Proc. Natl. Acad. Sci. USA
Vol. 89, pp. 7026–7030, August 1992
Cell Biology

A role for microtubules in sorting endocytic vesicles in rat hepatocytes

(receptor-mediated endocytosis/cytoplasmic dynein/vesicular transport/asialoglycoprotein)

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Communicated by G. Gilbert Ashwell, April 6, 1992

ABSTRACT **The vectorial nature of hepatocyte receptor-mediated endocytosis (RME) and its susceptibility to cytoskeletal disruptors has suggested that a polarized network of microtubules plays a vital role in directed movement during sorting. Using as markers a well-known ligand, asialoorosomucoid, and its receptor, we have isolated endocytic vesicles that bind directly to and interact with stabilized endogenous hepatocyte microtubules at specific times during a synchronous, experimentally initiated, single wave of RME. Both ligand- and receptor-containing vesicles copelleted with microtubules in the absence of ATP but did not pellet under similar conditions when microtubules were not polymerized. When 5 mM ATP was added to preparations of microtubule-bound vesicles, ligand-containing vesicles were released into the supernatant, while receptor-containing vesicles remained immobilized on the microtubules. Release of ligand-containing vesicles from microtubules was prevented by monensin treatment during the endocytic wave. Several proteins, including the microtubule motor protein cytoplasmic dynein, were present in these preparations and were released from microtubule pellets by ATP addition concomitantly with ligand. These results suggest that receptor domains within the endosome can be immobilized by attachment to microtubules so that, following monensin-sensitive dissociation of ligand from receptor, ligand-containing vesicles can be pulled along microtubules away from the receptor domains by a motor molecule, such as cytoplasmic dynein, thereby delineating sorting.**

Receptor-mediated endocytosis (RME) of asialoglycoproteins in hepatocytes is characterized by the vectorial movement of ligand-containing vesicles through the cytoplasm. The ligand–receptor complex is internalized from the cell surface into an acidic late endosomal compartment where the receptor and ligand dissociate and segregate, resulting in movement of ligand-containing vesicles onward toward lysosomal fusion, while receptor-containing vesicles recycle. Although the time course of compartmentalization and ligand–receptor sorting in this process has been well characterized (1–4), the mechanisms by which sorting is coupled to directed vesicular movement have been elusive. In previous studies (1, 2, 5, 6), reagents were developed to follow asialoglycoprotein and its receptor independently through the intracellular pathway.

The endocytic pathway is more or less linear and can be disrupted by drugs such as nocodazole or colchicine (1, 7, 8), suggesting that microtubules participate in the sorting and directed movement of endocytic vesicles (8–17). Microscopic immunolocalization of endosomes and lysosomes in association with microtubules has been demonstrated (8). Microtubule motor molecules, such as cytoplasmic dynein, which is present in hepatocyte cytoplasm (18, 19), have been shown to support ATP-sensitive, directional movement of vesicles and beads on taxol-stabilized microtubules (20, 21). In this study, *in vitro* affinity methods that have been used previously to study motor molecule association with microtubules (21–23) have been modified and extended to investigate the interaction of microtubules with endocytic vesicles containing asialoglycoprotein and/or its receptor. We demonstrate the direct binding of endosomal vesicles to microtubules in a manner that suggests a role for microtubules in segregation of ligand and receptor.

EXPERIMENTAL PROCEDURES

Materials. Human asialoorosomucoid (ASOR) was prepared from human orosomucoid (Sigma) by neuraminidase digestion (1) and either labeled with ^{125}I by a chloramine-T method (1) to a specific activity of 4000 cpm/ng for cell fractionation studies or electrostatically coupled to colloidal gold particles (24) for cellular localization studies. Taxol was a kind gift from Susan Horwitz (Albert Einstein College of Medicine). Rat hepatocytes were isolated and cultured as described (1, 5, 6).

