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 asynchronism is due to more than one rate-limiting step along the rough endoplasmic reticulum to *trans*-Golgi pathway.

EPITHELIAL cells are specialized to perform a variety of polarized functions that are in turn reflected by the polarized morphology and organization of their surface membrane domains, the basolateral and the microvillar (also termed brush border or apical) membranes. These domains possess unique sets of integral membrane proteins (34). Unraveling the biosynthesis of epithelial membrane proteins and the selective nature of their transport to the cell surface might therefore lead to an understanding of the processes underlying the biogenesis and maintenance of cell surface polarity.

Membrane glycoproteins are synthesized on ribosomes bound to the endoplasmic reticulum and are in most cases cotranslationally inserted into the lipid bilayer (30, 42, 43). During translocation through the endoplasmic reticulum, N-linked glycosylation and immediate trimming of the oligosaccharide side chains take place. The proteins are subsequently transported to and through the Golgi apparatus (6) where further processing of the N-linked and, in some instances, the addition of O-linked sugars occurs (19). After completion of the biosynthesis in the Golgi apparatus, the proteins are then transported to their spatially correct plasma membrane domains by a mechanism which is at present only poorly understood. The intracellular transport of newly synthesized secretory and membrane proteins was found to be asynchronous in many instances (7, 10, 21, 32, 44). These studies have suggested that the exit from the endoplasmic reticulum was the rate-limiting step for efficient transport. Among the many glycoproteins that can be found in the brush border membrane of intestinal epithelial cells, the hydrolases (in particular, peptidases and disaccharidases) are the major constituents (12, 18, 24, 33). Some of these hydrolases are expressed in the human intestinal epithelial cell line Caco-2 (14, 26). Using this cell line in studies of the biosynthesis of dipeptidylpeptidase IV (DPPIV),[1] a representate peptidase, and sucrase-isomaltase (SI), a representate disaccharidase, Hauri et al. (14) found that the time course of maturation of these two membrane proteins was asynchronous. Since the aim of the present study was to define the rate-limiting steps involved in this asynchronous intracellular protein transport, we found it necessary to design novel methods for the isolation of brush border membranes and of membranes derived from the Golgi apparatus. Using these new isolation techniques in conjunction with pulse–chase experiments, we can now provide evidence that the asynchronous transport of microvillar proteins is due to at least two major rate-limiting steps along the rough endoplasmic reticulum to *trans*-Golgi pathway.

Materials and Methods

Cell Culture and Labeling with [^{35}S]Methionine

Caco-2 cells were grown in Optilux petri dishes (Falcon Labware, Oxnard, CA) as described (14) or on Millipore filters (HATF0025) in mini-Marbrook chambers (11). The cells were subcultured weekly using the tryp-

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1. *Abbreviations used in this paper*: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; DPPIV, dipeptidylpeptidase IV; DPPIVc, complex-glycosylated form of dipeptidylpeptidase IV; DPPIVh, high-mannose form of dipeptidylpeptidase IV; SI, sucrase-isomaltase; SIc, complex-glycosylated form of sucrase isomaltase; SIh, high-mannose form of sucrase-isomaltase.

sin/EDTA method (26). Labeling with [^{35}S]methionine was carried out with cells grown on filters 5–15 d after confluency (14). In all experiments, a pulse time of 15 min was used except for the experiments with carbonyl cyanide *m*-chlorophenylhydrazone (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 [^{35}S]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 g/liter $CaCl_2$ and 0.059 g/liter $MgSO_4$. 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, Immunoisolation of Antigens, and SDS-PAGE

Microvillar hydrolases were localized by immunofluorescence on semithin cryosections (39) of filter grown Caco-2 cells using mAb HBB2/614/88 against SI and mAb HBB3/775/42 against DPPIV, as described (14). (Na^+/K^+)-ATPase was localized using mAb C 62.4 against the catalytic α-subunit of the dog enzyme (17) which was found to cross react with the human enzyme. Details of immunoisolation and SDS-PAGE were as described (14). SI was precipitated with a mixture of the four mAbs HBB2/614/88, HBB 3/705/60, HBB2/219/20, and HBB 1/691/79 directed against different epitopes, and DPPIV was precipitated with antibody HBB3/775/42. [^{32}S]Methionine-labeled proteins were visualized by fluorography using EN^{3}HANCE (New England Nuclear, Boston, MA). Bands on fluorograms were quantified by using a Camag LTC Scanner II connected to a Camag SP 4290 integrator. Alternately, [^{35}S]methionine-labeled proteins were excised from dried gels. The gel slices were digested with H_2O_2 at 50°C for 24 h followed by an overnight incubation in 2 ml Protosol (New England Nuclear). Finally, 8 ml of Instagel scintillation fluid were added and the radioactivity was determined in a 460-CD liquid scintillation counter. Both methods of quantification were compared and gave identical results.

