

## Actin-filaments Localize on the Sorting Endosomes of 3Y1 Fibroblastic Cells

Hiroyuki Nakagawa<sup>†</sup> and Shigeaki Miyamoto

Department of Biochemical Engineering and Science, Faculty of Computer Science and System Engineering, Kyushu Institute of Technology, Iizuka, Fukuoka 820-8502, Japan

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**ABSTRACT.** By immunofluorescence microscopic observation, monoclonal and polyclonal antibodies against a synthetic actin C-terminal peptide were found to stain too colloquial, ambiguous punctuate structures distributed throughout the cytoplasm of 3Y1 cells, independently of actin stress fibers. Antibody against rab5, a small GTP binding protein of the sorting endosome, and anti-actin antibody co-stained these punctuate structures. On the other hand, transferrin receptor, a well characterized marker of the sorting and recycling endosomes, colocalized with actin on the vesicular structures at the cell peripheral region but not at the perinuclear area where the recycling endosome localized. These observations suggest that actin molecules localize on the sorting endosomes. Tropomyosin, F-actin binding protein, also colocalized with actin on the sorting endosomes. From these results, we proposed that actin-filaments with tropomyosin constitute the membrane skeleton on the sorting endosome surface. This article is the first report to show that actin-filaments localize on the intact endosomes.

The biological macromolecules bound to their specific receptors on the cell surface are internalized into the sorting endosomes via the clathrin coated vesicles, then sorted and finally transported to the target organelle (29). The molecules to be degraded are transferred to the lysosomes via the late endosomes and the ones to be reused are returned to the cell membrane via the recycling endosome (20, 36, 42). Actin-filaments are thought to play important roles in these processes.

Cytochalasin D, an actin-filament depolymerizing molecule, inhibits the clathrin-coated vesicles from being pinched off from the cell membrane and the transport of many kinds of molecules from apical to basolateral membrane in epithelial cell lines (15, 21, 26). Also in a hepatoma cell line, cytochalasin D inhibits the transport of molecules from the endosome to the lysosome (13, 40). Genomic mutations of actin or actin binding proteins such as myosin I, fimbrin and cofilin inhibit endocytosis of yeast or *Dictyostelium* (14, 23, 25, 31). In mammalian cultured cells, myosin I molecules are scattered throughout cytoplasm similarly as vesicular structures (8), and transfection of motor-domain-truncated myosin I impaired endocytosis (12). These reports suggest that actin-filaments and actin

binding proteins play crucial roles in receptor-mediated endocytosis. Recently, Murphy *et al.* (1996) reported that treatment with cytochalasin D induced the recruitment of actin-filaments around the enlarged endosomes of the cells expressing GTPase-deficient rab5 (30). The cytoplasmic actin molecules have been reported to constitute not only stress fibers but also many punctuate structures (6), the localization of which resembles that of the endosomes. Highly purified endosome fraction contained a considerable amount of actin molecules (11, 41). These previous observations give rise to the possibility that actin molecules form a stable structure on the endosome surface.

To ascertain this, we first compared the localization of actin molecules with that of the endosomes by the use of immunofluorescence microscopy technique. We stained cultured fibroblasts with rhodamine-phalloidin and/or antibodies against actin and endosome markers. To clarify whether actin molecules at the sorting endosomes exist in a filamentous form, we used antibody against tropomyosin, a F-actin binding protein. To our knowledge this study is the first attempt to demonstrate the localization of actin-filament on intact endosomes.

### MATERIALS AND METHODS

#### Antibodies

Monoclonal (clone no. AC-40) and affinity-purified polyclonal antibodies against a synthetic actin C-terminal peptide (Ser-

<sup>†</sup> Corresponding Author: Department of Biochemical Engineering and Science, Faculty of Computer Science and System Engineering, Kyushu Institute of Technology, 680-4 Kawazu, Iizuka, Fukuoka 820, Japan.

Tel: +81-948-29-7832, Fax: +81-948-29-7801

Gly-Pro-Ser-Ile-Val-His-Arg-Lys-Cys-Phe) which is highly conserved sequence in vertebrate actin isoforms, and monoclonal anti-tropomyosin antibody (clone no. TM-311) were purchased from Sigma-Aldrich Japan K.K. (Tokyo, Japan). Monoclonal antibody against rat transferrin receptor (clone no. OX-26) was purchased from Chemicon International, Inc. (CA, USA). Monoclonal antibody against human rab5 (clone no. 15) was purchased from Transduction Laboratories, Inc. (KY, USA) (28). Monoclonal anti-tropomyosin, anti-transferrin receptor and anti-rab5 antibodies are reported to bind to the respective rat antigens by those distributors.

