INTRODUCTION

Most of the membrane-bounded organelles of higher eukaryotic cells rely on microtubule-based movement both to generate and to maintain their structure and position within the cell. Perhaps the best-studied aspect of these movements is the extension of tubular membrane structures from organelles such as the ER (Terasaki et al., 1986; Lee and Chen, 1988). ER membrane tubules form and elongate by moving along a microtubule track; these elongated tubules can also fuse with one another, giving rise to polygonal membrane networks. Formation of membrane tubules and networks has also been observed for the early endosome (Hopkins et al., 1990), mitochondria (Johnson et al., 1980), tubular lysosomes (Swanson et al., 1987), the intermediate compartment (Lippincott-Schwartz et al., 1990) and the trans-Golgi network (Cooper et al., 1990; Lippincott-Schwartz et al., 1991; Wood et al., 1991), suggesting that it may play an important role in membrane architecture and function.

The movement of membrane tubules is thought to be driven by the microtubule motors kinesin (Vale et al., 1985a), which moves towards the plus ends of microtubules (Vale et al., 1985b), and cytoplasmic dynein (Paschal et al., 1987), which moves towards the minus ends (Paschal and Vallee, 1987). The movement of elements of the Golgi apparatus towards the minus ends of microtubules at the microtubule-organising centre (Ho et al., 1989), for example, has been shown to require cytoplasmic dynein in a semi-intact cell system (Corthésy-Theulaz et al., 1992). In contrast, tubular lysosome extension requires kinesin activity, as the microinjection of anti-kinesin heavy chain antibodies inhibits this process (Hollenbeck and Swanson, 1990). The extension of ER tubules to the periphery of fibroblasts is thought to occur by movement along a microtubule track; these elongated tubules can also fuse with one another, giving rise to polygonal membrane networks.

SUMMARY

We have studied the microtubule-dependent formation of tubular membrane networks in vitro, using a heterologous system composed of *Xenopus* egg cytosol combined with rat liver membrane fractions enriched in either Golgi stacks or rough endoplasmic reticulum. The first step in membrane network construction involves the extension of membrane tubules along microtubules by the action of microtubule-based motor proteins. We have observed for both membrane fractions that 80-95% of moving tubule tips possess a distinct globular domain. These structures do not form simply as a consequence of motor protein activity, but are stable domains that appear to be enriched in active microtubule motors. Negative stain electron microscopy reveals that the motile globular domains associated with the RER networks are generally smaller than those observed in networks derived from a crude Golgi stack fraction. The globular domains from the Golgi fraction are often packed with very low density lipoprotein particles (the major secretory product of hepatocytes) and albumin, which suggests that motor proteins may be specifically enriched in organelle regions where proteins for export are accumulated. These data raise the possibility that the concentration of active motor proteins into specialised membrane domains may be an important feature of the secretory pathway.

Key words: microtubule motors; membrane traffic; video-enhanced microscopy
proposed to be the kinesin receptor for the ER (Toyoshima et al., 1992). So far, no candidates for cytoplasmic dynein receptors have been identified.

In order to understand the interaction of motor proteins with membranes in general, it is advantageous to be able to reconstruct the system faithfully outside the living cell. The formation of ER-like membrane networks can be reconstituted in vitro (Dabora and Sheetz, 1988; Allan and Vale, 1991) and, as is thought to be the case in vivo (Lee and Chen, 1988), is a two-step process involving membrane tubule extension along microtubules, followed by membrane fusion. Here, we have used a reconstituted system and video-enhanced DIC microscopy (VE-DIC) to investigate the process of network formation from two rat liver membrane fractions. We report that most moving membrane tubules possess a distinct, long-lived and highly motile globular domain at their tips. Negative stain electron microscopy reveals that the size and content of the globular domains in the RER and Golgi-enriched fractions is different, and that the Golgi-enriched fraction frequently contains motile globular domains that are packed with very low density lipoprotein (VLDL) particles. Immunofluorescence labelling of Golgi-enriched networks confirms that such globular domains may contain soluble secretory products, such as albumin, as well as the VLDL-associated protein, apolipoprotein B. These results suggest that the motor proteins may not be uniformly distributed along the membrane, but may selectively associate with regions containing concentrated secretory products. The enrichment of motors in particular domains may be an important feature of membrane tubule formation and may serve to enhance the delivery of secretory vesicles between membrane compartments.

