Autocrine Motility Factor Receptor Is a Marker for a Distinct Membranous Tubular Organelle

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Abstract. Autocrine motility factor (AMF) is secreted by tumor cells and is capable of stimulating the motility of the secreting cells. In addition to being expressed on the cell surface, its receptor, AMF-R, is found within a Triton X-100 extractable intracellular tubular compartment. AMF-R tubules can be distinguished by double immunofluorescence microscopy from endosomes labeled with the transferrin receptor, lysosomes labeled with LAMP-2, and the Golgi apparatus labeled with β-COP. AMF-R can also be separated from a LAMP-2 containing lysosomal fraction by differential centrifugation of MDCK cells and is found within a 100,000 *g* membrane pellet. By electron microscopic immunocytochemistry, AMF-R is localized predominantly to smooth vesicular and tubular membranous organelles as well as to a lesser extent to the plasma membrane and rough endoplasmic reticulum. AMF-R tubules have a variable diameter of 50–250 nm and can acquire an elaborate branched morphology. By immunofluorescence microscopy, AMF-R tubules are clearly distinguished from the calnexin labeled rough endoplasmic reticulum and AMF-R tubule expression is stable to extended cycloheximide treatment. The AMF-R tubule is therefore not a biosynthetic subcompartment of the endoplasmic reticulum. The tubular morphology of the AMF-R tubule is modulated by both the actin and microtubule cytoskeletons. In a similar fashion to that described previously for the tubular lysosome and endoplasmic reticulum, the linear extension and peripheral cellular orientation of the AMF-R tubule are dependent on the integrity of the microtubule cytoskeleton. The AMF-R tubule may thus form part of a family of microtubule-associated tubular organelles.

THE complex traffic of membrane proteins within the cell is determined by specific targeting signals in their protein sequences. The biosynthetic and intracellular trafficking routes of various receptors and proteins traverse multiple organelles and plasma membrane domains, however differential efficiency of the signals and mechanisms which regulate protein targeting can result in the predominant expression of a protein in a particular organelle. The steady state distribution of some proteins has thereby served as a marker to identify and define the relationship between intracellular organelles. Markers for the rough endoplasmic reticulum include calnexin and BiP (Bole et al., 1986; Degen and Williams, 1991; Wada et al., 1991; Hochstenbach et al., 1992) and the intermediate compartment and/or *cis*-Golgi network is labeled with p53, p58, and gp74 (Saraste et al., 1987; Schweizer et al., 1988; Alcalde et al., 1994). The Golgi apparatus can be identified by the presence of mannosidase or β-COP (Moreman and Touster, 1985; Donaldson et al., 1990; Duden et al., 1991; Serafini et al., 1991) and the *trans*-Golgi network (TGN) by TGN38 (Luzio et al., 1990). In the endocytic pathway, early endosomes and the early endosomal recycling compartment can be identified by the presence of the transferrin receptor (Hopkins and Trowbridge, 1983; Yamashiro et al., 1984). The late endosome has been defined as a mannose-6-phosphate receptor positive and LAMP positive organelle while the lysosome is a mannose-6-phosphate receptor negative, LAMP positive organelle (Geuze et al., 1988; Griffiths et al., 1988). Other distinct and, perhaps, function-specific endocytic organelles have been identified such as the class II compartment in antigen-presenting cells (Peters et al., 1991; Amigorena et al., 1994; Tulp et al., 1994).

Early endosomes, particularly the sorting endosome or CURL, exhibit a distinctive morphology with tubular extensions (Geuze et al., 1983; Marsh et al., 1986; Griffiths et al., 1989; Dunn and Maxfield, 1992) and tubular endosomes corresponding to the plasma membrane recycling compartment can be labeled by endocytosis (Wall et al., 1980; Wilson et al., 1987; Gruenberg et al., 1989; Tooze and Hollinshead, 1991). Overexpression of the small GTP-binding protein rab4 leads to the accumulation of endocytosed transferrin in a tubular endosomal recycling compartment (van der Sluijs et al., 1992). An extensive endosomal reticular

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network labeled by transferrin has been described to extend throughout the cytoplasm of HEp2 cells (Hopkins et al., 1990). Surface connected tubules implicated in the specialized endocytosis of β-VLDL have been recently described (Myers et al., 1993). Brefeldin A (BFA)[1] treatment induces the formation of tubular elements connecting the Golgi to the endoplasmic reticulum and the TGN to early endosomes (Cooper et al., 1990; Lippincott-Schwartz et al., 1991; Wood et al., 1991). Tubulation of the Golgi apparatus has been observed in the absence of BFA both in vivo and in vitro (Cluett et al., 1993).

Elongated tubular membrane organelles have been observed in both fixed tissue and cultured cells (Buckley, 1973; Phaire-Washington et al., 1980; Oliver, 1983; Beaudoin et al., 1985; Robinson et al., 1986; Swanson et al., 1987; Heuser, 1989; Luo and Robinson, 1992). Labeling of these tubular structures by either fluid phase endocytosis or cytochemically for lysosomal enzymes led to their appellation as lysosomal compartments. The lysosomal nature of the tubular lysosome was confirmed by the presence of the lysosomal membrane glycoprotein, lgp120, and shown to be associated with phagosomes and macropinosomes (Knapp and Swanson, 1990; Racoosin and Swanson, 1993). The morphology and cellular distribution of tubular lysosomes are microtubule dependent (Phaire-Washington et al., 1980; Swanson et al., 1987; Araki et al., 1993). A macrophage tubular compartment, continuous with phagolysosomes, exhibits similarities with tubular lysosomes and contains both the late endosome markers, M6PR and rab7, as well as lysosomal Lamp-2 suggesting that this tubular organelle is associated with a late endocytic compartment (Rabinowitz et al., 1992). The endoplasmic reticulum can also exhibit a highly tubular structure throughout the cytoplasm (Fawcett, 1981). Labeling of the endoplasmic reticulum in living cells with the dye DiOC6 reveals the extension and formation of an interconnected network of tubular elements (Terasaki et al., 1986; Lee and Chen, 1988; Lee et al., 1989). A similar tubular network is formed after the in vitro incubation of microsomes on a microtubule substrate (Dabora and Sheetz, 1988*b*; Vale and Hotani, 1988). While tubular organelles have been identified morphologically and labeled by endocytic tracers and protein markers of other endocytic organelles, a tubule-specific protein has yet to be identified.

The receptor for autocrine motility factor (AMF-R) was initially identified by its increased O-glycosylation after cell shape modulation of the metastatic capability of B16-F1 melanoma cells (Nabi and Raz, 1987, 1988). Antibodies against AMF-R are capable of stimulating both in vivo lung colonizing ability and in vitro cell motility (Nabi and Raz, 1987; Nabi et al., 1990; Watanabe et al., 1991*a*) leading to the identification of AMF-R as the receptor for AMF (Liotta et al., 1986; Silletti et al., 1991; Watanabe et al., 1991*b*). Increased expression of AMF-R correlates directly with the loss of E-cadherin in malignant bladder carcinomas and with a high incidence of recurrence and decreased survival in colorectal cancer (Nakamori et al., 1994; Otto et al., 1994). The AMF-R–mediated signaling pathway by which AMF stimulates cell motility involves a pertussis toxin sensitive G-protein, AMF-R phosphorylation, inositol phosphate production, and the lipoxygenase metabolite 12(S)-HETE (Stracke et al., 1987; Kohn et al., 1990; Nabi et al., 1990; Watanabe et al., 1991*b*; Timar et al., 1993). AMF-R exhibits a polarized cell surface distribution but intracellularly is found within elongated tubular vesicles (Nabi et al., 1992). We present evidence here that AMF-R is a marker for a distinct membranous tubular organelle.

Materials and Methods

Cells and Cell Culture

All cells were grown in an air-5% CO_2 incubator at constant humidity. MDCK II cells were grown in DMEM supplemented with 5% FCS, nonessential amino acids, vitamins, and glutamine (Gibco, Burlington, Ontario). The MDCK cells used exhibit a highly spread morphology when plated at low density at low passage number (<10). HeLa cells were obtained from Dr. Eric Cohen (Université de Montréal, Montreal, Quebec) and were grown in DMEM supplemented with 10% FCS, nonessential amino acids, glutamine, and a penicillin-streptomycin antibiotic mixture (Gibco).

Antibodies and Chemicals

Monoclonal antibody against AMF-R was used either in the form of ascites fluid or concentrated hybridoma supernatant (Nabi et al., 1990). Antibodies to MDCK LAMP-2 were as previously described (Nabi et al., 1991). Antibodies to β-COP were kindly provided by Jennifer Lippincott-Schwartz (NIH, Bethesda, MD) and to calnexin by John Bergeron (McGill University, Montreal, Quebec). Antibody to the human transferrin receptor (OKT9) was purchased from Ortho Diagnostics (Raritan, NJ) and to tubulin from ICN (Mississauga, Ontario).

