Intracellular Transport and Conformational Maturation of Intestinal Brush Border Hydrolases†

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ABSTRACT: Brush border hydrolases of the differentiated intestinal cell line Caco-2 are transported to the microvillar membrane at different rates. This asynchronism is due to at least two rate-limiting events, a pre- and an intra-Golgi step. The retardation of sucrase–isomaltase, a slowly migrating hydrolase, versus dipeptidylpeptidase IV, a rapidly transported enzyme, is neither due to differential trimming of N-linked carbohydrates nor due to oligomerization. In this study, the conformational maturation of biosynthetically labeled sucrase–isomaltase and dipeptidylpeptidase IV was probed by conformation-specific antibodies and proteases. These assays enabled us to correlate the conformational maturation of the two enzymes with their rates of transport. Furthermore, two naturally occurring mutants of sucrase–isomaltase with impaired intracellular transport displayed an immature conformation. It is proposed that differential kinetics of folding might be the underlying cause for both the pre- and the intra-Golgi steps of asynchronous intracellular transport. Furthermore, a proper tertiary structure might be a prerequisite for sucrase–isomaltase to leave the Golgi apparatus.

Studies of the maturation behavior of viral spike glycoproteins have led to the hypothesis that proper folding and oligomerization are critical for the export of membrane proteins from the endoplasmic reticulum (ER)[1] (Copeland et al., 1986; Doms et al., 1988; Gething et al., 1986; Hurtley et al., 1989; Kreis & Lodish, 1986; Lodish, 1989). Oligomerization alone does not appear to be sufficient for this transport step (Doms et al., 1988) nor does a successful exit from the ER garantee an efficient transport to the cell surface (Guan et al., 1984; Hardwick et al., 1986; Hauri et al., 1985a; Machamer et al., 1985; Naim et al., 1988; Zilberstein et al., 1980).

We are interested in mechanisms underlying the intracellular transport of brush border enzymes in intestinal epithelial cells. Digestive hydrolases are the major membrane glycoproteins of the apical microvillar membrane of intestinal epithelial cells (Kenny & Maroux, 1982; Semenza, 1986; Hauri, 1988). Using Caco-2 cells as a model system, we previously reported on the biosynthesis of sucrase–isomaltase (SIM) and dipeptidylpeptidase IV (DPPIV) (Hauri et al., 1985b). In these cells, DPPIV is synthesized as a high-mannose protein doublet (110 and 114 kDa) which is converted to a mature complex glycosylated 124-kDa protein. SIM is synthesized as a 210-kDa high-mannose protein which is processed to a 217-kDa complex glycosylated form. In Caco-2 cells, SIM appears and stays as a single polypeptide in the brush border, while in the intestine in vivo the enzyme is cleaved into a sucrase and an isomaltase subunit in the brush border by pancreatic proteases (Hauri, 1988). Despite cleavage, the two subunits remain attached to each other by noncovalent interactions. Transport to the site of complex glycosylation is slow for SIM and fast for DPPIV. This asynchronism is due to at least two rate-limiting steps, a pre- and an intra-Golgi event (Stieger et al., 1988), which are neither related to differential trimming of N-linked carbohydrates (Matter et al., 1989) nor to oligomerization (Jascur et al., 1991).

In this study, conformation-specific antibodies and protease assays were used to monitor the conformational maturation of SIM and DPPIV. We present evidence that the kinetics of conformational maturation of the two proteins correlate with the kinetics of their intracellular transport. Furthermore, the SIM of two patients with congenital sucrase–isomaltase deficiency (CSID) having a transport block of SIM in the Golgi apparatus (Naim et al., 1988) displayed a conformation different from wild-type SIM.

Materials and Methods

Cell Culture, Transport Block, and Inhibition of N-Linked Oligosaccharide Processing. Caco-2 cells were grown as described (Hauri et al., 1985b). Metabolic labeling with [^{35}S]methionine was carried out with cells grown on nitrocellulose filters (Stieger et al., 1988). Treatment with 1-deoxymannojirimycin (Boehringer Mannheim, Germany) was performed as previously described (Matter et al., 1989). Protein exit from the ER was blocked by a chase in a N_2 atmosphere using N_2-pregassed cell culture medium (Merisko et al., 1986).

Antibodies. Four different monoclonal antibodies against SIM were used: HBB 2/614, HBB 3/705, HBB 1/691, and HBB 2/219 (Hauri et al., 1985b). The first two mAbs were used for immunoprecipitation of SDS-denatured SIM and the latter two for purifying the native enzyme. It is important to note that HBB 2/219 and HBB 1/691 are unable to recognize the SDS-denatured antigen. Native DPPIV was immunoprecipitated with HBB 3/775 (Hauri et al., 1985b) and HBB 3/456 (Matter et al., 1990). The two mAbs do not recognize the SDS-denatured antigen. A polyclonal antibody against denatured DPPIV (α-den-DPPIV) was produced as follows. DPPIV was immunoprecipitated from Caco-2 membranes using mAb HBB 3/775, and the immunoprecipitate was subjected to SDS–PAGE. The DPPIV band was cut out,

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[1] Abbreviations: DPPIV, dipeptidylpeptidase IV; SIM, sucrase–isomaltase; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; CSID, congenital sucrase–isomaltase deficiency; DMN, 1-deoxymannojirimycin; ER, endoplasmic reticulum.

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homogenized, and mixed with complete Freund's adjuvant. The resulting emulsion was subcutaneously injected into a New Zealand White rabbit. Booster injections with antigen in incomplete Freund's adjuvant were given 1 and 6 months later by the same route. One week after the second booster, the rabbit was sacrified, and the serum was harvested. This serum was specific for the SDS-denatured DPPIV but unable to recognize the Triton X-100 solubilized native enzyme.

