

## Actin filaments facilitate two steps of endocytosis

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### SUMMARY

Recent reports have suggested a role for microtubules in the endocytic process and pointed out the role for actin filaments for the early steps of apical endocytosis in polarized epithelial cells. However, these studies do not address the respective contribution of these two types of filaments along the endocytic pathway. In addition, several studies failed to demonstrate the role for actin filaments in clathrin dependent endocytosis in non polarized cells. In the present study we have investigated the role for both filaments in the endocytosis of two ligands internalized via clathrin coated pits (transferrin and  $\alpha$ 2-macroglobulin) in a mouse hepatoma cell line. By immunocytochemical analysis with confocal microscopy, and biochemical analysis using a temperature sensitive step at 18°C, we have shown that actin filaments are involved in two steps of the

degradative pathway, and that microtubules are required at a stage in between. Actin filaments increase first, the uptake of ligands and second, their delivery to the degradative compartment, whereas microtubules are required to maintain the distribution of the late endocytic compartment in its juxtannuclear position and facilitate the delivery of the ligands to the degradative compartment upstream of the actin filament requirement. Furthermore actin filaments facilitate the recycling of transferrin from the perinuclear region to the plasma membrane. Our data indicate for the first time the sequential involvement of actin filaments and microtubules along one intracellular membrane trafficking pathway.

Key words: Actin, Endocytosis, Microtubule, Trafficking

### INTRODUCTION

Numerous macromolecules such as growth factors or lipoproteins bound to specific receptors at the cell surface are internalized by the cell via endocytosis. Initially, macromolecules are internalized into early endosomes and from there they are either recycled to the plasma membrane via tubular extensions or became concentrated within the vesicular main body of the sorting endosomes before being routed towards lysosomes (Dunn and Maxfield, 1992; Hopkins et al., 1990; Ghosh et al., 1994).

Several lines of evidence argue for a role for the cortical actin network in the endocytic process. Sako and Kusumi (1994), using video-enhanced contrast optical microscopy, have reported that transferrin and  $\alpha$ 2-macroglobulin bound to their receptors interact directly or indirectly with actin filaments below the cell surface. Although several attempts failed to demonstrate a role for the actin filaments in the endocytosis of transferrin, two recent studies have suggested a role for actin filaments in the clathrin dependent endocytosis of the  $\alpha$ -factor in yeast and the influenza virus at the apical domain of MDCK (Gottlieb et al., 1993; Kübler and Riezman, 1993). Evidences also suggested a role for microtubules in endocytosis. Microtubules are required in fibroblastic cells to maintain the cellular distribution of late endosomes in the vicinity of the microtubule organizing center (MTOC), as well as lysosomes (Matteoni and Kreis, 1987). In addition they increase the rate of the delivery of ligands to the degradative compartments

(EGF and asialoglycoproteins or the transmembrane proteins G of the VSV; Oka, 1983; Berg et al., 1985; Gruenberg et al., 1989; Brown et al., 1980).

Although these studies clearly indicate the participation of both microtubules and microfilaments in endocytosis, they do not address the respective contribution of these two types of filaments along the pathway to the lysosomes and the recycling pathway. In the present study we have investigated the role for both filaments in the endocytosis of two ligands internalized via clathrin coated pits in a mouse hepatoma cell line (BWTG3). The first, transferrin, is recycled to the plasma membrane from endosomes located at the cell periphery and in some cells from endosomes located in the juxtannuclear area (Hopkins, 1983; Hopkins et al., 1994). In contrast, the second,  $\alpha$ 2-macroglobulin dissociates from its receptor at low pH in the endosomes and is transferred to lysosomes where it is degraded (Tycko and Maxfield, 1982). By immunocytochemical analysis with confocal microscopy, and kinetic analysis, we have shown that actin filaments are involved in 2 steps of the degradative pathway, and that microtubules are required at a stage in between those which involve actin filaments. Actin filaments increase the uptake of ligands and their delivery to the degradative compartment, whereas microtubules are required to maintain the distribution of the late endocytic compartment in its juxtannuclear position and facilitate the delivery of the ligands to the degradative compartment upstream of the actin filament requirement.

## MATERIALS AND METHODS

### Drugs

Cytochalasin D and colchicine were purchased from Sigma. Microfilaments were depolymerized by incubating cells with 1  $\mu\text{M}$  of cytochalasin D in culture medium for 30 minutes at 37°C prior to the experiment. Microtubules were depolymerized by incubating cells with 30  $\mu\text{M}$  of colchicine in culture medium for 5 minutes at 4°C and 30 minutes at 37°C prior to the experiment. Both drugs were maintained throughout the experiment.

### Antibodies

The monoclonal antibody directed against  $\alpha$ -tubulin (N 356) was purchased from Amersham and mouse monoclonal antibody against the lysosomal transmembrane glycoprotein lgp110 was a generous gift from Prof. I. Mellman (Yale University New Haven, USA).

### Cell culture

The mouse hepatoma cell line BWTG3 previously characterized by Szpirer and Szpirer (1975) was obtained from Dr Weiss (Institut Pasteur, Paris France). BWTG3 cells were grown at 37°C under 10% CO<sub>2</sub>, in Coon's F12 modified medium (Seromed T085-05) supplemented with 10% fetal calf serum (Seromed; S 0115) and penicillin (10 U/ml)/streptomycin (10 mg/ml) (Seromed A2213).

### Immunofluorescence microscopy

For immunofluorescence analysis cells were grown for 2 days on coverslips, fixed with 3% paraformaldehyde, permeabilized with phosphate-buffered saline containing 0.01% saponin and analyzed by indirect immunofluorescence. Staining was performed by two sequential incubation steps. Cells were first incubated with purified IgG in the case of monoclonal antibodies or affinity-purified antibodies in the case of polyclonal antibodies at a concentration of 10  $\mu\text{g}/\text{ml}$ . They were secondly labeled with fluorescent anti-IgG antibodies, conjugated to either lissamine rhodamine B sulfonyl chloride (Molecular probes) or dichlorotriazinyl amino fluorescein (DTAF) (Research Inorganic) according to the method of Brandzaeg (1973). Fluorescent phalloidin conjugated to either rhodamine (Sigma P 5157) or fluorescein (Sigma P 5282), at 2 U per dish was used to label F actin. Acridine orange (2 mM; Sigma A-6014) in RPMI 1640 medium (Gibco 041-02402 M) was incubated with cells for 30 minutes in order to label the acidic compartment. After four washes with cold phosphate-buffered saline cells were immediately analyzed. Cells were viewed with an Axiophot microscope (Zeiss), or a confocal laser scanning microscope (CLSM, Leica) depending on the experiment.