#### Other materials

Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum were purchased from Dainippon Pharmaceutical Co., Ltd. (Osaka, Japan). Antibiotics and rhodamine-phalloidin were purchased from Sigma-Aldrich Japan K.K. (Tokyo, Japan).

#### Actin preparation

Actin was prepared from the acetone powder of rabbit back muscle according to the method of Spudich and Watt (1971) (33).

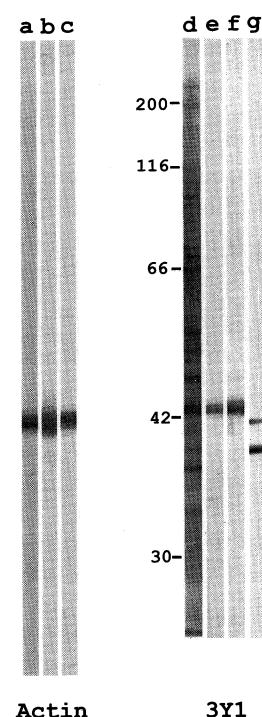
#### Cell culture and immunofluorescence microscopy

A rat embryonic fibroblast cell line 3Y1 (22, 43) was grown in DMEM supplemented with 10% FBS, 100 units/ml penicillin and 100  $\mu$ g/ml streptomycin (38). For indirect immunofluorescence microscopy, cells were plated on glass cover slips and cultured in 5% CO<sub>2</sub>/95% air at 37°C for 24 to 48 hours before use. Cells on glass cover slips were washed twice with PBS (137 mM NaCl, 2.7 mM Na<sub>2</sub>HPO<sub>4</sub>, 8.1 mM KCl, 1.5 mM KH<sub>2</sub>PO<sub>4</sub>) at 37°C, incubated with prewarmed 0.005% digitonin in intracellular buffer (110 mM KCl, 15 mM NaCl, 5 mM MgCl<sub>2</sub>, 1 mM EGTA, 0.5 mM PMSF, 10  $\mu$ M paclitaxel, 5 mM K-PO<sub>4</sub> buffer, and 50 mM HEPES-KOH buffer, pH 7.2) (16) for 20 seconds, fixed with prewarmed 3% formaldehyde in K-PBS (150 mM KCl, and 20 mM K-PO<sub>4</sub> buffer, pH 7.2) for 20 minutes at room temperature, washed 2  $\times$  5 minutes with PBS and incubated with methanol or acetone at -20°C for 30 seconds. Methanol treatment was essential for the immunofluorescence staining with the polyclonal anti-actin antibody used in this article. The following treatments were performed at room temperature, unless otherwise noted. Demembrated cells were washed 3  $\times$  5 minutes with PBS, blocked with 1% bovine serum albumin (BSA)-PBS for 20 minutes, incubated with the first antibodies diluted in 1% BSA-PBS for one hour or overnight at 4°C, washed 4  $\times$  5 minutes with PBS and incubated with FITC- or rhodamine-labeled anti-rabbit or anti-mouse IgG goat antibodies diluted in 1% BSA-PBS for one hour. Rhodamine-phalloidin used for staining actin-filaments was diluted in the second antibody solution. Immuno-stained cells were washed 6  $\times$  5 minutes with PBS, fixed with 3% formaldehyde in PBS for 5

minutes, washed with PBS and mounted in 0.1% *p*-phenylenediamine in 50% glycerol and 50 mM Na-CO<sub>3</sub> buffer, pH 8.0. All immunofluorescence observations were carried out under the confocal laser scanning microscope, Zeiss LSM410 (Carl Zeiss Jena, GmbH., Jena, Germany).