Cell Extraction, Immunoprecipitation, and SDS–PAGE. Metabolically labeled cells were rinsed twice with and harvested in PBS. Each sample was split into appropriate aliquots, and the cells were pelleted in a microfuge. The supernatant was discarded, and the cells were homogenized either in 1 mL of 100 mM Na_2HPO_4 (pH 8.0) containing 1% Triton X-100 and 40 μg/mL PMSF or in 200 μL of 30 mM triethanolamine (pH 8.0) containing 5 mM Na_2EDTA, 100 mM NaCl, and 40 μg/mL PMSF (buffer A). To the latter homogenate was added 50 μL of 10% SDS, and the sample was immediately boiled for 15 min. After the sample was cooled on ice, 750 μL of buffer A containing 2.5% Triton X-100 (buffer B) was added. Both samples were left on ice for 45 min and centrifuged at 104000*g* for 45 min. SIM and DPPIV were immunoprecipitated from the Triton X-100 extracts as described (Hauri et al., 1985b). The immunopurification of the two enzymes from SDS extracts was essentially as described by Mostov and Blobel (1983). The mouse mAbs were adsorbed to protein A–Sepharose (Pharmacia) via a polyclonal rabbit anti-mouse linker antibody (Nordic). The polyclonal α-den-DPPIV serum was bound directly to protein A–Sepharose. The beads were incubated with SDS extract for at least 1.5 h at 4 °C on an end-over-end shaker and washed 6 times with buffer A supplemented with detergents to a final concentration of 1% Triton X-100 and 0.2% SDS (buffer C). In some experiments, the sample was denatured by adding 50 μL of 10% SDS to 500 μL of a Triton X-100 extract. After being boiled for 15 min, the extract was chilled to 4 °C, diluted with 750 μL of buffer B, and used for immunoprecipitation as described above. Immunoprecipitates were analyzed by SDS–PAGE (Laemmli, 1970), and labeled proteins were visualized by fluorography. Fluorographs were quantified as previously described (Stieger et al., 1988). Whenever ratios of native precipitated to denatured precipitated enzymes were calculated, the two samples originated from the same [^{35}S]-methionine-labeled filter culture.

Protease Assays. [^{35}S]Methionine-labeled cells were extracted with Triton X-100 as described above but omitting PMSF. The extracts were incubated at 37 °C, and after 10 min, proteases in PBS were added. Added volume, one tenth of the sample volume. Final concentrations of proteases: DPPIV, 0.1–1000 μg/mL trypsin (Worthington); SIM, 6 μg/mL trypsin, 3 μg/mL proteinase K (Serva) or 23 μg/mL elastase (Serva). The digestion was stopped after 30 min by transferring the samples to 0 °C and adding 5 μL of 40 μg/mL PMSF. After 30 min at 0 °C, the digests were either brought to 1 mL with the Triton X-100–phosphate buffer or denatured with SDS and subjected to immunoprecipitation.

Analysis of SIM from CSID Patients. Biopsy tissue of CSID patients 2 and 6 of Naim et al. (1988) was homogenized in a glass–Teflon potter. An aliquot of each homogenate (1.4 mg of protein) was adjusted to a volume of 300 μL with distilled water and solubilized by the addition of 200 μL of 0.5 M K_2HPO_4 (pH 7.4) containing 2% Triton X-100. After 45 min on ice, the samples were centrifuged at 104000*g* for 1 h; 400 μL of solubilized membranes was radioiodinated by adding 10 μL of $Na^{125}I$ (1 mCi) and 20 μL of 20 mg/mL chloramine T. After 2 min at room temperature, the reaction was stopped with 20 μL of 20 mg/mL $Na_2S_2O_5$. Protein-bound iodine was seperated from free iodine by Sephadex-G25 column chromatography using 100 mM Na_2HPO_4 (pH 8.0) containing 1% Triton X-100 and 40 μg/mL PMSF as an elution buffer. Immunoprecipitation of native and denatured SIM was as described above.

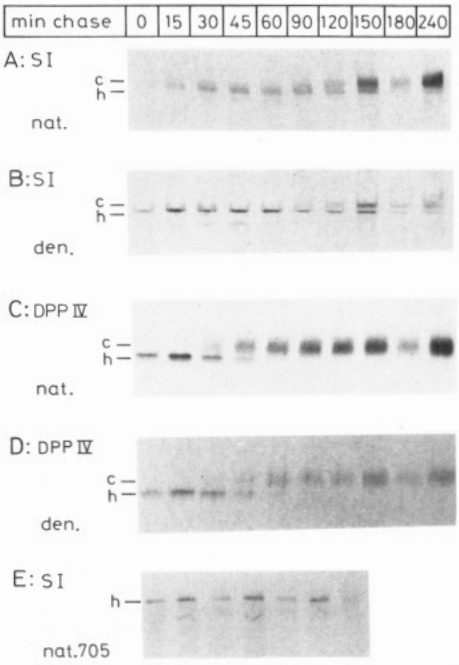

FIGURE 1: Immunoprecipitation of native and denatured antigens. Caco-2 cells were labeled with [^{35}S]methionine for 15 min and subsequently chased for the indicated intervals of time. The cells were harvested in PBS, and aliquots were then extracted either with Triton X-100 (A, C, E) or with SDS (B and D). These extracts were further subdivided into aliquots for immunoprecipitation. SIM and DPPIV were immunoprecipitated by using the following antibodies: (A) HBB 1/691 and HBB 2/219; (B) HBB 2/614 and HBB 3/705; (C) HBB 3/775 and HBB 3/456; (D) α-den-DPPIV serum; (E) HBB 3/705. Immunoprecipitates were analyzed by SDS–PAGE and fluorography. Note that this figure represents a single pulse–chase experiment; i.e., a given chase time represents one and the same culture in all the panels. h, high-mannose form; c, complex form; SI, SIM.

Results

Early Biosynthetic Forms of SIM and DPPIV Are Less Tightly Folded than Mature Hydrolases. Antibody and protease assays were established to probe for different conformations of early and late biosynthetic forms of SIM and DPPIV. The antibody assay is based on observations made in pulse–chase experiments. When Caco-2 cells were pulse-labeled with [^{35}S]methionine, chased for different intervals of time, and then solubilized with Triton X-100, the amount of SIM and DPPIV immunoprecipitable with antibodies specific for the native proteins increased with increasing times of chase (Figure 1A,C). When the cells were extracted by boiling in SDS and the two hydrolases were immunoprecipitated with antibodies against the denatured proteins, the amount of immunoprecipitated SIM and DPPIV remained constant (Figure 1B,D; Table I). Although immunoprecipitation under denaturing conditions was not 100% efficient, this constancy clearly indicated that the amount of immunoprecipitated denatured antigens is directly proportional to the total incorporation of [^{35}S]methionine into the two proteins. The results were the same irrespective of whether Triton X-100 extracts were boiled in SDS prior to immunoprecipitation or intact cells were directly extracted with SDS. This excludes the possibility that the increasing amount of immunopreci-

