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Structural basis for the inhibitory effect of brefeldin A on guanine nucleotide-exchange proteins for ADP-ribosylation factors

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ABSTRACT Protein secretion through the endoplasmic reticulum and Golgi vesicular trafficking system is initiated by the binding of ADP-ribosylation factors (ARFs) to donor membranes, leading to recruitment of coatomer, bud formation, and eventual vesicle release. ARFs are ≈20-kDa GTPases that are active with bound GTP and inactive with GDP bound. Conversion of ARF-GDP to ARF-GTP is regulated by guanine nucleotide-exchange proteins. All known ARF guanine nucleotide-exchange proteins contain a Sec7 domain of ≈200 amino acids that includes the active site and fall into two classes that differ in molecular size and susceptibility to inhibition by the fungal metabolite brefeldin A (BFA). To determine the structural basis of BFA sensitivity, chimeric molecules were constructed by using sequences from the Sec7 domains of BFA-sensitive yeast Sec7 protein (ySec7d) and the insensitive human cytohesin-1 (C-1Sec7). Based on BFA inhibition of the activities of these molecules with recombinant yeast ARF2 as substrate, the Asp^{965}–Met^{975} sequence in ySec7d was shown to be responsible for BFA sensitivity. A C-1Sec7 mutant in which Ser^{199}, Asn^{204}, and Pro^{209} were replaced with the corresponding ySec7d amino acids, Asp^{965}, Gln^{970}, and Met^{975}, exhibited BFA sensitivity similar to that of recombinant ySec7d (rySec7d). Single replacement in C-1Sec7 of Ser^{199} or Pro^{209} resulted in partial inhibition by BFA, whereas replacement of Gln^{970} in ySec7d with Asn (as found in C-1Sec7) had no effect. As predicted, the double C-1Sec7 mutant with S199D and P209M was BFA-sensitive, demonstrating that Asp^{965} and Met^{975} in ySec7d are major molecular determinants of BFA sensitivity.

ADP-ribosylation factors (ARFs) are members of the Ras superfamily of GTPases that are critical to vesicular trafficking, including exocytic protein transport (ER-to-Golgi and intra-Golgi) (1–2) and endocytosis (3), as well as synaptic vesicle formation (4, 5), peroxisome biogenesis (6), and phospholipase D activation (7, 8). Among these processes, the best characterized action of ARFs is in the initiation of budding of non-clathrin-coated vesicles from purified Golgi membranes. In eukaryotes, ARFs are abundant, ubiquitous, and highly conserved across phyla (9). Human and *Saccharomyces cerevisiae* ARF proteins share ≈74% amino acid sequence identity (10). Thus far, three yeast ARFs are known. Yeast ARF1 and ARF2 encode proteins with 96% identity and are probably overlapping in function (11), whereas yeast ARF3, the sequence of which is 60% identical to that of mammalian ARF6, is only 52% identical to yeast ARF1 and probably differs in function (12).

Although BFA was originally isolated and characterized as an antiviral compound, it has several other effects, including inhibition of protein secretion (13), stimulation of mono-ADP-ribosylation (14), inhibition of growth of various cancer cells (15), and activation of nuclear factor NF-κB (16). Best understood of these effects is the inhibition of an early step in regulated secretion (13). BFA blocks membrane export from the ER (17) and interferes with vesicle formation (18) by inhibiting GDP–GTP exchange on ARF (19, 20), thereby preventing binding of ARF and cytosolic coat proteins (including COPI) to donor membranes (13). With anterograde traffic stopped, net retrograde transport of Golgi components into the ER, mediated by Golgi tubulation, results in disruption of normal architecture (21). This can be reversed by the removal of BFA or inhibited by forskolin, an activator of adenylyl cyclase (13). Membrane tubulation, induced by BFA, has been also observed with more peripherally situated organelles, including endosomes, lysosomes, and the trans-Golgi network (TGN) (22). Consistent with these observations, BFA can also interfere with membrane trafficking outside of the early secretory pathway, including vesicle budding from the TGN (23), post-Golgi transport (24), and some endocytic pathways (25). Based on BFA inhibition, ARF has also been implicated in endocytosis (26) as well as clathrin coat assembly on the TGN (27).