Cell Incubation and Uptake of Ligand. Overnight cultured rat hepatocytes were washed twice with serum-free medium (SFM) (135 mM NaCl/1.2 mM $MgCl_2$/0.81 mM $MgSO_4$/27.8 mM glucose/2.5 mM $CaCl_2$/25 mM Hepes, pH 7.2) at 4°C. In some studies, gold–ASOR (1 μg/ml) was added to washed cells; otherwise, ^{125}I-ASOR (1 μg/ml) in 3 ml of SFM was added to each culture dish, which was incubated at 4°C for 60 min (1, 2, 5). Unbound ligand was removed by two washes with SFM, and cells were then incubated at 37°C for 45–60 min to permit a wave of endocytosis to proceed. After 60 min, cells that endocytosed gold–ASOR were fixed at room temperature and prepared for transmission electron microscopy as described (25). Cells allowed to internalize ^{125}I-ASOR were placed at 4°C to halt endocytosis (this also disrupts endogenous microtubules) and washed twice in SFM. In some experiments, cells were allowed to internalize ^{125}I-ASOR in the presence of the proton ionophore monensin (50 μM), which was added just prior to a 60-min incubation at 37°C.

Cell Fractionation and Isolation of Microtubule-Bound Components. After endocytosis was halted, cells were washed with SFM and then homogenized (20 strokes; tight Dounce) on ice in an equal volume of MEPS buffer (5 mM $MgSO_4$/5 mM EGTA/35 mM K^+Pipes/0.2 M sucrose, pH 7.1) containing 1 mM dithiothreitol and protease inhibitors (21). The homogenate was centrifuged for 10 min at 1000 ×

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Abbreviations: ASOR, asialoorosomucoid; RME, receptor-mediated endocytosis.

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g followed by 20 min at 40,000 × *g*. The supernatant was incubated at 37°C for 30 min with 1 mM GTP and 10 μM taxol to promote polymerization of endogenous tubulin. The suspension containing polymerized microtubules was then subjected to an intermediate centrifugation (19,000 × *g*; 15 min). This 19,000 × *g* pellet was washed three times by centrifugation and resuspension in MEPS buffer containing 10 μM taxol and was assayed by negative-stain electron microscopy (26) and SDS/PAGE to assess microtubule polymerization and the major structural and identifiable components associated with microtubules in the pellet. Bound vesicles were quantified by counting numbers of vesicles attached to microtubules in random fields and dividing by the total length of microtubules in the same field. Averages are from three experiments. Released vesicles were quantified by counting numbers of vesicles in random fields and dividing by the total area (in μm^2) per field. Averages are from two experiments with 11 fields total. The final 19,000 × *g* pellet was resuspended and repelleted in the presence or absence of 5 mM ATP and assayed as described above to determine the ATP sensitivity of vesicles bound to microtubules. ^{125}I-ASOR in various fractions was quantified or subjected to autoradiography. Asialoglycoprotein receptor was determined by immunoblot analysis (6); 1% Triton X-100 was added to some preparations for 10 min before centrifugation to ascertain the patency of components within membranes. In some experiments, vesicles released by ATP were treated on ice for 30 min with trypsin (100 μg/ml) (Sigma) in either the presence or the absence of 0.1% Triton X-100. Under these conditions, exposed ASOR is degraded as estimated by densitometry of an autoradiograph.

RESULTS

Interaction of ASOR-Containing Vesicles with Microtubules *in Situ*. As a control for our *in vitro* procedures, we localized ASOR in cultured hepatocytes morphologically 60 min after initiation of a single wave of endocytosis of gold-linked ASOR. Electron-opaque gold particles were present within vesicular structures, typical of late endosomes in these cells (Fig. 1). This is consistent with previous results (1) indicating that, at this time point, the majority of ligand and receptor is segregated in a late endosomal compartment, and ≈30% of ligand has reached the lysosomes. Microtubules, identified by their diameters and lengths, were observed in close proximity to the endocytic structures containing gold particles. In some instances, ligand was segregated to one pole of an endosome associated closely with a microtubule (Fig. 1, arrow).

