Apotransferrin and Holotransferrin Undergo Different Endocytic Cycles in Intestinal Epithelia (Caco-2) Cells

(Received for publication, January 24, 1997, and in revised form, April 7, 1997)

Marco Tulio Núñez, Claudia Núñez-Millacura, Marianela Beltrán, Victoria Tapia, and Xavier Alvarez-Hernandez

From Departamento de Biología, Facultad de Ciencias, Universidad de Chile, Casilla 653, Santiago, Chile and §Department of Medicine, Louisiana State University Medical Center at Shreveport, Shreveport, Louisiana 71130

Experiments have demonstrated that diferric transferrin and apotransferrin compete for the binding to basolateral transferrin receptors and that transferrin-mediated iron uptake by Caco-2 cells is inhibited by apotransferrin to a larger extent than that predicted solely by receptor competition. This inhibition can have important implications in determining the net exchange of iron between intestinal cells and the basolateral milieu. Accordingly, we further characterized the endocytic cycles of apotransferrin and diferric transferrin in Caco-2 cells. We found that after internalization both apotransferrin and diferric transferrin recycled to the cell exterior, but that apotransferrin had a protracted endocytic cycle. Confocal microscopy studies revealed a different cellular distribution of apotransferrin and diferric transferrin; both were found in a compartment close to the basal membrane, but apotransferrin reached as well regions closer to the apical membrane. Moreover, the intracellular distribution of transferrin receptors was influenced by the iron load of transferrin; cells incubated with apotransferrin presented a more apical distribution of transferrin receptors than cells incubated with diferric transferrin. These results indicate for the first time that the endocytic cycle of transferrin receptors in intestinal epithelial cells is determined by the iron content of transferrin. They explain also the marked inhibitory effect of apotransferrin on transferrin-mediated iron uptake by Caco-2 cells, since incubation of cells with apotransferrin resulted in the actual sequestration of the receptor in the cell interior.

Iron is an essential nutrient involved in cellular functions related to the binding and transport of oxygen, redox reactions, detoxification, and nucleotide synthesis (1). Excess iron is toxic, and healthy individuals maintain a balance between obligatory body iron losses and iron absorption (reviewed in Ref. 2). The mechanisms that establish body iron homeostasis are, therefore, of great importance. Although the mechanisms involved in the regulation of iron absorption are not well known, a model describing the sequential passage of iron from mucin to integrin and to mobilferin gives important clues on the molecular components involved in iron absorption (3). Using Caco-2 cells grown in bicameral inserts as a model of intestinal epithelia, it was shown that iron uptake through the basolateral endocytosis of iron-containing transferrin (Tf)alsa contributes importantly to the overall content of intracellular iron (4) and that the extent of apical iron uptake is inversely related to this content (5, 6).

The endocytic cycle of Tf has been thoroughly characterized in a variety of cells (reviewed in Ref. 7). In many cell types internalized Tf first reaches the sorting endosome (pH 6.2) located in the peripheral cytoplasm, then moves to the recycling endosome (pH 6.4) and from there back to the cell surface (8). To our knowledge, the endocytic cycle of apoTf has not been characterized. Using apoTf as ligand, an apical endocytic compartment, where basolateral internalized apoTf colocalized with an apical fluid face marker, was described (9). These findings were not complemented with studies using holoTf, to determine if both ligands have a different intracellular distribution, and the rationale for the use of apoTf was not given (9).