Secondary antibodies conjugated to either fluorescein, Texas red, or horseradish peroxidase were purchased from Jackson Laboratories (West Grove, PA). The fluorescent antibodies were designated for use in multiple labeling studies and no interspecies cross-reactivity was detected. To detect antibodies to AMF-R, secondary antibodies specific for the μ chain of rat IgM were used. Taxol was obtained from the National Products Branch of the National Cancer Institute (Bethesda, MD). Phalloidin conjugated to rhodamine was purchased from Molecular Probes (Eugene, OR). Except where otherwise indicated, all chemicals were purchased from ICN Biomedicals, Inc. (Costa Mesa, CA).

Immunofluorescence

Cells were plated sparsely on glass coverslips for 1–2 d, and then fixed by the addition of cold (−80°C) methanol directly to the coverslip, and then placed at −20°C for 15 min. In some experiments, cells were incubated with Ringer's solution (155 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 2 mM NaH_2PO_4, 10 mM glucose, 10 mM Hepes, pH 7.2) (Heuser, 1989) for 15 min at 37°C in a CO_2-free incubator, before methanol fixation. After fixation, cells were rinsed extensively with PBS (pH 7.4) supplemented with 0.1 mM Ca^{++} and 1 mM Mg^{++} (PBS/CM), and then incubated for ~30 min with PBS/CM containing 1% BSA (PBS/CM/BSA) to reduce nonspecific binding. All washings and incubations with both primary and secondary (FITC and Texas red conjugated) antibodies were done with PBS/CM/BSA. After labeling, the coverslips were mounted in Airvol (Air Products and Chemicals Inc., Allentown, PA) and viewed in a Zeiss Axiophot fluorescent microscope equipped with a 63× Plan Apochromat objective and selective filters. Images were photographed using Kodak T-Max 400 film.

Immunoblot

Cell lysates were separated by SDS-PAGE and blotted to nitrocellulose using a Mini-Protean apparatus (BioRad Labs, Hercules, CA). The blots were blocked with 20% skim milk in PBS/CM, incubated with the primary antibody, and then with the appropriate secondary antibody conjugated to horseradish peroxidase. The labeled bands were revealed by chemiluminescence using the ECL system (Amersham, Oakville, Ontario), and then ex-

1. *Abbreviations used in this paper*: AMF-R, receptor for autocrine motility factor; BFA, brefeldin A.

posed to preflashed Kodak X-Omat film. Prestained molecular weight markers were purchased from Sigma Chem. Co. (St. Louis, MO).

Triton X-100 Extraction

MDCK cells grown on either glass coverslips or tissue culture dishes were extracted with 1% Triton X-100 in PBS/CM containing protease inhibitors (1 mM PMSF, 10 μg/ml leupeptin, pepstatin A, and aprotinin) at 4°C for 30 min. Cells on coverslips were then fixed with cold methanol and immunofluorescently labeled with anti-AMF-R antibodies. The Triton X-100 extract of cells plated on tissue culture dishes was collected and the cellular material remaining attached to the plastic surface scraped into an equivalent volume of 1% SDS in PBS containing protease inhibitors. The total cellular content of AMF-R was determined by the lysis of unextracted cells with 1% SDS. Equal amounts by volume of the three lysates, diluted with sample buffer, were separated by SDS-PAGE, and blotted with antibodies to AMF-R.

Cell Homogenization

Ten 150-mm plates of MDCK cells, plated at a density of 2×10^6 cells per plate for 2–3 d until the cells were just confluent, were washed 4× with cold PBS, and then left on ice for 10 min before scraping the cells from the tissue culture dish. The scraped cells were then spun down at 1,500 rpm, resuspended in a homogenization buffer consisting of MEPS buffer (5 mM $MgSO_4$, 5 mM EGTA, 35 mM K^+Pipes, 0.2 M sucrose, pH 7.1) containing 1 mM DTT and protease inhibitors (Dabora and Sheetz, 1988*a*), recentrifuged, and then resuspended in approximately twice the cell volume of the homogenization buffer. The cells were homogenized by 12 strokes of a 1-ml Dounce homogenizer (tight pestle) on ice, and then centrifuged at 800 *g* at 4°C for 10 min. The pellet was resuspended in half the original volume of homogenization buffer, rehomogenized (12 strokes), and centrifuged at 800 *g*. The two supernatants were combined and centrifuged at 950 *g* to generate a postnuclear supernatant (PNS). The PNS was centrifuged at 20,000 *g* for 30 min (Biofuge 17) and the 20,000-*g* supernatant spun at 100,000 *g* for 60 min (RC M120; Sorvall Instruments, Wilmington, DE; fixed angle rotor) at 4°C. Proteins contained within the 100,000-*g* supernatant were precipitated with acetone at −20°C for 30 min and the 20,000-*g* pellet (20K P), the 100,000-*g* pellet (100K P), and acetone precipitate of the 100,000-*g* supernatant (100K S) were resuspended in 100 μl of a solution of 1% SDS, 50 mM Tris, pH 8, containing protease inhibitors. The samples were assayed for protein content using the BCA protein assay (Pierce, Rockford, IL). Equivalent amounts by volume of each of the samples (between 40 and 50 μg protein) were separated by SDS-PAGE and blotted with antibodies to AMF-R and LAMP-2.

Electron Microscopy

Cells grown on petri dishes were rinsed and incubated at 37°C in Ringer's solution for 15 min before fixing in Ringer's solution containing 2% paraformaldehyde and 0.2% glutaraldehyde for 30 min at 37°C. The fixed cells were rinsed in PBS/CM, scraped from the petri dish, and collected by centrifugation. The cell pellet was postfixed for 30 min with 1% osmium tetroxide in PBS/CM containing 1.5% potassium ferrocyanide (reduced osmium), dehydrated in a graded ethanol series and embedded in LR-White resin (Tamaki and Yamashina, 1994). Ultrathin sections (80 nm) were collected on formvar-coated grids, blocked for 1 h with 2% BSA and 2% goat serum in PBS/CM, and then incubated in PBS/CM/BSA at room temperature for 1 h with anti-AMF-R antibody followed by 12-nm gold conjugated anti-rat IgM (Jackson). The grids were washed with PBS/CM/BSA three times after the primary antibody, five times after the secondary antibody, and then washed three times with PBS/CM and twice with distilled water. The sections were then stained with 5% uranyl acetate for 15 min and visualized in a Philips 300 electron microscope.

Fields selected morphologically for the presence of a smooth tubule were photographed and the numerical density of particles associated with smooth tubules and vesicles, the rough endoplasmic reticulum, the plasma membrane, and the Golgi apparatus determined. The length of the limiting membrane of the indicated organelles was measured using a Sigma-Scan measurement system (Jandel Scientific, Corte Madera, CA) and the gold particles localized to these organelles counted. Rough endoplasmic reticulum was defined by the presence of a linear array of membrane-associated ribosomes. The Golgi apparatus was identified morphologically and the membrane length of associated saccules and vesicles measured. Control labeling was performed with 10 μg/ml nonimmune rat IgM antibodies (ICN), at a dilution equivalent to that of anti-AMF-R as determined by dot blot, and was analyzed similarly.

Drug Treatment

Brefeldin A (Calbiochem, San Diego, CA) was added at a concentration of 10 μg/ml directly to the cell culture medium for 30 min. Protein synthesis was inhibited by the addition of medium containing 100 μg/ml cycloheximide to the cells for the indicated time. Cycloheximide treatment reduced protein synthesis, measured by TCA precipitation of Triton X-100 extracts of [^{35}S]methionine-labeled cells, to 5% of the level of untreated cells after 1.5 h and to 2% after 14 h.

Actin cytoskeleton disruption was performed by the addition of 0.5 μg/ml cytochalasin D to the regular medium for 2 h at 37°C. Microtubule disruption was performed by the addition of 20 μM nocodazole in Ringer's solution for 30 min on ice to cells preincubated for 15 min with Ringer's solution at 4°C. Subsequent incubation with nocadozole or 10 μM taxol at 37°C for 30 min was preceded by a rapid rinse with warm Ringer's solution.

Results

Tubular Localization of AMF-R

The MDCK II cells used in this study exhibit a highly spread morphology at low passage which facilitated visualization of AMF-R distribution by fluorescence microscopy. Immunofluorescent labeling of MDCK cells with anti-AMF-R antibodies after direct fixation with cold methanol shows that AMF-R is localized to tubular and vesicular structures distributed throughout the cytoplasm. In some cells, the AMF-R labeling is localized to a dense perinuclear region. The tubular vesicles extend to and are oriented towards the periphery of the cell. A highly punctate nuclear label is also observed in certain cell preparations labeled immunofluorescently with the anti-AMF-R mAb (Fig. 1 *a*). The nuclear staining is not always observed suggesting that it is nonspecific.

