

## Effects of Colchicine on Localization of Alkaline Phosphatase in McA-RH 7777 Rat Hepatoma Cells

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We investigated the changes caused by microtubule disruption in cell contact-induced translocation of alkaline phosphatase (ALP) from the Golgi area to the plasma membrane in McA-RH 7777 cells. When the cells were treated with colchicine, the tubular structure of microtubules in the cytoplasm was lost. Colchicine treatment also resulted in the appearance of numerous dots containing mannosidase II (man II) throughout the cytoplasm. Moreover, ALP was distributed in small dots throughout the cytoplasm, as well as in all regions of the plasma membrane, although it was most concentrated at sites of intercellular contact. On the other hand, when the cells were incubated in basal medium after colchicine treatment, large spots containing ALP reappeared in the perinuclear cytoplasm more quickly than the accumulation of small dots containing man II. These findings suggest that colchicine causes disassembly of the Golgi complex into fragments, which scatter throughout the cytoplasm, but that it does not interfere with translocation of ALP to the plasma membrane. Furthermore, cytoplasmic ALP may be localized at sites other than the Golgi complex.

**Key words:** microtubule, colchicine, alkaline phosphatase, mannosidase II, McA-RH 7777

### I. Introduction

Glycoproteins in the plasma membrane are transported via the Golgi complex after synthesis in the rough-surfaced endoplasmic reticulum [1, 3, 13, 16, 19, 23–25]. Alkaline phosphatase (ALP) is a glycoprotein that is located in the plasma membrane [14, 17, 22], and in normal adult rat hepatocytes, it is predominantly localized in the bile canalicular domain of the plasma membrane [2, 4, 11, 28, 29].

Colchicine is an anti-microtubule agent that causes the disassembly of the tubular structure of microtubules in cells. Using immunocytochemistry, Hasegawa *et al.* demonstrated that colchicine interferes with intracellular transport of ALP in the apical plasma membrane of absorptive cells of the rat small intestine [15]. Furthermore, in primary cultures of adult or fetal rat hepatocytes, colchicine causes ALP to be distributed in a coarse granular distribution pattern in the cytoplasm [7, 10]. These reports suggest that microtubules

participate in the intracellular transport of ALP to the plasma membrane in polarized epithelial cells. However, there have been few reports on the intracellular transport of ALP in nonpolarized epithelial cells. McA-RH 7777 is a cell line derived from Morris rat hepatoma.

Our previous studies have shown that translocation of  $\gamma$ -glutamyltranspeptidase and ALP to the plasma membrane is promoted by cell-to-cell contact in McA-RH 7777 cells [8]. Moreover, using immunofluorescent double-staining for ALP and the Golgi marker mannosidase II (man II), we demonstrated that ALP is localized in the Golgi area of the cytoplasm in solitary McA-RH 7777 cells cultured at low concentration [9]. This indicates that contact between McA-RH 7777 cells promotes the translocation of ALP from the Golgi area of the cytoplasm to the plasma membrane. In the present study, we examined the changes caused by microtubule disruption in the translocation of ALP in McA-RH 7777 cells in order to better understand how contact between McA-RH 7777 cells induces translocation of ALP from the Golgi area of the cytoplasm to the plasma membrane.

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## II. Materials and Methods

### *Cell culture*

McA-RH 7777 cells derived from Morris rat hepatoma were purchased from DS Pharma Biomedical Co., Ltd. (Osaka, Japan) and cultured in  $\alpha$ -Eagle's medium ( $\alpha$ -MEM; DS Pharma Biomedical Co., Ltd.) containing 10% fetal bovine serum (basal medium). McA-RH 7777 cells were seeded at a concentration of  $6 \times 10^4$  cells/ml in 8-well collagen-coated BioCoat Culture Slides (Nippon Becton Dickinson, Tokyo, Japan), followed by culture in basal medium. For synchronized growth, 24 hr after the start of cell culture, McA-RH 7777 cells were cultured overnight in medium containing 2.5 mM thymidine. Medium was then replaced with basal medium and cells were incubated for an additional 8 hr. Medium was thereafter replaced with medium containing 1 mM hydroxyurea and cells were cultured overnight. After the cells were synchronized, medium was replaced with basal medium containing  $10^{-5}$  M colchicine, followed by incubation for 5, 15, 30 or 240 min. After synchronized growth, some cells were incubated for 4 hr in basal medium containing colchicine, and then for 0, 8, 12, 16, 20 or 26 hr in medium without colchicine.

