Purification and Mass Spectrometric Analysis of ADP-Ribosylation Factor Proteins from *Xenopus* Egg Cytosol†

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Received January 30, 1996; Revised Manuscript Received April 15, 1996⊗

ABSTRACT: The GTP analog GTPγS potently inhibits nuclear envelope assembly in cell-free *Xenopus* egg extracts. GTPγS does not affect vesicle binding to chromatin but blocks vesicle fusion. Fusion inhibition by GTPγS is mediated by a soluble factor, initially named GSF (GTPγS-dependent soluble factor). We previously showed that vesicles pretreated with GTPγS plus recombinant mammalian ARF1 were inhibited for fusion, suggesting that "GSF activity" was due to the ARF (ADP-ribosylation factor) family of small GTP-binding proteins. To ask if any soluble proteins other than ARF also inhibited vesicle fusion in the pretreatment assay, we purified GSF activity from *Xenopus* egg cytosol. At all steps in the purification, fractions containing ARF, but no other fractions, showed GSF activity. The purified GSF was identified as *Xenopus* ARF by immunoblotting and peptide sequence analysis. Reverse phase HPLC and mass spectrometry revealed that GSF contained at least three distinct ARF proteins, all of which copurified through three chromatography steps. The most abundant isoform was identified as ARF1 (62% of the total GSF), because its experimentally determined mass of 20 791 Da matched within experimental error that predicted by the sequence of the *Xenopus* ARF1 cDNA, which is reported here. The second-most abundant isoform (25% of GSF activity) was identified as ARF3. We concluded that ARF is most likely the only soluble protein that inhibits nuclear vesicle fusion after pretreatment with GTPγS.

In higher eukaryotes, the nuclear envelope disassembles early in mitosis (Fry, 1976). After mitosis, the process of nuclear reformation involves the binding and fusion of nuclear-derived vesicles at the chromosome surface. Both nuclear vesicle binding and fusion can be assayed *in vitro* using *Xenopus* egg extracts [reviewed by Wiese and Wilson (1993)]. Previous studies showed that, in the presence of a soluble activity, designated GSF[1] (GTP-dependent soluble factor), the slowly hydrolyzable analog of GTP, GTPγS, blocks nuclear vesicle fusion (Boman *et al*., 1992a). GSF activity was defined by a pretreatment assay; vesicles pretreated with GTPγS and cytosol (the source of GSF) were subsequently inhibited for fusion when tested in assembly reactions that did not contain GTPγS. By several criteria, the characteristics of GSF were similar to those of the ARF family of small GTPases.

The ARFs are a conserved family of small (20−21 kDa) GTP-binding proteins that interact reversibly with membranes; ARF·GDP is primarily soluble, whereas activated ARF·GTP binds tightly to membranes (Franco *et al.*, 1993; Boman & Kahn, 1995). The ARF protein family is proposed to regulate the coating and uncoating of transport vesicles in the secretory and endocytic pathways (Rothman, 1994). When ARFs bind GTPγS *in vitro*, they become activated irreversibly, inhibiting ER-to-Golgi and intra-Golgi transport, as well as homotypic endosome fusion (Balch *et al*., 1992; Taylor *et al.*, 1992; Kahn *et al*., 1992; Lenhard *et al.*, 1992). Transport inhibition by ARF·GTPγS correlates with the accumulation of ARFs and COP-coated vesicles on ER and Golgi membranes (Serafini *et al.*, 1991). Conversely, depletion of ARFs or treatment with brefeldin A, which blocks ARF activation, prevents both COP binding (Donaldson *et al*., 1991) and the subsequent formation of Golgi vesicles (Taylor *et al*., 1994). These data led to the proposal that ARF is itself a coat component (Serafini *et al*., 1991), but the mechanism by which ARF regulates coating is not understood. Interestingly, ARF was recently shown to stimulate the activity of phospholipase D, which hydrolyzes phosphatidylcholine to form choline and phosphatidic acid (Brown *et al*., 1993; Cockcroft *et al*., 1994). In addition, guanine nucleotide binding and dissociation rates for ARF are sensitive to specific phospholipids, such as phosphatidylinositol 4,5-bisphosphate (PIP_2; Terui *et al.*, 1994). These data suggest that the lipid composition of membranes is important for ARF action and that ARF may in turn manipulate that lipid environment. The relationship, if any, between ARF's effect on membrane lipids and its coat-recruiting activities has not yet been determined.

† Both laboratories contributed equally to this work, which was supported by American Cancer Society Grant CB-33B (K.L.W.), NIH Grant GM48646 (K.L.W.), and NIH Grant GM43378 (P.M.).

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⊗ Abstract published in *Advance ACS Abstracts,* June 1, 1996.

[1] Abbreviations: ARF, ADP-ribosylation factor; GSF, GTP-dependent soluble factor; GGBF, GTPγS-dependent Golgi binding factor; LC/MS, liquid chromatography-coupled mass spectrometry; DTT, dithiothreitol.

S0006-2960(96)00220-6 CCC: $12.00

In mammals, ARF proteins are encoded by at least six genes, ARF1−6 [reviewed by Boman and Kahn (1995)]. The human ARF1 and ARF3 proteins are 97% identical, and all ARF proteins from yeast to plants to mammals are >65% identical. This high degree of evolutionary conservation, and the fact that many cells express at least five different ARF genes, suggests that each ARF protein may have a distinct function. Consistent with this hypothesis, ARF6 associates preferentially with the plasma membrane and endosomes in living cells, whereas ARF1 appears to associate with ER and Golgi membranes, as determined by the overexpression of wild-type and dominant activating alleles *in vivo* (Zhang *et al.*, 1994; D'Souza *et al.*, 1995; Peters *et al.*, 1995). ARF3 and ARF4 also interact with ER and Golgi membranes. Except for differential binding of ARF1, ARF3, and ARF5 to Golgi membranes (Tsai *et al.*, 1992), it has not yet been possible to functionally distinguish between the ARF1, ARF3, ARF4, and ARF5 isoforms. For example, ARF1·GTPγS inhibits all *in vitro* vesicular transport assays tested thus far (Taylor *et al.*, 1992; Kahn *et al.*, 1992; Lenhard *et al.*, 1992).

