Dissection of the Asynchronous Transport of Intestinal Microvillar Hydrolases to the Cell Surface

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Abstract. Novel subcellular fractionation procedures and pulse-chase techniques were used to study the intracellular transport of the microvillar membrane hydrolases sucrase-isomaltase and dipeptidylpeptidase IV in the differentiated colon adenocarcinoma cell line Caco-2. The overall rate of transport to the cell surface was two-fold faster for dipeptidylpeptidase IV than for sucrase-isomaltase, while no significant differences were observed in transport rates from the site of complex glycosylation to the brush border. The delayed arrival of sucrase-isomaltase in the compartment where complex glycosylation occurs was only in part due to exit from the endoplasmic reticulum. A major slow-down could be ascribed to maturation in and transit of this enzyme through the Golgi apparatus. These results suggest that the observed asynchrony is due to more than one rate-limiting step along the rough endoplasmic reticulum to trans-Golgi pathway.

Materials and Methods

Cell Culture and Labeling with [35S]Methionine

Caco-2 cells were grown in Optilux petri dishes (Falcon Labsware, Oxnard, CA) as described (14) or on Millipore filters (HATF0025) in mini-Marbrook chambers (11). The cells were subcultured weekly using the tryp-
sin/EDTA method (26). Labeling with [35S]methionine was carried out with cells grown on filters 5-15 min after confuency (14). In all experiments, a pulse time of 15 min was used except for the experiments with carbonyl cyanide m-chlorophenyl hydrazone (CCCP). Pulse-chase experiments in the presence of CCCP were performed essentially according to Fries and Rothman (9) with the following modifications. The filter chambers were disassembled and the cells were pulse labeled with 250 μCi [35S]methionine for 5 min after a preincubation in methionine-free medium for 15 min. The filters were washed twice with 5 ml of ice-cold PBS containing 0.1% Triton X-100 and 0.059 g/liter MgSO4. The cells were then incubated in 5 ml of the same buffer containing 10 or 100 μM CCCP. 10 min later the cells were transferred to fresh buffer containing CCCP and returned to the 37°C incubator for 45 min before harvesting.

**Immunofluorescence, Immunolocalization of Antigens, and SDS-PAGE**

Microvillar hydrolases were localized by immunofluorescence on semithin cryosections (39) of filter grown Caco-2 cells using mAbs HB/B2/64/88 against SI and mAb HB/B3/77/42 against DPPIV, as described (14). (Na+/K+)-ATPase was localized using mAb C62.4 against the catalytic α-subunit of the dog enzyme (17) which was found to cross react with the human enzyme. Details of immunolocalization and SDS-PAGE were as described (14). SI was precipitated with a mixture of the four mAbs HB/B2/64/88, HB/B3/77/42, HB/B2/219/20, and HB/B1/69/79 directed against different epitopes, and DPPIV was precipitated with antibody HB/B3/77/42. [35S]Methionine-labeled proteins were visualized by fluorography using ENHANCE (New England Nuclear, Boston, MA). Bands on fluorograms were quantitated by using a Camag LTC Scanner II connected to a Camag SP 4290 integrator. Alternatively, [35S]methionine-labeled proteins were excised from dried gels. The gel slices were digested with H2O2 at 50°C for 5 min after a preincubation in methionine-free medium for 15 min. The filters were washed twice with 5 ml of ice-cold PBS containing 0.1 g/liter NaCl and centrifuged for 10 min at 2,000 rpm (370 g,r) in an SS34 rotor (Sorvall Instruments Div.). The supernatant was brought to exactly 30 ml with buffer B, and 4.66 ml stock isoosmotic Percoll (density of Percoll = 1.129, initial density = 1.048) was added. The Percoll gradient (see Fig. 5) was centrifuged for 41 min at 23,000 rpm (36,900 g,r) in an RC 2B centrifuge (Sorvall Instruments Div.) and the resulting gradient fractionated as follows. About 1.9 cm below the top of the gradient a sharp band was present. The position of this band was measured and the corresponding volume plus 1 ml (usually 10 ml in total) was discarded. The next 8 ml were pooled and processed for two gradients as follows. 4 g of the fraction was mixed with 2.5 g 60% (wt/wt) sucrose in 1 mM EDTA, 10 mM triethanolamine-acetic acid, pH 6.5. Metrizamide in a centrifuge tube and overlayed with 2.5 ml each of 17.5% and 11.5% (wt/wt) sucrose buffer Metrizamide and, finally, with buffer B. The gradient was run for 7 h at 23,000 rpm (70,600 g,r) at 8°C in a TST 41.14 rotor (Kontron Elektronik GmbH, Zürich). The 17.5:11.5% interphase was enriched in Golgi-derived membranes. This fraction, if necessary, was diluted with the buffer needed for the subsequent experiment and centrifuged for 1 h at 38,000 rpm (99,800 g,r) at 4°C in a TST 75.15 rotor (Kontron Elektronik GmbH).

