Vieira, J., & Messing, J. (1982) *Gene 19*, 259–268.
Viñas, O., Powell, S. J., Runswick, M. J., Iacobazzi, V., & Walker, J. E. (1990) *Biochem. J. 265*, 321–326.
von Heijne, G. (1986) *EMBO J. 5*, 1335–1342.
Walker, J. E., Gay, N. J., Powell, S. J., Kostina, M., & Dyer, M. R. (1987a) *Biochemistry 26*, 8613–8619.
Walker, J. E., Cozens, A. L., Dyer, M. R., Fearnley, I. M., Powell, S. J., & Runswick, M. J. (1987b) *Chem. Scr. 27B*, 97–105.
Walker, J. E., Powell, S. J., Viñas, O., & Runswick, M. J. (1989) *Biochemistry 28*, 4702–4708.
Wolstenholme, D. R., McFarlane, J. R., Okimoto, R., Clary, D. O., & Wahleithner, J. A. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 1324–1328.

Oligomerization and Intracellular Protein Transport: Dimerization of Intestinal Dipeptidylpeptidase IV Occurs in the Golgi Apparatus†

Thomas Jascur, Karl Matter,‡ and Hans-Peter Hauri*
Department of Pharmacology, Biocenter of the University of Basel, CH-4056 Basel, Switzerland
Received July 16, 1990; Revised Manuscript Received October 18, 1990

ABSTRACT: It was postulated that newly synthesized membrane proteins need to be assembled into oligomers in the endoplasmic reticulum in order to be transported to the Golgi apparatus. By use of the differentiated human adenocarcinoma cell line Caco-2, the general validity of this proposal was studied for small intestinal brush border enzymes which are dimers in most mammalian species. Chemical cross-linking experiments and sucrose gradient rate–zonal centrifugation revealed that dipeptidylpeptidase IV is present as a dimer in the brush border membrane of Caco-2 cells whereas the disaccharidase sucrase–isomaltase appears to be a monomer. Dipeptidylpeptidase IV was found to dimerize immediately after complex glycosylation, an event associated with the Golgi apparatus. Dimerization of this enzyme was inhibited by CCCP but did not depend on complex glycosylation of N-linked carbohydrates as assessed by the use of the trimming inhibitor 1-deoxymannojirimycin. It is concluded that dimerization of dipeptidylpeptidase IV occurs in a late Golgi compartment and therefore cannot be a prerequisite for its export from the endoplasmic reticulum.

The quaternary structure of integral plasma membrane proteins has become of central interest in the study of protein transport to the cell surface. From work with viral envelope proteins, the concept has emerged that assembly of these proteins into oligomers takes place in the endoplasmic reticulum (ER) and may be a prerequisite for their export from the ER and subsequent transport to the Golgi apparatus (Copeland et al., 1986, 1988; Gething et al., 1986; Kreis & Lodish, 1986; Doms et al., 1987, 1988; Boulay et al., 1988). Similar results have been obtained for endogenous membrane proteins of complex heterooligomeric structure [for a review, see Hurtley and Helenius (1989)]. However, one study is at variance with these general conclusions (Yewdell et al., 1988).

The biosynthesis of small intestinal brush border enzymes has been the subject of numerous investigations over the past years (Kenny & Maroux, 1982; Semenza, 1986; Hauri, 1988). These proteins are major constituents of the intestinal microvillus membrane and are involved in the terminal digestion of dietary carbohydrates and peptides. Some of them, including dipeptidylpeptidase IV (DPPIV)[1] and sucrase–isomaltase (SIM), are expressed in the differentiated human intestinal epithelial cell line Caco-2 (Pinto et al., 1983; Hauri et al., 1985). The enzymes are cotranslationally N-glycosylated to give the high-mannose precursors of 110 and 114 kDa for DPPIV, and 210 kDa for SIM. In the Golgi apparatus, they are processed to the complex glycosylated forms of 124 and 217 kDa, respectively. The intracellular transport of newly synthesized DPPIV and SIM in Caco-2 cells has been extensively studied. DPPIV is transported to the brush border membrane considerably faster than SIM, two rate-limiting steps being export from the ER and transit through the Golgi (Stieger et al., 1988).

Most brush border enzymes of rabbit, rat, and pig are known to exist as noncovalently linked homodimers in the microvillus membrane (Kenny & Maroux, 1982), but the intracellular site of their assembly into dimers is unknown. Therefore, the Caco-2 cell line expressing a number of human brush border hydrolases should provide a valuable system to assess the role of oligomerization for the intracellular transport of these endogenous plasma membrane proteins.

In the present paper, we provide evidence that in Caco-2 cells DPPIV is assembled into dimers in a late Golgi compartment and that transport through the Golgi apparatus, but not complex glycosylation, is required for dimerization of DPPIV. SIM, however, was found to be a monomer throughout its transport from the ER to the brush border

† Supported by the Swiss National Science Foundation.
* To whom correspondence should be addressed at the Department of Pharmacology, Biocenter of the University of Basel, Klingelbergstrasse 70, CH-4056 Basel, Switzerland.
‡ Present address: Department of Cell Biology, School of Medicine, Yale University, New Haven, CT 06510.

[1] Abbreviations: ApN, aminopeptidase N; DPPIV, dipeptidylpeptidase IV; SIM, sucrase–isomaltase; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; DMN, 1-deoxymannojirimycin; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; DSS, suberic acid bis(*N*-hydroxysuccinimide ester); DSP, 3,3′-dithiobis(propionic acid *N*-hydroxysuccinimide ester); EGS, ethylene glycol bis(succinimidyl succininate); MBS, *m*-maleimidobenzoic acid *N*-hydroxysuccinimide ester; DMS, dimethyl suberimidate.

0006-2960/91/0430-1908$02.50/0

membrane. We conclude that oligomerization is not a general prerequisite for export of membrane proteins from the ER.

Materials and Methods

Cell Culture, Metabolic Labeling, Immunoprecipitation, and SDS-PAGE. Caco-2 cell culture and labeling with [^{35}S]methionine were as described by Hauri et al. (1985) and Stieger et al. (1988). Immunoprecipitation of native antigens (Hauri et al., 1985) and of denatured antigens (Matter & Hauri, 1991) was described previously. For the analysis of native antigens, we used mAb HBB 3/775 against DPPIV, mAbs HBB 2/614, HBB 3/705, HBB 2/691, and HBB 2/219 against SIM, and HBB 3/153 specific for aminopeptidase N (ApN). Immunoprecipitates were analyzed by SDS-PAGE (Laemmli, 1970) and fluorography. Fluorographs were quantified as described (Stieger et al., 1988).