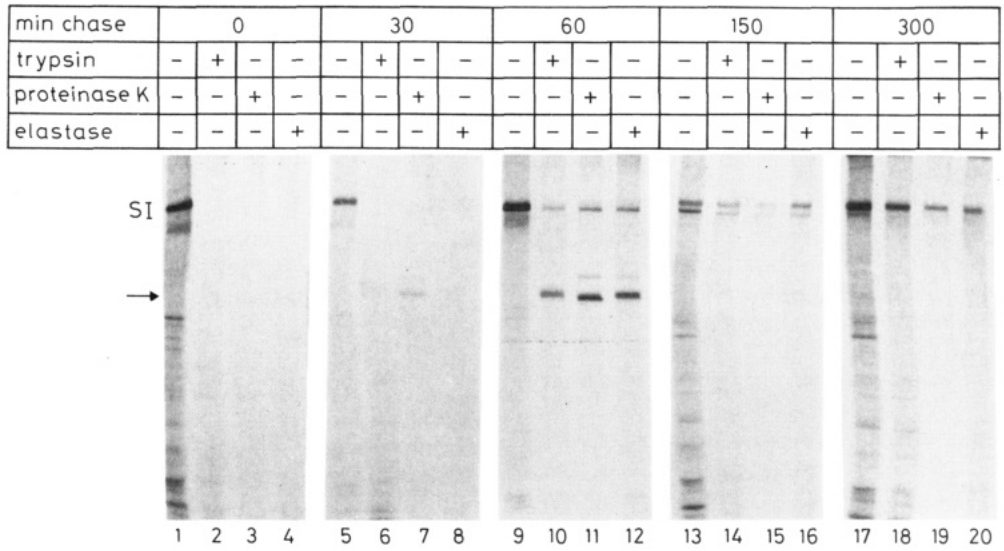

FIGURE 2: Protease sensitivity of SIM. Cells were metabolically labeled as in Figure 1. The Triton X-100 extracts were treated with the indicated proteases and subsequently denatured by boiling in the presence of SDS. SIM was immunoprecipitated by using mAbs HBB 3/705 and HBB 2/614 and analyzed by SDS–PAGE (7.5% gel). The arrow indicates the position of the main proteolytic fragment. The appearance of this fragment was monitored quantitatively in Figure 5A.

Table I: Immunoprecipitation of Denatured Antigens[a]

min chase	SIM ($x \pm 1$ SD)	DPPIV ($x \pm 1$ SD)
0	0.96 ± 0.29 ($n = 5$)	0.93 ± 0.28 ($n = 4$)
30	1.02 ± 0.13 ($n = 4$)	1.07 ± 0.20 ($n = 4$)
60	1.00 ± 0.25 ($n = 5$)	1.00 ± 0.15 ($n = 4$)
180	0.93 ± 0.28 ($n = 5$)	0.91 ± 0.25 ($n = 4$)
ON	1.03 ± 0.20 ($n = 5$)	1.09 ± 0.21 ($n = 3$)

[a] Caco-2 cells were labeled, chased, and boiled in SDS, and SIM and DPPIV were immunoprecipitated as described in Figure 1. In order to be able to compare different experiments, the value of a given chase time was first divided by the mean of all values obtained from a single experiment. These ratios derived from independent experiments (n) were used for calculating the above shown means (x) and the corresponding standard deviations.

pitable hydrolases is due to differential extraction properties of early and late biosynthetic forms as reported for influenza virus hemagglutinin (Skibbens et al., 1989). Figure 1 representing a single pulse–chase experiment shows some variation in band intensities. This is due to unequal [^{35}S]methionine incorporation of individual filter cultures which is inavoidable but does not affect the results because we directly compared the band intensities obtained with aliquots of the same homogenate after native and denaturing conditions of immunoprecipitation (see densitometric quantification below). Since both native SIM and DPPIV were immunopurified with two antibodies (either together or individually) which do not compete with each other (Hauri et al., 1985b; Matter et al., 1990), we conclude that at least two epitopes in both hydrolases undergo conformational changes.

Previously we have characterized mAb HBB 3/705 which recognizes high-mannose and complex glycosylated SIM under denaturing conditions. Surprisingly, under nondenaturing conditions of immunoprecipitation, only an early high-mannose form of this enzyme is bound by the antibody while later high-mannose and complex forms are no longer reactive (Hauri et al., 1985; also see Figure 1E). This suggests to us that at least one epitope of newly synthesized SIM has not acquired a mature conformation. We do not think that the loss of reactivity toward mAb HBB3/705 with time is due to posttranslational modifications since repeated freezing and thawing of mature SIM (having all the posttranslational modifications) already restore binding (K. Matter and H. P. Hauri, unpublished observation).

An alternative way to probe for the degree of folding of a protein is to test its sensitivity toward proteases. Newly synthesized high-mannose SIM was found to be highly sensitive to trypsin, proteinase K, and elastase (Figure 2, lanes 1–4) whereas complex glycosylated SIM was much more resistant to these proteases at the used concentrations (Figure 2, lanes 17–20). To our surprise, there was no direct rapid conversion from high to low sensitivity. Maximal resistance was acquired via a phase of intermediate sensitivity and occurred prior to complex glycosylation (Figure 2, lanes 9–12). Protease treatment of the intermediately sensitive high-mannose SIM (60 min of chase) resulted in a major fragment of about 120 kDa, whereas after 150 min of chase the high-mannose form showed maximal protease resistance (Figure 2, lanes 13–16). These results indicate that SIM undergoes a second conformational change before complex glycosylation.

The results with DPPIV were similar in one respect. Whereas the newly synthesized high-mannose enzyme was entirely degraded at intermediate and high trypsin concentrations, the complex glycosylated, mature protein was highly (albeit not completely) resistant to even high protease concentrations (Figure 3). In contrast to SIM, however, we never observed any fragments with DPPIV. It is well-known that less tightly folded proteins display increased protease sensitivity [e.g., see Eilers et al. (1988) and Vestweber and Schatz (1988)]. Overall these qualitative data suggest, therefore, that early biosynthetic forms of the two enzymes are less tightly folded than their mature forms.

Maturation to the Native Conformation Is Asynchronous. The aim of this study was to establish if there is a correlation between conformational maturation and efficiency of intracellular transport of brush border hydrolases to the site of complex glycosylation. For this purpose, the conformational changes of SIM and DPPIV were quantified by densitometric scanning of fluorographs and compared to the transport kinetics we previously established by subcellular fractionation (Stieger et al., 1988).

