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EVIDENCE THAT THE HYDROPHOBIC DOMAIN OF RAT RENAL γ-GLUTAMYLTRANSFERASE SPANS THE BRUSH BORDER MEMBRANE

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Lactoperoxidase and glucose oxidase catalyzed ^{125}I-iodination was used to specifically label isolated rat renal brush border membrane vesicles from either side of the membrane. Autoradiography of total membrane proteins demonstrated that asymmetric labeling was achieved. Specific immunoprecipitates of aminopeptidase M, an established transmembrane protein, and of γ-glutamyltransferase were isolated from vesicles solubilized with Triton X-100 or with papain. Following electrophoresis and autoradiography, the immunoprecipitates of the two solubilized forms of each enzyme derived from externally labeled vesicles exhibited the same intensity of labeling. In these experiments, the small subunit of the γ-glutamyltransferase was preferentially labeled suggesting that, compared to the large subunit, it is more exposed on the external surface of the membrane. With the samples derived from internally labeled vesicles, the Triton-solubilized form of each enzyme was intensely labeled, whereas the papain-solubilized forms contained insignificant amounts of radioactivity. Thus, the extent of contramembrane labeling was minimal. In these experiments, the large subunit of the γ-glutamyltransferase was preferentially labeled. The similarity of the labeling patterns obtained for aminopeptidase M and γ-glutamyltransferase suggests that the hydrophobic domain of the two amphipathic enzymes are selectively labeled from the internal surface and that the γ-glutamyltransferase may also be a transmembrane protein.

γ-Glutamyltransferase is an amphipathic heterodimeric protein that within rat kidney is primarily associated with the brush border membrane of the proximal tubule cells [1]. Purification of this enzyme, following its solubilization by partial proteolysis [2,3] or by treatment with detergents [3], yields two forms of the γ-glutamyltransferase that differ considerably in their physical properties. The papain-purified enzyme is soluble in aqueous buffers and has a molecular weight of 68000. In contrast, the Triton-purified γ-glutamyltransferase binds to detergent micelles, undergoes aggregation in the absence of detergent, and can be incorporated into phosphatidylcholine vesicle [4]. The molecular weight of the Triton-purified enzyme, after correcting for the mass of bound detergent, was estimated to be 87000 [3]. Sodium dodecyl sulfate polyacylamide gel analysis established that the small subunit of the γ-glutamyltransferase is unaltered by proteolytic solubilization, but that the large subunit of the papain-purified enzyme has a molecular weigth that is 20000 less than the large subunit of the Triton-purified enzyme [5]. These results suggest that the large subunit contains a proteolytically sensitive hydrophobic domain, that serves to anchor the γ-glutamyltransferase in the brush border membrane. More recent studies have indicated that this domain comprises the N-terminal portion of the large subunit [6].

Previous experiments [7–9], including studies carried out with ferritin-conjugated antibodies [10,11], have established that a significant propor-

tion of the γ-glutamyltransferase molecules are oriented with the hydrophilic and catalytically-active domain exposed on the external surface of the brush border membrane. The absolute asymmetry of this orientation was recently established by characterizing the proteolytic sensitivity of γ-glutamyltransferase and its interaction with specific Fab antibodies while associated with isolated brush border membrane vesicles [12].

Aminopeptidase M is a major protein component of the renal brush border membrane [13]. This peptidase also exhibits an absolute asymmetry of orientation [12,14] and it contains a hydrophobic domain [15–17]. By preloading brush border membrane vesicles with a photoactivatable macromolecular probe, Louvard et al. [18] were able to obtain evidence that the hydrophobic domain of aminopeptidase M spans the membrane. After photolysis, the amount of the reagent that was associated with detergent-solubilized aminopeptidase M was quantitated by specife immunoprecipitation. This amount was decreased 65% by further treatment with papain, indicating that the majority of the probe had reacted with the cytoplasmic segment of the hydrophobic domain of the aminopeptidase M. These results were confirmed by Booth and Kenny [19], who used brush border membrane vesicles preloaded with the hydrophilic, photosensitive probe, 3,5-di[^{125}I]iodo-4-azidobenzenesulfonate.