Chemical Cross-Linking. Proteins in membrane vesicles were cross-linked in 10 mM Na_2HPO_4-300 mM NaCl, pH 7.0, by adding either DSS or DSP (Sigma) to a final concentration of 0.875 mM. After 20 min at room temperature, the reaction was stopped with ethanolamine (45 mM final concentration). Triton X-100 extracts of Caco-2 cells were treated in the same way but in 100 mM Na_2HPO_4, 1% Triton X-100, and 40 μL/mL PMSF, pH 7.0. Cross-linking of DPPIV in intact cells was performed at 4 °C and achieved as follows. Labeled filter-grown cells were washed twice in PBS, and the chambers were disassembled. Thereafter the filters were transferred to a six-well plate containing Dulbecco's PBS, and the plate was placed on a horizontal shaker for 15 min. This rinsing step was repeated once with fresh Dulbecco's PBS. Then the cells were washed once with a triethanolamine buffer (10 mM triethanolamine, pH 7.4, 250 mM sucrose, and 2 mM $CaCl_2$) and transferred to fresh wells containing the cross-linker dissolved in the same buffer (DSS, 0.875 mM; DMS, 1 mM). After 30 min, the cells were washed twice for 15 min with the triethanolamine buffer containing additionally 50 mM ethanolamine. Thereafter the cells were harvested and solubilized with SDS. DPPIV was immunoprecipitated with a polyclonal antibody specific for the denatured antigen (Matter & Hauri, 1991).

In our search for SIM oligomers and high-mannose dimers of DPPIV, different conditions of solubilization and a number of different bifunctional reagents were tested. We do not report these experiments under Results since none of them modified the results obtained with the cross-linkers described above. For most of these experiments, a 10 mM triethanolamine–acetic acid buffer (pH 6.5–8.0) was used containing 20–300 mM NaCl and 1% detergent (Triton X-100, Nonidet P-40, octyl glucoside, $C_{12}E_8$, or CHAPS). In other experiments, Hepes, Tris, or phosphate buffers with different salt concentrations (NaCl or KCl; 20–400 mM) were used in conjunction with the different detergents. Yet in other experiments, the influence of divalent cations (Mg^{2+} and Ca^{2+}) and chelators (EDTA and EGTA) was studied. All these conditions (with the exception of the Tris and Hepes buffers) were combined with the cross-linking approach, and some were also tested by sucrose gradient centrifugation. Besides DSP and DSS, the following cross-linkers were used: dimethyl suberimidate, dimethyl 3,3′-dithiobis(propionimidate), 2-iminothiolane, bis(sulfosuccinimidyl suberate), 3,3′-dithiobis(sulfosuccinimidyl propionate), ethylene glycol bis(succinimidyl succinate), 1,5-difluoro-2,4-dinitrobenzene, *m*-maleimidobenzoyl-*N*-hydroxysulfosuccinimide ester, and 1,4-bis(maleimido)benzene. These cross-linkers vary in length from 3 to 16 Å and react with amino and sulfhydryl groups. Depending on their water solubility, these bifunctional agents were used at concentrations of 0.5–5 mM. None of these experiments gave an indication for the presence of oligomers other than those reported under Results.

Sucrose Gradient Rate-Zonal Centrifugations. Caco-2 cells grown on Millipore filters (Stieger et al., 1988) were labeled with 200 μCi of [^{35}S]methionine for various time periods and solubilized in 400 μL of 100 mM Na_2HPO_4, 1% Triton X-100, and 40 μg/mL PMSF, pH 7.0, for 1 h. After a 105000g_{av} 1-h spin, 350 μL of the supernatant was loaded onto 11.5-mL gradients [100 mM Na_2HPO, 1% Triton X-100, 40 μL of PMSF, and 5–20% (w/v) sucrose, pH 7.0] which had been formed with an LKB 11300 Ultrograd gradient mixer. The gradients were centrifuged in a Kontron 41.14 swingout rotor at 40 000 rpm (205000g_{av}) for 18 h at 8 °C. Each gradient was split into 18 fractions which were diluted with 0.5 mL of 100 mM Na_2HPO_4, 1% Triton X-100, and 40 μL/mL PMSF, pH 8.0, and analyzed by immunoprecipitation followed by SDS-PAGE.

Alternatively, sucrose gradients were run as follows. Labeled Caco-2 cells were harvested in solubilization buffer (10 mM triethanolamine, pH 7.2, 150 mM NaCl, 40 μg/mL phenylmethanesulfonyl fluoride, and 1% detergent). The experiment shown in this paper was performed with 1% Nonidet P-40. After 30 min on ice, the samples were spun in a Eppendorf centrifuge for 15 min, and the supernatants were loaded on continuous sucrose gradients (5–20% sucrose in 10 mM triethanolamine, pH 7.2, and 150 mM NaCl). The gradients were centrifuged in an SW56-Rotor (Beckman) at 45 000 rpm (198000g_{av}) for 6 h at 4 °C. Thereafter the gradients were divided into 12 equal fractions. These samples were boiled with SDS, and DPPIV was immunoprecipitated with a polyclonal antibody (Matter & Hauri, 1991).

Other Methods. The use of 1-deoxymannojirimycin (DMN) was as in Matter et al. (1989), the preparation of a crude membrane fraction was as described (Hauri et al., 1985), and Golgi membranes of Caco-2 cells were prepared according to Stieger et al. (1988). To inhibit intracellular protein transport, the Caco-2 cells were labeled with [^{35}S]methionine at 37 °C for 5 min, washed twice with ice-cold Dulbecco's PBS, and transferred to fresh Dulbecco's PBS containing 300 μM CCCP. After 10 min on ice, the cells were chased for 1 h at 37 °C (chase medium: Dulbecco's PBS–300 μM CCCP).

