

Brefeldin A-inhibited guanine nucleotide-exchange activity of Sec7 domain from yeast Sec7 with yeast and mammalian ADP ribosylation factors

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Contributed by Martha Vaughan, February 17, 1998

ABSTRACT The *Saccharomyces cerevisiae* Sec7 protein (ySec7p), which is an important component of the yeast secretory pathway, contains a sequence of ≈ 200 amino acids referred to as a Sec7 domain. Similar Sec7 domain sequences have been recognized in several guanine nucleotide-exchange proteins (GEPs) for ADP ribosylation factors (ARFs). ARFs are ≈ 20 -kDa GTPases that regulate intracellular vesicular membrane trafficking and activate phospholipase D. GEPs activate ARFs by catalyzing the replacement of bound GDP with GTP. We, therefore, undertook to determine whether a Sec7 domain itself could catalyze nucleotide exchange on ARF and found that it exhibited brefeldin A (BFA)-inhibitable ARF GEP activity. BFA is known to inhibit ARF GEP activity in Golgi membranes, thereby causing reversible apparent dissolution of the Golgi complex in many cells. The His₆-tagged Sec7 domain from ySec7p (rySec7d) synthesized in *Escherichia coli* enhanced binding of guanosine 5'-[γ -³⁵S]thio]triphosphate by recombinant yeast ARF1 (ryARF1) and ryARF2 but not by ryARF3. The effects of rySec7d on ryARF2 were inhibited by BFA in a concentration-dependent manner but not by inactive analogues of BFA (B-17, B-27, and B-36). rySec7d also promoted BFA-sensitive guanosine 5'-[γ -thio]triphosphate binding by nonmyristoylated recombinant human ARF1 (rhARF1), rhARF5, and rhARF6, although the effect on rhARF6 was very small. These results are consistent with the conclusion that the yeast Sec7 domain itself contains the elements necessary for ARF GEP activity and its inhibition by BFA.

The *Saccharomyces cerevisiae* Sec7 protein (ySec7p), which has been implicated in endoplasmic reticulum to Golgi and intra-Golgi transport, is an important component of the yeast secretory pathway (1–4). ySec7p contains a characteristic sequence motif (amino acids 827–1,017), which is referred to as the Sec7 domain (5). Analogous domains have been recognized also in bovine p200, which is a guanine nucleotide-exchange protein for ARF (6), yeast Gea1 and Gea2 (7), human cytohesin-1 (8) and ARNO (9), mouse GRP-1 (10), *Arabidopsis* EMB30 (5), and an ORF from *Caenorhabditis elegans* (11).

The 20-kDa GTP-binding ADP ribosylation factors (ARFs), initially identified as activators of cholera toxin-catalyzed ADP ribosylation (12), play an essential role in several pathways of intracellular vesicular transport (13, 14) and activate phospholipase D (15–17). Members of the ARF family are ubiquitous in eukaryotic cells. They include six mammalian ARFs that fall into three classes based on phylogenetic analysis, amino acid sequence, size, and gene structure as follows: class I, ARF1, ARF2, and ARF3; class II, ARF4 and ARF5; class III, ARF6 (18–20). It seems likely that the individual ARFs have different functions and sites of action in cells. They shuttle between cytosol and

intracellular membranes depending on whether GDP or GTP is bound. Thus far, three yeast ARFs are known (21–23). Two of them, yeast ARF1 and yeast ARF2, are believed to participate in vesicular trafficking in the Golgi system and have been localized to the Golgi by immunoreactivity (22, 24). The double deletion of yeast ARF1 and yeast ARF2 is lethal (22). In contrast, yeast ARF3, the sequence of which is 60% identical to that of mammalian ARF6, is not essential for cell viability and is not required for endoplasmic reticulum to Golgi transport (23).

