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Biochemistry

ADP-ribosylation factor is functionally and physically associated with the Golgi complex

(protein transport/GTP-binding protein/yeast/endoplasmic reticulum)

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ABSTRACT **ADP-ribosylation factor (ARF) is a ubiquitous, highly conserved 21-kDa GTP-binding protein, first identified in animal cells as the cofactor required for the *in vitro* ADP-ribosylation of the stimulatory regulatory subunit of adenylate cyclase, G_s, by cholera toxin. As the relevance of this activity to *in vivo* function is unknown, we have taken advantage of the conserved nature of ARF to study its function in *Saccharomyces cerevisiae*. Yeast cells bearing an *arf1* null mutation display a number of phenotypes suggesting a defect in the secretory pathway. Secreted invertase is only partially glycosylated, and there is a small internal accumulation of invertase. Genetic experiments revealed interactions between *ARF1* and other genes known to be involved in the secretory pathway, including *YPT1*, which encodes a different GTP-binding protein. In accord with these genetic results, immunofluorescence and immunoelectron microscopy show that ARF protein is localized to the Golgi apparatus in mammalian cells, in particular to the cytosolic surface of predominantly cis-Golgi membranes. Together, these results indicate that ARF functions in intracellular protein transport to or within the Golgi apparatus, a role not predicted by the previous *in vitro* biochemical studies.**

Since the demonstration that the *ras* oncogene protein binds GTP and has sequence homology to a larger family of GTP-binding proteins (for review, see ref. 1), many structurally related small GTP-binding proteins have been isolated. Despite intensive efforts, the *in vivo* functions of most of these proteins, including ras, are unknown. Recent evidence from work on *Saccharomyces cerevisiae* indicates that at least two small GTP-binding proteins, SEC4 (2) and YPT1 (3, 4), are involved in the processing and secretion of newly synthesized proteins from the endoplasmic reticulum (ER) to the cell surface. SEC4 and YPT1 are closely related by sequence but appear to function at different points in the yeast secretion pathway: YPT1 in ER–Golgi transport, SEC4 in post-Golgi transport. It has been proposed that the role of GTP-binding proteins in secretion might be to mediate the vectorial transport of secretory vesicles from one compartment to another (3, 5).

We are studying ADP-ribosylation factor (ARF), a small GTP-binding protein first identified and purified as the protein cofactor required for efficient *in vitro* ADP ribosylation of the stimulatory regulatory subunit of adenylate cyclase, G_s, by cholera toxin (6, 7). The purified protein was subsequently shown to have a single high-affinity binding site for guanine nucleotides, copurify complexed with GDP, and be active in the cholera toxin reaction only in the GTP-liganded state (8). Antibodies generated against a peptide derived from a region common to all known ARF sequences have been used to detect the presence of ARF in cell extracts of all eukaryotic organisms examined, including man, cow, mouse, frog, slime mold, yeast (9), fruit fly, and the plant *Arabidopsis thaliana* (unpublished observation). The determination of the DNA-derived protein sequences of bovine and yeast ARF has revealed that ARF is structurally related to both the p21 *ras* (including *YPT, SEC4, rho, ral, R-ras*, and many others) and G-protein α subunit families of GTP-binding proteins, although the regions in common are mostly limited to those responsible for guanine nucleotide handling (10).

Although ARF is among the best characterized of the small GTP-binding proteins in terms of *in vitro* biochemical properties, the relationship between these properties and *in vivo* function is obscure. Indeed, the cholera toxin assay in which ARF was identified would seem to have little relevance to the majority of organisms in which ARF is found. We have chosen to examine ARF function in *S. cerevisiae*, making use of the powerful yeast genetic system. We found that ARF protein is encoded by two genes in yeast, *ARF1* (10) and *ARF2*. The ARF1 and ARF2 proteins share >96% identity in amino acid sequence and are functionally homologous (T.S., M. A. Hoyt, D.B., and R.A.K., unpublished work). Neither *ARF1* nor *ARF2* alone is essential for viability, but deletion of both genes results in lethality. *arf1* null mutants display a number of phenotypes, including slow growth, cold sensitivity, and supersensitivity to fluoride ion, whereas *arf2* null mutants are indistinguishable from wild type (T.S., M. A. Hoyt, D.B., and R.A.K., unpublished work). The level of protein produced from *ARF1* is ≈10-fold higher than that from *ARF2* (T.S., M. A. Hoyt, D.B., and R.A.K., unpublished work), suggesting that the phenotypes observed in *arf1* null mutants are due to greatly reduced levels of ARF protein.