Results

The quaternary structure of DPPIV was probed by chemical cross-linking. A crude membrane fraction was prepared from Caco-2 cells after overnight labeling with [^{35}S]methionine. The membranes were cross-linked with the homobifunctional lysine-specific reagents disuccinimidyl suberate (DSS) or dithiobis(succinimidyl propionate) (DSP). DSP is an analogue of DSS which can be cleaved by reducing agents such as DTT. After cross-linking, the membranes were solubilized with Triton X-100, and DPPIV was immunoprecipitated and analyzed by SDS-PAGE in the presence or absence of DTT (Figure 1A). Cross-linking with DSS resulted in the appearance of a high-molecular weight band of about 210K. Cross-linking with DSP gave the same result as with DSS when analyzed under nonreducing conditions. When the samples were cross-linked with DSP and reduced with DTT after immunoprecipitation, all DPPIV-containing complexes were cleaved. Under these conditions, the only visible band was the DPPIV monomer, suggesting that the high molecular weight band represented the dimer of DPPIV. The complex is not due to disulfide bonds since DPPIV of control samples which were denatured without DTT migrated as a monomer on SDS gels (Figure 1A). These experiments suggest that

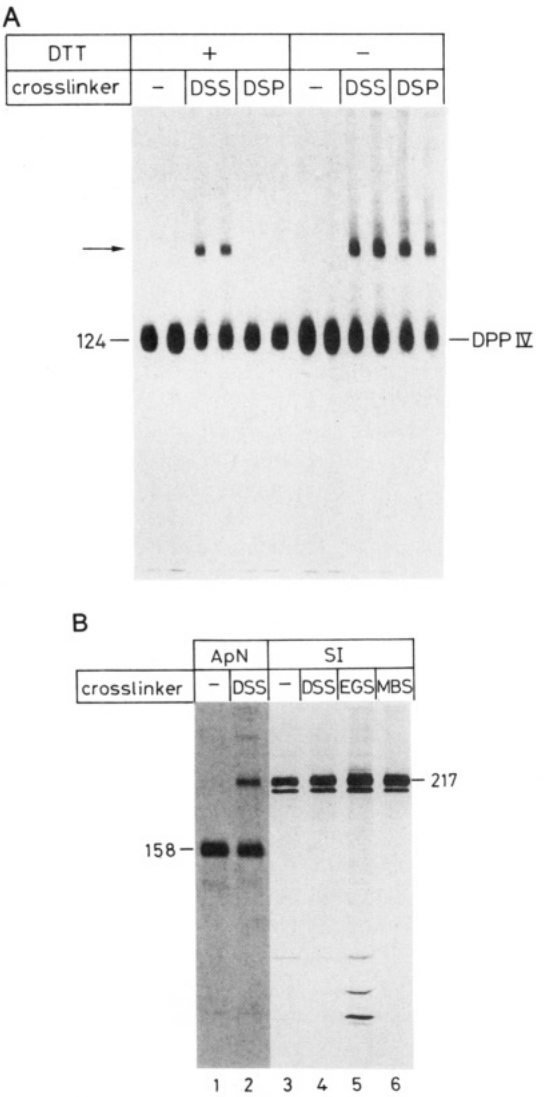

FIGURE 1: Chemical cross-linking of DPPIV (A), aminopeptidase N (ApN, B), and SIM (B) (fluorogram). After labeling of Caco-2 cells with [^{35}S]methionine for 4 h (SIM) or overnight (DPPIV and aminopeptidase N), a crude membrane fraction was prepared and cross-linked with DSS or DSP as indicated. The enzymes were immunoprecipitated with mAbs and analyzed by SDS–PAGE (4–10% gradient gels) under reducing (+DTT) and nonreducing (−DTT) conditions (A) or after reducing conditions only (B). The position of cross-linked DPPIV dimers is indicated by an arrow. Numbers at the margins of the gels indicate the molecular mass in kilodaltons. SI, SIM.

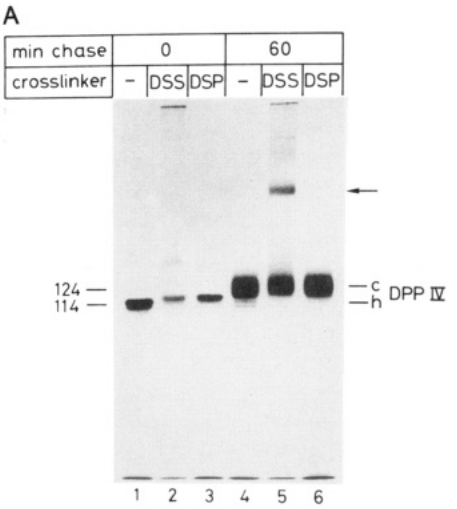

FIGURE 2: Time-dependent cross-linking of DPPIV. (A) Caco-2 cells were pulsed for 15 min with [^{35}S]methionine without (=0-min chase) or with a subsequent chase of 60 min. The total membrane fraction was cross-linked as indicated, DPPIV was immunoprecipitated, and the immunoprecipitates were analyzed by SDS–PAGE under reducing conditions followed by fluorography. The arrow indicates the position of DPPIV dimers. (B) Kinetics of complex glycosylation (×) and appearance of DSS-cross-linked dimers (○) of DPPIV. The ordinate indicates the percent of complex glycosylation or the percent of maximal cross-linking as determined by densitometric scanning of fluorograms. Longer chase periods did not increase the efficiency of cross-linking. Pulse–chase experiments were analogous to those of (A).

DPPIV is present (at least in part) as a dimer in the brush border membrane of Caco-2 cells.

Similarly, another peptidase, aminopeptidase N (ApN), was cross-linkable with DSS (Figure 1B). When SIM was subjected to the cross-linking procedure, no bands larger than the monomer were detectable on gels (Figure 1B). Other cross-linkers including *m*-maleimidobenzoic acid *N*-hydroxy-succinimide ester (MBS) and ethylene glycol bis(succinimidyl succinate) (EGS) while effective for DPPIV also failed to cross-link SIM. Mosimann et al. (1973) have reported that the quaternary structure of papain-solubilized rabbit SIM depends on the ionic strength. However, we tested a wide range of salt concentrations, detergents, and cross-linkers (see Materials and Methods for details) but were not able to find any dimers of human SIM.

In order to determine the intracellular site of the dimerization of DPPIV, the cross-linking approach was combined with pulse–chase labeling. The time course of dimerization allows one to draw conclusions on the intracellular location of dimer assembly since the kinetics of transport of newly synthesized DPPIV to the cell surface are known (Stieger et al., 1988). Figure 2A shows that complex glycosylated DPPIV but not its high-mannose precursor can be cross-linked into dimers. A faint band above the high-mannose monomer was consistently observed after cross-linking (lane 2). This band is most likely due to intramolecular rather than intermolecular cross-linking since it comigrated with the monomer on sucrose gradients (see Figure 6). Some radioactivity was observed which did not enter the separating gel after cross-linking. After DSP cross-linking followed by SDS–PAGE under reducing conditions, this band broke down at early and late chase times, and its absence from gels run with sucrose gradient fractions of non-cross-linked samples (see Figure 4) in contrast to cross-linked samples (see Figure 6) suggests an aggregate artifactually induced by the cross-linking procedure.