Electron Microscopy

Small samples of membrane suspensions were prefixed at room temperature by adding a stock solution of glutaraldehyde (8%) to a final concentration of 1%. After 15 min, the samples were centrifuged in a Microfuge or Airfuge (Beckman Instruments Inc., Palo Alto, CA). The supernatants were replaced by 1 or 2% glutaraldehyde in PBS. The fixed pellets were stored at 4°C until further processing. They were then washed with PBS and postfixed with 1% osmium tetroxide in PBS. The pellets were dehydrated and embedded in Epon B12. Gray-silver sections were cut with an Ultracut E (Reichert Jung, Vienna), poststained with saturated uranyl acetate in 50% ethanol and lead citrate (40), and studied with a Philips 300 or 420 T electron microscope.

Isolation of Brush Border Membranes

The method for the isolation of brush border membranes from Caco-2 cells was based upon procedures described by Lever (20) and Stieger and Murer (36). Cell monolayers were washed in situ once with 0.9% (wt/vol) NaCl and once with buffer A (300 mM D-mannitol, 5 mM EGTA, 12 mM Tris-HCl, pH 7.1, 40 μg/ml phenylmethylsulfonyl fluoride [PMSF] that was added from a stock solution, 40 mg/ml in ethanol). The cells were then scraped from the dish with a rubber policeman and suspended in 5 ml of buffer A (all volumes are given for one 100-mm dish) and centrifuged for 5 min at 560 g_{av}. The cells were carefully resuspended in 1 ml of buffer A and the osmolarity of the homogenization medium was lowered by the addition of 4 ml of ice-cold H_2O. The cells were equilibrated with nitrogen for 30 min at 35 bar in a minibomb cell disruption chamber (Kontes Co., Vineland, NJ) and then slowly released. The homogenate was degassed for 10 min and $MgCl_2$ was added to a final concentration of 10 mM. After 15 min on ice, the homogenate was centrifuged for 15 min at 5,000 rpm (2,300 g_{av}) and the resulting supernatant for 30 min at 15,000 rpm (20,700 g_{av}) in an SS34 rotor (Sorvall Instruments Div., Newton, CT). The pellet was resuspended in 1 ml of 60 mM D-mannitol, 5 mM EGTA-Tris, pH 7.1, 40 μg/ml PMSF, and then $CaCl_2$ was added to a final concentration of 10 mM. The suspension was left on ice for 15 min followed by two centrifugation steps as after addition of $MgCl_2$. The resulting brush border membrane vesicles (P4 fraction) were resuspended in the buffer required for the subsequent experiment.

For pulse-chase experiments, metabolically labeled cells of a single filter culture were pooled with unlabeled cells of three 100-mm culture dishes before subcellular fractionation.

Isolation of a Fraction Enriched in Golgi-derived Membranes

The cell monolayers were washed once with 0.9% (wt/vol) NaCl and once with 250 mM sucrose, 10 mM triethanolamine-acetic acid, pH 7.4 (buffer A). The cells were then scraped from the dish, suspended in 2 ml of buffer A per 100-mm dish, and centrifuged for 5 min at 560 g_{av}. The cell pellet was gently resuspended in 2 ml of 250 mM sucrose, 1 mM EDTA, 10 mM triethanolamine-acetic acid, pH 6.5 (buffer B), and centrifuged as above. The cells were then resuspended in 3 ml buffer B and homogenized by passing them 10 times through a ball-bearing homogenizer (2) with a clearance of 20 μm. The resulting homogenates were then pooled and centrifuged for 10 min at 2,000 rpm (370 g_{av}) 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 20,000 rpm (36,900 g_{av}) 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 g 60% ([wt/wt] 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] same buffer) Metrizamide and, finally, with buffer B. The gradient was run for 7 h at 23,000 rpm (70,600 g_{av}) 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_{av}) at 4°C in a TFT 75.13 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 Membane Fraction

