TRANSMEMBRANE DISPOSITION OF THE PHLORIZIN BINDING PROTEIN OF INTESTINAL BRUSH BORDERS

Amira KLIP, Sergio GRINSTEIN and Giorgio SEMENZA

Laboratorium für Biochemie der Eidgenössischen Technischen Hochschule, Universitätstrasse 16, CH 8092 Zürich, Switzerland

Received 13 December 1978
Revised version received 30 December 1978

1. Introduction

The absorptive epithelium of the small intestine is specialized in the transcellular transfer of a number of important nutrients, including sugars. The active transport step for D-glucose at the luminal side has been well characterized as a Na^+-dependent [1], phlorizin-sensitive [2] process. The main features of this uptake, including the competitive inhibition by phlorizin, are preserved in vesicular membrane preparations derived from brush borders [3,4]. In this in vitro system, [^{3}H]phlorizin binds with a K_d value that matches its K_i for the inhibition of D-glucose transport, and both values change in parallel upon changing the experimental conditions [4,5]. This strongly suggests that the [^{3}H]phlorizin binding site is (a part of) the D-glucose transport unit.

Different groups have approached the identification of the intestinal sugar transport component by either labeling [6,7] or reconstitution [8] techniques. Even though no unambiguous answer is yet available, it should nevertheless be possible to study the orientation of this transporter in the membrane by virtue of its phlorizin-binding properties. The binding of the glycoside is believed to occur predominantly to the outer surface of the brush border membrane, on the basis of its rapid onset and reversibility [4,5] and the report that the glycoside does not penetrate the luminal membrane of intact cells [9]. Even though, by analogy with other transport systems, it is reasonable to assume that the D-glucose transport molecule spans the membrane, it is at present unknown whether any portion of this protein actually extends into the cytoplasmic milieu.

We approached this problem experimentally by rendering brush border vesicles leaky, and then exposing their internal surface to proteases which are without effect on phlorizin binding when added externally. We found that [^{3}H]phlorizin binding to the D-glucose transport protein was abolished only when the vesicles had been pretreated with low concentrations of deoxycholate, which exposed the cytoplasmic surface of the membrane to trypsin. The results are interpreted as indicating that the transport unit exposes different regions at the outer and inner surface of the vesicles and is therefore transmembranal and asymmetric.

2. Materials and methods

Brush border membrane vesicles from rabbit small intestine were prepared by the Ca^{2+} precipitation method [10,11]. D-Glucose uptake by these membranes was measured by ultrafiltration [4] using 0.1 mM D-[1-^{3}H]glucose (Amersham). Binding of phlorizin to the membranes was measured by a modification of the method in [5] as follows: Aliquots (10 μl) of a membrane suspension (3–12 mg protein/ml) pre-equilibrated for at least 20 min with 300 mM D-mannitol, 0.75 mM dithioerythritol (DTE), 100 mM of either NaCl or KCl (see text), and 10 mM Tris–HCl (pH 7.0) were mixed with 10 μl 10 μM [G-^{3}H]-phlorizin (New England Nuclear, 2.06 Ci/mmol) in the same buffer. After 5 s vigorous vortexing and 10 s incubation at room temperature, binding was

Address correspondence to: Professor G. Semenza

stopped by addition of 2.5 ml ice-cold solution containing 250 mM KCl, 0.1 mM non-radioactive phlorizin (Roth), 1 mM Tris–HCl (pH 7.5) and immediately filtered through Sartorius filters (0.6 μm pore size). These were then washed once with 5 ml stopping solution and counted. Treatment of the vesicles (2–4 mg protein/ml) with potassium deoxycholate (DOC) was performed in the proportions indicated in the text in 250 mM KCl, 0.75 mM DTE, 10 mM Tris–HCl (pH 8.5) for 10 min at 0°C. The membranes were then centrifuged at 60 000 × *g* for 30 min, and the pellets washed once with 300 mM D-mannitol, 0.75 mM DTE, 10 mM Tris–HCl (pH 7.0).