Figure 4 shows the folding kinetics of SIM (A) and DPPIV (B) as monitored by the antibody approach. DPPIV matures with similar kinetics to the conversion of high-mannose to complex oligosaccharide side chains. By use of the protease approach, the conformational maturation was inseparable from complex glycosylation (Figure 5B). This implicates that the

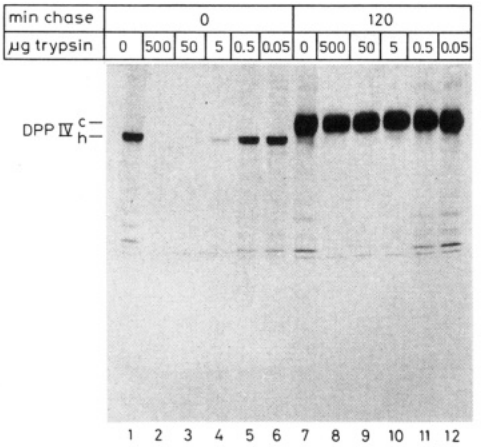

FIGURE 3: Trypsin sensitivity of DPPIV. Cells were labeled with [^{35}S]methionine for 15 min, chased as indicated, and extracted with Triton X-100. To 500 μL of extract was added the indicated amount of trypsin and incubated as described. Thereafter the digest was denatured by boiling in the presence of SDS. DPPIV was immunoprecipitated (α-den-DPPIV serum) and analyzed by SDS–PAGE (10% gel). h, high-mannose form; c, complex form.

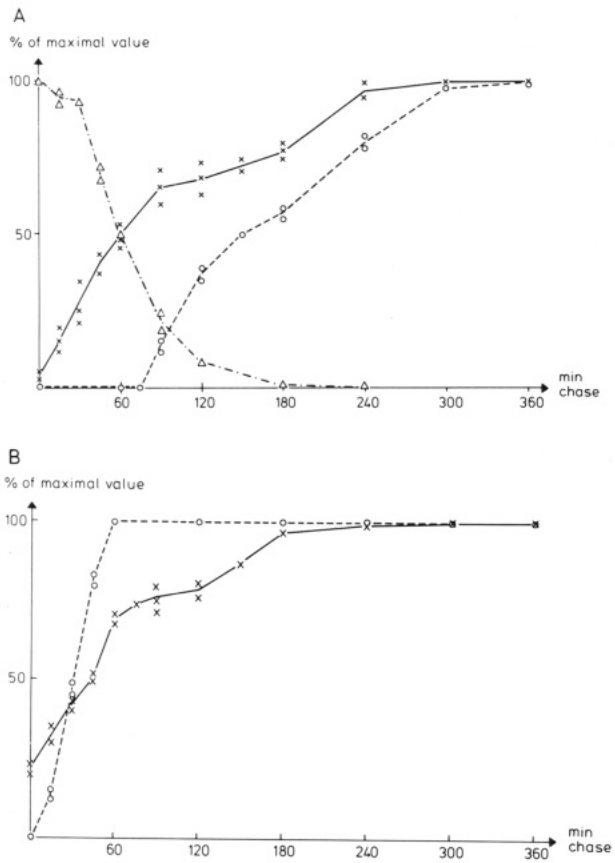

FIGURE 4: Conformational maturation monitored by conformation-specific antibodies. Pulse–chase experiments were performed as those in Figure 1. SIM (A) and DPPIV (B) were immunoprecipitated from Triton X-100 and SDS extracts and analyzed by SDS–PAGE. The resulting fluorographs were quantified, and the following ratios were calculated: (1) amount of immunoprecipitable antigen from Triton X-100 extracts divided by the amount obtained from SDS extracts (×); (2) amount of immunoprecipitable Triton X-100 solubilized SIM using mAb HBB 3/705 divided by the amount from SDS extracts (△); (3) percent of complex glycosylated protein (○), calculated from immunoprecipitations derived from SDS extracts. All values are given as percent of the corresponding maximal value.

two events occur simultaneously or very rapidly after one another. The conversion to complex glycosylated forms was found to occur at somewhat slower rates than previously reported (Hauri et al., 1985b; Stieger et al., 1988; Matter et al., 1989; Jascur et al., 1991). In these papers, the antigens were precipitated under nondenaturing conditions which slightly underestimates the early high-mannose forms while in immunoprecipitations under denaturing conditions, as used in the present study, the high-mannose and the complex glycosylated forms are recognized with the same efficiency.

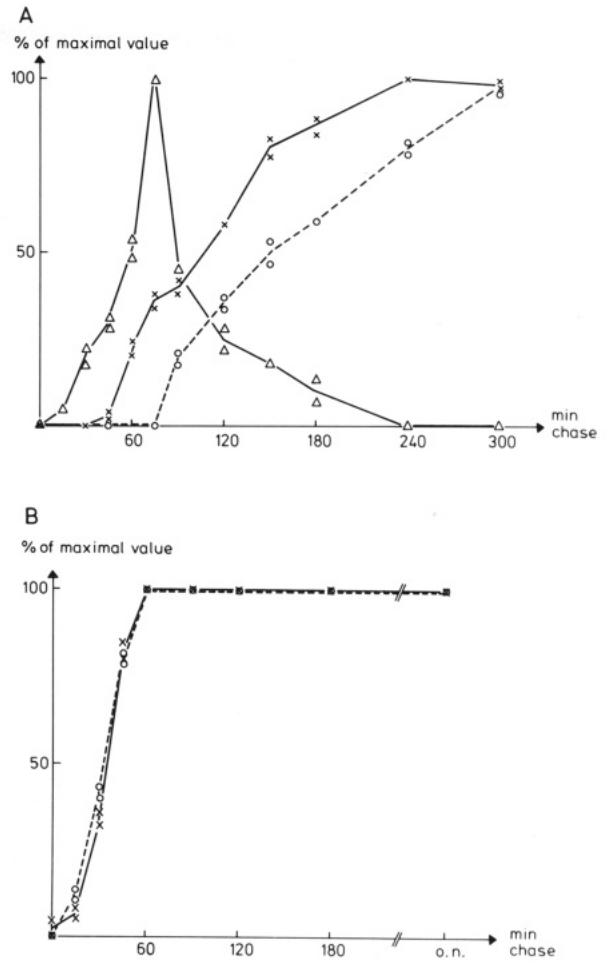

FIGURE 5: Conformational maturation monitored by differential protease sensitivity. Cells were labeled and extracted as described in Figures 2 and 3. SIM (A) was digested with proteinase K (3 μg/mL) and DPPIV (B) with trypsin (100 μg/mL). The denatured brush border enzymes were immunoprecipitated and analyzed by SDS–PAGE, and the resulting fluorographs were quantified. The following ratios were calculated: (1) the amount of intact antigen derived from digested divided by the amount derived from undigested samples (×); (2) the amount of SIM fragment (indicated by the arrow in Figure 2) divided by the amount of totally immunoprecipitated SIM from control samples (△); (3) percent of complex glycosylation of denatured immunoprecipitated antigen (○). All values are given as percent of the corresponding maximal value.