For the pulse-chase experiments with filter-grown cells, unlabeled Caco-2 cells grown on petri dishes were used as carriers as in the isolation procedure for brush border membranes.

**Isolation of Pre-Golgi Membrane Fraction**

This fraction was isolated from the same Percoll gradient as used for the isolation of the Golgi membrane (see Fig. 5). 1 ml at the bottom of the Percoll gradient (fraction V) was discarded. The next 8 ml (fraction IV) was collected and processed for 2 gradients. 4 g of the fraction was mixed with 3.27 g 60% (wt/wt) sucrose in 1 mM EDTA, 10 mM triethanolamine-acetic acid, pH 6.5. Metrizamide in a centrifuge tube and overlayed with 3.5 ml 22% (wt/wt) sucrose buffer Metrizamide followed by buffer B. The gradient was run for 7 h at 23,000 rpm (70,600 g,r) at 8°C in a TST 41.14 rotor (Kontron Elektronik GmbH). The 22:27% interphase (E II fraction) was collected and processed for 2 gradients. 4.0 g of the fraction was mixed with 2.0 g 60% (wt/wt) sucrose in 1 mM EDTA, 10 mM triethanolamine-acetic acid, pH 6.5. Metrizamide in a centrifuge tube and overlayed with 2.5 ml each of 17.5% and 11.5% (wt/wt) sucrose buffer Metrizamide and, finally, with buffer B. The gradient was run for 7 h at 23,000 rpm (70,600 g,r) at 8°C in a TST 75.15 rotor (Kontron Elektronik GmbH). The 22:27% interphase (E II fraction) was collected and designated pre-Golgi fraction.

**Enzyme Assays**

All measurements were performed at 37°C. Alkaline phosphatase (measured according to reference 37) and sucrase (measured according to reference 4) were used as marker enzymes for the brush border membrane. K+-stimulated p-nitrophenolphosphatase (measured according to reference 37; using buffers I and III) and KCN-resistant NADH oxidoreductase (determined as in reference 35) were used as markers for the basolateral membrane and the endoplasmic reticulum, respectively. With the exception of sucrase, all of these enzymes were measured using an LKB reaction rate analyzer 2086 Mark II. Glucosaminidase (measured according to reference 31) was used as a marker for lysosomes and galactosyltransferase (measured according to reference 40) with 0.5% (wt/wt) Triton X-100 and ovomucoid (as acceptor protein) was used to detect Golgi apparatus-derived membranes. Protein was determined with the Bio-Rad protein assay kit using protein standard I (Bio-Rad Laboratories, Cambridge, MA).

**Results**

**Polarized Expression of SI and DPPIV in the Microvillus Membrane of Caco-2 Cells**

Before undertaking biosynthetic studies, it was essential to establish the domain-specific location of DPPIV and SI in culture were pooled with unlabeled cells of three 100-mm culture dishes before subcellular fractionation.
Caco-2 cells grown on Millipore filters. In Fig. 1, immunofluorescent labeling of Caco-2 cryosections with enzyme-specific mAbs clearly showed that SI and DPPIV were detectable exclusively in the brush border membrane while the Na⁺/K⁺-ATPase α-subunit was associated only with the basolateral membrane. We conclude that the surface membrane of Caco-2 cells is polarized with respect to these three enzymes.

