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ARF-Induced Lysosomal Lysis *In Vitro*[1]

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Cytosol treated with guanosine 5′-*O*-(3-thiotriphosphate) (GTPγS) disintegrated lysosomes in a dose-dependent manner, as detected as the release of preloaded fluorescein isothiocyanate-dextran. The effect of GTPγS was suppressed by GTP or GDP, indicating a role of a GTP binding protein (G-protein) in the lysis [Sai, Y. *et al.* (1994) *Biochem. Biophys. Res. Commun.* 198, 869-877]. Gel filtration of cytosol and GTP-ligand blotting showed that a small GTP-binding protein participated in the lysosomal lysis. We partially purified the G-protein from rat liver cytosol and identified it as ARF1. GTPγS-stimulated lysis was reconstituted with ARF1 purified from bovine brain cytosol or recombinant ARF1. ARF bound to lysosomal membranes depending upon GTPγS in a dose-dependent manner. These results suggest that the transfer of ARF from the cytosol to the lysosomal membrane is necessary for GTPγS-stimulated lysis of lysosomes.

Key words: ARF, G-protein, lysis, lysosome.

Several types of GTP-binding protein (G-protein) have been identified, including heterotrimeric G-proteins, the elongation and initiation factors of protein synthesis, and the Ras superfamily. Monomeric low molecular weight G-proteins have molecular masses in the range of 20-30 kDa, and are characterized by their marked sequence homology with the Ras oncogene product. They have been categorized into four principal classes based on their degree of homology, namely, Ras, Rho, Rab, and the ARF families. In mammals they have been implicated in cellular proliferation, terminal differentiation, transformation, and other distinct biological processes, including degranulation (*1, 2*) and perturbation of lysosomal pH (*3*). Rho proteins are ADP-ribosylated by the C3 exoenzyme of *Clostridium botulinum*, in contrast to the other Ras superfamily members, and may play a role in controlling cytoskeletal organization (*4*). Rab and Arf proteins, which are distributed subcellularly throughout the exocytic and endocytic pathways in a distinctive manner, are associated with the membranes, and are likely to be regulators of vesicular trafficking among compartments (*5, 6*).

We identified distinct subsets of small G-proteins on rat liver lysosomal membranes (*7*). However, little is known of the role of the G-proteins in lysosomal function. We found that cytosol which had been treated with guanosine 5′-*O*-(3-thiotriphosphate) (GTPγS) disintegrated lysosomes in a dose-dependent manner, as detected as the release of preloaded fluorescein isothiocyanate-dextran (*8*). The effect of GTPγS was suppressed by GTP or GDP. This suggests that a G-protein participates in the lysis of lysosomes. In this study, we identified ARF1 as the G-protein that participates in GTPγS stimulated lysis.

EXPERIMENTAL PROCEDURES

Materials—Anti-ARF peptide antibody raised against a peptide corresponding to residues 23-36 of the ARF N-terminus was provided by Dr. Y. Kanaho (Tokyo Institute of Technology). *Escherichia coli* [BL21(DE3)]pLysS co-transfected with ARF/PET-20b and *N*-myristoyl transferase/pBB131 plasmids was provided by Dr. K. Nakayama (Universty of Tsukuba, Ibaraki). Fluorescein isothiocyanate-dextran (FD; Av. Mol. Wt.: 70,000) and Percoll were purchased from Sigma (St. Louis) and Pharmacia (Uppsala), respectively. Protease inhibitors of microbial origin were obtained from the Peptide Research Institute (Osaka). Most other chemicals were obtained from Sigma (St. Louis). FD-filled lysosomes and cytosol were prepared as described in a previous paper (*9*). The lysis assay was performed as reported (*9*).

[α-^{32}P]*GTP Binding to Protein Blots*—The assaying of [α-^{32}P]GTP-binding to protein blots was performed as described (*10, 11*). Fractions obtained on chromatography were boiled for 3 min in 50 mM Tris-HCl, pH 6.8, 7.5% glycerol and 1% sodium dodecyl sulfate (SDS), and then subjected to SDS (15%) gel electrophoresis (SDS-PAGE). The separated polypeptides were transferred to nitrocellulose, washed for more than 60 min with several changes of buffer comprising 50 mM Tris-HCl (pH 7.5), 5 mM

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Abbreviations: ARF, ADP-ribosylation factor; CAD, cationic amphiphilic drug; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; FD, fluorescein isothiocyanate-dextran; GTPγS, guanosine 5′-*O*-(3-thiotriphosphate); LysoPC, lysophosphatidylcholine; Mops, 3-[*N*-morpholino]propane sulfonic acid; NEM, *N*-ethylmaleimide; PA, phosphatidic acid; PAP, phosphatidic acid phosphohydrolase; PC, phosphatidylcholine; PI, phosphatidylinositol; PIP_2, phosphatidylinositol 4,5-bisphosphate; PLA_2, phospholipase A_2; PLC, phospholipase C; PS, phosphatidylserine; PVDF, polyvinylidene difluoride; SVE, 0.25 M sucrose, 1 mM EDTA (pH 7.0) and 0.1 (v/v)% EtOH; TGD buffer, 20 mM Tris/HCl (pH 7.4), 10% (v/v) glycerol, and 1 mM DTT; TMAH, tetramethylammonium hydroxide.

