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Small GTP-Binding Proteins on Rat Liver Lysosomal Membranes

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ABSTRACT. **GTP-binding proteins have been identified on the membranes of highly purified dextran-filled lysosomes (dextranosomes) and Triton-filled lysosomes (tritosomes) obtained from rat liver. Autoradiography of blots of lysosomal membrane proteins incubated with [α-^{32}P]GTP revealed the presence of several specific GTP-binding proteins with a relative molecular mass (M_r) predominantly in the range of 26–30 kDa. These GTP-binding proteins migrated slower in polyacrylamide gels than purified c-Ha-*ras* protein expressed in *E. coli*, whose apparent M_r was 23 kDa in the same blot. The relative contents of GTP-binding proteins in lysosomal membranes were comparable or greater than that of plasma membranes and of microsomes. Chemical extraction showed that lysosomal GTP-binding proteins were more tightly associated with the membranes than with microsomal GTP-binding proteins. The possible involvement of lysosomal GTP-binding proteins in cellular functions including vacuolar (lysosomal) acidification and organellar dynamics are discussed.**

Low molecular weight GTP-binding proteins are a rapidly growing family of monomeric proteins, with a molecular mass in the range of 20–30 kDa. These proteins are characterized by marked sequence homology with the *ras* oncogene product, as well as guanine nucleotide-binding and GTPase activity (for reviews see 8 and 15). They have been identified in various eukaryotes including mammalian and yeast cells (7, 8, 12, 18, 35), as specific guanine nucleotide-binding proteins by means of SDS-PAGE and blotting. Distinct subsets of low molecular weight GTP-binding proteins have been identified in various organelles including ER (31), Golgi (1, 19), plasma membranes (1, 30), secretory vesicles (39), endosomes (1), cytosol (30) and nuclear envelopes (34) and recently in peroxisomes (38). However, no study has yet been reported on GTP-binding proteins on lysosomes, except for those on the lysosome-related specific granules from human neutrophils (30).

The acidification of lysosome has been considered to play numerous and critical roles in maintaining the normal function of the endocytic and exocytic pathways. Interest in the role of *ras*-like small GTP-binding proteins on the maintenance of vacuolar H^+-ATPase which generates a proton gradient that is used for acidification of lysosomes (28), vacuolar dynamics and carcinogenesis has rapidly grown. For example, Gurich *et al.* (20) reported that GTP analogs affected acidification of endosomes derived from rabbit renal cortex, and Jiang *et al.* (21) reported that *ras* transformation results in significant perturbation of lysosomal pH.

Based upon this background, we investigated the presence of distinct subsets of low molecular weight GTP-binding proteins in rat liver lysosomal membranes as a first step towards understanding the role of GTP-binding proteins in intra-lysosomal pH regulation and lysosomal dynamics.

MATERIALS AND METHODS

Materials. Fluorescein isothiocyanate-dextran (FD, Av. Mol. Wt.: 70,000) and Percoll were purchased from Sigma (St. Louis) and Pharmacia (Uppsala), respectively. Uridine diphospho-D-[U-^{14}C]galactose (272.8 mCi/mmol) and [α-^{32}P]GTP (3,000 Ci/mmol) were purchased from DuPont-NEN (Boston). Protease inhibitors of microbial origin were obtained from the Peptide Research Institute (Osaka). Most other chemicals were obtained from Sigma (St. Louis). Recombinant c-Ha-*ras* protein expressed in *E. coli* was provided by Dr. S. Yokoyama (University of Tokyo).

Subcellular fractionation. Rat liver cells were fractionated by differential centrifugation according to the method of de Duve *et al.* (16). Percoll gradient centrifugation proceeded according to Arai *et al.* (2). Briefly, rats were injected *i.p.* with FD at a dose of 100 mg/100 g of body weight and starved overnight. Their livers were perfused with ice-cold 0.25 M sucrose and homogenized with four volumes of cold 0.25 M sucrose. The resulting 20% homogenate was centrifuged at 340 × g for 5 min, twice. The resulting post nuclear supernatant (PNS), was incubated at 37°C for 5 min in the presence of 1 mM

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Abbreviations: FD, FITC-dextran; PAGE, polyacrylamide gel electrophoresis; NAGA, β-*N*-acetyl-D-glucosaminidase; PNS, post nuclear supernatant; TMG, tritosomal membrane ghost.