The purpose of this study was to determine if the hydrophobic domain of the rat renal γ-glutamyltransferase can be labeled from the internal surface of the brush border membrane. This was accomplished by using lactoperoxidase and glucose oxidase to specifically ^{125}I-iodinate the internal and external surfaces of isolated rat renal brush border membranes. An analysis of the labeling of aminopeptidase M was also determined.

Experimental procedures

Materials. White male Sprague-Dawley rats (150–250 g) were obtained from Zivic Miller and were maintained on Purina rat chow. Carrier-free Na^{125}I was purchased from New England Nuclear. The papain-solubilized forms of γ-glutamyltransferase [3] and aminopeptidase M [20] were purified and used to prepare antiserum [12] as described previously. The antibodies were purified by precipitating with ammonium sulfate (50% saturation) and by chromatography on a DEAE Affi-Gel Blue (Pharmacia) column preequilibrated with 20 mM Tris-HCl, 28 mM NaCl, 0.02% NaN_3, pH 8.0. The eluant fractions containing antibodies were pooled, dialyzed against 150 mM NaCl, 50 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, pH 7.4 (Hepes buffer), concentrated and stored at −20°C. All other biochemicals were obtained from Sigma Co.

Preparation of brush border membrane vesicles. Rats were decapitated and their kidneys were excised and stored at −20°C until used. Brush border membranes were purified 10-fold from rat kidney cortex according to the procedure of Booth and Kenny [21] except that $CaCl_2$ was used instead of $MgCl_2$. To prepare vesicles with entrapped lactoperoxidase and glucose oxidase, the kidney cortex was initially homogenized in buffer containing 0.1 mg/ml of lactoperoxidase and of glucose oxidase and then carried through the normal isolation procedure. About 0.02% of the added activities were recovered in the vesicle preparation. The isolated vesicles were washed three times by repeated centrifugation at $16000 \times g$ for 20 min and resuspension in Hepes buffer without significantly reducing the specific activity of the entrapped lactoperoxidase and glucose oxidase. Incubation of loaded vesicles with papain, chymotrypsin or trypsin, neither solubilized nor inactivated the lactoperoxidase or glucose oxidase activities associated with the brush border membrane vesicles. γ-Glutamyltransferase [22] and aminopeptidase M [23] were assayed using γ-glutamyl-*p*-nitroanilide and leucine-*p*-nitroanilide as substrates, respectively. Protein concentration was determined by the method of Lowry et al. [24] using bovine serum albumin as the standard.

^{125}I-Labeling of brush border membrane vesicles. The iodination procedure was adapted from that described by Hubbard and Cohn [25]. Brush border membrane vesicles were resuspended in Hepes buffer to a protein concentration of 6 mg/ml. Interior labeling was achieved by stepwise addition of Na^{125}I (100 μCi/ml) and glucose (20 mM) to vesicles preloaded with lactoperoxidase and glucose oxidase. Exterior labeling was achieved by adding glucose oxidase and lactoperoxidase to un-

loaded vesicles followed by addition of $Na^{125}I$ and glucose. The iodination reaction was carried out in a hood at room temperature for 10 min. The reaction was stopped by adding ice-cold Hepes buffer which contained sufficient NaN_3 and NaI to bring the final concentrations to 50 mM and 100 mM, respectively. The iodinated vesicles were then washed five times by centrifugation at 16000 $\times g$ for 20 min followed by resuspension in Hepes buffer.