#### SDS-PAGE and Immunoblotting

Confluent culture of 3Y1 cells on a plastic dish (175 cm<sup>2</sup>) were washed twice with PBS, detached by a cell scraper and suspended in PBS. Cells were sedimented by the centrifugation of 800  $\times$  g for 10 minutes at room temperature. The precipitate was suspended in 500  $\mu$ l of a lysis solution (1% SDS, 1 mM sodium vanadate, 10 mM Tris-HCl, pH 7.4), boiled for 5 minutes and centrifuged by 20,000  $\times$  g for 20 minute at 30°C. The supernatant was mixed with one-third volume of an electrophoresis sample solution (4% SDS, 20% glycerol, 0.003% bromphenol blue, 4% 2-mercaptoethanol, 0.25 M Tris-HCl, pH 6.8) and subjected to SDS-PAGE according to the method of Laemmli (1970) (24), using 10% separating gels. Immunoblotting was performed essentially as described by Towbin *et al.* (1979) (37). The peptides separated in a gel were electri-



**Fig. 1.** Immunoblotting analysis of anti-actin and anti-tropomyosin antibodies specificity. Both mono- (lane b and e) and polyclonal (lane c and f) anti-actin antibodies reacted with purified rabbit skeletal-muscle actin (lane b and c) and the same single band of the whole SDS extract of 3Y1 cell (lane e and f). Monoclonal anti-tropomyosin antibody reacted with two isoforms of tropomyosin of 3Y1 cell (lane g). Lane a and d showed Coomassie-stained gels (a: purified rabbit actin, d: the whole SDS extract of 3Y1 cell). The mobilities of molecular weight markers are listed to the left of lane d as kilodalton.

cally transferred to the polyvinylidene difluoride membrane sheet. Horse radish peroxidase-conjugated anti-rabbit IgG and anti-mouse IgG goat antibodies were used for the second antibodies.