$CaCl_2$ to swell mitochondria, then centrifuged at 10,000×g for 30 min. The resulting pellet was resuspended in iso-osmotic (0.25 M sucrose) Percoll at a density of 1.10 g/ml (pH 7.4) and centrifuged at 60,000×g in a Hitachi RP30 rotor at 4°C for 15 min. Occasionally, the lysosomal layer (close to the bottom of the tube) was collected with a Pasteur pipette and centrifuged again in iso-osmotic Percoll under the same conditions. Fractions obtained by differential and Percoll gradient centrifugation (7 fractions of similar volume) were analyzed for enzyme and GTP-binding activities.

Isolation of intracellular organelles. Dextran-filled lysosomes (dextranosomes) were prepared as described by Arai *et al.* (2). Triton-filled lysosomes (tritosomes) were obtained as described (22) from the livers of rats given Triton WR-1339. Lysosomal membranes were obtained by five cycles of freeze-thawing in liquid nitrogen followed by centrifugation at 100,000×g for 1 hr. Sometimes, portions of the membrane pellets were extracted in either 0.1 M NaCl, 6 M urea, 0.1 M Na_2CO_3 (pH 11.5), 1 mM sodium citrate/acetate (pH 3.2) or 1% Triton X-100 for 30 min on ice with occasional vortex mixing. Extracted membranes were pelleted at 100,000×g for 30 min in a microultracentrifuge. Plasma membranes (10), microsomes (25), cytosol (16), mitochondria (16), peroxisomes (37) and nuclei (26) were prepared as described. Subcellular fractions and intracellular organelles were routinely prepared in the presence of 5 μg/ml each of protease inhibitors of microbial origin, pepstatin A, chymostatin, antipain and leupeptin.

Enzyme and protein assays. The following marker enzymes were assayed as follows: β-*N*-acetyl-D-glucosaminidase (NAGA, for lysosomes) using 4-methylumbelliferyl-β-*N*-acetyl-D-glucosaminide as the substrate (6); NADH-cytochrome *c* reductase (cyanide-insensitive, for microsomes) (16), cytochrome *c* oxidase (for mitochondria) (13), alkaline phosphatase (for plasma membrane) (29), and galactosyltransferase (for Golgi apparatus) (32) according to the published procedures. Protein was determined by the method of Bradford *et al.* (9), using bovine serum albumin as the standard.

[^{32}P]GTP binding to protein blots. The assay of [^{32}P]GTP-binding to protein blots was performed as described (30). Fresh subcellular fractions (15 μg) from rat liver were boiled for 3 min in 250 mM Tris-HCl, pH 6.8/7.5% glycerol/4% SDS, and applied to sodium dodecyl sulfate-polyacrylamide (15%) gel electrophoresis (SDS-PAGE) in the absence of β-mercaptoethanol. Separated polypeptides were transferred to nitrocellulose, washed for more than 60 min with several changes of buffer containing 50 mM Tris-HCl, 5 mM $MgCl_2$, 1 mM EDTA, and 0.3% Tween 20, pH 7.5, then incubated in the same buffer containing 1 μCi/ml [^{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 of exposure at −80°C.

RESULTS

Distribution of GTP-binding proteins in rat liver subcellular fractions after differential centrifugation. The distribution of GTP-binding proteins among subcellular organelles was analyzed on fractions obtained after systematic differential centrifugation (16) (Fig. 1). Proteins in each fraction were separated by SDS-PAGE, transferred to nitrocellulose and incubated with [α-^{32}P]GTP. Autoradiography revealed the presence of several specific GTP-binding proteins in the 21–30 kDa range (Fig. 2A). GTP-binding proteins were most abundant in the microsomal (P) fraction enriched with endoplasmic reticulum (NADH-cytochrome *c* reductase) and Golgi (galactosyltransferase), which may have contributed to the GTP-binding activity of this fraction. However, GTP-binding proteins were also detected in the light-mitochondrial (L) fractions enriched with the lysosomal marker enzyme, NAGA. A small amount of GTP-binding proteins was also detected in the heavy-mitochondrial (M) fraction enriched with mitochondria (cytochrome *c* oxidase) and in the cytosolic (S) fraction in decreasing order, but were almost undetectable in the nuclear (N) fraction. The specificity of the GTP-binding protein assay for GTP was evident from competi-

