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Tyrphostin A8 stimulates a novel trafficking pathway of apically endocytosed transferrin through Rab11-enriched compartments in Caco-2 cells

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Norouziyan F, Shen WC, Hamm-Alvarez SF. Tyrphostin A8 stimulates a novel trafficking pathway of apically endocytosed transferrin through Rab11-enriched compartments in Caco-2 cells. Am J Physiol Cell Physiol 294: C7–C21, 2008. First published October 24, 2007; doi:10.1152/ajpcell.00372.2006.—The potential application of transferrin receptors as delivery vehicles for transport of macromolecular drugs across intestinal epithelial cells is limited by several factors, including the low level of transferrin receptor-mediated transcytosis, particularly in the apical-to-basolateral direction. The GTPase inhibitor, AG10 (tyrphostin A8), has been shown previously to increase the apical-to-basolateral transcytosis of transferrin in Caco-2 cells. However, the mechanism of the increased transcytosis has not been established. In this report, the effect of AG10 on the trafficking of endocytosed transferrin among different endosomal compartments as well as the involvement of Rab11 in the intracellular trafficking of transferrin was investigated.

Confocal microscopy studies showed a high level of colocalization of FITC-transferrin with Rab5 and Rab11 in Caco-2 cells pulsed at 16°C and 37°C, which indicated the presence of apically endocytosed FITC-transferrin in early endosomes and apical recycling endosomes at 16°C and 37°C, respectively. The effect of AG10 on the accumulation of transferrin within different endosomal compartment was studied, and an increase in the transcytosis and recycling of internalized 125I-labeled transferrin, as well as a decrease in cell-associated 125I-labeled transferrin, was observed in AG10-treated Caco-2 cells pulsed at 37°C for 30 min and chased for 30 min. Moreover, confocal microscopy showed that FITC-transferrin exhibited an increased level of colocalization with Rab11, but not with Rab5, in the presence of AG10. These results suggest an effect of AG10 on the later steps of transferrin receptor trafficking, which are involved in subsequent recycling, and possibly transcytosis, of endocytosed transferrin in Caco-2 cells.

Rab proteins; recycling; transcytosis; guanosine 5′-triphosphatase inhibitor; AG10

THE INTRACELLULAR TRAFFICKING of plasma membrane proteins such as transferrin (Tf) has been studied extensively. Tf internalized from the plasma membrane via Tf receptor (TfR)-mediated endocytosis is first delivered to the peripheral early sorting endosomes via clathrin-coated vesicles in nonpolarized cells. In the acidic endosomal environment, the iron is dissociated from Tf and the apo-Tf-TfR complex is targeted to the recycling compartment. From this compartment, the apo-Tf-TfR complex is recycled back to the plasma membrane where apo-Tf is released from TfR (29, 38). The recycling compartment contains recycling receptors including TfR and low-density lipoprotein receptor, but not lysosomally directed ligands such as LDL or α2-macroglobulin. This endosomal compartment has been characterized as a tubular network, in contrast with the tubulovesicular sorting endosome, and is concentrated in the perinuclear area of nonpolarized cells, including Chinese hamster ovary cells (CHO), A1T20 cells, and HeLa cells (11, 15, 27, 30, 46, 62).

In polarized cells, the cell surface is separated into apical and basolateral plasma membrane domains. They have different lipid and protein composition, and endocytosis can occur from both domains (31, 34). In polarized cells, the basolaterally endocytosed TfR is efficiently sorted from the transcytotic pathway and recycled back to the basolateral membrane. Polarized epithelial cells contain distinct populations of apical early endosomes (AEEs) and basolateral early endosomes (Fig. 1). The majority of Tf (65%) was found to be recycled from basolateral early endosomes within 10 min after internalization from the basolateral membrane in MDCK cells (40). The existence of an endosomal compartment, which is accessible to both apically and basolaterally endocytosed ligands, has been reported in the polarized epithelial cells (17, 22, 52). In addition, transcytosing proteins such as IgA and recycling proteins such as Tf have been found in a “common” endosome (CE), which consists partly of tubules (32). This compartment, which seems to be an ubiquitous feature of the polarized epithelial cells, is termed the common recycling endosome in MDCK cells (41) or the subapical compartment in hepatocytes (19, 36, 44, 50). The CE has been identified as a tubularized endosomal system with a diameter of 60 nm in MDCK cells, and it is from there that the basolaterally endocytosed Tf-TfR complex recycles to the basolateral membrane (Fig. 1, pathway 8) (1, 9). On the other hand, the basolaterally internalized complex formed by polymeric immunoglobulin receptor and dimeric IgA on its route from the basolateral to the apical surface crosses the CE (Fig. 1, pathway 7) and moves to a population of 100- to 150-nm cup-shaped vesicles above the CE, which constitutes the apical recycling endosome (ARE) in MDCK cells (3, 12). It may reach the apical plasma membrane through the Tf recycling pathway (Fig. 1, pathway 4). In hepatocytes, the subapical compartment has been characterized as a subcompartimentalized endosomal compartment that is composed of 60- to 100-nm tubulovesicular structures (analogous to the CE in MDCK) and 150- to 200-nm cup-shaped vesicles in the apical region (analogous to the ARE in MDCK) (36).

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TfR has been considered in the targeting and delivery of a number of drugs including anticancer therapeutics (54). Furthermore, TfR-mediated transcytosis has been shown to be an effective approach for the delivery of proteins and peptides across the brain endothelium (18, 57) and intestinal epithelium (42). The transport of an insulin-Tf conjugate via TfR-mediated transcytosis was demonstrated across Caco-2 cells (39), and a hypoglycemic effect in the diabetic rats was reported after oral administration of the insulin-Tf conjugate (60). However, one of the problems associated with oral delivery via TfR-mediated transcytosis is that the majority of Tf is localized to the basolateral surface of the cells. To overcome the limitation of TfR-mediated transcytosis at such a low rate of transport, enhancers of apical-to-basolateral Tf transport have been studied. AG10 (tyrphostin A8, or 4-hydroxybenzylidenemalononitrile), a GTPase inhibitor, is an effective enhancer with potentially few side effects. The effect of AG10 on TfR-mediated transcytosis of the insulin-Tf conjugate has been demonstrated both in vivo and in vitro (59). However, the mechanism involved in the enhancement of TfR-mediated transcytosis in the presence of AG10 is still largely unknown.