The ARF proteins that inhibit Golgi transport *in vitro* (Melançon *et al.*, 1987) were purified from bovine brain cytosol as GTPγS-dependent Golgi binding factors (GGBFs; Taylor *et al.*, 1992). Two distinct peaks of activity, designated GGBF and GGBF* (higher and lower specific activity, respectively), were separated by hydrophobic interaction chromatography and later identified as ARF1 and ARF3 (Berger *et al.*, 1995). We showed that purified mammalian ARF1 binds to *Xenopus* nuclear vesicles and inhibits their fusion in the presence of GTPγS (Boman *et al.*, 1992b). Mammalian ARF3 also inhibited fusion but was less active. From these results, we hypothesized that GSF activity was due to endogenous *Xenopus* ARF protein(s). To test this hypothesis, and to ask if there were additional GTPγS-dependent soluble inhibitory factors, we purified GSF activity from *Xenopus* egg cytosol. A single peak of GSF activity was detected at each step in the purification, and the purified proteins were identified as ARFs by immunoblotting, peptide sequence analysis, HPLC, and mass spectrometry. We determined the complete cDNA sequence of *Xenopus* ARF1. By comparing the predicted mass of the ARF1 protein with our experimentally determined masses, we demonstrate that ARF1 is the most abundant isoform in purified GSF.

MATERIALS AND METHODS

Xenopus Egg Cytosol. Xenopus laevis egg extracts and demembranated sperm chromatin were prepared as described (Newmeyer & Wilson, 1991), except that the ATP regenerating system was not added to cytosol used to purify GSF. The protein concentration of the egg cytosol fraction was ~20 mg/mL, as determined using the Bradford protein assay with bovine serum albumin as a standard.

GSF Assay. GSF is defined as a soluble factor that binds to nuclear vesicles in the presence of GTPγS and inhibits vesicle fusion (Boman *et al.*, 1992a). All column fractions (except those from the Superdex75 column, which were already compatible) were dialyzed against buffer [85 mM sucrose, 50 mM KCl, 50 mM HEPES (pH 8), 2.5 mM magnesium acetate, and 1 mM DTT] before being assayed for GSF and GGBF activity. To assay GSF activity, column fractions (10−60 μL) were preincubated for 30 min with 2 μL of *Xenopus* membranes (protein concentration, ~25 mg/mL) and 0.1−1 mM GTPγS (lower concentrations of GTPγS were effective after the desalting step and anion exchange chromatography). The membranes were then pelleted by centrifugation at 30000*g* (Beckman TLA-100.2 rotor, 30 krpm, 15 min, 4 °C) and resuspended to their starting volume. The pretreated membranes were mixed with 20 μL of untreated cytosol containing 40 000 demembranated sperm pronuclei ("chromatin"; the substrate for nuclear vesicle binding; Newmeyer & Wilson, 1991), and reaction mixtures were incubated at 22−24 °C. The fusion of nuclear vesicles to form an enclosed nuclear envelope was assayed by light microscopy after 2 h as described (Boman *et al.*, 1992a). The rate of nuclear envelope growth is directly proportional to the rate of vesicle fusion. Fusion inhibition (GSF activity) was quantitated by calculating the surface area of at least 10 nuclei in each sample at the 2 h time point. For each set of assays, 2 μL of membranes was preincubated with 20 μL of *Xenopus* cytosol in the presence (positive control) or absence (negative control) of GTPγS. GSF activity is graphed as a downward peak to indicate fusion inhibition.

GGBF Assay. Column fractions were dialyzed before assaying GGBF activity, as described above. GGBF activity was assayed as the GTPγS-dependent inhibition of a well-characterized cell-free assay that reconstitutes intra-Golgi transport using mammalian cell extracts. Each column fraction was assayed in duplicate at a single time point in the absence or presence of 4 μM GTPγS, as described (Taylor *et al.*, 1992). Percent inhibition by GTPγS (GGBF activity) was calculated by taking the difference between transport measured in the absence and presence of GTPγS and dividing this difference by transport measured in the absence of GTPγS and finally multiplying by 100%. GGBF is graphed as an upward peak.

ARF Purification from Xenopus Cytosol. We used the purification protocol of Taylor *et al.* (1992) with minor changes. Three independent GSF preparations were done, with identical results. The results reported here are from one representative purification. All chromatography steps were performed at 4 °C on a Pharmacia FPLC system. *Xenopus* egg cytosol (50 mL, 1 g) was desalted using a Biogel P6-DG column (225 mL, 2.5 cm) equilibrated with desalting buffer [10 mM Tris (pH 8.0), 40 mM KCl, 1 mM $MgCl_2$, and 1 mM DTT]. Effluent was monitored at 280 nm. Protein-containing fractions were pooled and diluted with desalting buffer lacking KCl to achieve a conductivity equal to that of a 20 mM KCl standard.

The desalted cytosol was subjected to anion exchange chromatography on a column of Q Sepharose Fast Flow (85 mL, Pharmacia) equilibrated with a buffer lacking KCl [10 mM Tris (pH 8.0), 1 mM $MgCl_2$, and 1 mM DTT]. Bound proteins were eluted with a 450 mL linear gradient of KCl (0 to 0.5 M). Eluate was collected in 14 mL fractions; fractions 10−14 (KCl concentration ≈ 80−150 mM) contained activity and were pooled.

The Q Sepharose pool was concentrated to ~7.5 mL by ultrafiltration and adjusted to an $(NH_4)_2SO_4$ concentration of 1.1 M, and particulates were removed by filtration through a 0.22 μm filter before loading onto a hydrophobic interaction phenyl-Superose column (HR5/5, Pharmacia) equilibrated with 50 mM potassium phosphate (pH 7.2), 1.1 M $(NH_4)_2SO_4$, and 1 mM DTT. Protein was eluted with a linear

gradient of 1.1 to 0 M $(NH_4)_2SO_4$ and collected in 1.5 mL fractions. The elution buffer lacking $(NH_4)_2SO_4$ contained 1 mM $MgCl_2$. Fractions 9−13[$(NH_4)_2SO_4$ concentration ≈ 0.8−0.6 M] contained activity and were pooled.