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$MgCl_2$, 1 mM EDTA, and 0.3% Tween 20, and then incubated in the same buffer containing 1 μCi/ml [α-^{32}P]GTP for 1 h at 25°C. After washing with several changes of the same buffer, the blots were air-dried. GTP-binding proteins were visualized by autoradiography using Kodak X-OMAT film and enhancing screens after 1-5 days exposure at −80°C.

Immunodetection of ARF1—Proteins fractionated by SDS-PAGE were transferred to a polyvinylidene difluoride membrane (PVDF, 0.45 μM; Millipore), and then incubated with the anti-ARF1 peptide antibody. The bolt was then incubated with alkaline phosphatase-conjugated goat anti-rabbit IgG. Color development was performed with nitrobluetetrazolium/5-bromo-4-chloro-3-indolylphosphate. ARF signals were quantified by imaging with a scanner and integration of the images with analysis software (Molecular Analyst) from Bio-Rad.

Gel Filtration Column Chromatography of Cytosol on Superose 12—The cytosol was applied to a column of Superose 12 equilibrated with TGD buffer [20 mM Tris/HCl (pH 7.4), 10% (v/v) glycerol, 1 mM DTT] containing 1 μM GDP, 5 μg/ml protease inhibitor cocktail, and 0.15 M NaCl, and eluted with the same buffer at the flow rate of 0.25 ml/min. The resulting fractions were analyzed by means of the GTPγS-stimulated lysosomal lysis assay, the [α-^{32}P]GTP binding assay and immunoblotting involving the anti-ARF peptide antibody.

Sequential Ammonium Sulfate Precipitation of Cytosol—To the cytosol (50 ml, 326 mg of protein) was added ammonium sulfate to 20% saturation, followed by stirring for 30 min at 4°C and then centrifugation at 10,000×*g* for 30 min to separate the precipitate (0-20% saturation) from the supernatant. The procedure was repeated sequentially for the resultant supernatant by adding additional ammonium sulfate to separate the precipitate (20-40, 40-60, 60-80, and 80-90% saturation) from the final supernatant (90% saturation). The final supernatant and each of the precipitates dissolved in TGD buffer were incubated with 100 μM GTPγS, mixed separately with each of the non-treated fractions, and used in place of the GTPγS-treated cytosol in the subsequent lysis assay (*9*).

Partial Purification of G-Protein—To the cytosol (500 ml, 5 g of protein), solid ammonium sulfate was added to 40% saturation. After 30 min at 4°C, the precipitate was removed by centrifugation (12,000×*g*, 30 min) and discarded. The supernatant was brought to 80% ammonium sulfate saturation and then stirred for 30 min. The precipitate was collected as before. The pellet was dissolved in 100 ml of TGD buffer and then dialyzed overnight against 2 liters of TGD buffer. The clear supernatant was applied to a column of Q-Sepharose FF (Pharmacia, 18×1.6 cm) equilibrated with TGD buffer and eluted with a linear gradient of 0-1.0 M NaCl in TGD buffer. Fractions containing GTPγS-dependent lysis activity, eluted between 0.6-0.9 M NaCl, were pooled and dialyzed overnight against 1 liter of the same buffer, and the resulting dialysate was applied to a second column of heparin-Sepharose (Pharmacia, 0.5×10 cm) equilibrated with TGD buffer containing 1 μM GDP and 5 μg/ml protease inhibitors, and eluted with a linear 0 to 1.0 M NaCl gradient. The active fractions (eluted with 0.6-0.8 M NaCl) were pooled and dialyzed against TGD buffer. The dialysate was applied to a Mono Q column equilibrated with TGD buffer and eluted with a linear 0 to 1 M NaCl gradient. The activity was separated into two peaks.

Determination of the Peptide Sequences—Peptide sequence analysis was performed as described (*12*). The Mono Q fraction was subjected to SDS-PAGE and then transferred to a nitrocellulose filter for *in situ* digestion with lysyl endopeptidase. Peptides were separated by reverse-phase high performance liquid chromatography [C18 μ-Bondashere (Waters, 1×150 mm)] and sequenced with a peptide sequencer (Applied Biosystem model 476A sequencer).