Transport of SI and DPPIV from the Golgi Apparatus to the Cell Surface
Previous studies on the biogenesis of microvillar enzymes were performed with Caco-2 cells grown on petri dishes (14). Since surface polarity is more developed in cells grown on Millipore filters than on solid supports (1, 11), we applied the former system to Caco-2 cells. However, growth of the cells on Millipore filters might alter the biogenesis of the investigated membrane proteins. Therefore, it was important to establish that the previously observed asynchronism of protein transport also occurred in Millipore-grown cells. Fig. 2 shows the time course of conversion of the high-mannose to the complex-glycosylated forms of the two hydrolases in cell homogenates, an event that is mediated by the Golgi apparatus. Half maximal appearance of complex-glycosylated DPPIV and SI occurred at ~15-20 min and 130-140 min, respectively. These results are similar to those of our previous study and therefore suggest that growth of the Caco-2...
Table I. Specific Activities and Enrichment Factors of Marker Enzymes in Brush Border Membrane Vesicles from Caco-2 Cells

<table>
<thead>
<tr>
<th>Marker Enzyme</th>
<th>Homogenate</th>
<th>Isolated membrane</th>
<th>Enrichment factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkaline phosphatase</td>
<td>53.3 ± 19.5</td>
<td>1,103.4 ± 382.3</td>
<td>20.9 ± 2.8 (n = 8)</td>
</tr>
<tr>
<td>Sucrase</td>
<td>3.6 ± 0.8</td>
<td>68.3 ± 17.3</td>
<td>19.5 ± 3.5 (n = 5)</td>
</tr>
<tr>
<td>K⁺-stimulated p-nitrophenol phosphatase</td>
<td>4.8 ± 0.8</td>
<td>11.5 ± 8.9</td>
<td>2.5 ± 2.1 (n = 5)</td>
</tr>
<tr>
<td>KCN-resistant NADH-oxidoreductase</td>
<td>174.4 ± 22.3</td>
<td>62.4 ± 29.5</td>
<td>0.4 ± 0.1 (n = 8)</td>
</tr>
<tr>
<td>Glucosaminidase</td>
<td>58.5 ± 30.9</td>
<td>61.2 ± 41.7</td>
<td>1.0 ± 0.3 (n = 7)</td>
</tr>
<tr>
<td>Galactosyltransferase</td>
<td>0.18 ± 0.01</td>
<td>0.08 ± 0.04</td>
<td>0.4 ± 0.2 (n = 5)</td>
</tr>
</tbody>
</table>

* Mean ± 1 SD.  
* n, number of experiments.
Appearance and Maturation of DPPIV and SI in the Golgi Apparatus

To further dissect the asynchronous transport of SI and DPPIV, we developed a method for the isolation of a fraction enriched in Golgi-derived membranes (Fig. 5). Such a preparation, in conjunction with the pulse-chase technique, allows one to differentiate between pre-Golgi and intra-Golgi events. Caco-2 cells were homogenized under conditions that left the cisternae of the Golgi apparatus intact while most other membrane compartments vesiculated enabling us to isolate the Golgi elements on gradients. Fig. 6 shows a representative electron micrograph of the final fraction containing the expected drumstick profiles characteristic for the Golgi apparatus. The enzymatic characterization of this fraction, designated "Golgi fraction," is given in Tables III and IV. The fraction was enriched in galactosyltransferase activity, a marker enzyme for the Golgi apparatus, while enzyme activities for other cellular membranes were not enriched.

A pulse-chase protocol was used to study arrival at and transit through the Golgi fraction of the two hydrolases. A typical autoradiogram of such an experiment is given in Fig. 7. The flow kinetics are drawn in Fig. 8, a and b. The time required for half-maximal labeling of the high-mannose forms of the enzymes in the Golgi fraction was defined as the rate of their transport from the endoplasmic reticulum to the cis side of the Golgi apparatus. This rate, designated "apparent transport rate" (see Discussion), was found to be <15 min for DPPIV and ~45 min for SI. Thus, arrival of SIh (the high-mannose form of SI) in this fraction was delayed. However, this delay only in part accounted for the asynchronism of maturation to Slc (the complex-glycosylated form of SI) in the homogenate (Fig. 2). Fig. 8 b shows that the conversion of SIh to Slc was also substantially delayed in the Golgi fraction when compared to that of DPPIV (Fig. 8 a). In the Golgi fraction a much higher percentage of total SI exists as high-mannose forms than is true for total DPPIV. At the same time this high-mannose SI appears in the Golgi fraction more slowly than the high-mannose DPPIV. Half-maximal appearance of Slc in the Golgi fraction was observed after ~100-110 min. The Slc in the Golgi fraction was maximally labeled after ~180 min at which time the radioactivity of this enzyme in the brush border fraction was half-maximal.