Papain (Boehringer, Mannheim) was activated by a 30 min preincubation in 300 mM D-mannitol, 5 mM cysteine, 1 mM sodium ethylenediaminotetraacetate, 10 mM Tris–HCl (pH 6.8) under nitrogen. Proteolysis of the membranes was then performed at a proteinase:membrane protein (w/w) ratio of 1:50 in the same activating solution for 30 min at 37°C. The reaction was stopped by addition of a 30-fold molar excess of *N*-α-*p*-tosyl L-lysine chloromethylketone (TLCK, Sigma), and after 5 min at 37°C the suspension was diluted 3-fold with ice-cold activating solution, pelleted and washed repeatedly. Treatment of the membranes with trypsin (from bovine pancreas, Fluka AG) was performed at a proteinase:membrane protein (w/w) ratio of 1:50, at room temperature for 10 min in 300 mM D-mannitol, 0.75 mM DTE, 10 mM Tris–HCl (pH 7.5). The reaction was stopped as above.

Polyacrylamide (8.5%) gel electrophoresis in the presence of sodium dodecylsulfate (SDS) was performed as in [11]. For quantitation, the slabs were stained with Coomassie blue, dried and scanned in an Integraph CH 174 Densitometer. Protein was determined as in [12] using bovine gamma globulin as standard. Phospholipase A from *Naja naja* venom was prepared as in [13]. When indicated, the membranes were frozen in a dry ice–ethanol bath, followed by thawing at room temperature.

3. Results and discussion

3.1. *Sidedness and degree of sealing of the vesicles*

Studies pertaining to the orientation of a protein in the membrane require an accurate determination of the membrane sidedness. In the case of the intestinal brush border vesicles, this has been determined by measuring the accessibility of sucrase, an externally-located enzyme. Since no stimulation of the enzyme activity has been observed upon vesicle disruption with Triton X-100 [4,11], it has been concluded that all the sucrase molecules have access to the substrate. However, this criterion does not allow the distinction between sealed right-side-out from leaky vesicles. To determine the degree of leakiness of this preparation, the accessibility of an intravesicular marker was studied. Intact brush border vesicles were incubated with papain or trypsin and the electrophoretic pattern of the washed membranes was analysed (gels C,P and T, fig.1A).

In agreement with [4], the externally-facing enzymes sucrase and isomaltase (S and I in the figure) are almost quantitatively cleaved off the membrane by papain. In contrast, the other major protein of these vesicle preparations (M_r 45 000) was completely

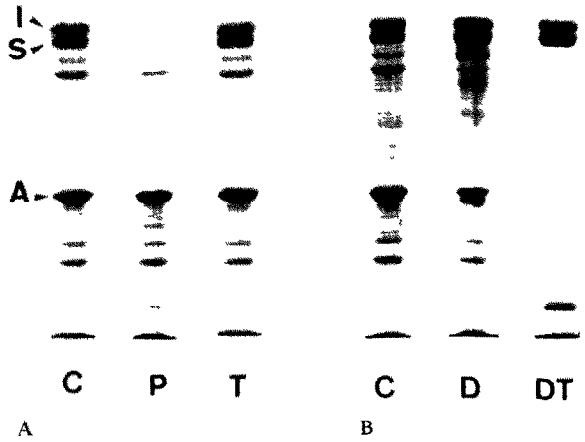

Fig.1. (A) Effects of papain on the protein composition of intestinal brush border membranes. Vesicles were incubated with or without papain or trypsin, as in section 2 and washed. The pellets were resuspended in the same volume of buffer and identical aliquots of these suspensions were applied to the gels. Gel (C), control vesicles; gel (P), papain-treated vesicles; gel (T), trypsin-treated vesicles. (B) Proteolysis of DOC-treated vesicles. Brush border vesicles were subjected to 0.5 mg DOC/mg protein as in section 2, washed and resuspended at 2 mg protein/ml. The suspension was then treated with trypsin. Gel (C), control vesicles; gel (D), DOC-treated vesicles; gel (DT), DOC-pretreated vesicles exposed to trypsin. I, isomaltase; S, sucrase; A, actin.

unaffected by the protease. This protein (A in fig.1) has been convincingly identified as actin [14], which is apparently trapped inside the vesicles when they form from the microvilli. In 3 experiments, > 90% of the actin resisted attack by papain as determined densitometrically.