### *Detection of microtubules*

McA-RH 7777 cells were fixed in cold absolute methanol for 10 min at  $-20^\circ\text{C}$ , washed with 0.01 M phosphate-buffered saline (PBS; pH 7.2) containing 0.85% NaCl and incubated for 1 hr at room temperature (RT) with 1:50 anti-rat  $\beta$ -tubulin monoclonal antibody (Chemicon International, Temecula, CA, USA) in PBS. Cells were then washed with PBS and incubated for 30 min at RT with 1:160 fluorescein isothiocyanate (FITC)-labeled anti-mouse IgG (Medical & Biological Laboratories Co., Ltd., Nagoya, Japan) in PBS. After immunostaining, cells were mounted in Fluoro-Guard Antifade Reagent (Bio-Rad, Hemel Hempstead, UK) and examined with a confocal laser scanning microscope (Radiance 2100, Bio-Rad or C1si, Nikon, Kawasaki, Japan).

### *Double staining for ALP and man II*

McA-RH 7777 cells were fixed in Zamboni solution (2% paraformaldehyde and 15% saturated picric acid in 0.1 M phosphate buffer, pH 7.4) for 10 min at RT. After washing with PBS containing 0.05% saponin, cells were immersed in 0.1% Triton X-100 in PBS for 5 min at RT. Cells were then immersed for 1 hr at RT in a solution containing 1:50 anti-rat ALP antiserum [5, 6] and 1:10,000 anti-rat man II monoclonal antibody (Covance Research Products, Richmond, CA, USA) in PBS. Next, cells were washed with PBS and reacted for 30 min at RT with 1:50 rhodamine-labeled anti-rabbit IgG and 1:160 FITC-labeled anti-mouse IgG antibody (Medical & Biological Laboratories Co., Ltd.) in PBS. All cells were mounted in Fluoro-Guard Antifade Reagent and examined with a confocal laser scanning microscope.

## III. Results and Discussion

### *Effects of colchicine on microtubules*

In McA-RH 7777 cells cultured in basal medium, microtubules extended from the nucleus to the cell surface (Fig. 1a). Treatment of the cells for 5 min with colchicine had little effect on tubular structures. After 15 min, some of the cells became slightly round, and the microtubules shortened, and after 30 min, microtubular structures were disrupted in most cells (Fig. 1b). After 4 hr of treatment with colchicine, the cells were roundish, and completely lacked microtubular structures. In addition, anti-tubulin staining revealed numerous dots with weak fluorescence throughout the cytoplasm (Fig. 1c). On the other hand, microtubular structures did not reappear in the cytoplasm until after 12 hr of incubation in basal medium following 4-hr colchicine treatment (Fig. 2a). However, from 16-hr incubation in basal medium after colchicine treatment, microtubular structures started to appear in the cytoplasm, and after 20-hr incubation in basal medium after colchicine treatment, microtubular structures were seen in most cells (Fig. 2b). At 26-hr incubation in basal medium after colchicine treatment, the distribution pattern of microtubules was similar to that in McA-RH 7777 cells cultured only in basal medium (Fig. 2c).

### *Effects of colchicine on localization of ALP and man II*

Double-staining for ALP and man II in McA-RH 7777 cells cultured in basal medium revealed that man II in the cytoplasm was present in aggregated, coarse dots near the Golgi complex (Fig. 3a). ALP was localized in numerous dots scattered throughout the cytoplasm and in the borders between adjacent cells (Fig. 3b). Sites showing positive reactions in the perinuclear cytoplasm were large and intensely stained (Fig. 3b) and most of the sites were in the same locations as man II staining (Fig. 3c). After 30 min of colchicine treatment, the man II-containing dots started to diffuse throughout the cytoplasm and became smaller than in cells cultured in basal medium (Fig. 3d). In some cells, large spots with intense staining for ALP in the perinuclear cytoplasm disappeared, and instead, ALP was observed in numerous small dots scattered throughout the cytoplasm, although the localization of ALP at the contacts between adjacent cells did not change (Fig. 3e, 3f). After 4 hr of treatment with colchicine, staining for man II in most cells showed numerous fine dots with weak fluorescence throughout the cytoplasm (Fig. 3g).