To ascertain the specificity of the nuclear label detected by immunofluorescence, cells were extracted with 1% Triton X-100 at 4°C, and then fixed with cold methanol for immunofluorescent labeling. After Triton X-100 extraction only the punctate nuclear label could be detected and the cytoplasmic tubular label had disappeared (Fig. 1 *b*). From parallel cultures, the Triton X-100 extract was collected and the petri dishes then scraped and the nonextracted material lysed in an equal volume of 1% SDS. AMF-R content of equivalent amounts by volume of these fractions was determined by immunoblot and compared to the total cellular AMF-R extracted with SDS (Fig. 1 *c*). All AMF-R detected was associated with the Triton X-100 extracted material and no AMF-R could be detected in the nuclear-associated material. The immunofluorescent nuclear labeling by the anti-AMF-R antibody is therefore nonspecific and AMF-R is present within a cytoplasmic Triton X-100 extractable organelle.

AMF-R Tubules Do Not Colocalize with Early Endosomes, Golgi Apparatus, or Lysosomes

Tubular early endosomes have been described as have tubular elements of the endosomal recycling compartment (Marsh et al., 1986; Gruenberg et al., 1989; Hopkins et al., 1990; Tooze and Hollinshead, 1991). Brefeldin A treatment induces the formation of elongated tubules between early endosomes and the TGN (Lippincott-Schwartz et al., 1991; Wood et al., 1991). To assess whether AMF-R is localized

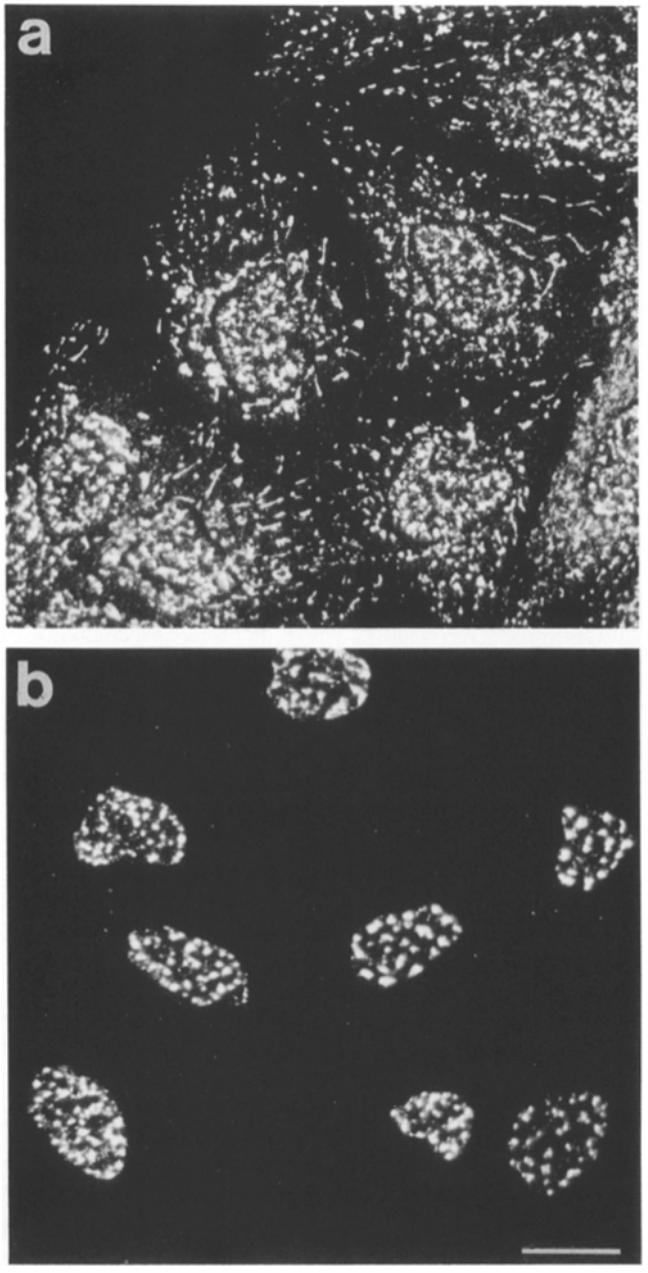

Figure 1. AMF-R is localized to Triton X-100 extractable tubules. MDCK cells were fixed with cold methanol directly (*a*) or after extraction with 1% Triton X-100 for 30 min at 4°C (*b*), and then immunofluorescently labeled for AMF-R. After treatment with Triton X-100, AMF-R tubules are extracted but the fluorescent nuclear label remains. In *c*, equal amounts of the total cellular extract (*Total*), Triton X-100 extracted material (*TX-100*) and the nonextracted material (*Non-Extr.*) were immunoblotted with antibodies to AMF-R (78 kD). Molecular mass markers from top to bottom: 180, 116, 84, 58, and 46 kD. Bar, 20 μm.

to early endosomes or to early endosome derived BFA-induced tubules, HeLa cells were double labeled with antibodies to AMF-R and the human transferrin receptor (Fig. 2). In HeLa cells, the transferrin receptor exhibited a characteristic punctate early endosomal distribution throughout the cytoplasm. After BFA treatment, elongated tubules were formed which localized primarily over the cell nucleus (Fig. 2 *d*). AMF-R tubules did not colocalize with the transferrin receptor neither before nor after BFA treatment. The BFA-induced tubules labeled with the transferrin receptor were narrower and more elongated than AMF-R tubules and did not extend to the cell periphery as did AMF-R tubules. In both HeLa (Fig. 2) and MDCK cells (not shown), the morphology and distribution of AMF-R tubules were apparently unaffected by treatment with BFA.

Previous studies have described tubular lysosomes labeled by fluid phase uptake (Swanson et al., 1987; Heuser, 1989). The perinuclear distribution of AMF-R might coincide with the Golgi apparatus. To determine whether AMF-R tubules colocalize with lysosomes or the Golgi apparatus, we used the organelle specific markers β-COP for the Golgi apparatus (Donaldson et al., 1990) and LAMP-2 for lysosomes (Nabi et al., 1991; Nabi and Rodriguez-Boulan, 1993). The tubular pattern of AMF-R labeling is clearly distinct from that of the Golgi apparatus (Fig. 3, *a* and *b*) or lysosomes (Fig. 3, *c* and *d*). The perinuclear Golgi label does not coincide with perinuclear AMF-R labeling. In the spread cytoplasmic region of the cells, overlap of AMF-R tubules with LAMP-2–labeled lysosomes is not observed. The AMF-R tubule is therefore not equivalent to either the Golgi apparatus or the lysosome.

Confirmation that AMF-R is not found within LAMP-2–containing lysosomes in MDCK cells was obtained from subcellular fractionation studies (Fig. 4). MDCK cells grown to near confluency yet still spread were scraped from tissue culture plates and homogenized. The postnuclear supernatant was centrifuged initially at 20,000 *g* and the 20,000-*g* supernatant was then centrifuged at greater than 100,000 *g*. The 20,000-*g* pellet (20K P), 100,000-*g* pellet (100K P), and 100,000-*g* supernatant (100K S) were analyzed for expression of LAMP-2 and AMF-R by immunoblot. LAMP-2 was localized predominantly to the 20,000-*g* lysosomal pellet and essentially no LAMP-2 was found in either the 100,000-*g* pellet or supernatant consistent with its lysosomal localization. AMF-R was localized to the 100,000-*g* pellet consistent with its presence in membranous components of the cell. The ability to distinguish AMF-R expression from LAMP-2 by both double immunofluorescence and subcellular fractionation indicates that, at least in MDCK cells, the AMF-R tubule cannot be considered to be a lysosomal organelle.