This fraction was isolated from the same Percoll gradient as used for the isolation of the Golgi fraction (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. 4.0 g of the fraction was mixed with 3.27 g 60% ([wt/wt] in 1 mM EDTA, 10 mM triethanolamine-acetic acid, pH 6.5) Metrizamide in a centrifuge tube and overlayed with 3 ml 22% ([wt/wt] same buffer) Metrizamide followed by buffer B. The gradient was run for 7 h at 23,000 rpm (70,600 g_{av}) at 8°C in a TST 41.14 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*-nitrophenylphosphatase (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/vol) 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

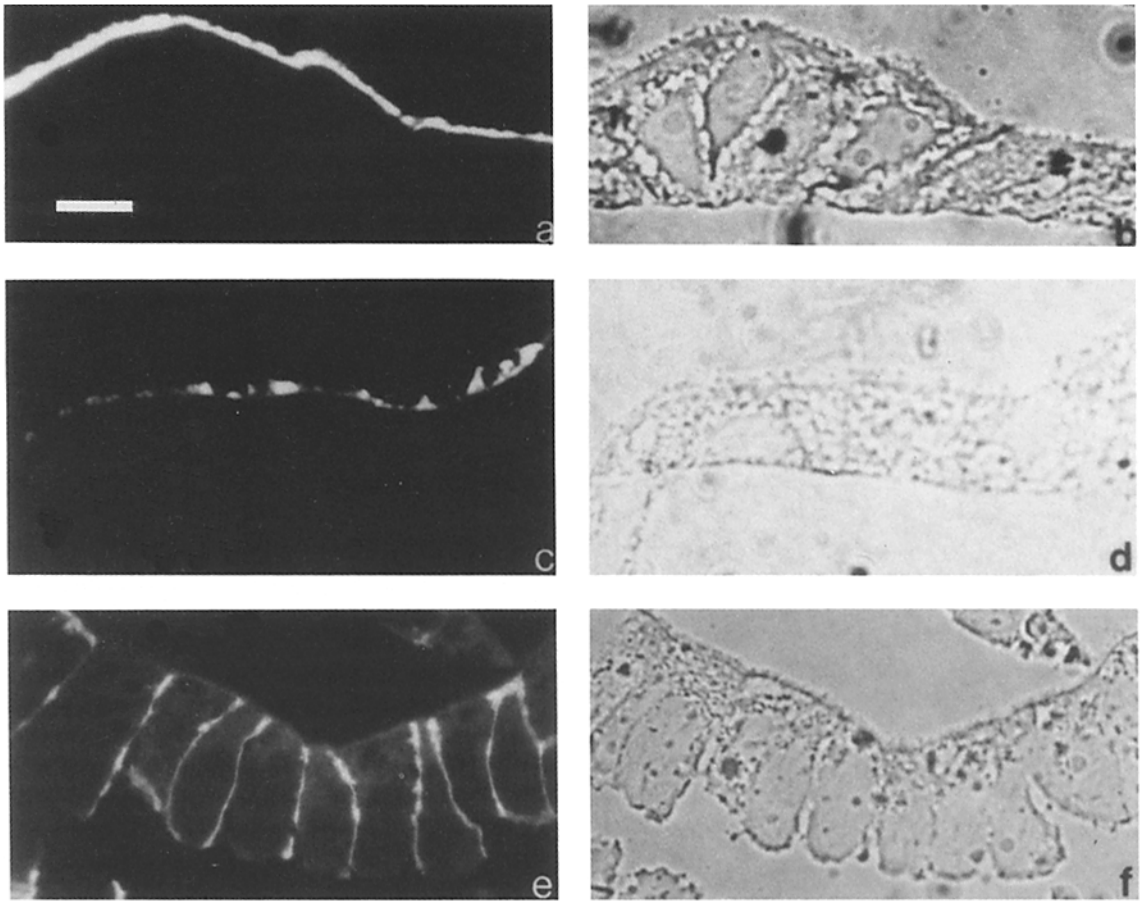

Figure 1. Immunofluorescent labeling of 1-μm cryosections of Caco-2 cell monolayers with mAb HBB 3/775/42 against DPPIV (*a*), mAb HBB 2/614/88 against sucrase-isomaltase (*c*), and mAb C62.4 against Na^+/K^+-ATPase (*e*). (*b*, *d*, and *f*) Corresponding phase-contrast micrographs. Note that the hydrolases are restricted to the apical cell surface, while Na^+/K^+-ATPase is confined to the lateral aspect of the cells. The lack of immunofluorescence for the latter enzyme of the basal cell surface may be due to the loss of this domain during scraping of the cells. Bar, 24 μm.

