Identification of a Lysosome Membrane Protein Which Could Mediate ATP-dependent Stable Association of Lysosomes to Microtubules*

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We have previously reported that purified thyroid lysosomes bind to reconstituted microtubules to form stable complexes (Mithieux, G., Audebet, C., and Rousset, B. (1988) Biochim. Biophys. Acta 969, 121–130), a process which is inhibited by ATP (Mithieux, G., and Rousset, B. (1988) Biochim. Biophys. Acta 971, 29–37). Among detergent-solubilized lysosomal membrane proteins, we identified a 50-kDa molecular component which binds to preassembled microtubules. The binding of this polypeptide to microtubules was decreased in the presence of ATP. We purified this 50-kDa protein by affinity chromatography on immobilized ATP. The 50-kDa protein bound to the ATP column was eluted by 1 mM ATP. The purified protein, labeled with 125I, exhibited the ability of interacting with microtubules. The binding process was inhibited by increasing concentrations of ATP, the half-maximal inhibitory effect being obtained at an ATP concentration of 0.35 mM. The interaction of the 50-kDa protein with microtubules is a saturable phenomenon since the binding of the 125I-labeled 50-kDa protein was inhibited by unlabeled solubilized lysosomal membrane protein containing the 50-kDa polypeptide but not by the same protein fraction from which the 50-kDa polypeptide had been removed by the ATP affinity chromatography procedure. The 50-kDa protein has the property to bind to pure tubulin coupled to an insoluble matrix. The 50-kDa protein was eluted from the tubulin affinity column by ATP. These findings support the conclusion that a protein inserted into the lysosomal membrane is able to bind directly to microtubules in a process which can be regulated by ATP. We propose that this protein could account for the association of lysosomes to microtubules demonstrated both in vitro and in intact cells.

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Interphase microtubules constitute the tracks supporting the intracellular movements of organelles (1-10). Microtubules are also involved in the positioning of vesicles inside the cell. Indeed, the intracellular distribution of subcellular vesicles is dependent on an intact microtubule framework (11-18) suggesting that, when not moving, organelles can bind to microtubules in a stable manner. It is now admitted that the microtubule-based translocations of organelles involve cytosolic protein motor systems hydrolyzing ATP, such as kinesin (19-22). The molecular basis of stable association of microtubules is a saturable phenomenon since the binding of the 125I-labeled 50-kDa protein was decreased in the presence of ATP. We purified this 50-kDa protein by affinity chromatography on immobilized ATP. The 50-kDa protein bound to the ATP column was eluted by 1 mM ATP. The purified protein, labeled with 125I, exhibited the ability of interacting with microtubules. The binding process was inhibited by increasing concentrations of ATP, the half-maximal inhibitory effect being obtained at an ATP concentration of 0.35 mM. The interaction of the 50-kDa protein with microtubules is a saturable phenomenon since the binding of the 125I-labeled 50-kDa protein was inhibited by unlabeled solubilized lysosomal membrane protein containing the 50-kDa polypeptide but not by the same protein fraction from which the 50-kDa polypeptide had been removed by the ATP affinity chromatography procedure. The 50-kDa protein has the property to bind to pure tubulin coupled to an insoluble matrix. The 50-kDa protein was eluted from the tubulin affinity column by ATP. These findings support the conclusion that a protein inserted into the lysosomal membrane is able to bind directly to microtubules in a process which can be regulated by ATP. We propose that this protein could account for the association of lysosomes to microtubules demonstrated both in vitro and in intact cells.