Electron Microscopic Localization of AMF-R to a Membranous Tubular Compartment

The lability of the AMF-R tubule to conventional fixatives led us to use a rapid cold methanol (−80°C) fixation method which maintains tubule morphology for immunofluorescence microscopy. Tubular lysosomes have been described as a highly labile organelle (Robinson et al., 1986) and Ringer's solution, a bicarbonate free buffer, has been used to study tubular lysosome expression (Heuser, 1989; Racoosin and Swanson, 1993). Our previous use of this solution to study AMF-R tubules (Nabi et al., 1992) directed us to establish a tubule fixation protocol for electron microscopy

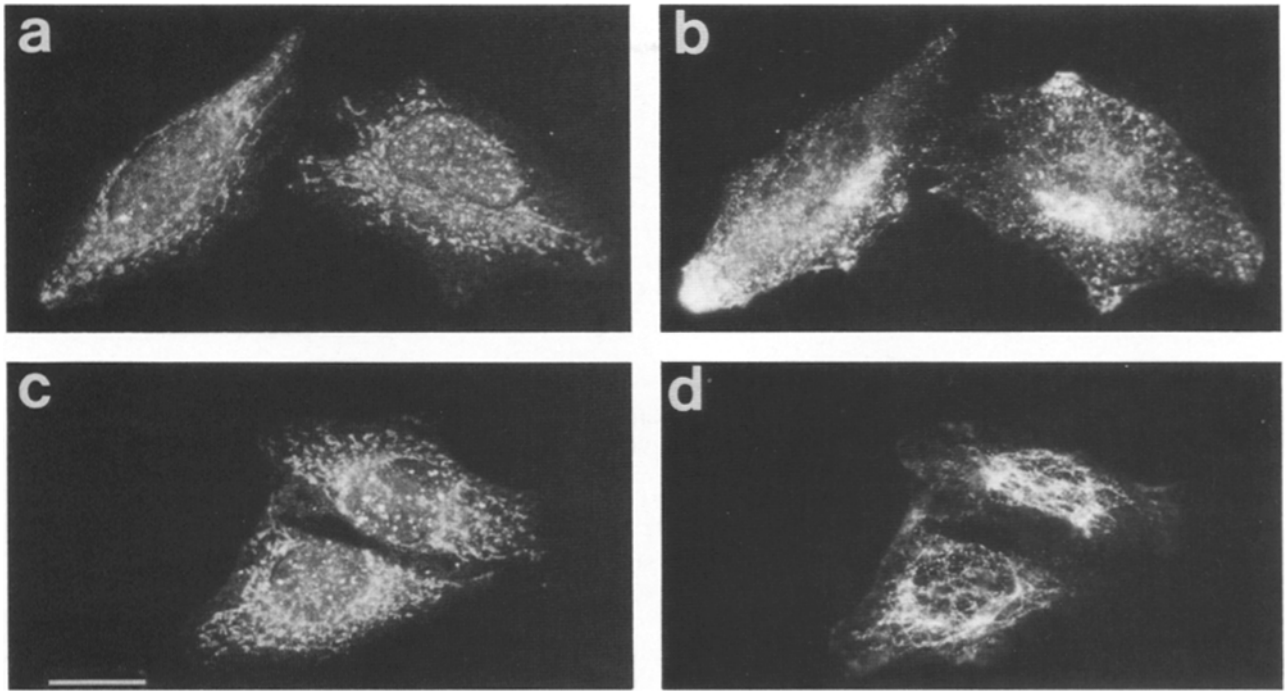

Figure 2. AMF-R tubules do not codistribute with endosomes containing the transferrin receptor. HeLa cells were either left untreated (*a* and *b*) or treated with 10 μg/ml BFA for 60 min (*c* and *d*). AMF-R (*a* and *c*) and the human transferrin receptor (*b* and *d*) were localized by double immunofluorescent labeling in the same cells using selective filters. Bar, 20 μm.

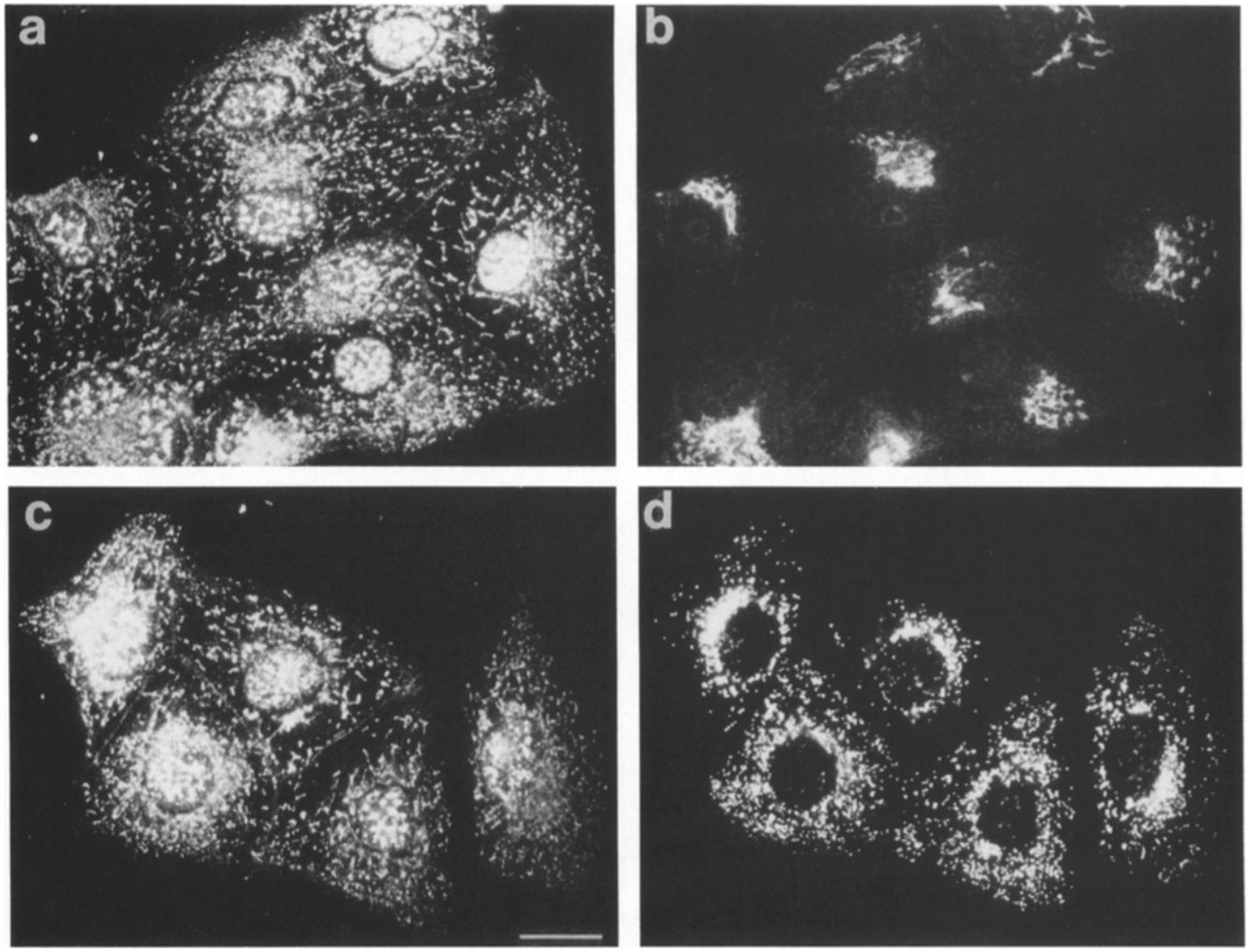

Figure 3. AMF-R tubules do not colocalize with the Golgi apparatus or lysosomes. MDCK cells were double immunofluorescently labeled for AMF-R (*a* and *c*) and either β-COP (*b*) or LAMP-2 (*d*). The left and right panels represent identical fields. Bar, 20 μm.

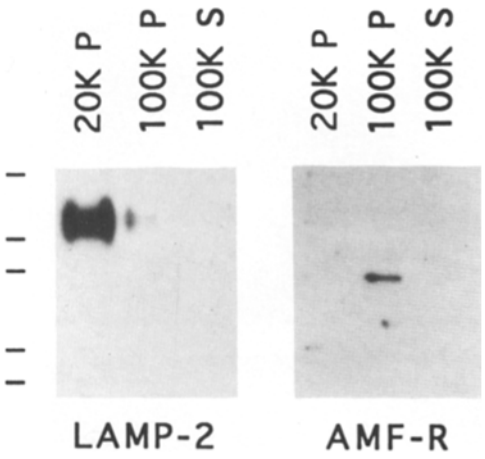

Figure 4. Localization of AMF-R and LAMP-2 to different subcellular fractions of MDCK cells. MDCK cells were homogenized and the postnuclear supernatant centrifuged sequentially at 20,000 *g* and 100,000 *g* to generate a 20,000-*g* pellet (*20K P*), a 100,000-*g* pellet (*100K P*), and a 100,000-*g* supernatant (*100K S*). These fractions were separated by SDS-PAGE and blotted with antibodies to LAMP-2 and AMF-R, as indicated. Molecular mass markers from top to bottom: 180, 116, 84, 58, and 46 kD.

using aldehyde fixatives based on Ringer's solution. MDCK cells were rinsed in Ringer's solution, and then fixed with 2% paraformaldehyde and 0.2% glutaraldehyde in Ringer's solution, and then processed and embedded in the hydrophilic LR-White resin. AMF-R labeling was weak but highly specific for membranous organelles.

Postembedding labeling with anti–AMF-R and 12-nm gold-conjugated anti–rat secondary antibodies revealed the specific cytoplasmic localization of AMF-R to a smooth membrane limited tubular organelle (Fig. 5). AMF-R labeled both isolated tubules (Fig. 5, *c*, *d*, and *e*) and vesicles (Fig. 5, *f* and *g*) as well as more elaborate tubular networks (Fig. 5, *a* and *b*). The average outside diameter of both the vesicular and tubular AMF-R-labeled compartments is variable and ranges from 50–250 nm. Quantification of the labeling by morphometry revealed that the majority of AMF-R labeling is found on smooth membranous tubules and vesicles (Table I). A lesser but apparently specific labeling was also found on rough endoplasmic reticulum defined by the presence of a linear array of membrane-associated ribosomes (see Fig. 5, *c* [*top*] and *d*). The significant AMF-R expression on the plasma membrane is consistent with the surface expression of AMF-R and its function as a motility factor receptor (Nabi et al., 1990; Silletti et al., 1991; Nabi et al., 1992). AMF-R labeling of the Golgi apparatus was minimal and essentially equivalent to the labeling detected with control nonimmune rat IgM antibody.

