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Isolation of a brefeldin A-inhibited guanine nucleotide-exchange protein for ADP ribosylation factor (ARF) 1 and ARF3 that contains a Sec7-like domain

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ABSTRACT **Brefeldin A (BFA) inhibited the exchange of ADP ribosylation factor (ARF)-bound GDP for GTP by a Golgi-associated guanine nucleotide-exchange protein (GEP) [Helms, J. B. & Rothman, J. E. (1992) *Nature (London)* 360, 352–354; Donaldson, J. G., Finazzi, D. & Klausner, R. D. (1992) *Nature (London)* 360, 350–352]. Cytosolic ARF GEP was also inhibited by BFA, but after purification from bovine brain and rat spleen, it was no longer BFA-sensitive [Tsai, S.-C., Adamik, R., Moss, J. & Vaughan, M. (1996) *Proc. Natl. Acad. Sci. USA* 93, 305–309]. We describe here purification from bovine brain cytosol of a BFA-inhibited GEP. After chromatography on DEAE–Sephacel, hydroxylapatite, and Mono Q and precipitation at pH 5.8, GEP was eluted from Superose 6 as a large molecular weight complex at the position of thyroglobulin (≈670 kDa). After SDS/PAGE of samples from column fractions, silver-stained protein bands of ≈190 and 200 kDa correlated with activity. BFA-inhibited GEP activity of the 200-kDa protein was demonstrated following electroelution from the gel and renaturation by dialysis. Four tryptic peptides from the 200-kDa protein had amino acid sequences that were 47% identical to sequences in Sec7 from *Saccharomyces cerevisiae* (total of 51 amino acids), consistent with the view that the BFA-sensitive 200-kDa protein may be a mammalian counterpart of Sec7 that plays a similar role in cellular vesicular transport and Sec7 may be a GEP for one or more yeast ARFs.**

The ARF (ADP ribosylation factor) family of 20-kDa GTP-binding proteins, originally named because of the ability to stimulate cholera toxin-catalyzed ADP ribosylation of $G_{s\alpha}$ (1), are now known as critical components of diverse intracellular vesicular trafficking pathways (2). ARF function depends on its alternation between inactive GDP- and active GTP-bound conformations. As ARF has no detectable GTPase activity and exchanges bound nucleotide very slowly at physiological concentrations of Mg^{2+}, its cycling between active and inactive forms is controlled by GTPase-activating proteins (GAP) and guanine nucleotide-exchange proteins (GEP). Protease-sensitive ARF GEP activity was found in Golgi membranes and was inhibited by the fungal metabolite brefeldin A (BFA) that blocks vesicular transport (3, 4). A cytosolic ARF GEP was also inhibited by BFA, but after purification from bovine brain and rat spleen, the GEP was no longer BFA sensitive (5, 6).

Available data are consistent with the possibilities that ARF GEP is not itself a target of BFA or that there are BFA-insensitive as well as BFA-sensitive forms of ARF GEP. We undertook to purify a BFA-sensitive GEP from bovine brain cytosol. As reported here, after ≈12,000-fold overall purification, a BFA sensitive-GEP was obtained, which behaved on gel filtration as a complex of ≈670 kDa. A component protein of ≈200 kDa was separated by SDS/PAGE and exhibited BFA-sensitive GEP activity after elution from the gel and renaturation. Amino acid sequences of peptides from this protein were very similar to those of Sec7 from *Saccharomyces cerevisiae* (7), consistent with the view that the BFA-sensitive 200-kDa ARF GEP is a mammalian counterpart of Sec7.

MATERIALS AND METHODS

Materials. DEAE–Sephacel was purchased from Pharmacia; hydroxylapatite (Bio-Gel HTP gel) was from Bio-Rad; phosphatidylserine was from Sigma; BFA was from Epicentre Technologies (Madison, WI); and 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF) was from Boehringer Mannheim. Sources of other materials have been published (5, 6).