The phenyl-Superose pool was concentrated to 1 mL by ultrafiltration and loaded onto a Superdex75 size exclusion column (10/30, Pharmacia) equilibrated with buffer containing 10 mM Tris (pH 7.9), 50 mM KCl, 1 mM $MgCl_2$, 1 mM DTT, and 10% w/v glycerol. The column was calibrated using albumin (68 kDa) and chymotrypsinogen (25 kDa) standards. A flow rate of 0.3 mL/min was used; the initial 4 mL of effluent was discarded, and fractions of 0.6 mL were collected thereafter. GSF and GGBF activities were detected in fractions 15−17, which corresponded to a protein peak at <25 kDa. The protein concentration in fractions 15 and 16 was ∼80 μg/mL, as measured using the BioRad protein assay with γ-globulin standards.

GTP Ligand Blots. Total proteins from each fraction were separated on 15% gels by SDS−PAGE and transferred to nitrocellulose filters. Filters were blocked, incubated with 2 μCi/mL [α-^{32}P]GTP for 50 min, and washed as described (Taylor *et al.*, 1992). Filters were air-dried and placed on a PhosphorImager screen (Molecular Dynamics). Radioactive bands at 20 kDa were quantitated using ImageQuant (Molecular Dynamics). All steps were performed at 22−24 °C.

Immunoblotting and Coomassie-Stained Gel. Proteins were separated on 12% gels by SDS−PAGE. Two gels were loaded with identical samples and run in parallel. One gel was stained using 0.05% Coomassie Brilliant Blue G and destained in 25% methanol and 7.5% acetic acid. Proteins from the second gel were transferred to a nitrocellulose filter and probed with monoclonal anti-ARF antibody 1D9 (a gift of Dr. R. Kahn; Cavenagh *et al.*, unpublished experiments). 1D9 was raised against human ARF1 and recognizes human ARFs 1, 3, 5, and 6 but not human ARF4 or any other small GTPases including the Arl (ARF-like) proteins. 1D9 recognizes ARF proteins of many species. Although the full range of its isoform recognition in other species is not known, 1D9 has similar affinities for the human and *Xenopus* ARF1. We used 1D9 to detect ARF proteins in *Xenopus* cytosol and to assay purified recombinant *Xenopus* ARF1. Bound antibodies were visualized using alkaline phosphatase-linked secondary antibodies. Isoform-specific antibodies, also obtained from Dr. R. Kahn, were used to probe fractions 16 and 17, which were resolved by 10 to 20% gradient SDS-PAGE and detected on blots by chemiluminescence.

HPLC Analysis of Purified GSF. Superdex75 fractions 15 and 16 were pooled and analyzed using a Rainin HPXL system. Samples were injected onto a Vydac narrow bore C_{18} column (2.1 × 250 mm) equilibrated in buffer A (6 mM HCl) plus 45% buffer B (90% acetonitrile in 6 mM HCl) and eluted over a 45% to 75% buffer B gradient (1% B/min) at a flow rate of 0.3 mL/min. Protein in the eluate was detected at 215 nm using a Dynamax UV-D detector (Rainin), and the resulting chromatogram was analyzed using the Dynamax v1.3.1 software package (Rainin).

Peptide Sequence Analysis. One-quarter (∼20 μg) of a single GSF preparation (Superdex75 fractions 15−17) was precipitated in 66% acetone using 150 μg of cytochrome *c* as a carrier. Proteins were resolved on a 7 to 15% gradient gel by SDS−PAGE and then electrophoretically transferred to nitrocellulose in low-SDS transfer buffer [0.01% SDS, 20 mM Tris (pH 8.5), 150 mM glycine, and 20% methanol]. The filter was stained with Ponceau S, and the GSF band was cut out and frozen in deionized water at −20 °C. Complete tryptic digests, HPLC separation, and peptide sequence analysis were done by Dr. William Lane (Chemical Laboratory, Harvard University, Cambridge, MA). Four peptide peaks were separable from background peaks; all four were retrieved and sequenced.

Mass Spectrometric Analysis. Mass determinations were carried out on an API-III triple quadrupole mass spectrometer (Sciex, Toronto, Canada). Unless noted, conditions were as described previously for electrospray analysis of bovine ARFs (Berger *et al.*, 1995). To separate proteins immediately before subjecting them to mass spectrometry, we directly coupled a liquid chromatograph (Model 140A, Applied Biosystems/Bodman, Ashton, PA) to the mass spectrometer. Liquid chromatography-coupled mass spectrometry (LC/MS) analysis of intact proteins was done using a 250 μm × 20 cm capillary column packed with POROS 120R resin (PerSeptive Systems, Cambridge, MA) equilibrated with buffer C (0.1% formic acid). Proteins were eluted by a 0 to 100% buffer D (80% acetonitrile and 0.1% formic acid) gradient (1.7% D/min) at a flow rate of 200 μL/min. A preinjector split was used to direct between 4 and 8 μL/min through the injector and column, directly onto the mass spectrometer.

The mass spectra of purified GSF (2 pmol/μL, 20−80 pmol/run) were acquired at unit resolution, scanning from 600 to 1600 Da/e with a dwell time of 0.55 ms and a 0.1 Da/e step size (6.0 s/scan) with an orifice voltage of 85−90 V. Data were further analyzed using a deconvolution algorithm that converts the families of ion peaks arising from each protein to a single peak that corresponds to the neutral species; the height of this single peak is a measure of the ion current associated with that protein. The relative abundance of the three main ARF species was then determined from their peak heights, using the sum of the three peak heights as a measure of total GSF signal. Programs provided with the Sciex instrument (Tune 2.4, MacSpec 3.2, Bio-MultiView 1.0b1.2, and MacBioSpec 1.0.1) were used for data collection and analysis.