Partial Purification of Bovine Brain ARF—ARF was partially purified from bovine brain cytosol by the method described by Moss *et al.* (*13*). Briefly, bovine brains were homogenized in buffer A [20 mM Tris/HCl (pH 9.0), 1 mM EDTA, 1 mM DTT, 1 mM NaN_3, 0.5 mM PMSF, 10% sucrose], filtered, and then centrifuged at 10,000×*g* for 1 h. Proteins in the cytosol fraction were precipitated with ammonium sulfate at 25-70% saturation and then dissolved in buffer B [20 mM potassium phosphate (pH 7.0), 1 mM EDTA, 1 mM DTT, 1 mM NaN_3, 1 mM benzamidine, 0.25 M sucrose]. After dialysis against buffer B, the proteins were subjected to CM-Sephadex FF column chromatography. The ARF fractions were adjusted to pH 5.35 with 0.1 M acetic acid and then applied to a CM-Sephadex FF column that had been equilibrated with buffer C [20 mM potassium phosphate (pH 5.35), 1 mM EDTA, 1 mM DTT, 1 mM NaN_3, 1 mM benzamidine, 0.25 M sucrose]. ARF fractions were neutralized to pH 7.0 and then dialyzed against buffer D [20 mM Tris/HCl (pH 8.0), 1 mM EDTA, 1 mM DTT, 1 mM NaN_3, 1 μM GDP, 5 mM $MgCl_2$, 1 mM benzamidine, 0.25 M sucrose]. The ARF fractions were applied to a hydroxylapatite column equilibrated with

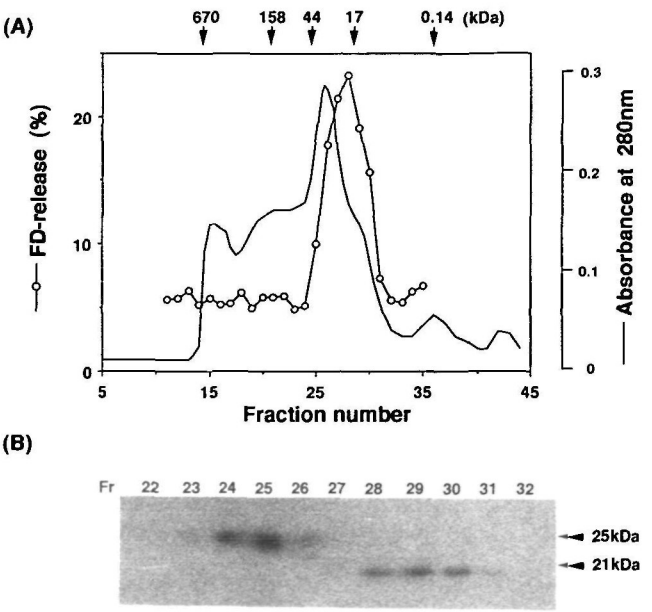

Fig. 1. **Molecular weight estimation of cytosolic G-proteins by gel-filtration.** Cytosol was loaded onto a Superose 12 column, and the lysis and [α-^{32}P]GTP binding activities of the fractions were monitored. For [α-^{32}P]GTP binding activity, each fraction was subjected to SDS-PAGE and then transferred to a nitrocellulose membrane, which was then incubated with [α-^{32}P]GTP and 0.1 mM ATP at 25°C for 1 h. (A) GTPγS-stimulated lysosomal lysis. (B) [α-^{32}P]GTP binding activity.

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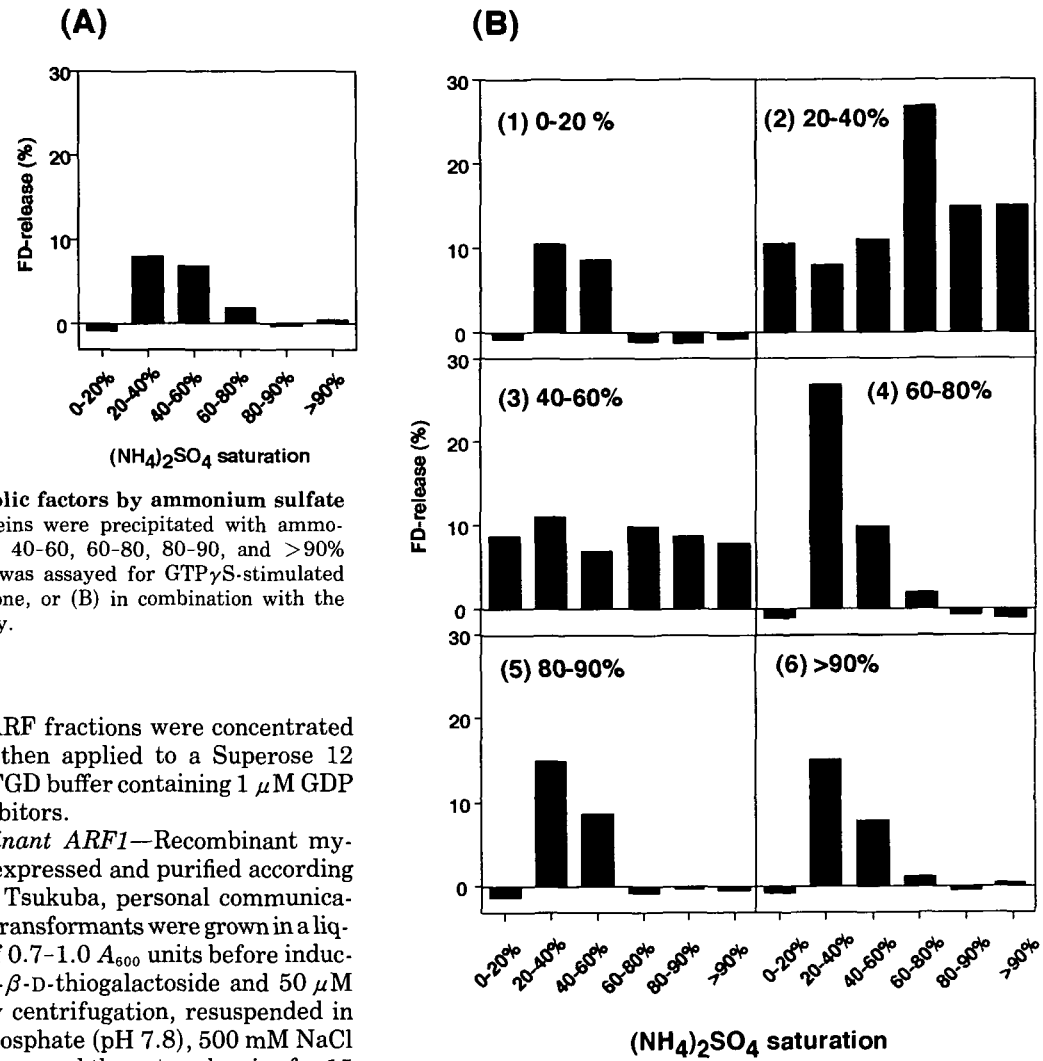