AMF-R Tubules Do Not Colocalize with the ER

To better define the relationship of the AMF-R tubule to the rough endoplasmic reticulum, we performed double immunofluorescent labeling of AMF-R tubules with the rough endoplasmic reticulum marker calnexin (Wada et al., 1991). Calnexin has been localized by electron microscopy exclusively to the nuclear membrane and rough endoplasmic reticulum (Hochstenbach et al., 1992). MDCK cells pretreated with Ringer's solution before fixation revealed the distribution of calnexin to an extended reticular network throughout the cytoplasm. Distinct tubular structures were not observed and the distribution of AMF-R–labeled tubules could clearly be distinguished from the more diffuse labeling of the rough endoplasmic reticulum with calnexin (Fig. 6, *a* and *b*). On rare occasions, AMF-R tubules could be observed to coincide with calnexin labeling (Fig. 6, *a* and *b*, *arrows*), consistent with the presence of AMF-R in rough endoplasmic reticulum observed by electron microscopy. To ensure that the AMF-R tubule is not a transient subcompartment of the endoplasmic reticulum in the biosynthetic pathway of AMF-R, we immunolabeled cells after cycloheximide treatment (Fig. 6, *c* and *d*). The tubular label of AMF-R was conserved even after 14 h of cycloheximide treatment. In contrast, the reticular nature of the endoplasmic reticulum was lost and calnexin was distributed diffusely throughout the cytoplasm. Under these conditions less than 2% of the protein synthesis of untreated cells could be detected. The continued tubular expression of AMF-R after cycloheximide treatment demonstrates that AMF-R is a stable component of this tubular organelle and that the tubular expression of AMF-R is not due to the presence of newly synthesized AMF-R in tubules of the endoplasmic reticulum. The ability

Table I. Quantitative Analysis of Postembedding Labeling of AMF-R

	Smooth tubules and vesicles	Rough endoplasmic reticulum	Plasma membrane	Golgi apparatus
AMF-R				
μm membrane	344.7	61.2	148.2	24.2
Gold particles/μm membrane	1.67 ± 0.20	0.58 ± 0.18	0.52 ± 0.13	0.17 ± 0.10
Control				
μm membrane	78.7	32.0	57.6	47.5
Gold particles/μm membrane	0.14 ± 0.06	0.02 ± 0.02	0.11 ± 0.07	0.27 ± 0.10

Gold particles associated with the indicated membrane organelles were counted and the density per μm membrane length determined. Control labeling was determined using a nonimmune rat IgM antibody.

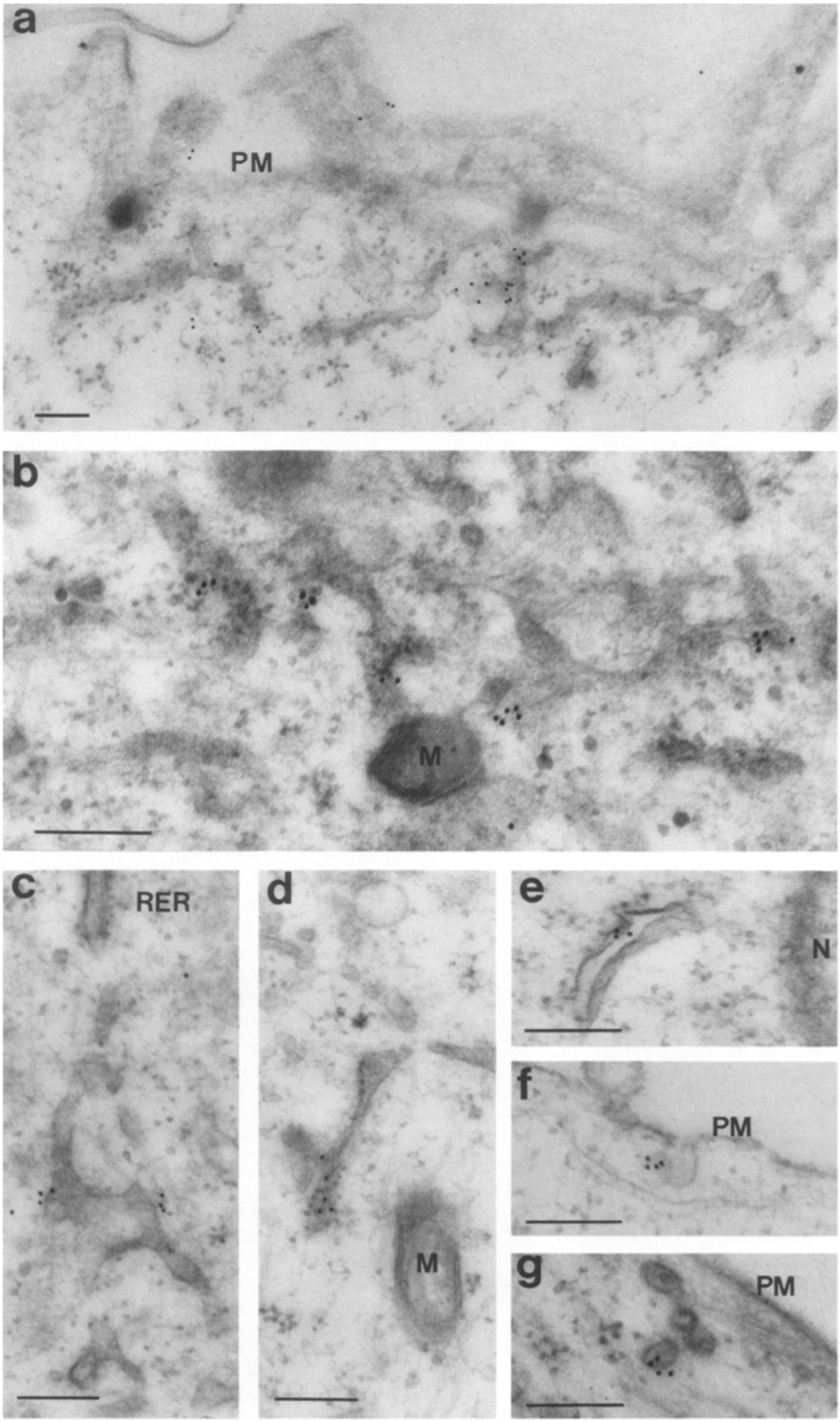

Figure 5. Electron microscopic localization of AMF-R. MDCK cells were fixed with 2% paraformaldehyde and 0.2% glutaraldehyde in Ringer's solution, osmicated, and embedded in LR white resin before postembedding labeling with anti-AMF-R and 12-nm gold-conjugated anti-rat IgM secondary antibodies. AMF-R is localized to smooth tubules which exhibit highly elaborate morphologies (*a* and *b*). AMF-R labels predominantly smooth tubules (*c* and *e*) but also some tubules which are partially coated with ribosomes (*d*). AMF-R also labels vesicular structures (*f* and *g*) as well as the plasma membrane including uncoated surface invaginations (*a* and *f*). The nucleus, plasma membrane (*PM*), mitochondria (*M*), and rough endoplasmic reticulum (*RER*) are indicated. Bars, 0.2 μm.