Purification of BFA-Sensitive GEP. Soluble proteins from bovine brain cortex (830 g) in 300 ml of buffer A (20 mM Tris, pH 8.0/1 mM EDTA/1 mM NaN_3/1 mM DTT/0.25 M sucrose) containing leupeptin, aprotinin, and soybean and lima bean inhibitors (each 1 μg/ml) with 0.5 mM AEBSF were precipitated with 45% saturated $(NH_4)_2SO_4$. Precipitated proteins (3.75 g) were dissolved in buffer B (buffer A plus 2 mM $MgCl_2$ and 0.5 mM AEBSF), dialyzed against the same buffer, and applied to a column (5 × 44 cm, 850 ml) of DEAE–Sephacel equilibrated with buffer B. After washing with 850 ml of buffer B containing 50 mM NaCl, proteins were eluted with a linear gradient of 50–250 mM NaCl in buffer B (total 3.4 liters). Fractions containing BFA-sensitive GEP activity (eluted with 160–190 mM NaCl) were pooled and adjusted to pH 7.5 and 200 mM NaCl (based on conductivity) before application to a column of hydroxylapatite (5 × 9 cm, 180 ml) equilibrated with buffer B containing 200 mM NaCl followed by elution with a linear gradient of potassium phosphate, pH 7.5 (0–300 mM, in the same buffer, total 1 liter). BFA-sensitive GEP activity was eluted with 130–190 mM potassium phosphate. These fractions were pooled, dialyzed against buffer B containing 30 mM NaCl, and applied to a column (1 × 10 cm) of Mono Q HR 10/10 (Pharmacia) equilibrated with buffer B containing 30 mM NaCl. After washing with 10 ml of the same buffer, bound proteins were eluted with a linear gradient of 30–500 mM NaCl in buffer B (total 70 ml). Fractions with BFA-sensitive GEP activity (usually about 330–420 mM NaCl) were pooled, dialyzed overnight against 25 mM 3-morpholinopropanesulfonic acid, pH 5.8/1 mM DTT/0.25 M sucrose/2 mM $MgCl_2$/0.5 mM AEBSF/1 mM EDTA/30 mM NaCl, and centrifuged (12,000 × *g*, 15 min). Precipitated proteins were washed with fresh dialysis buffer, dissolved in 250 μl of buffer A containing 1 M NaCl, and applied to a column (1 × 30 cm) of Superose 6 HR 10/30 (Pharmacia), followed by elution with

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Abbreviations: ARF, ADP ribosylation factor; GAP, GTPase-activating protein; GEP, guanine nucleotide-exchange protein; BFA, brefeldin A; AEBSF, 4-(2-aminoethyl)benzenesulfonyl fluoride; CTA, cholera toxin A subunit.

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buffer B containing 200 mM NaCl. BFA-sensitive GEP was eluted at the position of thyroglobulin (≈670 kDa).

SDS/PAGE and Electroelution of Proteins from Gel. Sample plus one-third volume of 4 × SDS/PAGE sample buffer (0.25 M Tris, pH 6.8/4% SDS/40% glycerol/10% mercaptoethanol/0.005% bromophenol blue) was incubated at room temperature for 30 min before transfer to the gel (4 or 8%). After electrophoresis at 4°C, part of the gel was divided into segments based on positions of prestained markers. The remainder of the gel was silver stained, and the molecular sizes represented in the gel segments were estimated by reference to molecular size markers. Proteins were eluted from gel fragments in elution buffer containing 25 mM Tris, 192 mM glycine, and 0.1% SDS, using an electro-eluter (Bio-Rad model 422) for 3 hr, and then electrodialyzed 3 hr in the same buffer without SDS, before dialysis overnight against buffer B. For other samples of GEP, electrophoresis was stopped just before proteins entered the separation gel; total protein was eluted and treated as were the separated proteins before assay.