Interaction of Microtubules with Endocytic Vesicles *in Vitro*. Our strategy involved isolating taxol-stabilized endogenous microtubules 60 min after the synchronous initiation of endocytosis of ^{125}I-ASOR. After 60 min, endocytosis was stopped and a 40,000 × *g* 20-min supernatant containing small vesicles and cytosol was obtained. As demonstrated in previous work (1, 2, 6) and by studies described below, a population of these vesicles represented endosomes containing ligand and/or receptor. When taxol was added to this 40,000 × *g* supernatant under polymerizing conditions, microtubules re-formed with an average length of >5 μm and in sufficient numbers for use in these studies. When the microtubules were pelleted at a lower force (19,000 × *g* for 15 min), resuspended, and washed extensively by subsequent centrifugation and resuspension, small vesicles consistently attached to and pelleted with them (Fig. 2*a*). Vesicles were not bound to other cytoskeletal elements, such as intermediate or actin filaments, as determined by negative-stain electron microscopy, and they were not pelleted in the absence of taxol under depolymerizing conditions for microtubules (data not shown). In electron micrographs (Fig. 2*a*), the microtubule-associated vesicles were somewhat heterogenous in size and appearance. They aligned along microtubules at an

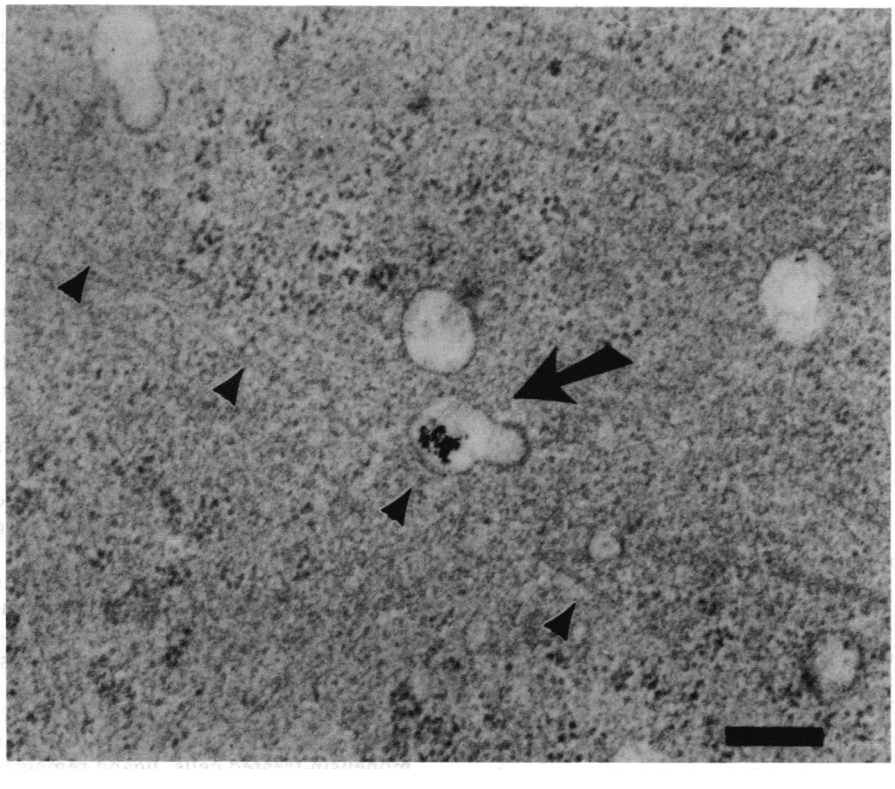

FIG. 1. Transmission electron micrograph of endocytosed gold–ASOR. After 60 min of single-wave endocytosis of gold-conjugated ASOR, endosomal vesicles containing label are found in close proximity to microtubules (arrowheads). Selected images (arrow) show label segregated to one pole of the endosome. (Bar = 200 nm.)