Figure 4. Appearance of newly synthesized DPPIV and SI in the brush border membrane fraction of Caco-2 cells. The pulse-chase protocol and the quantification of the radioactivity of gel slices was as in Fig. 2. The maximal relative amount of radioactivity associated with the enzymes (see Materials and Methods) was set to 100%.

Pre-Golgi Events

The above conclusion that the Golgi apparatus significantly contributes to the asynchronous protein transport critically depends on the purity of the Golgi fraction. For example, a copurification of a late endoplasmic reticulum compartment (i.e., transitional elements) with the Golgi fraction could lead to the above results even if exit from the endoplasmic
Table III. Specific Activities and Enrichment Factors of Marker Enzymes in a Fraction Enriched in Golgi-derived Membranes (FII) and in an Early Biosynthetic Fraction (Ell)

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Specific activity</th>
<th>Enrichment factor</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Homogenate</td>
<td>FII</td>
</tr>
<tr>
<td></td>
<td></td>
<td>FII</td>
</tr>
<tr>
<td>xylose transferase</td>
<td>0.281 ± 0.009</td>
<td>8.183 ± 0.811</td>
</tr>
<tr>
<td>K+-Stimulated p-nitrophenyl phosphatase</td>
<td>11.0 ± 2.3</td>
<td>1.9 ± 3.2</td>
</tr>
<tr>
<td>KCN-resistant NADH-</td>
<td>208.0 ± 31.8</td>
<td>115.8 ± 87.9</td>
</tr>
<tr>
<td>oxidoreductase</td>
<td>46.3 ± 7.6</td>
<td>45.7 ± 31.0</td>
</tr>
<tr>
<td>Glucosaminidase</td>
<td>188.6 ± 24.5</td>
<td>200.8 ± 44.5</td>
</tr>
</tbody>
</table>

The numbers indicate means ± 1 SD of four independent experiments.

Table IV. Yield and Recovery of Marker Enzymes in a Fraction Enriched in Golgi-derived Membranes (FII) and in an Early Biosynthetic Membrane Fraction (Ell)

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>FII</th>
<th>Ell</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.43 ± 0.08</td>
<td>6.1 ± 1.6</td>
<td>91.6 ± 11.9</td>
</tr>
<tr>
<td>xylose transferase</td>
<td>13.6 ± 2.0</td>
<td>0.5 ± 0.3</td>
<td>111.4 ± 6.9</td>
</tr>
<tr>
<td>K+-stimulated p-nitrophenyl phosphatase</td>
<td>0.1 ± 0.1</td>
<td>0.2 ± 0.2</td>
<td>85.4 ± 10.9</td>
</tr>
<tr>
<td>KCN-resistant NADH-</td>
<td>0.2 ± 0.1</td>
<td>18.6 ± 3.2</td>
<td>83.7 ± 5.5</td>
</tr>
<tr>
<td>oxidoreductase</td>
<td>0.4 ± 0.2</td>
<td>2.3 ± 1.1</td>
<td>87.0 ± 11.1</td>
</tr>
<tr>
<td>Glucosaminidase</td>
<td>0.5 ± 0.1</td>
<td>14.1 ± 3.1</td>
<td>105.4 ± 16.3</td>
</tr>
<tr>
<td>Alkaline phosphatase</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The numbers indicate means ± 1 SD of four independent experiments.
newly synthesized enzymes in this intracellular compartment.