A surprising finding is the observation that more complex glycosylated DPPIV was immunoprecipitable from protease-treated Triton X-100 extracts as compared to control samples (Figure 6A). Since this phenomenon is transient in nature (Figure 6B) and no proteolysis of DPPIV was detectable, it may reflect a transient association of DPPIV with another protein. Unfortunately, we were unable to directly demonstrate such an association. This finding explains, however, why the two curves in Figure 4B cross each other and the amount of immunoprecipitable native DPPIV still increases after 1 h of chase when complex glycosylation and conversion to protease resistance are already completed.

In contrast to DPPIV, the antibody (Figure 4A) as well as the protease approach (Figure 5A) revealed at least two maturation steps during the intracellular transport of SIM.

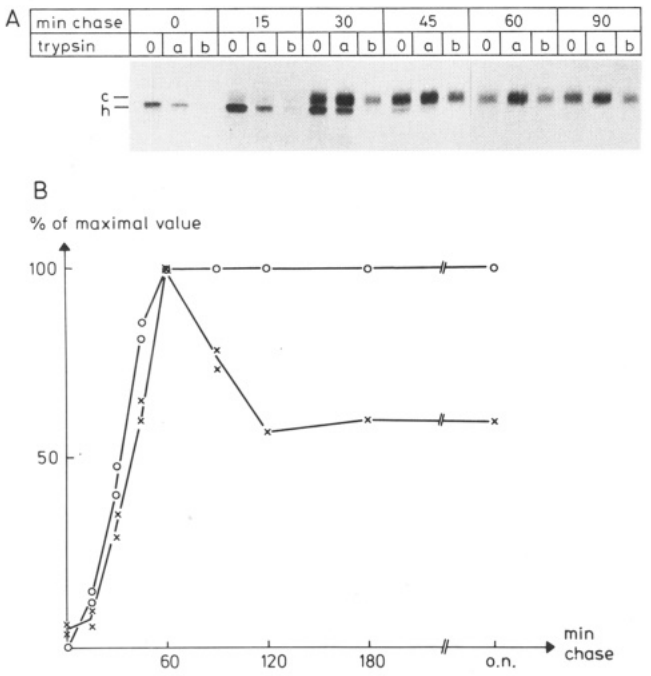

FIGURE 6: Immunoprecipitation of native DPPIV from digested extracts. The experiment was performed as in Figure 5B, but instead of denaturing the digests native DPPIV was immunoprecipitated with the mAbs HBB 3/775 and HBB 3/456. (A) Extracts were digested with either 10 μg/mL (a) or 100 μg/mL trypsin (b). h, high-mannose form; c, complex form. (B) The following ratios were calculated: the amount of immunoprecipitated DPPIV from digested (10 μg/mL trypsin) divided by the amount obtained from undigested samples (×) and the percent of complex glycosylated protein (○). All values are given as percent of the maximal value.

SIM is synthesized as an extremely protease-sensitive protein which is efficiently precipitated by antibody HBB 3/705 but inefficiently by antibodies against the native enzyme. In a first step, SIM is converted into a protein that is only weakly recognized by mAb HBB 3/705 but increasingly well with anti-native SIM antibodies. Remarkably, the latter process starts immediately after synthesis whereas the former has a lag time of 30 min. At 30 min of chase, proteolytic fragments beging to appear after protease treatment, suggesting that at least one domain is already tightly folded. In a second step, SIM is converted from an intermediate to a low protease-sensitive protein that is efficiently precipitable by anti-native SIM mAbs but not at all by HBB 3/705. Both steps of maturation precede the conversion to the complex glycosylated form.

Previous subcellular fractionation studies (Stieger et al., 1988) revealed that the high-mannose form of SIM in the Golgi apparatus reaches maximal levels at 75 min of chase exactly when the levels of the proteolytic fragment are maximal as shown in the present study. This suggests that the second step of conformational maturation might occur in the Golgi apparatus.

Protease Resistance Is Not Due to Processing of N-Linked Carbohydrates. It was previously shown that the Golgi mannosidase I inhibitor 1-deoxymannojirimycin (DMN) inhibits the conversion of high-mannose SIM and DPPIV to the endoglycosidase H resistant forms (Matter et al., 1989). To test whether the processing of N-linked carbohydrates confers protease resistance in the Golgi apparatus, we analyzed the conformation of SIM and DPPIV in cells that were pretreated with and chased in the presence of DMN. DMN was found to have no significant effect on the conformational maturation as assessed with anti-native mAbs (Figure 7A). The protease assay led to similar results (Figure 7B). The reduction in band