Assay of BFA-Inhibited GEP Activity. GEP was assayed by its effect on ARF stimulation of CTA-catalyzed ADP ribosylagmatine synthesis. Each sample was incubated with or without BFA (6 μg) with 10 μg of crude mixed ARF (primarily ARF1 and ARF3) prepared from rat spleen cytosol by gel filtration (8) at room temperature for 10 min in 90 μl of buffer A (with a final concentration of 10 mM DTT) containing 5 mM $MgCl_2$, 15 μg of BSA, 20 μg of phosphatidylserine, 0.1 μg each of leupeptin, aprotinin, and lima bean and soy bean trypsin inhibitors. For the second step, 10 μl of 100 μM GTPγS was added, and after incubation at 37°C for 40 min, the effect of activated ARF on CTA ADP ribosyltransferase activity was assayed (5). GEP activity (nmol/hr) is the difference between the increase in CTA activity produced by ARF alone and that by ARF plus GEP, i.e., CTA activity in the presence of both GEP and ARF minus the activity with ARF alone.

RESULTS

Purification of the BFA-inhibited GEP is described in *Materials and Methods*. Data from a representative preparation are summarized in Table 1. When the proteins precipitated from bovine brain cytosol with 45% saturated $(NH_4)_2SO_4$ were subjected to chromatography on DEAE–Sephacel, BFA-sensitive GEP was usually eluted at 160–190 mM NaCl (just after the bulk of the bound protein) with ≈30-fold purification (data not shown). In earlier studies (5), BFA-insensitive GEP was eluted from DEAE–Sephacel between 95 and 130 mM NaCl. After further purification on hydroxylapatite and Mono Q, BFA-sensitive GEP fractions were dialyzed at pH 5.8 (Table 1). BFA inhibition of GEP activity of the precipitated proteins is shown in Fig. 1. Although recovery of activity in the pH 5.8 precipitate was rather low and could perhaps be increased by modification of conditions, a 10-fold increase in specific activity was achieved. When these proteins were subjected to gel filtration on Superose 6 (Fig. 2*A*), BFA-sensitive GEP was eluted in the position of thyroglobulin (≈670 kDa). Proteins in samples of active fractions separated by SDS/PAGE are shown in Fig. 2*B*. Two of the silver-stained bands (200 and 190 kDa) appeared to correlate with the GEP activity (recorded at the bottom of Fig. 2*B*), whereas, several other bands in those fractions did not. Recovery from Superose 6 gel filtration was, unfortunately, only ≈10%, consistent with the notion that GEP may interact with the matrix (Table 1).

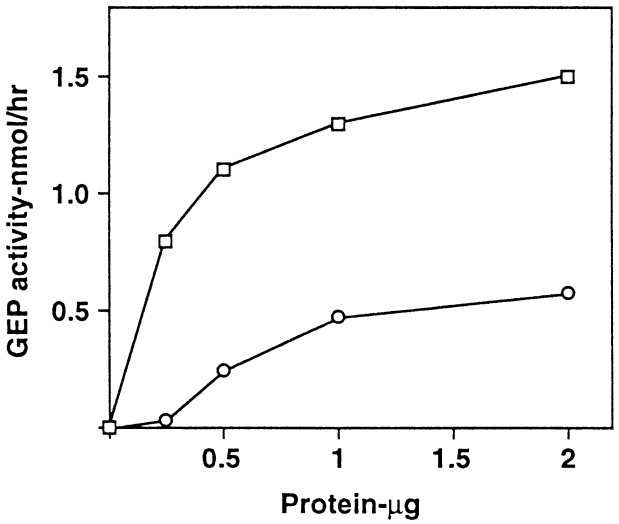

FIG. 1. BFA-inhibited GEP activity as a function of GEP protein. Protein precipitated at pH 5.8 after Mono Q chromatography was dissolved in buffer B containing 1 M NaCl. Protein was determined with the Bio-Rad assay. GEP activity of the indicated amount of protein was assayed without (□) and with (○) 6 μg of BFA. ARF activity without GEP (with or without BFA) was 0.36 nmol/hr. This experiment has been replicated twice.