Solubilization of iodinated brush border membrane vesicles. Iodinated brush border membrane vesicles were pelleted by centrifugation at 20000 $\times g$ for 20 min and resuspended in Hepes buffer containing 2% Triton X-100. The supernatant obtained following centrifugation at 60000 $\times g$ for 1 h was used as the Triton X-100-solubilized brush border membrane. Triton solubilization results in complete recovery of γ-glutamyltransferase and aminopeptidase activities. Papain (5 mg/ml) was activated by incubating with 20 mM dithiothreitol at 37°C for 15 min. Aliquots of brush border membranes were then incubated with 1/10 the amount of activated papain (w/w) for 1 h at 37°C. Papain digestion was terminated by the addition of sufficient iodoacetic acid to produce a final concentration of 20 mM. The samples were then centrifuged at 20000 $\times g$ for 20 min and the resulting supernatant was used as the papain-solubilized form of brush border membrane enzymes.

Immunoprecipitation of γ-glutamyltransferase and aminopeptidase M. Samples of Triton-solubilized, and papain-solubilized preparations of iodinated vesicles containing 9 units of γ-glutamyltransferase activity were mixed with sufficient anti-γ-glutamyltransferase antibodies to precipitate 10 units of enzyme. The mixtures were counted in a Packard gamma spectrometer and then incubated at 4°C overnight. The samples were then centrifuged at 20000 $\times g$ for 10 min, the resulting supernatants were assayed and found to contain less than 3% of the initial transpeptidase activity. The immunoprecipitates were resuspended in 1 ml of Hepes buffer containing 2% Triton X-100 and then pelleted by centrifugation at 20000 $\times g$ for 10 min. The pellets were then washed once with Hepes buffer and once with Hepes buffer containing 20 mM iodoacetic acid. After the precipitates were heated at 100°C for 10 min, 100 μl of the electrophoresis sample buffer (containing 6% SDS, 1.46 M β-mercaptoethanol, 10% glycerol, 0.0025% Bromophenol blue and 62.5 mM Tris-HCl, pH 6.8) was added. The immunoprecipitates were heated until dissolved, analyzed for radioactivity, and subjected to sodium dodecyl sulfate polyacrylamide slab gel electrophoresis (7.5% acrylamide) according to the method of Laemmli [26].

Samples of the solubilized preparations derived from iodinated vesicles containing 0.15 unit of aminopeptidase M activity were mixed with sufficient anti-aminopeptidase antibodies to precipitate 0.2 unit of enzyme. The immunoprecipitates were then processed following the same procedure used to immunoprecipitate the γ-glutamyltransferase.

The polyacrylamide slab gels were dried onto filter paper using a Bio-Rad Slab gel dryer. The dried gels were sandwiched between Kodak X-Omat R film and Dupont lightening-plus intensifying screen. Autoradiography was carried out at −70°C. The films were developed and scanned with a densitometer to determine the relative grain intensity of the exposed areas.

Results

Asymmetry of ^{125}I incorporation

The possibility of contramembrane labeling during experiments designed to label the internal surface of the brush border membrane was reduced by the addition of catalase. External labeling would occur in such experiments if a portion of the lactoperoxidase and glucose oxidase associated with the vesicles were tighthly bound to the external surface of the membrane. Since the lactoperoxidase-catalyzed iodination utilizes hydrogen peroxide generated during the oxidation of glucose, the alternative consumption of hydrogen peroxide by externally-added catalase would effectively block external iodination. As shown in Table I, the addition of 0.2 mg of catalase during the exterior labeling of vesicles decreased the extent of ^{125}I-incorporation 7-fold. In contrast, the addition of catalase during the interior labeling procedure resulted in a slight increase (1.9-fold) in the extent of labeling. The observation that ^{125}I-incorporation during interior labeling was not inhibited by the addition of catalase suggests that little contramembrane labeling occurs under these condi-

TABLE I

EFFECT OF CATALASE ON ^{125}I-INCORPORATION INTO BRUSH BORDER MEMBRANE VESICLES

Exterior and interior labeling of 0.35 ml samples of brush border membrane vesicles were carried out as described in Experimental procedures in either the absence (−) or presence (+) of 0.2 mg of catalase. All samples contained approx. 1 munit and 10 munits of lactoperoxidase and glucose oxidase, respectively.