The appearance of cross-linkable dimers of DPPIV was studied by pulse–chase experiments and densitometric scanning of fluorograms (Figure 2B). We found that dimerization followed soon after complex glycosylation, half-maximal dimerization trailing half-maximal complex glycosylation by approximately 6 min. Throughout the chase period, a high-mannose dimer never became apparent. When the samples

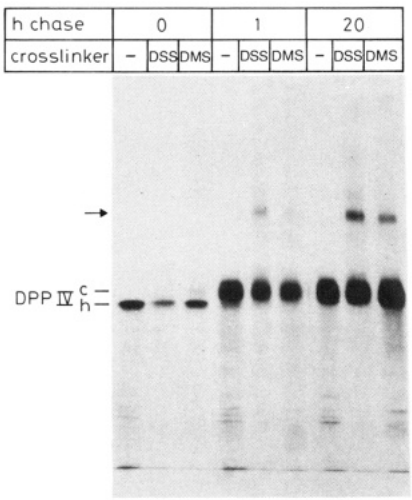

FIGURE 3: In situ cross-linking of DPPIV. Caco-2 cells were metabolically labeled for 30 min with [^{35}S]methionine and chased for 0, 1, or 20 h. Thereafter intact cells were cross-linked with either DSS or DMS. After cross-linking, the cells were harvested and boiled in SDS, and DPPIV was immunoprecipitated by using an anti-DPPIV polyclonal antibody. The immunoprecipitates were analyzed by SDS–PAGE (4–10% gradient gel) and fluorography. The arrow indicates the position of dimers. h, high-mannose form; c, complex form.

were cross-linked with DSP and the cross-linking reagent was cleaved with DTT after immunoprecipitation, no other proteins were found to coprecipitate at any time point.

SIM was present as a monomer at any time after its synthesis (not shown). This renders it unlikely that a putative SIM dimer dissociates after its export from the endoplasmic reticulum and thereby escapes detection at longer chase times. Doms et al. (1986) have shown, however, that influenza hemagglutinin forms labile trimers first in the endoplasmic reticulum, which are later stabilized within or after passage through the Golgi complex. Therefore, we tested a wide range of cross-linking conditions using different detergents at different ionic strength as described under Materials and Methods. All these conditions were tested with water-soluble and water-nonsoluble homo- and heterobifunctional agents of different length. None of these conditions led to the detection of high-mannose dimers of DPPIV.

In a final attempt to find high-mannose dimers, intact cells were cross-linked with the membrane-permeable bifunctional agents DSS and DMS. Cells labeled for 30 min with [^{35}S]-methionine were directly cross-linked or first chased for 1 or 20 h. Again, no high-mannose dimers of DPPIV were found (Figure 3). Cross-linking with whole cells was somewhat less efficient than with homogenates or detergent extracts. Nevertheless, a high-mannose dimer would clearly be visible, if present, because the high-mannose form runs as a sharp band in contrast to the complex glycosylated form (see Figure 8).

A possible reason for the failure to detect high-mannose dimers may be that the mAb fails to recognize high-mannose dimers since it precipitates the monomeric high-mannose form less efficiently than the complex protein. To circumvent this problem, immunoprecipitations were also performed under denaturing conditions with the same result (e.g., Figure 3, and above described experiments). By this procedure, different biosynthetic forms of DPPIV can be immunoprecipitated with the same efficiency (Matter & Hauri, 1991). Overall, these data led us to conclude that the high-mannose form of DPPIV does not form dimers.

Chemical cross-linking has two major disadvantages. First, it is not a quantitative approach. Therefore, we do not know

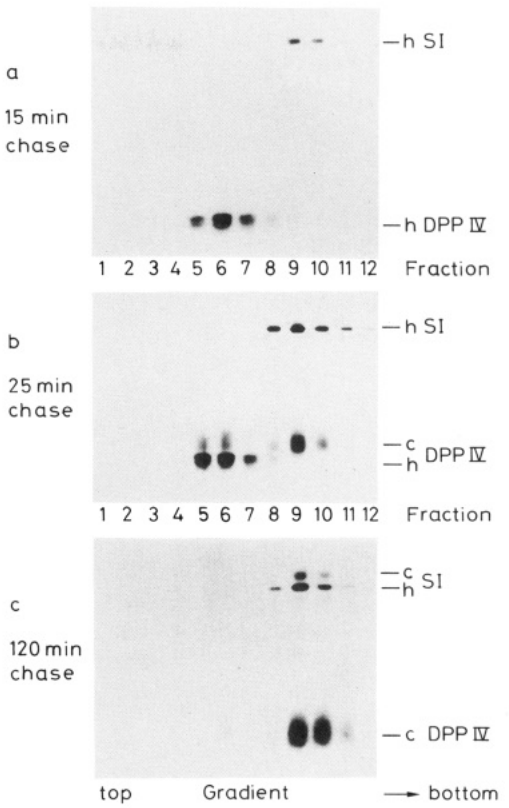

FIGURE 4: Rate–zonal centrifugation analysis of DPPIV and SIM. Caco-2 cells were labeled for 15 min with [^{35}S]methionine and chased for 15 (a), 25 (b), or 120 min (c) with unlabeled methionine in excess. Triton X-100 extracts were subjected to sucrose gradient rate–zonal centrifugation. Each gradient was split into 18 equal-volume fractions, and DPPIV and SI were immunoprecipitated and analyzed by SDS–PAGE (6% gels) and fluorography. Shown are the 12 top fractions. The remainder six fractions were empty and are not shown. h, high-mannose form; c, complex form; SI, SIM.

whether all complex-type DPPIV molecules are dimerized. Second, nonspecific cross-linking has to be considered as discussed above. Because of these limitations, we chose sucrose gradient rate–zonal centrifugation as an alternative approach to elucidate the oligomeric state of DPPIV and SIM. Caco-2 cells were pulse-labeled and then chased for various times. Triton X-100 extracts were subjected to sucrose gradient centrifugation, and the gradients were then fractionated into 18 equal aliquots, fraction 1 representing the top of the gradient. From each fraction, DPPIV and SIM were quantitatively immunoprecipitated and analyzed by SDS–PAGE (Figure 4). After 15 min of chase, DPPIV was present in a high-mannose form, mainly in fraction 6. After a 25-min chase, a second peak appeared in the position of fractions 9 and 10 which contained only complex glycosylated DPPIV. However, fraction 6 by that time point contained both high-mannose and complex glycosylated DPPIV. Finally, after 2 h of chase, all DPPIV was found in fractions 9 and 10. SIM was coimmunoprecipitated as a reference since it did not undergo a shift in the gradient. Even after overnight labeling the complex form of SIM comigrated with high-mannose SIM on the gradient (not shown), suggesting that SIM did not form oligomers under the conditions used.