The report that expression of human ARF4, as well as yeast ARF1 or yeast ARF2, could rescue Sec7 mutant yeast cells in an allele-specific manner provided evidence for a functional interaction of ARF and ySec7p (25). ARFs, like other GTPases, cycle between inactive GDP-bound and active GTP-bound conformations (26). Activation of ARFs (i.e., conversion of ARF-GDP to ARF-GTP) is promoted by guanine nucleotide-exchange proteins (GEPs), several of which have been identified. Complementation studies in yeast led to the discovery of Gea1 and Gea2 (7), GEPs that possess Sec7 domains and are inhibited by brefeldin A (BFA), a fungal fatty acid metabolite that reversibly interferes with protein secretion while causing apparent disintegration of the Golgi complex (27–29). The 47-kDa protein named ARNO (9), suggested to be a human homologue of Gea1, is BFA-insensitive ARF GEP and the ARNO Sec7 domain itself stimulated nucleotide exchange. Assuming that the Sec7 domain might be responsible for nucleotide exchange activity, cytohesin-1, which also contains a Sec7 domain and had been described as a regulator of $\beta 2$ -integrin in human lymphocytes (30), was synthesized in *Escherichia coli* and shown to be BFA-insensitive ARF GEP (31). More recently, the deduced amino acid sequence of a 200-kDa BFA-sensitive mammalian ARF GEP (p200) cloned from a bovine brain cDNA library revealed that it also contains a Sec7 domain (6). All of these observations are consistent with the view that the Sec7 domains are activators of guanine nucleotide exchange for ARFs.

We report herein that purified rySec7d itself exhibits BFA-sensitive ARF GEP activity. It catalyzed guanine nucleotide exchange on ryARF1 and ryARF2, and unmyristoylated recombinant human ARF1, ARF5, and ARF6 (rhARF1, rhARF5, and rhARF6, respectively) but not ryARF3.

EXPERIMENTAL PROCEDURES

Materials. Guanosine 5'-[γ -³⁵S]thio]triphosphate ([³⁵S]-GTP[γ S]; 1,250 Ci/mmol; 1 Ci = 37 GBq) was from NEN Life Science Products. [adenine-¹⁴C]NAD (252 mCi/mmol) was purchased from Amersham Life Sciences. PCR reagents and

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0027-8424/98/954204-5\$0.00/0

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Abbreviations: ARF, ADP ribosylation factor; GEP, guanine nucleotide exchange protein; BFA, brefeldin A; GTP[γ S], guanosine 5'-[γ -thio]triphosphate; ry, recombinant yeast; rySec7d, recombinant yeast Sec7 domain; rh, recombinant human; CTA, cholera toxin A subunit. [†]To whom reprint requests should be addressed at: Room 5N-307, Building 10, 10 Center Drive, MSC 1590, National Institutes of Health, Bethesda, MD 20892-1590.

restriction enzymes, unless otherwise indicated, and 4-(2-aminoethyl) benzenesulfonyl fluoride were from Boehringer Mannheim. Phosphatidylserine was from Sigma. BFA was from Epicentre Technologies (Madison, WI). Analogues of BFA, B-17, B-27, and B-36, were prepared by Andrew Green (Université Joseph Fourier, Grenoble, France).

Preparation of Recombinant Yeast and Human ARF Proteins. For the preparation of ry- and nonmyristoylated rhARFs, competent BL21 (DE3) *E. coli* were transformed with an ampicillin-resistance plasmid pET3a containing yeast ARF1, ARF2, or ARF3 or human ARF1, ARF5, or ARF6 DNA. For large-scale production of recombinant proteins, flasks of prewarmed LB broth (500 ml) containing carbenicillin (50 $\mu\text{g}/\text{ml}$) were each inoculated with 25 ml from an overnight culture of transformants. After the cultures reached an OD_{600} of approximately 0.6, isopropyl β -D-thiogalactopyranoside (Gold Biotechnology, St. Louis) was added (final concentration, 0.5 mM), followed by incubation for 2 h at 37°C. Cells were then harvested and lysed by sonification in TE buffer (10 mM Tris-HCl, pH 8.0/1 mM EDTA) containing lysozyme (5 mg/ml; Boehringer Mannheim). After centrifugation (100,000 \times g, 35 min, 4°C), the supernatant was applied to a column (2.4 \times 100 cm, V_t = 240 ml) of Ultrogel Aca54 (IBF Biotechnics, Columbia, MD) equilibrated with buffer containing 20 mM Tris-HCl (pH 8.0), 1 mM EDTA, 1 mM NaN_3 , 1 mM DTT, 0.25 M sucrose, 5 mM MgCl_2 , 100 mM NaCl, and 0.5 mM 4-(2-aminoethyl) benzenesulfonyl fluoride. Proteins also were eluted with the buffer. Samples of fractions were assayed for ARF activity and protein content (Bio-Rad assay). Purity was assessed by SDS/PAGE in 14% gels and staining with Coomassie blue. Fractions containing ARF activity were pooled and stored in small portions at -20°C.