There is a relationship between apoTf and iron flux in intestinal epithelia cells, since basolateral apoTf increases the apical to basolateral iron flux in iron-deficient Caco-2 cells (10). Furthermore, the binding of iron-containing Tfs to Tf receptors was competitively inhibited by apoTf, and apoTf inhibited Tf-mediated iron uptake in Caco-2 cells (4). This inhibition was larger than expected solely by receptor competition, suggesting that apoTf affects other step(s) in the iron uptake cycle. From the above, it follows that the ratio apoTf/hoToTf in the basal medium should determine the amount of cellular iron in intestinal epithelia cells, which in turn could determine the extent of body iron absorption. In this study we decided to further investigate the behavior of both Tf and the Tf receptor when Tf was internalized in the apo or in the iron-containing form. We found that both diFeTf and apoTf recycled to the cell exterior, but apoTf had a protracted endocytic cycle. Confocal microscopy studies revealed that diFeTf and apoTf reached different intracellular compartments. Moreover, Tf receptors also carried an endocytic cycle that was dependent on whether the ligand was apoTf or diFeTf. These results indicate for the first time that the endocytic cycle of the Tf receptor in intestinal epithelia cells depends on its bound ligand.

EXPERIMENTAL PROCEDURES

Cells—Caco-2 cells, from the American Type Culture Collection (no. HTB37, Rockville, MD), were cultured in Dulbecco’s minimal essential medium (Life Technologies, Inc., catalog no. 430-2100EC) supplemented with 10% fetal bovine serum (Sigma). Culture medium was changed every 3 days. For transport and binding experiments, cells were grown on 0.33-cm² polycarbonate cell culture inserts, with 0.4-

This paper is available on line at http://www.jbc.org

1 The abbreviations used are: Tf, transferrin; FITC, fluorescein isothiocyanate; diFeTf-FITC, diferric transferrin labeled with fluorescein isothiocyanate; RITC, rhodamine isothiocyanate; apoTf-RITC, apoTf labeled with rhodamine isothiocyanate; MOPS, 3-(N-morpholino)propanesulfonic acid.
Basolateral Endocytosis of Transferrin by Caco-2 Cells

Figure 1. Endocytosis of apoTf and diFeTf, pulse-chase kinetics. Insert-grown Caco-2 cells were pulsed for 15 min at 37 °C with 125I-labeled apoTf or 125I-labeled diFeTf, followed by a chase with unlabeled apoTf or diFeTf. At the times shown, the 125I radioactivity remaining in the cell was determined. Shown is one of three similar experiments.

Figure 2. Colocalization of apoTf and diFeTf in Caco-2 cells. Caco-2 cells grown in transparent inserts were simultaneously incubated from the basolateral side for 60 min at 37 °C with diFeTf-FITC and apoTf-RITC. Cells were then fixed with glutaraldehyde and examined with a confocal microscope. Optical sections were taken from the apical plate and the optical cuts from apical to basolateral.

Confocal Microscopy—Cells grown in transparent inserts (Costar) were incubated from the basolateral side for 60 min at 37 °C with saline (50 mM Na-MOPS, 94 mM NaCl, 7.4 mM KCl, 0.74 mM MgCl₂, 1.5 mM CaCl₂, pH 7.4) containing 0.1 mM deferrioxamine. Cells were then fixed with 3% paraformaldehyde, treated with 0.5% Triton X-100, and reacted with OKT9 monoclonal anti-Tf receptor antibody (the kind gift of Dr. A. Orellana). Second antibody was goat anti-mouse IgG1 (Sigma) labeled with RITC. Fluorescence was determined in the confocal microscope as described above.

Quantification of Light Intensity—Fluorescence intensity due to Tf or apoTf-RITC was measured and analyzed in the confocal microscope using the software provided with the microscope.
were divided into 10 equal sections following the basal to apical axis, and the relative intensity of the bands was determined using the SigmaScan program (Jandel Scientific, San Rafael, CA).

Data Analysis—Curve fitting was done using the GraphPad Prism program (GraphPad Software Inc., San Diego, CA). The experiments shown were repeated two to four times. Variability between experiments was 10%.