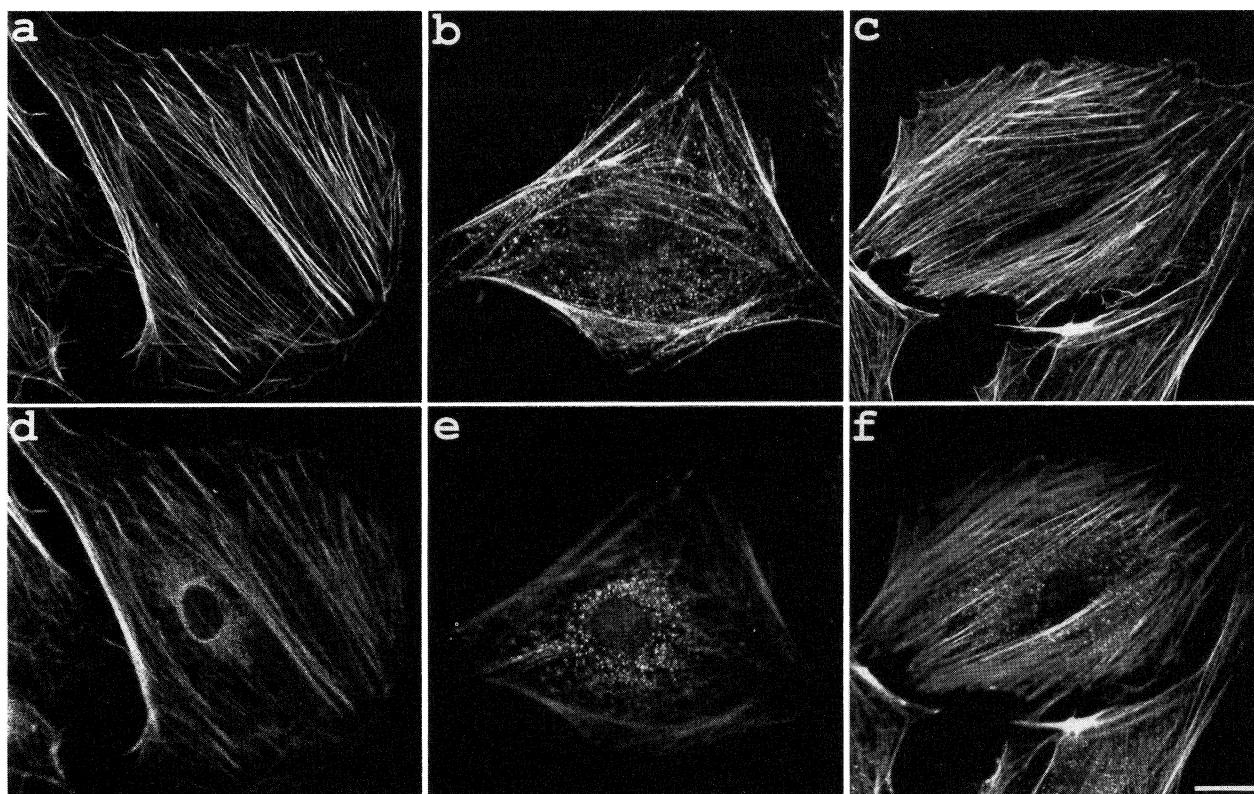
## RESULTS

### Actin Localization in the Cytoplasm of 3Y1 Cells

We immunoblotted the purified rabbit skeletal-muscle actin and the whole SDS extract of 3Y1 cell with the mono- and polyclonal anti-actin and monoclonal anti-tropomyosin antibodies to ascertain their specificity (Fig. 1). Both antibodies against actin reacted with the purified actin and the same single band of 3Y1 cell extracts. Anti-tropomyosin antibody reacted with two isoforms of tropomyosin of 3Y1 cell. In non-muscle cells, tropomyosin isoforms have variant molecular weights in the range of 30 to 40 kDa (27).

To clarify the distribution of actin molecules in the cytoplasm of 3Y1 cells, we stained them with rhodamine-phalloidin and anti-actin antibodies. When the focal plane was set at the basal level of 3Y1 cells, the

following features were observed: 1) rhodamine-phalloidin and both anti-actin antibodies stained many stress fibers (Fig. 2a-c); 2) in addition to them, many punctuate structures were observed with polyclonal anti-actin antibody (Fig. 2b); and 3) the fluorescence from the many stress fibers stained with monoclonal anti-actin antibody was too intense to distinguish from that from punctuate structures (Fig. 2c). To visualize them more clearly, we selected the cell having less stress fibers (Fig. 2b). Next we set the focal plane at the nuclear equator to observe the middle part of the cytoplasm. Only diffuse fluorescence of rhodamine-phalloidin was observed in the cytoplasm different from that of the basal level (Fig. 2d). On the other hand, both poly- and monoclonal anti-actin antibodies stained many punctuate structures throughout the cytoplasm (Fig. 2e and f). To wash out soluble materials, we permeabilized the living cell membrane by brief treatment with the digitonin solution (see Materials and Methods), which is reported not to affect cytoplasmic organelles' function and structure (16, 34). A diffuse background fluorescence was reduced by this treatment, but