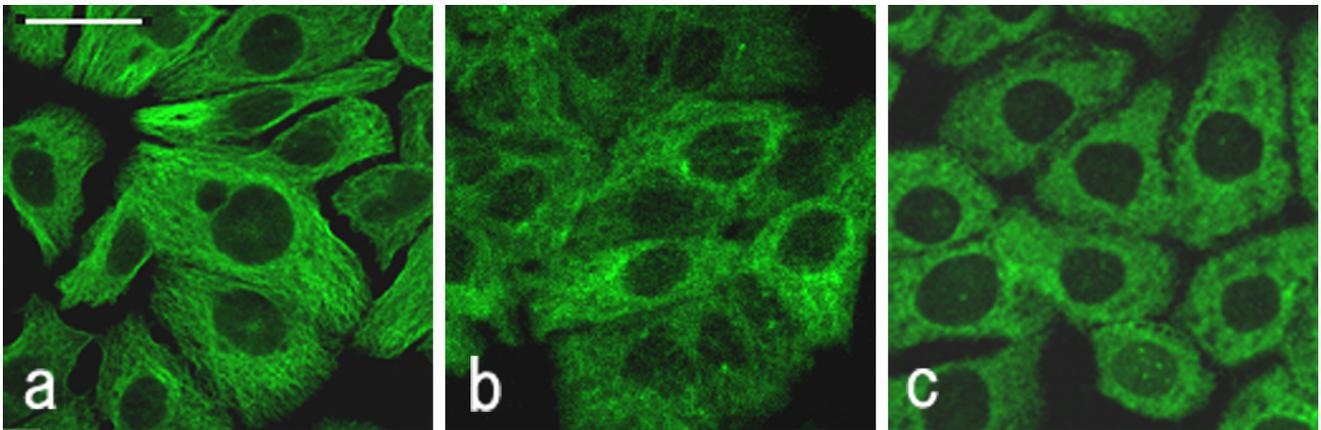
On staining for ALP, there were numerous small dots of ALP in the cytoplasm, and the intensity of ALP staining at intercellular contacts became more intense (Fig. 3h, 3i). In some cells, ALP was localized in areas of the plasma membrane not in contact with other cells (Fig. 3h). On the other hand, distribution patterns of ALP and man II did not show any change in most cells, except some aggregation of man II-containing dots around the nucleus, until after 12 hr of incubation in basal medium following 4-hr treatment with colchicine, as compared with cells treated for 4 hr with colchicine alone (Fig. 4a–4c). However, at 20-hr incubation

in basal medium after colchicine treatment, large spots with intense staining for ALP in the perinuclear cytoplasm were seen in numerous MCA-RH 7777 cells (Fig. 4e), while man II remained as dots scattered throughout the cytoplasm in numerous MCA-RH 7777 cells (Fig. 4d). At 26-hr incubation in basal medium after colchicine treatment, ALP and man II showed similar localization patterns as those in MCA-RH 7777 cells cultured only in basal medium (Fig. 4g, 4h).

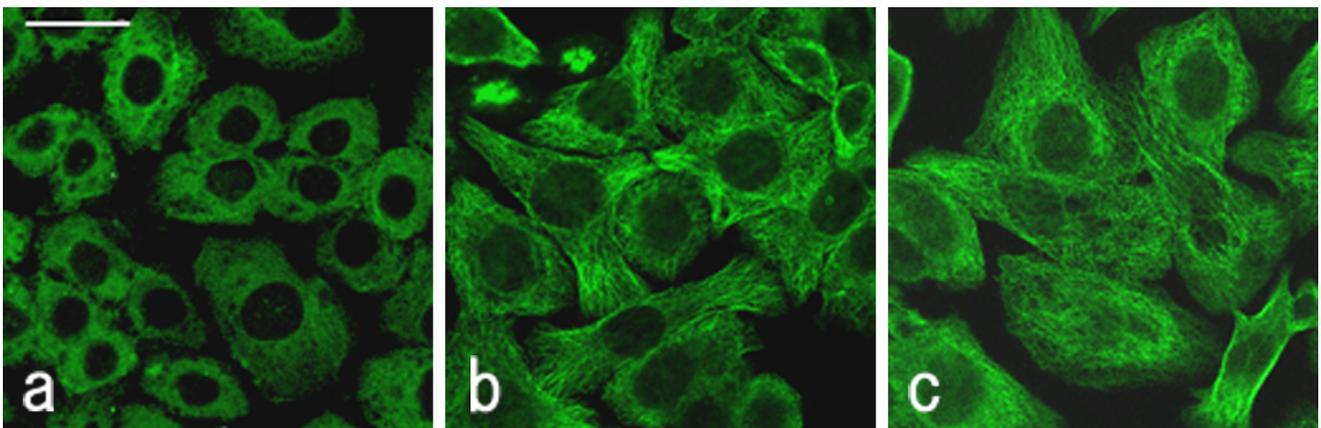
In the present study, we showed that colchicine causes the disassembly of microtubules in MCA-RH 7777 cells, resulting in the scattering of small dots containing man II, a Golgi marker, throughout the cytoplasm. Man II is localized in the Golgi stack (cis and medial elements of the Golgi complex) but not in GERL and its derivatives (trans element of the Golgi complex) [20]. We used Trans-Golgi Network