In this report, the effect of AG10 on Tf intracellular trafficking was used to investigate the intracellular processing of endocytosed FITC-Tf conjugates to identify endosomal compartments involved in Tf intracellular pathways in Caco-2 cells. We also study the effects of AG10 on the colocalization of various Rab proteins with endocytosed Tf during its intracellular trafficking and transcytosis in Caco-2 cells. We found that Rab11 is involved in the apical recycling of Tf in Caco-2 cells. Furthermore, AG10 stimulates the trafficking pathway of the apically endocytosed Tf through the Rab11-containing endosomal compartment. On the other hand, the basolaterally endocytosed Tf did not have access to the Rab11-containing endosomal compartment in Caco-2 cells, unless in the presence of AG10. Therefore, results from this report will provide information regarding not only the difference in TfR processing between apical and basolateral membrane, but also the effect of AG10 on the intracellular trafficking of Tf. Elucidating the role of the intracellular controllers of the recycling and transcytosis of Tf, such as Rab proteins, is potentially important for the development of Tf as a vehicle for the increase of intestinal absorption of protein drugs.

**MATERIAL AND METHODS**

*Cell culture.* C2BBe1, a subclone of Caco-2 cells, was obtained from the American Type Culture Collection. The cells were grown in DMEM (Invitrogen) supplemented with 50 U/ml penicillin, 50 μg/ml streptomycin, nonessential amino acids, L-glutamine, and FBS (10%).

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**Table 1. Summary of the involvement of Rab proteins Rab5 and Rab11 in Tf intracellular trafficking**

<table>
<thead>
<tr>
<th>Rab5</th>
<th>Rab11</th>
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<tbody>
<tr>
<td><strong>Localization</strong></td>
<td><strong>Pericentriolar recycling endosome in nonpolarized cells (14, 37, 48), apical recycling endosome in Caco-2 and MDCK cells (5, 53), trans-Golgi network (7, 49)</strong></td>
</tr>
<tr>
<td>Plasma membrane, clathrin-coated vesicles, early endosomes (4, 6, 55)</td>
<td></td>
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<tr>
<td><strong>Function</strong></td>
<td><strong>Regulation of Tf recycling from pericentriolar recycling endosome in nonpolarized cells (48), involvement in Tf recycling from apical recycling endosomes in Caco-2 cells (current study)</strong></td>
</tr>
<tr>
<td>Coat propagation and fission of coated vesicles from plasma membrane (20), docking and fusion of coated vesicles with early endosomes (4, 16, 28); regulation of homotypic fusion between early endosomes (2, 4, 13), and ligand sequestration (28)</td>
<td></td>
</tr>
<tr>
<td><strong>Colocalization with Tf</strong></td>
<td>In Caco-2 cells pulsed at 37°C (current study)</td>
</tr>
<tr>
<td>In Caco-2 cells pulsed at 16°C (current study)</td>
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</table>

Sources are given in parentheses. Tf, transferrin.
Rabbit polyclonal antibodies against Rab5 and Rab11 were purchased from Santa Cruz Biotechnology. The rabbit polyclonal antibody against ZO-1 and Alexa-Fluor-labeled secondary antibodies were purchased from Molecular Probes. AG10 was obtained from Calbiochem.

Preparation of dferric, radiolabeled, and FITC-labeled Tf. To prepare dferric Tf, 100 μl ferric ammonium citrate in H2O with a stock concentration of 10 mg/ml was added to 1 ml of apo-Tf in PBS solution (20 mg/ml) under constant stirring. After incubation of the mixture at 37°C for 2 h, the iron-loaded Tf was dialyzed against 2 L PBS at 4°C for 16 h.

To radio-label Tf, the dferric Tf was added to Na125I in PBS, followed by the addition of freshly made chloramine T and sodium metabisulfite in PBS solution. The reaction was terminated by addition of potassium iodide to the reaction tube. The radiolabeled Tf was separated by gel filtration through a 10 × 0.5 cm Sephadex G-50 column.

FITC (Sigma) was conjugated to Tf with a molar ratio of 50:1 in 1 M sodium bicarbonate pH 9.0 under constant stirring for 1 h at 25°C. The untreated FITC was removed by dialysis against 2 L PBS at 4°C for 16 h. Protein concentration and the degree of labeling were calculated by using formulas provided by Molecular Probes (Eugene, OR).

Pulse-chase experiments using 125I-labeled Tf. The 2-wk-old confluent Caco-2 cells grown on 24-mm Transwell filters (Costar, Cambridge, MA), which displayed a transepithelial electrical resistance (TEER) of 100 ohms/cm² as measured by an epithelial voltohmmeter (EVOM; World Precision Instrument, West Haven, CT), were incubated with serum-free DMEM containing 0.1% BSA and 20 mM HEPES for 1 h at 37°C to deplete endogenous Tf. The cell monolayers were further incubated in the presence or absence of AG10 (500 μM) for 2 h at 37°C. 125I-labeled Tf [2–4 μg/ml, 2 × 10⁶ counts per minute (cpm/ml)] was bound to the apical surface of the cells in the presence or absence of AG10 on ice for 15 min, which was followed by incubation at either 16°C or 37°C for 30 min. After removal of the unbound and surface-bound 125I-labeled Tf with ice-cold PBS (containing 0.1 mM Ca²⁺ and 0.05 mM Mg²⁺) and acid (0.15 M NaCl, 0.5 M acetate, pH 2.2) washes on ice, the cells were chased for 0, 2, or 30 min in the presence of 10-fold Tf at 37°C. At the end of the 30-min chase period, the unbound and surface-bound FITC-Tf was removed by medium changes, PBS and acid washes, and the cells were fixed by using 3.7% paraformaldehyde (Sigma) in PBS, which was followed by quenching and permeabilization with 0.2% saponin for 5 min. The cells were then blocked with 10% FBS in PBS and incubated with appropriate primary antibodies diluted in 1.5% FBS in PBS. Alexa-Fluor 568 goat anti-rabbit antibody was used for the secondary detection of the proteins. The nucleus was labeled by incubation with 4',6-diamidino-2-phenylindole, dilactate (DAPI, dilactate, Invitrogen) dye for 15 min on a platform shaker. The efficiency of “acid washes” in removing the surface-bound FITC-Tf was demonstrated in control experiments, in which the cells were incubated with FITC-Tf for 30 min at 37°C and were not subjected to the acid washes at the end of the pulse period.