Coat-protein association with donor membranes requires formation of the active GTP-bound ARF, which is accelerated by ARF guanine nucleotide-exchange proteins (GEPs), whereas GTPase-activating proteins (GAPs) enhance GTP hydrolysis to produce inactive ARF-GDP (28), resulting in coat protein dissociation. Several ARF GEPs that differ in molecular size and BFA sensitivity have been identified (28). The larger (≈200-kDa) BFA-sensitive ARF GEPs include p200 from bovine brain (29) and yeast Gea1 (30), whereas the smaller (≈50-kDa) ARF GEPs, including cytohesin-1 (31), ARNO (32), and GRP1 (33), are BFA-insensitive. All ARF GEPs share a so-called Sec7 domain (Sec7d), of ≈200 amino acids that is sufficient for GEP activity (32, 34–36). Sec7 is a yeast gene product that is essential for protein secretion (37). Crystal structures of the human ARNO Sec7 domain (38, 39) and an ARF1/Gea2 Sec7 domain complex have been published (40) as well as information on the solution structure of C-1Sec7 interacting with ARF1 lacking the first 17 amino acids (41).

Although BFA inhibition of a Golgi-localized ARF GEP activity was first reported in 1992 (19, 20), it was five more years before a BFA-inhibited GEP was purified and cloned (29). We recently reported essentially complete inhibition of the GEP activity of recombinant ySec7d with 0.4 mM BFA, indicating that the Sec7 domain itself contains the elements necessary for BFA inhibition as well as ARF GEP activity (35). The present study was undertaken to define more precisely the region(s) of ySec7d required for BFA inhibition, using chi-

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Abbreviations: ER, endoplasmic reticulum; ARF, ADP-ribosylation factor; GEP, guanine nucleotide-exchange protein; BFA, brefeldin A; rySec7d, recombinant yeast Sec7 domain; C-1 Sec7, cytohesin-1 Sec7 domain; TGN, trans-Golgi network; GTP[γS], guanosine 5′-[γ-thio]-triphosphate.

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meric proteins composed of segments of ySec7d and C-1Sec7, which is not inhibited by BFA. It was concluded and confirmed by using mutants containing single-, double-, and triple-amino acid replacements in C-1Sec7 that Asp^{965} and Met^{975} in ySec7d are major determinants of BFA sensitivity.

MATERIALS AND METHODS

Materials. BL21 (DE3) competent cells and pET-14b vector were purchased from Novagen; plasmid purification kits and Ni-nitrilotriacetic acid (Ni-NTA) agarose from Qiagen (Chatsworth, CA); DH5α-competent cells from Life Technologies (Grand Island, NY); glutathione Sepharose 4B, pGEX-2T, and thrombin from Amersham Pharmacia; *Pfu* DNA polymerase and pCR-Script Amp SK(+) cloning kit from Stratagene; cycle sequencing kits from Perkin–Elmer; Rapid DNA Ligation kit, PCR nucleotide mix, restriction enzymes, lysozyme, and 4-(2-aminoethyl)benzenesulfonyl fluoride from Boehringer Mannheim; guanosine 5′-[γ-[^{35}S]thio]triphosphate (1,250 Ci/mmol, 1 Ci = 37 GBq) from NEN; guanosine 5′-[γ-thio]triphosphate (GTP[γS]) and phosphatidylserine from Sigma; BFA from Epicentre Technologies (Madison, WI). B36, an analogue of BFA prepared by Andrew Green (Université Joseph Fourier, Grenoble, France), was a gift of Julie Donaldson (National Heart, Lung, and Blood Institute, National Institutes of Health).

Preparation of Recombinant Yeast ARF2 Protein. yARF2 was cloned in an ampicillin-resistance plasmid vector (pET7, Novagen), which was used to transform competent BL21 (DE3) *Escherichia coli* (35). For large-scale production of recombinant proteins, flasks of prewarmed Luria–Bertani (LB) broth (500 ml) containing carbenicillin (50 μg/ml) were inoculated with 25 ml from an overnight culture of transformants. After the cultures reached an OD_{600} of $\approx$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). After centrifugation (100,000 $\times$ *g*, 35 min, 4°C), the supernatant was applied to a column (1.7 $\times$ 100 cm) of Ultrogel AcA 54 (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 were eluted with the same buffer. Fractions containing ARF activity were pooled and stored in small portions at −20°C.

Preparation of rySec7d and C-1Sec7. His_6-tagged rySec7d and C-1Sec7 were prepared as described by Sata *et al.* (35) and Pacheco–Rodriguez *et al.* (36), respectively. Briefly, yeast Sec7 domain (residues 827-1017) was cloned from a yeast cDNA library (CLONTECH) by PCR amplification using primers based on the yeast Sec7 gene sequence and ligated to the pET-14b expression vector by using *Nde*I and *Bam*HI restriction sites. rySec7d protein, with a His_6-tag, was expressed in *E. coli* strain BL21 (DE3) and purified to homogeneity on Ni-NTA agarose columns. C-1Sec7 (residues 62–249) was cloned into pQE30 by using *Kpn*I and *Hin*dIII restriction sites and purified on Ni-NTA resin (Qiagen, Chatsworth, CA). Proteins (0.25–1 mg/ml) were stored in small portions at −20°C.