(TGN) 38 as a marker for the trans element of the Golgi complex, and demonstrated that TGN38 shows the same distribution pattern as man II after colchicine treatment in MCA-RH 7777 cells (unpublished data). This confirms that the Golgi complex is disassembled into small fragments that are scattered throughout the cytoplasm [27]. In addition, the large spots in the perinuclear cytoplasm with intense staining for ALP observed in MCA-RH 7777 cells cultured in basal medium were lost in colchicine-treated cells, and ALP was scattered as small dots throughout the cytoplasm; however, the levels of ALP at intercellular contacts were not substantially changed until 2 hr after colchicine treatment.

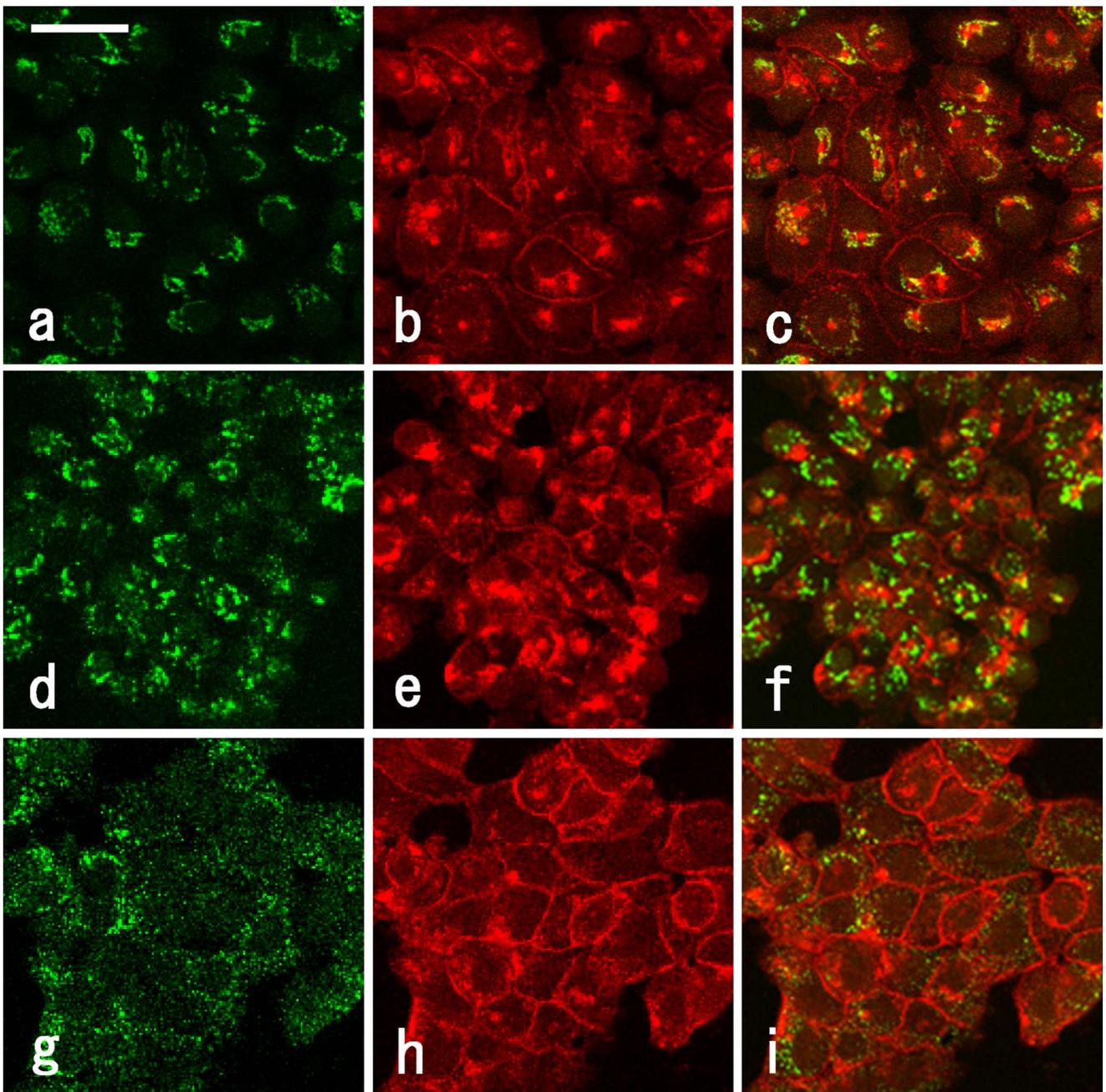
In cells treated with colchicine for 4 hr, man II and ALP in the cytoplasm was distributed in small dots, and staining for ALP at the intercellular contacts became more intense