Construction of Recombinant His₆-Tagged Yeast Sec7 Domain Expression Vector. Sequence encoding the Sec7 domain (amino acids 827–1,017) was amplified from a yeast cDNA library (CLONTECH) by PCR using the forward primer 5'-AGC-CATATGAGGAAACTGCTTTATCGGAA-3' (underlined sequence indicates *NdeI* restriction site) and reverse primer 5'-GCCGGATCCTTATGAAAGCATTCGCTGATG-3' (underlined sequence is a *BamHI* restriction site). The PCR product was gel-purified and subcloned into pCR-Script Amp SK(+) vector, yielding pCRySec7. The resulting plasmid was purified and sequenced. The first segment (encoding amino acids 827–953) and second segment (encoding amino acids 953–1,017) of the PCR product were separately inserted into expression vector pET-14b (Novagen), which had been digested with *NdeI* and *BamHI* restriction enzymes, because the sequence of the yeast Sec7 domain has an *NdeI* restriction site at amino acid 953. pCRySec7 was digested with *NdeI* and *BamHI*, yielding two different sized inserts. Each insert was gel-purified before the two were ligated (according to the manufacturer's instructions) into pET-14b vector, which encodes an N-terminal His₆ sequence followed by a thrombin cleavage site sequence and three cloning sites, yielding pET-14b/ySec7.

Preparation of His₆-Tagged rySec7d. Competent BL21 (DE3) *E. coli* were transformed with plasmid pET-14b/ySec7 and selected for ampicillin resistance. Transformants were grown overnight in 10 ml of LB broth containing ampicillin (50 $\mu\text{g}/\text{ml}$). A sample (5 ml) of overnight culture containing expression plasmid was inoculated into 100 ml of LB medium containing ampicillin and incubated at 37°C until OD_{600} reached approximately 0.6 (about 2 h). After isopropyl β -D-thiogalactopyranoside was added to a final concentration of 0.4 mM, the cultures were incubated for an additional 3 h at 37°C and bacteria were collected by centrifugation (5,000 \times g, 5 min, 4°C). For lysis, bacterial pellets were suspended in 4 ml of ice-cold 20 mM Tris-HCl (pH 8.0), containing 300 mM NaCl, 10% glycerol, protease inhibitor mixture (PharMingen; 10 $\mu\text{l}/\text{ml}$), and lysozyme (10 mg/ml), before sonification. All subsequent procedures were done at 4°C. After cellular debris was removed by centrifugation (17,000 \times g, 15

min), the clear lysate was incubated (1 h) with 1 ml of Ni-nitrilotriacetic acid agarose (Qiagen, Chatsworth, CA), which was then washed extensively with 20 mM Tris-HCl, pH 8.0/300 mM NaCl/10% glycerol/20 mM imidazole/0.5 mM 4-(2-aminoethyl) benzenesulfonyl fluoride, until no protein was detected by Bio-Rad protein assay of 200- μl samples. Bound protein was eluted with 80 mM Tris-HCl, pH 8.0/100 mM EDTA/2 M NaCl and dialyzed for 3 h against 20 mM Tris-HCl, pH 8.0/1 mM EDTA/1 mM DTT/5 mM MgCl_2 /30 mM NaCl/1 mM NaN_3 /10% glycerol. This His₆-tagged Sec7 domain (0.25–1.0 mg/ml) was divided into small portions and stored at -20°C. Two different preparations were used for the experiments reported herein.

Nucleotide Binding Assay. GTP[γ S] binding to purified ryARF or rhARF protein was assessed by using a rapid filtration technique (32). Briefly, 0.5–2 μg (25–100 pmol) of ARF and 4 μM [³⁵S]GTP[γ S] were incubated at 4°C, 24°C, or 30°C with rySec7d and other additions as indicated in 20 mM Tris-HCl, pH 8.0/1 mM DTT/3 mM MgCl_2 /1 mM EDTA containing 50 μg of BSA and 20 μg of phosphatidylserine (total volume, 100 μl). At the indicated times, samples were transferred to nitrocellulose filters in a manifold (Millipore) for rapid filtration followed by washing six times, each wash with 2 ml of ice-cold buffer (40 mM Tris-HCl, pH 8.0/100 mM NaCl/3 mM MgCl_2 /2.5 mM DTT). Filters were then dried before addition of scintillation fluid for radioassay. Data are presented as means \pm SEM of values from triplicate assays. Error bars smaller than symbols are not shown. All observations have been replicated at least twice with different preparations of recombinant proteins.