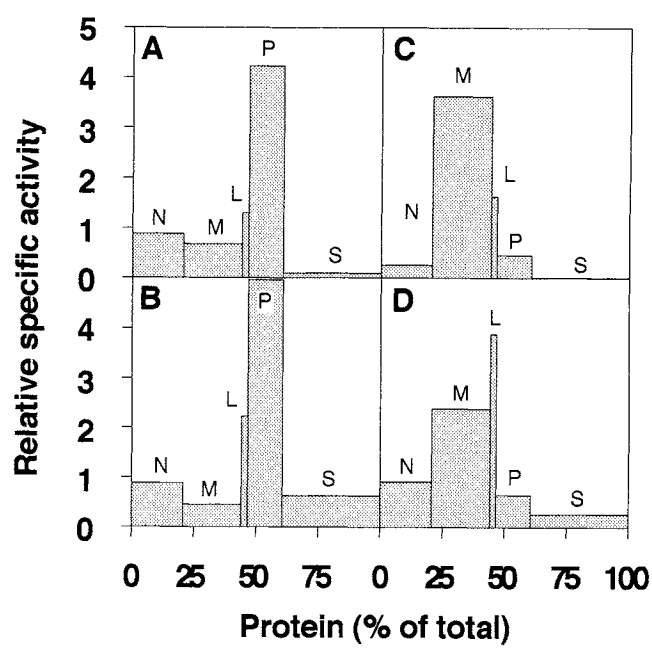

Fig. 1. Subcellular distribution of organelle markers in differential centrifugation. Rat livers homogenized in 0.25 M sucrose/1 mM EDTA, pH 7.4/0.1% EtOH containing 5 μg/ml of protease inhibitors were fractionated by differential centrifugation according to de Duve *et al.* (15) and analyzed for the indicated markers as described under "MATERIALS AND METHODS". (A) NADH-cytochrome *c* reductase; (B) galactosyltransferase; (C) cytochrome *c* oxidase; (D) NAGA.

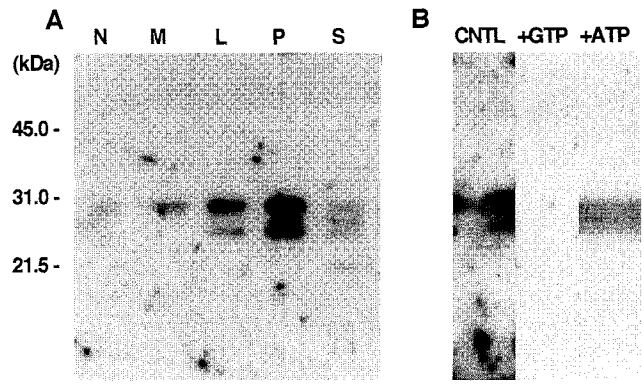

Fig. 2. Subcellular distribution of GTP-binding proteins in differential centrifugation (A) and specificity of the GTP-binding protein assay (B). Subcellular fractions (10 μg/lane) from differential centrifugation (Fig. 1) were applied to non-reducing SDS-PAGE, electroblotted onto nitrocellulose and probed with [α-^{32}P]GTP as described under "MATERIALS AND METHODS". GTP-binding proteins were visualized by autoradiography. (A) Lanes: N, nuclear fraction; M, heavy mitochondrial fraction; L, light mitochondrial fraction; P, microsome fraction; S, cytosol fraction. (B) Light mitochondrial fractions were probed with [α-^{32}P]GTP under different conditions. Lanes: CNTL, standard condition; +GTP, with 10 μM GTP; +ATP, with 100 μM ATP.

tion studies, in which 10 μM GTP and 100 μM ATP completely blocked and hardly affected respectively, the binding profile of [α-^{32}P]GTP (Fig. 2B).

However, differential centrifugation alone did not reveal whether or not the GTP-binding proteins in the light mitochondrial fraction were due to cross-contamination by the microsomal fraction, although in some cases, marker enzymes for Golgi and ER were almost undetectable in the light mitochondrial fraction (data not shown). Therefore, a more sophisticated evaluation was required to clarify these issues, and the results are shown below. In the following studies, 10 μM ATP was included in the assay mixture to reduce non-specific adsorption of [α-^{32}P]GTP (Fig. 2B).