### Fluorescent coupled human $\alpha$ 2-macroglobulin and human transferrin

Iron-loaded human apo transferrin (Sigma T2252) and  $\alpha$ 2-macroglobulin (Sigma M7151) were coupled either with DTAF or lissamine rhodamine B sulfonyl chloride as previously described (Maxfield et al., 1978). Conjugated proteins were separated from free fluorochromes by Sephadex G50 gel filtration in phosphate buffered saline. The specific binding of the coupled proteins to the cell surface receptors was determined by competition experiments using a 100-fold higher concentration of non coupled proteins.

### Iodination of human $\alpha$ 2-macroglobulin and transferrin

Human  $\alpha$ 2-macroglobulin and human transferrin loaded with iron (Ciechanover et al., 1983) were iodinated using chloramine T (Sigma C 9887) as described by Mosher et al. (1977). After iodination, more than 95% of the radioactivity was precipitable by 10% trichloroacetic acid. The specific activity was 0.43 mCi/mg for  $\alpha$ 2-macroglobulin and 0.66 mCi/mg for transferrin. The specificity of binding of radiolabeled proteins was determined by competition with non labeled ligand; 100-

fold concentrations of non labeled ligand reduced the binding of radiolabeled ligand at 4°C by more than 80%.

### Ligand internalization, kinetic of uptake and kinetic of degradation

Cells ( $10^6/3.5 \text{ cm}^2$ ) grown for two days on coverslips or in wells were rinsed three times with RPMI 1640 containing 1 mg/ml of bovine serum albumin (BSA), and incubated twice for 15 minutes in the same medium at 37°C. Then fluorescent (1) or radiolabeled (2) ligands were internalized according to the following conditions: (1) cells were incubated for 30 minutes with DTAF- $\alpha$ 2-macroglobulin (20  $\mu\text{g}/\text{ml}$ ) and/or rhodamine-transferrin (100 nM) in RPMI 1640 containing 1 mg/ml BSA at 37°C or 18°C. Unbound fluorescent ligand was removed by three washes with cold phosphate-buffered saline and cells were fixed directly, or incubated with prewarmed RPMI 1640 during specified times and before being fixed with paraformaldehyde. (2) For ligand uptake, cells were incubated for 15 minutes in a precooled medium at 4°C and for 1 hour in precooled medium containing iodinated  $\alpha$ 2-macroglobulin (10  $\mu\text{g}/\text{ml}$ ) or transferrin (100  $\mu\text{M}$ ). Cells were then rinsed 6 times with cold phosphate-buffered saline containing 0.5 mM calcium chloride and 0.5 mM magnesium chloride and the radiolabeled ligand replaced by unlabeled ligand;  $\alpha$ 2-macroglobulin (10  $\mu\text{g}/\text{ml}$ ) or transferrin (100 nM). Bound radiolabeled ligand was internalized for selected intervals at 37°C. Following these incubations, medium containing the released ligand was collected and cells rapidly chilled to arrest ligand movement. Residual cell surface bound ligand was stripped by treating cells for 2 minutes with 1 volume of cold RPMI 1640, pH 2.8, followed by 2 volumes of cold RPMI 1640, pH 9, for one minute. The intracellular labeled ligand was recovered in cells lysed with 0.8% of Triton X-100. The total amount of ligand was determined by adding radioactivity in the medium containing the released ligand, the medium containing the cell surface bound ligand and the cell lysate. Internalized ligand was expressed as a percentage of the total amount of ligand.

To follow transferrin recycling, radiolabeled transferrin (100  $\mu\text{M}$ ) was internalized for 30 minutes at 37°C. Then cells were rinsed 6 times with cold phosphate-buffered saline containing 0.5 mM calcium chloride and 0.5 mM magnesium chloride, and the residual cell surface bound ligand was stripped at low pH as described above. Cells were then incubated at 37°C in RPMI 1640 containing unlabeled ligand (100 nM). At selected intervals, medium containing the released ligand was collected. At the end of the experiment, cells were lysed as described above. Recycled transferrin was expressed as a percentage of the total amount of endocytosed ligand.

To determine the kinetics of  $\alpha$ 2-macroglobulin degradation, radiolabeled  $\alpha$ 2-macroglobulin was internalized as described for the transferrin recycling experiment without acidic treatment. The medium containing the released ligand was precipitated with 10% trichloroacetic acid (TCA), and the amount of  $\alpha$ 2-macroglobulin degraded was collected in the soluble TCA fraction. No radioactivity was detected in the TCA soluble fraction obtained from the cell lysate. The cell associated and the supernatant trichloroacetic acid precipitable and non precipitable radioactivity were determined with a  $\gamma$  counter. The amount of radioactivity contained in the TCA soluble fraction corresponding to the degraded  $\alpha$ 2-macroglobulin was expressed as a percentage of the total amount of endocytosed  $\alpha$ 2-macroglobulin.

## RESULTS

### The distribution of internalized transferrin and $\alpha$ 2-macroglobulin at one pole of the nucleus requires intact microfilaments and microtubules

To study the role for actin filaments and microtubules along the endocytic pathway, BWTG3 cells, derived from a mouse