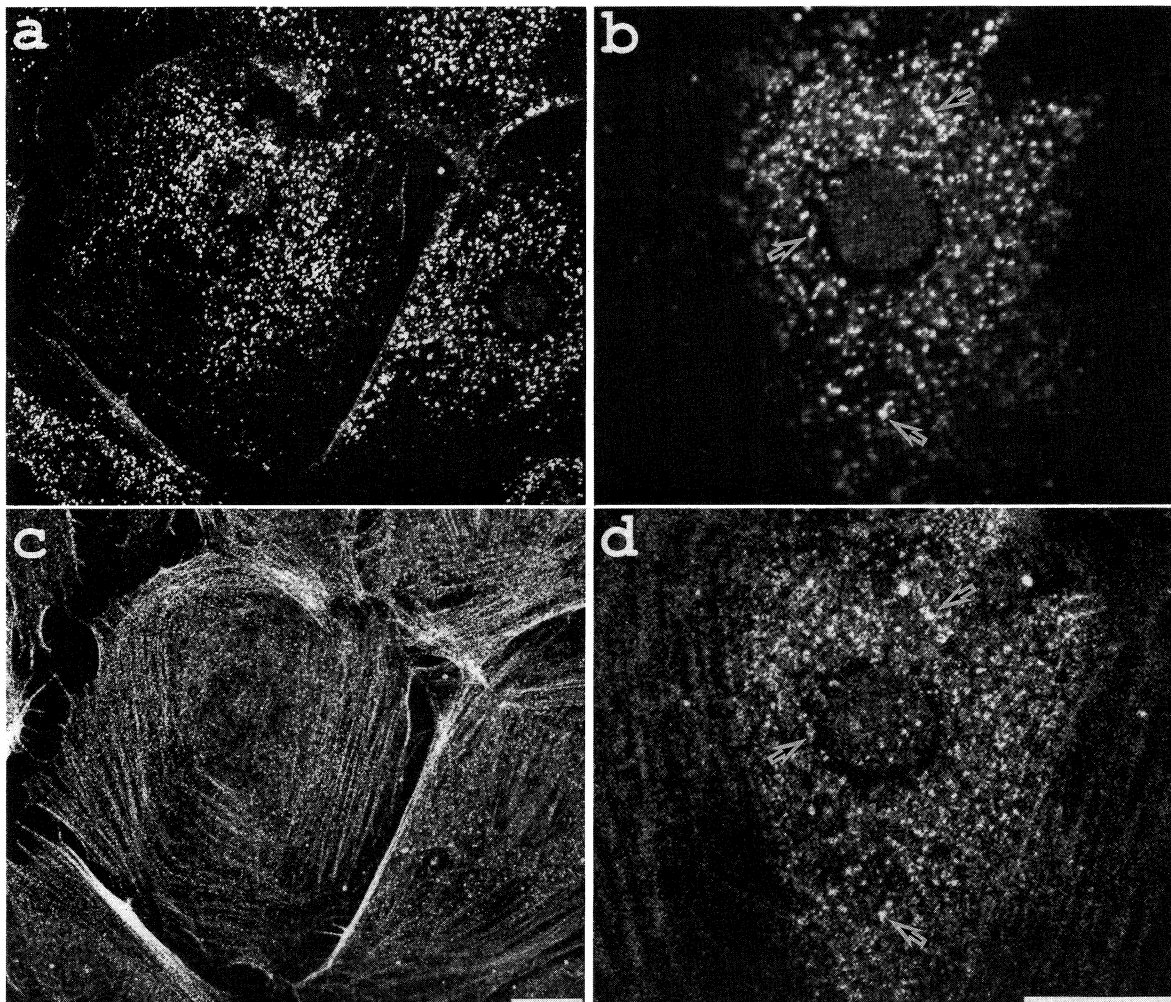
**Fig. 2.** Localization of actin in 3Y1 cells. Cells were stained with rhodamine-phalloidin (a and d), polyclonal (b and e), or monoclonal (c and f) antibodies against actin. Rhodamine-phalloidin stained the stress fibers (a) and the diffusive matters around the nucleus (d), but not the punctuate structures observed with both anti-actin antibodies. Many punctuate structures in addition to the stress fibers were immunostained with both poly- (b and e) and monoclonal (c and f) anti-actin antibodies. The focal plane was set at the basal level of cells (a-c). To observe the middle part of the cytoplasm, the focal plane was set at the nuclear equator (d-f). Bar, 20  $\mu$ m.

the fluorescence of the punctuate structures stained with anti-actin antibodies was retained in the cytoplasm. These results showed that actin molecules constituted stable punctuate structures in addition to stress fibers and/or bound to a cytoplasmic organelle.

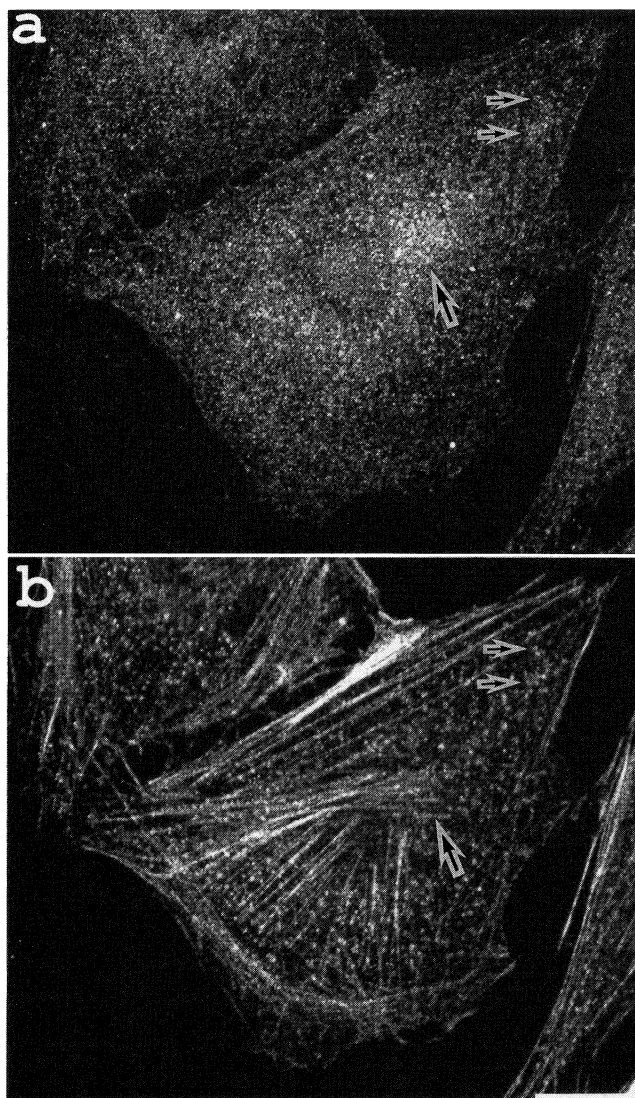
#### **Rab5 and transferrin receptor colocalized with the punctuate structures of actin**