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

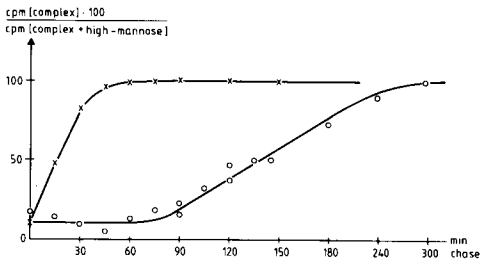

Figure 2. Conversion of the high-mannose to complex-glycosylated forms of DPPIV (X) and SI (o) in homogenates of Caco-2 cells. Shown are results from pulse–chase experiments with [^{35}S]methionine (pulse, 15 min). After various time intervals of chase, the enzymes were immunoprecipitated and separated by SDS-PAGE. Gel slices corresponding to high-mannose or complex-glycosylated forms of the enzymes were cut out of dried gels, solubilized, and radioactivity was measured in a β-counter.

Table I. Specific Activities and Enrichment Factors of Marker Enzymes in Brush Border Membrane Vesicles from Caco-2 Cells

	Specific activity		
	Homogenate	Isolated membrane	Enrichment factor
	mU/mg protein		
Alkaline phosphatase	53.3* ± 19.5	1,103.4 ± 382.3	20.9 ± 2.8 (n = 8)
Sucrase	3.6 ± 0.5	68.3 ± 17.3	19.5 ± 3.5 (n = 5)
K^+-stimulated p-nitrophenyl phosphatase	4.8 ± 0.8	11.5 ± 8.9	2.5 ± 2.1 (n = 5)
KCN-resistant NADH-oxidoreductase	174.4 ± 22.3	62.4 ± 29.5	0.4 ± 0.1 (n = 8)
Glucosoaminidase	58.5 ± 30.9	61.2 ± 41.7	1.0 ± 0.3 (n = 7)
Galactosyltransferase	0.18 ± 0.01	0.08 ± 0.04	0.4 ± 0.2 (n = 5)

* Mean ± 1 SD.
n, number of experiments.

cells on filters rather than on petri dishes does not significantly alter the overall maturation pattern of the two enzymes under investigation. To determine whether transport of the two enzymes from the site of complex glycosylation in the Golgi apparatus to the cell surface is also asynchronous, we developed a method for the isolation of the brush border membrane from Caco-2 cells. Although seemingly similar to previously published methods for the isolation of brush border membranes from intact tissue, it is worth noting that homogenization conditions and the order of the precipitation steps (i.e., Mg^{++} before Ca^{++}) were found to be critical for obtaining highly purified brush border membranes from Caco-2 cells. Tables I and II show that the membrane fraction isolated using this method was enriched in brush border marker enzymes SI and alkaline phosphatase, while contamination by intracellular membranes was minimal. Due to the small number of labeled cells it was advantageous to use carrier dishes (unlabeled Caco-2 cells grown in petri dishes) for the pulse–chase experiment. The clearly asynchronous appearance of DPPIV and SI in the brush border fraction is illustrated in Fig. 3. It is also notable that the autoradiograms lacked any band in the region of the high-mannose form of these proteins, providing additional evidence for the purity of the membrane fraction. The kinetics of the appearance of DPPIV and SI in the brush border membrane of the Caco-2 cells is shown in Fig. 4. The difference between the time of half-maximal appearance in the brush border membrane and the half-maximal appearance of the complex-glycosylated forms in the homogenate was used to calculate a transport rate of 40–60 min for SI and 60–75 min for DPPIV. Thus the transport kinetics from the site of complex glycosylation in the Golgi apparatus to the brush border membrane were similar for the two hydrolases.