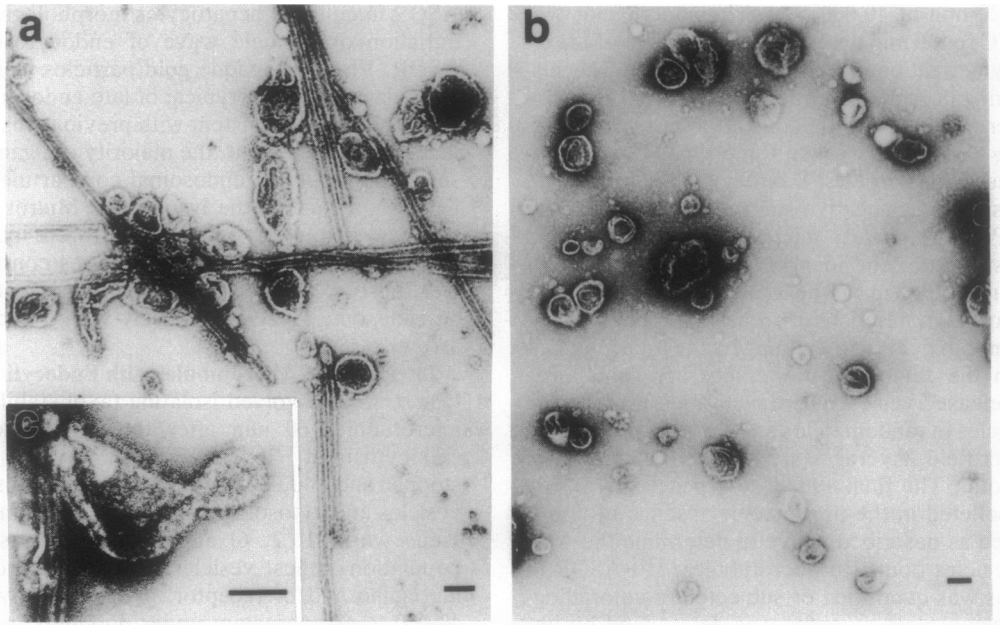

FIG. 2. Negative-stain electron micrographs of hepatocyte vesicles bound to microtubules and released by ATP. After 60 min of single-wave endocytosis of ASOR, cells were homogenized and a 40,000 × *g* supernate was prepared. Taxol was added and taxol-solubilized microtubules pelleted at 19,000 × *g*. (*a*) The 19,000 × *g* pellet washed three times in MEPS buffer containing 10 μM taxol. Several classes of vesicles repeatedly copellet with the microtubules. Some vesicles seem directly attached to the microtubule wall. (*b*) Supernate after addition of 5 mM ATP. A subpopulation of the vesicles is released. (*c*) The pellet after ATP treatment still contains vesicles bound to microtubules. In selected images, some vesicles that are attached to microtubules resemble the *in situ* endosome. (Bars = 100 nm.)

average density of 1.6 per μm. Some vesicles bound *in vitro* to microtubules resembled strongly those seen *in vivo* (compare Figs. 1 and 2*c*).

Analysis of the microtubule pellet for endocytosed components revealed that populations of vesicles containing ^{125}I-ASOR associated with microtubules. This attachment was stable after several washes by resuspension (Fig. 3*a*, lane 2). Endocytic vesicles containing receptor were also identified in the washed microtubule pellet by immunoblot (Fig. 3*b*, lane 2) using a polyclonal antibody raised in rabbit that has been shown to primarily recognize the H1 subunit of the asialoglycoprotein receptor (6). In the absence of taxol under depolymerizing conditions for microtubules, neither ^{125}I-ASOR nor its receptor was detected in the pellet. If endocytosis was stopped 5 min after initiation, little or no internalized ASOR pelleted with the microtubules (data not shown). The supernate of the final microtubule wash contained few vesicles (2.2 ± 1.6 per μm^2) and undetectable ^{125}I-ASOR or receptor. Therefore, some late endocytic vesicles containing ligand and/or receptor bound tightly to microtubules *in vitro*, perhaps by connections representing components of the mechanism by which these vesicles segregate and sort.

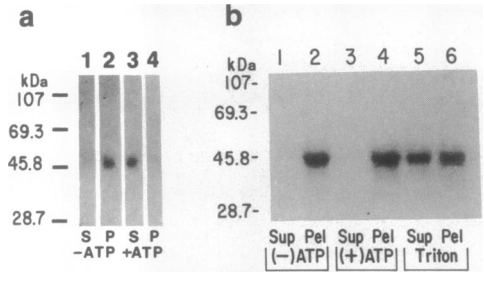

FIG. 3. Assay of receptor and ligand content of vesicles bound to microtubules 60 min after initiation of endocytosis. (*a*) Autoradiographic analysis of the ligand ^{125}I-ASOR in the fractions corresponding to Fig. 2. SDS/7.5% PAGE. Ligand is not detected in the supernatant after buffer wash (−ATP, lane 1), but it is found in the 19,000 × *g* pellet (lane 2). When 5 mM ATP is added, ligand moves to the supernatant (+ATP, lane 3). Thus, ligand-containing vesicles are bound to microtubules in an ATP-sensitive manner. (*b*) Immunoblot analysis of the asialoglycoprotein receptor. Parallel samples from SDS/10% PAGE are subjected to immunoblotting (6). Receptor also pellets in the 19,000 × *g* fraction in the absence of ATP (−ATP, lane 2) and is not detected in the supernatant (lane 1). When 5 mM ATP is added, in contrast to ligand, receptor remains wholly in the pellet (+ATP, lane 4). Addition of 1% Triton X-100 releases a portion of the receptor into the supernatant (lane 5). Thus, vesicles containing receptor are bound to the microtubules in an ATP-insensitive manner.