MATERIALS AND METHODS

Materials
Sucrose (BRL, Gaithersburg, MD, USA) was ultra pure grade and HEPES (BDH, Poole, Dorset, UK) was biochemical grade. Vanadate-free ATP was purchased from Sigma (Poole, Dorset, UK). Creatine phosphate was obtained from Boehringer Mannheim (Lewes, East Sussex, UK). Osmium tetroxide and collodion (nitrocellulose) solutions were purchased from TAAB (Reading, Berks., UK). Sheep anti-human apolipoprotein B antiserum, which also recognises rat apolipoprotein B (Graham et al., 1991), was purchased from Boehringer Mannheim. Sheep anti-rat albumin was supplied by Cappel/Organon Teknika (Turnhout, Belgium). Rhodamine-conjugated concanavalin A (Rh-ConA) was obtained from Vector Laboratories (Peterborough, Cambs, UK), and fluorescein-conjugated donkey anti-sheep IgG was obtained from Sigma. All other reagents were obtained from either Sigma or BDH.

Preparation of Xenopus egg cytosol
Laid eggs are arrested in metaphase II of meiosis and can be induced to enter interphase by electrical activation or treatment with calcium ionophores (e.g. see Murray, 1991). Concentrated interphase extracts can also be prepared from meiotically arrested eggs by using buffers without EGTA (Newmeyer and Wilson, 1991). We have found that more-dilute interphase cytosol can be prepared from meiotic eggs in the presence of EGTA as described below. Omitting the EGTA had no effect on the results obtained. Eggs were collected and the jelly coats removed (Murray, 1991). The eggs were carefully washed with acetate buffer (100 mM potassium acetate, 3 mM magnesium acetate, 5 mM EGTA, 10 mM HEPES, 150 mM sucrose, adjusted to pH 7.4 with KOH) and allowed to settle under gravity in a glass homogeniser. Excess buffer was removed and replaced with 1.5 egg volumes of acetate buffer plus 10 µg/ml protease inhibitors (chymostatin, pepstatin, leupeptin), 0.2 mM PMSF and 2 µg/ml cytochalasin D. The eggs were homogenised by several passes with a hand-held glass or Teflon pestle. The homogenate was centrifuged in a Sorvall HB-4 rotor at 11,000 g for 10 minutes at 4 °C in 14 ml Falcon polypolypropene culture tubes. The resulting supernatant was collected by puncturing the tube and then further clarified by centrifugation in a Beckman SW55 rotor at 150,000 g for 1 hour at 4 °C. The high speed supernatant was stored in liquid nitrogen. After thawing, MgATP and creatine phosphate were added to 1 mM and 7.5 mM, respectively, and the high speed supernatant was centrifuged in a Beckman TL-100 rotor at 55,000 rpm (117,000 gav) for 30 minutes at 4 °C. The resulting cytosol fraction (7-10 mg/ml protein) was kept on ice and remained active for more than 4 hours.

Preparation of rat liver membrane fractions
A fraction enriched in Golgi stacks was prepared from rat liver as described (Allan and Vale, 1991). A rough endoplasmic reticulum fraction was isolated from male or female rat liver as follows. All steps were performed at 4 °C. Finely minced liver was suspended in 2 volumes of 0.25 M sucrose in acetate buffer (plus 10 µg/ml protease inhibitors) per gram wet weight, and was then homogenised using a Polytron homogeniser at low speed. A post-mitochondrial supernatant was prepared by centrifugation at 600 g for 10 minutes followed by 10,000 gav for 10 minutes in a Sorvall SS34 rotor. The post-mitochondrial supernatant was layered onto a step gradient consisting of 12 ml 1.1 M sucrose, 12 ml 1.4 M sucrose and 6.5 ml 1.7 M sucrose (all prepared in acetate buffer containing 1 µg/ml protease inhibitors and 0.2 mM PMSF) and centrifuged in a Beckman SW28 rotor at 100,000 g for 2 hours at 4 °C. RER was collected from the 1.7 M/1.4 M interface and diluted with acetate buffer plus 0.2 mM PMSF to a sucrose concentration of approximately 0.4 M. The RER fraction was concentrated by centrifugation at 100,000 g for 1 hour in a Beckman SW28 rotor, and resuspended to 10-15 mg/ml in 0.25 M sucrose in acetate buffer plus 10 µg/ml protease inhibitors and 0.2 mM PMSF. Both RER and crude Golgi stack fractions were stored in liquid nitrogen.