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EXPERIMENTAL PROCEDURES

Purification and Solubilization of Lysosomal Membrane Protein—Lysosomes were purified from pig thyroid according to the procedure previously described (25) and modified by the use of a hyperosmotic medium from the beginning of the purification (23). After osmotic pressure-dependent lysis, the lysosomal membranes were sequentially washed in 10 mM Tris-HCl, pH 7.4 (buffer T), then in the same buffer containing 0.6 M NaCl, and again in buffer T. Washings were made after centrifugation of the membranes at 100,000 × g for 30 min at 4 °C. The final lysosomal membrane pellets were thoroughly resuspended in buffer T containing 0.5% CHAPS* at a concentration of 1 mg of protein/ml. The mixture was maintained at room temperature

* The abbreviations used are: CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonate; LMP, soluble lysosomal membrane protein; Mes, 4-morpholinolinosulfonic acid; EGTA, [ethylenebis(oxyethylene)]tetraacetic acid; BSA, bovine serum albumin; SDS, sodium dodeyl sulfate; PAGE, polyacrylamide gel electrophoresis.

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30 min, and then insoluble material was removed by centrifugation at 100,000 \( \times g \) for 30 min at 25 °C. Soluble lysosomal membrane protein (LMP) represented 35–45% of the starting membrane protein.

**Purification of Microtubule Proteins**—Microtubule protein was purified from rat brain by two cycles of temperature-dependent assembly-disassembly (26). Polymerizing steps were performed in buffer A (100 mM Mes, 1 mM EGTA, 0.5 mM MgCl\(_2\), pH 6.4) supplemented by 1 mM GTP and 4 mM glycerol. Tubulin was further purified from twice-cycled microtubule protein by phosphocellulose chromatography (27).

**Coupling of Tubulin to Affi-Gel 10**—Affi-Gel 10 (Bio-Rad) was sequentially washed with isopropyl alcohol and distilled water (5 ml/ml of gel). One mg of phosphocellulose-purified tubulin in buffer A was added to 1 ml of gel and placed at 4 °C for 4 h under gentle agitation. The mixture was then supplemented with 1 mM ethanamine, pH 8.0 (0.1 ml/ml of gel) for 1 h to block remaining reactive sites. The gel suspension was then placed in a column and washed with 10 bed volumes of buffer A.

**ATP Affinity Chromatography**—ATP affinity chromatography was performed using a column containing 1 ml of swollen ATP-agarose gel having an 8-carbon spacer (A 9264, Sigma). The LMP fraction (about 1 mg of protein) was diluted 5 times to reach a CHAPS concentration of 0.1% and applied on the gel at a flow rate of 1 ml/min at room temperature. The column was sequentially washed with: (a) 2 ml of buffer T containing 0.1 M Na\(_2\)CO\(_3\) and (c) 10 ml of buffer T. The column was then eluted with 2 ml of 1 mM ATP in buffer T. After extensive washing with buffer T containing 1.5 M NaCl, the ATP column was kept at 4 ºC in 0.05% azide in buffer T for reuse.

**Labeling Procedures**—Lysosome membranes were labeled with \(^{125}\)I using the Bolton-Hunter reagent (Amersham, Les Ulis, France). Pelleted membranes (1 mg of protein) were suspended in 50 mM phosphate buffer, pH 7.0, and incubated with 0.5 mcg of \(^{125}\)I-labeled Bolton-Hunter reagent. After 30 min at 0 ºC, the reaction was stopped by the addition of 10 mM glycine. \(^{125}\)I-Labeled membranes were washed once in buffer T and \(^{125}\)I-labeled membrane proteins (125-I-LMP) were extracted by CHAPS treatment as described above. The specific radioactivity of 125-I-LMP was 0.15 mcg/mg of protein.

The ATP affinity-purified fraction, desalted and concentrated, was labeled with \(^{125}\)I using the IODO-GEN reagent (Pierce, Cambridge, United Kingdom). Two mg of the ATP-purified protein in 50 \( \mu l \) of buffer T were mixed with 0.25 mg of \(^{125}\)I-iodide in a conical tube coated with IODO-GEN. After 2 min at 0 ºC, the mixture was diluted to 500 \( \mu l \) with buffer T and transferred on a PD-10 Sephadex column preequilibrated with BSA to separate residual \(^{125}\)I-iodide from the labeled protein. \(^{125}\)I-Labeled ATP affinity-purified protein (specific activity 300-500 mcg/mg) was recovered in liquid nitrogen in the presence of 0.5 mg/ml BSA in buffer T.