	Exterior labeling		Interior labeling	
	− Catalase	+ Catalase	−Catalase	+ Catalase
Total cpm	$3.57 \cdot 10^7$	$3.64 \cdot 10^7$	$3.46 \cdot 10^7$	$3.07 \cdot 10^7$
cpm in vesicles	$3.04 \cdot 10^6$	$4.39 \cdot 10^5$	$4.32 \cdot 10^5$	$7.41 \cdot 10^5$
% incorporation	8.5	1.2	1.3	2.4
Ratio +/−		0.14		1.9

tions. The addition of catalase was included in all subsequent interior labeling experiments.

The achievement of asymmetric labeling was also verified by sodium dodecyl sulfate polyacrylamide gel analysis of the total membrane proteins obtained from externally and internally labeled vesicles (Fig. 1). The two samples exhibit identical banding patterns when stained for proteins, however, autoradiographic analysis revealed asymmetric labeling patterns. The external labeling occurred primarily in proteins of high molecular weight. One of the major proteins labeled from the external surface migrates with an electrophoretic mobility corresponding to aminopeptidase M. In contrast, internal labeling results in iodination of primarily low molecular weight proteins. One of the major proteins labeled from the internal surface migrates with an apparent molecular weight of 42000 and is presumed to be actin.

Analysis of externally labeled vesicles

Previous studies using freeze-fracture electron microsopy [14] and various immunoprecipitation procedures [12] have established that the isolated preparation of rat renal brush border membranes consists of approx. 90% right-side out and 10% inverted vesicles. Treatment with papain results in the release of the catalytically active hydrophilic domains of the enzymes from only right-side out vesicles. The activities associated with inverted vesicles are removed by centrifugation. In contrast, Triton-solubilization releases intact amphipathic proteins from both right-side out and inverted vesicles. If the hydrophobic domain spans the membrane, its extent of labeling in the small proportion of inverted vesicles should contribute only slightly to the total radioactivity associated with the immunoprecipitate of the Triton-solubilized form of the enzyme. As a result, the amount of radioactivity associated with the immunoprecipitates of the two solubilized forms of the proteins obtained from externally labeled membranes should be nearly identical.

Specific antibodies were initially used to precipitate aminopeptidase M from the solubilized preparations of externally labeled vesicles (Fig. 2). The slightly greater mobility observed for the papain-treated form ($M_r = 135000$) compared to the Triton-solubilized form ($M_r = 150000$) is characteristic of the hydrophilic and amphipathic forms of rat renal aminopeptidase M, respectively [27]. The amount of radioactivity specifically associated with each of the solubilized forms of the enzyme appeared to be very similar. This was confirmed by densitometric analysis of the autoradiogram. The relative area associated with the papain-solubilized form of aminopeptidase M was equal to 83% of the area associated with the Triton-solubilized form. The two immunoprecipitates contained approx. 1% of the total radioactivity originally incorporated into the externally labeled vesicles.

The immunoprecipitates of the two solubilized forms of γ-glutamyltransferase also exhibited a similar intensity of labeling (Fig. 2). Densitometric analysis again confirmed that the relative com-