Preparation of Chimeric and Mutated cDNA Constructs for Bacterial Expression. An overlap extension PCR was used to produce ySec7d/C–1Sec7 chimeric proteins. For example, chimera C209/Y was constructed by amplifying a 5′ fragment from C-1Sec7 cDNA by using the forward primer (*i*) 5′-ACGTACTC*CCCGGG*AATGGACCCTAAA-3′ (italicized sequence is a *Sma*I restriction site) together with the reverse primer (*ii*) 5′-CTCTTGTAAAGAGGGCTT*ATCTTTGACATT*-3′ (ySec7d sequence is italicized) spanning the desired parts of C-1Sec7 and ySec7d. A 3′ fragment was amplified from ySec7d cDNA by using the reverse primer (*iii*) 5′-ACGTACGTG*GAATTC*TCATTCAGAAATTAACTT-3′ (italicized sequence is an *Eco*RI restriction site) and a second C-1Sec7/ySec7d primer (*iv*) 5′-AAAGATAAGCCC*TCTTTACAAGAGTTTTTA*-3′ (sequence of ySec7d is italicized). The two resulting fragments, which overlapped by 24 bp, were isolated and gel-purified. In a second PCR, 100–120 ng of each fragment were pooled, fused, and amplified by using the (*i*) and (*iii*) primers. The resulting product was subcloned into pCR-Script Amp SK(+) vector, and the fragment excised with *Sma*I and *Eco*RI was gel-purified before ligation to the pGEX-2T expression vector. All mutated sequences were confirmed by automated sequencing.

A similar procedure was employed to generate point mutations. For example, for the C(S199D) mutation, two PCRs, one with primers (*i*) (from above) and (*v*) 5′-GTTGTGCAG*ATC*GGTGTTCAACATGATGAT-3′ (mutated codon italicized) and one with (*vi*) 5′-ACGTACGTG*GAATTC*TCAGTCTTCTGGGAT-3′ (*Eco*RI restriction site sequence italicized) and (*vii*) 5′-AACACC*GAT*CTGCACAACCCCAATGTC-3′ (mutated codon italicized) were performed by using C-1Sec7 cDNA as template. A final PCR with equimolar amounts of the two products (399 and 177 bp, isolated by agarose gel electrophoresis) as templates and primers (*i*) and (*vi*) were carried out to produce the final mutant DNA (558 bp), which was subcloned into pCR-Script Amp SK(+) vector. The fragment excised with *Sma*I and *Eco*RI was gel-purified and ligated to the pGEX-2T vector before confirmatory sequencing.

Expression of Chimeric and Mutant Proteins in *E. coli*. Each construct was introduced into DH5α *E. coli*, which was cultured overnight before addition to a 25$\times$ volume of LB medium with ampicillin. After incubation at 30°C until OD_{600} reached $\approx$0.6 (about 2.5 h), recombinant protein synthesis was induced by adding isopropyl β-D-thiogalactopyranoside (final concentration 0.1 mM), followed by incubation for 2 h at 30°C. After centrifugation (4,000 $\times$ *g*, 5 min, 4°C), the bacterial pellet was dispersed in 25 ml of ice-cold PBS containing lysozyme (100 μg/ml), 0.5 mM 4-(2-aminoethyl)benzenesulfonyl fluoride, and 3 mM $MgCl_2$, lysed by sonification after freezing and thawing, and centrifuged (17,000 $\times$ *g*, 15 min). A slurry (50%) of glutathione Sepharose 4B (1.25 ml) was added to the supernatant followed by incubation at 4°C for 2 h with continuous rocking. After beads were washed three times with 10 ml of ice-cold PBS, the protein product was released from the glutathione *S*-transferase fusion protein by incubation of beads with thrombin (20 units/ml) for 4 h at 4°C, although digestion was monitored by SDS/PAGE. Proteins ($\approx$0.1–0.5 mg/ml) were stored at −20°C in PBS containing 0.5 mM 4-(2-aminoethyl)benzenesulfonyl fluoride, 3 mM $MgCl_2$, and 2 mM DTT.