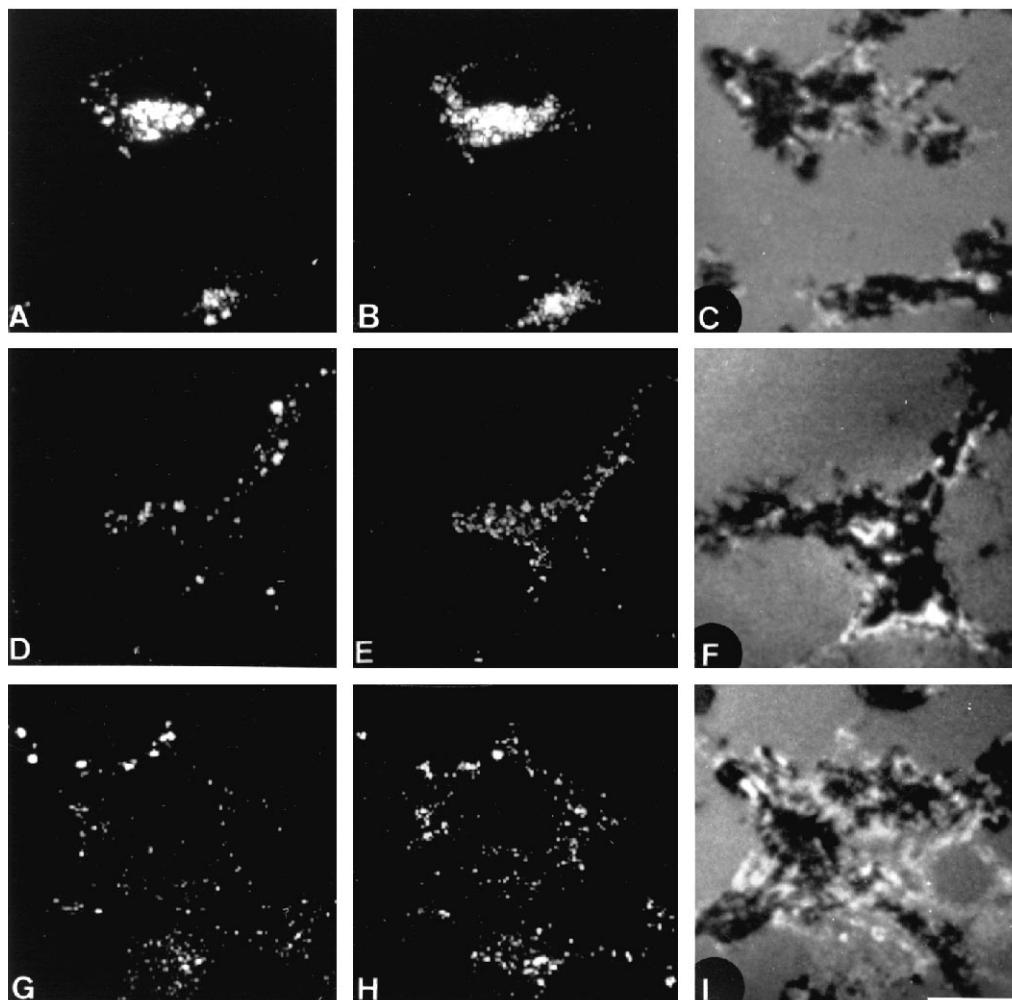
hepatoma cell line, were treated with cytochalasin D (1  $\mu$ M), or colchicine (30  $\mu$ M) prior to the internalization of transferrin and/or  $\alpha$ 2-macroglobulin. The distribution of endocytosed ligands, analyzed by confocal laser microscopy (CLSM), showed that within 5 minutes ligands were mainly internalized in early endosomes located at the cell periphery. In cells treated with cytochalasin D or colchicine the distribution of the ligands was unchanged (data not shown). After 30 minutes of internalization, transferrin and  $\alpha$ 2-macroglobulin were predominantly distributed at one pole of the nucleus as previously observed (Matteoni and Kreis, 1987; Hopkins, 1983; Hopkins et al., 1994) (Fig. 1A,B,C). In contrast, when cells have been treated with colchicine or cytochalasin D prior to endocytosis, transferrin and  $\alpha$ 2-macroglobulin were no longer concentrated in a juxtannuclear position but rather dispersed throughout the cytoplasm indicating that both microtubules and actin filaments affect the distribution of internalized ligands in this region (Fig. 1D,E,F and G,H,I). At the concentrations used, both drugs did not affect the cell shape or cell spreading (Fig. 1C,F,I). We also checked that cytochalasin D did not affect the organization of microtubules at these concentrations (as judged by immunofluorescent staining with antibody directed against  $\alpha$ -tubulin), and that colchicine did not affect the organization of microfilaments (data not shown).

We have observed that  $\alpha$ 2-macroglobulin and transferrin

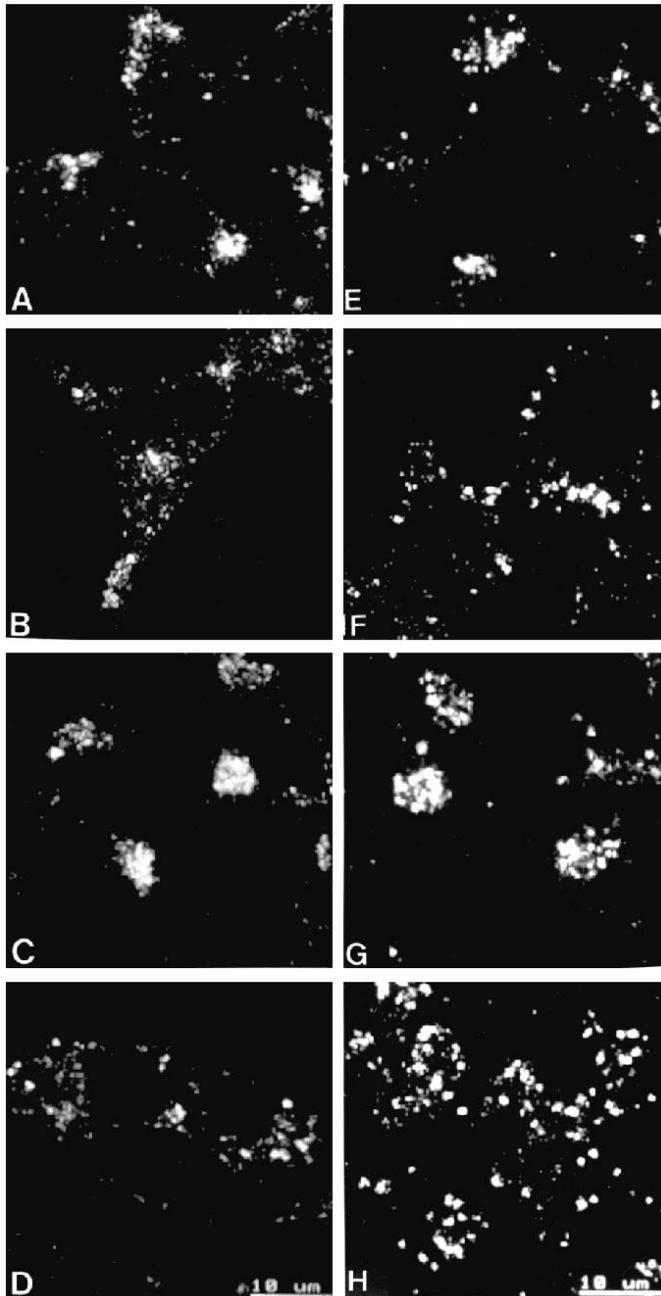
internalized at 18°C were clustered in the juxtannuclear region (Fig. 2A and E). In this region the sorting endosomes and some of the recycling endosomes are found (Salzman and Maxfield, 1989; Hopkins et al., 1994). Both ligands were redistributed throughout the cytoplasm when cells were warmed up at 37°C suggesting that  $\alpha$ 2-macroglobulin moves on along the endocytic pathway to reach lysosomes whereas transferrin recycles (Fig. 2A versus B, and E versus F). Indeed lysosomes were dispersed throughout the cytoplasm of the hepatoma cell line (see Fig. 4). The redistribution of the two ligands also occurred when microtubules were depolymerized before warming cells to 37°C (Fig. 2D,H). In contrast, when microfilaments were depolymerized it was inhibited suggesting that microfilaments facilitate the exit of the ligands from the juxtannuclear compartments (Fig. 2C,G).