The used type of sucrose gradients can be considered to be isokinetic (Martin & Ames, 1961; Burgi & Hershey, 1963). Therefore, it was possible to calculate the sedimentation constant for SIM, which was found to be 9.5 S. This value is in good agreement with the finding of Mosimann et al. (1973), who reported an s_{20w}, value of 9.6 S for monomeric rabbit SIM, confirming the monomeric nature of SIM in

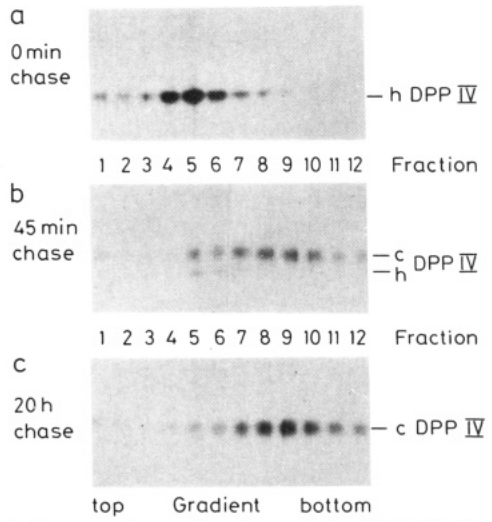

FIGURE 5: Rate–zonal centrifugation analysis of DPPIV. Caco-2 cells were labeled for 30 min with [^{35}S]methionine and chased for 0 min (a), 45 min (b), or 20 h (c). Cells were extracted with Nonidet P-40, and the extracts were analyzed on detergent-free sucrose gradients. Each gradient was split into 12 equal-volume fractions, and DPPIV was immunoprecipitated with a polyclonal antibody after boiling in SDS. The immunoprecipitates were analyzed by SDS–PAGE and fluorography. h, high-mannose form; c, complex form.

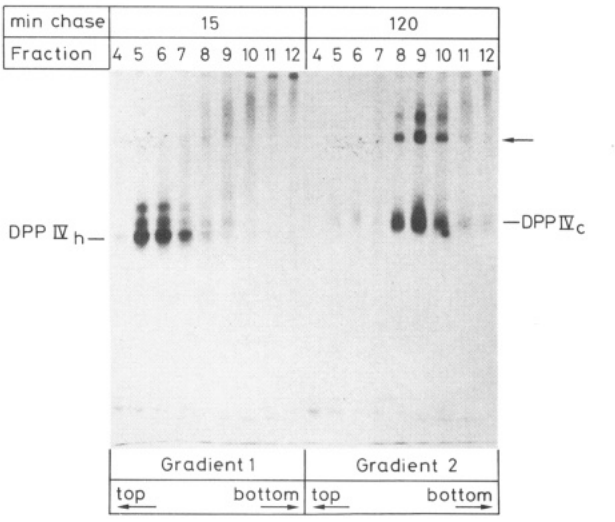

FIGURE 6: Correlation of cross-linking and sedimentation properties of DPPIV. The pulse–chase protocol was as in Figure 3 with the exception that the Triton X-100 extracts were cross-linked with DSS before rate–zonal centrifugation. Shown is the fluorogram of a 6% gel. The arrow indicates the position of DPPIV dimers.

Caco-2 cells. Because no specific bands larger than the monomer were detectable even at 0-min chase, it is unlikely that we have missed an early oligomerization step. Furthermore, assuming that a dimer has the same density and a similar shape as the monomer, it can be calculated that a dimer should migrate 1.59 times faster through an isokinetic gradient than a monomer. This is consistant with DPPIV monomer appearing in fraction 6 and a dimer peak between fractions 9 and 10 (calculated ratio of migration 1.63). This interpretation is confirmed by the fact that the proposed dimer of DPPIV (with a molecular weight of 248 K) cosedimented with SIM (M_r 217K). Therefore, we conclude that the slowly and the rapidly sedimenting DPPIV peaks represent the monomeric and the dimeric enzyme forms, respectively.

Since the "monomer" fractions comprised both high-mannose and complex glycosylated DPPIV whereas the "dimer" fractions contained only complex glycosylated DPPIV, these experiments are in line with the cross-linking experiments in which dimerization took place only after complex glycosylation. As in the cross-linking experiments, we never observed high-mannose dimers on the sucrose gradients even at shorter chase times (see Figure 5). It is important to note that the kinetics of dimerization of DPPIV were identical irrespective of whether determined by cross-linking or by sucrose gradient centrifugation. As illustrated in Figure 4, after 25 min of chase, 41% of the total DPPIV was complex glycosylated and 32% was dimers; in another experiment, after 35 min of chase, 72% was complex glycosylated and 55% was dimers. Both results are in agreement with the time course of dimerization obtained by cross-linking (Figure 2B). Futhermore, the sucrose gradient experiments showed that after 2 h of chase, when the cross-linking efficiency of DPPIV reached its maximum, all DPPIV was present as dimers on sucrose gradients. As with cross-linking, different detergent conditions were also tested for the sucrose gradient centrifugation. Figure 5 shows an example in which labeled cells were solubilized with Nonidet P-40 and the state of dimerization was analyzed by detergent-free gradients. High-mannose DPPIV behaved always as a monomer (Figure 5; complex and high-mannose forms do not cofractionate). Interestingly, under no conditions did we detect higher aggregates of high-mannose DPPIV (which would be found at the bottom of the gradients). This suggests that the higher aggregates found with the cross-linking approach are artificial. Since the fractions were immunoprecipitated with a polyclonal mAb after denaturing with SDS, all existing molecular forms of DPPIV can be assumed to be recognized with the same efficiency. Experiments with other detergents such as octyl glucoside or $C_{12}E_8$ gave identical results (not shown).