Fig. 2. **Separation of cytosolic factors by ammonium sulfate precipitation.** Cytosolic proteins were precipitated with ammonium sulfate at 0-20, 20-40, 40-60, 60-80, 80-90, and >90% saturation, and each fraction was assayed for GTPγS-stimulated lysosomal lysis, either (A) alone, or (B) in combination with the incubated fraction, respectively.

buffer D, and the eluted ARF fractions were concentrated with a Centricon 10 and then applied to a Superose 12 column equilibrated with TGD buffer containing 1 μM GDP and 5 μg/ml protease inhibitors.

Preparation of Recombinant ARF1—Recombinant myristoylated ARF was overexpressed and purified according to Nakayama (University Tsukuba, personal communication) and Ref. *14*. Briefly, transformants were grown in a liquid medium to a density of 0.7-1.0 A_{600} units before induction with 1 mM isopropyl-β-D-thiogalactoside and 50 μM myristic acid, collected by centrifugation, resuspended in 10 ml of 20 mM sodium phosphate (pH 7.8), 500 mM NaCl containing 10 mg of lysozyme, and then stored on ice for 15 min. The cells were lysed by ultrasonication in the presence of 0.3% Triton X-100, followed by three cycles of thawing and centrifugation at 10,000$\times g$ for 15 min at 4°C. The resultant supernatant was applied to an Ni-loaded chelating Sepharose FF column. After washing unbound materials out with 20 mM sodium phosphate (pH 6.0), 500 mM NaCl, ARF was eluted with imidazole elution buffer [20 mM sodium phosphate (pH 6.0), 500 mM NaCl, and 300 mM imidazole], and then applied to a Superose 12 column equilibrated with TGD buffer containing 10 mM GDP and eluted with the same buffer. The ARF-containing fractions were pooled and used for the lysosomal lysis assay.

Interaction of ARF with Lysosomes—Samples of lysosomes (50 μg of protein) were incubated at 37°C in a 6 ml reaction mixture consisting of buffer [0.1 M KCl, 0.2 M sucrose, 10 mM $MgCl_2$, 20 mM 3-[*N*-morpholino]propane sulfonic acid (Mops)-tetramethylammonium hydroxide (TMAH) (pH 7.0)], 1 mM ATP and cytosol, which had been treated with various amounts of GTPγS. After incubation, a 1 ml aliquot was removed for determination of GTPγS-stimulated lysosomal lysis. The rest was centrifuged at 100,000$\times g$ for 1 h to sediment lysosomal membranes. The membranes were washed with the buffer and collected by centrifugation (100,000$\times g$, 1 h). The samples were used for the immunodetection of ARF.

RESULTS

A Low Molecular Weight G-Protein Is Involved in Lysosomal Lysis—To determine the molecular weight of the G-protein participating in the GTPγS-stimulated lysis, rat liver cytosol was fractionated by gel filtration on a Superose 12 column. However, fractions eluted from this column failed to show any lysis activity unless fresh cytosol was present, suggesting a requirement for additional cytosolic factor(s). Therefore, fractions first incubated with 100 μM GTPγS were mixed with lysosomes and fresh cytosol containing other cytosolic factor(s). GTPγS-stimulated lysis activity was eluted as a broad band corresponding to approximately 20 kDa from the Superose 12 column (Fig. 1). [α-^{32}P]GTP blot analysis showed that [α-^{32}P]-GTP bound to the 21 kDa (*28-30*) and 25 kDa bands (*24, 25*) among the active bands. However, [α-^{32}P]GTP blot analysis revealed few apparent signals in the fractions (*26, 27*) containing the peak activity. These results suggest that there are a couple of small G-proteins collaboratively participating in lysosomal lysis.