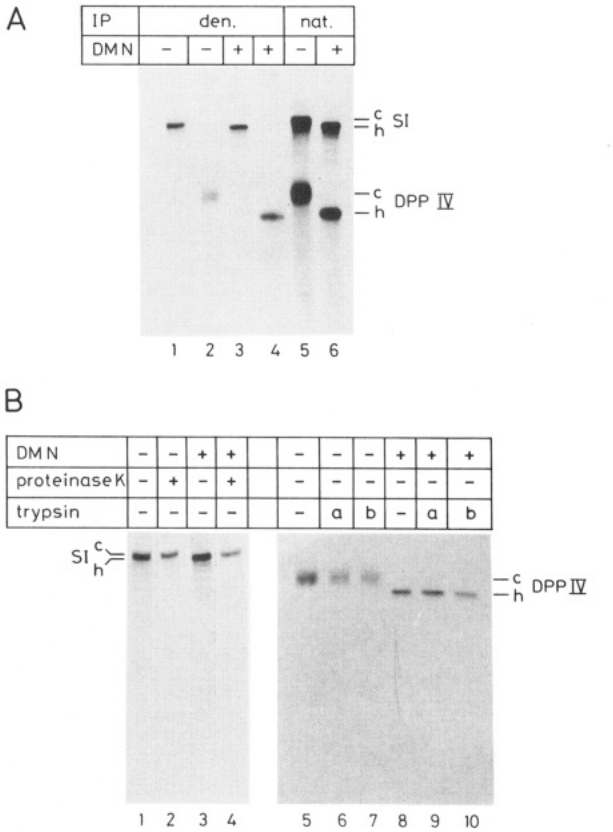

FIGURE 7: Effect of N-linked carbohydrate trimming on maturation. Cells preincubated in the presence or absence of DMN were labeled with [^{35}S]methionine for 15 min and chased for 5 h. (A) The cells were extracted either with Triton X-100 or with SDS. SIM and DPPIV were immunoprecipitated with antibodies specific for the denatured (lanes 1–4; SIM and DPPIV were precipitated individually) or native (lanes 5 and 6; SIM and DPPIV were precipitated together) antigens. (B) Triton X-100 extracts were digested with proteases (DPPIV with trypsin: a, 10 μg/mL; b, 100 μg/mL) and immunoprecipitated after denaturing the antigens by SDS and heat. h, high-mannose form; c, complex form; SI, SIM.

intensity after protease treatment is in the same range irrespective of the presence or absence of DMN (compare the reduction from lane 5 to lane 7 with that from lane 8 to lane 10, or the reduction from lane 5 to lane 6 with that of lane 8 to lane 9). In this particular experiment, the reduction of the SIM band after protease treatment was somewhat higher in the presence of DMN than in its absence, but it is clear that SIM is not completely sensitive to protease when complex glycosylation is inhibited by DMN nor does the major fragment appear.

We conclude, therefore, that protease resistance is not due to complex glycosylation and that the conformational maturation of SIM and DPPIV does not appear to be induced by the processing of N-linked oligosaccharides. Since the earliest forms of both hydrolases are already deglucosylated (Matter et al., 1989), it was not necessary to perform these experiments also with a glucosidase inhibitor.

It cannot be excluded, however, that another posttranslational modification is involved in brush border enzyme maturation. For example, it is known that in Caco-2 cells SIM and DPPIV also carry O-linked carbohydrates (Matter et al., 1989). SIM harbors its sites for O-linked glycosylation mainly in the membrane stalk (Hunziker et al., 1986; Green et al., 1987). This renders it less likely that O-glycosylation is the underlying cause of the two-step maturation to protease-resistant SIM. Another potential modification is sulfation. Danielsen (1987) has indeed shown that some brush border

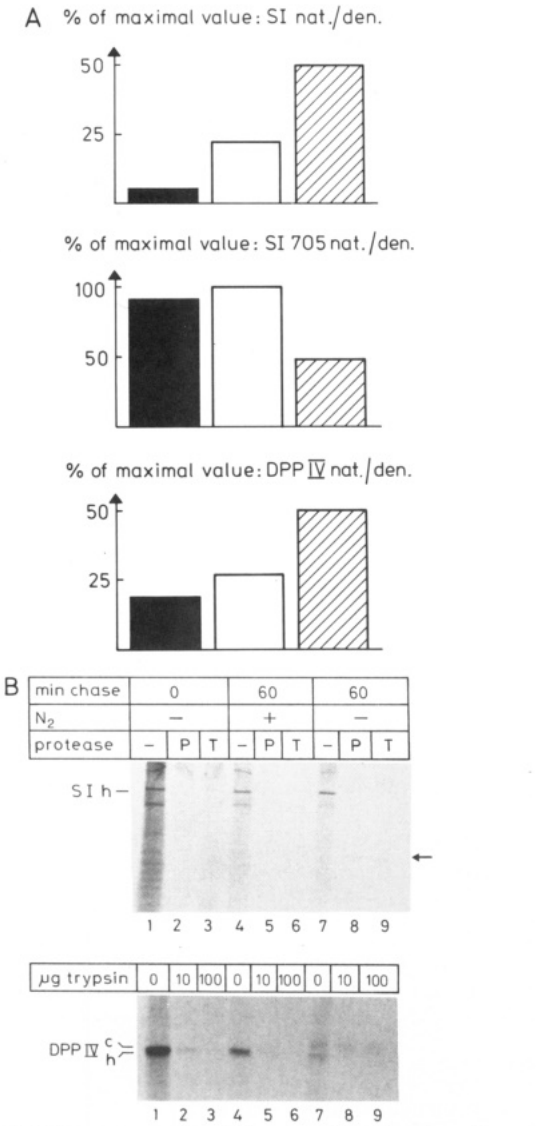

FIGURE 8: Inhibition of protein exit from the ER. Caco-2 cells were pulse-labeled for 5 min with [^{35}S]methionine and chased for 1 h in a CO_2/O_2 atmosphere (hatched bars) or in a N_2 atmosphere (open bars) or directly extracted with detergent (black bars). Thereafter the experiment was continued as in Figure 1 (A) or as in Figures 2 and 3 (B). nat., native antigen; den., denatured antigen; P, proteinase K; T, trypsin; h, high-mannose form; c, complex form; SI, SIM. The arrow in (B) indicates the position of the proteolytic fragment of SIM. The values represent the mean of two experiments.

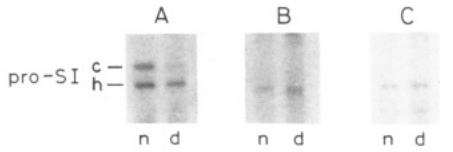

FIGURE 9: Immunoprecipitation of native and denatured SIM from biopsies of CSID patients. Homogenized biopsies derived from case 2 (B), from case 6 (C), or from a control subject (A) were solubilized with Triton X-100, and the extracts were radioiodinated. The radiolabeled SIM was immunoprecipitated under native conditions with mAbs HBB 1/691 and HBB 2/219 or first denatured with SDS and heat prior to immunoprecipitation with mAbs HBB 2/614 and HBB 3/705. Immunoprecipitates were analyzed by SDS–PAGE and autoradiography. Shown are only those regions of gels which contain pro-SIM. n, native; d, denatured; c, complex form; h, high-mannose form; SI, SIM.

hydrolases are sulfated at tyrosine residues. Tyrosine sulfation, however, is a trans-Golgi event (Baeuerle & Huttner, 1987) and therefore cannot account for the apparent conformational changes revealed by antibodies and proteases.