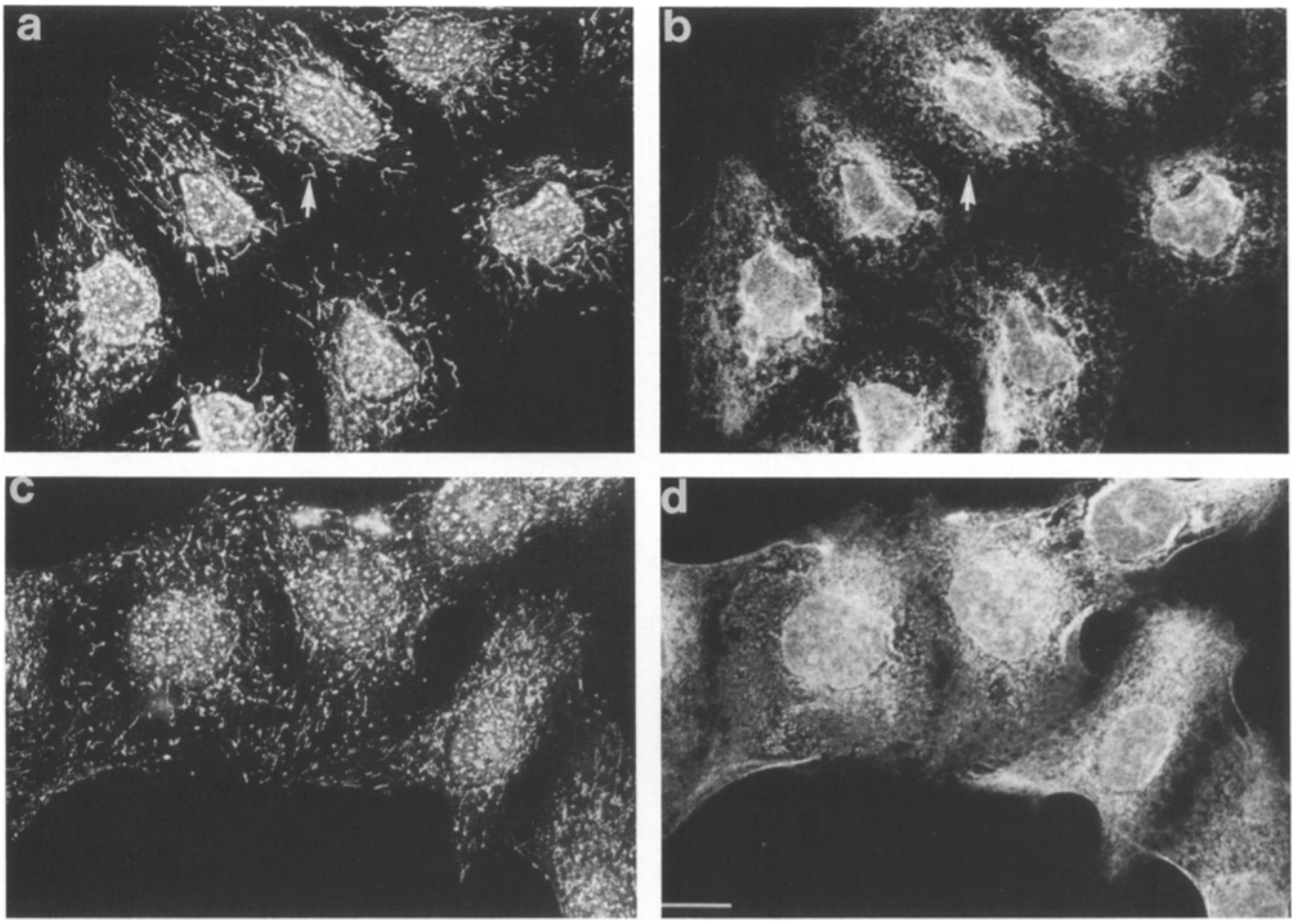

Figure 6. AMF-R tubules do not colocalize with the calnexin-labeled rough endoplasmic reticulum. MDCK cells left untreated (*a* and *b*) or treated with 100 μg/ml cycloheximide for 14 h (*c* and *d*) were double immunofluorescently labeled for AMF-R (*a* and *c*) and calnexin (*b* and *d*). Cells were incubated with Ringer's solution for 15 min at 37°C before cold methanol fixation. Rarely, colocalization of AMF-R and calnexin in the same tubular structure can be observed in untreated cells (*arrows*). Bar, 20 μm.

to clearly distinguish AMF-R from calnexin-labeling demonstrates that this tubular organelle is not a subcompartment of the rough endoplasmic reticulum.

Cytoskeletal Interactions of AMF-R Tubules

The AMF-R tubule is therefore a tubular membranous compartment which can be distinguished from early endosomes, lysosomes, the Golgi apparatus, and the rough endoplasmic reticulum. Its tubular morphology, observed both by immunofluorescence and electron microscopy, is highly suggestive of an interaction with the cellular cytoskeleton.

In polarized epithelial cells, actin fibers are oriented circumferentially around the cell periphery, compared to the actin cytoskeleton of fibroblasts, in which actin stress fibers are oriented the long of the cell parallel to the direction of cell movement (Takeuchi, 1987). This is observable even in the sparsely plated highly spread MDCK cells used in this study in which the actin fibers exhibit a circumferential distribution around the cell periphery (Fig. 7 *b*). Disruption of the actin cytoskeleton by cytochalasin D treatment of MDCK cells did not disrupt AMF-R tubule morphology, but rather resulted in the increased elongation and extension of labeled tubules to the cell periphery (Fig. 7 *c*). Cytochalasin D treatment of MDCK cells induced the formation of cellular extensions containing individual microtubules which coincided with tubular AMF-R labeling (Fig. 8). Similarly, in untreated cells, AMF-R tubules codistribute and exhibit a colinearity with microtubules, however, the dense microtubule staining makes it impossible to identify the association of AMF-R tubules with individual microtubules (see Fig. 9, *a* and *b*). These results suggest that, while not necessary to maintain AMF-R tubule morphology, the actin cytoskeleton may regulate the elongation and extension of AMF-R tubules along microtubules to the cell periphery.

To better study the effect of microtubule disruption on AMF-R tubule morphology, we treated MDCK cells with the microtubule disrupting drug nocodazole in Ringer's solution (Fig. 9). Preincubation in Ringer's solution favors the formation of more elongated tubules such that the effects of microtubule disrupting drugs were better observed. After a 15-min preincubation at 37°C in Ringer's solution, MDCK cells were treated with 20 μM nocodazole for 30 min at 4°C, and then with nocodazole at 37°C to disrupt the microtubule integrity of the cells. Loss of microtubule integrity was associated with the loss of the peripheral orientation of AMF-R tubules and the formation of vesicles and shorter unoriented

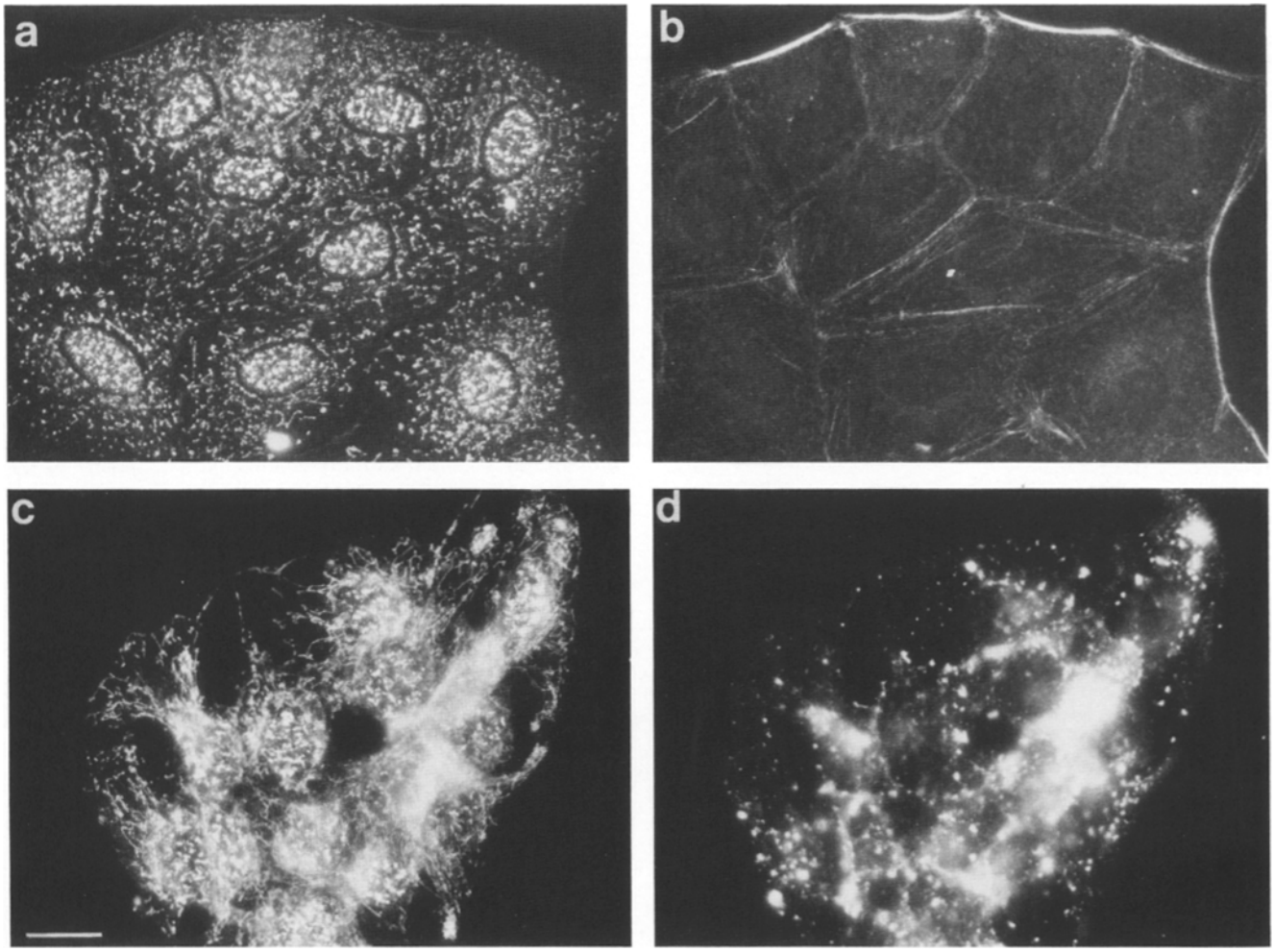

Figure 7. Disruption of the actin cytoskeleton of MDCK cells induces the elongation of AMF-R tubules. MDCK cells were either left untreated (*a* and *b*) or treated with 0.5 μg/ml cytochalasin D for 2 h at 37°C (*c* and *d*) to disrupt the actin cytoskeleton. AMF-R labeling was revealed with FITC-conjugated anti-rat secondary antibodies (*a* and *c*) and actin with rhodamine-conjugated phalloidin (*b* and *d*). Bar, 20 μm.