Image processing. The images were collected from different fields of the prepared slides by using a confocal laser-scanning microscope (LSM 510 Meta NLO imaging system; Zeiss) equipped with argon, red HeNe, and green HeNe lasers for standard confocal fluorescence microscopy and a Ti-Sapphire tunable Coherent Chameleon laser (720–950 nm) for multiphoton excitation of DAPI. The ability of this system to acquire fluorescence emission signals resolved within narrow ranges in multitrack mode and the use of singly labeled control samples ensured the validity of colocalization studies. The acquisition parameters for the collection of images were kept constant between samples to allow comparison of the images taken from different fields and slides. The analysis of the confocal microscopy image by using computer software has already been described by Nunez et al. (33). Caco-2 cell monolayers were optically sectioned in the z-axis from the apical to the basolateral side of the cell. The first z-section from the apical surface of the cells was captured when FITC-Tf (green) was observed. The step size in the z-axis was 0.4 μm. Analysis of the extent of colocalization between markers of interest in confocal microscopy images was done by using the Enhanced Colocalization tool available with the Zeiss LSM 510 software. To quantify the colocalization between FITC-Tf and Rab proteins, the image fields were thresholded. The whole image field was defined as a region of interest, and the system calculated mean intensities + 2 × SD to threshold the images. Colocalization coefficients representing the relative number of colocalizing pixels in either channel compared with the total number of pixels above the threshold irrespective of their intensity were measured through the image sequence in the z-series. To analyze the extent of the colocalization between FITC-Tf and the Rab proteins, we used the colocalization coefficient of FITC determined in each z-section of a z-series that shows the relative number of colocalizing FITC pixels compared with the total number of FITC pixels above the threshold in the z-sections. The average colocalization coefficient of FITC-Tf at any specific height in the cells was obtained by using the FITC-Tf colocalization coefficients of the z-sections, which were collected from different fields of the prepared
slide and localized at the same height above either the nucleus or the tight junction. For statistical analysis, the SE was calculated. Student’s t-test was used to analyze significance ($P \leq 0.05$). In the images labeled with the same fluorophore to detect ZO-1 and Rab protein staining, the single cells were thresholded twice, with and without ZO-1 staining. To obtain the colocalization coefficient, ZO-1 staining was omitted and the inner part of the cells was defined as the region of interest. To look at the distribution of the tight junction, the mean intensity for Alexa-Fluor 568 pixels in the cells thresholded with ZO-1 was considered. Three-dimensional views of the $z$-series were obtained by using the projection function, which calculated a series of projections after the rotation of the data package around the $y$-axis. The separate profile for each channel along a three-dimensional view was obtained by using the Profile function. Two-dimensional deconvolution views of the $yz$- and $xz$-plane were generated by using the ortho function. All images were compiled by using Adobe Photoshop software (version 7.0; Adobe Systems, Mountain View, CA).

RESULTS

FITC-Tf was colocalized with Rab5 and Rab11 in Caco-2 cells pulsed at 16°C and 37°C, respectively. The transport of endocytosed synaptic vesicle proteins to the perinuclear region has been shown to be inhibited at temperatures lower than 20°C (8). To study the effect of the temperature on the intracellular location of the internalized Tf in Caco-2 cells, the cells were pulsed at the apical plasma membrane with FITC-Tf for 30 min at two different temperatures: 16°C and 37°C. The surface-bound FITC-Tf was removed by sequential neutral and acid washes, and the cells were then labeled with antibodies against Rab5 or Rab11. The acid washes contributed to the removal of FITC-Tf, which may bind to microvilli, from the apical cell surface, which have a length of 1.32 μm (data not shown). The average short and long microvilli in C2BBe1 subclone cells were reported to be 0.6 μm and 1.6 μm, respectively (35).

Consistent with the previous observations showing the effect of the temperature on the accumulation of Tf in different endosomal compartments, FITC-Tf showed a high level of colocalization with Rab5 in the cells pulsed at 16°C. Galleries of the optical sections from the apical to the basolateral plasma membrane of the cells are presented in Fig. 2, A and B, and in Fig. 3, A and B. The three-dimensional views of these galleries were obtained by using the projection function (Fig. 2, C and D, and Fig. 3, C and D). Comparing the galleries of the optical

![Fig. 2](http://apcell.physiology.org/)