Conclusions drawn from subcellular fractionation studies critically depend on the extent of cross contamination by membranes of other organelles. For instance, contamination of our brush border fraction with Golgi membranes would affect the observed transport rates. The high enrichment factor of marker enzymes for brush border membranes and the low enrichment factor for galactosyltransferase provide strong evidence that cross contamination by Golgi membranes in our preparations is negligible. It has recently been shown that the brush border of the human small intestine can be labeled with polyclonal antibodies raised against galactosyltransferase (28). However, it was not determined in that study whether or not the immunoreacting material had galactosyltransferase activity. It is important to note that we were unable to measure significant galactosyltransferase activity levels in Caco-2 brush border membranes (Table I) while in the galactosyltransferase-enriched Golgi fraction the brush border enzyme activities were low. We therefore believe that galactosyltransferase is still valuable as a marker enzyme for detecting Golgi-derived membranes (see also reference 3).

Our suggestion that the Golgi apparatus significantly contributes to the asynchronous transport is only valid if the Golgi fraction is not significantly contaminated by elements of the endoplasmic reticulum that are involved in protein biosynthesis and transport. This was indeed the case as shown in experiments in which protein exit from the endoplasmic reticulum was blocked by CCCP. Under these conditions no newly synthesized DPPIV was detectable in the Golgi fraction. This shows that the Golgi fraction was sufficiently pure to study the arrival of brush border enzymes in the Golgi apparatus.

Figure 7. Appearance of newly synthesized DPPIV and SI in the Golgi fraction of Caco-2 cells. Golgi fractions were prepared after different time intervals of chase. The hydrolases were immunoprecipitated with a mixture of anti-enzyme antibodies and the immunoprecipitates were separated by SDS-PAGE. Note that the apparent intensities of the bands are not necessarily comparable between individual lanes due to variability in overall incorporation of radioactivity among the cultures.

Table V. Apparent Intracellular Transport Rates of Newly Synthesized Microvillar Hydrolases as Deduced from Pulse-Chase Experiments

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>ER to brush border</th>
<th>ER to Golgi</th>
<th>ER to site of complex glycosylation</th>
<th>Site of complex glycosylation to brush border</th>
</tr>
</thead>
<tbody>
<tr>
<td>SI</td>
<td>180-190</td>
<td>45</td>
<td>130-140</td>
<td>40-60</td>
</tr>
<tr>
<td>DPPIV</td>
<td>80-90</td>
<td>&lt;15</td>
<td>15-20</td>
<td>60-75</td>
</tr>
</tbody>
</table>

* Time for half-maximal appearance of mature enzyme in the brush border membrane fraction (Fig. 4).
† Time for half-maximal appearance of the high-mannose form in the Golgi fraction (Fig. 8).
§ Time for half-maximal appearance of the complex-glycosylated form in the homogenate (Fig. 2).
‖ Transport rate from ER to brush border minus transport rate from ER to site of complex glycosylation.

Figure 8. Arrival and maturation of newly synthesized DPPIV (A) and SI (B) in the Golgi fraction of Caco-2 cells deduced from pulse-chase experiments (quantification from fluorograms). The highest amount of radioactivity (high-mannose plus complex forms) in either DPPIV or SI in the Golgi fraction relative to the amount of radioactivity in the enzymes in the corresponding homogenate was set to 100%. (×) High-mannose forms; (○) complex forms.

Figure 9. Appearance of newly synthesized high-mannose DPPIV (Δ, ▲) and SI (○, ⌠) in the pre-Golgi fraction (——) as compared to the Golgi fraction (— — —). For purposes of clarity the maximal relative amount of radioactivity in the high-mannose forms was set to 100%.
The apparent rates calculated for the endoplasmic reticulum to cis-Golgi transport and for the transport from the endoplasmic reticulum to the site of complex glycosylation are probably somewhat lower than the actual rates. Actual rates could only be determined under conditions that would completely prevent complex glycosylation and protein exit from the Golgi apparatus but that would not affect transfer from the endoplasmic reticulum to the Golgi complex. Such conditions have not been found yet. However, in the Golgi fraction, a much higher percentage of the total SI exists as high-mannose forms than is true for total DPPIV. This high-mannose SI appears in the Golgi complex more slowly than the high-mannose DPPIV. These results clearly indicate an intragolgi rate-limiting step of the conversion of high-mannose to complex SI.