Assay of Cholera Toxin A Subunit (CTA)-catalyzed ADP Ribosylagmatine Formation. ARF activity was assayed by its effect on the GTP[γ S]-dependent activation of cholera toxin-catalyzed ADP-ribosylagmatine formation (32). Assays containing ryARF2 (0.5 μg) with or without 0.5 μg of rySec7d in 100 μl of 10 μM GTP[γ S]/20 mM Tris-HCl, pH 8.0/1 mM EDTA/1 mM NaN_3 /10 mM DTT/0.25 M sucrose/5 mM MgCl_2 , 15 μg of BSA, and 20 μg of phosphatidylserine were incubated at 37°C for 40 min. Components needed to quantify ARF stimulation of CTA catalyzed ADP-ribosylagmatine formation were then added in 300 μl (total) to yield final concentrations of 50 mM potassium phosphate (pH 7.5), 6.5 mM MgCl_2 , 20 mM DTT, ovalbumin (0.1 $\mu\text{g}/\text{ml}$), 0.2 mM [adenine-¹⁴C]NAD (0.05 μCi), 10 mM agmatine, phosphatidylserine (0.13 mg/ml), 50 μM ATP, and 20 μM Cibachrome blue F3G-A (Fluka) with 2 μg of cholera toxin. The reaction product [¹⁴C]ADP-ribosylagmatine was isolated for radioassay by using AG1-X2 (Bio-Rad).

RESULTS

Effect of rySec7d on Activation of ryARF1, ryARF2, and ryARF3. rySec7d in the presence of GTP[γ S] catalyzed the activation of yeast ARF1 and yeast ARF2 as assayed by their effects on CTA-catalyzed ADP-ribosylagmatine synthesis (Fig. 1). Activation of CTA by yeast ARF3 was greater than that by the other two yeast ARFs in the absence of Sec7 domain and was not further increased by incubation with it. These experiments were carried out at 37°C before the time course of GTP[γ S] binding had been determined.

Effect of rySec7d on GTP[γ S] Binding to ryARF2. Fig. 2 shows the time course of rySec7d-stimulated [³⁵S]GTP[γ S] binding to ryARF2 at different temperatures. With incubation at 30°C, the stimulated rate was slowing by 1 min, although the low rate of binding by ryARF2 alone was essentially constant for 20 min (Fig. 2A). At 24°C also, the magnitude of the rySec7d effect was declining well before 1 min (Fig. 2B). To determine whether it was becoming inactivated with time, fresh rySec7d was added to some reactions after 15 s (Fig. 2B). Because the rate of GTP[γ S] binding was increased by adding fresh rySec7d, it may be inferred that rySec7d was being inactivated under assay conditions at 24°C and 30°C.

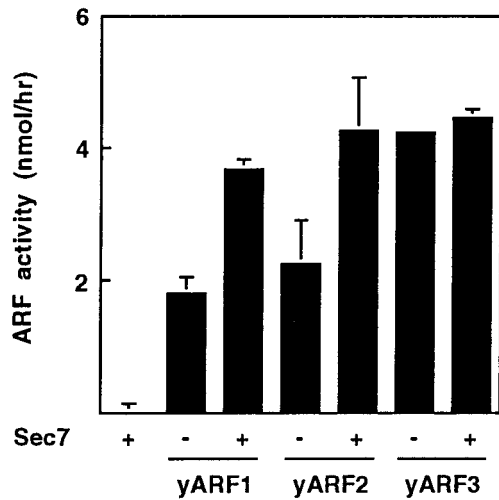


FIG. 1. Effect of rySec7d on activation of CTA by ryARF1, ryARF2, and ryARF3. First, 0.5 μg of ryARF1, ryARF2, or ryARF3 and 10 μM GTP[γS] were incubated without or with 0.5 μg of rySec7d at 37°C for 40 min in 20 mM Tris-HCl (pH 8.0) containing 1 mM EDTA, 1 mM NaN_3 , 10 mM DTT, 0.25 M sucrose, 5 mM MgCl_2 , 15 μg of BSA, and 20 μg of phosphatidylserine. Components needed to quantify ARF stimulation of cholera toxin-catalyzed ADP ribosyl-gmatine formation were then added, and incubation was continued for 60 min at 30°C before separation of the product for radioassay. ARF activity is the difference between CTA activity with ARF and that without ARF. The experiment was repeated twice.