**Fig. 2.** A high extent of colocalization between FITC-Tf and Rab5 was observed in Caco-2 cells pulsed at 16°C. Caco-2 cells pulsed with FITC-Tf (green) at 16°C and 37°C for 30 min were immunolabeled with primary and fluorescent secondary antibodies against Rab5 (red). The nucleus was labeled by using 4',6-diamidino-2-phenylindole (DAPI; blue). Images selected from a series of optical sections from the apical toward the basolateral membrane of the cells pulsed at 16°C (A) and 37°C (B) are shown. The three-dimensional (3D) views of the cells pulsed at 16°C (C) and 37°C (D) are presented. The apically peripheral distribution of FITC-Tf is shown in the 3D view of the cells pulsed at 16°C (arrow). E: the average colocalization coefficient (CC) of FITC-Tf colocalized with Rab5 at different heights representing the distance from the nucleus (N) of the cells is shown. The plotted $z$-sections are within the optical $z$-series localized from −2.0 μm to 8.8 μm relative to the $z$-section containing the first sign of nucleus labeling (arrow) and toward the apical cell membrane. Error bars show SEs of an $n = 15$ ($n$ corresponds to the number of image fields per slide). BLM, basolateral membrane; APM, apical membrane.
sections of the cells pulsed at 16°C and 37°C, we found FITC-Tf colocalized with Rab5 to a high degree in the first three z-sections of the gallery of the cells pulsed at 16°C (Fig. 2, A and B), which is more noticeable in one of the cells presented in this gallery. These z-sections are localized about 4.6 μm to 6.7 μm above the intracellular level at which the nucleus begins to appear. An apically peripheral distribution of FITC-Tf, which was absent in the cells pulsed at 37°C, is shown in the three-dimensional view of the gallery of the Caco-2 cells pulsed at 16°C and immunolabeled with the antibody against Rab5 (Fig. 2C). On the other hand, a high degree of colocalization between FITC-Tf and Rab11 was observed in the cells pulsed with FITC-Tf at 37°C. As shown in Fig. 3B, FITC-Tf was colocalized with Rab11 in the z-sections localized only 2 μm to 3 μm above the nucleus of the cells pulsed at 37°C. The three-dimensional views of the galleries confirm also the FITC-Tf presence above the nucleus in the cells pulsed at 37°C (Figs. 2D and 3D). The colocalization between FITC-Tf and Rab11 in Caco-2 cells pulsed at 37°C is shown in Fig. 3D.

The average colocalization coefficient of the FITC-Tf colocalized with the Rab proteins, which was obtained by analyzing the images collected from different fields of the prepared slides, was plotted versus the z-sections arranged on the basis of their distance from the nucleus toward the apical surface of the cells (Figs. 2E and 3E). In the collected z-series, the first z-section from the apical surface in which the nucleus was first observed was set to zero, negative numbers move toward the basolateral membrane, whereas positive numbers move toward the apical membrane. Consistent with the results obtained from the galleries of the optical sections of the cells, the highest degree of the colocalization between FITC-Tf and Rab5 was found close to the apical membrane of the cells pulsed at 16°C. This was determined in ~70% of the fields analyzed in the z-sections localized from 6.4 μm to 7.2 μm above the z-section containing the first sign of nucleus labeling (arrow), and toward the apical cell membrane. FITC-Tf was colocalized with Rab5 and Rab11 in two different intracellular locations above the tight junction. To determine the intracellular location of the z-sections con-
taining the highest level of the colocalization between FITC-Tf and the Rab proteins, the tight junction was labeled by using antibody against ZO-1. Staining of ZO-1 allowed us to follow the FITC-Tf distribution in single cells, and it also eliminated the effect of the uneven filter membrane and different cell height.

Galleries of the optical sections from the apical to the basolateral plasma membrane of the cells pulsed at 16°C and 37°C are shown in Figs. 4A and 5A. Consistent with the results obtained from the cells without any tight junctional labeling, the intracellular location of the FITC-Tf colocalized with Rab5 and Rab11 is different in the cells pulsed at 16°C and 37°C (Figs. 4A and 5A). In the cells pulsed at 16°C, a high level of colocalization between FITC-Tf and Rab5 was observed in the z-sections localized apically and distal from the tight junction. On the other hand, FITC-Tf was colocalized with Rab11 in the z-sections localized above the tight junction in the cells pulsed at 37°C. The intracellular location of the FITC-Tf colocalized with the Rab proteins relative to the tight junction is also shown in the two-dimensional deconvolution views of the yz- and xz-plane (Figs. 4B and 5B). These results were also confirmed by analyzing the distribution of FITC-Tf, the Rab proteins, and the ZO-1 along the three-dimensional views of the galleries (Figs. 4C and 5C). The distribution profile for each channel was obtained by using the Profile function. At 16°C, the green and red peaks that represent FITC-Tf and Rab5 overlapped far from the peaks representing the ZO-1 and the nucleus (Fig. 4C). On the other hand, an overlap between the peaks representing FITC-Tf and Rab11 was observed adjacent to the ZO-1 in the cell pulsed at 37°C (Fig. 5C). The animated

![Fig. 4.](http://ajpcell.physiology.org/) Colocalization between FITC-Tf and Rab5 is higher in the z-sections close to the apical membrane of the cells. Caco-2 cells pulsed with FITC-Tf (green) at 16°C and 37°C for 30 min were immunolabeled with appropriate primary and fluorescent secondary antibodies against Rab5 and ZO-1 (both red). The nucleus was labeled by using DAPI (blue). A: images between the apical membrane and the tight junction selected from a series of optical sections of a single cell pulsed at 16°C are shown. B: the ortho image from the optical section no. 4 of the single cell is shown. C: the distribution profiles for FITC-Tf, Rab5, and ZO-1 pixels along the 3D projection of the single cell are presented. D: the average colocalization coefficient of FITC-Tf and the average mean intensity (MI) of both Rab5 and ZO-1 pixels at different heights, representing the distance from the tight junction of the cells pulsed at 16°C and 37°C, are shown. The z-sections containing the tight junctional labeling are shown (arrows). The plotted z-sections are within the optical z-series localized from -2.0 μm to 8.4 μm relative to the z-section containing the first sign of tight junctional labeling, and toward the apical cell membrane. Error bars show SEs of n = 13–18 (n corresponds to the number of cells analyzed per slide).
three-dimensional projections are also available in the online version of this article (supplemental movies 1 and 2).