#### Microtubules but not microfilaments are required to maintain distribution of the acidic compartment

The endocytic compartments are acidic and with the exception of the early endosomes they can be labeled with acridine orange. In non treated cells and cells treated with cytochalasin D the acidic compartment labeled with acridine orange formed a juxtannuclear 'cap' with a few vesicular profiles (Fig. 3A,B). In contrast, when microtubules were depolymerized with colchicine, this compartment became fragmented and



**Fig. 1.** Effect of cytochalasin D and colchicine on the distribution of internalized transferrin and  $\alpha$ 2-macroglobulin over 30 minutes. The distribution of rhodamine-conjugated  $\alpha$ 2-macroglobulin (A,D,G) and DTAF-conjugated transferrin (B,E,H), simultaneously internalized for 30 minutes at 37°C, was analyzed by CLSM on optical sections of 0.5  $\mu$ m through the nucleus in non treated cells (A,B,C), cells treated with cytochalasin D (D,E,F), or with colchicine (G,H,I). Micrographs (C,F,I) show the shape of corresponding cells analyzed by reflection with the CLSM. Note that ligands are concentrated in the vicinity of the nucleus in non treated cells whereas they are more dispersed in cells treated with cytochalasin D or colchicine. Bar, 10  $\mu$ m.

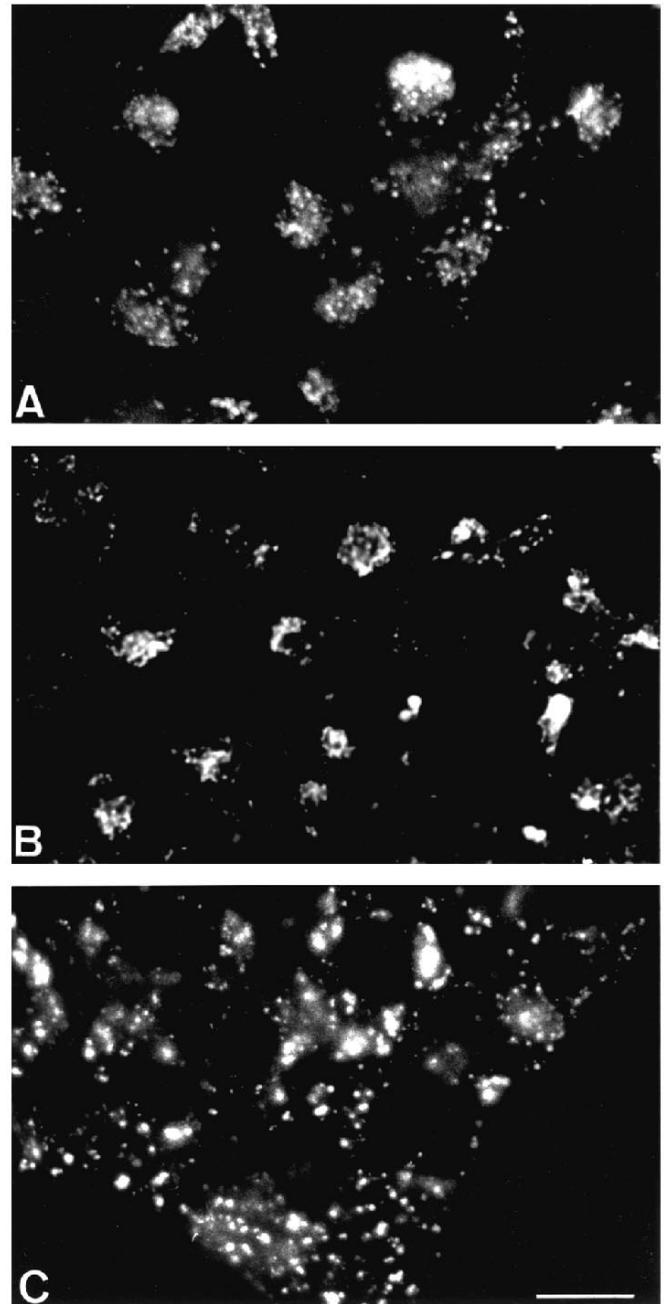


**Fig. 2.** Intracellular distribution of  $\alpha$ 2-macroglobulin and transferrin accumulated at 18°C and chased at 37°C is affected by cytochalasin D treatment. DTAF-conjugated transferrin (A,B,C,D) and rhodamine-conjugated  $\alpha$ 2-macroglobulin (E,F,G,H) were internalized in separate experiments for 30 minutes at 18°C, chased for 10 minutes at 18°C in order to be accumulated in the juxtannuclear compartment and 20 minutes at 18°C without treatment or in the presence of cytochalasin D or colchicine. Then cells were chased for 30 minutes at 18°C (A,E) or 37°C without treatment (B,F) or in the presence of cytochalasin D (C,G) or colchicine (D,H). The intracellular distribution of  $\alpha$ 2-macroglobulin and transferrin was analyzed by CLSM after paraformaldehyde fixation at 4°C. The ligands remained in the juxtannuclear location in cells kept at 18°C (A,E) or at 37°C in the presence of cytochalasin D (C,G), whereas they were scattered throughout the cytoplasm in non treated cells chased at 37°C (B,F) or cells treated with colchicine and chased at 37°C (D,H) Bar, 10  $\mu$ m.