Distribution of GTP-binding proteins on subcellular fractions of Percoll density gradient. The Ca^{2+}-treated PNS fraction from rats given dextran was separated on a Percoll density gradient, which separated lysosomes from all other cellular organelles. The lysosomal maker enzyme, NAGA and fluorescein isothiocyanate-dextran (FD), sedimented close to the bottom, whereas the other marker enzymes such as alkaline phosphatase (plasma membranes), NADH-cytochrome *c* reductase (endoplasmic reticulum), cytochrome *c* oxidase (mitochondria, not shown) and galactosyltransferase (Golgi) were all retained close to the top fraction (Fig. 3A). Figure 4A shows the distribution of GTP-binding proteins along the Percoll gradient. GTP-binding proteins were distributed mostly in the upper fractions, probably due to the distribution of plasma membranes, endosomes, endoplasmic reticulum and Golgi in these

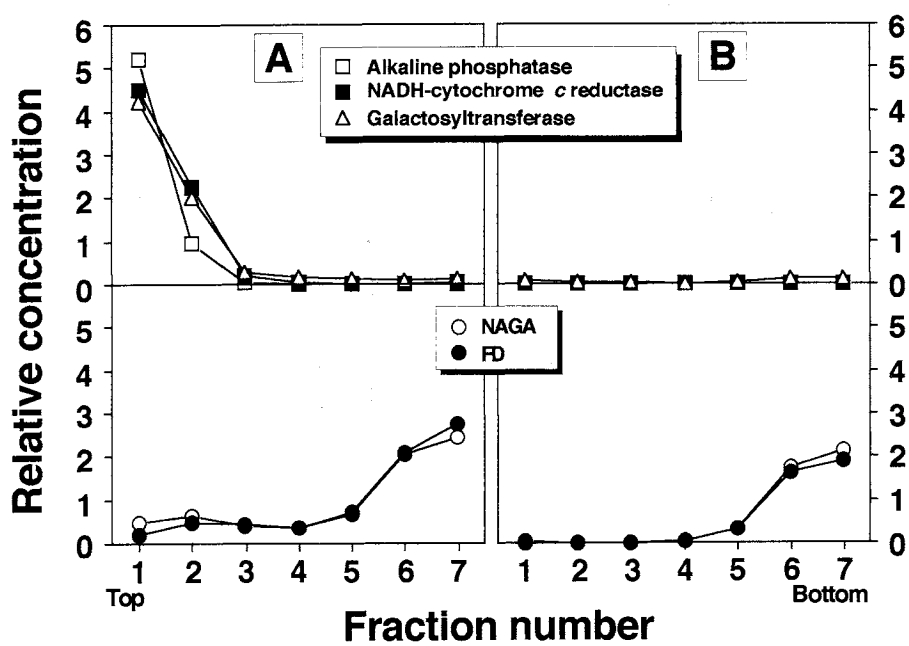

Fig. 3. Subcellular distribution of organelle markers in Percoll density gradients. Fractions from the following Percoll density gradients were analyzed for the indicated markers as described under "MATERIALS AND METHODS". (A) Ca^{2+}-treated PNS was centrifuged at 60,000 × g for 15 min in iso-osmotic Percoll at a density of 1.10 g/ml (pH 7.4) (for details, see "MATERIALS AND METHODS"). (B) The turbid layer (lysosomal fraction) close to the bottom of the first gradient (A) was collected and centrifuged again under the same conditions.

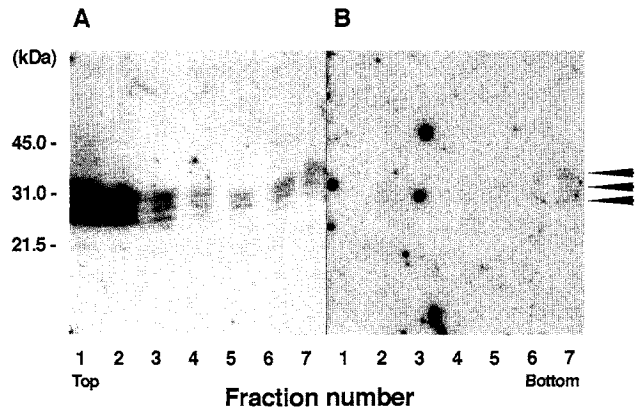

Fig. 4. Subcellular distribution of GTP-binding proteins in Percoll density gradients. Fractions (15 μl/lane, each) from the Percoll density gradients (Fig. 3A and 3B) were analyzed for GTP-binding protein as described in the legend for Fig. 2, except that 10 μM ATP was included in the assay buffer.