The z sections arranged on the basis of their distance from the tight junction were plotted versus both the average mean intensity of the red pixels and the average colocalization coefficient of the FITC-Tf, which were obtained by analyzing a number of single cells. After adjustment of the level of the collected z-sections relative to the tight junction, the first z-section containing the highest mean intensity of the red pixels was set to zero. The distribution of the red pixels through the cells is shown (Figs. 4D and 5D), which represent both the Rab labeling and the ZO-1 labeling, and the peak of the graph correlates with the presence of the tight junction in the particular z-sections in the cells as detected in the images collected by confocal microscope. The average thickness of the z-sections containing ZO-1 staining shown in Figs. 4D and 5D was 1.22 μm. Staining of ZO-1 did not affect the observed colocalization between FITC-Tf and the Rab proteins (Figs. 2E, 3E, 4D, and 5D). The highest level of colocalization between FITC-Tf and Rab5 at 16°C was observed in the z-sections with an average distance of 6 μm above the tight junction (Fig. 4, A and D). On the other hand, the z-sections localized about 2.0 to 2.8 μm above the tight junction contained the highest degree of the colocalization between FITC-Tf and Rab11 at 37°C (Fig. 5, A and D). The distribution of Rab5 and Rab11 was not affected by the temperature (data not shown).

The effect of AG10 on transcytosis, recycling, and cell association of Tf was observed only in the cells pulsed at 37°C. The recycling, transcytosis, and cell association of Tf was measured in the presence and absence of AG10. As shown in Caco-2 cells, which were pulsed with 125I-labeled Tf at 16°C, the recycling and transcytosis of Tf were not affected by the treatment with AG10, but the cells pulsed at 37°C showed a statistically significant increase in the recycling and transcytosis of Tf in the presence of AG10 (Fig. 6, A and B). The cell
AG10 did not show any significant effect on recycling, transcytosis, and cell association of Tf at 16°C. No significant changes in recycling (A), transcytosis (B), and cell association of Tf (C) were observed in AG10 (500 μM)-treated Caco-2 cells that were pulsed for 30 min at 16°C and chased for 30 min at 37°C. On the other hand, recycling and transcytosis of Tf increased and cell-associated Tf decreased in the presence of AG10 in the cells pulsed at 37°C. Dotted line with triangles indicates control cells pulsed at 37°C; dotted line with squares indicates AG10-treated cells pulsed at 37°C; solid line with triangles indicates control cells pulsed at 16°C; solid line with squares indicates AG10-treated cells pulsed at 16°C. One of three similar experiments is shown. Error bars show SEs of an n = 3.

AG10 did not show any effect on the integrity of tight junction. The integrity of the tight junction was tested by measuring TEER. Consistent with a published report (59), treatment with AG10 up to 6 h had no effect on the TEER, i.e., 100 ohms/cm² in both treated and control Caco-2 cell mono-

AG10 increased colocalization between FITC-Tf and Rab11. To define the involvement of Rab11 in the effect of AG10 on Tf distribution in Caco-2 cells, the cells were pulsed with FITC-Tf at the apical membrane in the presence or absence of AG10 (500 μM) for 30 min at 37°C, followed by a chase in the continued presence or absence of AG10 for 30 min at 37°C. The galleries of the optical sections from the apical toward the basolateral cell membrane of the nontreated and AG10-treated Caco-2 cells are shown in Fig. 9, A and B. In the z-sections located above the nucleus, a higher degree of colocalization between FITC-Tf and Rab11 was observed in the cells treated with AG10 than in nontreated cells (Fig. 9, A and B). Both colocalization coefficient and the mean intensity of FITC-Tf obtained from the average of three different experiments were plotted versus the z-sections arranged on the basis of their distance from the nucleus (Fig. 9, C and D). The intracellular level at which the nucleus begins to appear was determined by

Fig. 6. AG10 did not show any significant effect on recycling, transcytosis, and cell association of Tf at 16°C. No significant changes in recycling (A), transcytosis (B), and cell association of Tf (C) were observed in AG10 (500 μM)-treated Caco-2 cells that were pulsed for 30 min at 16°C and chased for 30 min at 37°C. On the other hand, recycling and transcytosis of Tf increased and cell-associated Tf decreased in the presence of AG10 in the cells pulsed at 37°C. Dotted line with triangles indicates control cells pulsed at 37°C; dotted line with squares indicates AG10-treated cells pulsed at 37°C; solid line with triangles indicates control cells pulsed at 16°C; solid line with squares indicates AG10-treated cells pulsed at 16°C. One of three similar experiments is shown. Error bars show SEs of an n = 3.

Fig. 7. AG10 decreased the uptake of Tf at 37°C but did not show any effect on Tf uptake at 16°C. Caco-2 cells were pulsed with 125I-labeled Tf at 37°C and 16°C with and without AG10 (500 μM) were then chased for 10 or 30 min at 37°C. At the end of the chase period, the surface-bound Tf was removed by using an acid-wash solution, and the radioactivity [in counts per minute (cpm)] in the acid-wash fractions and cell lysis was counted. In the presence of AG10, Tf uptake decreased significantly in the cells pulsed at 37°C (A). AG10 did not show any effect on Tf uptake in cells pulsed at 16°C (B). The amount of surface-bound Tf was not affected in the cells pulsed at 16°C and 37°C (B). Solid bar, control cells pulsed at 37°C; dark shaded bar, AG10-treated cells pulsed at 37°C; light shaded bar, control cells pulsed at 16°C; open bar, AG10-treated cells pulsed at 16°C. Error bars show SEs of an n = 3.
AG10 (500 μM) are shown. The 3D views of the cells in the absence (B) and with AG10 (E) are labeled by using DAPI. Images selected from a series of optical sections from the apical toward the basolateral plasma membrane of the cells incubated without (A) and with AG10 (B) are shown. The 3D views of the cells in the absence (C) and presence of AG10 (D) are presented. One of two similar experiments is shown. Con, control.