Cloning of Xenopus ARF1. A *Xenopus* stage I/II oocyte cDNA library packaged in λgt10 (the kind gift of Dr. J. Battey, NIDCD, NIH, Bethesda, MD) was screened by low-stringency hybridization to a probe that contained the entire coding region of human ARF1. Hybridizations were done at 22−24 °C in a solution containing 40% formamide and 4x SSC. cDNAs that tested positive through three rounds of screening were sequenced manually using the femtomole DNA Sequencing System (Promega). The GenBank accession number for *Xenopus* ARF1 is U31350.

RESULTS

GSF activity was defined by a pretreatment assay in which *Xenopus* egg membranes were pre-exposed to cytosol plus GTPγS and then pelleted and assayed for nuclear envelope assembly in the presence of untreated cytosol plus chromatin (Boman *et al*., 1992a). *Xenopus* egg cytosol contains ARF immunoreactivity and the factors responsible for inhibiting nuclear envelope assembly in the presence of GTPγS (GSF activity). Our goal in purifying GSF activity from *Xenopus* cytosol was to determine if GSF activity is restricted to *Xenopus* ARF proteins, by asking if there are other cytosolic

factors with GSF activity. Our GSF purification protocol was modeled after that used to purify GGBF activity (inhibition of mammalian intra-Golgi transport in the presence of GTPγS) from bovine brain extracts (Taylor *et al.*, 1992), because GSF and GGBF exhibited similar physical and biochemical characteristics. Because *Xenopus* cytosol also contains GGBF activity (data not shown), we assayed both GSF and GGBF activities, as well as GTP binding to 20 kDa proteins (by [α-^{32}P]GTP ligand blots), during purification.

GSF Purification. GSF activity was purified using four chromatography steps: desalting, anion exchange, hydrophobic interaction, and size exclusion. For clarity, the inhibitory GSF activity is graphed as a downward peak to indicate fusion inhibition, whereas GGBF activity and 20 kDa GTP-binding proteins are graphed as upward peaks. Fractions that contained activity were pooled and loaded on the subsequent column. The results reported here are from a typical purification; the purification was performed a total of three times with identical results.

Desalting the cytosol had no effect on GSF activity; this was expected, since dialysis of the cytosol also had no effect on GSF activity (data not shown). A single peak of GSF activity was eluted from ion exchange chromatography; moreover, all three activities (GSF, GGBF, and GTP binding by 20 kDa proteins) coeluted in this single peak (Figure 1A). The coincidence of the *Xenopus* GGBF activity and 20 kDa GTP-binding proteins differs markedly from this step of GGBF purification from bovine brain cytosol (Taylor *et al.*, 1992), in which the peak of GGBF activity is offset from the majority of 20 kDa GTPases.

The Q Sepharose fractions (10−14) that contained activity were processed as described in Materials and Methods and loaded onto the phenyl-Superose column. GSF activity again eluted in a single peak that also contained GGBF activity and the 20 kDa GTP-binding proteins (Figure 1B). This single peak of *Xenopus* GSF/GGBF activity is in sharp contrast to the clear separation of GGBF activity into two distinct peaks (corresponding to ARF1 and ARF3) during this step in purification from bovine brain extracts (Taylor *et al.*, 1992). Fractions 9−13, which contained activity, were pooled and separated by size. The Superdex75 size exclusion column yielded again a distinct peak of 20 kDa proteins (fractions 15−17) that contained both activities (Figure 1C), with fraction 16 being the most active in all assays. *Xenopus* GSF remained active through multiple freeze-thaw cycles, exposure to 1.1 M ammonium sulfate, and prolonged storage at 4 °C.

The purification of *Xenopus* GSF/ARF is summarized in Table 1. The pooled GSF fractions from each purification step were again assayed for GSF activity to determine the amount of protein required for half-maximal inhibition of fusion (IC_{50}). By comparing the IC_{50} values (per milligram) of each purified pool to that of desalted cytosol, we estimate an overall 64-fold purification, with a recovery of 1.7% of the starting GSF activity. Most of the loss of activity occurred during the phenyl-Superose step. Such loss could indicate the separation of ARF from an additional factor that is not essential, but which stimulates ARFs activity (see Discussion). From our calculations, we estimate that GSF/ARF proteins comprise approximately 0.5% of the total soluble protein in egg cytosol. This is comparable to other eukaryotic cells; ARF represents ~1% of bovine brain cytosol (Sewell & Kahn, 1988) and 0.1−1% of human tissues (R. Kahn, personal communication).