RESULTS

Endocytosis of ApoTf and DiFeTf, Pulse-Chase Kinetics—We recently reported that iron uptake from 0.1 mM diFeTf was 65% inhibited by 0.5 mM apoTf, while the expected inhibition, based on receptor competition, was only 25% (4). One possibility for this extra inhibition was different cell handling of these Tfs. Hence, we determined the kinetics of the endocytic cycle of apoTf and diFeTf in Caco-2 cells (Fig. 1). Internalized diFeTf will be named “diFeTf,” although it should lose most of its iron during endocytosis (4). DiFeTf presented a characteristic endocytic cycle kinetics with a $t_{1/2}$ of about 25 min. ApoTf, instead, exhibited a protracted cycle with a $t_{1/2}$ of about 60 min (Fig. 1).

Simultaneous Subcellular Localization of Internalized ApoTf and DiFeTf—We next studied the intracellular localization of internalized apoTf and diFeTf by confocal microscopy (Fig. 2). A high degree of colocalization (yellow spots) were observed in a basal region, while in more apical regions a predominance of the pseudo-red apoTf fluorescence was observed (Fig. 2A). A gallery of optical cuts (Fig. 2B) and integration of the signal in the z axis (Fig. 2C) showed a distinctive pattern of distribution for both labels, with a preferential basal distribution for diFeTf and a more apical distribution for apoTf. Densitometric analysis indicated that diFeTf distributed mainly in the first half of the basal to apical axis, while apoTf distributed also into the second third of the axis (Fig. 3).

Effect of Hyperosmolarity on the Internalization of ApoTf and DiFeTf—We observed differences in endocytic cycle kinetics and intracellular localization of apoTf and diFeTf could be due to different mechanisms of internalization, e.g. clathrin-medi-
ated or fluid phase endocytosis. Since only clathrin-mediated endocytosis is inhibited by hyperosmolarity (14), we tested its effect on the internalization of diFeTf or apoTf (Fig. 4). Internalization rates (molecules of Tf internalized $\times h^{-1} \times \text{insert}^{-1} \times 10^{-4}$) under isotonic and hypertonic conditions were 18.2 and 8.1 for apoTf and 45.5 and 18.8 for diFeTf, respectively (Fig. 4). Hence, hyperosmolarity inhibited to the same extent the internalization of apoTf and diFeTf, although the internalization rate of diFeTf was 2.5-fold larger than the rate of apoTf internalization. These results indicate that in Caco-2 cells both apoTf and diFeTf undergo clathrin-mediated internalization, and hence are internalized bound to the Tf receptor.

**Intracellular Localization of Tf Receptors when Cells Were Incubated with apoTf or DiFeTf—**A different intracellular distribution of apoTf and diFeTf would result if one of the ligands, e.g. apoTf, dissociated from its receptor after internalization. Hence, we probed the location of Tf receptors after basolateral incubation of insert-grown Caco-2 cells with either apoTf or diFeTf. We found that, while incubation with diFeTf resulted in a receptor distribution mostly in the first half of the basal to apical axis, incubation with apoTf produced a more even distribution of receptors in this axis (Fig. 5). Thus, the distribution of Tf receptors followed closely in each case that of apoTf or diFeTf, an indication that these Tfs carried on their endocytic cycles bound to Tf receptors.

**DISCUSSION**

In its simplest form, iron absorption can be described as the regulated passage of luminal iron through the intestinal epithelium. This passage is regulated by the intracellular iron levels of intestinal epithelial cells (6), and its adequate functioning maintains body iron homeostasis. It is therefore important to advance knowledge of the mechanisms responsible for the regulation of intracellular iron concentration in intestinal epithelial cells. With this in mind, we characterized the endocytic cycle of apoTf and diFeTf in polarized Caco-2 cells.