To test if there exists a direct correlation between cross-linking behavior and sedimentation properties, we combined both methods. Detergent extracts of pulse–chase-labeled cells were cross-linked with DSS and then analyzed by sucrose gradient centrifugation. Figure 6 shows that after a short chase DPPIV was not cross-linked and was therefore found in the monomer peak. After 2 h of chase, DPPIV showed the typical cross-linking pattern and appeared in the dimer peak. Interestingly, in these experiments, we obtained high molecular weight aggregates at the bottom of the gradients which did not enter the gel. This strongly supports the above conclusion that the high molecular weight aggregates seen by PAGE were cross-linking artifacts. Additional bands appeared with a somewhat higher molecular weight than the DPPIV dimer on the gel run with the 2-h chase sample. Since the bands had the same sedimentation properties as the dimer, they are most likely not due to oligomers larger than dimers but to differentially cross-linked dimers (e.g., intramolecular cross-links, different number of cross-links, different number of reacted amino groups).

Collectively, the results obtained by two different methods are in agreement and argue that in Caco-2 cells DPPIV dimerizes about 6 min after complex glycosylation. By this time, most DPPIV is in the Golgi apparatus which suggests that the Golgi may be the intracellular compartment in which dimerization occurs. In order to establish the site of dimerization more precisely, we applied the cross-linking approach to a subcellular fraction of Caco-2 cells that was highly enriched in Golgi membranes (Stieger et al., 1988). Our finding that in this Golgi fraction DPPIV was cross-linkable to dimers in a similar way as in the cell homogenate established the Golgi apparatus as the site of dimerization of DPPIV (Figure 7).

On the basis of the observation that assembly into dimers occurred soon after complex glycosylation, we wondered if

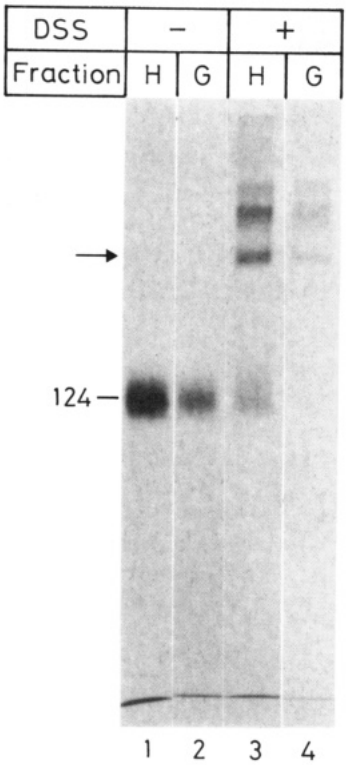

FIGURE 7: Cross-linking of DPPIV in a Golgi (G) and homogenate (H) fraction (fluorogram). Caco-2 cells were labeled with [^{35}S]-methionine overnight, and detergent-solubilized DPPIV was cross-linked, immunoprecipitated, and analyzed by SDS–PAGE (6% gel). The arrow indicates the dimer position.

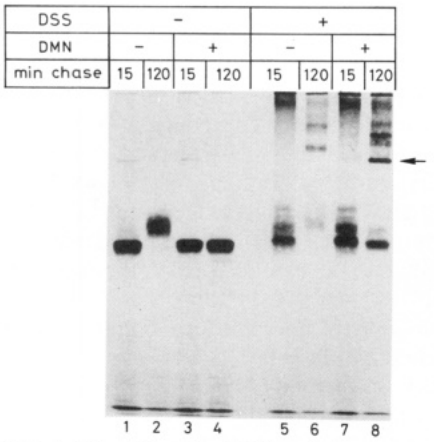

FIGURE 8: Effect of the trimming inhibitor 1-deoxymannojirimycin (DMN) on dimerization of DPPIV (fluorogram). The arrow indicates the position of the "high-mannose" (i.e., endo H sensitive) dimer of DPPIV.

terminal glycosylation of N-linked carbohydrates was required for dimerization of DPPIV. Trimming inhibitors like 1-deoxymannojirimycin (DMN), an inhibitor of Golgi–α-mannosidase I, provide convenient tools for blocking complex glycosylation without affecting other cellular processes (Fuhrmann et al., 1984). DMN blocks terminal N-glycosylation but does not interfere with transport of DPPIV to the cell surface (Matter et al., 1989). Cells were preincubated for 3 h with 2 mM DMN, pulse-labeled for 15 min with [^{35}S]methionine, and chased for 15 or 120 min. The quaternary structure of DPPIV was then analyzed by cross-linking a Triton X-100 extract of whole cells. Figure 8 shows that DMN completely inhibited complex glycosylation. After 2 h of chase in the presence of DMN, the DPPIV still displayed the typical high-mannose doublet band. However, at

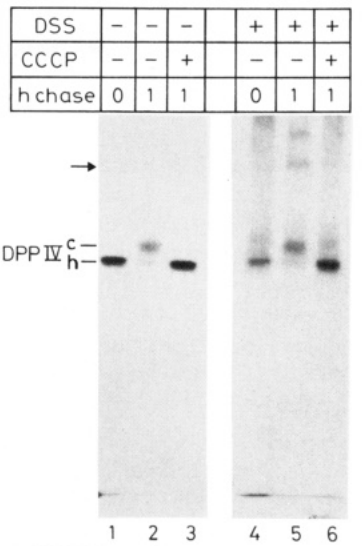

FIGURE 9: Effect of CCCP on dimerization of DPPIV. Cells were pulse-labeled for 5 min with [^{35}S]methionine and then incubated for 10 min in ice-cold Dulbecco's PBS either with or without 300 μM CCCP. Chase was for the indicated time intervals in the presence or absence of 300 μM CCCP. After solubilization and cross-linking with DSS, the DPPIV was immunoprecipitated and analyzed by SDS–PAGE (fluorogram of a 6% gel).

that time, DPPIV was cross-linkable to a dimer in the high-mannose form, suggesting that complex glycosylation is not required for dimerization.

Is transport to the Golgi apparatus a prerequisite for dimerization of DPPIV? Exit of newly synthesized proteins from the ER requires energy in the form of ATP and can be blocked by CCCP, an uncoupler of oxidative phosphorylation (Tartakoff & Vasalli, 1977; Fries & Rothman, 1980). Cells were pulse-labeled with [^{35}S]methionine for 5 min at 37 °C followed by a 10-min incubation on ice in the presence of CCCP and a chase at 37 °C in the presence of CCCP. The oligomeric state of DPPIV was determined by cross-linking as in the previous experiments. Figure 9 shows that after 60 min of chase the maturation of DPPIV to the complex glycosylated form was inhibited by CCCP. No dimers were formed under these conditions, suggesting that transport to the Golgi apparatus may be a requirement for dimerization.