GTPγS Binding Assay, GEP activity, i.e., the acceleration of replacement of ryARF2-bound GDP, was assessed by measuring [^{35}S]GTP[γS] binding by using a rapid filtration technique (35). Briefly, 1 μg (50 pmol) of ryARF2 and 4 μM [^{35}S]GTP[γS] were incubated for 3 h at 4°C without or with the indicated amount of rySec7d, C-1Sec7, or chimeric or mutant proteins and other additions as indicated in medium containing 20 mM Tris·HCl (pH 8.0), 1 mM DTT, 3 mM $MgCl_2$, 1 mM EDTA, 30 μg of BSA, and 10 μg of phosphatidylserine (total volume, 100 μl). After 3 h, samples were transferred to nitrocellulose filters in a manifold (Millipore) for rapid filtration followed by washing six times, each time 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 dried before addition of scintillation fluid for radioassay. Data are means $\pm$ SEM of values from triplicate assays in a representative experiment. All observations have been replicated at least twice with different preparations of recombinant proteins.

RESULTS

Effect of BFA on rySec7d- or C-1Sec7-Stimulated GTP[γS] Binding to ryARF2. We initially investigated the effect of BFA on rySec7d- or C-1Sec7-stimulated GTP[γS] binding to ryARF2 (Fig. 1*A*). As reported (35), enhancement of GTP[γS] binding to ryARF2 by rySec7d was inhibited by BFA, which did not inhibit C-1Sec7-stimulated binding. It had been noted that C-1Sec7 had BFA-insensitive GEP activity toward yARF2, although cytohesin-1 did not (36). We therefore used C-1Sec7 sequence for construction of chimeric molecules to identify the BFA-interacting sites of ySec7d.

Determination of the Region(s) of rySec7d Required for BFA Inhibition. We initially made three ySec7d/C-1Sec7 chimeric proteins from which the N-terminal 14 amino acids of the Sec7 domain were deleted. Y924/C, in which 84 amino acids in the N-terminal region of C-1Sec7 (residues 75–158) were replaced with the corresponding sequence from ySec7d (Fig. 1*B*), catalyzed BFA-insensitive enhancement of GTP[γS] binding to ryARF2, whereas the activity of C158/Y, in which the C-terminal 91 amino acids of C-1Sec7 (residues 159–249) were replaced with the corresponding segment of ySec7d, was inhibited by BFA. The acceleration of nucleotide binding by both of these chimeras was, however, less than that by rySec7d (Fig. 1*A*). These data indicate that the C-terminal 91 amino acids of ySec7d contain structure important for BFA sensitivity. Chimera C194/Y, in which the C-terminal 55 amino acids (residues 195–249) were changed to the corresponding sequence in ySec7d (Fig. 1*B*), also showed BFA-sensitive activation of GTP[γS] binding to ryARF2 (Fig. 1*A*). The fact that the percentage inhibition of C158/Y and C194/Y exchange activity by BFA was 80–90%, similar to that with rySec7d, suggested that the C-terminal region (residues 961-1015) of ySec7d contains the determinants of BFA sensitivity.

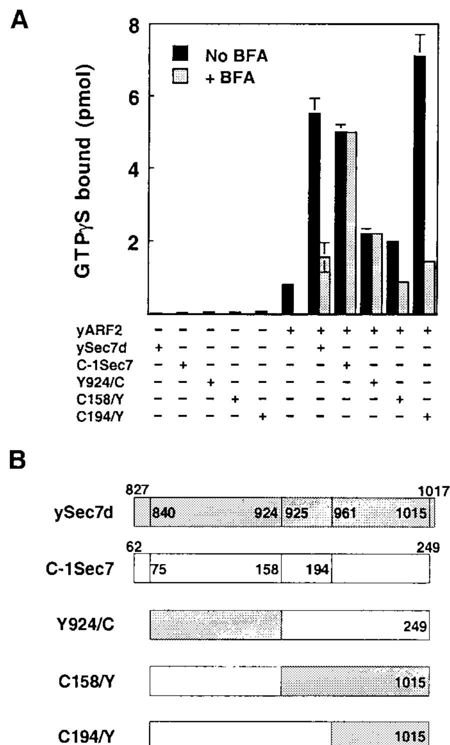

FIG. 1. Determination of ySec7d region required for BFA inhibition by using ySec7d/C-1Sec7 chimeras. (*A*) ryARF2 (50 pmol) and rySec7d (42 pmol), C-1Sec7 (45 pmol), or 50 pmol of each chimeric protein were incubated with 4 μM [^{35}S]GTP[γS] in a total volume of 100 μl without (black bars) or with (gray bars) 6 μg of BFA (0.2 mM) for 3 h at 4°C before radioassay of protein-bound [^{35}S]GTP[γS]. ryARF2 and GEPs were also incubated individually as controls (first six lanes). The experiment was repeated twice, with similar results. Data are means ± SEM of values from triplicate assays. Errors <0.03 pmol are not shown. (*B*) Diagram of ySec7d, C-1Sec7, and chimeric molecules.