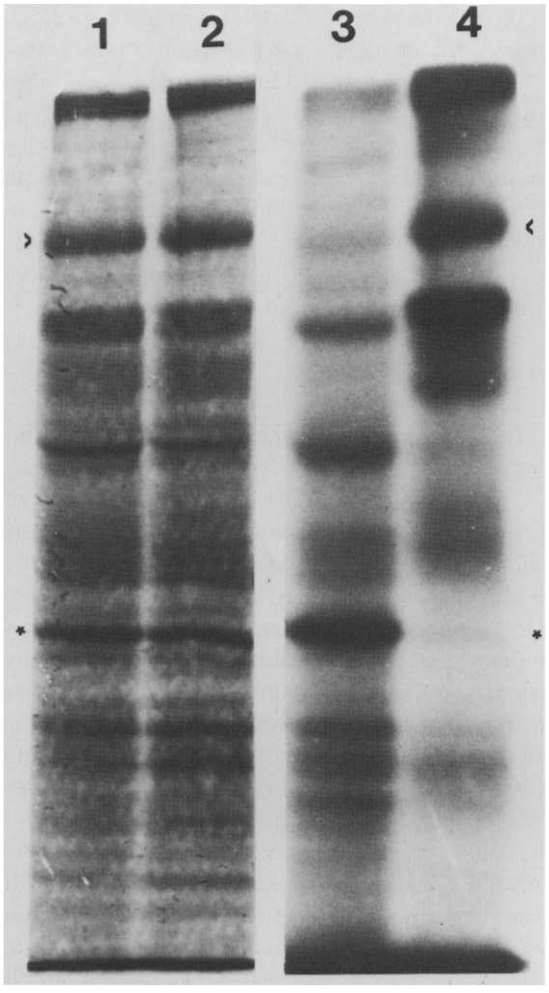

Fig. 1. Polyacrylamide gel electrophoresis and autoradiography of lactoperoxidase-catalyzed ^{125}I-labeled total brush border membrane proteins. Aliquots of iodinated brush border membranes (50 μg) were precipitated with −20°C acetone, dried under vacuum and resuspended in 100 μl of sample gel buffer containing 6% sodium dodecyl sulfate. The samples were then heated at 100°C for 10 min and loaded onto a 7.5% polyacrylamide slab gel with a 3% polyacrylamide stacking gel both containing 0.1% SDS. Electrophoresis was carried out for 4 h with a current of 25 mA. The gel stained for proteins is shown in lanes 1 and 2 and the corresponding autoradiogram is shown in lanes 3 and 4. Lanes 2 and 4 are samples labled at the external surface, whereas lanes 1 and 3 are samples labled at the internal surface. The ★ and < indicate the electrophoretic mobility of actin and aminopeptidase M, respectively

bined areas associated with the large and small subunits of each form of enzyme was nearly identical. However, in each case 2.2-fold more radioactivity was associated with the small subunit of the γ-glutamyltransferase than with the large subunit. This suggests that compared to the large subunit, the small subunit of the γ-glutamyltransferase may be exposed to a greater extent on the external surface of the brush border membrane. The radioactivity included in each of the immunoprecipitates comprised approximately 0.5% of the total radioactivity incorporated into externally labeled vesicles. The relative difference in the percent of the total radioactivity incorporated into aminopeptidase M versus the γ-glutamyltransferase is consistent with their relative percent composition within the brush border membrane; the isolation of γ-glutamyltransferase requires a 2-fold greater purification than the isolation of aminopeptidase M [3,20].

Analysis of internally labeled vesicles

By carrying out an analysis of vesicles labeled specifically from the internal surface, it should be possible to determine if the hydrophobic domain of an amphipathic protein spans the membrane. If all the vesicles were right-side out, only transmembranous portion of the hydrophobic domain would be labeled from the internal surface. However, in inverted vesicles, the catalytically active hydrophilic domain would be labeled from the internal surface. As a result, amphipathic enzymes immunoprecipitated from the Triton-solubilized membranes may be labeled in both the hydrophilic and hydrophobic domains. In contrast, immunoprecipitates of papain-solubilized enzymes should contain only the unlabeled catalytically active hydrophilic domain.

Analysis of specific immunoprecipitates obtained from internally labeled vesicles indicated that the Triton-solubilized forms of aminopeptidase M and of γ-glutamyltransferase were intensely labeled, whereas the papain-solubilized forms contained no apparent radioactivity (Fig. 3). The patterns were quantitated by densitometric analysis. In both cases, the relative areas associated with the papain-solubilized enzymes were less than 5% of the area associated with the Triton-solubilized forms. In contrast to the results observed with the externally labeled membranes, the ratio of radioactivity associated with the small and large subunits of the Triton-solubilized γ-