*Table II. Yield and Recovery of Marker Enzymes in Isolated Brush Border Membranes from Caco-2 Cells**

	P4	Total‡	
Protein	0.33 ± 0.03	82 ± 10	(n = 6)
Alkaline phosphatase	6.8 ± 1.0	97 ± 15	(n = 6)
Sucrase	6.7 ± 1.4	76 ± 10	(n = 4)
K^+-stimulated p-nitrophenyl phosphatase	0.88 ± 0.71	75 ± 13	(n = 5)
KCN-resistant NADH-oxidoreductase	0.10 ± 0.03	88 ± 4	(n = 6)
Glucosaminidase	0.33 ± 0.03	84 ± 7	(n = 6)
Galactosyltransferase	0.14 ± 0.06	85 ± 10	(n = 5)

* The values are given as percentage of the amount determined in the homogenate and represent means ± 1 SD.
‡ Sum of recoveries determined in each fraction.
n, number of experiments.

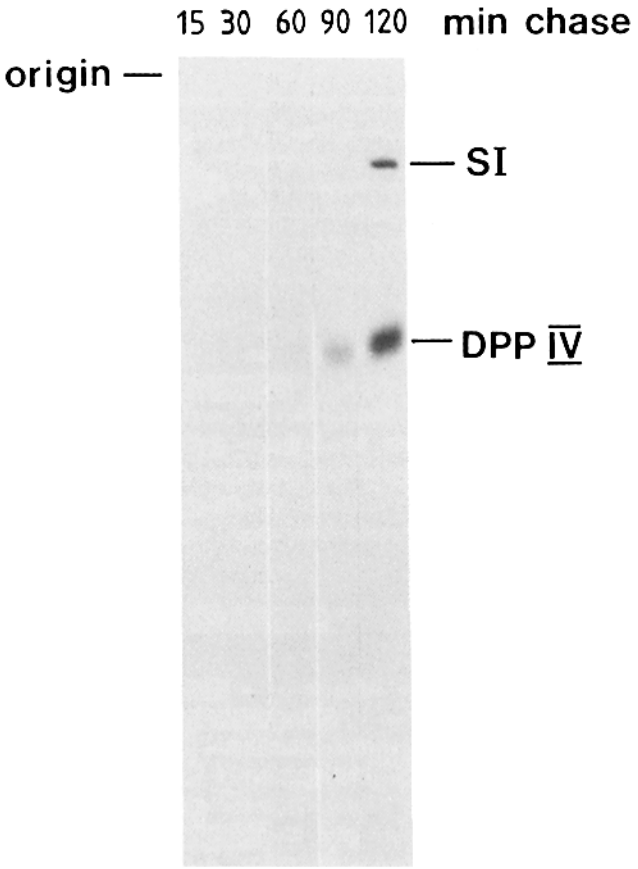

Figure 3. Appearance of newly synthesized DPPIV and SI in the brush border membrane fraction of Caco-2 cells. The cells were pulse labeled with [^{35}S]methionine and the hydrolases were immunoprecipitated from the subcellular fraction after various times of chase with a mixture of mAbs against SI and DPPIV.

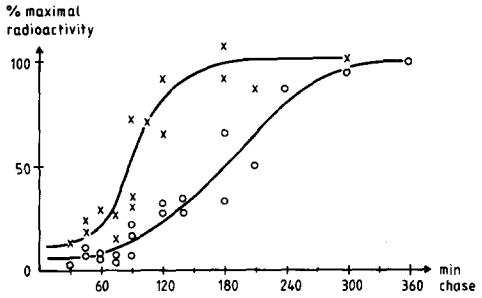

Figure 4. Appearance of newly synthesized DPPIV and SI in the brush border membrane fraction of Caco-2 cells. The pulse-chase protocol and the quantification of the radioactivity of gel slices was as in Fig. 2. The maximal relative amount of radioactivity associated with the enzymes (see Materials and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

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

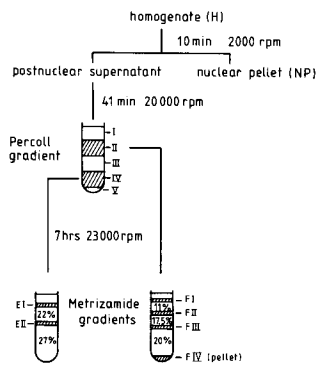

Figure 5. Flow diagram for the isolation of the Golgi fraction (*FII*) and a pre-Golgi membrane fraction (*EII*).