Maturation of SIM and DPPIV in the Endoplasmic Reticulum. A comparison of the kinetics of the conformational maturation steps with the known transport kinetics to and through the Golgi apparatus suggests that some maturation steps may occur at a post-ER stage. In order to determine more precisely which events occur in the ER, we blocked protein exit from this organelle by chasing the pulse-labeled cells in a N_2 atmosphere for 1 h. Under these conditions, DPPIV remained in the high-mannose form and did not acquire protease resistance. DPPIV of control cells was converted to the complex glycosylated, protease-resistant protein within 1 h (Figure 8B, lower panel). A similar result was obtained when DPPIV of N_2-treated cells was probed with conformation-specific antibodies (Figure 8A). The ratio of native to denatured immunoprecipitable DPPIV was higher in control cells chased for 1 h in CO_2/air than in cells which were not chased, or chased for 1 h in a N_2 atmosphere. After a chase under N_2, the antibodies specific for the native protein precipitated somewhat more DPPIV than in cells not chased at all. This may reflect a very early stage of maturation, but other interpretations cannot been ruled out at present. Nevertheless, this experiment indicates that DPPIV arrested in the ER exhibits conformational properties similar to the newly synthesized protein.

For SIM, the results were somewhat different. After a chase in a N_2 atmosphere, less SIM was immunoprecipitable with anti-native SIM mAbs as compared to the CO_2/air controls, but it was more than from nonchased cells. On the other hand, HBB 3/705 precipitated SIM from N_2-treated and from nonchased cells with equal efficiency. When SIM was blocked in the ER by N_2, it was not able to acquire the partially protease-resistant conformation (Figure 8B, upper panel). These data suggest that HBB 3/705 is specific for SIM as long as the enzyme remains in the ER, but the first conformational changes of SIM already occur in the ER as revealed by the anti-native SIM mAbs. This is in line with the results of Figure 4A which showed increasing binding of the anti-native SIM mAbs before the recognition by HBB 3/705 drops. Furthermore, partial protease resistance is a post-ER event since it is prevented in N_2-treated cells. The conversion to partial protease resistance was also prevented by CCCP, a well-known inhibitor of protein exit from the ER, or by a chase at 15 °C, which leads to a transport block in a post-ER pre-Golgi compartment (Saraste & Kuismanen, 1984) (not shown). We conclude therefore that the conformational change leading to partial protease resistance occurs in the cis-Golgi.

Overall we can differentiate between four conformational SIM variants: (1) the newly synthesized ER form which is protease sensitive and efficiently recognized by HBB 3/705 but inefficiently by anti-native SIM mAbs; (2) a second, more mature ER form which is still completely protease sensitive and efficiently recognizable by HBB 3/705 but has an increased affinity for anti-native SIM mAbs; (3) an early immature Golgi form that displays intermediate protease resistance as revealed by the appearance of a proteolytic fragment; and (4) a later mature Golgi form with increased resistance to proteases, that is efficiently precipitable with anti-native SIM mAbs, is not recognized by HBB 3/705, but has not yet undergone complex glycosylation.

Transport-Deficient Mutants of SIM Do Not Attain Mature Conformation in Congenital Sucrase–Isomaltase Deficiency. If conformational changes are indeed important for intracellular protein transport, one would predict that transport-deficient mutant proteins must be conformationally immature. Recently, several naturally occurring SIM mutants

have been found in biopsy tissue from patients suffering from congenital sucrase–isomaltase deficiency (CSID) (Hauri et al., 1985a; Lloyd & Olsen, 1987; Naim et al., 1988). In some of these cases, SIM is unable to migrate to the brush border membrane. Unfortunately, it was not possible to reinvestigate all of these cases, but we have analyzed two of them [cases 2 and 6 of Naim et al. (1988)]. In both cases, electrophoretically normal SIM is synthesized but arrested in the Golgi apparatus and does not undergo complex glycosylation.

Homogenized biopsies were solubilized with Triton X-100, and the extracts were radioiodinated. The radiolabeled extracts were immunoprecipitated with mAbs against the native protein or denatured by boiling in the presence of SDS followed by immunoprecipitation with antibodies against the denatured protein. In both cases, the ratio of native to denatured immunoprecipitated antigen was reduced as compared to the control (Figure 9), suggesting that SIM was unable to adopt a mature conformation. Due to the low amount of material at our disposition, it was not possible to also analyze the protease sensitivity. Naim et al. (1988) have reported that mAbs HBB 1/691 and HBB 2/219 are unable to recognize SIM of case 2 while we now find some reduced reactivity with these antibodies. This discrepancy is probably due to a higher specific radiolabeling achieved in the present study. That the mutant forms of SIM do not accumulate in the Golgi may be due to degradation of the improperly folded and arrested proteins. The immature conformation of SIM in the two patients is in line with the hypothesis that a proper tertiary structure is required for efficient passage through the Golgi apparatus and to exit this organelle.

Discussion

In this paper, we provide evidence that the newly synthesized brush border enzymes SIM and DPPIV undergo conformational changes at various steps of the exocytic pathway as probed by conformation-specific antibodies and proteases.

The antibody approach is based on the observation that the affinity of the two enzymes for antibodies against the native conformation increases with time of chase whereas the amount of enzyme precipitated under denaturing conditions remains constant. Therefore, the ratio of antigen precipitated with anti-native antibodies to antigen precipitated with anti-denatured antibodies indicates the degree of conformational maturation. To circumvent the problem of unequal [^{35}S]-methionine incorporation, this ratio was always calculated from samples of the same homogenate. The second approach was the measurment of changes in protease sensitivity. Trypsin was found most suitable for DPPIV, and three different proteases revealed three SIM variants. It has recently been shown that incompletely folded and mature proteins can be distinguished on the basis of protease sensitivity (Geering et al., 1987; Williams et al., 1988).