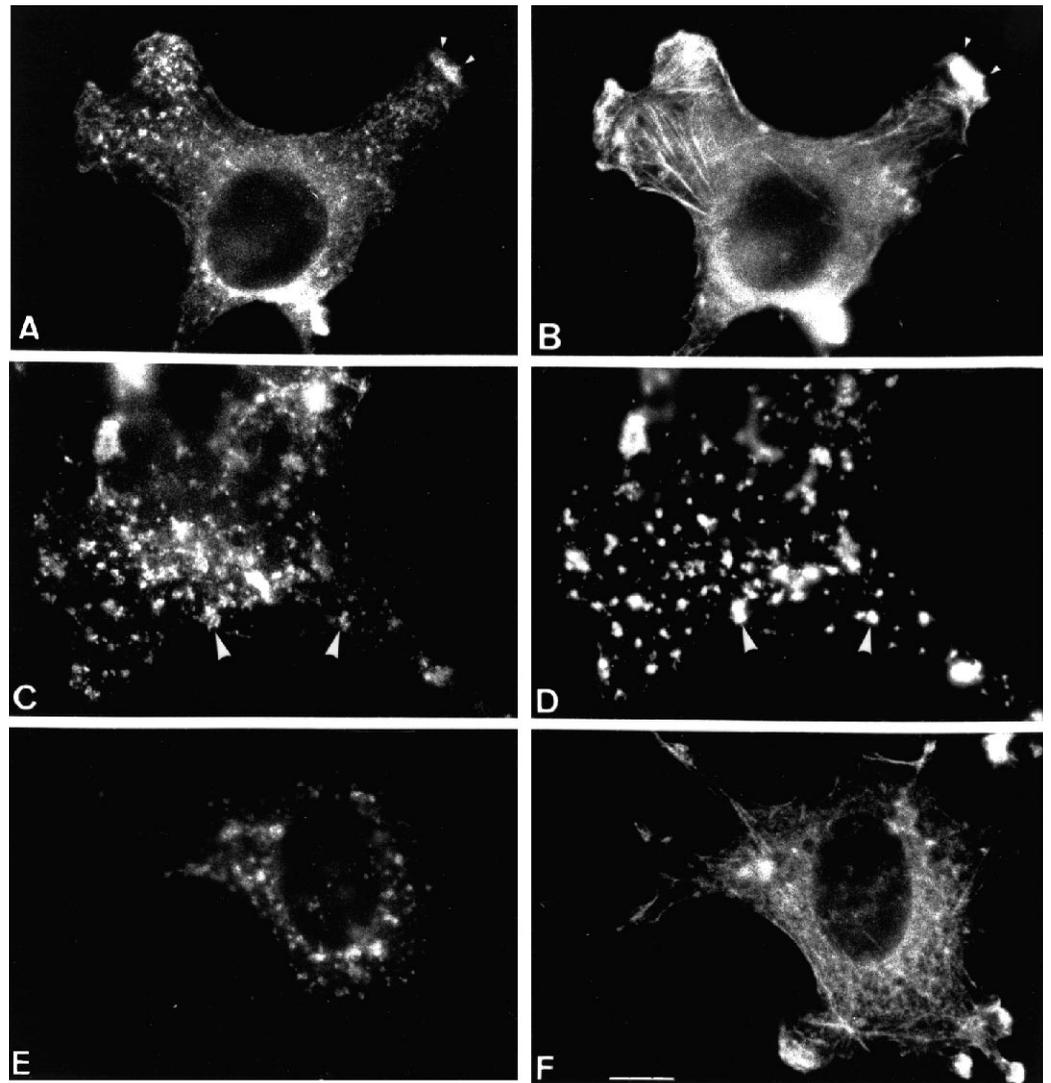
dispersed, indicating that microtubules but not microfilaments are required to maintain the structure of these endocytic compartments (Fig. 3C).

**Lysosomes are co distributed with collapsed actin filaments after cytochalasin D treatment**

We next studied the cellular distribution of lysosomes depending on the presence of microtubules or microfilaments



**Fig. 3.** Effect of colchicine and cytochalasin D on the distribution of the acidic compartment. The acidic compartment was visualized in non treated cells (A), cells treated with cytochalasin D (B), or colchicine (C) by labeling them with acridine orange. Some punctate structures dispersed throughout the cytoplasm, were also observed. However, the rapid fading of this vital staining and the low fluorescent intensity of the punctate structures compare to the juxtannuclear staining make these structures hardly visible on the photographs. Bar, 4  $\mu$ m.



**Fig. 4.** Effect of colchicine and cytochalasin D on the distribution of lysosomes. Cells were double labeled with a monoclonal antibody directed against lgp 110, a lysosomal transmembrane glycoprotein (A,C,E), and rhodamine-conjugated phalloidin (B,D,F). Lysosomes were scattered throughout the cytoplasm in non treated cells (A,B), cells treated with cytochalasin D (C,D) or with colchicine (E,F). They were detected in lamellipodia of non treated cells (A,B; small arrows) but not in cells treated with colchicine. They were codistributed with collapsed actin filaments in cells treated with cytochalasin D (C,D; see arrows). Bar, 2  $\mu$ m.

using a specific antibody directed against a transmembrane glycoprotein, lgp 110. The punctate structures, stained with antibodies directed against lgp 110 were dispersed throughout the cytoplasm and in membrane extensions such as lamellipodia in both non treated cells and cells treated with cytochalasin D, however, they were dispersed to a lesser extent in cells treated with colchicine (Fig. 4A,C,E). They were not detected in membrane extensions in these last conditions. Surprisingly the punctate structures seemed to be accumulated together with actin filaments in lamellipodia of non treated cells doubly stained with phalloidin and antibodies directed against lgp110 (Fig. 4A versus B). Furthermore in cells treated with cytochalasin D these structures were codistributed with collapsed actin filaments (Fig. 4C versus D). The distribution of cathepsin D, a lysosomal hydrolase concentrated mainly into lysosomes, was similar to the distribution of lgp110 in control cells and similarly affected in cells treated by both drugs (data not shown).

#### **Microfilaments but not microtubules facilitate the cellular uptake of transferrin and $\alpha$ 2-macroglobulin**

The dispersion of the internalized ligands, in the absence of

microtubules or microfilaments might correspond either to a delay for the transport of the ligands from the cell surface to the endocytic compartment located in the cell center or to a disorganization of late endocytic compartments. To decide between these two possibilities we compared the kinetic uptake of the two ligands in the presence and in the absence of actin filaments and/or microtubules.

The kinetics of transferrin uptake was similar in untreated cells and cells treated with colchicine but was delayed with a lag time of 2 minutes in cells treated with cytochalasin D (Fig. 5A). The mean half-life for transferrin uptake was 2 minutes in control cells and cells treated with colchicine and 4 minutes in cells treated with cytochalasin D. The maximal amount of internalized transferrin was decreased in cells treated with cytochalasin D. Furthermore cytochalasin D treatment increased up to  $30.1 \pm 4.4\%$  ( $n=11$ ) the amount of transferrin bound at the cell surface (and up to 40% the number of transferrin receptors) but did not modify the affinity constant of transferrin for its receptor.

Transferrin uptake in Hep-2 cells was also delayed when actin filaments were depolymerized by cytochalasin D (Fig. 5C). Similar results were obtained with BHK cells (data not

shown) indicating that these effects were not restricted to the hepatoma cell line. After internalization, transferrin is recycled from endosomes to the plasma membrane. As it has been pre-

viously reported, most of internalized transferrin was recycled within 20 minutes (Fig. 5D). Depolymerization of actin filaments did not affect these kinetics (Fig. 5D).