No change in the electrophoretic pattern was discernible when the membranes were treated with trypsin (fig.1A, gel T). This is expected in the case of sucrase and isomaltase, which are constantly exposed to trypsin under physiological conditions. Indeed, sucrase and isomaltase were also unaffected even when the membranes were disrupted by DOC in the presence of trypsin, reflecting a lack of susceptibility rather than accessibility to the protease. This was not the case for actin, which was almost completely digested by either trypsin or papain in the presence of DOC. This finding is in agreement with an intravesicular localization of actin. Assuming that actin is equally distributed among the vesicles, it is possible to calculate that > 90% of the vesicles were sealed in the right-side-out configuration.

The disorganization of the membrane occurred gradually upon addition of increasing amounts of DOC. As shown in fig.2, the ability of the vesicles to accumulate D-glucose in the presence of a NaSCN gradient was completely abolished by pretreatment with 1 mg DOC/mg protein, and a partial reduction of the overshoot was observed with lower DOC concentrations. This reduced transport capacity is probably associated with the breakdown of membrane continuity rather than with inactivation of the glucose transport system, as evidenced by the concomitant reduction in the equilibrium value (40 min points, fig.2) which is a measure of the secluded volume of the vesicles, and by the fact that phlorizin binding was preserved (see below). Additional evidence that the vesicular membrane is made leaky by DOC was obtained by analysing the protein composition of detergent-treated vesicles. Figure 1B shows that a large fraction of the actin originally present inside the vesicles (band A in gel C) has been released following DOC treatment (gel D; a detailed account of the effects of DOC on the protein pattern will be presented elsewhere). When DOC-treated membranes such as those of gel D were incubated with trypsin, most of the remaining actin was lost from the sedimenting membranes (gel DT), due to either its degradation

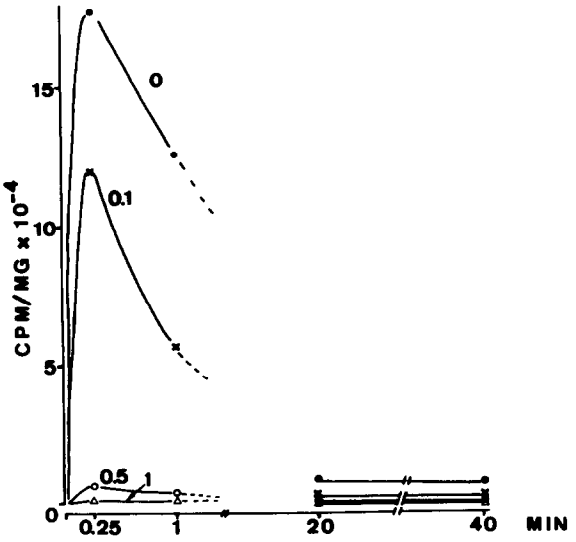

Fig.2. Time course of glucose uptake into brush border vesicles pre-treated with varying concentrations of DOC. The vesicles were treated with the amount of detergent indicated in the figure (in mg/mg protein), centrifuged and washed as in section 2. D-glucose uptake was measured in the presence of a NaSCN gradient as in [11]. Abscissa: time course, in minutes. Ordinate: D-[^{3}H]glucose taken up/mg protein.

or to proteolysis of its means of attachment to the membrane. Similar results were obtained with papain (not illustrated) yet neither enzyme was able to release actin from sealed vesicles. Taken together, these results strongly suggest that the vesicles, which are originally sealed and right-side-out, can be opened by addition of DOC, reducing their ability to accumulate substrates, and allowing the penetration of externally added proteases which can now cleave intravesicular proteins that were previously protected. Similar effects of DOC and other bile salts have been reported for both natural [15] and artificial [16] membranes.