In this paper, we present genetic and biochemical evidence that ARF, like SEC4 and YPT1, is involved in secretion in yeast and that ARF protein is localized to the Golgi apparatus in mammalian cells. ARF is not closely related to SEC4 and YPT1 and represents a new class of secretion-associated GTP-binding protein. Our results indicate that ARF and YPT1 proteins might function at similar points in the secretion pathway, suggesting that certain steps in secretion require the action of multiple GTP-binding proteins.

MATERIALS AND METHODS

Media, Strains, Plasmids, and Antibodies. Media for yeast growth and sporulation were as described by Sherman *et al.* (11), except for 5-fluoroorotic acid plates (12). The strains used in secretion experiments were DBY1034 *MATa his4-539 lys2-801 ura3-52*, DBY5330 *MATa his3-Δ200 leu2-3,112 trp1-1 ura3-52 arf1::HIS3* (pRB720), DBY5331 *MATa his3-Δ200*

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Abbreviations: ARF, ADP-ribosylation factor; ER, endoplasmic reticulum.

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leu2-3,112 trp1-1 ura3-52 arf1::HIS3 (pRB1297), DBY1803 *MATa his4-539 lys2-801 ura3-52 ypt1-1*, DBY5332 *MATα his3-Δ200 leu2-3,112 ura3-52 sec18-1*, and DBY5333 *MATα his3-Δ200 leu2-3,112 ura3-52 sec18-1 arf1::URA3*. The strains used to characterize genetic interactions between *ARF1* and *YPT1* were DBY5334 *MATα his3-Δ200 leu2-3,112 lys2-801 ura3-52 ypt1-1* and DBY5335 *MATα his3-Δ200 leu2-3,112 trp1-1 ura3-52 arf1::HIS3*. The two *arf1* disruption mutations used are identical in the amount of *ARF1* coding sequence removed, differing only in the inserted marker (T.S., M. A. Hoyt, D.B., and R.A.K., unpublished work). Plasmids used were pRB720, a yeast *CEN* vector; pRB1297 and pRB1298, yeast *CEN* and 2μ vectors, respectively, with a 1.6-kb *Eco*RI-*Pst* I fragment containing the *ARF1* gene (10); and pRB319 and pRB320, yeast *CEN* and 2μ vectors, respectively, with the *YPT1* gene (13). Genetic techniques were as described (14). Anti-ARF antibodies (R-5) were raised in rabbits against a 14-amino acid peptide present in a conserved region of the protein (residues 23–36 of bovine ARF1) and affinity purified as described in Kahn *et al.* (9). Immunoblots of total cell lysates of NIH 3T3 cells with the affinity-purified anti-ARF antibodies confirmed recognition of ARF protein with no significant cross-reactivity. The anti-invertase antibody was the gift of Randy Schekman (33).

Invertase Induction, Assay, and Immunodetection. Invertase synthesis was induced by shifting exponentially growing cells from yeast extract/peptone (YEP) medium containing 5% glucose to YEP containing 0.1% glucose for 3 hr. External invertase was assayed at 37°C by using intact cells as described by Goldstein and Lampen (15); units of activity are μmol of glucose released per min. Internal invertase was assayed in spheroplast lysates prepared by digestion with Zymolyase 100,000 at 5 μg/ml in 1.4 M sorbitol/50 mM potassium phosphate, pH 7.5/25 mM 2-mercaptoethanol and subsequent lysis with 0.5% Triton X-100. Yeast protein extracts for immunoblotting were prepared and electrophoresed as described (16). Proteins were electrophoretically transferred to nitrocellulose, and invertase was detected by incubation with anti-invertase rabbit serum followed by ^{125}I-labeled protein A (17).