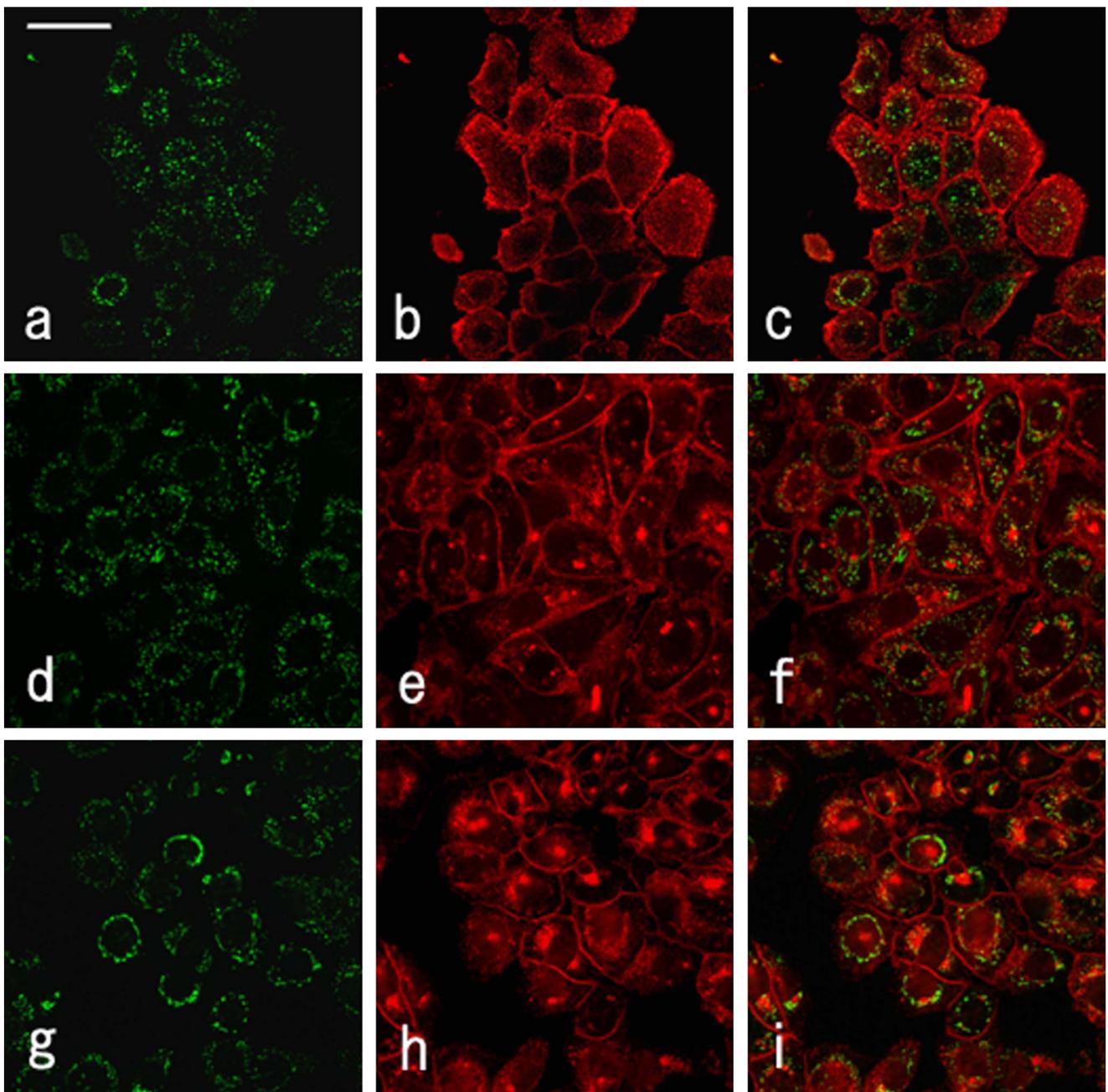
**Fig. 1.** Changes in microtubules in MCA-RH 7777 cells after colchicine treatment. (a) MCA-RH 7777 cells in base medium. Microtubules extend from the nucleus to cell surface. (b) MCA-RH 7777 cells after 30 min of treatment with colchicine. Microtubular structures are disrupted in most cells. (c) MCA-RH 7777 cells after 4 hr of colchicine treatment. Microtubular structures are completely lost, and numerous dots with weak fluorescence are present throughout the cytoplasm. Bar=30  $\mu$ m.