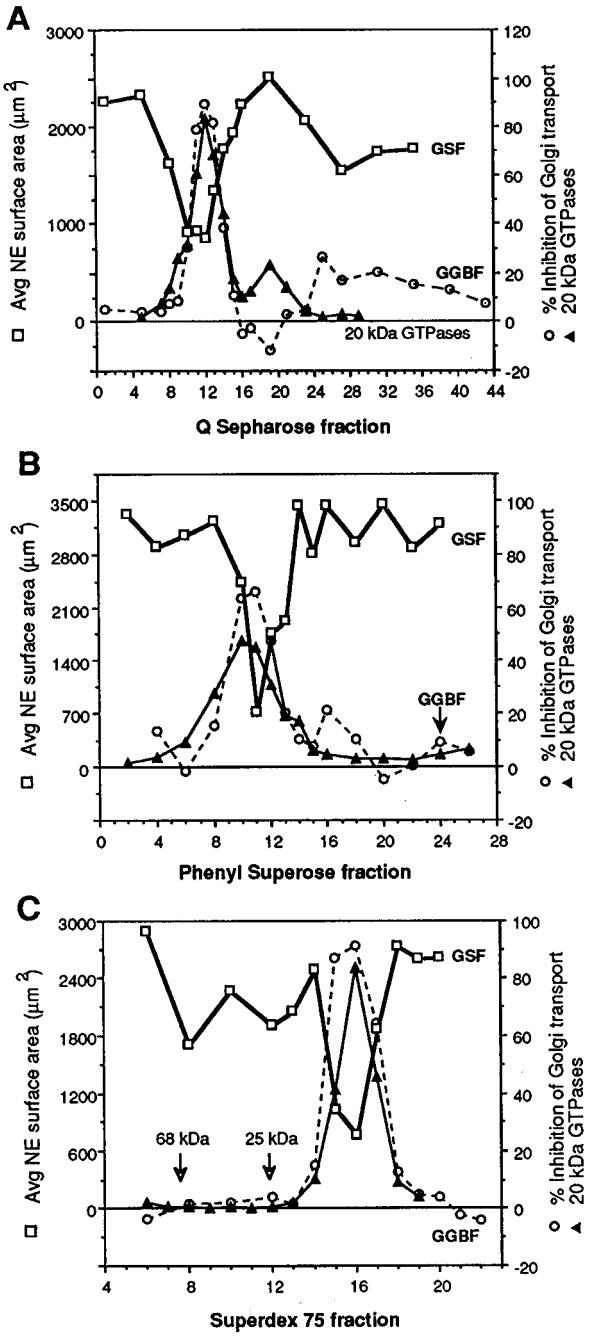

FIGURE 1: Purification of GSF activity. FPLC column fractions were assayed for three parameters: GSF activity *in vitro*, GGBF activity *in vitro*, and 20 kDa GTP-binding proteins on [α-^{32}P]GTP ligand blots. The inhibitory GSF activity is shown as a downward peak, whereas GGBF activity and 20 kDa GTPases are shown as upward peaks; all three activities cofractionated throughout purification. (A) Anion exchange chromatography (Q Sepharose Fast Flow). Fractions 10−14 contained activity. (B) Hydrophobic interaction chromatography (phenyl-Superose). Fractions 9−13 contained activity. (C) Size exclusion (Superdex75). The elution times of molecular mass standards, albumen (68 kDa) and chymotrypsinogen (25 kDa), are shown. Fractions 15−17 contained the GSF and GGBF activity peaks and the peak of 20 kDa GTP-binding proteins.

The purified GSF fractions from size exclusion chromatography contained one major Coomassie-staining band of 20 kDa (Figure 2A). To determine if purified GSF coeluted with ARF immunoreactivity, Superdex75 fractions 13−19 were immunoblotted with ARF monoclonal antibody 1D9

Table 1: Purification of *Xenopus* ARF/GSF

pooled fraction	volume (mL)	yield (mg)	IC_{50} (μg)[a]	recovery (%)[b]
desalting	66.0	990	270	100
Q Sepharose	66.0	92	35	72
phenyl-Superose	7.5	7.1	17	11
Superdex75	1.8	0.13	2.1	1.7

[a] IC_{50} is defined as the amount of each sample required during membrane pretreatment with GTPγS to cause half-maximal inhibition of nuclear vesicle fusion. [b] Percent recovery is defined as $[(IC_{50}/\text{total protein})_{\text{desalted cytosol}}/(IC_{50}/\text{total protein})_{\text{pooled fraction}}] \times 100$.

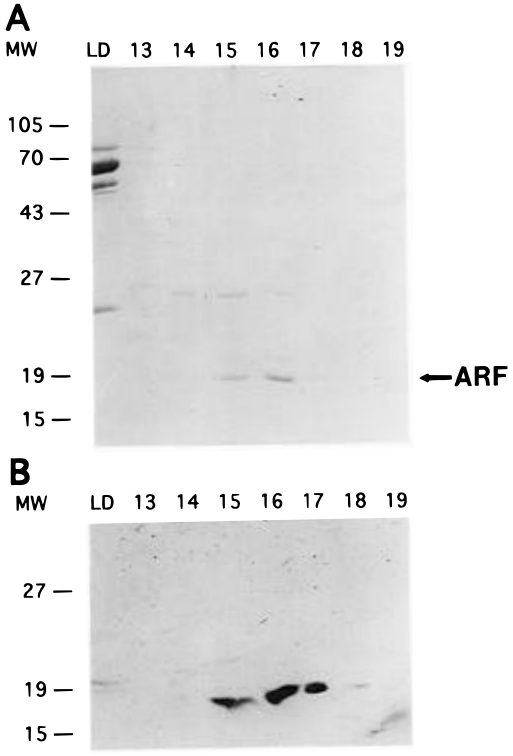

FIGURE 2: Purity of Superdex75 active fractions. (A) Total proteins from Superdex75 fractions 13−19, resolved by 12% SDS−PAGE and stained with Coomassie Brilliant Blue. (B) Duplicate gel transferred to nitrocellulose and probed with monoclonal antibody 1D9, which recognizes multiple human ARF isoforms (see Materials and Methods). The major component of fractions 15−17 is a 20 kDa protein that is recognized by monoclonal antibody 1D9 and correlates with the peak of GSF activity. One contaminating band (27 kDa) is present primarily in fraction 15 and does not bind GTP or correlate with GSF activity. Silver staining by three different methods revealed no additional contaminating bands; a silver-stained gel was not shown because ARF does not stain well with silver: MW, molecular mass markers; LD, Superdex75 load (10 μL = 95 μg); and 13−19, Superdex75 fractions 13−19 (8 μL each).