Immunocytochemical Localization of ARF. NIH 3T3 cells were plated in 35-mm culture dishes that had been precoated with Cell Tak (Biopolymers, Farmington, CT). For immunofluorescence, the cells were fixed by using 3.7% formaldehyde and then incubated with affinity-purified rabbit anti-ARF (50 μg/ml) followed by affinity-purified goat anti-rabbit IgG labeled with rhodamine (50 μg/ml, Jackson ImmunoResearch), as described by Willingham and Pastan (18). Cells were prepared for EM immunocytochemistry as described (19, 20). Affinity-purified rabbit anti-ARF (50 μg/ml) was localized by using the ferritin bridge procedure (19), followed by postfixation in glutaraldehyde and OsO_4, embedding in Epon 812, and postsectioning staining with lead citrate and bismuth subnitrate (19).

RESULTS

An *arf1* Mutation Causes a Secretion Defect. The precedent for the involvement of small GTP-binding proteins in secretory protein transport established by work on *SEC4* and *YPT1* led us to examine this process in yeast *arf* mutants. We measured the glycosylation and secretion of invertase as indicators of secretory pathway function. Invertase is expressed from the *SUC2* gene by two transcripts: one constitutive and the other induced by low glucose growth conditions (21). The protein produced by the constitutive transcript is cytosolic, whereas that produced by the regulated transcript has a signal sequence and is secreted (21). Glycosylation of invertase was studied by inducing invertase synthesis in cultures of a wild-type strain and various mutant strains. The proteins in crude extracts of these cultures were then separated by SDS/PAGE, and the mobility of invertase was determined by immunoblotting with anti-invertase antibodies (Fig. 1).

The wild-type strain displays the characteristic pattern of invertase staining (22); the nonglycosylated cytoplasmic form migrates as a faint distinct band, whereas the glycosylated, secreted form migrates more slowly and appears as a broad smear (Fig. 1, lane a). The *sec18-1* mutation blocks secretion from the ER to the Golgi apparatus at the nonpermissive temperature; thus, invertase receives only core glycosylation (22). Invertase from the *sec18-1* mutant strain migrates normally when induction takes place at 22°C and migrates as a more rapidly migrating triplet of distinct bands at 35°C (compare Fig. 1, lanes f and g). Interestingly, the *arf1::HIS3* strain produces invertase that migrates with a mobility intermediate between that of core-glycosylated and fully glycosylated forms (Fig. 1, lane b). This defect is largely complemented by a yeast centromere plasmid bearing the *ARF1* gene. There is no apparent difference between the invertase produced by the *arf1::HIS3* strain at 14°C and that produced at 30°C, despite the partial cold-sensitivity displayed by *arf1::HIS3* mutants (Fig. 1, lanes b and e). A mutation in the GTP-binding protein *YPT1* has also been shown to cause a partial glycosylation defect in yeast cells (3). The invertase glycosylation pattern produced by *arf1::HIS3* and *ypt1-1* strains is very similar (Fig. 1, lanes b and d).

To show that the form of invertase seen in the *arf1* null mutant has actually traversed the secretory pathway, we induced invertase synthesis in a *sec18-1 arf1::URA3* double mutant (the *arf1*::HIS3 and *arf1::URA3* alleles differ only in the inserted marker). The double mutant has the *arf1::URA3* phenotype at the permissive temperature for *sec18-1* but has the *sec18-1* phenotype at the nonpermissive temperature (Fig. 1, lanes h and i). Thus, *sec18-1* is epistatic to *arf1::URA3*. This result demonstrates that invertase must pass through the *SEC18*-dependent secretory pathway to achieve the level of glycosylation found in the *arf1::URA3* mutant and that the increased mobility of invertase is unlikely to be due to proteolysis.

The *arf1* null phenotypes described above exist in the presence of wild-type *ARF2*. To examine the phenotype of cells devoid of ARF protein, we used a strain that has the chromosomal *ARF1* and *ARF2* genes deleted but carries an episomal copy of *ARF1* under control of the inducible *GAL1* promoter (T.S., M. A. Hoyt, D.B., and R.A.K., unpublished