Video-enhanced differential interference contrast (VE-DIC) microscopy
Rat liver RER or crude Golgi fraction membranes were added to Xenopus egg cytosol (to a final concentration of 0.2-0.3 mg/ml membrane protein) and incubated in simple microscope flow cells for 5 minutes in a humid chamber, as described (Allan and Vale, 1991). During this time, microtubules polymerised spontaneously from motor proteins or microtubule-associated proteins adsorbed onto the glass. The movement of membranes along microtubules was assayed only on these attached microtubules. Movement remained active for several hours if the flow cell was kept in a humid chamber, and was not affected by 30 minutes of continuous microscopic observation. Motility was followed in real time using a Zeiss standard microscope fitted with DIC optics, a 1.4 NA achromatic aplanatic oil immersion condenser, and a plan ×100 1.25 NA lens. Light from a 100 W mercury vapour lamp was passed through both water and glass heat-filters (LOT-Oriel Ltd, Leatherhead, Surrey, UK) and a 546 nm narrow band interference filter, before being relayed to the microscope via a 1 mm silica optical fibre (Technical Video Ltd., Woods Hole, MA, USA; Inoué, 1986). DIC images were projected onto a Hamamatsu Newvicon camera (C2400-07) using a ×12 eye-piece. Background subtraction, image contrast enhancement and two-frame rolling averaging were performed in real time using a Hamamatsu Argus 10 image processor. Sequences were recorded onto S-VHS tape using a Panasonic S-VHS AG 7330 recorder. Images were digitised
either directly from the Argus 10 output signal (after background subtraction and contrast enhancement), or from video tape, using a Quick Capture board (Data Translation, Wokingham, Berks, UK) in an IBM-AT-compatible computer. The digitised images were then imported into NIH-IMAGE (a public domain program) on a Macintosh Iici for further image processing. Final images were transferred into Aldus Pagemaker and printed using a Canon colour laser writer.

Electron microscopy
Electron microscope grids were placed on 12 mm×12 mm coverslips and coated with 1% colloidion (nitrocellulose). After this treatment the grids were firmly attached to the coverslip, which could then be used to form a flow chamber by placing on a microscope slide with small pieces of 0.14 mm thick plastic as spacers. The chambers were filled with 20 µl of sample and incubated in a humid chamber for 20-40 minutes to allow membrane network formation. The motility observed using nitrocellulose-coated coverslips was very similar to that seen with uncoated glass surfaces. Excess membrane and cytosol were washed away by gently flowing 100 µl of acetate buffer containing 20 µM taxol through the chamber, leaving the microtubules and membrane networks intact on the nitrocellulose surface. The networks were fixed by flowing through 200 µl of 2% paraformaldehyde plus 0.25% glutaraldehyde in PIPES buffer (80 mM PIPES/KOH, 1 mM MgCl₂, 1 mM EGTA, 150 mM sucrose, pH 6.8), followed by incubation for 30 minutes at room temperature in a humid chamber. The networks were further stabilised by flowing through 200 µl of 1% osmium tetroxide (diluted from a 4% stock with PIPES buffer) and incubating for 20 minutes at room temperature in a humid chamber. Both fixation and osmication of the membrane networks was required to maintain the morphology of both microtubules and membranes prior to negative staining. The flow cells were disassembled after washing extensively with water, and the grids were carefully removed. The grids were negatively stained with 1.5% sodium phosphotungstate plus 10 µg/ml bacitracin (Sigma; Poole, Dorset, UK) to promote spreading of the stain. Specimens were observed using a Philips 301 electron microscope. The membrane networks were fixed in the early stages of formation so that the percentage of membrane tubules that were moving at the time of fixation was maximal. Membrane tubules which had an expanded region at their tips that was in close proximity to microtubules were photographed if they were of approximately 1 µm or more in length (most were considerably longer).

Immunofluorescence microscopy
Networks for immunofluorescence microscopy were prepared as described for electron microscopy, except that the coverslips were uncoated; 0.075 mm plastic spacers were used; and fixation was performed by flowing through 100 µl of 0.2% glutaraldehyde in acetate buffer. After fixation for 20 minutes at room temperature, the flow chambers were rinsed with 100 µl acetate buffer and the coverslips were lifted off the spacers with forceps. Excess buffer was blotted using filter paper and the networks were post-fixed by gentle immersion in methanol at −20°C for 5 minutes. This step served to attach the networks firmly to the glass surface and to permeabilise the membrane networks. In some instances, networks were washed with acetate buffer plus taxol, and then fixed in methanol directly, without glutaraldehyde fixation. The coverslips were then incubated in 1 mg/ml NaB₃H₄ in phosphate buffered saline (PBS) for 3× 4 minutes. After blocking for 30 minutes in 0.2% fish skin gelatin/PBS, coverslips were incubated for 20 minutes with anti-rat albumin or anti-apolipoprotein B antisera diluted 1/700 and 1/100-1/200, respectively, and then with Rh-ConA diluted 1/500 in PBS. After washing twice with PBS, the coverslips were incubated for 10 minutes with fluorescein-conjugated anti-sheep antibodies diluted 1/50 in fish skin gelatin/PBS. After washing twice with PBS, the coverslips were incubated for 10 minutes with Rh-ConA diluted 1/500 in PBS. After 3×5 minute washes in PBS, coverslips were mounted in polyvinyl alcohol mounting medium (Osborn and Weber, 1982) containing 25 mg/ml 1,4-diazabicyclo[2.2.2]octane to reduce photo-bleaching. Samples were observed with a Bio-Rad MRC-600 confocal microscope using the 488DF10 and 568DF10 single-channel excitation filters. Identical confocal settings were used for all samples, and similar contrast-enhancement was subsequently applied to all images. Digitised images were processed and printed as described for VE-DIC images.