To further study the effect of AG10 on the Tf trafficking, Caco-2 cells were pulsed for a shorter period of time. It has been shown that Tf traverses through endosomes containing Rab5, Rab5/Rab4, and Rab4/Rab11 in A341 cells (43). Therefore, Caco-2 cells were pulsed with FITC-Tf in the absence or presence of AG10 (500 μM) for 2 min at 37°C, followed by a chase of 2 min at 37°C in the continued absence or presence of AG10. Optical sections, selected from the galleries of the nontreated and AG10-treated Caco-2 cells pulsed at 37°C for 2 min and chased for 2 min, are shown in Fig. 10, A–D. The z-sections are localized at 2 μm above the tight junction of the cells. The average colocalization coefficient of FITC-Tf was plotted versus the z-sections arranged on the basis of their distance from the tight junction (Fig. 10E). Interestingly, a high level of colocalization between FITC-Tf and Rab5 was observed in Caco-2 cells. About 60% of the internalized FITC-Tf was colocalized with Rab5 in the z-section localized at 3.2 μm distal from the tight junction (Fig. 10E). Consistent with the results obtained in the cells pulsed with FITC-Tf at 37°C for 30 min with no chase, the highest level of colocalization between FITC-Tf and Rab11 was observed in the z-sections that are located about 2.4 μm to 2.8 μm above the tight junction (Fig. 10E). However, FITC-Tf was colocalized with Rab5 to a higher extent than with Rab11. As we have already shown, the colocalization between FITC-Tf and Rab11 increased in the presence of AG10 (500 μM) (Fig. 9). In the z-sections localized about 1.2 μm to 2.0 μm above the tight junction, a 25% increase in the extent of the colocalization between FITC-Tf and Rab11 was observed (Fig. 10E). On the other hand, AG10 did not show any effect on the colocalization between FITC-Tf and Rab5 in Caco-2 cells pulsed for 2 min at 37°C and chased for 2 min (Fig. 10, C–E). The average mean intensity of Rab11 plotted versus the z-sections is also shown in 10E. AG10 did not show any significant effect on the distribution pattern of the Rab11 in the apically located z-sections, specifically, z-sections localized 2.0 to 2.8 μm above the tight junction of Caco-2 cells.

We have been interested in using AG10 to increase the oral delivery of protein and peptides via TIR-mediated transcytosis in intestinal epithelial cells (42). Therefore, in the above experiments, we focused on the trafficking pathways for Tf internalized at the apical cell membrane. However, we find it necessary to study the intracellular pathways of the Tf internalized at the basolateral cell membrane and to also determine the effect of AG10 on the trafficking of the basolaterally endocytosed Tf. Therefore, Caco-2 cells were pulsed with FITC-Tf at the basolateral cell membrane in the absence or presence of AG10 (500 μM) for 30 min at 37°C, and they were then labeled with antibodies against Rab11. To determine the exact location of FITC-Tf, the cells were labeled with antibody against ZO-1. The nucleus was also labeled by using DAPI. Some optical sections, obtained from the galleries of the nontreated and AG10-treated Caco-2 cells, from the apical toward the basolateral membrane of the cells are shown in Fig. 11. In the cells in which FITC-Tf was endocytosed at the basolateral membrane, the FITC-Tf was mainly found in the z-sections localized basolaterally and under the intracellular level at which the tight junction was localized (Fig. 11A). However, in the presence of AG10, the basolaterally endocytosed FITC-Tf had access to the z-sections localized apically and above the tight junction (Fig. 11B). The individual channels, green and red channels, of the images which were shown in Fig. 11, A and B, were collected from the intracellular level localized above the tight junction of the cell; the superimposed images are shown in Fig. 11, C and D. In the control cell monolayer, the display for the green channel did not show the presence of FITC-Tf in the z-section localized ~1.0 μm above the intracellular level at which ZO-1 begins to appear (Fig. 11C). On the other hand, we observed that the FITC-Tf was colocalized with Rab11 in the z-section located ~1.6 μm above the tight junction (Fig. 11D, arrows). As shown in Fig. 11E, the average colocalization coefficient of the FITC-Tf increased significantly in the z-sections localized apically and above the tight junction. FITC-Tf was detected in the z-sections localized about 2.0 μm to 2.4 μm above the tight junction, only in the cells that have been treated with AG10 (Fig. 11F).

**DISCUSSION**

The effect of the temperature on the intracellular trafficking of Tf, as well as its colocalization with the Rab proteins, was studied in cultured Caco-2 cells. Consistent with the published reports regarding the effect of the low temperatures below 20°C on intracellular trafficking of the endocytosed ligands (8), we observed that approximately 50–60% of FITC-Tf labeling was colocalized with Rab5 in the apically located z-sections of the cells pulsed at 16°C (Table 1 and Fig. 2E). The transfer of Tf to the late endosomal compartments that contain Rab11 was
blocked (Fig. 1, pathway 3), as evidenced by the failure to observe marked colocalization of these two markers under these conditions. Consequently, the recycling and transcytosis of Tf occurred mostly from endosomal compartments that contain Rab5 (Fig. 1, pathway 2). As expected, Rab5 showed a vesicular staining, and the endosomal compartment containing Rab5, which was accessible to FITC-Tf bound to the apical surface of the cells, was localized close to the apical membrane (Fig. 4, A–D). This observation indicates the presence of FITC-Tf in the AEEs. The peripheral colocalization between FITC-Tf and Rab5, which was observed through the cytoplasm, may depend on the role of Rab5 in the vesicular transport among surface membranes and the endosomal compartments (Table 1). A population of Rab5 observed in the level of the tight junction was not accessible to the FITC-Tf bound to the apical or the basolateral surface of the cells (data not shown).