Discussion

The results of the cross-linking experiments performed in the present study suggest that DPPIV and also ApN are dimers in the brush border membrane of Caco-2 cells. Cleavage of DSP-cross-linked DPPIV did not reveal any other proteins associated with the high molecular weight form of DPPIV. Furthermore, the time course of cross-linking demonstrated that dimerization of DPPIV was almost concomitant with complex glycosylation. Half-maximal dimerization followed half-maximal complex glycosylation after $\approx$6 min, indicating that assembly of DPPIV dimers might occur within the Golgi apparatus.

Sucrose gradient centrifugation fully confirmed the results obtained by cross-linking. These experiments showed that monomeric high-mannose DPPIV was converted to monomeric complex glycosylated DPPIV prior to dimerization while SIM remained a monomer throughout the entire chase. Since the absence of high-mannose DPPIV and SIM oligomers may be due to inappropriate assay conditions, we tested a variety of buffers at different ionic strengths and a number of different detergents in the density gradient assay as well as in the cross-linking experiments (using bifunctional agents different

in size and chemical properties). However, all the conditions tested gave identical results in respect to oligomerization. The results of SIM are at variance with previous studies in which it was reported that pig and rabbit SI are dimers (Mosimann et al., 1973; Cowell et al., 1986). It is possible that this discrepancy is due to species differences as already known for aminopeptidase N (Kenny & Maroux, 1982). While the present paper was in preparation, Beaulieu et al. (1989) reported that all biosynthetic forms of Caco-2 SIM had similar sedimentation rates on sucrose gradients which is in accordance with the observations of the present study.

The intracellular site of DPPIV dimerization is most likely the Golgi apparatus as DPPIV of a Golgi-enriched fraction was cross-linkable to dimers in a similar way as in the total cell homogenate. The Golgi apparatus as a central modifying organelle offers several processes to be the trigger of dimerization at the molecular level. For example, complex glycosylation of N-linked carbohydrates would appear to be an attractive covalent modification that might lead to dimerization of DPPIV. Inhibition of complex glycosylation by DMN revealed that the high-mannose form of DPPIV has a potential to form stable dimers, but only after its passage through the Golgi apparatus. This shows that complex glycosylation is not a prerequisite for dimerization of DPPIV. Dimerization might be related to other covalent modifications, such as O-linked glycosylation or tyrosine sulfation (Huttner, 1987; Danielsen, 1987), or might require a characteristic environment (Griffiths et al., 1983; Mellman et al., 1986). It seems unlikely that a prolonged stay in the ER would lead to dimer assembly since we were unable to detect high-mannose dimers after cross-linking when protein export from the ER was inhibited by CCCP.

Trimerization of viral envelope proteins was postulated to be essential for their export from the ER (Copeland et al., 1986, 1988; Gething et al., 1986; Kreis & Lodish, 1986; Doms et al., 1987, 1988; Boulay et al., 1988; Hurtley et al., 1989). It was observed that trimerization preceded export from the ER and that mutant proteins which remained in the ER, in some cases, stayed as monomers. Therefore, it was concluded that trimerization is a prerequisite but not sufficient for exit from the ER (Doms et al., 1988). On the other hand, Yedell et al. (1988) reported that trimerization of influenza hemagglutinin occurred at a later step during intracellular transport, probably in the Golgi apparatus. The reason for this discrepancy is unknown.

In general, plasma membrane glycoproteins tend to be oligomers, and it is assumed that they oligomerize in the ER (Hurtley & Helenius, 1989). Examples for endogenous plasma membrane proteins that may oligomerize in the ER are the insulin receptor (Bischoff et al., 1988), the acetylcholine receptor (Smith et al., 1986), the T cell receptor (Clevers et al., 1988), (Na^+,K^+)-ATPase (Tamkun & Fambrough, 1986), MHC class I and class II antigens [for a review, see Carlin and Merle (1986)], and membrane-bound IgM (Hombach et al., 1988). While the data concerning the subcellular site of assembly of these proteins are consistent with assembly in the ER, post-ER assembly sites such as the post-ER pre-Golgi compartment or cis-Golgi have not been ruled out formally. On the other hand, it is clear that these proteins oligomerize before they reach the trans-Golgi.

Although our data are fully compatible with dimerization of DPPIV occurring in the Golgi apparatus, the possibility has to be considered that dimerization may already occur in a post-ER pre-Golgi intermediate compartment, also designated exosomes (Saraste & Kuismanen, 1984), salvage compartment (Warren, 1987), or p53 compartment (Schweizer et al., 1990). If one assumes that exit from the ER is the rate-limiting step and that movement of DPPIV from the intermediate compartment to the cis-Golgi and subsequently to the trans-Golgi are comparatively rapid processes, only a minor fraction of high-mannose DPPIV dimer could have been present at any time, and not been easily detected. Although such a possibility cannot entirely be excluded, it appears unlikely to us because high-mannose DPPIV was easily visible in an isolated Golgi fraction (Stieger et al., 1988).

We believe that our methods would have been sensitive enough to visualize oligomerization of high-mannose SIM and DPPIV if it existed. Therefore, we conclude that oligomerization is unlikely to be of general importance for protein exit from the ER. In light of our results, we believe that it is premature to propose that membrane proteins generally oligomerize in the ER and that oligomerization is a prerequisite for their exit from the ER. It rather appears that, depending on the protein, oligomerization may occur in the ER (e.g., VSV G protein), in the Golgi apparatus (e.g., DPPIV), or not at all (e.g., SIM). Moreover, even for proteins that assemble in the ER, direct evidence for the necessity of oligomerization for transport is lacking. Very recently Danielsen (1990) has provided cross-linking evidence that both the transient and the mature forms of pig aminopeptidase N can form dimers. Most notably, dimerization of the transient form did not begin until 5–10 min after synthesis, and its rate was similar to the rate of transport of peptidases to the Golgi (Stieger et al., 1988). Pig lactase, on the other hand, was only cross-linkable in its mature form. These data are in line with our observation that at least some brush border enzymes dimerize beyond the ER.

Is the different oligomerization behavior of DPPIV and SIM in any way related to their asynchronous intracellular transport (Hauri et al., 1985)? It has previously been shown that this asynchronism is due to at least two rate-limiting steps, i.e., exit from the ER and the conversion of the high-mannose to the complex forms in the Golgi apparatus (Stieger et al., 1988). Collectively, our data argue against oligomerization as the underlying cause of the asynchronous transport of DPPIV and SIM, since oligomerization occurs after the asynchronous transport steps in the case of DPPIV, the rapidly transported hydrolase.