RESULTS
Motile globular domains drive membrane tubule extension and network formation
We have shown previously that interphase Xenopus egg cytosol promotes the microtubule-based movement of vesicles and membrane tubules in vitro from a heterologous source such as a crude rat liver Golgi fraction (Allan and Vale, 1991). These movements can be followed in real time using VE-DIC microscopy. As membrane tubules extend and cross other tubules, membrane fusion occurs (Fig. 1C and D), resulting in the formation of tubular membrane networks (Fig. 1A; and Dabora and Sheetz, 1988; Vale and Hotani, 1988; Allan and Vale, 1991). An unusual feature of the moving membrane tubules in the crude rat liver Golgi fraction is that the tips of the tubules often possess a distinct globular domain (Fig. 1B-D). These globular domains were highly motile, and were able to move continuously for considerable periods of time, switching from one microtubule track to another (Fig. 1B-D). The membrane tubules extending behind the globular domains were able to form static interactions with microtubules (Fig. 5H) and with components on the glass surface (Fig. 1D,E). It can be seen from Fig. 1 that the motile globular domains form only a small proportion of the total membrane network surface area. By recording the number of moving domains per field versus the length of membrane tubule networks, we estimated that the globular domains accounted for only 4-8% of the membrane surface area after a 20 minute incubation (assuming a membrane tubule diameter of 100 nm and an average globular domain diameter of 300 nm). Taken together, these results suggest that active motor proteins are concentrated in the globular domains.

While motile globular domains at the membrane tubule tips were common when the rat liver crude Golgi fraction was used, they were rarely seen during membrane tubule extension from endogenous Xenopus egg membranes (for example, see Fig. 2 in Allan and Vale, 1991). We were therefore interested in investigating the process of network formation using another membrane source. A rough endoplasmic reticulum fraction was prepared from rat liver and was incubated with Xenopus egg cytosol as described in Materials and Methods. After a delay of 10-20 minutes, membrane tubules were observed to extend along microtubules as shown in Fig. 2B-D. The delay was apparently required for the fusion of the rough microsomes into larger membrane structures prior to tubule extension. As was observed using the crude Golgi fraction, many of the moving RER tubules possessed globular domains at their tips which were highly motile and able to switch microtubule tracks (Fig. 2B-D). The RER tubules also formed extensive networks on the coverslip surface (Fig. 2A).
Again, the globular domains appeared to form only a small fraction of the total network surface area (Fig. 2), which we estimated to be 1-2.5% after a 30 minute incubation (assuming a membrane tubule thickness of 100 nm and a globular domain diameter of 200 nm). Motile globular domains were therefore a common feature in membrane tubule extension and network formation from both rat liver RER and crude Golgi stack fractions, and also from rat liver SER fractions (V. Allan, unpublished data).

Most moving membrane tubules possess a globular domain

To assess the proportion of tubules that extended with a globular domain at the leading end, sequences recorded onto video tape were subsequently replayed and every clear tubule movement was scored for tip morphology. For both rat liver membrane fractions the majority of moving tubules had a globular domain leading (Table 1), and this proportion remained constant irrespective of the length of incubation (data not shown). Such domains were not obligatory for movement, however, as 15-20% of RER tubules and 6-11% of crude Golgi fraction tubules moved without any visible swelling at their tips (Table 1). It is not clear whether these two methods of tubule extension show the existence of two different types of membrane network in each case, or whether one network has the capacity to extend membrane tubules by both mechanisms, but with 80-95% of movements involving a distinct tip domain. It is also possible that different motor proteins drive the movement of the blunt and globular tips. We have not been able to establish the direction of tubule movement in this system, however, because any microtubules of defined polarity present at the start of an assay become obscured by polymerization of free microtubules before membrane tubule extension occurs (Allan and Vale, 1991).

Motile globular domains are stable structures

One question raised by these observations was whether the globular domains were formed transiently by the action of motor proteins or whether they were long-lived structures. Close observation of tubule extension suggested that the domains were stable, as they were occasionally observed to detach from their associated membrane tubule and to continue moving (Fig. 3). In contrast, the released membrane tubules recoiled rapidly and were apparently re-absorbed into the membrane network (Fig. 3, arrowhead). A similar re-absorption of the membrane tubule occurred when the globular domain dissociated from the microtubule without losing its attachment to the membrane tubule. When this happened, however, the globular domains remained associated with the membrane network and were free to undergo linear diffusional movement along the membrane tubules in the network (Fig. 4A-C). Such diffusing globular domains were able subsequently to reattach to a microtubule and begin moving (Fig. 4D,E). In addition to being able to diffuse linearly along the membrane tubules, the globular domains could also progress along a membrane tubule by moving on an underlying microtubule (data not shown) and therefore did not have to be at a tubule tip in order to move. The evidence that the domains do not depend upon motor protein-generated force for their existence was strengthened by the observation that changes in the tension in the associated membrane tubule had no effect on either the motility or the morphology of the globular domains (Fig. 4F-H). Most of the globular domains maintain their shape and size while moving, as observed by VE-DIC, but occa-
Motile domains in membrane tubule extension

 occasional a domain could be seen to increase in size over time (data not shown). Taken together, these results suggest that the motile globular domains observed in these rat liver membrane networks are pre-existing structures, enriched in active motor proteins, which are capable of many cycles of detachment from and reattachment to microtubules.