In an attempt to slow the rate of rySec7d-stimulated GTP[γS] binding and stabilize the activity of rySec7d to permit more precise quantification, incubations were carried out at 4°C, which yielded a constant rate of binding for 3 h (Fig. 2C). Under those conditions, GTP[γS] binding to ryARF2 was accelerated in a concentration-dependent manner by rySec7d (Fig. 3).

Effects of rySec7d on GTP[γS] Binding to Recombinant Yeast and Human ARFs. The rate of GTP[γS] binding to ryARF1, like that to ryARF2 was significantly accelerated by rySec7d, but binding to ryARF3 was not affected (Fig. 4A). Even 2 μg of rySec7d, 10 times the amount used in this experiment, did not accelerate binding to 50 pmol of ryARF3 (data not shown). This lack of effect on GTP[γS] binding by yeast ARF3 and its failure to increase ryARF3 activity in the CTA assay are consistent with the knowledge that the function of yeast ARF3, although at present not defined, clearly differs from that of yeast ARF1 and yeast ARF2.

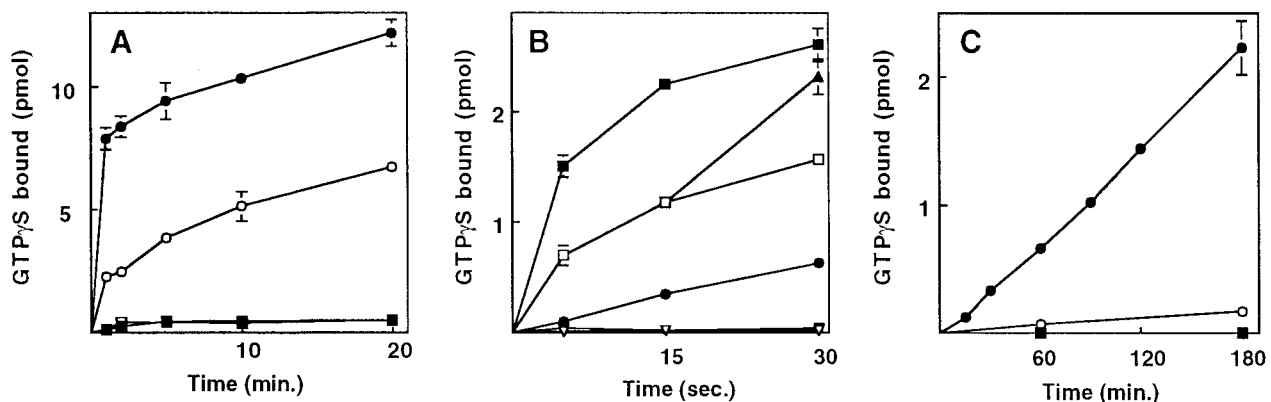


FIG. 2. Effect of temperature on rySec7d stimulation of GTP[γS] binding to ryARF2. (A) ryARF2 (1 μg , 50 pmol) and 4 μM [^{35}S]GTP[γS] were incubated at 30°C without (■ and □) or with (● and ○) 0.2 μg (8.3 pmol) of rySec7d for the indicated time before collection of proteins for radioassay. Some samples (□ and ○) also contained 6 μg of BFA (0.2 mM). (B) ryARF2 (50 pmol) and 4 μM [^{35}S]GTP[γS] were incubated at 24°C without (●) or with 0.2 μg (□) or 0.4 μg (■) of rySec7d for the indicated time. To some samples, after incubation for 15 sec with 0.2 μg of rySec7d, a second addition of 0.2 μg was made (▲). rySec7d, 0.2 μg (▼) or 0.4 μg (▼) was also incubated without ARF. (C) ryARF2 (50 pmol) and 4 μM [^{35}S]GTP[γS] were incubated at 4°C without (○) or with (●) 0.2 μg of rySec7d for the indicated time. rySec7d (0.2 μg) was also incubated without yeast ARF2 (■).

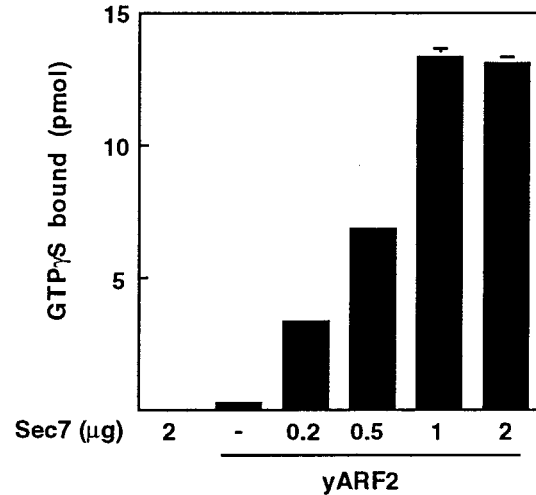


FIG. 3. Effect of rySec7d concentration on binding of GTP[γS] to ryARF2. ryARF2 (50 pmol) and 4 μM [^{35}S]GTP[γS] were incubated with the indicated amount of rySec7d at 4°C for 3 h before radioassay of protein-bound [^{35}S]GTP[γS]. rySec7d (2 μg) incubated alone bound no GTP[γS].