The distribution of fluorescent punctuates stained with anti-actin antibodies resembled that of the early endosomes (Fig. 2) (7). To compare their localizations, we double-stained 3Y1 cells with polyclonal anti-actin and either monoclonal anti-rab5 or anti-transferrin receptor antibodies. Rab5, a small GTP binding protein, is thought to regulate the sorting endosome function (4,

7). Ullrich *et al.* (1996) reported that rab5 was associated with the sorting endosomes (39). Transferrin receptor is a well characterized recycling membrane protein in receptor-mediated endocytosis and localizes at both of the sorting and recycling endosomes (20, 29). Anti-rab5 antibody stained many vesicular structures throughout the cytoplasm (Fig. 3a and b). At the basal level of cells, anti-actin antibody stained stress fibers and many small dotty structures scattered throughout the cytoplasm (Fig. 3c). The distribution of these structures appeared to be similar to that of the vesicles stained with anti-rab5 antibody (Fig. 3a). When the focal plane was set at the middle part of the cytoplasm, clearly actin was shown to colocalize with the rab5 positive vesicles (Fig. 3b and d, arrows). Interest-



**Fig. 3.** Rab5 localization to the punctuate structures of actin. Cells were immunostained with monoclonal anti-rab5 (a and b) and polyclonal anti-actin (c and d) antibodies. At the basal level of cells, rab5 distributed as vesicular structures (a). On the same focus plane, many dotty structures of actin scattered over the cytoplasm in addition to the stress fibers (c). The distribution of rab5 positive vesicles was similar to that of the dotty structures of actin. When the focal plane was set at the middle part of the cytoplasm, actin clearly colocalized with rab5 positive vesicles (b and d, arrows). Bar, 20  $\mu$ m.



**Fig. 4.** Transferrin receptor localization in 3Y1 cells. Cells were double-stained with monoclonal anti-transferrin receptor (a) and polyclonal anti-actin (b) antibodies. Anti-transferrin receptor stained vesicular structures (a). Anti-actin also stained many punctuate structures in addition to the stress fibers (b). Colocalization of transferrin receptor and actin was observed at the cell peripheral region (small arrows), but not at the perinuclear area (large arrows). The focal plane was set at the basal level of cells. Bar, 20  $\mu$ m.

ingly, actin colocalized with transferrin receptor at the cell peripheral vesicles (Fig. 4, small arrows) but not at the vesicles around the nucleus where the recycling endosomes localized (Fig. 4, large arrows). From these results, we concluded that actin-filaments localize on the sorting endosome vesicles but not on the recycling endosomes.

