeast two-hybrid analysis of DCB and HUS interactions in mammalian ArfGEFs. AH109 yeast cells were co-transformed with the expression plasmids for bait (BD) and prey (AD) fusion proteins and selected on double synthetic dropout (−Trp/−Leu/−His/−Ade). 1, DCBGBF1/DCBGBF1; 2, DCBGBF1/DCBBIG1A; 3, DCBGBF1/HUSGBF1; 4, DCBBIG1/HUSBIG1; 5, DCBBIG1/HUSBIG2; 6, DCBGBF1/HUS-Sec7GBF1; 7, DCBGBF1/DCB-HUSGBF1; 8, DCBGBF1/DCB-HUS-Sec7GBF1; 9, DCBGBF1/Sec7GBF1; 10, DCBGBF1/GBF1; 11, DCBGBF1/DCB-HUSGBF1; 12, DCBBIG1/DCB-HUSGBF1; 13, GBF1/DCB-HUS-Sec7GBF1; 14, HUSGBF1/DCB-HUS-Sec7GBF1; 15, HUSGBF1/GBF1; 16, HUSGBF1/DCBGBF1; 17, HUSGBF1/DCBGBF1; 18, HUSGBF1/DCBGBF1; 19, HUSGBF1/DCBBIG2; 20, HUSGBF1/Sec7GBF1; 21, DCB-HUSGBF1/3A; 22, HUSGBF1/3A; 23, DCBBIG1/3A.
brane curvature-sensing BAR domain found in amphiphysins and Arfaptin/POR, an Arf effector (27). We thus investigated the binding of DCBBIG1 to liposomes of various compositions using a sedimentation assay (Table 1 and Fig. 4A). However, no such interaction could be observed regardless of the liposome composition, suggesting that the DCB homodimer does not have membrane-binding properties on its own.

The Sec7 domain did not interact with the constructs tested in our yeast two-hybrid analysis (Table 2, A and B, and Fig. 3, sectors 9 and 20). To further analyze whether the N terminus could regulate the catalytic exchange activity of the Sec7 domain, we used recombinant proteins and fluorescence kinetics assay. We first analyzed the effect of excess DCBBIG1 on the exchange rate of the Sec7 domain of human BIG1 (Sec7BIG1) using Δ17Arf1 as substrate. No inhibition or stimulation of the exchange rate by DCBBIG1 could be observed, regardless of whether Δ17Arf1, Sec7BIG1, or both had been preincubated with DCBBIG1 (Fig. 4B). We then analyzed the catalytic activity of a BIG1 construct spanning the DCB-HUS-Sec7 domains using the same fluorescence assay. This construct was active at stimulating GDP/GTP exchange on Δ17Arf1 (Fig. 4C), and it was inhibited by brefeldin A with a Ki of 23.9 ± 7.2 μM, which is similar to that measured for the Sec7 of BIG1 alone (23). To confirm that the DCB-HUS tandem had no effect on the catalytic activity, we took advantage of a unique thrombin cleavage site located at residue 622 between the HUS and Sec7 domains, which allowed us to generate free DCB-HUSBIG1 and Sec7BIG1 by limited proteolysis. Exchange rates measured with a BIG1 peptide concentration of 0.5 μM were in the same range for the uncleaved and cleaved fragments (0.073 ± 0.005 and 0.098 ± 0.012 s⁻¹, respectively), suggesting that the DCB-HUS tandem does not have a simple one-to-one regulatory activity toward the Sec7 domain.

The N terminus of large ArfGEFs interacts with several large ArfGEF protein partners (reviewed in Ref. 1). We thus investigated whether the DCB/HUS structure may be required for protein-protein interactions. To this end, we took advantage of the fact that the N terminus of GBF1 binds to 3A, a protein from enteroviruses that blocks host cell secretion by inhibiting GBF1 function (28). The cytosolic portion of 3A (residues 1–60) interacts with DCB-HUSGBF1 and DCB-HUS-SEC7GBF1 in the yeast two-hybrid assay (Fig. 3, sector 21). In contrast, no interaction was observed with individual DCB or HUS domains (Fig. 3, sectors 22 and 23). This is consistent with data showing that deletion of either the first 50 amino acids of GBF1 or deletion of the HUS domain and downstream sequences abolishes interaction with the 3A protein in the mammalian two-hybrid system (29). These results show that portions of both the DCB and HUS domains of GBF1 are required for binding to the viral 3A protein and suggest the possibility that an integral DCB-HUS structure is necessary for binding of the 3A protein.