**Fig. 2.** Changes in microtubules in MCA-RH 7777 cells incubated in basal medium after 4-hr colchicine treatment. (a) MCA-RH 7777 cells incubated for 8 hr in basal medium after 4-hr colchicine treatment. Microtubular structures do not yet reappear in the cytoplasm. (b) MCA-RH 7777 cells incubated for 20 hr in basal medium after 4-hr colchicine treatment. Microtubular structures appear in the cytoplasm in most cells. (c) MCA-RH 7777 cells incubated for 26 hr in basal medium after 4-hr colchicine treatment. The distribution pattern of microtubules is similar to that in MCA-RH 7777 cells cultured in basal medium alone. Bar=30  $\mu$ m.



**Fig. 3.** Localization of man II and ALP in McA-RH 7777 cells after colchicine treatment. **(a)** Localization of man II in McA-RH 7777 cells in basal medium. Man II is observed in aggregated, coarse dots in the Golgi area of the cytoplasm. **(b)** Localization of ALP in McA-RH 7777 cells in basal medium. Most of the large spots with intense staining for ALP are colocalized at the same sites as staining for man II. ALP is also localized in numerous small dots scattered throughout the cytoplasm and at sites of intercellular contact. **(c)** Merge of **(a)** and **(b)**. **(d)** Localization of man II in McA-RH 7777 cells after 30-min treatment with colchicine. Dots with specific staining for man II are somewhat smaller than in cells cultured in basal medium and are scattered throughout the cytoplasm. **(e)** Localization of ALP in McA-RH 7777 cells after 30 min of colchicine treatment. Large spots with strong staining for ALP in the Golgi areas disappear in some cells, and staining is observed in numerous small dots scattered in the cytoplasm, but there is no change in the localization of ALP at sites of intercellular contact. **(f)** Merged image of **(d)** and **(e)**. **(g)** Localization of man II in McA-RH 7777 cells after 4-hr treatment with colchicine. Numerous fine dots with weak staining for man II are scattered throughout the cytoplasm. **(h)** Localization of ALP in McA-RH 7777 cells after 4-hr treatment with colchicine. Staining for ALP in the cytoplasm is present as numerous small dots. Staining for ALP is stronger at sites of intercellular contact. ALP is also present on the plasma membrane in regions not in contact with other cells. **(i)** Merged image of **(g)** and **(h)**. Bar=50  $\mu$ m.