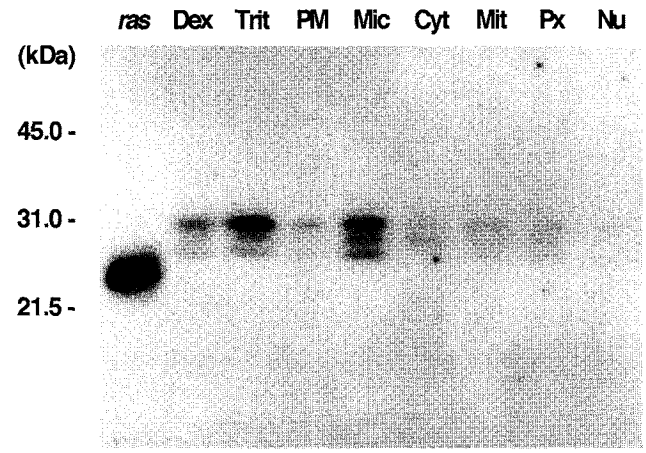

Fig. 5. GTP-binding proteins of various organelles isolated from rat liver and recombinant c-Ha-*ras* protein. Twenty micrograms of each fraction and 5 μg of *ras* recombinant protein expressed in *E. coli* were analyzed for GTP-binding as described in the legend to Fig. 4. Lanes: *ras*, c-Ha-*ras* recombinant protein; Dex, dextranosomes; Trit, tritosomes; PM, plasma membranes; Mic, microsomes; Cyt, cytosol; Mit, mitochondria; Px, peroxisomes; Nu, nuclei.

fractions. However, some GTP-binding activity was also found in the lower fraction, where most of the lysosomal marker enzyme but none of the marker enzymes for other organelles were localized.

When the bottom fraction was centrifuged for the second time, only the lysosomal markers, NAGA and FD, were found close to the bottom, and no markers for other organelles were detected (Fig. 3B). In this gradient, most of the GTP-binding activity was distributed along with the lysosomal markers (Fig. 4B). These results indicated the genuine localization of GTP-binding proteins on rat liver lysosomes.

Comparative study of GTP-binding proteins among various rat liver subcellular organelles. GTP-binding was assayed in various subcellular organelles isolated from the rat liver, including highly purified lysosomes (dextranosomes and tritosomes), plasma membranes,