GEP Activity of 200-kDa Protein. To demonstrate BFA-inhibited GEP activity, proteins were eluted from gels after SDS/PAGE and renatured before assay. As SDS at concentrations of >0.005% inhibited GEP activity, the eluted proteins were dialyzed extensively against buffer B before assay. When a gel (8%) was divided into segments representing proteins of ≈30–47, 48–57, 58–66, 130–170, and 171–200 kDa, assay of electroeluted and renatured proteins from each detected BFA-inhibited GEP activity only in the 171- to 200-kDa samples (data not shown). There was no GEP activity in the segment that included 60-kDa proteins, i.e., the size of a BFA-insensitive GEP purified by Tsai *et al.* (5). SDS/PAGE in 4% gel separated the 200-kDa protein clearly from other bands. The eluted 200-kDa protein exhibited GEP activity that was inhibited by BFA, as was the GEP activity of the proteins that were eluted before entering the separating gel (Fig. 3).

ARF Specificity of Purified GEP. For assay of GEP during purification, a crude fraction of mixed ARFs prepared from rat spleen cytosol by gel filtration (8) was used as substrate. To

Table 1. Purification of BFA-inhibited GEP

Purification step	Total protein, mg	Specific activity, unit/μg	Purification (-fold)	Total units ($\times 10^3$)
DEAE–Sephacel	388	0.077	1*	29.9 (100)†
Hydroxylapatite	124	0.33	4.3	40.9 (137)
Mono Q	42	0.50	6.5	21.0 (70)
pH 5.8 precipitate	2	5.0	65	10.0 (33)
Superose 6	0.03	33	429	0.99 (3)

Procedure is described in *Materials and Methods*. One unit is the amount of GEP required to double activity of a standard amount of ARF (10 μg of crude mixed ARF preparation) in the CTA assay. Purification has been replicated four times.

*In other experiments, GEP from DEAE–Sephacel was purified ≈28-fold over the $(NH_4)_2SO_4$ precipitate.

†In parentheses, recovery relative to preparation from DEAE–Sephacel = 100%.