**Fig. 4.** Localization of man II and ALP in MCA-RH 7777 cells incubated in basal medium after 4-hr colchicine treatment. **(a)** Localization of man II in MCA-RH 7777 cells incubated for 8 hr in basal medium after 4-hr colchicine treatment. Man II is localized in numerous small dots scattered throughout the cytoplasm in most cells, although man II-containing dots aggregate around the nucleus in a few cells. **(b)** Localization of ALP in MCA-RH 7777 cells incubated for 8 hr in basal medium after 4-hr colchicine treatment. Intense staining for ALP is recognized at intercellular contacts and small dots containing ALP is observed in the cytoplasm. In some cells, positive reactions for ALP are also observed in areas of the plasma membrane not in contact with other cells. **(c)** Merged image of **(a)** and **(b)**. **(d)** Localization of man II in MCA-RH 7777 cells incubated for 20 hr in basal medium after 4-hr colchicine treatment. Small dots containing man II are still scattered throughout the cytoplasm in other cells, although they aggregate in the perinuclear cytoplasm in some cells. **(e)** Localization of ALP in MCA-RH 7777 cells incubated for 20 hr in basal medium after 4-hr colchicine treatment. Large spots with intense staining for ALP reappear in the perinuclear cytoplasm in numerous cells. **(f)** Merged image of **(d)** and **(e)**. **(g)** Localization of man II in MCA-RH 7777 cells incubated for 26 hr in basal medium after 4-hr colchicine treatment. **(h)** Localization of ALP in MCA-RH 7777 cells incubated for 26 hr in basal medium after 4-hr colchicine treatment. Localization patterns of man II and ALP are similar to those in MCA-RH 7777 cells cultured only in basal medium. **(i)** Merged image of **(g)** and **(h)**. Bar=50  $\mu$ m.

than in cells cultured in basal medium. Weak staining for ALP was also observed in areas of the plasma membrane not in contact with other cells. Based on these results, we concluded that, in McA-RH 7777 cells, cytoplasmic ALP normally exists in the Golgi areas, but when the Golgi complex is disassembled, it is released from the Golgi areas and transported to the cell membrane via small vesicles as part of an unknown pathway.

Microtubules are known to mediate intracellular transport, and they are disassembled by anti-microtubular reagents, such as colchicine and nocodazole. Colchicine has been reported to interfere with the normal transportation of ALP to the apical domain of the plasma membrane in polarized epithelial cells [7, 10, 15, 21]. In the present study, we examined whether colchicine inhibits the translocation of ALP to the plasma membrane at sites of intercellular contact in cultures of McA-RH 7777 cells. Even after colchicine treatment, the localization of ALP at intercellular contacts was the same as in cells cultured in basal medium, although the remainder of the ALP was scattered in fine dots throughout the cytoplasm. This demonstrates that colchicine does not inhibit the transportation of ALP to the plasma membrane at sites of intercellular contact.

Durand-Schneider *et al.* showed that in primary cultures of rat hepatocytes, colchicine inhibits the transport of apical plasma membrane proteins, but it does not inhibit the transport of lateral plasma membrane proteins [12]. This suggests that microtubules participate only in the transport of apical plasma membrane proteins in polarized epithelial cells. Accordingly, it is thought that in McA-RH 7777 unpolarized rat hepatoma cells, microtubules do not participate in ALP transport to the plasma membrane at sites of intercellular contact. On the other hand, microtubules are known to play a role in maintaining the composition of the Golgi complex in the cytoplasm [18, 27].

Large spots in the perinuclear cytoplasm with intense staining for ALP that were observed in McA-RH 7777 cells cultured in basal medium were lost in colchicine-treated cells, and ALP was scattered as small dots throughout the cytoplasm. During incubation in basal medium after colchicine treatment, these spots reformed in the perinuclear cytoplasm more quickly than the accumulation of small dots containing man II in the Golgi areas. It is therefore possible that they are not components of the Golgi complex.

It was recently reported that a water channel protein, aquaporin-2, which is translocated to the apical plasma membrane of Madin-Darby canine kidney (MDCK) cells by forskolin treatment, colocalizes with the endosomal marker proteins EEA1 and Rab11 in the cytoplasm after forskolin washout [26]. Similarly, in the present study, large spots of ALP in the perinuclear cytoplasm or small dots containing ALP scattered throughout the cytoplasm may be related to a type of endosome. In a future study, we plan to examine the relationship between localization of cytoplasmic ALP and endosomes.

#### IV. Acknowledgments

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