On the other hand, FITC-Tf showed a higher level of colocalization with Rab11 in the cells which were pulsed at 37°C (Fig. 3, A–E). This observation indicated the transfer of FITC-Tf from Rab5-containing endosomal compartments to a Rab11-containing endosomal compartment in the cells pulsed at 37°C (Fig. 1, pathway 3). The existence of a population of cup-shaped vesicles containing Rab11 and Rab25, which constitute the ARE, has been reported in the apical region of MDCK cells (5, 12). We found Rab11 in the vesicles in the apical region of Caco-2 cells and above the ZO-1 staining, which could be analogous to the ARE in MDCK cells (Fig. 4, A–D). Studies in MDCK cells and rat hepatocytes suggested the subdivision of a single endosomal compartment (subapical compartment) into CE and ARE (9, 51). The involvement of Rab11 in the recycling of internalized Tf has been shown in nonpolarized cells (Table 1). Ren et al. (37) reported the inhibition of Tf recycling from perinuclear recycling endosomes in TRVb cells expressing the dominant-positive (Q70L) or dominant-negative (S25N) mutant forms of Rab11.

Tyrphostins are a group of low-molecular-weight organic compounds that have been used to specifically inhibit the GTPase activity of protein tyrosine (10, 23, 24, 61). The effect of AG10 (tyrphostin A8) on the transport of insulin-Tf conjugate has already been shown. The TfR-mediated transcytosis of insulin-Tf increased by 20-fold in AG10-treated Caco-2 cells (59). An enhancement in the hypoglycemic effect of orally administered insulin-Tf conjugate was also reported in diabetic rats (59).

To investigate the effect of AG10 on intracellular processing of Tf among the endosomal compartments, we made use of the observation that endocytosed Tf accumulates in the endosomal compartments containing Rab5 and Rab11 at 16°C and 37°C,
respectively (Table 1 and Figs. 2–5). The cells were pulsed with 125I-labeled Tf at two different temperatures, and, consistent with the results obtained from confocal microscopy, the passage of the endocytosed Tf from endosomal compartments containing Rab5 to those containing Rab11 was blocked at 16°C and the majority of Tf (~90%) was chased out of the cells within the first 10 min (Fig. 1, pathway 2, and Fig. 6A). As shown in Fig. 6A, in the cells pulsed with 125I-labeled Tf at 37°C, the recycling of Tf followed the criteria of the slow-phase recycling, i.e., an average of only 20% of Tf was recycled in the first 10 min (Fig. 1, pathway 4). The effect of AG10 was pronounced at 37°C, and the results obtained from pulse-chase and uptake assays suggested that AG10 affects the late process of Tf transport, after the exit of Tf from endosomal compartments containing Rab5. The significant increase in the apical-to-basolateral transcytosis of Tf (Fig. 6B) in the presence of AG10 may explain the effect of AG10 on the transport of insulin-Tf conjugate in Caco-2 cells, as previously reported (58). The increase in the mean intensity of the apically endocytosed FITC-Tf, which was observed in the more basolateral regions of the Caco-2 cells treated with AG10 (Fig. 9D), may also be explained by the effect of AG10 on TIR-mediated transcytosis. The amount of cell-associated Tf decreased by an average of ~40% after a 30-min chase in the presence of AG10 (Fig. 6C). As shown in Fig. 7A, AG10 decreased the uptake of Tf by 15% after a 10-min chase, which can be explained by the increase in the amount of recycled and transcytosed Tf in Caco-2 cells. From the uptake assay in the presence and absence of AG10, there was no significant difference in the amount of surface-bound Tf, as shown in the radioactivity recovered in the acid-wash fractions (Fig. 7B). Since a high level of the cellular uptake of Tf at 37°C has been shown in many cell types and the existence of an internal TIR pool has been suggested (21, 26), a potential effect of AG10 on the TIR internal pool can be considered. In the presence of AG10, the internalized Tf-TIR complexes may be depleted from Caco-2 cells resulting in an increase in both recycling and transcytosis of Tf. The effect of AG10 on the late processes of Tf trafficking was confirmed by the demonstration of a higher degree of colocalization between FITC-Tf and Rab11 in the presence of AG10 (Fig. 9), suggesting the involvement of Rab11 in the recycling of apically endocytosed Tf at 37°C and possibly explaining the effect of AG10 on apical recycling of Tf (Fig. 6A). In the presence of AG10, Tf was transported to a higher degree in the endosomal compartments containing Rab11 before the apical delivery. AG10 may increase the transport of Tf.
Tf-TfR complexes from intracellular compartments containing the internalized Tf at 37°C, which are localized before the Rab11-containing endosomal compartment, to the endosomal compartments from which transcytosis and recycling of Tf occur. This may explain as well the observed high colocalization between the apically endocytosed FITC-Tf and Rab11 in the presence of AG10 (Fig. 9C). An overloading of these endosomal compartments may potentially result in an increase in both transcytosis and recycling of Tf.