Pulse-chase experiments indicated that internalized apoTf took twice as long as diFeTf to externalize back to the basal medium. Therefore, apoTf not only competes with diFeTf for binding to the Tf receptor (4) but it actually sequesters the Tf receptor in intracellular compartments, rendering it unavailable for iron uptake. Blood plasma has a relation of apoTf to FeTf that varies from about 2 in individuals in iron balance to 10 or more in individuals with anemia secondary to iron deficiency (16). Because of the high concentration of Tf in plasma (about 35 $\mu$M), even in conditions of iron deficiency, there should be enough FeTf in plasma to provide for a full supplement of iron to intestinal cells (4). The simple mechanism of apoTf-mediated receptor competition/sequestration described here should effectively down-regulate Tf-mediated iron uptake in these cells. Thus, enterocytes of iron-deficient individuals would have lower intracellular iron levels and hence enhanced iron absorption.

Confocal microscopy revealed differences in the distribution of internalized apoTf and diFeTf and of Tf receptors. It is puzzling why apoTf and diFeTf carry out different endocytic cycles since diFeTf delivers its iron in the process (4). The similar intracellular distribution found for Tfs and Tf receptors indicate that both apoTf and diFeTf carry on their endocytic cycles bound to their receptors. Two alternatives come to mind to explain these different distributions. It may be that after reaching a common basal compartment, e.g. the early endosome (7), the apoTf-Tf receptor complex could be targeted to an apical compartment, and from there back to the basolateral membrane. DiFeTf, on the other hand, could recycle back to the basolateral membrane directly from the basal compartment. Another possibility is that both apoTf and diFeTf pass through the apical compartment but that apoTf is retained longer than diFeTf in this compartment. Although the molecular bases that underlie this process are unknown, its functioning should help to regulate intracellular iron levels and iron absorption by intestinal cells.

In summary, we determined that apoTf and diFeTf undergo different endocytic cycles in polarized Caco-2 cells. The protracted cycle of apoTf sequesters the Tf receptor inside the cell, making it less available for iron uptake. We propose that this mechanism regulates the extent of iron uptake by making it a function of plasma iron levels, and that it is part of the mechanism that determines the net flow of iron between the intestinal cell and blood plasma.

**REFERENCES**

1. Wrighglesworth, J. M., and Baum, H. (1980) in Iron in Biochemistry and Medicine II (Jacobs, A., and Worwood, M, eds) pp. 29–86, Academic Press, New York
2. Flanagan, P. R. (1990) Acta Paediatr. Scand. Suppl. 361, 21–30
3. Conrad, M. E., and Umbreit, J. N. (1993) Am. J. Hematol. 42, 67–73
4. Nuñez, M. T., Tapia, V., and Arredondo, M. (1996) J. Nutr. 126, 2151–2158
5. Alvarez-Hernandez, X., Nichols, G. M., and Glass, J. (1991) Biochim. Biophys. Acta 110, 205–208
6. Tapia, V., Arredondo, M., and Nuñez, M. T. (1996) Am. J. Physiol. 271, 6443–6447
7. Gruenberg, J., and Maxfield, F. R. (1995) Curr. Opin. Cell Biol. 7, 552–563
8. Mayor, S., Presley, J. F., and Maxfield, F. R. (1994) J. Cell Biol. 121, 1257–1269
9. Hughson, E. J., and Hopkins, C. R. (1990) J. Cell Biol. 110, 337–348
10. Mayor, S., Presley, J. F., and Maxfield, F. R. (1994) J. Cell Biol. 121, 1257–1269
11. Young, S., Bonford, A., and Williams, R. (1984) Biochem. J. 219, 505–510
12. Balti, P. K., and Harris, W. R. (1990) Arch. Biochem. Biophys. 281, 251–256
13. McFarlane, A. S. (1963) J. Cell Biol. 21, 90
14. Heuser, J. E., and Anderson, R. G. W. (1989) J. Cell Biol. 108, 389–400
15. Jing, S., Spencer, T., Miller, K., Hopkins, C., and Troubridge, I. S. (1990) J. Cell Biol. 110, 283–294
16. Williams, J., and Moreton, K. (1980) Biochem. J. 185, 483–488