Location of BFA Interaction Sites in the C-terminal One-Third of ySec7d. We made three different ySec7d/C-1Sec7 chimeric proteins, focusing on the last 55 residues of ySec7d (Fig. 2*B*). In C209/Y and C226/Y, the C-terminal 40 or 23 amino acids, respectively, were replaced with corresponding sequences of ySec7d (Fig. 2*B*); both chimeras enhanced GTP[γS] binding to ryARF2, and neither was inhibited by BFA (Fig. 2*A*). In contrast, CYC, in which the sequence (Ser^{199}–Pro^{209}) of C-1Sec7 was changed to the corresponding sequence of ySec7d (Fig. 2*B*), had BFA-sensitive activity, with a percentage inhibition by BFA similar to that of rySec7d (Fig. 2*A*). Taken together, these data confirm that the Asp^{965}–Met^{975} sequence, which is located just after the "block 2" region (38) of ySec7d, is a major determinant of its BFA sensitivity.

Identification of Specific Amino Acids of ySec7d Responsible for BFA Inhibition. Comparison of several ARF GEP sequences revealed clear differences between BFA-sensitive (ySec7d, Gea1, and p200) and BFA-insensitive ARF GEPs (C-1Sec7, GRP1, and ARNO) in the region corresponding to Asp^{965}–Met^{975} of ySec7d (Fig. 3 *Upper*). Asp^{965}, Gln^{970}, and Met^{975} of ySec7d were present in all of the BFA-sensitive ARF GEPs, whereas the corresponding amino acids, Ser^{199}, Asn^{204}, and Pro^{209} of C-1Sec7 were found in all of the BFA-insensitive ARF GEPs. We therefore made mutants in which these three amino acids were replaced (Fig. 3 *Lower*). CYC(SNP), in which Asp^{965}, Gln^{970}, and Met^{975} of the BFA-sensitive chimeric CYC were changed to the corresponding amino acids (Ser, Asn, and Pro) of C-1Sec7, accelerated GTP[γS] binding to ryARF2, but

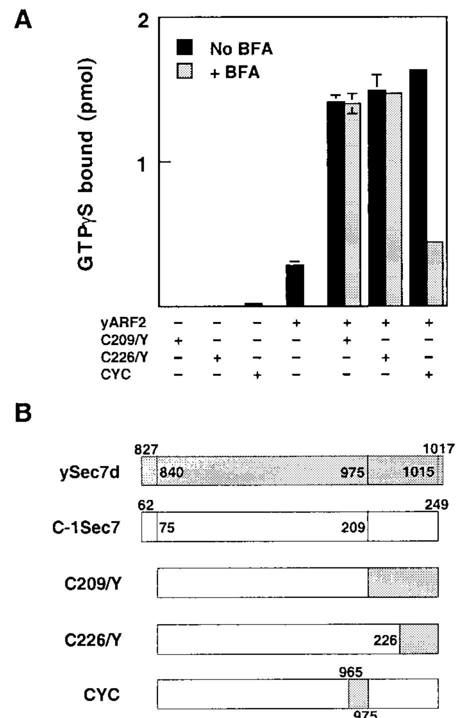

FIG. 2. Localization of determinants of BFA inhibition in the C-terminal one-third of ySec7d. (*A*) ryARF2 and 50 pmol of each chimeric GEP were incubated with 4 μM [^{35}S]GTP[γS] without (black bars) or with (gray bars) 6 μg of BFA for 3 h at 4°C before collection of proteins for radioassay. ryARF2 and chimeric GEPs were also incubated individually as controls. Two independent experiments were performed with similar results. Errors <0.01 pmol are not shown. (*B*) Diagram of ySec7d/C-1Sec7 chimeras. Data are presented as in Fig. 1.