Structure of membrane tubule tips

Although these specialised domains shared the feature of being highly motile, when examples of extending tubules were compared (Fig. 5), it was clear that the globular domains varied from a simple ball-like structure to more complex forms. From these VE-DIC images it appeared that the RER globular domains (Fig. 5A'-N') were in general both smaller and simpler than those seen in the crude Golgi membrane fraction (Fig. 5A-N). This suggested that while highly motile globular structures were a common feature in the movement of rat liver membrane tubules, their morphology varied with organelle type.

VE-DIC is an excellent technique for studying the motile properties of membranes, but it provides little detailed structural information. We therefore used negative stain electron microscopy to investigate the ultrastructure of membrane tubule tips. Examples of the range of structures found in the Golgi fraction and RER membrane networks are shown in Figs 6 and 7, respectively, and correspond quite well with those observed by VE-DIC. The crude Golgi fraction tubules, for example, can possess small (Figs 5H and 6A-C), medium (Figs 5B,L and 6l) and large globular domains (Figs 5E,K and 6G,H), as well as apparently double domains (Figs 5D,F,N and 6D-F). The most complex and largest of the structures observed at the tips of Golgi fraction membrane tubules (e.g. Fig. 5C,J) are not shown in Fig. 6, however, as they were too heavily negatively stained. The structure of RER tubules observed by VE-DIC (Fig. 5A'-N') and the ultrastructure revealed by negative stain electron microscopy (Fig. 7) were also found to correspond. With this membrane fraction, however, most tubule tips possessed single globular domains of variable size (Fig. 7), which were generally smaller than those at the tips of crude Golgi fraction membrane tubules.

It is clear from Figs 6 and 7 that the domains at membrane tubule tips may take a number of different forms. The most common structure observed in the Golgi fraction membrane tubules was a typical example of such a tubule from a shorter incubation (15 minutes). The motile globular domain (asterisk) moved continuously, and was able to switch microtubule tracks. The membrane tubule also formed a static attachment to the coverslip (arrow). Bars, 5 µm.

![Image](image1.png)

**Table 1. Percentage of tubules extended by a globular domain**

<table>
<thead>
<tr>
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<th>% With a globular domain</th>
<th>% Without a globular domain</th>
<th>Number counted</th>
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<tbody>
<tr>
<td>Golgi fraction</td>
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<tr>
<td>Supernatant 1</td>
<td>93.8</td>
<td>6.2</td>
<td>178</td>
</tr>
<tr>
<td>Supernatant 2</td>
<td>89.3</td>
<td>10.7</td>
<td>140</td>
</tr>
<tr>
<td>RER fraction</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Supernatant 2</td>
<td>85.4</td>
<td>14.6</td>
<td>171</td>
</tr>
<tr>
<td>Supernatant 3</td>
<td>79.5</td>
<td>20.5</td>
<td>73</td>
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Sequences of membrane network formation were recorded onto video tape and all clear examples of membrane tubule movement along microtubules were scored for tip morphology. Any tubule with either a clear swelling at its tip, or a domain that gave greater contrast by VE-DIC, was counted as possessing a globular domain. Total duration of sequences analysed: 146 minutes for Golgi fraction tubule extension; 126 minutes for RER. Supernatants 1, 2 and 3 were prepared from different batches of eggs.

*Xenopus egg cytosol promotes the formation of rat liver rough endoplasmic reticulum networks. After 1 hour of incubation in the presence of ATP and cytosol, an extensive membrane network has formed from a rough microsome fraction (A). At this stage, many of the membrane tubules were stationary, but the moving membrane tubules usually possessed globular domains at their tips. (B-E) A typical example of such a tubule from a shorter incubation (15 minutes). The motile globular domain (asterisk) moved continuously, and was able to switch microtubule tracks. The membrane tubule also formed a static attachment to the coverslip (arrow). Bars, 5 µm.
tubules consisted of one or more particle-filled domains, as shown in Fig. 6E-I. Such structures are under-represented in Fig. 6, as heavy negative staining around the globular domain made many examples unsuitable for photographic reproduction. Even with dense negative stain, however, it was possible to distinguish the particulate content of most of these domains (data not shown). The electron-lucent particles had an average diameter of 52.5 ± 12.4 nm (s.d.; n=45). Particles of similar size and appearance have previously been shown to be very low density lipoprotein (VLDL) particles (Stein and Stein, 1967; Mahley et al., 1968, 1970; Claude, 1970; Alexander et al., 1976; Rusiñol et al., 1993), which are the major secretory product of hepatocytes. These are found clustered in the lumen of the cis-Golgi and associated tubular network and onward through the Golgi stack (see Discussion).