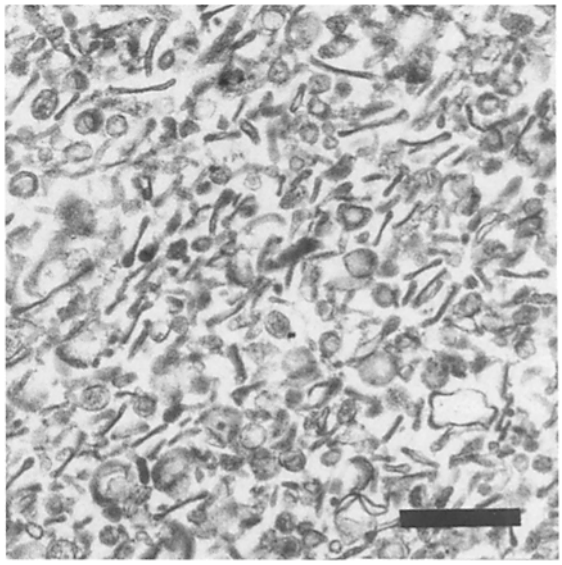

Figure 6. Representative electron micrograph of the FII Golgi fraction. Note the electron dense drumstick profiles characteristic for the Golgi apparatus.

of maturation to SIc (the complex-glycosylated form of SI) in the homogenate (Fig. 2). Fig. 8 *b* shows that the conversion of SIh to SIc 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 SIc in the Golgi fraction was observed after ~100–110 min. The SIc 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. When the enzyme approached maximal levels in the brush border fraction (after 300 min) the SIc in the Golgi fraction had decreased by only ~40%, suggesting the existence of a SIc pool that is not immediately exported from the Golgi apparatus. The kinetic behavior of DPPIV is strikingly different from that of SI. Conversion of DPPIVh (the high-mannose form of DPPIV) to DPPIVc (the complex form of DPPIV) was rapid taking ~15 min only. The disappearance of DPPIVc from this fraction was biphasic. A rapid disappearance was observed up to 120 min at which time appearance of DPPIVc in the brush border fraction was close to maximal. This rapid disappearance is therefore most likely due to delivery of DPPIVc to the brush border. At later time points, the radioactivity in DPPIVc disappeared more slowly. Overall these results suggest that the intracellular transport of SI is delayed at a pre-Golgi as well as at an intra-Golgi stage.

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 (EII)

	Specific activity			Enrichment factor	
	Homogenate	FII	EII	FII	EII
	mU/mg				
Galactosyltransferase	0.281 ± 0.009	8.183 ± 0.811	0.024 ± 0.013	29.1 ± 2.2	0.09 ± 0.05
K^+-Stimulated *p*-nitrophenyl phosphatase	11.0 ± 2.3	1.9 ± 3.2	0.3 ± 0.3	0.14 ± 0.25	0.03 ± 0.03
KCN-resistant NADH-oxidoreductase	208.0 ± 31.8	115.8 ± 87.9	622.3 ± 41.6	0.54 ± 0.36	3.1 ± 0.52
Glucosaminidase	46.3 ± 7.6	45.7 ± 31.0	17.8 ± 6.7	0.95 ± 0.59	0.38 ± 0.11
Alkaline phosphatase	188.6 ± 24.5	200.8 ± 44.5	445.5 ± 50.8	1.02 ± 0.09	2.4 ± 0.2

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

reticulum would be the only rate-limiting step. Due to the lack of an established marker protein for transitional elements, it is presently not possible to directly assess such a cross contamination. However, it appears unlikely that transitional elements would cofractionate with our Golgi fraction since, depending on the homogenization procedure, they can be expected to have similar properties to the smooth endoplasmic reticulum (i.e., if vesiculated) or to the rough endoplasmic reticulum (i.e., if still attached to portions of the rough endoplasmic reticulum). Both these fractions are denser than the Golgi cisternae and were found to migrate to a lower position on metrizamide gradients.