**Dimerization of Large ArfGEFs in Vivo**—The above analysis suggests that the DCB domain supports the dimerization of large ArfGEFs and organizes a structure that can bind protein partners. We thus assessed the dimerization and function of this domain in cells for two large ArfGEFs of the GBF group.

We first analyzed the formation of human GBF1 dimers in mammalian cells by pull-down assays (Fig. 5A). We found out that full-length GBF1 can easily be isolated as a dimer from...
mammalian cells. Next, we examined dimer formation between the full-length GBF1 and forms of GBF1 deleted of the DCB domain alone or of both the DCB and HUS domains. Clearly, whereas deletion of the DCB domain alone reduced somewhat the formation of a dimer with full-length GBF1, both the DCB and HUS domains had to be deleted to nearly abolish dimer formation. Thus, the DCB and the HUS domains are both involved in the dimerization of GBF1.

We then analyzed the effect of deleting the DCB domain of Gea1p, a member of the GBF group of large ArfGEFs in yeast, using a plasmid shuffle strategy. The strain used contains the wild-type GEA1 gene on a low-copy plasmid. The Gea1p-DCB allele was introduced into a low copy DCB/H9004 plasmid. The Gea1p-DCB protein was expressed and was not degraded (Fig. 5B, left). Clones failed to grow at 30 °C upon the loss of the wild-type GEA1 gene when the geadΔDCB plasmid became the sole copy of the redundant GEA1 and GEA2 genes (Fig. 5B, right). This result indicates that the DCB domain of Gea1p is essential for yeast viability.

DISCUSSION

A Conserved DCB/DCB and DCB/HUS Structure in Eukaryotic Large ArfGEFs and the Related Mon2p Family—In this study, we investigated the domain/domain interactions within the BIG and GBF groups of large ArfGEFs, which we predicted previously to share a common architecture (15). Based on biochemical and yeast two-hybrid analyses of mammalian BIG1, BIG2, and GBF1, we establish that all three members share a similar DCB–HUS organization upstream of their Sec7 domains, in which the DCB domain interacts with itself and with the HUS domain. The DCB/HUS interaction requires the highly conserved HUS box, a five-amino acid motif found in all members of the BIG and GBF groups of ArfGEFs.

Because of its bipartite organization, the DCB–HUS tandem provides different ways for large ArfGEFs to form multimers. One is through the DCB/DCB interaction, which is an obligate intermolecular interaction. Since the DCB domain forms a strong homodimer in vitro, we propose that it supports constitutive homodimerization of large ArfGEFs. The existence of this interaction in native BIG and GBF ArfGEFs is supported by its formation in a range of yeast two-hybrid GBF1 constructs, the dimerization of the recombinant BIG1 and BIG2 constructs, and our in vivo data on GBF1. It is also consistent with the molecular weight of several large ArfGEFs of both the BIG and GBF groups as measured by size exclusion chromatography, including yeast Gea1p (30), human BIG1 and BIG2 (31, 32), and plant GNOM (16). All elute as large molecular weight complexes, which, given the uncertainty of this technique for nonglobular proteins, is consistent with their association as homodimers.

In contrast, the DCB/HUS interaction can occur either between two monomers (intramolecular) (Fig. 6A) or within a single ArfGEF polypeptide (intramolecular) (Fig. 6B). An intramolecular DCB/HUS interaction would provide a second contribution to dimerization in addition to the DCB/DCB interaction. This possibility is supported by our co-immunoprecipitation results, which show that the ΔDCB form of human GBF1 formed a dimer with full-length GBF1 almost as efficiently as full-length GBF1 in mammalian cells, whereas deletion of both DCB and HUS domains practically eliminated dimerization with full-length GBF1. Interestingly, the HUS box has an unusual level of sequence conservation and content of polar residues within a protein interface, pointing to a potential for the DCB/HUS interaction to open up and expose the HUS box (Fig. 6C). The HUS box could then carry out other functions, allowing in particular the formation of ArfGEF tetromers through three-dimen-
sional domain swapping (Fig. 6D). An interesting corollary is that this could allow large ArfGEFs to form heterotetramers, which are more likely to form than heterodimers, given the stability of the homodimeric DCB/DCB interaction. BIG1 and BIG2 have been shown to co-immunoprecipitate in human cells (32), which could thus be mediated by the formation of heterotetramers containing one BIG1 homodimer and one BIG2 homodimer.