That human SIM does not form oligomers at any time of its life cycle might be related to its primary structure. SIM is composed of two highly homologous peptide segments, the isomaltase and the sucrase domain, and it has been postulated that this enzyme has evolved by gene duplication of an ancestral isomaltase (Hunziker et al., 1986). This means that SIM could be considered a "pseudo-dimer". Therefore, it will be interesting to assess the maturation of the tertiary structure of this enzyme.

ACKNOWLEDGMENTS

We thank Kaethy Bucher for excellent technical assistance and Urs A. Meyer for his continuous support.

Registry No. Dipeptidylpeptidase IV, 54249-88-6.

REFERENCES

Beaulieu, J.-F., Nichols, B., & Quaroni, A. (1989) *J. Biol. Chem. 264*, 20000–20011.

Bischoff, J., Libresco, S., Shia, M. A., & Lodish, H. F. (1988) *J. Cell. Biol. 106*, 1067–1074.

Boulay, F., Doms, R. W., Webster, R. G., & Helenius, A. (1988) *J. Cell Biol. 106*, 629–639.

Burgi, E., & Hershey, A. D. (1963) *Biophys. J. 3*, 309–321.

Carlin, B. E., & Merlie, J. P. (1986) in *Protein Compartmentalisation* (Strauss, A. W., Boime, I., & Kreil, G., Eds.) pp 71–86, Springer-Verlag, New York.
Clevers, H., Alarcon, B., Wileman, T., & Terhorst, C. (1988) *Annu. Rev. Immunol. 6*, 629–662.
Copeland, C. S., Doms, R. W., Bolzau, E. M., Webster, R. G., & Helenius, A. (1986) *J. Cell Biol. 103*, 1179–1191.
Copeland, C. S., Zimmer, K. P., Wagner, K. R., Healey, G. A., Mellman, I., & Helenius, A. (1988) *Cell 53*, 197–209.
Cowell, G. M., Tranum-Jensen, J., Sjöström, H., & Norên, O. (1986) *Biochem. J. 237*, 455–461.
Danielsen, E. M. (1987) *EMBO J. 6*, 2891–2896.
Danielsen, E. M. (1990) *Biochemistry 29*, 305–308.
Doms, R. W., Keller, D. S., Helenius, A., & Balch, W. E. (1987) *J. Cell Biol. 105*, 1957–1969.
Doms, R. W., Ruusala, A., Machamer, C., Helenius, J., Helenius, A., & Rose, J. K. (1988) *J. Cell Biol. 107*, 89–99.
Fries, E., & Rothman, J. E. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 3870–3874.
Fuhrmann, U., Bause, E., Legler, G., & Ploegh, H. (1984) *Nature 307*, 755–758.
Fulcher, I. S., Ingram, J., & Kenny, A. J. (1986) *FEBS Lett. 205*, 323–327.
Gething, M.-J., McCammon, K., & Sambrook, J. (1986) *Cell 46*, 939–950.
Griffiths, G., Quinn, P., & Warren, G. (1983) *J. Cell Biol. 96*, 835–850.
Hauri, H.-P. (1988) *Subcell. Biochem. 12*, 155–219.
Hauri, H.-P., Sterchi, E. E., Bienz, D., Fransen, J. A. M., & Marxer, A. (1985) *J. Cell Biol. 101*, 838–851.
Hombach, J., Leclercq, L., Radbruch, A., Rajewsky, K., & Reth, M. (1988) *EMBO J. 7*, 3451–3456.
Hunziker, W., Spiess, M., Semenza, G., & Lodish, H. F. (1986) *Cell 46*, 227–234.
Hurtley, S. M., & Helenius, A. (1989) *Annu. Rev. Cell Biol. 5*, 277–307.
Hurtley, S. M., Bole, D. G., Hoover-Litty, H., Helenius, A., & Copeland, C. S. (1989) *J. Cell Biol. 108*, 2117–2126.
Huttner, W. B. (1987) *Trends Biochem. Sci. 12*, 361–363.
Kenny, A. J., & Maroux, S. (1982) *Physiol. Rev. 62*, 91–128.
Kreis, T. E., & Lodish, H. F. (1986) *Cell 46*, 929–937.
Laemmli, U. K. (1970) *Nature 227*, 680–685.
Lippincott-Schwartz, J., Bonifacino, J. S., Yuan, L. C., & Klausner, R. D. (1988) *Cell 54*, 209–220.
Martin, R. G., & Ames, B. N. (1961) *J. Biol. Chem. 236*, 1372–1379.
Matter, K., & Hauri, H.-P. (1991) *Biochemistry* (following paper in this issue).
Matter, K., McDowell, W., Schwarz, R. T., & Hauri, H.-P. (1989) *J. Biol. Chem. 264*, 13131–13139.
Mellman, I., Fuchs, R., & Helenius, A. (1986) *Annu. Rev. Biochem. 55*, 663–700.
Mosimann, H., Semenza, G., & Sund, H. (1973) *Eur. J. Biochem. 36*, 489–494.
Norên, O., Sjöström, H., Danielsen, E. M., Cowell, G. M., & Skovbjerg, H. (1986) in *Molecular and Cellular Basis of Digestion* (Desnuelle, P., Sjöström, H., & Norên, O., Eds.) pp 335–365, Elsevier, Amsterdam.
Pinto, M., Robine-Leon, S., Appay, M.-D., Kedinger, M., Triadou, N., Dussaulx, E., Lacroix, B., Simon-Assmann, P., Haffen, K., Fogh, J., & Zweibaum, A. (1983) *Biol. Cell. 47*, 323–330.
Saraste, J., & Kuismanen, E. (1984) *Cell 38*, 535–594.
Schweizer, A., Fransen, J. A. M., Matter, K., Kreis, T. E., Ginsel, L., & Hauri, H.-P. (1990) *Eur. J. Cell Biol.* (in press).
Semenza, G. (1986) *Annu. Rev. Cell Biol. 2*, 255–313.
Smith, M. M., Lindstrom, J., & Merlie, J. P. (1987) *J. Biol. Chem. 262*, 4367–4376.
Stieger, B., Matter, K., Baur, B., Bucher, K., Hoechli, M., & Hauri, H.-P. (1988) *J. Cell Biol. 106*, 1853–1861.
Tamkun, M. M., & Fambrough, D. M. (1986) *J. Biol. Chem. 261*, 1009–1019.
Tartakoff, A. M., & Vasalli, P. (1977) *J. Exp. Med. 146*, 1332–1345.
Warren, G. (1987) *Nature 327*, 17–18.
Yewdell, J. W., Yellen, A., & Bächi, T. (1988) *Cell 52*, 843–852.