An unexpected but interesting observation is the long apparent residence time of part of the complex-glycosylated hydrolases in the Golgi fraction. This is not likely due to cross contamination by brush border membranes since the radioactive corresponding marker enzyme activities are low in the Golgi fraction. In particular, the radioactivity corresponding to DPPIV in this fraction continues to decrease at a time when there is no further concomitant increase in the brush border membrane. Since the turnover of brush border enzymes in cell culture is slow (Hauri, H.-P., unpublished data), this suggests that part of the newly synthesized enzymes of this pool never reaches the cell surface but is rather degraded in the Golgi apparatus or transported to other intracellular organelles like the lysosomes. Fransen et al. (8) have demonstrated by immunolectron-microscopy that in human small intestinal biopsies a mAb against SI could label lysosomes in addition to organelles of the biosynthetic pathway. However, it is not known whether this lysosomal SI was directly imported from the Golgi apparatus and hence bypassed the cell surface or whether it originated from endocytosis.

Some of the present data are in line with the suggestions of Danielsen and Cowell (5) who postulated that SI and aminopeptidase N in hog intestinal organ cultures are synchronously transported from the site of complex glycosylation to the brush border membrane. However, we disagree with the conclusions of these authors that a pre-Golgi event is the only rate-limiting step for the efficient transport of the hydrolases to the cell surface. Our results strongly suggest that the asynchronous transport of these hydrolases is also due to intra-Golgi events. In the study of Danielsen and Cowell (5), the rate of conversion from transient (high mannose) to mature (complex-glycosylated) form was assumed to be a measure for transport to the Golgi apparatus. However, this event clearly is a mediain (29) to trans-Golgi function (27) and therefore does not reflect initial arrival at the Golgi apparatus.

It is currently unknown to what extent the present observations on the role of the Golgi apparatus in the asynchronous transport can be generalized for endogenous membrane proteins. To our knowledge there is only one previous study dealing with the asynchronous migration to the cell surface of endogenous membrane glycoproteins (44). Although the authors of that study concluded that the different transport rates of two closely related histocompatibility antigens are due to an event associated with the endoplasmic reticulum, their subcellular fractionation data do not strictly rule out the contribution of an intra-Golgi event. It is important to note that for secretory proteins, the endoplasmic reticulum rather than the Golgi apparatus was found to be the rate-limiting step for migration (10, 21, 25, 32). Thus, it is likely that fundamental differences exist between the transport of secretory and membrane proteins.

The molecular basis for asynchronous protein transport is unknown. In intestinal epithelial cells, slowly transported microvillar glycoproteins like SI (14), lactase–phlorizin hydrolase (23), or maltase–glucoamylase (22) share a number of common properties that are distinct from the rapidly migrating peptidases. These three enzymes are disaccharidases that are synthesized as single-chain, two–active-site polypeptides (33). At least one of them, SI, but probably all three, appear to have evolved by partial duplication of ancestor genes coding for one–active-site enzymes (16). Gene duplication may have interfered, to some extent, with the efficient maturation of the enzymes by making protein folding and/or glycosylation more complicated. This speculation is supported by results from studies on patients suffering from hereditary sucrase–isomaltase deficiency. Minor alterations in sucrase-isomaltase (probably point mutations) that are not detectable by SDS-PAGE were found to lead to an inhibition of transport at the level of the Golgi apparatus (13). The rapidly transported peptidases, on the other hand, including DPPIV, aminopeptidase N, angiotensin I–converting enzyme, and PABA-peptide hydrolase are synthesized as single-chain, one–active-site polypeptides (12, 33, Sterchi, E., H. Naim, and H.-P. Hauri, unpublished data). Furthermore, they are in general smaller (up to twofold) than the major disaccharidases.

In conclusion, the present study suggests that transit through the Golgi apparatus in addition to exit from the endoplasmic reticulum is rate limiting in the migration of two microvillar hydrolases to the cell surface. The molecular basis of the asynchronous enzyme transport remains to be elucidated. Furthermore, the Golgi apparatus may play an im-
An important role in regulating the surface expression of these enzymes at a posttranslational level.

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