**Microtubule Binding Experiments**—Microtubules (2.5 mg of microtubule protein/ml) were assembled for 30 min at 37 ºC and sheared by four to five passages through the needle (25 G) of a syringe and kept for 5 min at 25 ºC. One-hundred \( \mu l \) of the microtubule suspension were mixed with the \(^{125}\)I-labeled protein (either LMP or ATP affinity-purified protein) in a total volume of 400 \( \mu l \) of buffer A containing 10 mg/ml BSA. When present, ATP was added just before microtubules. After 30 min at 25 ºC, the incubation mixtures were layered on a 1 M NaCl cushion in buffer T. Pellets and supernatants were counted for radioactivity in a Packard scintillation \( \gamma \) counter and analyzed for protein by SDS-PAGE and autoradiography.

**Other Methods**—Protein was assayed according to the method of Lowry using BSA as a standard. PAGE was performed on 9% polyacrylamide gel in the presence of SDS according to Laemmli (28) including slight modifications (29).

**RESULTS**

**Attempts to Evidence Solubilized Lysosomal Membrane Protein with Microtubule Binding Activity**—Microtubules were incubated with \(^{125}\)I-LMP, in the absence or in the presence of ATP, with the aim to detect LMP component(s) which could bind to microtubules in a process dependent on ATP. SDS-PAGE and autoradiography analyses of the labeled polypeptide composition of microtubule pellets revealed that only few polypeptides of LMP fraction associated to preformed microtubules. They represented about 8% of the total \(^{125}\)I-LMP radioactivity. In the absence of microtubules, no significant radioactivity could be observed as sedimentable material. In the presence of 1 mM ATP, the amount of \(^{125}\)I-labeled polypeptide bound to microtubules was decreased by about 40% (range 30–61), as estimated by densitometric analysis (Fig. 1). This effect of ATP was selective since ATP did not significantly decrease the radioactivity associated with the microtubule pellet. Since ATP in the same concentration range was shown to prevent the formation of microtubule-lysosome complexes (24), this result strongly suggested that this polypeptide, the molecular mass of which was about 50 kDa, could account for the binding of intact lysosomes to microtubules.

**Purification of the 50-kDa Protein by ATP Affinity Chromatography**—Data presented above indicate that the 50-kDa polypeptide likely exhibits binding site(s) for ATP. We have thus tried to purify this protein by specific retention-elution on an ATP-agarose matrix. LMP were applied on the ATP affinity column. Several LMP were retained on the ATP-agarose matrix. Most of them were found in the 0.1 M NaCl wash, an elution step carried out to elute proteins bound to the affinity matrix via electrostatic interactions. The elution of the column with 1 mM ATP yielded a protein fraction which contained a major polypeptide (Fig. 2). This polypeptide co-migrated on SDS-PAGE with the 50-kDa component identified in Fig. 1. It is noteworthy that neither AMP nor ADP at the same concentration gave rise to the elution of this polypeptide. The 50-kDa polypeptide represented about 80% of the protein of the ATP-eluted fraction (BSA excluded) as judged by Coomassie Blue staining. From protein determinations and densitometric analysis, we estimated that about 1 \( \mu g \) of the 50-kDa polypeptide was obtained from 1 mg of total membrane protein.