A region homologous to the DCB and HUS domains is present in a novel eukaryotic protein family, Mon2p/Ysl1p/SF21, that is related to the large ArfGEFs (33–35) but lacks the Sec7 nucleotide exchange domain (34, 35). Yeast Mon2p has been shown to localize to late Golgi/endosomes (33–35) and to bind Arl1p (33), a close relative of Arf proteins. We propose that members of the Mon2p family feature a DCB-HUS structure, including a DCB homodimerization domain (Fig. 1A) and a HUS domain with a candidate HUS box containing the central (Y/F)D motif (Fig. 1C) and capable of forming a DCB/HUS interaction. DCB/DCB-mediated dimerization is in agreement with the co-immunoprecipitation of yeast Mon2p as a homodimer (34). The presence of the DCB-HUS module without an associated Sec7 domain in these proteins is consistent with a structural function that is independent of the biochemical nucleotide exchange activity.

The Role of the DCB-HUS Structure in Large ArfGEF Interactions—The N terminus of large Golgi ArfGEFs has been reported to interact with various protein partners. Notably, the N terminus of GBF1 interacts with Rab1 (36) and regions of mammalian BIGs encompassing DCB-HUS interact with AMY-1 (12), protein kinase A (10), FKBP13 (11), the Exo70 subunit of the exocyst (37), and the HSC70 chaperone (12). The requirement of 3A protein for both the DCB and HUS domains suggests that the two-domain DCB-HUS structure could mediate these protein-protein interactions in addition to its dimerization function. Furthermore, phenotypic data in vivo have shown that the DCB-HUS tandem is necessary and sufficient to define the subcellular localization of p200/BIG1 to Golgi membranes (17, 18). Interestingly, this is also the case for the DCB-HUS homology region of the related protein Mon2p (34). Furthermore, mutation of the HUS box in Gea2p in yeast, which is related to the large ArfGEFs (33–35) but lacks the Sec7 nucleotide exchange domain (34, 35). Yeast Mon2p has been shown to co-immunoprecipitate in human cells (32), which could thus be mediated by the formation of heterotetramers containing one BIG1 homodimer and one BIG2 homodimer.