3.2. *Phlorizin binding and the effect of proteases*

Phlorizin binds to at least two distinct types of sites in intact brush border membranes [4,5]: A low affinity, cation-insensitive type, and a high affinity, Na^+-dependent type, that can be competitively displaced by transported monosaccharides and which is thought to represent specific binding to the sugar 'carrier'. The effect of trypsin treatment on phlorizin

Table 1
Effect of trypsin on phlorizin binding to brush border vesicles before and after DOC treatment

Preparation	Treatment	Specific	Unspecific
Intact vesicles	Control	15 207 ± 838 (5)	10 152 ± 1448 (5)
	Trypsin	17 128 ± 1480 (5)	9596 ± 1549 (5)
DOC treated vesicles	Control	32 937 ± 5508 (8)	4116 ± 674 (8)
	Trypsin	4948 ± 2033 (8)	4356 ± 701 (8)

Binding figures are given in cpm/mg protein. 1 pmol = 965 cpm. The values are the mean ± 1 SE of no. expts in parenthesis. The vesicles were treated with 0.5 mg DOC/mg protein as in section 2. Unspecific binding was determined in Na^+-free medium. 'Specific' binding was obtained by subtracting the unspecific from the total binding, measured in 100 mM Na^+. Although several explanations can be offered for the reduced unspecific binding after DOC, no experimental evidence supporting any of them is yet available

binding to intact and DOC-treated vesicles is shown in table 1. Neither the specific nor the unspecific binding to normal vesicles were altered by the protease. Similar results were obtained [4] using papain instead of trypsin. DOC treatment not only preserved specific phlorizin binding, but in fact yielded higher specific activities, presumably as a result of the selective release of intravesicular proteins (see fig.1B) unrelated to glycoside binding.

Strikingly, disruption of the membranes by DOC renders the binding sensitive to trypsin. The same ratio of protease to protein which caused no effect in intact vesicles, drastically reduced (85%) specific binding in the DOC-treated membranes (table 1). In contrast, the Na^+-independent (unspecific) binding was not significantly diminished, indicating that in this regard the effect of the protease is selective. These results can be explained if it is assumed that a trypsin-sensitive portion of the sugar transporter is exposed to the inside but not to the outside of brush border vesicles, and that cleavage results in loss of binding activity. Alternatively, a major structural change of the membrane brought about by the addition of DOC could expose a part of the phlorizin-binding molecule on the outside face that was previously inaccessible to the action of the protease. The DOC action cannot be the result of complete disintegration of the membranous structure because:

(i) The experiments were carried out with a particulate fraction that sediments at 60 000 × g for 30 min;

(ii) This fraction is retained by 'millipore' filters. Drastic alteration of the protein conformation is also unlikely since specific (i.e., Na^+-dependent and D-glucose protectable) phlorizin binding is preserved after DOC treatment.

Further evidence indicating that the effect of DOC is associated with membrane opening, rather than with conformational changes, was obtained by correlating the degree of leakiness induced by the detergent with the appearance of trypsin sensitivity. The degree of sealing was manipulated by the addition of various concentrations of detergent, and estimated by measuring the fractional reduction in sugar transport (see fig.2). Aliquots from the same samples were also used to measure specific phlorizin binding before and after trypsinization. The data are pooled in fig.3. A highly significant correlation (r^2 = 0.98) was found between the loss of membrane continuity and the acquisition of trypsin sensitivity. The fact that the slope of the line (0.79) is smaller than one, can be explained in at least two ways. First, it is possible that a fraction of the vesicles become leaky to small molecules such as Na^+ or glucose but not to larger ones, such as the protease. Secondly, it is conceivable that the conditions of the experiment, such as the duration of the exposure to trypsin, are not sufficient for the complete degradation of the phlorizin-binding protein(s), resulting only in partial inactivation of the available molecules. Similar results were obtained when the sugar uptake at equilibrium, rather than the overshoot values, were used for the correlation. These results