**Interaction of the 50-kDa ATP Affinity-purified Polypeptide with Microtubules**—The protein fraction, purified on the ATP affinity column, which mainly contained the 50-kDa polypeptide, was labeled with \(^{125}\)I and studied for its capacity to bind with microtubules. In the absence of ATP, 25 ± 1.5% (n = 4) of the total radioactivity of the \(^{125}\)I-labeled ATP affinity-purified protein fraction was found in the microtubule pellet. No radioactivity was sedimentable when microtubules were not present. Upon addition of increasing concentrations of ATP (0.1–5 mM), the radioactivity bound to microtubules progressively decreased to a value of about 8%. The concentration of ATP which induced a 50% decrease of the binding was about 0.35 mM whereas 80–85% of the maximal inhibition of the binding was obtained at an ATP concentration of 1 mM. PAGE and autoradiography analyses revealed that the radioactivity bound to the microtubule pellets corresponded to \(^{125}\)I-labeled 50-kDa polypeptide (Fig. 3C). The \(^{125}\)I-labeled ATP affinity-purified protein fraction also contained a small amount of BSA used as carrier protein during the purification steps (see the legend of Fig. 2) and some other contaminants in trace amounts (Fig. 3B). These contaminants did not bind to microtubules and remained in the supernatants, irrespective of the ATP concentration (Fig. 3D).

The amount of \(^{125}\)I-labeled 50-kDa polypeptide bound to microtubules as a function of ATP concentration was deter-
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**FIG. 1. Identification of a lysosomal membrane protein which binds to microtubules in an ATP-dependent manner.** $^{125}$I-LMP (1.15 mCi, 8 μg of protein) were incubated with preassembled microtubules (250 μg of microtubule protein) in the absence and in the presence of 1 mM ATP for 30 min at 25 °C. Microtubules were pelleted by centrifugation and analyzed by PAGE in the presence of SDS and autoradiography. Left panel, Coomassie Blue staining of the protein present in the pellet in the absence of ATP (lane a) and in the presence of ATP (lane b). Lanes c, d, and e, autoradiographic analysis of total $^{125}$I-LMP (c), and $^{125}$I-LMP present in the microtubule pellet in the absence of ATP (d) and in the presence of ATP (e). Half of the pellets was analyzed in each case. Numbers on the right refer to the migration of molecular weight markers. The arrowheads indicate the top of the gel and the front of migration. The arrow points to the polypeptide with ATP-dependent microtubule binding activity. Right panel, densitometric scans of lane d (control) and lane e (ATP).

**FIG. 2. Purification of the 50-kDa polypeptide by ATP affinity chromatography.** Analysis by SDS-PAGE. Lysosomal membranes (2.5 mg of protein) were solubilized by CHAPS; 1.1 mg of LMP were recovered and applied onto the ATP column. Lane a, total purified lysosomes (150 μg of protein loaded on the gel); lane b, soluble lysosomal protein (150 μg of protein); lane c, total lysosomal membranes (150 μg of protein); lane d, lysosomal membrane protein not solubilized by CHAPS (150 μg of protein); lane e, total LMP fraction (150 μg of protein); lane f, LMP fraction not retained by the column (100 μg of protein); lane g, protein fraction retained on the column and eluted by 100 mM NaCl (the total fraction loaded on the gel: 70 μg); lane h, protein fraction eluted by 1 mM ATP (totality loaded). From densitometric analysis, it was estimated that the 50-kDa polypeptide present in the ATP eluate (indicated by the arrow) represents 2–3 μg of protein. The other polypeptide seen in lane d is BSA used as carrier protein to coat the tubes and saturate the PD-10 column during the preparation of the ATP-eluted fraction; a significant loss of the 50-kDa protein was observed in the absence of carrier protein.

Analyzing the radioactivity of the 50-kDa labeled spots on polyacrylamide gels. The 50-kDa polypeptide contained 38% of the $^{125}$I radioactivity incorporated into protein of the ATP-eluted fraction by the IODO-GEN labeling procedure. In the absence of ATP, 65–70% of the $^{125}$I-labeled 50-kDa polypeptide were able to bind to microtubules (a value in complete agreement with the percentage of the radioactivity of the $^{125}$I-labeled ATP affinity-purified protein fraction which was found associated with microtubules, i.e. 25%). Increasing ATP concentration (0–5 mM) progressively decreased the binding of the $^{125}$I-labeled 50-kDa protein to microtubules from 65–70% to 15–20%. A representative experiment is shown in panel A of Fig. 3.