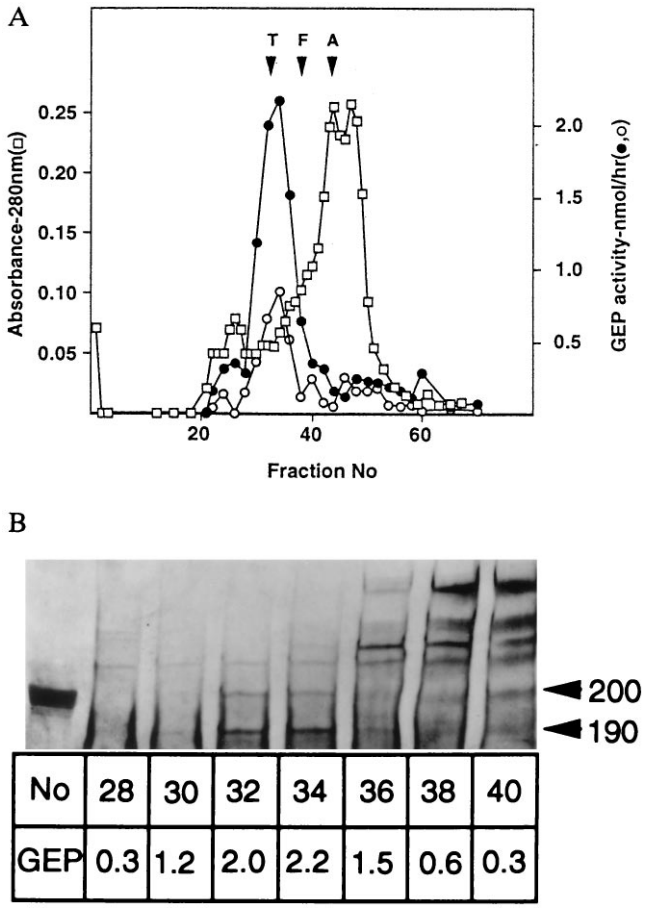

FIG. 2. Chromatography of BFA-sensitive GEP on Superose 6. Protein precipitated at pH 5.8 from pooled active Mono Q fractions was dissolved in buffer B containing 1 M NaCl and applied to a column (1 × 30 cm) of Superose 6, which was eluted with buffer B containing 200 mM NaCl (0.4 ml/min). (*A*) Samples (20 μl) of column fractions (0.4 ml) were assayed for GEP activity without (●) or with (○) 6 μg of BFA. Protein concentration was quantified as absorbance at 280 nm (□). Positions of elution of thyroglobulin (T, 669 kDa), ferritin (F, 440 kDa), and bovine serum albumin (A, 67 kDa) are indicated by arrowheads. (*B*) Samples (30 μl) of the indicated fractions were subjected to SDS/PAGE in a 4% gel followed by silver staining. Positions of 190- and 200-kDa proteins are indicated on the right. The first lane contains ≈0.5 μg of myosin (200 kDa). Fraction numbers (No) and GEP activity (nmol/h/20 μl) are indicated below each lane. ARF activity was 1.2 nmol/hr.

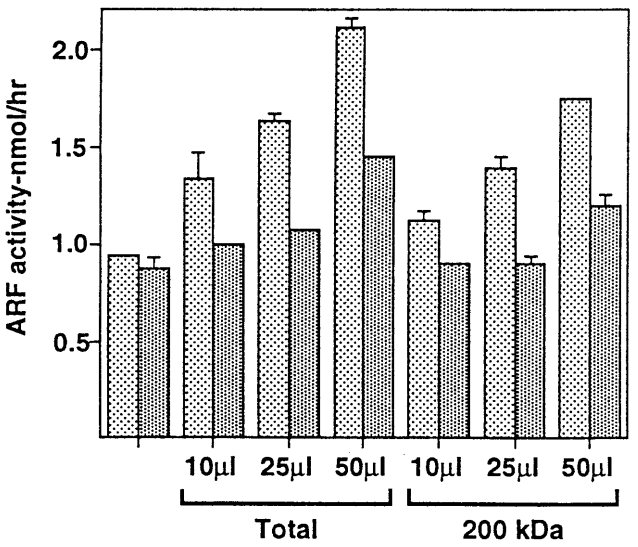

FIG. 3. Activity of BFA-inhibited GEP eluted from gel after SDS/PAGE. A sample (≈100 units) of pH 5.8 precipitate was treated with SDS sample buffer at room temperature for 30 min before separation of proteins by SDS/PAGE (4% gel). After electrophoresis, the segment of gel containing protein of ≈200 kDa (referring to position of prestained marker myosin) was excised. Protein was electroeluted for 3 hr in buffer containing 0.1% SDS, followed by electrodialysis for 3 hr in the same buffer without SDS and then dialyzed against buffer B. Another sample of ≈100 units (Total) was treated the same way except that electrophoresis was stopped and elution carried out just before proteins entered the separation gel. GEP activities of indicated amounts of the total and 200-kDa protein fractions are shown. The first of each pair of columns is activity without BFA and the second with 6 μg of BFA.

evaluate the substrate specificity of the partially purified GEP, recombinant myristoylated (mARF3, mARF5) and nonmyristoylated (rARF3, rARF5) proteins were used (Table 2). GEP apparently activated mARF3 more effectively than it did nonmyristoylated rARF3. It failed, however, to activate ARF5 whether or not myristoylated, although the ARF5 preparations themselves were clearly active. Both ARF1 and ARF3 purified from bovine brain were good substrates for the BFA-sensitive GEP (Table 2). These observations are consistent with the conclusion that the purified GEP activates class I (ARF1 and -3) but not class II (ARF5) ARFs.