Influence of ATP on Vesicle Binding to Microtubules. Resuspending the pellet in buffer containing 5 mM ATP resulted in release of a subpopulation of vesicles (3.8 ± 0.6 per μm^2) (Fig. 2*b*), representing ≈20% of the total bound vesicles as determined morphometrically, and a substantial portion of the ^{125}I-ASOR. After ATP treatment, there was a reduction in the amount of ligand remaining with the microtubule pellet (Fig. 3*a*, lane 4), indicating that the population of ligand-containing vesicles was specifically and selectively released by ATP. Ligand was protected from limited proteolysis by trypsin but >70% degraded when treated with trypsin in the presence of 0.1% Triton X-100, indicating that it was contained within the released vesicles.

In contrast, the receptor remained microtubule associated and was insensitive to release by 5 mM ATP (Fig. 3*b*, lane 4). Extraction of the ATP pellet in 1% Triton X-100, followed by centrifugation, resulted in release of much of the receptor into the supernatant, showing that the receptor was largely, if not entirely, contained in the pelleting vesicles.

Role of Ligand–Receptor Segregation on Vesicle Binding to Microtubules. Monensin treatment used to prevent acidification and subsequent ligand–receptor dissociation (5, 6) during endocytosis did not affect the initial binding of ligand- or receptor-containing vesicles present in the 19,000 × *g* pellet. When ATP was added to the microtubule pellet derived from monensin-treated cells, ligand remained with the pellet (Fig. 4). Ligand could then be released into the supernate by the addition of Triton X-100, consistent with the release of receptor under similar conditions (Fig. 3*b*).

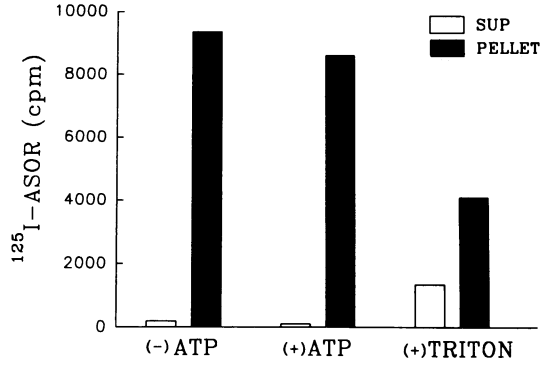

FIG. 4. Effect of monensin on ligand release by ATP. Monensin (50 μM) added at the initiation of single-wave endocytosis of ^{125}I-ASOR remained in the solution until the homogenization step at 60 min. (−)ATP, ^{125}I-ASOR was found primarily in the 19,000 × *g* pellet; (+)ATP, ^{125}I-ASOR was not released into the supernate; (+)Triton, addition of 1% Triton X-100 released about one-quarter of the label, consistent with the release of receptor shown in Fig. 3*b*.

Identification of Cytoplasmic Dynein. Since ATP-sensitive attachment of vesicles to microtubules could be mediated in part by microtubule motor proteins, we assayed our preparations for cytoplasmic dynein. SDS/PAGE analysis revealed that cytoplasmic dynein was released by ATP from microtubules (Fig. 5*a*) along with ligand-containing vesicles. Cytoplasmic dynein was identified (*i*) by heavy-chain comigration with similar chains from protozoan axonemal dynein, (*ii*) by ATP-sensitive release from microtubules (22), and (*iii*) by vanadate-sensitive UV photocleavage (27). UV photocleavage at the V1 site (Fig. 5*b*) reduced the amount of cytoplasmic dynein heavy chain and gave rise to two new fragments at ≈260 and ≈195 kDa, consistent with previously reported values (28). In addition, several other unidentified proteins were released from microtubules by ATP (Fig. 5), including bands at 210 and 110 kDa. The 210-kDa protein may be MAP 4 (18).