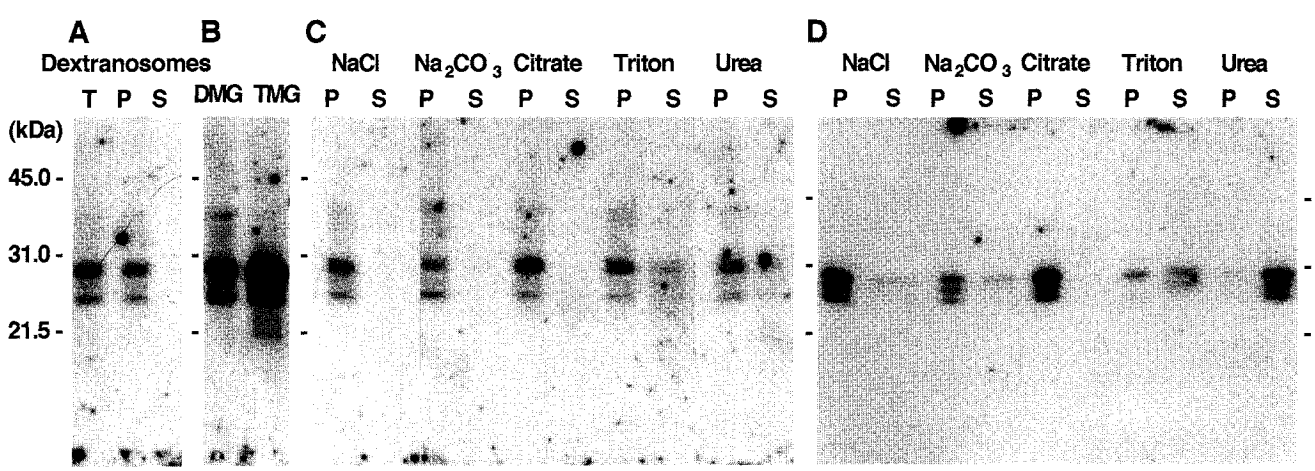

Fig. 6. Association of lysosomal GTP-binding proteins with membranes (A, B) and extraction of lysosomal and microsomal GTP-binding proteins from membranes (C, D). The following fractions were analyzed for GTP-binding proteins as described in the legend for Fig. 4. (A) Lysosomes (dextranosomes, 20 μg) were freeze/thawed five times and centrifuged at 100,000 × g for 60 min. The membrane precipitates were resuspended in the initial volume. Lanes: T, total dextranosomes; P, membrane precipitates; S, supernatant. (B) Lanes: DMG, membranes from dextranosomes (20 μg/lane); TMG, membranes from tritosomes (20 μg/lane). (C) Portions of the membrane precipitates (Panel A, 20 μg) were further extracted with 0.1 M NaCl, 0.1 M Na_2CO_3 (pH 11.5), 1 mM Na-citrate/acetate (pH 3.2), 1% Triton X-100 or 6 M urea. The precipitates (P) were separated from the supernatants (S) and resuspended in the initial volume. (D) Microsomes (20 μg) were extracted with the same solution as in (C).

microsomes, cytosol, mitochondria, peroxisomes, and nuclei, along with recombinant c-Ha-*ras* protein expressed in *E. coli* (Fig. 5). Relatively high GTP-binding activity was detected both in the plasma membrane and in microsomes that are known to contain small GTP-binding proteins. However, comparable or higher GTP-binding activity was detected in the dextranosomes and tritosomes. Faint bands were detected in the cytosol and mitochondria, the most faint being in the peroxisomes. On the other hand, GTP-binding activity was hardly detectable in the isolated nuclei. The molecular mass of the major GTP-binding proteins was quite similar among subcellular organelles, ranging from 26–30 kDa. On the other hand, purified *ras* protein showed considerable GTP-binding activity at a molecular mass of about 23 kDa under our conditions.

Association of GTP-binding proteins with lysosomal membranes and their extraction. Dextranosomes were further processed by five cycles of freeze-thawing followed by centrifugation to separate the membranes from the contents. Portions of the membrane pellets were extracted, along with microsomal fractions, with one of the following solutions; 0.1 M NaCl, 0.1 M Na_2CO_3 (pH 11.5), 1 mM sodium citrate/acetate (pH 3.2), 1% Triton X-100, or 6 M urea. As shown in Fig. 6, GTP-binding proteins were localized on lysosomal membranes and were not detectable in the lysosomal contents (Fig. 6A). Tritosomal membrane ghosts (TMG) contained comparable amounts of GTP-binding proteins to those of dextranosomal membranes (Fig. 6B). The GTP-binding proteins on dextranosomal membranes were partially extracted only by 1% Triton X-100 and 6 M urea (Fig. 6C). In contrast, the GTP-binding proteins in microsomes were extracted mostly and totally by 1% Triton X-100 and 6 M urea respectively, from their membranes (Fig. 6D). These results suggest that lysosomal GTP-binding proteins are closely associated with membranes, but are different from microsomal GTP-binding proteins in their solubility in urea.

DISCUSSION

In this study we identified GTP-binding proteins predominantly in the range of 26–30 kDa in lysosomal fractions, by specific binding of [α-^{32}P]GTP after SDS-PAGE and transfer to nitrocellulose. Longer exposure of the film with the blot also revealed the much smaller GTP-binding proteins of about 20–23 kDa in the lysosomal fractions (data not shown). All these proteins seem to have originated from the lysosomes themselves, because 1) they co-migrated with lysosomal markers during cell fractionation on Percoll density gradients, 2) they were closely associated with lysosomal membranes, which exclude nonspecific absorption of G-proteins from cytosol and/or other subcellular organelles, 3) they were associated with membranes in a mode that is different from that of microsomal GTP-binding proteins and 4) there was a relatively high abundance of GTP-binding proteins on lysosomal membranes on a protein basis, which also excludes the possibility that they originated from contamination by other subcellular organelles.