Amino Acid Sequence of Purified GEP. To obtain purified GEP for amino acid sequencing, proteins in the active fractions from Superose 6 chromatography were precipitated with 10% trichloroacetic acid, separated by SDS/PAGE in 4% gel, transferred to PVDF membrane, and stained with Ponceau red. The segment of membrane that contained the 200-kDa protein was cut out for sequencing of peptides separated after tryptic digestion (Harvard Microchemistry Facility). Amino acid sequences of nine peptides were used to search a protein data base. One of the peptides (pk 57) was found to have significant similarity to Sec7 protein sequence. The other eight peptides were then directly compared with Sec7 using GENE WORKS, and three had significant sequence identity (Fig. 4). Of

Table 2. Effect of partially purified BFA-inhibited GEP on activity of class I and class II ARFs

Exp.	ARF amount	ARF activity, nmol/hr: No GEP	ARF activity, nmol/hr: Plus GEP	Ratio, + GEP/ − GEP
1	Mixed, 10 μg	0.72	2.2	3.2
	mARF3, 1 μg	1.0	2.1	2.1
	2 μg	1.8	3.6	2.0
	4 μg	3.1	5.6	1.8
	rARF3, 1 μg	0.56	0.77	1.4
	2 μg	1.1	1.5	1.4
	4 μg	2.1	3.0	1.4
2	Mixed, 10 μg	0.80	2.0	2.5
	mARF5, 0.1 μg	0.49	0.53	1.1
	0.2 μg	0.73	0.78	1.1
	rARF5, 0.5 μg	0.66	0.52	0.8
	1.0 μg	1.3	1.3	1.0
3	Mixed, 10 μg	0.77	2.0	2.6
	mARF5, 0.5 μg	2.0	1.8	0.9
	1.0 μg	3.8	4.0	1.1
4	ARF1, 0.2 μg	0.44	1.1	2.5
	ARF3, 0.2 μg	1.2	2.3	1.9

Recombinant myristoylated (mARF3, mARF5) and nonmyristoylated (rARF3, rARF5) proteins were prepared by published methods (20). Mixed ARF is the preparation from rat spleen cytosol used for assays during GEP purification. ARF1 and ARF3 were purified from bovine brain (21). GEP precipitated at pH 5.8 (1 μg per assay) was used.

		Amino acid	Identity %
		†* *†**† ** *	
Sec7	891	AIMHAFVDEFDFIGM	
pk57		EVMYAYVDQHDFSGK	47(67)
		* ††* †††****	
Sec7	994	PRDFLEGLFNEIANN	
pk118		PEEYLSAIYNEIAGK	40(73)
		**† * **†	
Sec7	1374	EELSGFEFQH	
pk54		GELANFRFQK	50(70)
		† †	
Sec7	1384	DFLKPFEYTVQ	
pk80		DFLRPFEHIMK	55(73)

FIG. 4. Amino acid sequences of peptides from 200-kDa protein aligned with Sec7 sequences. Protein sequence databases (nonredundant version) were searched with amino acid sequences of nine peptides from the 200-kDa protein using the BLAST algorithm (19) available on the Internet from the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov). One peptide (pk57) was found to have significant similarity to Sec7. Alignment of sequences of the eight other peptides with Sec7 using GENE WORKS identified three with significant identity to Sec7. In parentheses is percentage similarity of sequences. *, Identical amino acids; †, conservative differences.

51 amino acids in the four peptides, 24 (47%) were identical and 12 (24%) represented conservative differences.

DISCUSSION

Tsai *et al.* (5) reported that BFA-sensitive GEP in bovine brain cytosol behaved as a macromolecule of ≈700 kDa. After purification, however, the GEP was no longer inhibited by BFA and behaved as a molecule of ≈60 kDa on gel filtration and SDS/PAGE (5). More recently, a ≈55-kDa BFA-insensitive GEP that was not associated with a large protein complex was purified from rat spleen cytosol (6). The purified BFA-inhibited GEP described here was eluted from Superose 6 as a molecule of ≈670 kDa. After SDS/PAGE, the 200-kDa protein component was demonstrated to possess the BFA-sensitive GEP activity. A 190-kDa protein appeared also to be correlated with activity. The relationship between these two proteins and the subunit composition of the larger complex remain to be defined. Does the 670-kDa complex contain subunits in addition to the 190- and 200-kDa proteins, e.g., other enzymes such as a phospholipase D? What are the stoichiometries of the 190- and 200-kDa proteins in the complex? Is the 190-kDa subunit perhaps a proteolytic product of the 200-kDa GEP? Although numerous questions remain, it appears clear that in addition to the smaller BFA-insensitive GEP proteins purified earlier (5, 6), a BFA-inhibited ARF GEP(s) of larger size does exist.