To confirm that the globular tip domains may contain VLDL components, we have developed methods for fixing and permeabilising the membrane networks for immunofluorescence analysis. Antibodies to apolipoprotein B, the major protein component of VLDL particles, revealed that the protein was indeed localised in globular domains at the tips of membrane tubules (Fig. 8A-F, arrowheads), but was below the level of detection in the tubules themselves. Rhodamine-conjugated ConA provided a convenient marker for the whole membrane network. We have also used antibodies to localise a soluble secretory protein, albumin. Like

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**Fig. 3.** Globular domains can detach from membrane tubules. Two video sequences of membrane tubule extension from crude Golgi fraction networks show two motile globular domains (asterisks) with associated membrane tubules, which moved along microtubules (A,B,E). The tension generated by this process is evident in the deflection of the joining membrane tubule shown in B (arrow). The globular domains were subsequently detached from the membrane tubules (C,F) and continued moving along microtubules (C,D,F,G) while the membrane tubules recoiled elastically and were apparently reabsorbed into the membrane network. In C, a short length of membrane tubule is still visible (arrowhead). Bar, 2 µm.

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**Fig. 4.** Neither motor activity nor continued membrane tubule tension are needed for the maintenance of globular domains. A globular domain (asterisk) attached to a membrane network underwent rapid linear diffusional movement along a membrane tubule (A-C). Between the frames shown, the domain moved bi-directionally, but with an overall bias in one direction. The same domain subsequently bound to a microtubule and began moving (D), resulting in extension of a membrane tubule (arrowheads). The tubule was oriented parallel to the DIC shear direction and is therefore not imaged well. In A-C another moving membrane tubule can be seen (arrows) which moved out of the field of view by 7 s (D). Images from a different network (F-G) show that the movement and morphology of a globular domain (asterisk) was unaffected by the tension in its associated membrane tubule (small arrows), which was slack in F and G, but taut in H. A large moving vesicle (open arrows) was also present. Both sequences show Golgi fraction networks. Bar, 2 µm.
apolipoprotein B, albumin was also localised in many globular tip domains (Fig. 8G-K, arrowheads), but, in contrast, was present at low levels in the membrane tubules. The morphology of the tubule tips corresponds well with that observed both by DIC and EM, with tubules possessing single (Fig. 8A–E, I and K), double (Fig. 8G,J) or multiple (Fig. 8F,H) globular domains. Taken together, these results suggest that membrane tubule domains containing both particulate and soluble secretory components may become enriched in active motor proteins.

Not all the Golgi fraction tubule tips observed by negative stain EM contained visible VLDL particles, however. Some tips possessed a globular domain that had collapsed during fixation and staining, and which was devoid of obvious contents (Fig. 6C,J). Other simple domains had maintained their shape, but their contents were not penetrated by the negative stain (Fig. 6A,B). Both these types of tip domain were common in the RER networks (Fig. 7), which had correspondingly fewer globular domains containing VLDL particles (Fig. 7F,G). These particles had an average diameter of 44.7 ± 12.4 nm (s.d.; n=26). A third type of domain that was only observed in the Golgi fraction tubules was a combination of a small, VLDL-containing domain at the tip of the tubule, with a flattened, multi-lamellar region of membrane just behind it (Fig. 6D,E). Such structures may correspond to those identified by immunofluorescence, where only part of the complex, an expanded tubule tip contained albumin or apolipoprotein B (arrows in Fig. 8F,H,J).

One question raised by these images is how are the globular domains attached to the membrane tubule? For a number of RER tubules, the membrane of at least part of the globular domain was in direct continuity with the tubule membrane (Fig. 6A,B). In other cases, for both membrane fractions, the superimposition of globular domain and tubule tip made it impossible to determine whether their membranes were continuous (Figs 6C,J and 7B). On the other hand, many of the Golgi fraction globular domain and tubule membranes appeared not to be continuous (Fig. 6C,F-J), as were a few of the RER tubules and tips (Fig. 7F-H). It is not clear whether this reflects damage caused during fixation and staining, or whether the globular domains can attach firmly enough, without membrane fusion, to transmit sufficient force from the motors in the globular domain to the attached membrane to allow tubule extension. It is also possible that such tubules correspond to those that will shortly lose their globular domains, as shown in Fig. 3.