Several categories of guanine nucleotide-binding proteins have been identified so far, including the heterotrimeric G-proteins, the elongation and initiation factors of protein synthesis, and the *ras* superfamily. Relatively well understood are the high molecular weight (heterotrimeric) G-proteins, which regulate signal transduction at the plasma membrane (16). These GTP-binding proteins consist of α, β and γ subunits, among which, the α subunit (39–52 kDa) possesses the GTP-binding site.

Monomeric low molecular weight GTP-binding proteins are a second family. They have a molecular mass in the range of 20–30 kDa, and are characterized by marked sequence homology with the *ras* oncogene product. They have been categorized into three principle branches based on their degree of homology, namely, the *ras*, the *rho* and the *rab* families (14). Some of these proteins bind GTP after SDS-PAGE and transfer to nitrocellulose. This feature distinguishes them from their high molecular weight counterparts. The *ras* oncogene products, p21 *ras*, are associated with the plasma membrane and have a conserved carboxy terminal cysteine that is posttranslationally polyisoprenylated. In mammals they are implicated in cellular proliferation, terminal differentiation, transformation and other distinct biological processes including degranulation (4, 5) and perturbation of lysosomal pH (21). *Rho* proteins are ADP-ribosylated by the C3 exoenzyme of *Clostridium botulinum* in contrast to other *ras* superfamily members and may play a role in controlling the cytoskeletal organization of PC12 cells (27, 33). *Rab* proteins, which are distributed throughout the exocytic and endocytic pathways in a distinctive subcellular manner, are associated with the membranes, and are likely regulators of vesicular trafficking among compartments (for review, see 3). Unlike their distant cousins, the heterotrimeric G-proteins and the elongation and initiation factors of protein synthesis, the functions of the *ras* superfamily proteins are not well understood except that they are thought to be regulated by an exchange reaction of GDP/GTP.

Lysosomal GTP-binding proteins resemble these small GTP-binding protein families in the following respects: 1) they are detectable on nitrocellulose, 2) the molecular mass is in the range of 26–30 kDa, and 3) they are associated with membranes. However, closer association of lysosomal GTP-binding proteins are distinguishable from *rab*3A proteins on bovine adrenal chromaffin granules (14), *rab*6 protein on human HEp2

Golgi (19) and *SEC*4 protein on yeast secretory vesicles (18); namely, they were totally solubilized by Triton X-100 although partially or slightly extracted by urea. Furthermore, their molecular masses are higher than that of the *ras* product (Fig. 5). To identify the functions of lysosomal GTP-binding proteins, it is important to determine the family to which they belong. Therefore we are currently examining their reactivity to a variety of specific antibodies and various toxins. Our preliminary data indicated that lysosomal GTP-binding proteins detected on nitrocellulose in the range of 26–30 Da are neither reactive to anti-*ras* antibody nor ADP-ribosylated by the C3 exoenzyme of *Clostridium botulinum*. There are several possible roles of GTP-binding proteins on lysosomal membranes. 1) Direct or indirect effects on the proton-pump and/or ion-channels: GTP analogs affect the endosomal proton-pump (20) and increase lysosomal pH in *ras*-transformed NIH-3T3 cells (21). 2) Fusion of lysosomes with endocytotic vesicles, lysosomes themselves, autophagic vecuoles or plasma membranes (like endosome-endosome fusion (11, 24), and exocytosie (5)). 3) Sorting lysosomal proteins through fusion between lysosomes and transport vesicles derived from the Golgi apparatus (3). 4) Participation in membrane recycling between lysosomes and plasma membranes. 5) Regulation of lysosomal phospholipase and phospholipid kinase activity (like heterotrimeric G-proteins on the plasma membrane phospholipase C (17)). 6) Direct import of proteins to be degraded within lysosomes (as suggested for mitochondrial (23) and nuclear envelope (36) GTP-binding proteins).

Further studies are required to identify the vital functions played by lysosomal GTP-binding proteins in cellular physiology.

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