tubules (Fig. 9, *c* and *d*). Nocodazole treatment at 4°C followed by warming to 37°C in the presence of the microtubule stabilizing drug taxol induced the formation of short microtubule fibers and a loss of the oriented tubular morphology of AMF-R–labeled tubules (Fig. 9, *e* and *f*). Interestingly, microtubule disruption is associated with the loss of the linear morphology of AMF-R tubules and the formation of "curly" tubules. This is far more evident in the taxol-treated cells in which tubule length was maintained, although not linearly. The results obtained with nocodazole and taxol clearly indicate that the linear extension and peripheral cellular orientation of AMF-R tubules is microtubule dependent and identifies the AMF-R tubule as a microtubule-associated organelle. The actin and microtubule cytoskeletons may coordinately interact to regulate AMF-R tubule morphology and extension to the cell periphery.

Discussion

The autocrine motility factor receptor, AMF-R, is expressed on the cell surface, where it functions as a motility factor receptor, as well as intracellularly within elongated tubules (Nabi et al., 1992). We show here that AMF-R tubules are Triton X-100 extractable and after cell homogenization AMF-R is localized to a 100,000-*g* membrane fraction. By immunoelectron microscopy, AMF-R is localized predominantly to smooth membrane tubules and vesicles with an outer diameter which varies from 50–250 nm. To a lesser extent, AMF-R is localized to the plasma membrane and the rough endoplasmic reticulum. These results are consistent with the plasma membrane localization of AMF-R as well as its localization to an intracellular membranous tubular organelle. After disruption of the actin cytoskeleton of MDCK cells, AMF-R tubules extended into peripheral cellular extensions where they exhibited a highly elongated morphology and can be observed to coalign with microtubules. Disruption of the microtubule cytoskeleton by nocodazole treatment or its modulation by sequential nocodazole and taxol treatment results in the loss of the extended linear morphology and peripheral orientation of AMF-R tubules. AMF-R is therefore a marker for a membranous tubular compartment whose morphology can be regulated by modulation of the microtubule and actin cytoskeletons.

By immunofluorescence microscopy, AMF-R tubules do

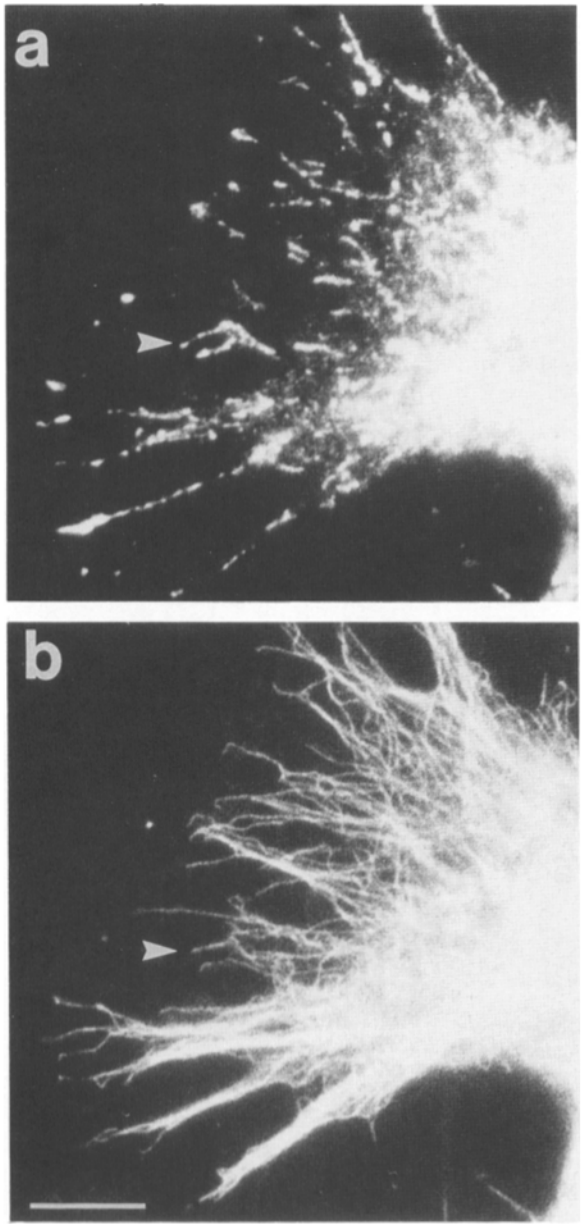

Figure 8. AMF-R tubules are associated with individual microtubules. MDCK cells treated with 0.5 μg/ml cytochalasin D were labeled for AMF-R (*a*) and tubulin (*b*) and a peripheral region containing cellular extensions was enlarged to show the association of AMF-R vesicles with individual microtubules. The arrowheads reveal a distinctive motif in which AMF-R tubules can be seen to directly coincide with single microtubules. Bar, 10 μm.

not colocalize with transferrin receptor–labeled early endosomes nor with BFA-induced tubules connecting the TGN and early endosomes. The diameter of AMF-R tubules is larger (50–250 nm) than tubular endosomes labeled by fluid phase or transferrin endocytosis (30–60 nm) (Tooze and Hollinshead, 1991; Hopkins et al., 1994). We also do not observe colocalization of perinuclear AMF-R label with the Golgi apparatus labeled with an antibody to β-COP (Donaldson et al., 1990). Tubular lysosomes have been defined as lysosomal based on the presence of lysosomal acid phosphatase and the lysosomal membrane glycoprotein, lgp120 (Swanson et al., 1987; Luo and Robinson, 1992; Racoosin and Swanson, 1993). However, AMF-R tubules are clearly distinguished from lysosomes labeled with the lysosomal membrane glycoprotein LAMP-2 (Nabi and Rodriguez-Boulan, 1993) by double immunofluorescent labeling as well as by the ability to separate AMF-R from LAMP-2–containing lysosomes by differential centrifugation. The variable size of AMF-R tubules (50–250 nm in diameter) is similar but not equivalent to that observed for the tubular lysosome (70–100 nm) (Phaire-Washington et al., 1980; Swanson et al., 1987; Araki et al., 1993). A tubular morphology similar to that described for the AMF-R tubule has been described for the basal lysosome in exocrine acinar cells (Oliver, 1983) and for the snake-like tubule in the exocrine pancreas (Beaudoin et al., 1985). Interestingly, these similar tubular organelles are labeled cytochemically by trimetaphosphatase and β-NADPase reactions but not by acid phosphatase reactions. As we observe for the AMF-R tubule, the basal lysosome appears to be closely related to the endoplasmic reticulum (Oliver, 1983). The absence of an acid phosphatase reaction for these tubules of exocrine cells supports their identity with the AMF-R tubule as a similar nonlysosomal tubular organelle.

The smooth AMF-R–labeled tubules can be distinguished morphologically from ribosome-studded tubules of the rough endoplasmic reticulum by their more elaborate morphology with evident membrane infoldings (Fig. 5, *a* and *b*). However, AMF-R does label rough tubules and AMF-R tubules can be detected which contain both smooth and rough membranes (Table I; Fig. 5 *d*). The endoplasmic reticulum labeled in living cells reveals a highly interconnected tubular network consisting of linear tubules, a polygonal reticulum and triple junctions (Terasaki et al., 1986; Lee and Chen, 1988; Lee et al., 1989). While isolated tubules of the endoplasmic reticulum labeled fluorescently with DiOC6 resemble AMF-R tubules (Terasaki et al., 1986), we do not observe an interconnected tubular network labeled by antibodies to AMF-R. The AMF-R tubule is clearly not a subcompartment of the rough endoplasmic reticulum as AMF-R tubular labeling can be distinguished from that of calnexin. Furthermore, AMF-R tubule distribution is resistant to cycloheximide treatment indicating that AMF-R is a stable constituent of this tubule which should not be considered a biosynthetic organelle. It is possible that the AMF-R tubule is related to smooth endoplasmic reticulum, an organelle associated with lipid metabolizing cells that has been identified morphologically in liver and other tissues (Fawcett, 1981). Further definition of the relationship of the AMF-R tubule and the endoplasmic reticulum should prove interesting.