Binding of GTP[γS] by rhARF1 or rhARF5 (Fig. 4B) was also stimulated by rySec7d, although to a lesser extent than that by ryARF1 and ryARF2. rhARF5 appeared to be a somewhat better substrate than rhARF1. The effect of rySec7d on GTP[γS] binding to rhARF6 was much smaller (Fig. 4B) but was consistent and was greater when 4 μg of rySec7d was used (data not shown).

Data from assays at 4°C yielded K_m values of 0.52, 0.58, 7.1, 5.0, and 12.5 μM for ryARF1, ryARF2, rhARF1, rhARF5, and rhARF6, respectively (data not shown). For ryARF experiments 0.2 μg of rySec7d was used and for those with rhARFs, 2 μg of rySec7d was used.

Effect of BFA or BFA Analogues on rySec7d-Stimulated GTP[γS] Binding to ryARF2. GTP[γS] binding to ryARF2 in the presence of rySec7d was inhibited by BFA in assays at 30°C (Fig. 2A). To assess more quantitatively the BFA sensitivity of rySec7d, the effect of BFA concentration on its enhancement of GTP[γS] binding to ryARF2 was examined at 4°C, when rates of binding were constant for 3 h (Fig. 5). The time course data are not shown but based on values at 3 h, BFA inhibition was concentration-dependent and essentially complete with 0.4 mM (12 μg).

To evaluate the specificity of inhibition, we tested analogues of BFA that have been reported (33) to have no effect on Golgi

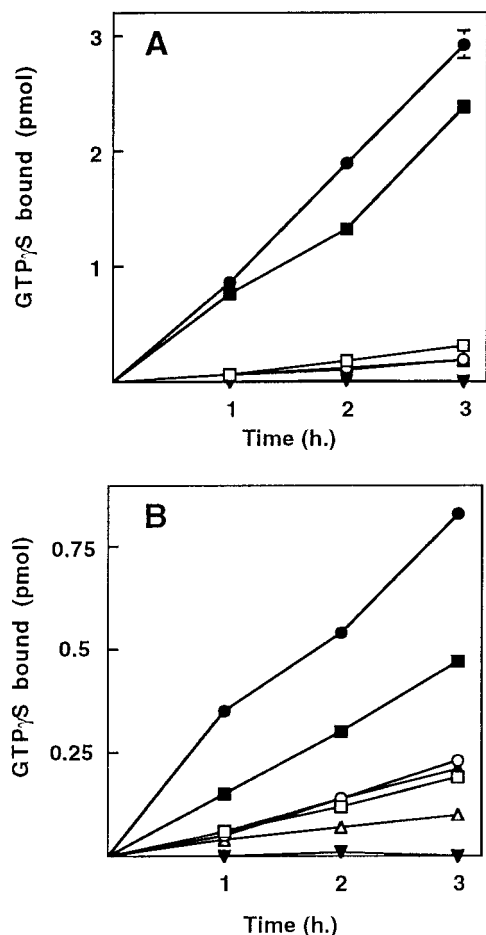


FIG. 4. Effect of rySec7d on GTP[γS] binding to ryARFs and rhARFs. (A) Fifty picomoles of ryARF1 (□ and ■), ryARF2 (○ and ●), or ryARF3 (△ and ▲) and 4 μM [³⁵S]GTP[γS] were incubated without (□, ○, and △) or with 0.2 μg (■, ●, and ▲) of rySec7d for the indicated time before radioassay of protein-bound [³⁵S]GTP[γS]. rySec7d (0.2 μg, ▼) did not bind GTP[γS]. (B) One hundred picomoles of rhARF1 (□ and ■), rhARF5 (○ and ●), or rhARF6 (△ and ▲) were incubated without (open symbols) or with (solid symbols) 2 μg of rySec7d and 4 μM [³⁵S]GTP[γS] before radioassay of protein-bound [³⁵S]GTP[γS]. rySec7d (2 μg; ▼) was incubated as control.

structure or on the distribution of coatomer β subunit (β-COP) in intact cells (Table 1). In these assays at 30°C, percentage inhibition by BFA (9 μg) was less than it was at 4°C (and similar to that shown in Fig. 2A), but analogues B-17, B-27 (a stereoisomer at the C-7 position), and B-36 had no effect on rySec7d activity.