Separation of G-Proteins and Other Factor(s) by Ammonium Sulfate Fractionation—The lysis reaction consists of

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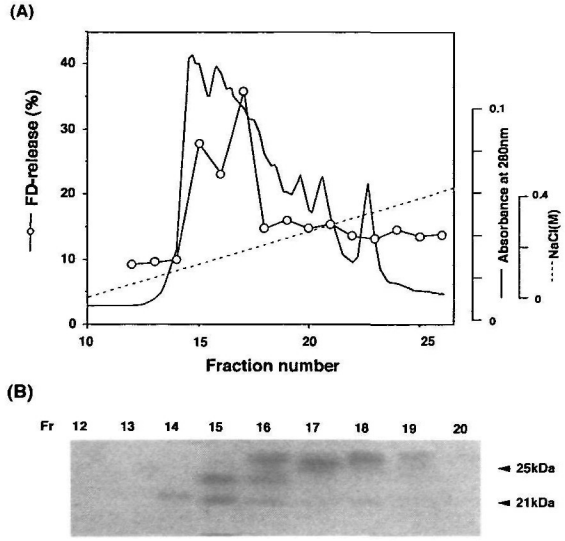

Fig. 3. **Separation of cytosolic G-proteins on Mono Q.** Proteins precipitated with ammonium sulfate at 40-80% saturation were chromatographed sequentially on Q-Sepharose and heparin-Sepharose, and the active fractions were subsequently loaded onto a Mono-Q column. For [α-^{32}P]GTP-binding activity, aliquots of fractions were subjected to SDS-PAGE, and then transferred to PVDF membranes, which were incubated with [α-^{32}P]GTP and 0.1 mM ATP at 25°C for 1 h, and then autoradiographed. (A) GTPγS-stimulated lysosomal lysis. (B) [α-^{32}P]GTP binding activity.

two steps, (i) activation of cytosolic G-protein(s) by GTPγS, and (ii) ATP-dependent disintegration of lysosomes by the activated cytosol. This suggests that cytosolic factor(s) other than G-protein(s) are involved. The cytosolic factors were separated by ammonium sulfate precipitation. Namely, cytosol was separated into six fractions (0-20, 20-40, 40-60, 60-80, 80-90, and >90% saturated fractions) with ammonium sulfate. None of the fractions alone showed significant activity (Fig. 2A). But the activity appeared on combination of the fractions. As clearly shown in Fig. 2B (2) and (4), the combination of the 20-40% and 60-80% ammonium sulfate saturation fractions showed the highest activity. [α-^{32}P]GTP ligand blotting assays showed that, although G-proteins appeared in all fractions, they were especially enriched in the 60-80% ammonium sulfate saturated fraction (data not shown). The other factor(s) [putative partner protein(s)] was abundant in the 20-40% saturated fraction.

ARF1 Participates in GTPγS-Stimulated Lysosomal Lysis—Since lysosome-disintegration activity was detected on the combination of G-protein(s) and other cytosolic factor(s), the chromatographic fractions were first preincubated with GTPγS, and then mixed with the 20-40% ammonium sulfate saturated fraction, which is enriched with the other factor(s), and subjected to the lysosomal lysis assay to determine the active G-protein. The G-protein that participated in the GTPγS-stimulated lysis was partially purified by standard chromatography. The 40-80% ammonium sulfate saturated fraction of cytosol was applied to Q-Sepharose FF, heparin-Sepharose, and Mono Q columns, sequentially. A chromatogram obtained with the Mono Q anion exchange column is shown in Fig. 3. The activity was separated into two peaks. The first peak showed a strong signal for [α-^{32}P]GTP binding at 21 kDa

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                  10          20          30          40
r ARF1   MGnifanLFk qLFGKKEMRI LMVGLDAAGK TTILYKLKLG
b ARF2   MGnvfekLFk sLFGKKEMRI LMVGLDAAGK TTILYKLKLG
h ARF3   MGnifgnLlk sLiGKKEMRI LMVGLDAAGK TTILYKLKLG
h ARF4   MGltissLFs rLFGKKqMRI LMVGLDAAGK TTILYKLKLG
h ARF5   MGltvsaLFs riFGKKqMRI LMVGLDAAGK TTILYKLKLG
h ARF6   MGkvlsk     iFGnKEMRI LMlGLDAAGK TTILYKLKLG

                  50          60          70          80
r ARF1   EiVTTI PTIG FNVETVEYKN IsFTVWDVGG QDkIRPLWRH
b ARF2   EiVTTI PTIG FNVETVEYKN IsFTVWDVGG QDkIRPLWRH
h ARF3   EiVTTI PTIG FNVETVEYKN IsFTVWDVGG QDkIRPLWRH
h ARF4   EiVTTI PTIG FNVETVEYKN IcFTVWDVGG QDrIRPLWRH
h ARF5   EiVTTI PTIG FNVETVEYKN IcFTVWDVGG QDkIRPLWRH
h ARF6   qsVTTI PTvG FNVETVtYKN vkFnVWDVGG QDkIRPLWRH