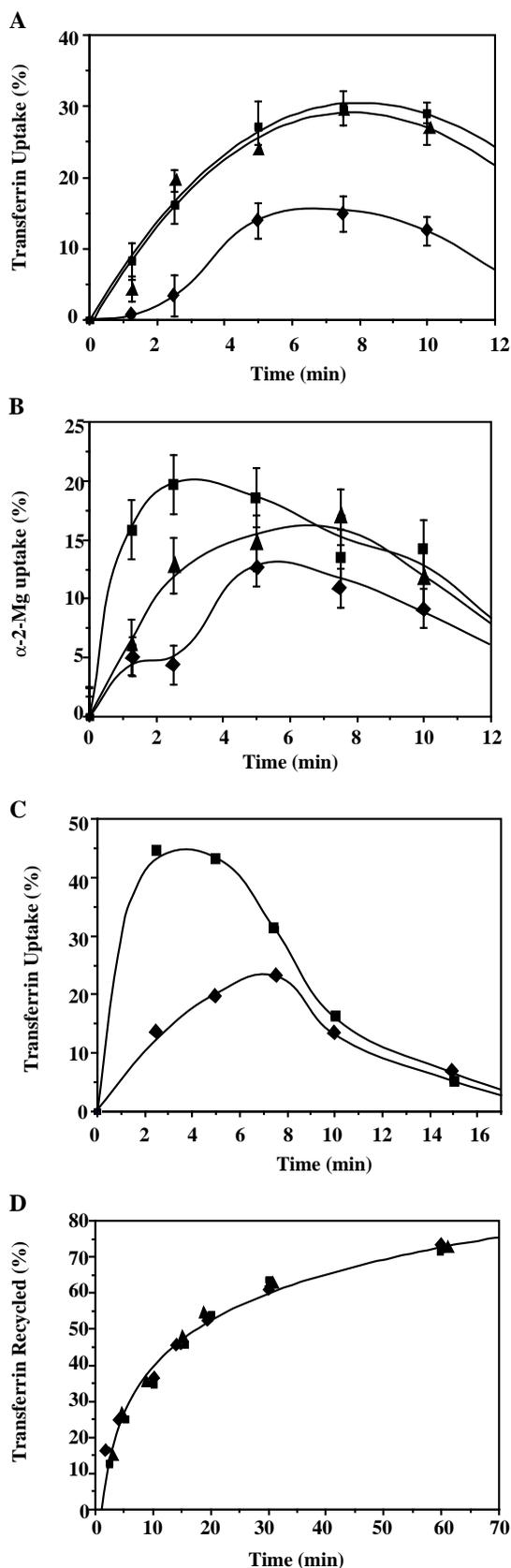
The kinetics of  $\alpha 2$ -macroglobulin uptake, similar to that of transferrin, is delayed with a lag time of 2 minutes when actin filaments were depolymerized and with a lag time of 1 minute when microtubules were depolymerized (Fig. 5B). The amount of  $\alpha 2$ -macroglobulin bound to the cell surface is also increased up to 30% when the cells were treated with cytochalasin D. Altogether, these results indicated that the depolymerization of actin filaments impaired the first step of ligand internalization and lead to its accumulation at the cell surface.

### Microfilaments and microtubules are both required for the delivery of $\alpha 2$ -macroglobulin to the degradative compartment

We next examined whether actin filaments or microtubules could participate in movement along the degradative pathway by following the degradation of internalized radiolabeled  $\alpha 2$ -macroglobulin. The percentage of degraded  $\alpha 2$ -macroglobulin over the total amount internalized after 30 minutes of internalization and 40 minutes of chase decreased from 35% in non treated cells, to 27% in cells treated with colchicine, 15% in cells treated with cytochalasin D and 15% in cells treated with cytochalasin D and colchicine (Fig. 6A). The degradation of  $\alpha 2$ -macroglobulin was significantly inhibited up to 22% in cells treated with colchicine ( $n=4$ ;  $P<0.0075$ ), and 55% in cells treated with cytochalasin D ( $n=3$ ;  $P<0.003$ ) or with cytochalasin D and colchicine ( $n=2$ ;  $P<0.02$ ) (Fig. 6B). These results indicate that both actin filaments and microtubules facilitate the delivery of  $\alpha 2$ -macroglobulin to the degradative pathway.

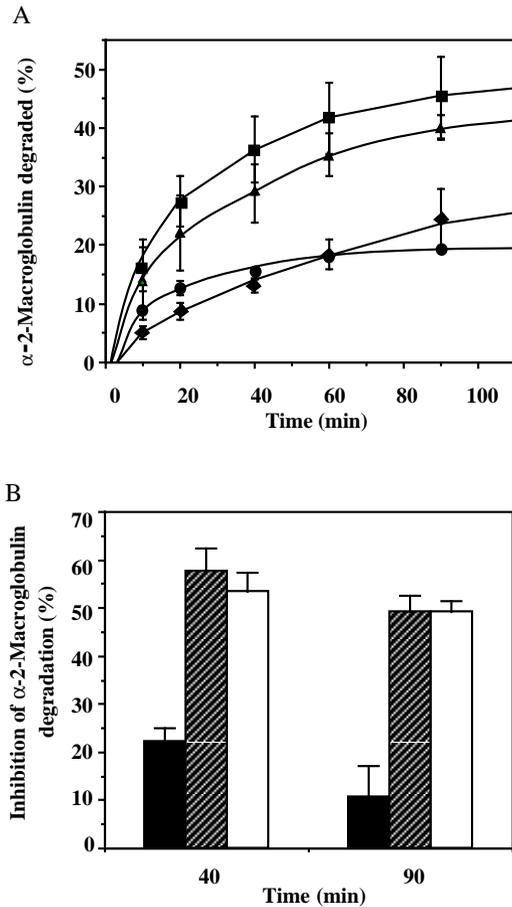
### Actin filaments and microtubules act in sequence in the delivery of $\alpha 2$ -macroglobulin to the degradative compartment

Since cytochalasin D and colchicine do not have a cumulative effect on  $\alpha 2$ -macroglobulin degradation the two types of filaments might be required stepwise for the delivery of the



**Fig. 5.** Effect of cytochalasin D on the internalization of transferrin and  $\alpha 2$ -macroglobulin and the recycling of transferrin. (A,C) Kinetics of  $^{125}\text{I}$ -transferrin endocytosis in BWTG3 cells (A) and in HEp-2 cells (C): cells were not treated (■), or treated with colchicine (▲) or cytochalasin D (◆) for 30 minutes at  $37^\circ\text{C}$  prior the internalization of transferrin as described in Materials and Methods. Drugs were maintained during transferrin internalization. The internalized transferrin was normalized to the total amount of transferrin bound to the cells at time 0 (A,  $n=3$ ; mean  $\pm$  standard error). (B) Kinetics of  $^{125}\text{I}$ - $\alpha 2$ -macroglobulin endocytosis: BWTG3 cells were not treated (■), or treated with colchicine (▲) or cytochalasin D (◆) for 30 minutes at  $37^\circ\text{C}$  prior to the internalization of  $\alpha 2$ -macroglobulin as described in Materials and Methods. Drugs were maintained during  $\alpha 2$ -macroglobulin internalization. The internalized  $\alpha 2$ -macroglobulin was normalized to the total amount of  $\alpha 2$ -macroglobulin bound at time 0 ( $n=2$ ; mean  $\pm$  standard error). (D) Kinetics of transferrin recycling:  $^{125}\text{I}$ -transferrin was internalized for 30 minutes at  $37^\circ\text{C}$  in control cells (■), cells treated with cytochalasin D (◆), and cells treated with colchicine (▲) as described in Materials and Methods. Then after treating or not the cells with cytochalasin D, transferrin released in the supernatant was determined at selected intervals and normalized to the total internalized transferrin ( $n=2$ ).