DISCUSSION

The data reported herein demonstrate that rySec7d catalyzes GTP[γS] binding by ryARF1 and ryARF2 and to a lesser extent by rhARF1, rhARF5, and rhARF6 but not ryARF3. The rate of nucleotide binding by the two yeast ARFs (50 pmol) was increased 7- to 15-fold by rySec7d (8.3 pmol), whereas 10 times as much rySec7d increased GTP[γS] binding by rhARF5 (100 pmol) only 3.5-fold. Among the three rhARFs tested, rhARF5 was the best substrate. The effect of rySec7d on ryARF2 was inhibited in a concentration-dependent manner by BFA but not by BFA analogues B-17, B-27, and B-36. Thus, the yeast Sec7 domain contains both the catalytic site responsible for guanine nucleotide-exchange activity on ARFs and the structure responsible for sensitivity to BFA. In addition, it appears that the yeast Sec7 domain itself contains some determinants of ARF specificity. The ARF specificity of the

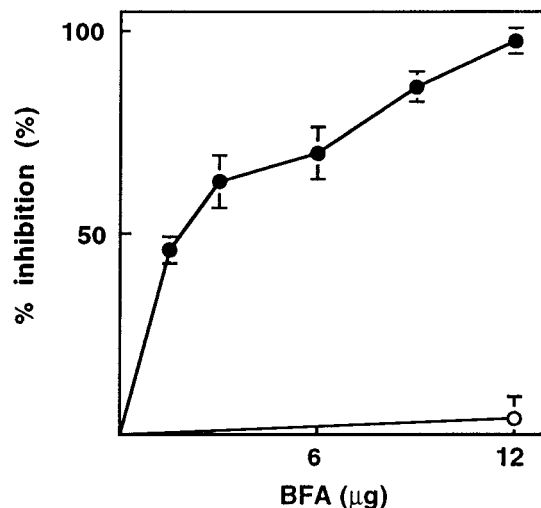


FIG. 5. Inhibition of rySec7d-stimulated GTP[γS] binding to ryARF2 by BFA. Fifty picomoles of ryARF2 and 4 μM [³⁵S]GTP[γS] were incubated with 0.2 μg of rySec7d and the indicated amounts of BFA (●) or MeOH (○) for 3 h at 4°C before radioassay of protein-bound [³⁵S]GTP[γS]. The decrease in binding caused by BFA is expressed as percentage inhibition of binding in its absence. All assays contained 1.2% CH₃OH, the amount added with 12 μg of BFA.

much larger (~200-kDa) intact Sec7 protein has not been established. Comparison of the catalytic properties of Sec7 and its Sec7 domain should provide clues to the existence of other regulatory elements in the native structure.

In addition to ARNO, with a Sec7 domain previously reported to display ARF GEP activity (9), several other proteins that contain Sec7 domains and display ARF GEP activity have been described. One of these is cytohesin-1, the Sec7 domain of which was reported to act as a positive regulator of αLβ2 integrin function (30). GRP-1, a protein very similar to ARNO and cytohesin-1, was described more recently (10). Other ARF GEPs containing Sec7 domains [i.e., Gea1 and Gea2 in yeast (7) and p200 from bovine brain (6)] are perhaps more similar to Sec7 in that they are much larger proteins than ARNO, cytohesin-1, and GRP-1 and are inhibited by BFA. The other three are not and presumably, therefore, do not function in BFA-inhibited vesicular trafficking. Interaction of the products of the *GEA* genes and those of other components of the secretory pathway in *Saccharomyces* were demonstrated (7). The growth defect of the temperature-sensitive *gea1-4* mutant was suppressed by expression of multicopy yeast ARF1 and yeast ARF2 but not yeast Sec7, and multicopy Gea1 did not suppress the temperature-sensitive defect of a Sec7 mutant. In addition, both the growth defect of *gea1-4* and the deletion of both *GEA* genes failed to be complemented by the expression of ARNO in yeast, although it did suppress the dominant-negative effects of a yeast ARF2