Extension to the cell periphery of the endoplasmic reticulum and tubular lysosome is microtubule dependent (Terasaki et al., 1986; Swanson et al., 1987; Lee et al., 1989; Hollenbeck and Swanson, 1990). Similar to these two tubular organelles, after nocodazole treatment and loss of microtubule integrity, AMF-R tubules are shorter and show a distinct loss of orientation towards the periphery of the cell. As described for the endoplasmic reticulum (Lee et al., 1989), the linear extension and peripheral orientation of AMF-R tubules was not supported by short taxol-stabilized microtubules and AMF-R tubule morphology was maintained, even enhanced, after actin cytoskeleton disruption.

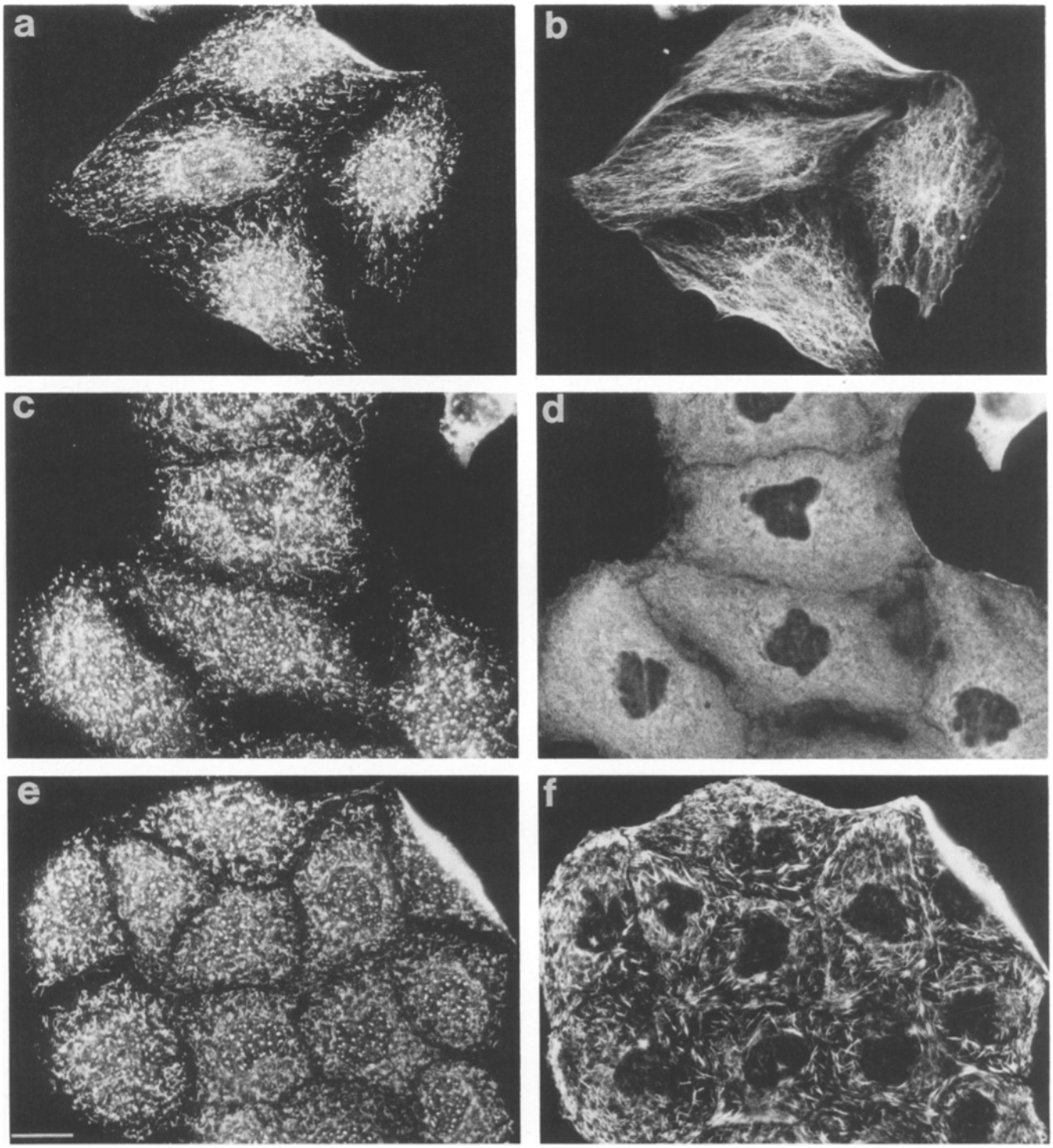

Figure 9. Linear extension and peripheral orientation of AMF-R tubules is microtubule dependent. MDCK cells were preincubated in Ringer's solution for 15 min (*a* and *b*) or incubated with 20 μM nocodazole at 4°C before warming to 37°C in the continued presence of nocodazole (*c* and *d*) or in the presence of 10 μM taxol (*e* and *f*). Loss of microtubule integrity due to nocodazole treatment and the formation of short taxol stabilized microtubule fibers are both associated with a similar loss of the linear extension and peripheral orientation of AMF-R tubules. Bar, 20 μm.

After cytoplasmic acidification, both the AMF-R tubule and tubular lysosome lose their tubular morphology and form vesicles which move to the periphery of the cell (Heuser, 1989; Nabi et al., 1992). These observations suggest that the AMF-R tubule is related to this endocytic organelle and that the rapid intracellular movement of these organelles occurs along microtubules (Heuser, 1989). The role of kinesin in tubular lysosome extension to the cell periphery was demonstrated by the inhibitory effect of microinjected anti-kinesin antibodies, similar to that of nocodazole treatment (Hollen-

beck and Swanson, 1990). Our results suggest, as described for the endoplasmic reticulum and the tubular lysosome, that the extension of the AMF-R tubule to the cell periphery is mediated by the microtubule cytoskeleton.

We propose that the AMF-R tubule constitutes, along with the tubular lysosome and endoplasmic reticulum, a member of a family of microtubule-associated tubular organelles. These organelles may act in various cellular functions to target cellular components to peripheral regions of the cell. Differential morphology and extension throughout the cytoplasm of the endoplasmic reticulum may enhance its surface area and increase the efficiency of protein synthesis (Rajasekaran et al., 1993). The tubular lysosome or tubular compartment of the macrophage may serve to deliver lysosomal components to newly internalized phagosomes or pinosomes (Knapp and Swanson, 1990; Rabinowitz et al., 1992; Racoosin and Swanson, 1993). The identity of AMF-R as a receptor for a motility factor with a defined role in cell motility and tumor metastasis implicates the AMF-R tubule in the motile process.

Forward cell locomotion is associated with cytoskeletal force-generating mechanisms, insertion of membrane at the leading edge, and the coordinate establishment and disruption of cell-substrate adhesive contacts (Singer and Kupfer, 1986). Disruption of microtubules by specific drugs has long been known to inhibit cell movement (Vasiliev et al., 1970; Gail and Boone, 1971; Goldman, 1971). Newly synthesized VSV G protein has been shown to be targeted to the leading edge of motile fibroblasts (Bergmann et al., 1983). Nocodazole treatment disrupts the polarity of G protein insertion into the plasma membrane implicating microtubules in the determination of the directionality of pseudopodial extension (Rogalski et al., 1984). Extension of microtubules into neuronal growth cones after cytochalasin D treatment suggests that the actin cytoskeleton network can restrict microtubule extension into peripheral lamella (Forscher and Smith, 1988). Directed microtubule extension towards growth cone target interactions are apparently guided by localized F-actin assembly (Lin and Forscher, 1993). The increased elongation and extension of AMF-R tubules to the cell periphery after cytochalasin D treatment of MDCK cells may reflect a restrictive role for the actin cytoskeleton on the extension of microtubules and AMF-R tubules to the cell periphery. The AMF-R tubule is a candidate for a motility specific compartment targeted along microtubules to the leading edge of motile cells. AMF-R endocytosis and intracellular targeting to this tubular organelle might thus be involved in the function of AMF-R as a motility factor receptor. However, the AMF-R tubule is apparently not equivalent to the transferrin receptor labeled tubular endosome targeted to the leading edge of migrating fibroblasts (Hopkins et al., 1994).

Autocrine motility factor receptor is described here as a marker for a distinct microtubule-associated tubular membranous organelle. The AMF-R tubule can be distinguished from early endosomes, lysosomes, the endoplasmic reticulum, and the Golgi apparatus identified by specific markers. The AMF-R tubule is a smooth membranous structure which can form extended tubular networks. The availability of a marker for this tubular organelle will allow the further characterization of the nature of this organelle as well as the determination of its role in cell motility.

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