ARF5 was apparently not a substrate for the purified BFA-inhibited ARF GEP described here that utilized both hARF1 and hARF3 (class I). The BFA-insensitive ARF GEP purified earlier from bovine brain (5) and rat spleen (6) similarly utilized class I ARFs in preference to ARF5. That may not be unexpected, as BFA inhibition of ARF5 binding to Golgi membranes was not observed at the same time that BFA inhibited the binding of ARF1 and ARF3 (8). No GEP activities toward class II and class III ARFs have been described, although they presumably exist. If the yeast Sec7 is a GEP, it may be less ARF-specific than are the mammalian ARF GEP proteins thus far characterized, all of which appear to utilize preferentially class I ARFs. Overall, for whatever reason, there may be somewhat less strict ARF specificity in *Saccharomyces* than there is, for example, in mammalian cells, since, at least to some extent, yeast ARF mutants can be complemented by any of the mammalian or other ARFs that have been tested (9). The ability to complement yeast ARF1 mutants has, in fact become a secondary criterion for designation of a protein as an ARF.

Based on the similarities in amino acid sequences of the 200-kDa GEP protein and yeast Sec7 (7), it may be suggested that Sec7 is also a GEP. Sec7 was identified in a collection of conditionally lethal mutants (10) as one of the genes involved in Golgi vesicular trafficking (7). The product of the Sec7 gene is a phosphoprotein of ≈230 kDa (7) that moves between cytosolic and membrane fractions (11). It was recently reported that expression of human ARF4 was able to rescue the growth of two different Sec7 mutant yeast alleles, albeit with somewhat different degrees of effectiveness (12). The two mutants were also rescued by overexpression of yeast ARF1 or yeast ARF2 in an allele-specific manner (12). These data are consistent with the notion that Sec7 is an ARF GEP.

Rescue of the yeast Sec7 mutants by hARF4 (12) is of special interest as there is virtually no information on the specific distribution or function of this ARF in mammalian cells, despite the general belief that all of the ARF proteins are involved in some aspect of intracellular vesicular transport. Because ARF4 and ARF5 (mammalian class II) are more similar to each other than they are to class I and class III ARFs or to known yeast ARFs (2), it was somewhat surprising that the expression of hARF5 failed to suppress either of the two Sec7 mutants. The interpretation of this finding is, however, unclear since Deitz *et al.* (12) were unable to measure hARF5 protein in the yeast.

Deduced amino acid sequences of other gene products contain regions of ≈157 amino acids, referred to as Sec7 domains (13), that are very similar to amino acids 860-1016 of *Saccharomyces* Sec7. These include a *Caenorhabditis elegans* open reading frame (14) and a human protein B2-1, which has been found in T natural killer cells but not in T helper cells (15). In addition, the product, EMB30, of a gene that was identified as essential for proper development of the *Arabadopsis* embryo contains a Sec7 domain. The *embo30-1* mutant contains a lysine replacement for glutamate 658, an amino acid that is conserved in the four Sec7 domains thus far identified (13). Shevell *et al.* (13) discussed in depth possible relationships between the functions of EMB30 and Sec7, neither of which is itself well understood, although Franzusoff and coworkers (16–18) have provided considerable information about Sec7 involvement in yeast Golgi vesicular transport. It will be important to determine whether the protein products of the Sec7 domain proteins (and Sec7) have GEP activity. Of the four GEP peptides in Fig. 4, two (pk 57 and pk 118) are aligned with Sec7 domain sequences, the others with a more C-terminal part of the Sec7 molecule. All of our observations are consistent with the view that the BFA-sensitive 200-kDa protein may be a mammalian counterpart of Sec7 that plays a similar role in cellular vesicular transport and Sec7 may be a GEP for one or more yeast ARFs.

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