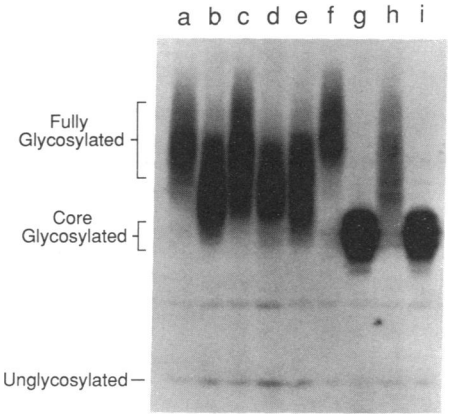

FIG. 1. Invertase produced in wild-type and mutant strains. Invertase synthesis was induced by incubation in low-glucose medium and detected with an anti-invertase antibody. Lanes: a, wild type at 30°C; b, *arf1::HIS3* at 30°C; c, *arf1::HIS3* (pARF1) at 30°C; d, *ypt1-1* at 30°C; e, *arf1::HIS3* at 14°C; f, *sec18-1* at 23°C; g, *sec18-1* at 35°C; h, *sec18-1 arf1::URA3* at 23°C; and i, *sec18-1 arf1::URA3* at 35°C.

work). This strain grows on medium containing galactose as the carbon source but ceases growth after a shift to glucose-containing medium. Cells of this mutant strain and of wild type were grown in galactose-containing medium, shifted to medium containing 5% glucose for 7 hr to deplete the cells of ARF protein, and then induced for invertase synthesis. Immunoblotting of lysates from such cells shows mostly the core-glycosylated form of invertase (data not shown), like that seen in *sec18-1* cells at the nonpermissive temperature.

To determine whether the defect in glycosylation seen in *arf1* null cells is accompanied by a defect in secretion of invertase to the cell surface, the internal and external levels of invertase were assayed. Fig. 2 shows that the kinetics of induction are similar in wild-type and *arf1::HIS3* cells of similar genetic background and that the amount of invertase secreted is approximately the same. The *arf1::HIS3* mutant does exhibit a small accumulation of internal invertase relative to wild type; after 90 min of induction, 26% of total invertase activity is internal in the mutant, whereas only 13% of total invertase is internal in wild-type cells. This partial defect is similar to that observed by Segev *et al.* (3) for the *ypt1-1* mutant, consistent with the glycosylation defect being very similar in these two mutants.

Genetic Interactions Between *arf1* and Known Secretion-Defective Mutants. The secretion defect of *arf1* mutant cells, by analogy with known mutants (23), suggests that ARF plays a role in protein transport from the ER to the Golgi apparatus, or within the Golgi apparatus. We performed genetic experiments with *arf1*, *ypt1-1*, and *sec* mutants to identify any relationships that might exist. As both YPT1 and ARF1 are low-molecular-weight GTP-binding proteins, we first investigated whether these two proteins are functionally related. First, *ypt1-1* and *arf1::HIS3* mutants were transformed with high- and low-copy number plasmid vectors containing either no insert, a *YPT1* insert, or an *ARF1* insert. In each case the mutant defect was complemented by the plasmid bearing a wild-type copy of the gene for which the strain was mutant but not by plasmids without an insert nor with an insert of the other gene. Thus, *YPT1* and *ARF1* cannot substitute for one another and are not functionally homologous.

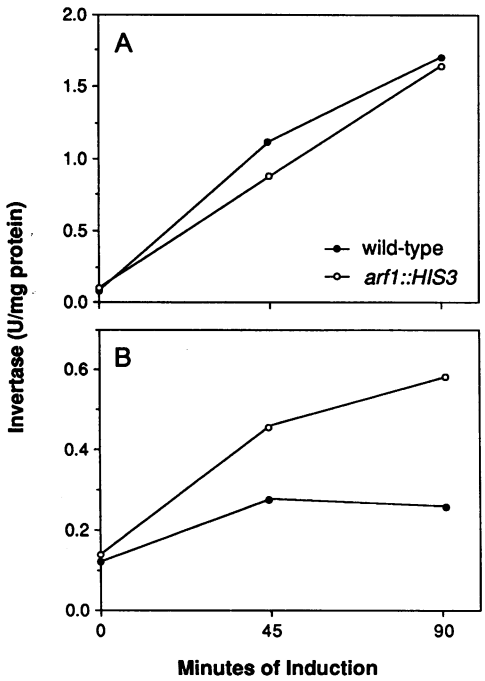

FIG. 2. External (*A*) and internal (*B*) levels of invertase in wild-type and *arf1::HIS3* cells. Invertase was assayed as described.