Collectively our results led to a complex picture of maturation for SIM. The conformational maturation of SIM occurs slowly. SIM is synthesized as a highly protease-sensitive protein that is very inefficiently recognized by antibodies against the native conformation. Within 30 min of chase, the binding efficiency increases from 5% to 30% maximal reactivity. This increase is the first recognizable sign of a conformational change. However, as long as SIM remains in the ER, it keeps its original conformation in respect to two parameters: its binding efficiency to mAb HBB 3/705 and its protease sensitivity. Under conditions allowing SIM to exit from the ER, a third SIM form appears with intermediate protease sensitivity (i.e., proteolytic fragments appear) and increased affinity for anti-native antibodies (i.e., about 70% of mature SIM). On the other hand, this SIM form only poorly reacts with mAb HBB 3/705. The third SIM form was not observed when protein exit from the ER was blocked by anoxia or CCCP. Since certain conformational changes are energy dependent (Doms et al., 1987), one might argue that ATP depletion rather than blocked protein exit may inhibit this maturation step. This is unlikely since the cells chased at 15 °C, which leads to protein accumulation in a post-ER compartment (Saraste & Kuismanen, 1984), the maturation to the third SIM form did not occur. Strikingly, the time of maximal accumulation of the proteolytic fragments coincides with that of maximal accumulation of high-mannose SIM in a fraction enriched in Golgi membranes [75 min of chase; see Stieger et al. (1988)], suggesting that the third SIM form is localized in the cis-Golgi. Moreover, the appearance of proteolytic fragments suggests that some domains of SIM are already tightly folded in the cis-Golgi. It is possible that these fragments represent sucrase and isomaltase subunits. The relationship between folding and protease sensitivity has elegantly been documented for mitochondrial proteins (Eilers et al., 1988; Vestweber & Schatz, 1988). Finally, SIM is converted to a mature form in respect to antibody binding and protease sensitivity. It is important to note that this maturation step precedes complex glycosylation. We believe that it occurs in the medial Golgi.

DPPIV exhibits a much simpler maturation behavior. Only two structural variants were found, an early completely trypsin-insensitive form and a mature form with high trypsin resistance. The latter form is more efficiently precipitated by anti-native mAbs than the former. The conversion to protease resistance was contemporaneous with complex glycosylation and did not take place in ATP-depleted cells. These results are strong evidence that the observed event occurs in the Golgi apparatus. Interestingly, this conformational maturation preceded dimerization which occurs immediately after complex glycosylation (Jascur et al., 1991). This sequence of events is plausible since one would expect a protein to acquire a proper tertiary structure before oligomerization. Because DPPIV is transported much more rapidly to and through the Golgi apparatus, it is possible that the resolution of the used techniques is insufficient to reveal a stepwise maturation as found for SIM. Indeed, the fact that DPPIV in cells chased under anoxia has a slightly higher affinity for the anti-native mAbs than in cells which were pulse-labeled for only 5 min may indicate such subtle changes.

An unexpected finding was that in a certain time window of chase the native DPPIV was more efficiently immunoprecipitated from trypsin-digested than from undigested Triton X-100 extracts. Since the electrophoretic behavior was not altered by the protease treatment, the most likely interpretation is that DPPIV is temporarily associated with another protein which interfers with the antibody recognition. This event occurs at a time when DPPIV is expected to leave the Golgi apparatus on the basis of subcellular fractionation. We were not able, however, to provide direct experimental evidence for such an interaction.

It is interesting to discuss the described conformational maturation in respect to the asynchronous transport of SIM and DPPIV to and through the Golgi complex (Stieger et al., 1988) and in context of the importance of proper folding for the intracellular transport of viral proteins [Copeland et al., 1986, 1988; Doms et al., 1988; Gething et al., 1986; Hardwick et al., 1986; Schuy et al., 1986; for a review, see Hurtley and Helenius (1989)]. An attractive hypothesis is that SIM does not leave the ER efficiently since its folding to an exit-com-

petent conformation (the second conformational variant) occurs slowly. On the other hand, this process may be fast for DPPIV, leading to the observed rapid transport to the Golgi apparatus. The finding that SIM does not oligomerize at all and DPPIV only in a late Golgi compartment (Jascur et al., 1991) points to the possibility that conformational maturation of the tertiary structure rather than oligomerization is essential for the exit of SIM and DPPIV from the ER. Thus, brush border hydrolases are different from enveloped viral glycoproteins which oligomerize in the ER (Copeland et al., 1986; Gething et al., 1986; Hardwick et al., 1986; Kreis & Lodish, 1986; Schuy et al., 1986). Differential folding in the ER might explain the first rate-limiting step in the intracellular transport of brush border hydrolases (Stieger et al., 1988). Our finding of continuing conformational maturation in the Golgi apparatus may offer an explanation why exit from the ER does not guarantee successful transport all the way to the cell surface (Guan et al., 1986; Hardwick et al., 1986; Hauri et al., 1985a; Naim et al., 1988; Zilberstein et al., 1980). Furthermore, it is tempting to speculate that the second rate-limiting step for the transport of SIM to the trans-Golgi is related to a slow conversion from the intermediate to the high protease-resistant conformation.

If the hypothesis that protein folding and intracellular transport are causally related is correct, one would predict that transport-arrested brush border enzymes in naturally occurring mutant phenotypes are conformationally immature. CSID in humans is the only brush border disease in which this prediction can be tested at the present time. It has been shown that in several cases of CSID SIM is synthesized as a protein with an electrophoretically normal molecular weight but is unable to reach the cell surface (Hauri et al., 1985a; Lloyd & Olsen, 1987; Naim et al., 1988). In one of these patients, SIM accumulated as an enzymatically barely active high-mannose molecule that was efficiently recognized by mAb HBB 3/705, and it was suggested that this abnormal SIM was arrested in the ER. The preservation of the HBB 3/705 immunoreactivity in Caco-2 cells by manipulations that prevent protein exit from the ER supports this suggestion. Unfortunately, it was not possible to reinvestigate this patient. However, we have analyzed the conformation of SIM in two other CSID patients with impaired transport at the level of the Golgi apparatus. In both patients, no SIM was transported to the brush border, and no complex glycosylated SIM forms were found (Naim et al., 1988). Here we showed that the transport-defective SIM of these mutants was not properly folded on the basis of its reduced affinity to anti-native mAbs. This result is in line with the notion that proper folding might be a prerequisite for SIM to undergo complex glycosylation and efficient transport to the brush border membrane.

While all our data fit with our proposal that conformational changes are related to the efficiency of intracellular protein transport, a direct causal relationship awaits further experimental proof. The precise function of protein folding for the transport of SIM and DPPIV may be revealed more clearly by extending the analysis of transport-deficient mutant phenotypes.

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