                  90          100         110         120
r ARF1   YfQNTqGLIF VVDSNDRE  Rv nEAReELmrM LaEDELRDAv
b ARF2   YfQNTqGLIF VVDSNDRE  Rv nEAReELtrM LaEDELRDAv
h ARF3   YfQNTqGLIF VVDSNDRE  Rv nEAReELmrM LaEDELRDAv
h ARF4   YfQNTqGLIF VVDSNDRE  Rv qEvadELqkM LlvDELRDAv
h ARF5   YfQNTqGLIF VVDSNDRE  Rv qEsadELqkM LqEDELRDAv
h ARF6   YytgTqGLIF VVDcaDRd  Ri dEARqELhri indrEmRDAi

                  130         140         150         160
r ARF1   LLVFANKQDL PnAMnaaEiT dKLGLhSLRh RnWYIQaTCA
b ARF2   LLVFvNKQDL PnAMnaaEiT dKLGLhSLRq RnWYIQaTCA
h ARF3   LLVFANKQDL PnAMnaaEiT dKLGLhSLRh RnWYIQaTCA
h ARF4   LLlFANKQDL PnAMaisEmT dKLGLqSLRn RtWYvQaTCA
h ARF5   LLVFANKQDm PnAMpvsElT dKLGLqhLRs RtWYvQaTCA
h ARF6   iLiFANKQDL PdAMkphEiq eKLGLtriRd RnWYvQpsCA

                  170         180
r ARF1   TSGDGLYEGL DWL  SNqL rNqk     181
b ARF2   TSGDGLYEGL DWL  aNqL KNqk     181
h ARF3   TSGDGLYEGL DWL  SNqL KNqk     181
h ARF4   TqGtGLYEGL DWL  SheLsKr       180
h ARF5   TqGtGLYdGL DWL tSNeLsKr       180
h ARF6   TSGDGLYEGL tWLsdyiyks         175
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Fig. 4. **Comparison of the amino acid sequences of ARFs and the peptides derived from the 21 kDa protein.** The deduced amino acid sequences of mammalian ARFs are shown with capital letters representing amino acid identity and gaps introduced to facilitate alignment. The sequences in boxes correspond to those of the peptides obtained on lysyl endopeptidase-digestion of the 21 kDa protein. rARF1 rat ARF1 (*15*); bARF2, bovine ARF2 (*15*); hARF3, human ARF3 (*15*); hARF4, human ARF4 (*15*); hARF5, human ARF5 (*15*); hARF6, human ARF6 (*15*).

(Fig. 3B), probably corresponding to the first active fractions on the Superose 12 gel chromatography.

Fractions that contained the 21 kDa polypeptide were subjected to SDS-PAGE and then transferred to a PVDF membrane. The 21 kDa band material was digested *in situ* with lysyl endopeptidase and the peptide fragments were separated by HPLC. Analysis of the three fragments derived on digestion of the 21 kDa protein yielded the sequences shown in Fig. 4. The derived amino acid sequence exactly matched that predicted from the cDNA sequence of rat ARF1 (*15*).

To confirm the participation of ARF in GTPγS-stimulated lysis, the lysis reaction was reconstituted using bovine brain ARF or recombinant myristoylated ARF1 in place of rat cytosol for treatment with GTPγS. Figure 5 shows a Superose 12 chromatogram of partially purified bovine brain ARF. Each fraction in the chromatogram was first treated with 100 μM GTPγS and then incubated with lysosomes, in the presence of ATP and rat liver cytosol [as

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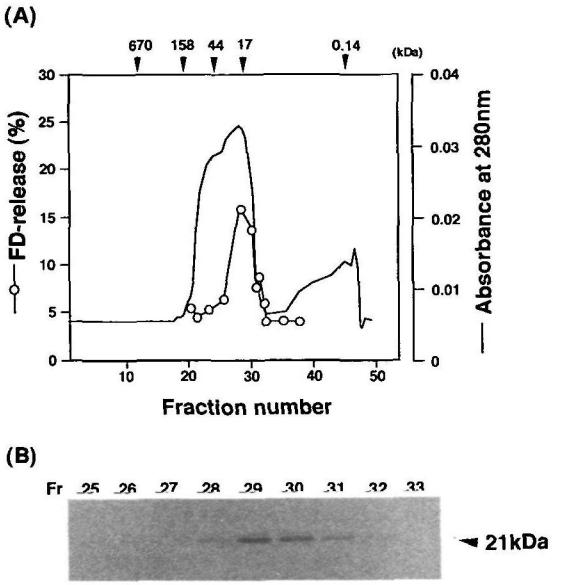

Fig. 5. **Reconstitution of GTP-stimulated lysosomal lysis with bovine brain ARF.** Bovine brain ARF was partially purified by sequential standard column chromatographies on CM-Sepharose, hydroxylapatite, and Superose 12. In place of cytosol, the final Superose 12 fractions were treated with GTPγS and then subjected to lysis with additional cytosol. (A) GTPγS-stimulated lysosomal lysis. (B) Western blotting with anti-ARF antibody.