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             965                 975
ySec7d      |D| L H S S |Q| I K N K |M|
Gea1        |-| S - N P |-| V - D H |-|
p200        |-| - - - P |-| V - - - |-|
C-1Sec7     |S| - - N P |N| V - D - |P|
GRP1        |S| - - N H |N| V R D - |P|
ARNO        |S| - - N P |N| V R D - |P|

ySec7d       D  L H S S  Q  I K N K  M
CYC(SNP)     S  - - - -  N  - - - -  P

C-1Sec7      S  L H N P  N  V K D K  P
C(DQM)       D  - - - -  Q  - - - -  M
C(DM)        D  - - - - - - - - - -  M
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FIG. 3. Alignment of Asp[965]–Met[975] sequence of ySec7d with corresponding regions of other GEPs. Asp[965], Gln[970], and Met[975] are highly conserved among BFA-sensitive ARF GEPs, whereas the corresponding Ser[199], Asn[204], and Pro[209] in C-1Sec7 are conserved in BFA-insensitive ARF GEPs (boxed with solid lines). CYC(SNP) was made by replacing Asp[965], Gln[970], and Met[975] in chimera CYC with Ser[199], Asn[204], and Pro[209] as in C-1Sec7. In C-1Sec7, Ser[199], Asn[204], and Pro[209] were replaced with the corresponding amino acids in ySec7d to yield C(DQM). The double mutant C(DM) contains Asp replacing Ser[199] and Met replacing Pro[209] in C-1Sec7. Mutated residues are in boldface. Numbers above the sequence correspond to positions in ySec7p. Hyphen indicates amino acid identical to that in the sequence at the top of each group.

was not inhibited by BFA (Fig. 4). CYC(SNP) activity was proportional to protein at least up to 1 μg (data not shown). In contrast, C(DQM), in which Ser[199], Asn[204], and Pro[209] were replaced by the corresponding amino acids (Asp, Gln, and Met) in ySec7d, was inhibited by BFA (Fig. 4), consistent with the possibility that Asp[965], Gln[970], and/or Met[975] of ySec7d could be responsible for BFA sensitivity.

Y(Q970N) with Gln[970] of ySec7d replaced by Asn (as found in C-1Sec7) was still inhibited by BFA (Fig. 5). With single amino acid replacements in C-1Sec7, C(N204Q) remained BFA-insensitive, whereas C(S199D) and C(P209M) were rendered BFA-sensitive, although percentage inhibition of GEP activity by BFA was less than that with rySec7d (Fig. 5). To confirm that Asp[965] and Met[975] in ySec7d are crucial for BFA sensitivity, a double mutant of C-1Sec7, C(DM), in which the corresponding residues were replaced, was prepared. C(DM) exhibited BFA sensitivity similar to that of rySec7d (Figs. 6 and 7), and its activity was unaffected by an inactive analogue B36

FIG. 4. Effect on BFA sensitivity of three residue replacements in C-1Sec7 and CYC. ryARF2 (50 pmol) and 50 pmol of each GEP mutant were incubated with 4 μM [^{35}S]GTP[γS] without (black bars) or with (gray bars) 6 μg of BFA (0.2 mM) for 3 h at 4°C before assay of protein-bound [^{35}S]GTP[γS]. ryARF2 and mutants were also incubated individually as controls. Data are presented as in Fig. 1. The experiment was repeated twice with similar results. Errors <0.02 pmol are not shown.

FIG. 5. Specific amino acids in the ySec7d Asp[965]–Met[975] sequence required for BFA inhibition. Ser[199], Asn[204], and Pro[209] in C-1Sec7 were replaced by the corresponding amino acids in ySec7d (Asp[965], Gln[970], and Met[975], respectively) to produce C(S199D), C(D204Q), and C(P209M). Gln[970] in ySec7d was changed to Asn in Y(Q970N). ryARF2 (50 pmol), 50 pmol of each mutant protein, and 4 μM [^{35}S]GTP[γS] were incubated for 3 h at 4°C without (black bars) or with (gray bars) 6 μg of BFA (0.2 mM) before assay of protein-bound [^{35}S]GTP[γS]. ryARF2 and mutants were also incubated individually as controls. Data are presented as in Fig. 1. The experiment was repeated twice with similar results. Errors <0.05 pmol are not shown.

(Fig. 6). BFA inhibition was concentration-dependent and almost complete with 0.4 mM (Fig. 7).

DISCUSSION

Based on observations that the GEP activities of ySec7d and C-1Sec7 are BFA-sensitive and insensitive, respectively, we defined the interaction sites of ySec7d with BFA *in vitro* by using chimeric and mutant proteins with sequences based on differences in the primary structures of ySec7d and C-1Sec7. Assays of several chimeric proteins indicated that the Asp[965]–Met[975] sequence in ySec7d may be responsible for its BFA sensitivity. Several ARF GEPs were divided into two groups, based on the identity of these 11 amino acids. The Asp[965], Gln[970], and Met[975] present in ySec7d were highly conserved among BFA-sensitive ARF GEPs, whereas the corresponding amino acids, Ser[199], Asn[204], and Pro[209] in C-1Sec7 were found in all of the BFA-insensitive ARF GEPs. The studies with the