In the second experiment we attempted to construct an *arf1::URA3 ypt1-1* double mutant by crossing two single mutants and dissecting tetrads from the resulting diploid strain. In this cross there was a pattern of spore viability that suggested that the double mutant is inviable; all tetrads with four viable spores were parental ditype for the *ARF1* and *YPT1* genes, whereas in tetrads with less than four viable spores, the missing spore(s) could be inferred to be the double mutant (12 tetrads). We confirmed that the combination of an *arf1* null mutation with *ypt1-1* is a mitotic lethal by constructing a strain of the genotype *arf1::HIS3 ypt1-1* in the presence of a plasmid bearing the wild-type *YPT1* gene and the *URA3* gene as a genetic marker (13). Five double mutants were obtained in this way; each was Ura^+, indicating the presence of the *YPT1* plasmid. The double mutants require the *YPT1* plasmid for viability, as demonstrated by an inability to grow on medium containing 5-fluoroorotic acid, which selects against *URA3* function (12). Both *arf1::HIS3* and *ypt1-1* single-mutant segregants originally containing the plasmid can lose the plasmid and grow on the same medium.

We also tested whether any of the characterized yeast *sec* mutants display genetic interactions with *arf1*. Double mutants were constructed with *arf1::URA3* and *sec7-1*, *sec12-4*, *sec13-1*, *sec14-1*, *sec16-1*, *sec17-1*, *sec18-1*, *sec19-1*, *sec20-1*, *sec21-1*, *sec22-3*, *sec23-1* (24), as well as *bet1-1* and *bet2-1* (25). In most cases the double mutant is viable and has the phenotypes of the parental single mutants unchanged. For *sec21-1* and *bet2-1*, however, the double mutant with *arf1::URA3* is usually inviable; rare double mutants that are recovered grow extremely slowly at 26° and are unable to grow at higher temperatures at which the single mutants are viable. The *arf1::URA3 sec7-1* double mutant is viable but is similarly more temperature-sensitive than the *sec7-1* mutant.

Immunocytochemical Localization of ARF. As the phenotypes of yeast *arf* mutants suggest a role in protein secretion, we examined the localization of ARF protein in cells that have morphologically well-defined secretory structures. Immunofluorescence localization in NIH 3T3 cells by using affinity-purified anti-ARF antibodies (9) showed a pattern characteristic of stacked Golgi cisternae [Fig. 3*B*; (18)]; normal globulin controls showed no localization (Fig. 3*A*). Similar results were obtained in the human adrenal carcinoma cell line, SW13 (data not shown). By immunoelectron microscopy with the same antibodies, ARF staining in NIH 3T3 cells was confined to the cytoplasmic faces of Golgi stack membranes (Fig. 3 *C* and *D*). Very little staining was found in other parts of the cells, although quantitative determination was not done. The cis versus trans faces of the Golgi stacks could, in some cases, be discerned by the membrane interruptions induced in the cholesterol-rich trans membranes by the saponin incubations used in the localization procedure (19). In some cases, it appeared that the ARF staining was mainly on the cytoplasmic face of the cis elements, rather than the trans elements, although a clear distinction could not always be made, and some trans elements also showed small amounts of ARF staining.

DISCUSSION

We have used two different experimental approaches to begin defining ARF function *in vivo*: (*i*) Analysis of the phenotype of yeast *arf* mutants suggests that ARF is involved in transport of proteins to or within the Golgi apparatus. (*ii*) Immunolocalization of ARF protein in animal cells indicates that ARF is localized to the Golgi apparatus. Given the underlying consistency of these results, the sequence conservation seen among ARF genes from very different species probably reflects functional conservation and ARF involvement in protein secretion in all eukaryotes.