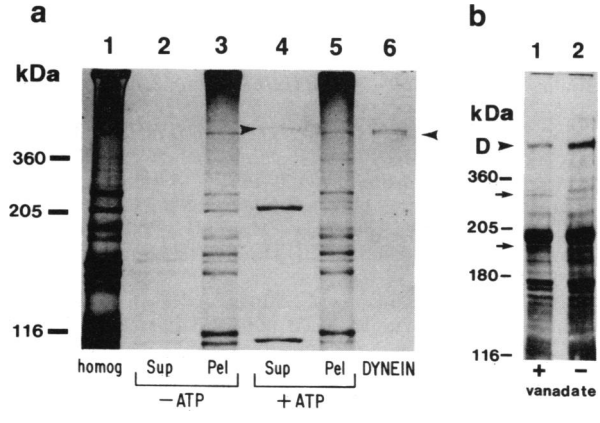

FIG. 5. Identification of cytoplasmic dynein. (*a*) Release of dynein heavy-chain parallels release of ligand-containing vesicles. Coomassie blue-stained SDS/5% PAGE of fractions corresponding to Fig. 3*a*. In the presence of 5 mM ATP (+ATP, lane 4), large proteins that comigrate with 22S axonemal dynein standards (lane 6) (22) are released into the supernate. (*b*) Vanadate-sensitive UV photocleavage of hepatocyte cytoplasmic dynein. Silver-stained SDS/4% PAGE. The MEPS supernate released in the presence of ATP was subjected to V1 UV photocleavage in the presence of 50 μM sodium vanadate (27). After 60 min of UV exposure in the presence of vanadate (lane 1), the intensity of the dynein heavy chain(s) (D) is reduced as compared to a control after UV exposure in the absence of vanadate (lane 2). Cleavage products are seen only in lane 1 as indicated by arrows. Equal volumes of ATP supernate were loaded in both lanes.

DISCUSSION

Interaction of various galactose-terminating glycoproteins with their specific hepatocyte receptor results in internalization of the ligand–receptor complex and processing along the pathway of RME. This pathway is vectorial and can be divided into a number of discrete but interrelated steps (Fig. 6). It stands to reason that if polarized microtubular networks are organized within the cell, then polarized processes such as receptor–ligand segregation may be orchestrated into this network. This is an idea shared by many groups (1, 9–11, 15, 18, 20). Our results suggest that segregation of ligand and receptor into functionally distinct late endosomal compartments occurs in association with microtubules. We hypothesize that receptor-containing domains of the segregating endosome bind to microtubules and are temporarily immobilized, while ligand-containing domains bind to microtubules through another mechanism that permits their release in the presence of ATP. This *in vitro* ATP-mediated release of microtubule-bound vesicles is characteristic of microtubule motor-based motility systems (8, 9, 20, 23). Although only 20% of the vesicles bound to microtubules are released by ATP in these experiments, this population includes a significant amount of the ligand-containing vesicles but virtually none of the receptor-containing vesicles, suggesting that vesicle binding to microtubules is a specific process that occurs during a limited time along the endocytic path. This could account for the relatively tight association of only a fraction of the ligand- or receptor-containing vesicles with microtubules, while the majority of vesicles would have