FIG. 6. Effect of rySec7d and C(DM) on [^{35}S]GTP[γS] binding and its inhibition by BFA or its analogue. ryARF2 and 1 μg of rySec7d (42 pmol) or the double mutant, C(DM) (50 pmol) were incubated for 3 h at 4°C with 4 μM [^{35}S]GTP[γS] without (black bars) or with (gray bars) 9 μg of BFA or the inactive analogue B36 (0.3 mM) before radioassay of protein bound [^{35}S]GTP[γS]. ryARF2, rySec7d, and C(DM) were also incubated individually as controls. Data are presented as in Fig. 1. The experiment was repeated twice with similar results. Errors <0.05 pmol are not shown.

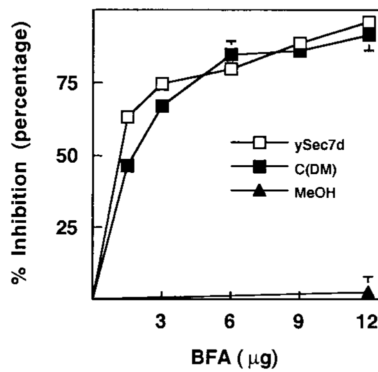

FIG. 7. Inhibition of rySec7d- or C(DM)-stimulated [^{35}S]GTP[γS] binding to ryARF2 by BFA. ryARF2 and 1 μg of rySec7d (□) or C(DM) (■) and 4 μM [^{35}S]GTP[γS] were incubated with the indicated amounts of BFA or MeOH (▲) for 3 h at 4°C before radioassay of protein-bound [^{35}S]GTP[γS]. The decrease in binding caused by BFA is reported as percentage inhibition of binding in its absence. All assays contained 1.2% (vol/vol) CH_3OH. Data are means ± SEM of values from triplicate determinations. Error bars smaller than symbols are not shown. The experiment was repeated twice.

triple replacement mutants C(DQM) or CYC(SNP) suggested that Asp^{965}, Gln^{970}, and/or Met^{975} in ySec7d could be responsible for BFA sensitivity. Whereas the single mutants C(S199D) and C(P209M) were partially inhibited by BFA, C(N204Q) and Y(Q970N) remained BFA-insensitive and sensitive, respectively. This could mean that the residue corresponding to Gln^{970} in ySec7d or Asn^{204} in C-1Sec7 is not important for BFA-inhibited GEP activity or only that the difference of one carbon atom in otherwise similar acidic amino acids can be tolerated at this site. BFA inhibition of the double mutant C(DM) was very similar to that of rySec7d, indicating that Asp^{965} and Met^{975} in ySec7d are critical determinants of the BFA sensitivity.

The Sec7-domain active site has a bipartite structure (38, 39), with a hydrophobic groove (including block 2 or motif 2) and an adjacent hydrophilic loop (block 1 or motif 1). It is notable that although Asp^{965} and Met^{975} lie outside of the block 2 region, BFA inhibited stimulation of GTP[γS] binding by rySec7d or the mutant C(DM) almost completely at a concentration of 0.4 mM (Fig. 7). Although BFA is a small molecule (M_r = 280.37) relative to the Sec7 domain, it may affect the interaction of Met^{194} (ARNO numbering) in the block 2 region with ARF indirectly by interacting with Asp^{965} and Met^{975}. Indeed, Met^{194} has been proposed to be a part of the core region of the active site and to form direct interactions with ARF. Its replacement with lysine or alanine (M194K or M194A) essentially abolished GEP activity of the Sec7 domain (38, 39). The negatively charged Asp^{965} and the hydrophobic Met^{975} also could be involved in nucleotide exchange by ySec7d. Consistent with this proposal, mutation in C-1Sec7 of the charged residues Asp^{207}, Lys^{208}, and Arg^{219} (which surround the hydrophobic groove) decreased GEP activity 50–80% (41). In addition, the BFA-sensitive ARF GEP activity of p200 was modulated by elements nearer the N terminus that enhanced catalytic activity ≈100-fold and also increased the sensitivity to BFA (N. Morinaga, R. Adamik, J. Moss, and M.V., unpublished data), suggesting that additional determinants of BFA sensitivity may reside outside of the Sec7 domain.