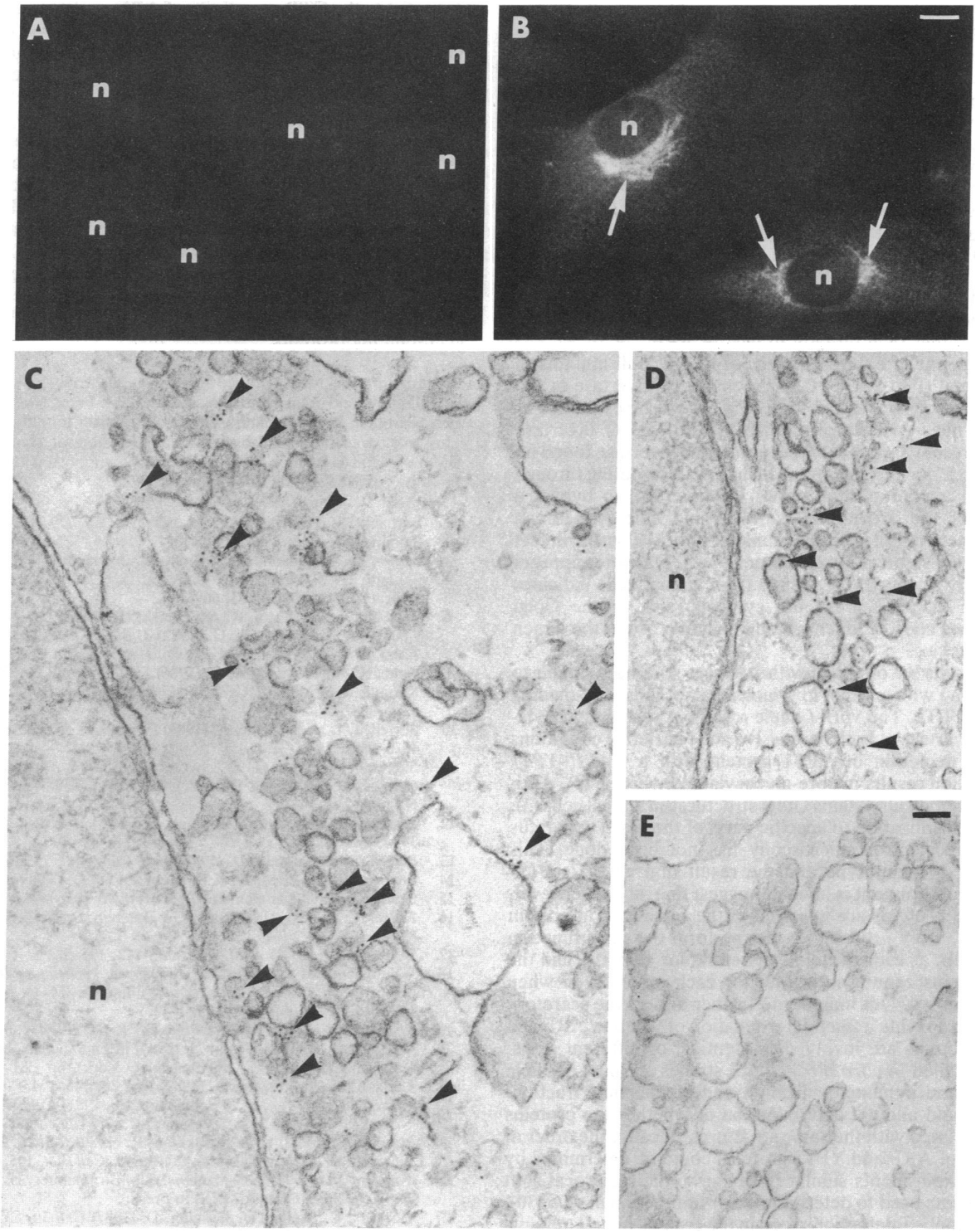

FIG. 3. NIH 3T3 cells were fixed and processed for immunofluorescence (*A* and *B*) or EM immunocytochemistry (*C–E*), as described by using rabbit anti-ARF serum (*B*, *C* and *D*) or normal rabbit globulin (*A* and *E*). (*B*) The perinuclear Golgi stack pattern seen with anti-ARF (arrows) is a pattern not seen in the normal globulin control (*A*). (*C* and *D*) Ferritin cores (arrowheads) marking the position of anti-ARF antibodies can be seen on the cytoplasmic face of Golgi membranes; ferritin is virtually absent from the same area of a cell from the normal globulin control sample (*E*). n, nucleus. (*A* and *B* ×700, Bar = 10 μm; *C* and *E* ×68,600, Bar = 0.1 μm.)

This role of ARF in secretion is in marked contrast to the previously defined role of ARF as cofactor in the *in vitro* cholera toxin-catalyzed ADP ribosylation of G_s, located virtually exclusively in the plasmalemma *in vivo* (for review, see ref. 26). Although we currently lack the information to fully reconcile these differing results, we believe that the results from *in vivo* experiments presented here reflect the fundamental role of ARF in cellular physiology. Our results do not rule out the possibility that ARF has more than one function. For example, while ARF is largely localized in Golgi stacks, it may also be present in other cellular compartments. Indeed, the presence of a soluble ARF protein in bovine tissues (9) is good evidence for other cellular locations and, possibly, functions for ARF in animal cells.