The binding of the $^{125}$I-labeled 50-kDa polypeptide to microtubules was a very rapid process; the binding was maximum after 5 min of incubation before centrifugation and remained constant for at least 45 min. The addition of ATP after 15 min of incubation of microtubules with the $^{125}$I-labeled ATP affinity-purified protein fraction induced the dissociation of the $^{125}$I-labeled 50-kDa polypeptide from microtubules; binding values resulting from radioactivity measurements were the same when ATP was present since the beginning of the incubation or added after 15 min.

The binding of the $^{125}$I-labeled 50-kDa polypeptide to microtubules was significantly decreased in the presence of purified unlabeled 50-kDa protein at a concentration of 1 μg/ml; it was also inhibited by the addition of unfractionated LMP at a concentration expected to contain about 1 μg/ml 50-kDa polypeptide, but not by the LMP fraction lacking the 50-kDa polypeptide (removed by filtration on the ATP affinity column). Results of a representative experiment are reported in Table I. These data indicate that the interaction of the 50-kDa protein with microtubules is a saturable phenomenon.

The 50-kDa Protein Binds to Pure Tubulin in an ATP-dependent Manner—In order to test whether the 50-kDa polypeptide could bind to microtubules through a direct interaction with tubulin dimers, we have studied the interaction of the 50-kDa polypeptide with pure tubulin immobilized on an agarose matrix. The ATP affinity-purified protein fraction was mixed with BSA as a carrier and applied to the tubulin affinity column. The results reported in Fig. 4 show that BSA was not retained by the tubulin column. On the contrary, the 50-kDa polypeptide interacted with the affinity matrix; it was
not eluted by extensive washing with buffer T. The 50-kDa polypeptide was eluted from the tubulin affinity column with 1 mM ATP.

**DISCUSSION**

In our preceding papers, we reported the characteristics of the interaction phenomenon taking place *in vitro* between highly purified lysosomes and reconstituted microtubules (23, 24). In this work, we report the identification of a lysosomal membrane protein (*a*) which exhibits an ATP-binding site and a tubulin-binding site, and (*b*) which is able to bind to preformed microtubules in an ATP-dependent manner. This protein has the property of an integral membrane protein since: (i) it remains associated to the lysosomal membrane through multiple steps of sedimentation along the lysosome purification procedure; (ii) it is not removed from the membrane by high salt treatment (0.6 M NaCl); and (iii) it is solubilized by means of a detergent. The 50-kDa protein, inserted into the lysosomal membrane, may account by itself for the *in vitro* interaction of lysosomes with microtubules. It is noteworthy that the ATP-dependent inhibition of the interaction of the 50-kDa polypeptide with microtubules and the ATP-dependent inhibition of the formation of lysosome microtubule complexes appeared as closely related phenomena. Indeed, the two ATP effects were observed in the same

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**TABLE I**

**Competitive binding between labeled and unlabeled 50-kDa polypeptide**

| Protein fraction added | Concentration *125I*-labeled 50-kDa protein bound to microtubules |
|------------------------|---------------------------------------------------------------|
|                        | **µg/ml** | % of control |
| None                   | 100       |
| LMP                    | 50 (0.125) | 97          |
|                        | 460 (1.15) | 42          |
| LMP*                   | 50        | 102         |
|                        | 460       | 96          |
| 50-kDa ATP affinity-purified protein | 0.1 | 100 |
|                        | 1.0       | 36          |