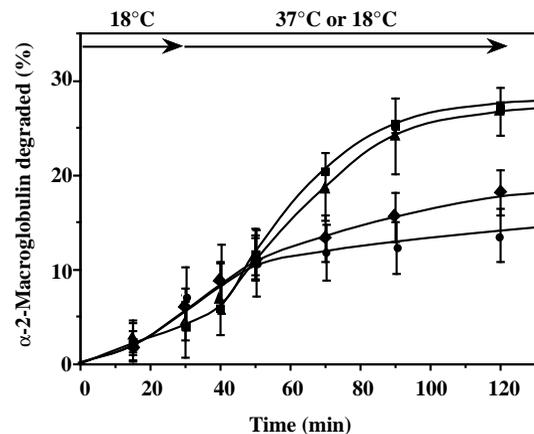
ligand to the degradative compartment. To determine at which step of the degradative pathway actin filaments and microtubules might be involved, we took advantage of the temperature sensitive block at 18°C. It has been demonstrated that at 18°C, endocytosed ligands are accumulated in the late



**Fig. 6.** Effect of cytochalasin D, cytochalasin D and colchicine, or colchicine on the degradation of  $\alpha 2$ -macroglobulin.  $^{125}\text{I}$ - $\alpha 2$ -macroglobulin was internalized for 30 minutes at 37°C in non treated cells (■) or cells previously treated with cytochalasin D (◆) or colchicine (▲) or with both drugs (●) as described in Materials and Methods. Then, cells were rinsed free of ligands and reincubated at 37°C for specified times in the presence or not of cytochalasin D, colchicine or both drugs, similar to the treatment received before. The amount of radioactivity contained in the TCA soluble fractions corresponding to the degraded  $\alpha 2$ -macroglobulin was normalized to the total amount of endocytosed  $\alpha 2$ -macroglobulin. (A) The mean percentage of degraded  $\alpha 2$ -macroglobulin as a function of time for non treated cells and cells treated with cytochalasin D or colchicine ( $\pm$  standard deviation;  $n=4$ ) or cytochalasin D and colchicine ( $\pm$  standard deviation;  $n=2$ ). (B) The percentage of inhibition of  $\alpha 2$ -macroglobulin degradation in cells treated with colchicine (dark columns) or cytochalasin D (striped columns) or cytochalasin D and colchicine (white columns). It corresponds to the rate of  $\alpha 2$ -macroglobulin degraded in cells treated by the drugs over the rate of  $\alpha 2$ -macroglobulin degraded in control cells after 40 and 90 minutes of chase. After 40 minutes of chase, the percentage of  $\alpha 2$ -macroglobulin degraded is significantly decreased to below 22% in cells treated with colchicine ( $n=4$ ;  $P<0.0075$ , paired Student's  $t$ -test), 55% in cells treated with cytochalasin D ( $n=4$ ;  $P<0.003$ , paired Student's  $t$ -test) and 53% in cells treated with cytochalasin D and colchicine ( $n=2$ ;  $P<0.02$ , paired Student's  $t$ -test).

endocytic compartment and are not degraded (Dunn et al., 1980; Salzman and Maxfield, 1989). This block is reversible and ligands are delivered to the degradative compartment when cells are warmed up at 37°C.

We examined whether actin filaments or microtubules are required to deliver  $\alpha 2$ -macroglobulin accumulated in the juxtanuclear compartment at 18°C (see above, Fig. 1 and Fig. 2), in the degradative compartment. Radiolabeled  $\alpha 2$ -macroglobulin was internalized at 18°C and after depolymerization of microfilaments or microtubules chased at 37°C. Only 10% of the radiolabeled  $\alpha 2$ -macroglobulin internalized and maintained for 1 hour at 18°C was degraded when microfilaments and microtubules remained intact (Fig. 7) whereas lysosomal enzymes were still active at this temperature (data not shown). The percentage of  $\alpha 2$ -macroglobulin that was degraded increased 3-fold in non treated cells as well as in cells treated with colchicine when  $\alpha 2$ -macroglobulin was internalized for 30 minutes at 18°C, then chased for 30 minutes at 18°C prior to being chased for 90 minutes at 37°C. In contrast, the percentage of degraded  $\alpha 2$ -macroglobulin in cells treated with cytochalasin D was similar to the percentage measured in non treated cells loaded and chased at 18°C (Fig. 7). These results indicated that the integrity of microfilaments for the delivery of ligands to lysosomes was required downstream of the juxtanuclear compartment.



**Fig. 7.** Effect of cytochalasin D and colchicine on the degradation of  $\alpha 2$ -macroglobulin when the ligand was first internalized at 18°C and then chased at 37°C to the degradative compartment.  $^{125}\text{I}$ - $\alpha 2$ -macroglobulin was internalized for 30 minutes at 18°C, chased for 10 minutes at 18°C in order to be accumulated in the late endocytic compartment and 20 minutes at 18°C without treatment or in the presence of cytochalasin D or colchicine. Then cells were kept at 18°C (●) or were chased at 37°C without any drugs (■) or in the presence of cytochalasin D (◆) or colchicine (▲) similar to the previous treatment. Degraded  $\alpha 2$ -macroglobulin corresponding to the radioactivity determined in the soluble TCA fraction was normalized to the total amount of  $^{125}\text{I}$ - $\alpha 2$ -macroglobulin internalized at time 0 and expressed as a function of the chase times (mean  $\pm$  standard deviation). In non treated cells or cells treated with colchicine, chased at 37°C, the percentage of  $^{125}\text{I}$ - $\alpha 2$ -macroglobulin degraded increased significantly as compared to cells kept at 18°C ( $P<0.006$ ,  $n=3$ ;  $P<0.02$ ,  $n=2$ , respectively, paired Student's  $t$ -test). In contrast, the increased percentage of  $\alpha 2$ -macroglobulin degradation is significantly inhibited in cells treated with cytochalasin D ( $P<0.006$ ,  $n=3$ , paired Student's  $t$ -test).