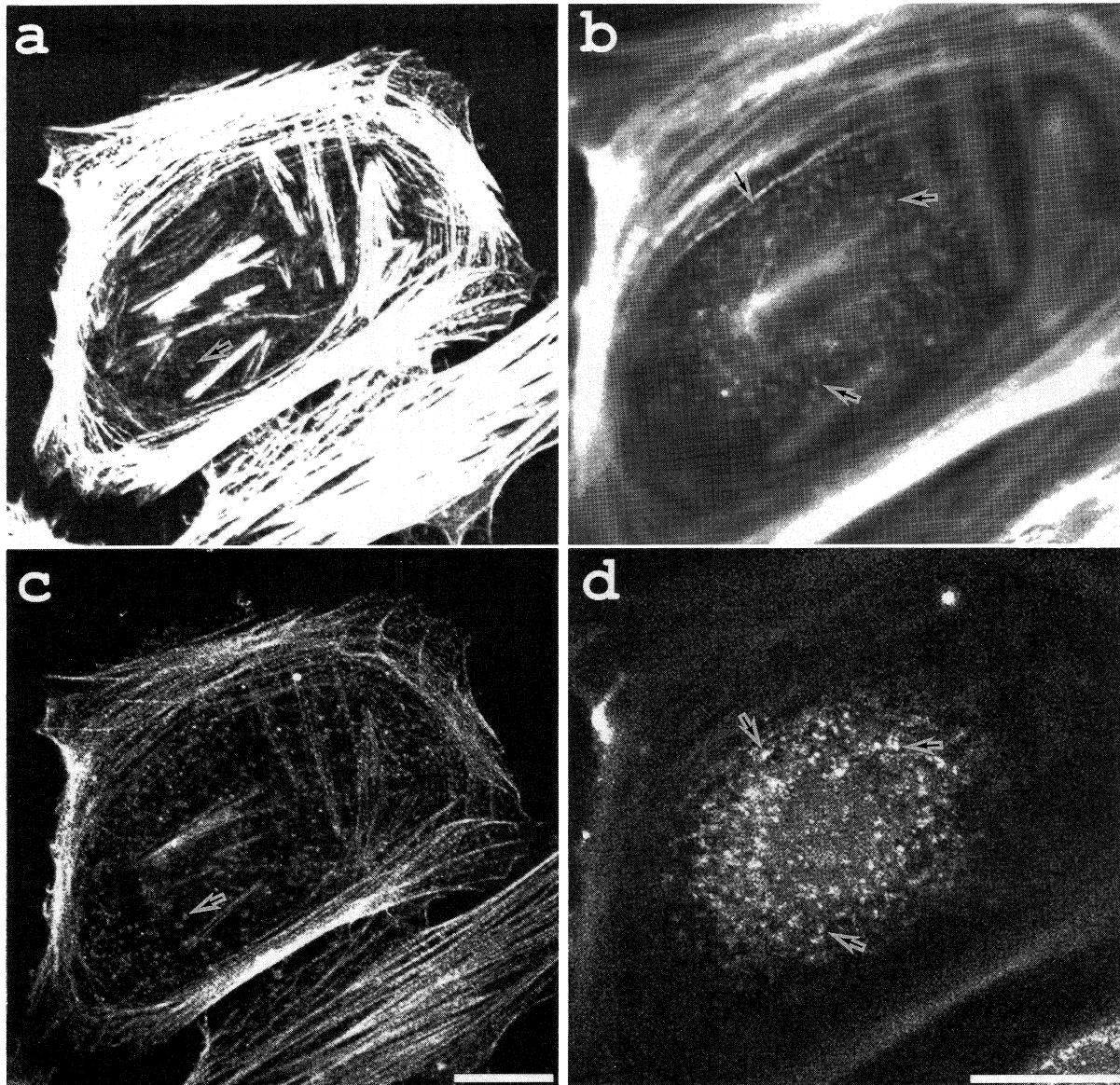
#### Tropomyosin localization to the punctuate structures of actin

To clarify whether actin molecules on the sorting endosomes constitute filaments or are only aggregated is important to understanding their roles. Tropomyosin is well known to bind to F-actin and stabilize them not only in muscle cells but also in non-muscle cells (27, 35). We therefore performed a double-staining of 3Y1 cells with monoclonal anti-tropomyosin and polyclonal anti-actin antibodies. Although, at the basal level of cells, only stress fibers appeared to be stained with anti-tropomyosin antibody in the normally contrasted image (data not shown), many fluorescent punctuates became observable in the enhanced image allowing us to visualize weak fluorescence (Fig. 5a). In the same cell, actin also colocalized with these fluorescent punctuates of tropomyosin (Fig. 5a and c, arrows). In the middle part of the cytoplasm, the fluorescent punctuates of tropomyosin appeared in the normally contrasted image (Fig. 5b). Colocalization of actin with tropomyosin at these punctuate structures was clearly observed (Fig. 5b and d, arrows). These results suggest that, at least, actin-filaments with tropomyosin exist on the sorting endosome vesicles.