Assuming that at least part of the transitional elements would vesiculate under our homogenization conditions and, thus, probably cofractionate with smooth endoplasmic reticulum, we have isolated a crude membrane fraction which is enriched in the smooth endoplasmic reticulum marker KCN-resistant NADH-oxidoreductase but low in galactosyltransferase (Tables III and IV). Pulse-chase experiments showed that this fraction contained high-mannose forms of both enzymes, which had been labeled earlier than the corresponding high-mannose forms in the Golgi fraction (Fig. 9). While radioactivity in DPPIVh immediately decreased starting at the earliest chase time, the radioactivity in SIh only decreased after a 60-min chase. These results indicate that this membrane fraction termed pre-Golgi fraction contains a compartment which is intermediate to rough endoplasmic reticulum and Golgi apparatus and therefore the data are in line with the suggestion that the delayed transport of SI is in part due to a pre-Golgi event.

To further characterize the pre-Golgi and the Golgi fractions, pulse-chase experiments were performed in the presence of CCCP. CCCP, an uncoupler of oxidative phosphorylation, is known to block the exit of proteins from the endoplasmic reticulum without affecting Golgi morphology (38). CCCP had indeed no effect on the subcellular fractionation of Caco-2 cells (not shown). Fig. 10 shows that in the presence of CCCP, DPPIVh is not converted to the complex form in the pre-Golgi fraction (which also contains some brush border membranes; see Table III), while neither DPPIVh nor DPPIVc appeared in the Golgi fraction. Similar results were obtained with SI (not shown). These results suggest that the pre-Golgi fraction contains kinetically late endoplasmic reticulum membranes, probably transitional elements, and that the Golgi fraction is not detectably contaminated by elements of the endoplasmic reticulum.

Discussion

The present study suggests that the observed asynchronous transport of SI and DPPIV to the cell surface is due to at least two rate-limiting steps; i.e., exit from the endoplasmic reticulum and transit through the Golgi apparatus. The transport from the site of complex glycosylation, an event that is associated with the Golgi complex, to the brush border was similar for the two enzymes. We believe that the slightly different transport rates are in fact not significantly different. Furthermore, the data suggest that in the Golgi complex there is a slowly turning over pool of hydrolases, as indicated by the unexpectedly long residence time of a fraction of the

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

	FII	EII	Total
Protein	0.43 ± 0.08	6.1 ± 1.6	91.6 ± 11.9
Galactosyltransferase	13.6 ± 2.0	0.5 ± 0.3	111.4 ± 6.9
K^+-stimulated *p*-nitrophenyl-phosphatase	0.1 ± 0.1	0.2 ± 0.2	85.4 ± 10.9
KCN-resistant NADH-oxidoreductase	0.2 ± 0.1	18.6 ± 3.2	83.7 ± 5.5
Glucosaminidase	0.4 ± 0.2	2.3 ± 1.1	87.0 ± 11.1
Alkaline phosphatase	0.5 ± 0.1	14.1 ± 3.1	105.4 ± 16.3

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

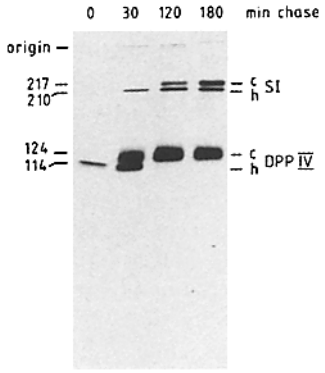

Figure 7. Appearance of newly synthesized DPPIV and SI in the Golgi fraction of Caco-2 cells. Golgi fractions were prepared after different time intervals of chase. The hydrolases were immunoprecipitated with a mixture of anti-enzyme antibodies and the immunoprecipitates were separated by SDS-PAGE. Note 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

Enzyme	ER to brush border*	ER to Golgi‡	ER to site of complex glycosylation§	Site of complex glycosylation to brush border‖
	min	*min*	*min*	*min*
SI	180–190	45	130–140	40–60
DPPIV	80–90	<15	15–20	60–75

* 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.

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). The transport rate of SI to the brush border has most recently been confirmed by pulse-chase experiments in conjunction with a novel surface protease assay (Eilers, U., and H.-P. Hauri, manuscript in preparation).

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.