(Figure 2B), which recognizes human ARFs 1, 3, 5, and 6, as well as ARF homologs in many species (see Materials and Methods). The 20 kDa band in purified GSF was clearly recognized by this antibody. The amount of this 20 kDa ARF protein peaked in fraction 16, as did the level of GSF activity. In contrast, a minor band of ~27 kDa did not correlate with the peak of GSF activity (Figure 1C), did not immunoreact with 1D9 (Figure 2B), did not bind GTP on [α-^{32}P]GTP ligand blots (data not shown), and therefore cannot be responsible for GSF activity. We concluded that GSF activity, the cytosol- and GTPγS-dependent inhibition of nuclear vesicle fusion, is due to endogenous *Xenopus* ARF protein(s). Moreover, the observation of a single peak of GSF activity throughout the purification implied that ARF

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GSF
hARF1  MGNIFANLFKGLFGKKEMRILMVGLDAAGKTTILYKLKLGEIVTTIPTIG  50
bARF2  MGNVFEKLFKSLFGKKEMRILMVGLDAAGKTTILYKLKLGEIVTTIPTIG  50
hARF3  MGNIFGNLLKSLIGKKEMRILMVGLDAAGKTTILYKLKLGEIVTTIPTIG  50
hARF4  MGLTISSLFSRLFGKKQMRILMVGLDAAGKTTILYKLKLGEIVTTIPTIG  50

                ————1————        ——————2——————
GSF             NISFTVWDVGGQDK       hyfqn-qglifvv-sn
hARF1  FNVETVEYKNISFTVWDVGGQDKIRPLWRHYFQNTQGLIFVVDSNDRERV 100
bARF2  FNVETVEYKNISFTVWDVGGQDKIRPLWRHYFQNTQGLIFVVDSNDRERV 100
hARF3  FNVETVEYKNISFTVWDVGGQDKIRPLWRHYFQNTQGLIFVVDSNDRERV 100
hARF4  FNVETVEYKNICFTVWDVGGQDRIRPLWKHYFQNTQGLIFVVDSNDRERI 100
                *       *

                ——3——|——4——
GSF             MLAEDELRDAVLLVFANK
hARF1  NEAREELMRMLAEDELRDAVLLVFANKQDLPNAMNAAEITDKLGLHSLRH 150
bARF2  NEAREELTRMLAEDELRDAVLLVFAVKQDLPNAMNAAEITDKLGLHSLRH 150
hARF3  NEAREELMRMLAEDELRDAVLLVFANKQDLPNAMNAAEITDKLGLHSLRH 150
hARF4  QEVADELQKMLLVDELRDAVLLLFANKQDLPNAMAISEMTDKLGLQSLRN 150
                **        *  *

GSF
hARF1  RNWYIQATCATSGDGLYEGLDWLSNQLRNQK 181
bARF2  RNWYIQATCATSGDGLYEGLDWLSNQLKNQK 181
hARF3  RNWYIQATCATSGDGLYEGLDWLANQLKNKK 181
hARF4  RTWYVQATCATQGTGLYEGLDWLSNEL SKR 180
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FIGURE 3: Amino acid sequences of tryptic peptides. The entire amino acid sequences of human ARF1, ARF3, and ARF4 and bovine ARF2 are shown (Sewell & Kahn, 1988; Bobak *et al.*, 1989). All four peptides obtained from gel-purified, trypsin-digested *Xenopus* GSF/ARF are positioned above the mammalian ARFs. The complete sequence could not be determined for peptide 2 (lowercase); unknown positions are designated by hyphens. The GSF peptides are identical to those of human ARF1 and ARF3 but clearly distinct from those of ARF2 and ARF4 at positions indicated by asterisks. Human ARF5 and ARF6 differ from GSF to a greater extent and are not shown. Mammalian ARF1 and ARF3 differ from each other only near their N- and C-termini.

may be the only soluble protein that can inhibit nuclear vesicle fusion under these conditions (see Discussion).

Purified GSF Contains at Least Three ARF Proteins. The 20 kDa protein(s) in Superdex fractions 15−17 was conclusively identified as ARF(s) by peptide sequence analysis. Four tryptic peptides from the purified GSF were isolated and sequenced (no additional peptide peaks were resolved in quantity sufficient for sequencing). All four *Xenopus* peptides were identical to the corresponding regions of human ARF1 and ARF3 (Figure 3) and clearly different from bovine ARF2 and human ARF4 (Figure 3) and human ARF5 and ARF6 (data not shown). Only N- and C-terminal peptides would have allowed us to distinguish between ARF1 and ARF3, since these proteins are 97% identical at the amino acid level and differ only at their N- and C-termini. Unfortunately, these peptides were not recovered, perhaps due to myristoylation in the case of the N-terminal peptide. The four sequenced peptides nevertheless allowed us to conclude that ARF1, ARF3, or both were the major isoforms present in *Xenopus* egg cytosol.

To further characterize ARF species present in the GSF fraction, and determine their relative abundance, we analyzed the purified GSF using HPLC and mass spectrometry. Reverse phase HPLC analysis showed that purified GSF fractions contained at least three ARF proteins (Figure 4): one abundant species (63% of the total) and two minor species (24 and 13% of the total). These three peaks eluted at apparent acetonitrile concentrations of 57.2, 57.9, and 58.8%, consistent with ARF proteins from other species (Taylor *et al.*, 1992; see Figure 4). In contrast to bovine ARF1 and ARF3, which are well-resolved by this method (see arrows in Figure 4), the most abundant isoforms of *Xenopus* ARFs nearly coeluted.

Liquid chromatography-coupled mass spectrometry (LC/MS) analysis of purified GSF also revealed three distinct species (Figure 5). Figure 5A shows a representative mass

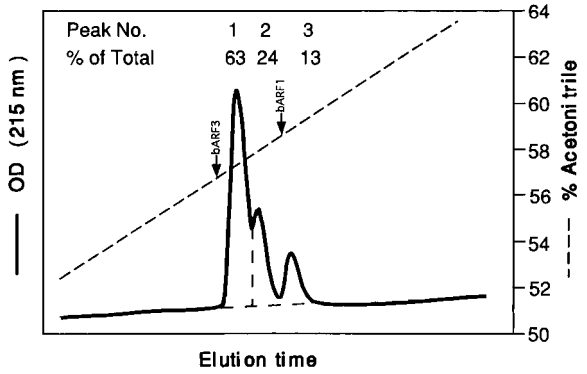

FIGURE 4: Reverse phase HPLC analysis of purified GSF reveals three proteins with elution properties characteristic of mammalian ARFs. Purified GSF (4 μg of total protein) was applied to an analytical reverse phase HPLC column and eluted with a linear gradient of acetonitrile. The region of the chromatogram from 50 to 64% acetonitrile is shown. Three peaks eluted at apparent acetonitrile concentrations of 57.2, 57.9, and 58.8% and respectively represent 63, 24, and 13% of the total absorbance in this region of the gradient. The data shown are representative of analysis carried out in duplicate and are similar to results obtained with a second GSF preparation.

spectrum on which various charged forms of the major ARF species are labeled with their corresponding mass/charge (*m*/*z*) values. Closer examination indicated the presence of two additional species of lower mass and abundance (Figure 5B). Deconvolution of the spectrum in Figure 5A confirms the presence of three species with masses as indicated (Figure 5C). The averages of four such independent mass determinations are listed in Table 2. Strikingly, the relative amounts of the three species were essentially identical to those obtained by HPLC analysis. A fourth minor protein with a mass of 20 678 Da, which comprised less than 2% of the total GSF signal, was observed in three out of four LC/MS runs.