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**Fig. 3. ATP-dependent interaction of the 50-kDa protein with microtubules.** The *125I*-labeled ATP affinity-purified protein fraction (0.2 µCi, 70 ng of protein) was incubated in buffer A containing BSA (10 mg/ml) for 30 min at 25 °C with preassembled microtubules (250 µg of microtubule protein) in the absence and in the presence of increasing concentrations of ATP. Microtubules were then sedimented, and the pellets were counted for radioactivity. Half of the microtubule pellets and a fourth of the supernatants (to avoid the overloading of the gel with BSA) were analyzed by SDS-PAGE and autoradiography. To allow a quantitative comparison of autoradiograms, the time of exposure of gels corresponding to supernatants was 20 times higher than that of the gels of microtubule pellets. The *125I*-labeled 50-kDa protein was quantified in pellets and supernatants by counting the radioactivity of the 50-kDa polypeptide spots on polyacrylamide gels. Panel A, percent of 50-kDa protein found in the microtubule pellets versus ATP concentration. Panel B, autoradiogram showing the composition of the *125I*-labeled ATP affinity-purified fraction. Panel C, autoradiographic analysis of microtubule pellets resulting from incubations in the presence of various concentrations of ATP: 0, 0.1, 0.5, 1, 2, and 5 mM ATP from the left lane to the right lane, respectively. Panel D, autoradiographic analysis of the corresponding supernatants.

**Fig. 4. Interaction of the 50-kDa polypeptide with pure tubulin coupled to Affi-Gel 10.** The ATP affinity-purified protein fraction obtained from 2 mg of membrane protein (corresponding to about 2 µg of the 50-kDa polypeptide) was mixed with 60 µg of BSA in a total volume of 500 µl of buffer T and applied on the tubulin affinity column (1 ml of gel). The flow-through was collected within 1 ml. After washing with 20 ml of buffer T, the column was eluted with 1 ml of 1 mM ATP in buffer T. The flow-through fraction and the ATP-eluted fraction were analyzed by SDS-PAGE. Lane a, 60 µg of BSA and 2 µg of the 50-kDa protein were loaded as a control; lane b, fraction which flowed through the column; lane c, fraction eluted by ATP.
The interaction of the 50-kDa polypeptide with microtubules is a saturable process. A concentration of 1 μg/ml unlabeled 50-kDa protein decreased the binding of 125I-labeled 50-kDa protein by about 60%. The apparent dissociation constant of the 50-kDa protein-microtubule complex should thus be close to or slightly lower than 2 × 10^{-8} M.

We propose that this 50-kDa protein could be involved in the establishment of stable associations of lysosomes to microtubules which could determine the intracellular distribution of lysosomes. The positioning of lysosomes inside the cell has indeed been reported to be highly dependent upon the microtubular organization (17, 30–34). Lysosomes are generally clustered close to the centrosome in a region of the cell which is characterized by a high density of microtubules.

The present results support and further substantiate our previous tentative model (24) in which we proposed that a given vesicle could be subjected to two different types of interaction with microtubules: stable association or motile association. The shift from one type of association to the other could be regulated. In this respect, it is interesting to notice that ATP (without hydrolysis) which causes the dissociation of lysosome-microtubule stable complexes (24) promotes the formation of “transient” vesicle-microtubule complexes and sustains vesicle translocation through its hydrolysis (2, 6, 19, 21). A given regulatory factor could, in this way, control two complementary mechanisms ensuring the distribution and the dynamics of vesicles. Different aspects of our model are in keeping with previous findings from other laboratories. The requirement for a specific mechanism for organelle segregation around the centrosome has been put forward by Matteoni and Kreis (31). The activity of ATP as a dissociating agent for microtubule-secretory granule complexes was previously reported by Sherline et al. (35) and Suprenant and Dentler (36).

Motile microtubule vesicle associations and stable microtubule vesicle associations would take place through different molecular components. The former process involves translocator units such as kinesin (19–22) and microtubule-associated protein 1C (37, 38) corresponding to soluble or cytosolic protein components when not operative; the latter process could be based on anchoring factors, present on the vesicle membrane, such as the 50-kDa protein complexes (24) described here. The functions of these factors would be to mediate the adhesion and then the immobilization of the vesicles onto the microtubules. We recently proposed to name such proteins immobilins (17). There is now a need for similar studies on other intracellular vesicles to determine whether the 50-kDa protein or another protein with a similar function exists on the membrane of other organelles, which could be subjected to immobilization or movement depending on regulatory signals.

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