## DISCUSSION

### What is the functional significance of the involvement of actin filaments in the uptake of ligands?

We have shown that the depolymerization of actin filaments with cytochalasin D delayed the uptake of the two ligands and lead to their accumulation at the cell surface. This effect was similar in two other cell types (BHK, HEp-2) indicating a general involvement of actin filaments in the first step of receptor mediated endocytosis. Although it is well established that depolymerization of actin filaments inhibits patching of ligand-receptor complexes at the cell surface, more recent work failed to demonstrate a role for the actin filaments in an early step in the endocytosis of ligands such as transferrin in mammalian cells (Salisbury et al., 1980; Wolkoff et al., 1984; Sandvig and Van Deurs, 1990). In one of these reports cytochalasin B instead of cytochalasin D (which is less efficient) was used whereas in the other the kinetics of transferrin uptake was not distinguished from its binding to the cell surface. In contrast, in our experiments the ligands were first bound at the cell surface at 4°C prior to determining the kinetics of their uptake at 37°C. The different experimental conditions might explain the discrepancy between our observations and these earlier studies. Indeed Ciechanover et al. (1983) have shown that the half-life of the binding of the transferrin to the cell surface is twofold longer than the half-life of its uptake indicating that the binding of the ligand to the cell surface can be a rate limiting step.

We cannot rule out that disorganization of the cortical actin network by cytochalasin D has an indirect effect on the early steps of endocytosis. However, the accumulation of transferrin receptors at the surface of cells treated with cytochalasin D is consistent with the data of Sako and Kusumi (1994), proposing that transferrin and  $\alpha$ 2-macroglobulin receptors at the cell surface could interact directly or indirectly with cytoskeleton components, and those of Gottlieb et al. (1993) and Kubler and Riezman (1993) showing an accumulation of coated pits when actin filaments are depolymerized. Altogether they strongly suggest a model in which actin filaments participate in the formation of early endosomes. Three steps at least lead to the formation of this organelle, the two last steps occurring also in clathrin independent endocytosis (Watts and Marsh, 1992), as follows: (1) the capping of ligand receptor together with the recruitment of clathrin lattice; (2) the invagination of the membrane into a constricted pit; (3) the severing of the neck of the invaginated membrane to form a vesicle. According to Takei et al. (1995) and Hinshaw and Schmid (1995) dynamin participates in the fission of the vesicles in a clathrin dependent or independent fashion. Actin filaments together with specific actin binding proteins could participate in the clustering of ligands and/or membrane invagination whereas dynamin could participate in the severing of the membrane invagination. The observation of cortical actin patches associated with the cell surface invaginations of the plasma membrane and the inhibition of the  $\alpha$  factor uptake in *Saccharomyces cerevisiae* when Sec 6 (an actin binding protein homologous to fimbrin) was deleted support this hypothesis (Mulholland et al., 1994; Kubler and Riezman, 1993). Alternatively dynamin and actin could both participate in a complex molecular mechanism leading to

membrane invagination and severing of the newly formed invagination.

### Actin filaments and microtubules are involved stepwise in membrane trafficking along the degradative pathway

Depolymerization of microtubules in the hepatoma cell line BWTG3 impaired the distribution of internalized ligands in the juxtannuclear area, the redistribution of the acidic compartment and to some extent lysosomes since we have not detected Igp110 associated structures in lamellipodia of cells treated with colchicine. Depolymerization of actin filaments also impairs the distribution of the ligands in the juxtannuclear area but does not seem to affect the distribution of the acidic compartment. In addition depolymerization of the microtubules and actin filaments inhibits the degradation of endocytosed  $\alpha$ 2-macroglobulin. However, the depolymerization of microtubules affects the delivery of the ligands upstream of the temperature sensitive block at 18°C whereas actin filaments are required downstream of the temperature sensitive block. Thus our data are consistent with a possible role for microtubules in the transport of ligands within the endosomal compartment, and actin filaments between endosomes and lysosomes. They are in agreement with *in vitro* experiments demonstrating that microtubules and dynein increase fusion events between endosomes (Aniento et al., 1993) but do not affect fusion events between endosomes and lysosomes (Mullock et al., 1994). The mechanism by which microtubules and dynein facilitate *in vivo* the transport of the ligands through the endosomal compartment is presently unclear. As proposed by Aniento et al. (1993), they may facilitate the rate of vesicular traffic, alternatively they may facilitate the rate of endosomes maturation, and/or stabilize their structure according to the model proposed by Mayor et al. (1993) and Stoorvogel et al. (1991).

The enrichment of lysosomes together with actin filaments in membrane extensions and their co distribution with collapsed actin filaments after cytochalasin D treatment also support our hypothesis although we cannot rule out the alternative possibility that actin filaments participate in the biogenesis of the lysosomes. However, the observations of Van Deurs et al. (1995) rather favour our hypothesis. They have shown that depolymerization of microfilaments leads to the accumulation of fluid phase markers in multivesicular bodies which are devoid of lysosomal markers and also decreases the degradation and the delivery of ricin internalized via non coated pits in these structures.

According to Hopkins et al. (1994) and Ghosh et al. (1994), transferrin accumulated in the pericentriolar area is sorted from the degradative compartment and recycled back to the plasma membrane. The inhibition by cytochalasin D of the dispersion of the transferrin accumulated in the perinuclear area would be consistent with the requirement of actin filaments to sort the recycled ligands from the degradative pathway. Semi quantitative electron microscopic studies from Hopkins et al. (1994) have indicated that the separation between the recycled ligands and ligands routed to the degradative pathway occurs within tubules. Thus an attractive hypothesis would be that actin filaments might be required, to form these tubules and/or to cluster ligands in these tubules and consequently facilitate the sorting of recycled ligands from ligands delivered to the degradative compartment.

In conclusion we have provided morphological and kinetic evidences to support the proposal that actin filaments facilitate the uptake of ligands internalized via coated pits, and the sorting of recycled ligands concentrated in the perinuclear area from the degradative pathway. Are actin filaments involved in a similar molecular mechanism in both steps? The identification of the actin binding proteins involved in these two steps may provide an answer. In this respect myosin Is which have been shown to interact between membrane domains and actin filaments are good candidates for acting in association with actin to exert tension on membrane domain in order to elongate or invaginate membrane domains. In addition our data indicate for the first time the sequential involvement of actin filaments and microtubules along one intracellular membrane trafficking pathway. The identification of the two types of motor proteins associated either with microtubules or actin on the same endosome as predicted by the *in vitro* experiment of Kuznetsov et al. (1992) raises important questions about the strict regulation of these motors proteins.

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