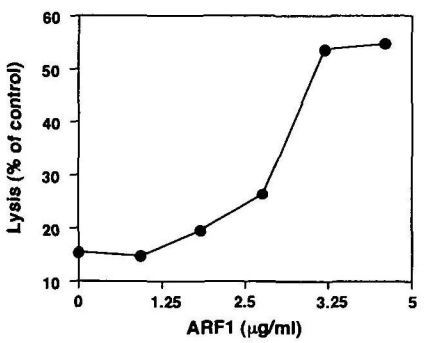

Fig. 6. **Stimulation of lysosomal lysis by recombinant ARF1.** Purified recombinant ARF1 was treated with 100 μM GTPγS, and then incubated with lysosomes in the presence of ATP and the 40% saturated ammonium sulfate fraction of rat liver cytosol [as a source of cytosolic factor(s)]. The lysosomal lysis is shown as a percentage of a control that was incubated with GTPγS-treated cytosol of rat liver.

a source of cytosol factor(s)]. The eluted fractions corresponding to a molecular mass of about 20 kDa showed significant lysis activity. The activity pattern coincided with that of ARF abundance on Western blotting with anti-ARF antibody. Figure 6 shows the results with recombinant myristoylated ARF1, showing the dose-dependent stimulation of the lysosomal lysis. These results strongly suggested that ARF participated in the GTPγS-stimulated lysis.

GTPγS Induced ARF Binding to Lysosomal Membranes—ARF is required for coated-vesicle budding. ARF binds reversibly to Golgi membranes *in vitro* in a manner that is affected by guanine nucleotides. GTPγS, a stable analog of GTP, promotes the association of ARF with the membranes (*16, 17*). To determine whether or not ARF

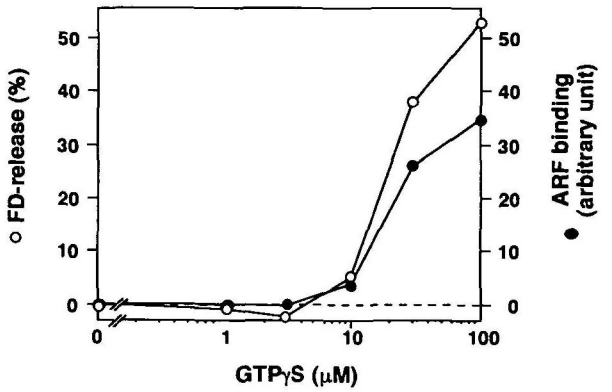

Fig. 7. **GTPγS-stimulated binding of ARF to lysosomes.** After preincubation for 10 min at 37°C with various concentrations of GTPγS, cytosol was incubated with lysosomes and ATP for 20 min at 37°C. GTPγS-stimulated lysosomal lysis and GTPγS-dependent binding of ARF to lysosomes were monitored. The amount of ARF bound to lysosomal membranes was detected by Western blotting with anti-ARF antibody as described under "EXPERIMENTAL PROCEDURES."

binding to lysosomal membranes is required for GTPγS-stimulated lysis, we examined whether or not the stimulation of lysis parallels ARF binding to lysosomal membranes in the GTPγS dose response. No ARF was detected on lysosomal membranes. As shown in Fig. 7, soluble ARF bound to the lysosomal membranes in a GTPγS-dose dependent manner. The dose response of the GTPγS-stimulation of the lysis well paralleled the binding of ARF to lysosomal membranes.

DISCUSSION

We described a new phenomenon, *i.e.* lysosomes isolated from rat liver were disintegrated on incubation with cytosol treated with GTPγS. This reaction seems to be lysosome-specific, because no other organelle isolated from rat liver, including mitochondria, peroxisomes and Golgi, or red blood cells showed significant lysis (data not shown). This phenomenon, which we refer to as "GTPγS-dependent lysosomal lysis," consists of two consecutive steps, (i) activation of the cytosol by GTPγS, and (ii) ATP-dependent disintegration of lysosomes.

The first reaction, which is specific for GTPγS, most probably involves G-protein(s). Gel-filtration and GTP-ligand blotting studies of cytosol suggest that at least two low molecular weight G-proteins participate in the GTPγS-stimulated lysis. We showed that (i) ARF1 was one of the responsive G-proteins, (ii) the ARF1 purified from bovine brain cytosol or recombinant ARF1 could replace the cytosol in GTPγS-stimulated lysis, and (iii) ARF bound to lysosomal membranes depending upon GTPγS in a dose-dependent manner. These results suggest that the transfer of ARF from the cytosol to lysosomal membranes is a necessary step for GTPγS-stimulated lysis.