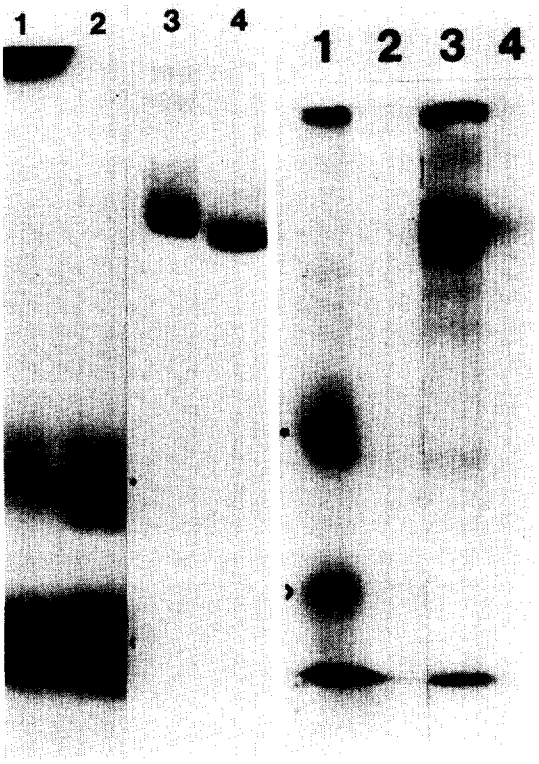

Fig. 2. (Left). Autoradiograph of the polyacrylamide slab gel containing the immunoprecipitates of γ-glutamyltransferase (lanes 1 and 2) and aminopeptidase M (lanes 3 and 4) derived from externally labeled vesicles. The enzymes were solubilized by treatment with Triton (lanes 1 and 3) or with papain (lanes 2 and 4). The ★ and > indicate the large and small subunits of γ-glutamyltransferase, respectively.

Fig. 3. (Right). Autoradiograph of the polyacrylamide slab gel containing the immunoprecipitates of γ-glutamyltransferase (lanes 1 and 2) and aminopeptidase M (lanes 3 and 4) derived from internally labeled vesicles. The enzymes were solubilized by treatment with Triton (lanes 1 and 3) or with papain (lanes 2 and 4). The * and > indicate the large and small subunits of γ-glutamyltransferase, respectively.

glutamyltransferase derived from internally labeled vesicles was only 0.70.

In order to establish that the hydrophobic domain of aminopeptidase M and of γ-glutamyltransferase are labeled from the internal surface, the effect of papain-treatment on the extent of radioactivity incorporated into immunoprecipitates derived from Triton-solubilized vesicles was determined. Papain proteolysis of the solubilized amphipathic enzymes hydrolyzes the segment connecting the hydrophobic and hydrophilic domains. Since the antibodies were prepared against the hydrophilic, papain-purified forms of the enzymes, the reduction in the extent of total radioactivity incorporated into immunoprecipitates should reflect the proportion of radioactivity incorporated into the hydrophobic domain. As shown in Table II, treatment with papain reduces by 40–60% the radioactivity contained in the immunoprecipitates derived following Triton-solubilization of brush border membrane vesicles labeled from the internal surface.

Discussion

The immunoprecipitates of γ-glutamyltransferase and aminopeptidase M solubilized from externally labeled vesicles by treatment with Triton or with papain contained a similar percentage of the total incorporated radioactivity. Electrophoretic and autoradiographic analysis also established that the γ-glutamyltransferase samples contained a similar distribution of radioactivity. These results are consistent with the fact that the catalytically active hydrophilic domain constitutes a large proportion of the mass of the two amphipathic enzymes and that this domain is oriented on the outside surface of nearly all of the vesicles.