To further investigate the effect of the duration of the pulse period on the colocalization between the apically endocytosed FITC-Tf and Rab proteins, Caco-2 cells were pulsed with FITC-Tf in the absence or presence of AG10 (500 μM) for 2 min at 37°C and were then chased in the continued absence or presence of AG10 for 2 min. As shown in Fig. 10, FITC-Tf was colocalized with Rab5 in a higher extent than with Rab11. However, the highest level of the colocalization between FITC-Tf and Rab5 was observed in Caco-2 cells pulsed at 37°C for 2 min without any chase (data not shown). The presence of the Rab5 in the z-sections localized above the tight junction indicates that Rab5 leaves AEE in the cells pulsed at 37°C. This may be due to the involvement of Rab5 in the vesicular transport between AEE and the endosomal compartments that are involved in the later steps of Tf trafficking. At 37°C, pathway 3 is not blocked, and Rab5 may be involved in the transport of vesicles containing FITC-Tf out of AEE and toward CE and ARE. It may also be explained by the existence of a subcompartmental structure for the endosomes. A 19% overlap between Rab5 and Rab11 has been reported in A431 cells (43). A small population of Rab5 may be localized in the ARE but not at the same domain as Rab11. Most important, AG10 did not show any effect on the colocalization between Tf-TfR complexes from intracellular compartments containing the internalized Tf at 37°C, which are localized before the Rab11-containing endosomal compartment. Caco-2 cells were pulsed with FITC-Tf (green) at the basolateral membrane for 30 min at 37°C in the absence or presence of AG10 (500 μM), which was followed by the removal of the unbound and surface-bound FITC-Tf by multiple PBS and acid washes. The cells were immunolabeled with primary and fluorescent secondary antibodies against Rab11 and ZO-1 (both red). The nucleus (blue) was labeled by using DAPI. Images selected from a series of optical sections from the apical toward the basolateral membrane of the cells pulsed with FITC-Tf in the absence (A) and the presence of AG10 (B) are shown. Displays for the individual channels, green and red channels, and also for the superimposed images of the z-sections localized above the tight junction of the nontreated cell (C) and AG10-treated cell (D) are shown. The colocalization coefficient of the FITC-Tf colocalized with Rab11 (E) and the mean intensity of the FITC-Tf (F) at different heights, representing the distance from the tight junction of the cells, are shown. The plotted z-sections are within the optical z-series localized from 0.0 μm to 2.4 μm relative to the tight junction and toward the apical cell membrane. Error bars show SEs of an n = 12 (n corresponds to the number of cells analyzed per slide).
FITC-Tf and Rab5 (Fig. 10, C–E). Consistent with previous results (Fig. 9), AG10 increased the colocalization between FITC-Tf and Rab11 by 20% (Fig. 10, A, B, and E). This confirms the effect of AG10 on the trafficking of the apically endocytosed Tf in which Rab11 is involved. AG10 did not show any effect on the distribution pattern of Rab11 in Caco-2 cells. This was confirmed by quantitative analysis of the mean intensity of Rab11 through the optical sections from the apical to the basolateral side of the cells (Fig. 10E). A condensed Rab11-containing endosomal compartment, which was observed in the AG10-treated cells, was also found in the control cells. However, the effect of AG10 on Rab11 protein needs further investigation.

As shown in the present study, the apically endocytosed FITC-Tf was colocalized with Rab11 at 37°C in Caco-2 cells (Table 1; Fig. 3, B, D, and E; and Fig. 5, A–D). However, others have reported that there was no colocalization between the basolaterally endocytosed Tf and Rab11 in MDCK cells (3, 53). Therefore, the Tf trafficking endocytosed from the basolateral membrane in Caco-2 cells was further investigated in the absence or presence of AG10. Consistent with the reported observations by Brown et al. (3) in MDCK cells, the basolaterally endocytosed FITC-Tf did not reach the Rab11-containing endosomal compartment localized above the tight junction of Caco-2 cells (Fig. 11). This finding indicates the existence of different intracellular pathways for the apically and basolaterally endocytosed Tf in Caco-2 cells. The majority of the basolaterally endocytosed Tf recycles back to the basolateral cell membrane after the exit from CE (Fig. 1, pathway 8) and does not have access to the Rab11-containing endosomal compartment above the tight junction. On the other hand, the apically endocytosed Tf, after the exit from CE, moves toward the Rab11-containing endosomal compartment and reaches the apical membrane through the pathway 4 (Fig. 1). Interestingly, in AG10-treated Caco-2 cells, a fraction of the basolaterally endocytosed FITC-Tf was transported to the apically localized z-sections above the tight junction (Fig. 11B). In the presence of AG10, the basolaterally endocytosed FITC-Tf was colocalized with Rab11, which, as expected, was localized in the z-sections above the tight junction (Fig. 11, D and E). The pulse-chase experiment also showed that the effect of AG10 on 125I-labeled Tf trafficking is not limited to the apically endocytosed Tf. AG10 increases the recycling and also the basolateral-to-apical transcytosis of the basolaterally endocytosed Tf in Caco-2 cells (F. Norouziyan, unpublished observations). The observed colocalization between FITC-Tf and Rab11 may be due to the effect of AG10 on the basolateral-to-apical transcytosis of Tf. Interestingly, the basolaterally endocytosed Tf may reach the apical membrane in the presence of AG10 through pathway 4, which normally is accessible only to the apically endocytosed Tf.

In summary, in the present study, we took advantage of the effect of temperature on the colocalization between the apically endocytosed Tf and Rab proteins, Rab5 and Rab11, to study the effect of AG10 on the intracellular trafficking of Tf. The effect of AG10 on Tf intracellular trafficking was observed only at 37°C, but not 16°C, which indicates that the effect of AG10 on Tf trafficking occurred after the exit of Tf from the AEE. We found that, in Caco-2 cells, the apically endocytosed Tf crosses the Rab11-containing endosomal compartment after the exit from the CE on its way toward the apical membrane. The transport of the apically endocytosed Tf to the Rab11-containing endosomal compartment was stimulated in AG10-treated Caco-2 cells, which may be due to the involvement of Rab11 in the apical recycling of Tf. By shortening the pulse period to 2 min at 37°C, and also a chase of 2 min, a colocalization between Rab5 and the apically endocytosed FITC-Tf was observed above the tight junction. This may be due to the involvement of Rab5 in the transport of the vesicles containing Tf or the existence of a subcompartmental structure for the Rab5 and Rab11-containing endosomes. The level of the colocalization between Rab5 and FITC-Tf was not affected in the presence of AG10. The existence of different intracellular pathways for the apically and basolaterally endocytosed Tf was shown in Caco-2 cells. The basolaterally endocytosed Tf did not have access to the Rab11-endosomal compartment. Interestingly, in the presence of AG10, FITC-Tf was found in the apically located z-sections and was further colocalized with Rab11. This may be explained by the effect of AG10 on the transcytosis of the basolaterally endocytosed Tf, which may reach the apical membrane also through pathway 4.

Tyrophostins specifically inhibit the GTPase activity of protein tyrosine kinases (10, 23, 24, 61), and AG10 was also reported to inhibit the GTPase activity of transducin and calcineurin (25, 56). The ability of tyrophostins to inhibit the GTP-utilizing enzymes raises the possibility of their inhibitory effect on GTPase proteins such as Rab proteins. The involvement of Rab proteins in the effect of AG10 on Tf intracellular trafficking needs further investigation.

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