The yeast *ARF1* and *ARF2* genes produce nearly identical proteins that are functionally equivalent, but *ARF1* contrib-

utes ≈90% of the total ARF protein in wild-type cells (T.S., M. A. Hoyt, D.B., and R.A.K., unpublished work). It is worth noting then that the secretion phenotypes of the *arf1* null mutant, observed in the presence of a wild-type *ARF2* gene, represent the effect of a dramatic reduction in the level of ARF protein rather than the true ARF null phenotype. We attempted to determine the effect of a complete absence of ARF protein by examining cells depleted of ARF by growth for several generations under conditions in which ARF synthesis was repressed. Although the phenotype of these cells suggested that the true ARF null defect is more similar to that of the well-characterized ER to Golgi *sec* mutants, this type of experiment must be interpreted with caution. A potential problem is that the observed phenotype might be due to secondary effects of growth with diminishing amounts of ARF, rather than directly to lack of ARF.

The amount of external invertase in *arf1* null mutants is near normal (Fig. 2); yet the percentage of invertase in the incompletely glycosylated state (Fig. 1) can be estimated at >80%. Thus, this partially glycosylated form of invertase must represent a significant portion of the invertase found on the cell exterior. Explanations include (*i*) a rerouting through the Golgi apparatus that allows the invertase to bypass a portion of the processing pathway and emerge with an incomplete array of glycosidic linkages or (*ii*) a partial defect in the glycosylating enzymes themselves. The latter appears less likely, as the EM evidence from animal cells indicates that ARF is present on the cytosolic side of the Golgi membranes and the glycosylating enzymes are in the lumen of the stacks.

The results we obtained with ARF are strikingly similar to those found with another low-molecular-weight GTP-binding protein, YPT1. The *ypt1-1* allele results in partial glycosylation of invertase at both permissive and restrictive conditions (3), and depletion of YPT1 protein with a *GAL10-YPT1* construction results in core-glycosylated invertase (4). Anti-YPT1 antibody also stains Golgi structures in animal cells (3), although localization to specific parts of the Golgi apparatus by immunoelectron microscopy has not been done. This similarity of phenotype and the result that an *arf1::HIS3 ypt1-1* double mutant is inviable suggest that, at a crude level, these two proteins are involved in the same step in protein transport. They probably do not perform the same function in this step, as each protein is essential for viability, and the two proteins cannot substitute for each other, even when overexpressed. Our inability to further dissect the secretory pathway prevents a finer determination of whether ARF and YPT1 proteins are involved in identical or different steps. More detailed *in vitro* biochemical studies are now possible (27–29), and immunolocalization and biochemical fractionation should allow a determination of whether the proteins are associated with the same structures. Ideally, the order of function of ARF and YPT1 proteins could be determined by *in vitro* experiments similar in design to the reciprocal-shift experiments used to determine order of function *in vivo* (30).

The genetic interactions seen in the crosses of *arf1* mutants to secretion-defective mutants identify a small set of genes whose function is most affected by altering ARF level: *SEC21*, *BET2*, and *SEC7*. Although these results suggest some form of *in vivo* interaction between the gene products of these genes and ARF protein, no understanding of that interaction is revealed. Recently, Bacon *et al.* (31) have shown similar genetic interactions between *ypt1* mutants and a similar subset of secretion mutants. It would be interesting to know whether the gene products of *SEC21*, *BET2*, or *SEC7* can activate the GTPase activity of ARF or YPT1, analogous to the effect of GTPase activating protein on *ras* (32). We have detected an ARF GTPase activating protein activity in yeast cell extracts in an *in vitro* assay (unpublished observations), and extracts from mutant strains can be tested in this assay.

In summary, several characteristics of ARF make it a good candidate for a GTP-binding mediator of vectorial vesicle transport as has been discussed (3, 5): (*i*) ARF is localized to the secretion apparatus, (*ii*) ARF is present in both soluble and membrane-bound forms, and (*iii*) ARF requires a GTPase activating factor to detectably hydrolyze GTP. Although direct evidence to support such a role for ARF is lacking, the yeast studies define an excellent system in which to test hypotheses relating to the function of ARF and other small GTP-binding proteins.

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