Irrespective of how the motile domains are attached to the membrane tubules, it is clear that they can drive membrane tubule formation. Some evidence of that interaction was obtained from the negatively stained networks, as the attachment of the tip domain to microtubules was so close in some cases that it resulted in deformation of the membrane either by
Fig. 6. Ultrastructure of membrane tubule tips in crude Golgi fraction membrane networks. Membrane networks were formed on nitrocellulose-coated EM grids and fixed as described in Materials and Methods. Fixation of the networks was performed after an incubation of 20 minutes (A,D,E,I), 30 minutes (B,C,F,H,J) or 60 minutes (G). The grids were stained with either 1.5% sodium phosphotungstate (A-I) or 2% sodium phosphotungstate (J), both including 10 µg/ml bacitracin. The particles (arrowheads in F) within some tip domains (F-I) probably correspond to VLDL particles. The tips of some membrane tubules appear to flatten where they come into close contact with microtubules (arrows in B,C,D,H). Bar, 200 nm.
Fig. 7. Ultrastructure of RER membrane tubule tips. Membrane networks were formed on nitrocellulose-coated EM grids and fixed as described in Materials and Methods. Fixation of the networks was performed after an incubation of 30 minutes (A,B,D,H,J) or 40 minutes (C,E,G,I). The grids were stained with either 1.5% sodium phosphotungstate (A-G,I,J) or 2% sodium phosphotungstate (H), both including 10 μg/ml bacitracin. VLDL particles (arrowheads) can be seen in two tubule tips (F,G). Most globular domains, however, do not possess any obvious contents and may appear collapsed (B,C,D,J). Flattening of the membranes that are in close apposition with microtubules can be seen in some membrane tubules (arrows in F,G,J). Bar, 200 nm.
flattening (Figs 6B-D,H and 7F,G,J) or by distortion of the membrane towards the point of microtubule binding (Figs 6E,I and 7G).

**DISCUSSION**

**Selective attachment of active motor proteins to sub-domains of membrane tubules**

We have studied the process of membrane tubule movement along microtubules using a cell-free assay. Xenopus egg cytosol not only promotes the active movement of endogenous Xenopus egg ER (Allan and Vale, 1991), but also drives membrane tubule extension from exogenous membrane sources such as rat liver crude Golgi fractions (Allan and Vale, 1991; this study) and rat liver RER (this study). We report here that 80-95% of membrane tubule movements in both rat liver fractions are driven by a highly motile globular domain at the tubule tip (Figs 1-4). These domains, which are stable structures, represent only a small fraction of the total surface area of the membrane tubules and networks. In addition, we have shown, using negative stain electron microscopy, that they can form close interactions with microtubules (Figs 6B-D,H and 7F-H). These results argue that active motor proteins interact selectively with these domains.

The motile globular domains described here appear to be stable structures enriched in active motor proteins, which are not generated solely by motor action. As evidence for this conclusion, the domains maintain their VE-DIC morphology irrespective of whether: (i) the attached tubule is under tension; (ii) the domain is moving along a microtubule; or (iii) the domain detaches from the membrane tubule. Electron microscopic and immunofluorescence studies indicate that a proportion of the globular domains, particularly in the networks derived from the crude Golgi fraction, contained secretory products in the form of VLDL particles (Figs 6-8) and albumin (Fig. 8). This finding suggests that the same membrane structure can be enriched in both active motor proteins and proteins en route through the exocytic pathway. The globular structure of the tubule tip domains is not simply due to the presence of bulky secretory products, however, as many of the domains associated with RER tubules (Fig. 7)
had no obvious particulate contents, but were still enlarged compared with the attached membrane tubule. It is possible that these domains contain soluble secretory products, such as albumin.

How is motor activity concentrated in these specialised domains? One possibility is that the activators of motor proteins, rather than the actual motors, are concentrated in such domains. However, the localisation of motor proteins in other systems supports the idea that motors themselves may not be uniformly distributed. For example, the immunofluorescence localisation of both kinesin (Pfister et al., 1989; Wright et al., 1991; Henson et al., 1992) and cytoplasmic dynein (Koonce and McIntosh, 1990; Pfarr et al., 1990; Gill et al., 1991; Lin and Collins, 1992) reveals a predominantly punctate pattern for both motors, not an even distribution throughout larger organelles such as the ER or Golgi apparatus. One possible explanation for these results is that the antibodies used may not have recognised the motor proteins or isoforms associated with these large organelles. A second possibility, however, is that the motor proteins may indeed be clustered within discrete domains. In support of the latter proposal, it has been observed, by immunoelectron microscopy, that kinesin is localised in patches on the mitochondrial outer membrane (Leopold et al., 1992). On the basis of pharmacological data, we have previously proposed that the movement of tubules derived from the crude Golgi fraction was driven by kinesin or a related protein (Allan and Vale, 1991). It has not been possible, so far, to localise specific motor proteins on the membrane tubules using immunofluorescence, perhaps due to a combination of poor cross-reactivity of available antibodies to frog motor proteins following fixation, and the small number of motors that may be sufficient to generate movement.