The Predominant Isoform in Xenopus GSF Is ARF1. To determine which isoform, ARF1 or ARF3, was the most abundant in purified GSF, we determined the complete cDNA sequence of *Xenopus* ARF1 (Figure 6; see Materials and Methods). *Xenopus* ARF1 is highly homologous to human ARF1; the nucleotide sequences are 82% identical in the coding region, and the predicted *Xenopus* protein (181 amino acids; Figure 6) differs at a single residue (methionine replacing isoleucine at position 4). This remarkable evolutionary conservation between the human and amphibian proteins reinforces the idea that ARF1 plays a crucial role in cells.

The correspondence of the predicted mass of *Xenopus* ARF1 (20 795 Da, assuming cleavage of the initiating methionine and subsequent N-myristoylation) with the observed mass of the major ARF species (20 791 Da) provides definitive identification of this species as *Xenopus* ARF1. A similar slight mass discrepancy (4 Da) was observed during mass spectrometric analysis of bovine brain ARFs and thus appears to be characteristic of ARFs.[2]

[2] Previous mass spectrometric analysis of the N-terminal peptide of bovine ARF1 and ARF3 established their uniform modification with a single $C_{14:0}$ fatty acid (Berger *et al.*, 1995). The 4 Da discrepancy between the predicted and measured masses of intact *Xenopus* and bovine ARFs therefore does not result from acylation with unsaturated acyl chains as described for other proteins [e.g., see Neubert *et al.* (1992)].

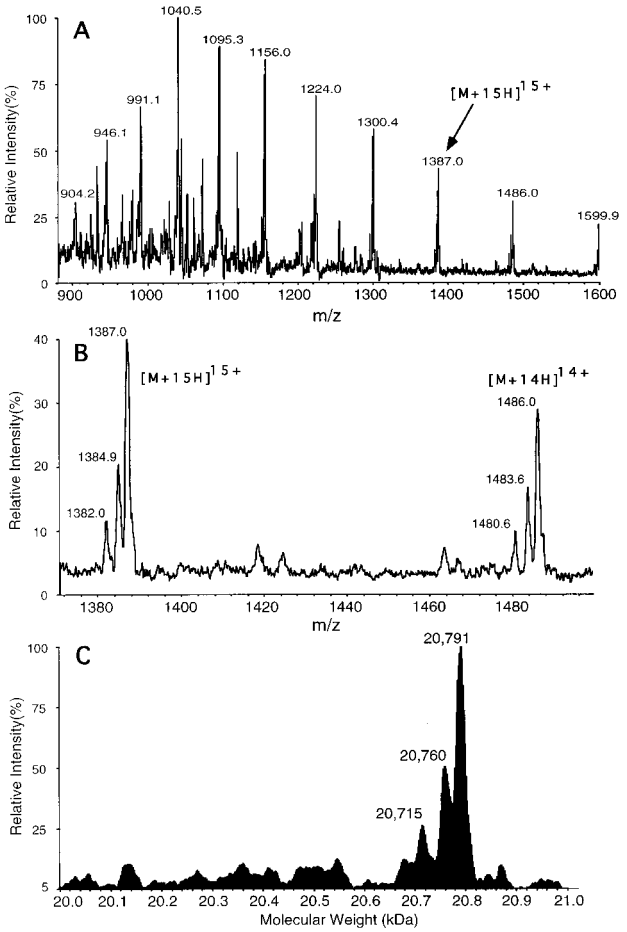

FIGURE 5: Electrospray MS analysis of purified GSF reveals three proteins with masses characteristic of mammalian ARFs. Purified GSF (3.2 μg) was analyzed by LC/MS as described in Materials and Methods. (A) A representative mass spectrum of GSF obtained by summation of individual spectra collected during elution of the protein. The +15 charged form of GSF is indicated. (B) A region from 1370 to 1500 *m*/*z* of the spectrum in panel A was expanded to reveal the presence of multiple species in the +14 and +15 charged forms of GSF. (C) The spectrum in panel A was processed by the hypermass module of the MassSpec 3.2 program to generate a deconvoluted mass spectrum in the ARF mass region (20−21 kDa). The symmetry of the peaks in panels B and C is entirely consistent with homogeneous acylation of *Xenopus* ARFs.

Table 2: Analysis of Purified GSF by Liquid Chromatography-Coupled Electrospray Mass Spectrometry (LC/MS)

observed mass (Da)[a]	relative signal intensity (%)[b]	assignment[c]
20 791 ± 1	61 ± 2	xARF1
20 761 ± 1	26 ± 2	xARF3
20 715 ± 1	13 ± 1	(xARF5)

[a] Four independent mass measurements (LC/MS) on 20−80 pmol of the same preparation of GSF were carried out as described in Materials and Methods. The mean masses (± standard error) are reported. [b] The percent of total GSF ion signal present in each of the three GSF peaks was calculated as described in Materials and Methods. [c] See text for discussion of protein assignments.