Table 1. Effect of BFA analogues on rySec7d-stimulated [³⁵S]GTP[γS] binding to ryARF2

BFA or analogue	% inhibition
Vehicle	4.34 ± 0.87
BFA	54.15 ± 4.31
B-17	10.95 ± 6.14
B-27	4.66 ± 1.67
B-36	7.10 ± 3.76

Fifty picomoles of ryARF2 and 4 μM [³⁵S]GTP[γS] were incubated with vehicle, BFA (9 μg), or analogues of BFA (B-17, B-27, or B-36) (9 μg of each) and 2 μg of rySec7d for 20 min at 30°C, as in Fig. 2A, before radioassay of protein-bound [³⁵S]GTP[γS]. All assays contained 1.2% CH₃OH. Data are means ± SEM of values from triplicate determinations. The experiment was replicated twice.

mutant (7). These observations are consistent with the idea that different Sec7-related proteins function with different ARF substrates, despite sharing similar Sec7 domains.

It seems to be agreed that myristoylated ARFs are better than nonmyristoylated ARFs as GEP substrates, perhaps chiefly because they are more readily associated with membranes (or other proteins), which facilitates the specific ARF-GEP interaction. Individual ARFs are affected differently by specific phospholipids in terms of both guanine nucleotide binding and interactions with other proteins. The true physiological specificity of the multiple ARF GEPs remains to be established. To do this likely will require, in addition to detailed studies of intact cells, extensive *in vitro* work with individual myristoylated ARFs to characterize the complex effects of phospholipids and other experimental conditions on guanine nucleotide binding and release as well as on their specific interactions with GEPs and numerous other proteins.

Until quite recently, by far the largest part of the data on ARF properties and functions was derived from and applied to class I ARFs, specifically mammalian ARF1 and ARF3. It is not surprising, since these were the first to be discovered and are, it appears, the most abundant (facts that are probably not unrelated). Conditions for assays of function that were developed for those ARFs are, of course, not necessarily optimal for class II or class III ARFs. Although recognized, this problem is not always addressed experimentally. For example, ARF1 and ARF3 have been the most commonly used substrates for assay of GEP activity and ARNO was initially described as a GEP that used myristoylated, but not nonmyristoylated, ARF1 as substrate (9). There is now convincing evidence from studies with intact cells, as well as *in vitro*, that ARNO is a BFA-insensitive GEP for ARF6 (34). This conclusion is consonant with earlier reports that BFA had no effect on the intracellular localization of ARF6 (35) and observations that implicated ARF6 in the regulation of plasma membrane-cytoskeletal structure, possibly related to endocytosis (36, 37).

rySec7d apparently had a small but significant effect on GTP[γ S] binding by rhARF6, although no activity could be detected with ryARF3. As reported, the amino acid sequences of these two ARFs are more similar (60% identical) than those of any other yeast and human ARFs (23). It seems that yeast ARF3, unlike the other two yeast ARFs, does not function in endoplasmic reticulum to Golgi transport. It is also immunologically distinct from all six mammalian ARFs (23). (It does stimulate cholera toxin-catalyzed ADP ribosylation in the presence of GTP, which is by definition a property of all ARFs.) ARF6 is also the most structurally divergent member of the mammalian ARF family (18) and appears to function in peripheral plasma membrane/endosomal vesicular trafficking and in regulating plasma membrane-cytoskeletal architecture, somewhat like the Rho-related GTPases (35–38). It is possible that the small effect of rySec7d on GTP[γ S] binding by rhARF6 is another example of failure of the *in vitro* assay used to reflect accurately the ARF specificity that obtains *in vivo*.

ARNO, like the very similar cytohesin-1 and GRP-1, contains in addition to the catalytic Sec7 domain, a C-terminal pleckstrin homology (PH) domain (9). The PH domains of GRP-1 and cytohesin 1 specifically bind phosphatidylinositol 3,4,5-trisphosphate (10). It was suggested that this product of phosphatidylinositol 3-kinase-catalyzed modification of the much more abundant phosphatidylinositol 4,5-bisphosphate could serve to localize and activate ARF6 at sites involved in transduction of extracellular signals (34). Because rhARF6 was a poor substrate and yeast ARF3 was not detectably affected by rySec7d, it is tempting to speculate that a yeast homologue of ARNO is a BFA-insensitive GEP for yeast ARF3.

We thank Dr. Vincent C. Manganiello for critical review of the manuscript and Ms. Carol Kosh for expert secretarial assistance.

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