How could the motors be recruited to specialised domains? It seems unlikely that an enrichment would be observed if the motors bound only to membrane lipids, since in some cases the tip domain membranes are clearly continuous with the membrane tubule, and might be expected to share the same lipid composition, although the existence of lipid microdomains cannot be ruled out. Mechanisms for motor enrichment in specific regions are more easily imagined if the motor proteins are assumed to bind to membranes via integral membrane protein receptors (Toyoshima et al., 1992; Yu et al., 1992), which in turn might interact directly with concentrated secretory material within the lumen or with other membrane components that are themselves clustered in domains earmarked for membrane traffic. It is interesting to note that two types of highly motile structures contain concentrated secretory products: namely, the constitutively secreted VLDL particles described here, and the regulated secretory granules studied by a number of groups (Iida et al., 1988; Kreis et al., 1989; Rothwell et al., 1989; Burkhardt et al., 1993). The presence of such bulky aggregates cannot be the sole requirement for motor recruitment, however, as many of the RER domains we have observed do not contain such material. Alternatively, motors could interact with a coat protein that is associated with newly forming secretory vesicles. However, whether the clathrin or non-clathrin vesicle coat proteins (Schmid, 1993) play a role in clustering of motor proteins, or whether they prevent motor binding (Pelham, 1991), remains to be established.

Possible relevance to membrane traffic in vivo

One important question is: does the phenomenon we describe here also occur in living cells? Real-time observation of ER network formation in vivo indeed revealed that a proportion of membrane tubules extended with a distinct globular domain at their tips (Lee and Chen, 1988). The other extending tubules possessed no obvious tip domain, as we have observed for 20% of RER tubule movements. We therefore believe that our in vitro assay is reproducing faithfully the motility observed for ER networks in vivo. Other membrane networks may also move using motors concentrated in selected membrane subdomains. For instance, multi-vesicular bodies within tubular endosomal networks have been observed to move in living cells (Hopkins et al., 1990), although the nature of the filament system involved in this movement was not established.

The globular membrane structures that we observe may also be part of the normal secretory process in hepatocytes. The apoprotein components of VLDL are synthesised in the RER while the lipid components are concentrated into particles in the SER; the two are thought to be combined when they reach the transitional elements of the ER (Stein and Stein, 1967; Alexander et al., 1976). While VLDL particles are absent from the SER, they are present singly in the SER and either singly or in small clusters in the transitional elements. As would be predicted from these observations, most of the RER tubules that we have described here have no particulate content at their tips (Fig. 7). The remaining tubules which do contain VLDL may be derived from transitional ER, or from contaminating SER, intermediate compartment or Golgi apparatus membranes. Once the VLDL particles reach the cis-Golgi cisterna and related tubular network, they are found in large clusters at the tips of tubules and in the dilated cisternal rims (Stein and Stein, 1967; Claude, 1970). This clustering is maintained throughout the Golgi apparatus, resulting finally in the formation of VLDL-filled secretory vesicles (Stein and Stein, 1967; Claude, 1970). The VLDL-filled tip structures that we have observed in tubules derived from the crude Golgi fraction membranes (Fig. 6) are very similar to those seen in vivo.

In organelles of both the exo- and endocytic pathways, clustering of motors might facilitate the inclusion (or exclusion) of a motor protein complex in regions where vesicle budding is occurring. Because the choice of active motor protein included in any budded vesicle will determine whether the vesicle moves towards the cell centre or periphery, the sorting of motor proteins as well as vesicle contents may be an important step in membrane traffic. Possessing the appropriate motor protein should facilitate delivery of a newly formed transport vesicle to the next membrane compartment.

Microtubule-based motors could also play other roles in membrane traffic besides the movement of transport vesicles. For instance, the concentration of motors or their receptors might be an early event that precedes the recruitment of luminal content, and might also facilitate subsequent sorting steps. Satir and co-workers (Goltz et al., 1992) have proposed that cytoplasmic dynein may be involved in the sorting of ligand from receptors within the endosome. It is also conceivable that motor-generated force could aid the budding process in some situations. The clustering of motors into domains where material for transport is concentrated could, in fact, play a dual role: firstly, the motors associated with the forming bud
could be used to generate the movement and dynamics of the organelle itself; and secondly, after the vesicles bud off, the motors could transport the vesicles to the next organelle in the pathway. On a larger scale, restricting motor proteins to particular regions of an organelle might facilitate the formation or the function of specialised domains within a continuous membrane structure. The interplay between microtubule-based movement, organelle structure and membrane traffic is clearly complex, but in vitro reconstitution systems, such as that described here, should prove useful in investigating these interactions.

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