Additional Isoforms. The second-most abundant species (mass of 20 761 Da, 25% of the total GSF) was identified as *Xenopus* ARF3, on the basis of its relative abundance as determined by HPLC and LC/MS. Abundance was critical in this determination because the peptide sequence analysis revealed only peptides that are shared by both ARF1 and ARF3, and ARF1 and the putative ARF3 together comprised 87% of the sample. The mass of the putative ARF3 is 80

FIGURE 6: cDNA sequence and predicted amino acid sequence of *Xenopus* ARF1. The open reading frame encodes 181 amino acids; the predicted mass of the singly myristoylated protein after removal of the initiating methionine is 20 795 Da. The GenBank accession number is U31350.

Da larger than that of the human/rat ARF3, which has a mass of 20 681 Da (Berger *et al.*, 1995); we therefore predict that *Xenopus* ARF3 differs by at least one amino acid from its mammalian counterpart.

Xenopus and mammalian ARFs exhibited two reproducible differences in chromatographic behavior during purification. Our sequence data and mass determinations can now explain these differences on hydrophobic interaction columns (HPLC and phenyl-Superose). In particular, the single substitution of methionine for isoleucine at position 4 of *Xenopus* ARF1 may be responsible for its decreased affinity for both columns. As noted above, we also deduce that *Xenopus* ARF3 differs from mammalian ARF3 by at least one amino acid, which might alter its hydrophobicity and cause it to nearly coelute with ARF1.

DISCUSSION

The purification of a single peak of GSF activity suggested that ARFs may be the only type of soluble GTPase that inhibits fusion when used to pretreat membranes in the presence of GTPγS. This finding will greatly assist our ability to dissect and interpret the potential roles of different GTPases during nuclear assembly. For example, when *Xenopus* cytosol is depleted of ARFs (and other small proteins) by size fractionation, vesicle fusion in the depleted cytosol is still inhibited when GTPγS is added to the reaction mixture, suggesting the involvement of a second type of GTPase (T. M. Gant and K. L. Wilson, unpublished experiments). Interestingly, this second unidentified putative GTPase appears to be located on the membranes and to stimulate fusion when provided with GTP, suggesting that it (unlike ARF) may play a positive role in fusion (C. Wiese and K. L. Wilson, unpublished experiments). The GSF purification results presented here strongly suggest that other soluble GTPases, such as rho (Boivin & Beliveau, 1995; Takaishi *et al.*, 1995), either are not efficiently recruited to nuclear vesicles during pretreatment with GTPγS or, if recruited, do not inhibit nuclear vesicle fusion.

The low recovery of GSF activity suggested that an auxiliary factor(s) that modulates ARF function in the GSF assay might have been resolved during purification, most likely during chromatography on phenyl-superose or Superdex75, where the percent recovery was low (Table 1). The existence of an "ARF-potentiating factor" was first discovered when ARF was purified from bovine brain cytosol using the mammalian intra-Golgi GGBF assay (Taylor, 1993). This ARF-potentiating factor is insensitive to GTPγS in the absence of ARFs but acts synergistically to inhibit transport in the presence of ARF1·GTPγS. We therefore infer that the relatively low recovery of GSF activity in our purification may have been due to the loss of one or more *Xenopus* ARF-potentiating factors, which remain to be identified.

The question of how ARF·GTPγS inhibits nuclear vesicle fusion is interesting, because it could occur by one of several distinct mechanisms: by activating phospholipase D (the biophysical and biochemical consequences of phospholipase D activation are not yet understood), by recruiting cytosolic coatomer (COP1) complexes, or perhaps by interacting with downstream effectors that have not been identified. The connection between ARF and phospholipase D is intriguing because phospholipase D is also activated by rho (a small GTPase distinct from ARF; Keen & Beck, 1989; Singer *et al.*, 1995) or by suboptimal amounts of ARF plus additional unidentified cytosolic proteins (Singer *et al*., 1995). If fusion is inhibited by ARF·GTPγS because it activates phospholipase D, then either rho and the other potential phospholipase D activators do not bind membranes during pretreatment or their effects on phospholipase D are weaker or more reversible than the effects of ARF. Further experiments will be required to determine if ARF·GTPγS inhibits nuclear vesicle fusion by activating phospholipase D or if inhibition involves coatomer proteins.

This work allowed us to identify the ARF isoforms that are present in *Xenopus* egg cytosol. ARF1 and ARF3 were the most abundant isoforms, representing 62 and 25%, respectively, of the total ARF. The ratio of ARF1 to ARF3 in the *Xenopus* preparation (2.5 to 1) is nearly opposite that found in bovine brain (three times more ARF3 than ARF1; Berger *et al.*, 1995), the only other tissue for which this information is available. The predominance of ARF1 in egg and ARF3 in brain most likely reflects the importance, in each cell type, of vesicle transport events that specifically require a given isoform. We did not conclusively identify the third LC/MS peak (mass of 20 714 Da), but it is likely to be ARF5 on the basis of the use of isoform-specific antibodies to human ARFs. Although we do not know how sensitive these antibodies are to *Xenopus* ARFs, immunoblots confirmed that GSF fraction 16 contained ARF1 as the most abundant species, ARF3 (less abundant), and low but detectable amounts of ARF5 (A. L. Boman, unpublished experiments). In addition, antibodies specific for human ARF4 gave a barely detectable signal, which might cor-

respond to a fourth LC/MS species with a mass of 20,678 Da; this 20 678 Da peak, which is not included in Table 2, was detected in three out of four runs, with a relative signal intensity of less than 2%. When the cDNA sequences of additional *Xenopus* ARFs are determined in the future, the abundance in *Xenopus* eggs of ARF isoforms that match our LC/MS data can be predicted directly.

ACKNOWLEDGMENT

We thank Drs. Natalie Ahn and Katheryn Resing (HHMI, University of Colorado, Boulder, CO) for providing access to their mass spectrometry facility. We are grateful to Dr. Katheryn Resing for performing preliminary analysis of GSF and for providing expert advice on data analysis. We thank Dr. William Lane for the peptide sequence analysis and Dr. J. Battey for providing the cDNA library. We are indebted to Dr. Rick Kahn for providing antibody 1D9 and the isoform-specific ARF antibodies and to Rick Kahn and Tracey Michelle Gant for valuable discussions and comments on the manuscript.

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BI960220I