When γ-glutamyltransferase was iodinated from the external surface of the vesicles, the amount of radioactivity incorporated into the small subunit was 2.2-fold greater than that incorporated into the large subunit. In contrast, when the labeling is carried out from the internal surface of the vesicles,

TABLE II

EFFECT OF PAPAIN TREATMENT ON THE EXTENT OF RADIOACTIVITY IMMUNOPRECIPITATED FROM INTERNALLY LABELED AND TRITON-SOLUBILIZED BRUSH BORDER MEMBRANE VESICLES

Brush border membrane vesicles were labeled from the internal surface and then solubilized as described in Experimental procedures. The amount of ^{125}I precipitated with specific antibodies are expressed as a percent of total label incorporated.

Sample	Amino peptidase M (%)	γ-Glutamyltransferase (%)
Triton-solubilized	0.41	0.18
Triton-solubilized, papain-treated	0.24	0.077

a greater proportion of the label was incorporated into the large subunit. In addition, amino acid analysis indicates that the large subunit contains three times as many tyrosine residues as the small subunit [28]. Thus, these results suggest that the small subunit of the γ-glutamyltransferase is more exposed on the external surface of the membrane. The large subunit could either be masked due to its interactions with the small subunit or it may be more deeply buried within the membrane. The latter possibility is consistent with the finding that the large subunit of γ-glutamyltransferase contains the hydrophobic membrane binding domain [3–6].

The observation that papain-solubilized forms of aminopeptidase M and γ-glutamyltransferase obtained from internally labeled vesicles contained insignificant amounts of radioactivity, indicates that contramembrane labeling did not occur. The finding that different proteins are selectively labeled from the internal or external surface of the vesicles (Fig. 1) also indicates that contramembrane labeling is minimal. The addition of catalase to the internal labeling experiments may have contributed to this specificity. The ability of added catalase to stimulate the extent of labeling achieved by the intrapped glucose oxidase and lactoperoxidase was highly reproducible. This unexpected, but beneficial effect presently lacks an explanation.

The difference in the extent of labeling observed between the Triton-solubilized and the Triton-solubilized, papain-treated forms of the two enzymes indicates that labeling of the hydrophobic domain from the internal surface of right-side out vesicles is significant. If all of the brush border membrane vesicles were right-side out, then only the hydrophobic domain, associated with the large subunit of the γ-glutamyltransferase, should be labeled from the internal surface. The results of the internal labeling experiments indicate that the large subunit is preferentially but not exclusively labeled. The labeling of the small subunit that is observed in these experiments probably occurs in inverted vesicles. Compared to the hydrophobic membrane binding portion, the catalytically active domain is much greater in size. As a result, the relative surface area of the enzymes exposed on the internal surface of inverted vesicles may greatly exceed the surface area exposed on the internal surface of right-side out vesicles. Therefore, labeling of inverted vesicles could contribute a much greater proportion of the total label incorporated in the Triton-solubilized sample than anticipated from their percent composition of the total vesicle population.

Lactoperoxidase-catalyzed iodination has been used to indicate that various proteins span the plasma membrane of the erythrocyte. In such experiments, the cystoplasmic segments of transmembrane proteins were specifically labeled either by sealing the reagents inside membrane ghosts [29,30] or by external labeling of inside-out vesicles [31]. This method was also used to label both surfaces of lymphocyte [32] and thymocyte [33] plasma membrane vesicles. However, the major limitation to this method is that it results in significant intramembrane labeling of the fatty acyl side chains of the membrane lipids [19]. As a result, lactoperoxidase-catalyzed iodination cannot be used to definitively establish that a protein is exposed on the cytoplasmic surface of the brush border membrane. However, the similarity of the results observed with γ-glutamyltransferase and aminopeptidase M, a well characterized transmembrane protein [18,19] suggests that the hydrophobic domain of the γ-glutamyltransferase may also span the membrane.

Recent experiments using specific inhibitors of γ-glutamyltransferase have suggested that this enzyme may participate directly in the ability of the kidney to accumulate γ-glutamyl peptides [34]. The finding that the hydrophobic domain of the γ-glutamyltransferase spans the brush border membrane makes possible the direct involvement of this protein in the transport of such compounds. These observations should serve to further stimulate efforts to isolate and to characterize the hydrophobic domain of the γ-glutamyltransferase.

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