#### DISCUSSION

Bucci *et al.* (1992) and Murphy *et al.* (1996) reported that overexpression of rab5 induced an enlargement of the early endosome (4, 30). Murphy *et al.* (1996) also found that actin-filaments localized on these abnormal endosomes (30). However, it is still unclear whether actin-filaments localize on the intact endosomes or not. In this article, we present the first evidence that actin-filaments localize on the intact sorting endosomes. Many punctuate structures in the cytoplasm were stained with anti-actin antibodies (Fig. 2). Similar structures have been reported by Cao *et al.* (1993) (6). They observed bead-like structures of actin scattered throughout the cytoplasm of cultured fibroblast. Judging from the distribution of the actin beads described in their article, the punctuate structures we observed were the same as theirs. From the binding of an exogenous G-actin binding protein to the beads, they suggested that the actin molecules on the beads were nonfilamentous. But they did not try to observe the localization of endogenous F-actin binding protein, such as tropomyosin, on the beads. We also observed that rhodamine-phalloidin was not able to stain actin punctuates with detectable intensity as they previously did (Fig. 2). However, in this study, we demonstrated that tropomyosin, a F-actin binding protein, colocalized with actin on the sorting endosomes. This result strongly suggests that at least a part of the actin molecules on the sorting endosomes, if not all, exists in a filamentous form, but that the densi-





**Fig. 5.** Tropomyosin localization to the punctuate structures of actin. Cells were double-stained with monoclonal anti-tropomyosin (a and b) and polyclonal anti-actin (c and d) antibodies. At the basal level of cells, many punctuate structures were stained with anti-tropomyosin in addition to the stress fibers (a). These punctuate structures were also co-stained with anti-actin antibody (a and c, arrows). In the middle part of the cytoplasm, tropomyosin and actin were clearly colocalized at the punctuate structures (b and d, arrows). Bar, 20  $\mu$ m.

ty of the actin-filaments on the endosome surface may be too low to be detectably stained by rhodamine-phalloidin. Therefore, the immunofluorescent intensity of anti-tropomyosin at the sorting endosome was lower than that of the stress fiber.

Since actin-filament and tropomyosin are the major components of the erythrocyte cell membrane skeleton based on spectrin network (3), we concluded that the same kind of membrane skeleton localizes also on the sorting endosome surface. The existence of a spectrin-

based membrane skeleton of cytoplasmic organelle has also been reported for the Golgi complex (1, 2, 10, 19). Holleran *et al.* (1996) has shown that Arp1 (actin related protein 1) filaments localized to the Golgi complex but actin did not (19). To date localization of tropomyosin to the cytoplasmic organelle has never been reported. Arp1 filaments are thought to link the Golgi complex to microtubules via cytoplasmic dynein as a component of dynactin complex (5, 19), and also are speculated to play a pivotal role of spectrin network on

the Golgi surface as actin does under the cell membrane (19). Interestingly, Arp1 localization appears to be restricted to the Golgi complex (19). The membrane skeleton of the recycling endosome has remained unknown. In the present study, we showed that actin-filaments localize themselves restrictively to the sorting endosome. These results suggest that membrane skeleton components of the sorting endosome are different from those of the Golgi complex. As to why their components differ, one possible explanation is the difference of cytoskeleton that they bind to. In a well spreading fibroblastic cell, the Golgi complex localizes around the microtubule organizing center (MTOC) (9). To maintain localization, they developed a membrane skeleton that binds to microtubules radiating from the MTOC. In contrast the sorting endosomes localize beneath the cell membrane (17). Actin-filament meshworks exist under the cell membrane but there are few microtubules there (18, 32). This leads us to speculate that the sorting endosomes bind to actin-filament meshworks via their binding proteins to maintain localization, and that they have a developed membrane skeleton having actin-filaments.

The cell membrane skeleton has been reported to form specialized membrane domains where specific membrane proteins migrate. These domains are thought to participate in the sorting process of membrane proteins into clathrin-coated pits (3). A novel class of clathrin-coated vesicles has been reported to bud off from the sorting endosome (34). The membrane skeleton may engage in a membrane protein sorting mechanism of the sorting endosome, which has been reported to be of complex tubulovesicular shape (17, 34). How they regulate their shapes still remains an open question, but their membrane skeleton is thought to contribute to such regulation. Biochemical analysis and ultrastructural observation of their surface structure ought to provide further evidence to elucidate these questions, as well as interesting information for understanding the intracellular vesicle transport system.

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