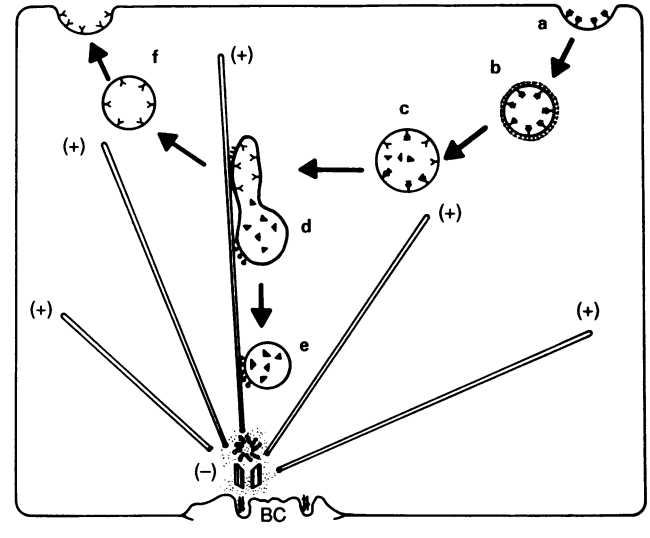

FIG. 6. Hypothetical pathway of vesicle sorting along microtubules during RME of ASOR in hepatocytes. ASOR binds to its receptor at the basolateral surface of the hepatocyte. When endocytosis is initiated, receptor–ligand complexes in coated pits (a) are internalized into coated vesicles (b), which subsequently lose their clathrin coats (c). Microtubules in the hepatocyte are organized in a polarized manner, possibly as shown (9). Maturing endocytic vesicles associate tightly with microtubules (d) such that their receptor-containing domains cannot be released by ATP addition; at the same time, although independently, receptor and ligand dissociate. Ligand-containing vesicles are segregated (e) and can be released from the microtubules in an ATP-sensitive manner. We propose that while acid-mediated dissociation of ligand and receptor is proceeding, one pole of the vesicle bulges as it is moved along the microtubule by a minus end-directed motor (e.g., cytoplasmic dynein) (d) toward lysosomes, leaving the immobilized receptor domain behind. Dissociated ligand distributes into this vesicular space proportionally to its volume and fission follows. Finally, receptor-containing vesicles recycle (f). BC, bile canaliculus.

progressed to different stages in the pathway that are not dependent on microtubule association.

Previous studies revealed that microtubule disruptors such as nocodazole and colchicine inhibited particular steps in the pathway of RME (1, 7, 8). Incubation of cells in colchicine resulted in reduced lysosomal degradation of ligand (7). Surface receptor or ligand internalization was not reduced, but intracellular segregation of ligand and receptor was inhibited (1), which is consistent with the interpretation presented here. Experiments performed in the presence of monensin showed that attachment of endosomes to microtubules is independent of receptor–ligand dissociation. When ligand dissociation is prevented, vesicles released by ATP addition do not contain ligand. Ligand remains bound to receptor (5, 6) in vesicles that associate with microtubules in an ATP-independent manner. These results suggest that the ATP-insensitive binding to microtubules of a domain of maturing endosomes containing receptor, either shortly before or shortly after receptor–ligand dissociation, is a necessary step in receptor–ligand segregation. ATP-sensitive binding presumably occurs at a second pole in the segregating endosome into which ligand moves only after dissociation (as in Fig. 1).

Cytoplasmic dynein is present in our preparation and is released by ATP together with the ligand-containing vesicles. A few other proteins, whose importance remains to be determined, are also released by ATP. The possibility that ATP-sensitive release of ligand-containing vesicles is mediated by ATP-sensitive binding of cytoplasmic dynein to the microtubules is an attractive one. It is consistent with several current immunofluorescence studies (8, 14, 19), as well as with our observation that, in cultured hepatocytes, lysosomes are elongated along and possibly bound to microtubules *in vivo* (P.M.N., unpublished observation). In support of this hypothesis, ASOR delivery to lysosomes from endosomes is inhibited (29), and receptors can redistribute to the cell interior (30) when the ATPase inhibitor vanadate is present during uptake.

Cytoplasmic dynein (18) is a two-headed molecule in which ATP binding and hydrolysis are localized to the globular heads. Dynein could be bound stably to the nonreceptor domain of the endosomes, while one or more of its heads interacted with the microtubule. In the absence of ATP, dynein–tubulin rigor binding would permit the nonreceptor endosomal domain as well as the receptor domain to pellet with the microtubules. *In vitro*, ATP binding to the dynein heads would cause dissociation of the dynein–tubulin complex to release the dynein and the nonreceptor endosomal domain, which would normally contain the dissociated ligands, while the receptor-containing domain would not be released. In the cell, the dynein might walk along the microtubule as a minus end-directed motor (20, 23, 31), pulling the endosome apart. This would provide an appropriate directionality to the endocytic process and the force-generating mechanism required for vesicle fission during ligand–receptor segregation.

We wish to thank K. Shifteh and C. Stavropoulos for their assistance and K. Barkalow and T. Hamasaki for helpful discussion.

This work was supported by National Institutes of Health Grant DK-41918.

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