ARFs, originally identified as co-factors required for the cholera toxin-catalyzed ADP-ribosylation of Ga_s (*18*), have grown in number, resulting in the formation of an ARF family consisting of 17 structurally related gene products that includes 6 ARF proteins and 11 ARF-like proteins. The ARF proteins are divided into three classes on the basis of

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their size and amino acid identity. ARFs 1 to 3 (181 amino acids) class 1, ARFs 4 and 5 (180 amino acids) class II, and ARF 6 (175 amino acids) forms class III (*19, 20*). The best characterized ARF protein is ARF1. It is localized in the Golgi apparatus and plays a central role in intra-Golgi transport (*21, 22*). It is involved in the recruitment of cytosolic coat proteins to Golgi membranes during the formation of transport vesicles (*23*). The ARFs have also been implicated in Golgi transport, endoplasmic reticulum (*24*), nuclear vesicle fusion (*25*), and endosome fusion (*26, 27*). Apart from their role in transport activation, ARF proteins are known to be activators of phospholipase D (PLD) in cell-free preparations (*28, 29*). PLD hydrolyzes phosphatidylcholine (PC) to produce phosphatidic acid (PA) and choline. PA affects several cellular events, including secretion, DNA synthesis, and cell proliferation, and is formed in response to a number of agonists, neurotransmitters and growth factors. The role of ARFs in vesicular trafficking may be related to their ability to stimulate PLD (*30, 31*). The speculation that PLD activity is important for protein transport is supported by the observation that such activity is higher in membrane fractions rich in Golgi. Furthermore, basal PLD activity appeared to be constitutive and no longer sensitive to ARF in Golgi membranes obtained from cells resistant to brefeldin A, an inhibitor of protein transport through the Golgi apparatus (*32*). The hydrolysis of PC by PLD may regulate fusion and/or fission processes. Indeed, the ratio of PI to PC seems to be important for vesicular trafficking, and a mutant yeast deficient in a PI transfer protein can be rescued by mutations that decrease the amount of PC. PLD can decrease the concentration of PC and therefore influence the PI : PC ratio in membranes (*33*). Wortmannin, an inhibitor of phosphatidylinositol 3-kinase (PI3-kinase), inhibits proper trafficking of lysosomal acid hydrolases in mammalian cells showing a requirement for PI3-kinase activity. The actual role played by PIs in membrane trafficking remains to be clarified, although they have now been implicated in a number of exocytic and endocytic transport reactions (*34*). We showed that neomycin inhibited GTPγS-stimulated lysosomal lysis. Neomycin binds strongly and selectively to inositol phospholipids, leading to the inhibition of their metabolism. Neomycin inhibits the stimulation of PLD by phosphatidylinositol 4,5-bisphosphate (PIP_2). PIP_2 is a cofactor of membrane PLD in the brain, the synthesis and hydrolysis of which have been suggested to be important determinants of the regulation of PLD in signal transduction and membrane transport (*35*). The activation of PLD and subsequent production of PA are early key events in the formation of coatmer-coated vesicles from Golgi (*36*). The fusion and/or fission may also be influenced by PA derived through PC hydrolysis by PLD. We are now studying whether or not PLD activity is stimulated by ARF1 during GTPγS-stimulated lysis.

Cationic amphiphilic drugs (CADs) showed inhibitory activity toward GTPγS-stimulated lysis of lysosomes (*9*). CADs inhibit both cytosolic and microsomal NEM-sensitive phosphatidic acid phosphohydrolase (PAP) (*37, 38*). Spermine is a naturally occurring polyamine that has been implicated in the regulation of PAP activity. PAP may be regulated by the increase in the amount of phosphatidate arising from PLD stimulation. PAP catalyzes the hydrolysis of PA to diacylglycerol (DAG). The DAG and PA balance in the membrane is also important for the appropriate response(s) to extracellular signals (*39*). PAP appears to be involved in the control of this balance. It is unclear whether or not the PLD/PAP system participates in GTPγS-stimulated lysosomal lysis.

We showed that cytosolic factor(s) other than ARF participate in the GTPγS-stimulated lysis. Some proteins reportedly interact with ARF to regulate catalytic activities. GTP hydrolysis by ARF is dependent on a GTPase-activating protein (GAP) (*40, 41*), and PLD activation by ARF requires a 50 kDa cytosolic cofactor (*42, 43*). These factors might also participate in the GTPγS stimulated lysis.

What is the mechanism underlying this new phenomenon? As GTPγS-activated ARF binds to lysosomes and may stimulate PLD on lysosomal membranes (or cytosol), it might disturb the lipid composition of lysosomal membranes. It is possible that local changes in the concentrations of these lipids alter the structure of the lipid bilayer, and thereby facilitate budding and docking/fusion processes (*44*). Under physiological conditions, the mechanism might be related to the lysosomal dynamics within the cells: when organelle membranes fuse, they may pass through a transition state that is unstable unless the fusion reaction is stopped. This step could be regulated by the G-proteins cycle (between active and inactive states). In our system, however, lysosomes were disintegrated on irregular activation of ARF by GTPγS, which may be related to the perturbation of lysosomal membranes. The GTPγS-stimulated lysis of lysosomes we observed *in vitro* might be a reflection of some essential unit reaction related to lysosomal dynamics (possibly related to membrane fusion and/or fission) played by G-proteins, and, therefore, might help us clarify the role of G-proteins in lysosomal function. This remains to be explored in the future, for example, through *in vitro* reconstitution studies.

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