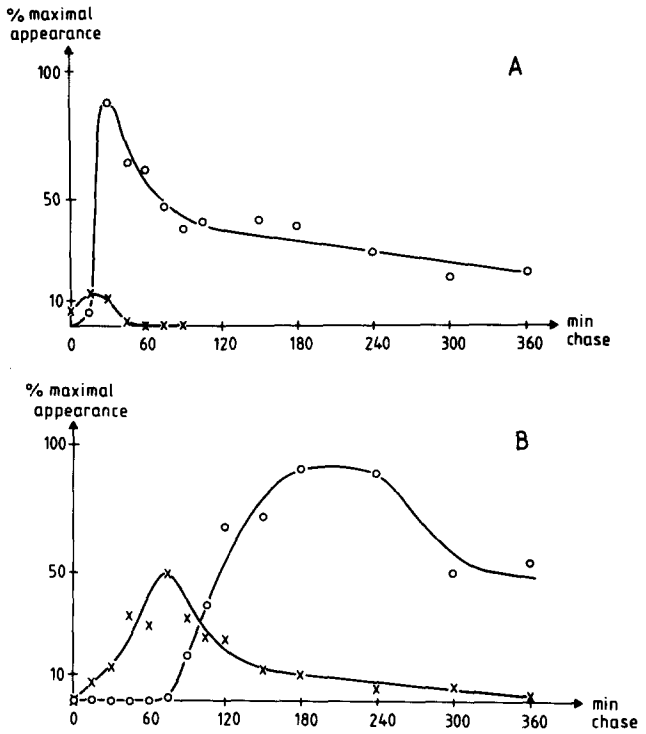

Figure 8. Arrival and maturation of newly synthesized DPPIV (*A*) and SI (*B*) in the Golgi fraction of Caco-2 cells deduced from pulse-chase experiments (quantification from fluorograms). The highest amount of radioactivity (high-mannose plus complex forms) in either DPPIV or SI in the Golgi fraction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

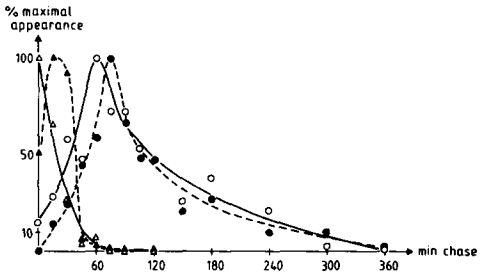

Figure 9. Appearance of newly synthesized high-mannose DPPIV (△, ▲) and SI (○, ●) in the pre-Golgi fraction (——) as compared to the Golgi fraction (– – –). For purposes of clarity the maximal relative amount of radioactivity in the high-mannose forms was set to 100%.

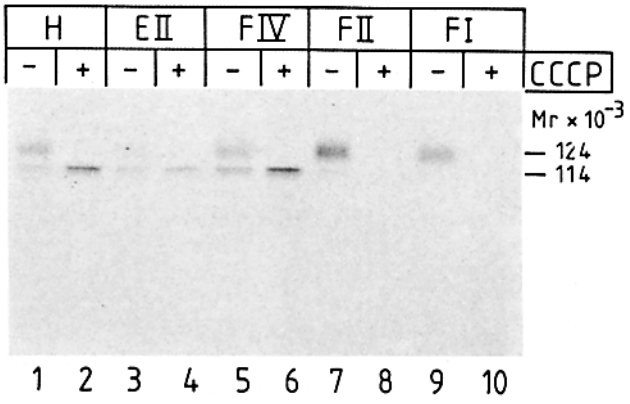

Figure 10. Effect of CCCP on the appearance of DPPIV in subcellular fractions of Caco-2 cells. Shown is a fluorogram from a pulse-chase experiment carried out as described under Materials and Methods. After a 45-min chase the cells were processed for subcellular fractionation. Fractions were solubilized and the enzyme was immunoprecipitated and separated by SDS-PAGE. (Lanes *1* and *2*) Homogenate; (lanes *3* and *4*) pre-Golgi fraction (*EII*); (lanes *5* and *6*) fraction *FIV*; (lanes *7* and *8*) Golgi fraction (*FII*); (lanes *9* and *10*) fraction *FI*.

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 intra-Golgi 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 corresponding marker enzyme activities are low in the Golgi fraction. In particular, the radioactivity corresponding to DPPIVc 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 either degraded in the Golgi apparatus or transported to other intracellular organelles like the lysosomes. Fransen et al. (8) have demonstrated by immunoelectron-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 medial- (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-

portant role in regulating the surface expression of these enzymes at a posttranslational level.

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