BFA has been widely used to investigate membrane transport mechanisms and pathways. It inhibits some ARF1 GEPs and has been shown to prevent ARF1 binding to Golgi membranes *in vitro* (19, 20). Effects of BFA are, however, not limited to the Golgi apparatus. Membrane tubulation induced by BFA has been also observed involving peripheral organelles, including endosomes, lysosomes, and the TGN (22). Consistent with these observations, the formation of vesicles from the TGN (23), post-Golgi trafficking of the mannose-6-phosphate receptor (24), the maturation of secretory granules (42), and some endocytotic pathways (25, 26) are all sensitive to BFA. Indeed, ARF1 has been proposed to regulate recruitment to membranes of three different coat complexes, COPI, AP-1, and AP-3 independently (1, 2, 4). COPI-coated vesicles are believed to mediate anterograde intra-Golgi transport and retrograde Golgi to ER transport (1), whereas AP-1 and AP-3 have been linked to vesicle budding from the TGN (2) and transport within the endosomal–lysosomal system (4), respectively. As seen by immunofluorescence microscopy, COPI, AP-1, and AP-3 localization displayed differential sensitivity to BFA, implying the existence of distinct ARF GEPs that control the assembly of different coats (5). The observation that binding of both ARF1 and AP-3 to membranes was inhibited by BFA *in vitro*, by ≈70% and ≈50%, respectively, is consistent with this proposal (5). Differences in the BFA sensitivity of ARF1 GEPs may be reflected in different effects of the drug on specific ARF1-regulated events. Evidence that ARF1 may recruit distinct coat protein complexes by more than one mechanism, is provided by the finding that added exogenous phospholipase D induced recruitment of coatomer to membranes and also stimulated release of nascent secretory vesicles from the TGN (43, 44). Through activation of phospholipase D (7, 8), ARF may indirectly alter membrane lipid composition to facilitate binding of different coat proteins.

It is unclear why no specific GEPs for ARFs of class II (ARFs 4 and 5) or class III (ARF6) have been purified. Indeed, it is possible that assay conditions usually used, which were developed for class I ARFs, are not really appropriate for class II or class III ARFs. A lack of effect of BFA on the intracellular localization of ARF6 (45) was reported and ARF6 was identified in association with the plasma membrane as well as with uncharacterized endosomal structures (3, 45–47). On the other hand, when MDCK cells were treated with BFA, AP-3 was dispersed from punctate structures to the cytosol, indicating that its location was sensitive to BFA (5). These observations suggest that the ARF GEP regulating AP-3 binding via ARF1 or another BFA-sensitive ARF, differs from an ARF6 GEP, at least in its BFA sensitivity, despite a localization similar to that of a putative ARF6 GEP.

However, because the subcellular localization of ARF6 appears to differ considerably in different cells (48), it is imperative that the distribution of ARF6, or better, its colocalization with a second relevant molecule, be demonstrated in each specific system studied. All BFA-insensitive ARF GEPs identified thus far have been found exclusively in the cytoplasm in unstimulated cells (49–52). Stimulation with phosphatidylinositol 3-kinase, insulin, and EGF (or NGF), respectively, resulted in a PH-domain-dependent translocation of cytohesin-1 (49), ARNO (50) and GRP1 (51) to the plasma membrane. Overexpression of ARNO (or ARNO 3) in mammalian cells induced a phenotype resembling that associated with BFA inhibition of ARF1 activation without affecting other intracellular compartments, probably because of the continuous production and consumption of COPI-containing vesicles (52, 53).

It is far from clear that all of the effects of BFA on cell structure and function are caused by the interference with ARF activation and coat protein assembly (21). Indeed, there is evidence that an ADP-ribosylation reaction may also play a role in the cellular effects of BFA. BFA induces the mono-ADP-ribosylation of a 50-kDa cytosolic protein (BARS-50) by activating a specific endogenous mono-ADP-ribosyltransferase (14). BARS-50 can bind GTP, and its interaction with the G protein βγ subunit complex ($G_{\beta\gamma}$) resulted in inhibition of ADP-ribosylation of BARS-50 (54). Because ARF and certain PH domains can interact directly with $G_{\beta\gamma}$ (55, 56), and $G_{\beta\gamma}$ can interfere with the activation and binding of ARF and

β-COP to Golgi membranes (57), BARS-50 might be postulated to play a regulatory role in ARF-mediated membrane transport. Its state of ADP-ribosylation, which can be controlled by BFA, may alter its regulatory properties. Consistent with these speculations is a recent report that depletion of NAD^+ (the ADP ribose donor) or pharmacological blockade of the BFA-dependent ADP-ribosylation prevented Golgi disassembly by BFA in permeabilized cells (58). It is beginning to seem increasingly likely that multiple mechanisms are involved in the effects of BFA.

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