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REFERENCES

1. D’Souza-Schorey, C., and Chavrier, P. (2006) Nat. Rev. Mol. Cell Biol. 7, 347–358
2. Jackson, C. L., and Casanova, J. E. (2000) Trends Cell Biol. 10, 60–67
3. Shin, H. W., and Nakayama, K. (2004) J. Biochem. (Tokyo) 136, 761–767
4. Zhao, X., Lasell, T. K., and Melancon, P. (2002) Mol. Biol. Cell. 13, 119–133
5. Shintosuka, C., Waguri, S., Wakasugi, M., Uchiyama, Y., and Nakayama, K. (2002) Biochem. Biophys. Res. Commun. 294, 254–260
6. Garcia-Mata, R., Szul, T., Alvarez, C., and Sztul, E. (2003) Mol. Biol. Cell 14, 2250–2261
7. Geldner, N., Anders, N., Wolters, H., Keicher, J., Kornberger, W., Muller, P., Delbarre, A., Ueda, T., Nakano, A., and Jurgens, G. (2003) Cell 112, 219–230
8. Niu, T. K., Pfeifer, A. C., Lippincott-Schwartz, J., and Jackson, C. L. (2005) Mol. Biol. Cell 16, 1213–1222
9. Shin, H. W., Morinaga, N., Noda, M., and Nakayama, K. (2004) Mol. Biol. Cell 15, 5283–5294
10. Li, H., Adamik, R., Pacheco-Rodriguez, G., Moss, J., and Vaughan, M. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 1627–1632
11. Padilla, P. I., Chang, M. J., Pacheco-Rodriguez, G., Adamik, R., Moss, J., and Vaughan, M. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 2322–2327
12. Ishizaki, R., Shin, H. W., Iguchi-Ariga, S. M., Ariga, H., and Nakayama, K. (2006) Genes Cells 11, 949–959
13. Kawamoto, K., Yoshida, Y., Tamaki, H., Torii, S., Shintosuka, C., Yamashina, S., and Nakayama, K. (2002) Traffic 3, 483–495
14. Cox, R., Mason-Gamer, R. J., Jackson, C. L., and Segev, N. (2004) Mol. Biol. Cell 15, 1487–1505
15. Mouratou, B., Biou, V., Joubert, A., Cohen, J., Shields, D. I., Geldner, N., Jurgens, G., Melancon, P., and Cherfils, J. (2005) BMC Genomics 6, 20
16. Grebe, M., Gadea, J., Steinmann, T., Kientz, M., Rahfeld, J. U., Salchert, K., Koncz, C., and Jurgens, G. (2000) Plant Cell 12, 343–356
17. Mansour, S. J., Skaug, J., Zhao, X. H., Giordano, J., Scherer, S. W., and Melancon, P. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 7968–7973
18. Park, S. K., Hartnell, L. M., and Jackson, C. L. (2005) Mol. Biol. Cell 16, 3786–3799
19. Whitmore, L., and Wallace, B. A. (2004) Nucleic Acids Res. 32, W668–W673
20. Giertz, D., St jean, A., Woods, R. A., and Schiestl, R. H. (1992) Nucleic Acids Res. 20, 1425
21. Schneider, S., Buchert, M., and Hovens, C. M. (1996) BioTechniques 20, 960–962
22. Bigay, J., Gounon, P., Robineau, S., and Antonny, B. (2003) Nature 426, 565–566
23. Zeeh, J. C., Zeghouf, M., Grauffel, C., Guibert, B., Martin, E., Dejaegere, A., and Cherfils, J. (2006) J. Biol. Chem. 281, 11805–11814
24. Peyroche, A., Courbezette, R., Rambourg, A., and Jackson, C. L. (2001) J. Cell Sci. 114, 2241–2253
25. Volland, C., Urban-Grimal, D., Geraud, G., and Haguenaüer-Tsapis, R. (1994) J. Biol. Chem. 269, 9833–9841
26. Sheen, V. L., Ganesh, V. S., Topcu, M., Sebire, G., Bodel, A., Hill, R. S., Grant, P. E., Shugart, Y. Y., Imotola, J., Khoury, S. J., Guerrini, R., and Walsh, C. A. (2004) Nat. Genet. 36, 69–76
27. Peter, B. J., Kent, H. M., Mills, I. G., Vallis, Y., Butler, P. J., Evans, P. R., and McMahon, H. T. (2004) Science 303, 495–499
28. Wessels, E., Duijsings, D., Niu, T. K., Neumann, S., Oorschot, V. M., de

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29. Wessels, E., Duijsings, D., Lanke, K. H., Melchers, W. J., Jackson, C. L., and van Kuppeveld, F. J. (2007) J. Virol. 81, 5238–5245
30. Peyroche, A., Paris, S., and Jackson, C. L. (1996) Nature 384, 479–481
31. Morinaga, N., Tsai, S. C., Moss, J., and Vaughan, M. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 12856–12860
32. Yamaji, R., Adamik, R., Takeda, K., Togawa, A., Pacheco-Rodriguez, G., Ferrans, V. J., Moss, J., and Vaughan, M. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 2567–2572
33. Jochum, A., Jackson, D., Schwarz, H., Pipkorn, R., and Singer-Kruger, B. (2002) Mol. Cell Biol. 22, 4914–4928
34. Efe, J. A., Plattner, F., Hulo, N., Kressler, D., Emr, S. D., and Deloche, O. (2005) J. Cell Sci. 118, 4751–4764
35. Gillingham, A. K., Whyte, J. R., Panic, B., and Munro, S. (2006) J. Biol. Chem. 281, 2273–2280
36. Monetta, P., Slavin, I., Romero, N., and Alvarez, C. (2007) Mol. Biol. Cell 18, 2400–2410
37. Xu, K. F., Shen, X., Li, H., Pacheco-Rodriguez, G., Moss, J., and Vaughan, M. (2005) Proc. Natl. Acad. Sci. U. S. A. 102, 2784–2789