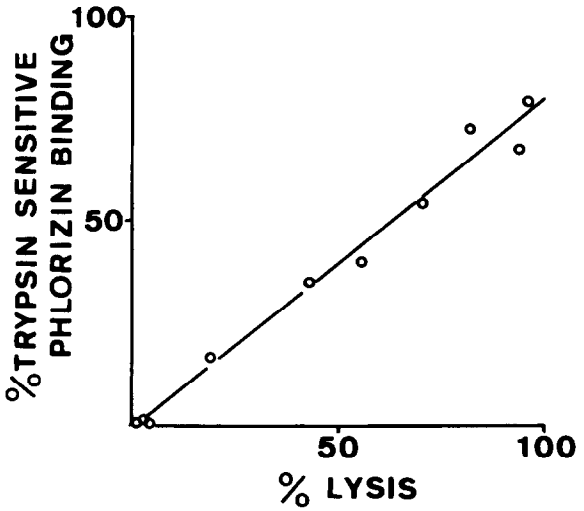

Fig.3. Induction of trypsin sensitivity by DOC as a function of the degree of vesicle lysis. Varying fractions of the vesicles were opened by treatment with increasing amounts of DOC (0–0.8 mg/mg protein) and washed. Lysis was then estimated by the decrease in D-glucose uptake measured at 1 min, and is expressed as % of the control (abscissa). Binding of phlorizin was measured in paired samples, one of which was incubated with trypsin. The fractional decrease in specific binding induced by the enzyme is plotted in the ordinates. The points are the mean of duplicate determinations, obtained in two experiments. The line was fitted by the least squares method.

suggest an internal site of action of the enzyme.

Additional support for this conclusion could in principle be obtained by disrupting the membrane by procedures other than the use of DOC, which are unlikely to produce the same structural changes, and then studying the trypsin sensitivity of phlorizin binding. Unfortunately, our attempts to disrupt the vesicles by freezing and thawing were completely unsuccessful: the uptake of D-glucose by vesicles subjected to 3 successive freeze–thaw cycles was indistinguishable from that of control vesicles, and phlorizin binding was, accordingly, insensitive to proteolysis. Exposure to Triton X-100 (0.06 mg/mg protein) or SDS (0.1 mg/mg protein) followed by centrifugation and washing, rendered the preparation leaky to D-glucose, but simultaneously completely abolished the specific binding of phlorizin, precluding measurement of the effects of trypsin. Similar results were obtained after incubating the vesicles for 20 min at 37°C with 1 : 20 (w/w) of a crude phospholipase A preparation from *Naja naja*. The vesicles also proved to be remarkably resistant to osmotic shock. Diluting vesicles suspended in mannitol–Tris buffer (320 mOsmol/l) into 80 vol. distilled water produced no detectable changes in either D-glucose uptake or phlorizin binding. However, limited success was obtained by pre-equilibrating the vesicles with 2 M glycerol prior to dilution. In 3 similar experiments, 39%, 45% and 46% of the vesicles were lysed, as judged by the reduction of D-glucose uptake. When these membranes were exposed to trypsin, after the osmotic shock, 18%, 53% and 17% of the specific binding became susceptible to the action of the protease.

These results support the view that the phlorizin-binding molecule contains a cytoplasmic portion which is susceptible to tryptic hydrolysis. It is, however, impossible at this stage to completely rule out the possibility that both DOC and osmotic lysis expose a trypsin-sensitive part of the molecule by mechanisms other than membrane opening.

The precise location of the phlorizin-binding moiety of the sugar transport system has not been well defined, but on the basis of the available evidence (see section 1), it is generally believed that binding occurs at the outside surface of both intestinal and renal brush borders [9,17]. If the trypsin-sensitive part of the transporter is indeed confined to the cytoplasmic face of the membrane, this would imply that the protein is asymmetric and that it spans the bilayer. In this event, the mechanism by which trypsin at the cytoplasmic surface affects [^{3}H]phlorizin binding to the opposite surface, remains to be explored.

Finally, our findings could explain the discrepancy between the results in [18], where phlorizin binding was reported reduced by trypsin or papain in kidney brush border membranes prepared in hypoosmotic buffers, and those in [19], where no effect of the proteases on the binding of the glycoside to a similar preparation obtained in isoosmotic conditions was detected. These conflicting results can be rationalized by assuming that at least a fraction of the membranes used by the former group were broken.

Acknowledgements

We are indebted to Drs D. Diedrich, M. Kessler and

H. U. Lutz for helpful suggestions and critical review of the manuscript. This work was partially supported by SNSF, Berne.

References

[1] Bihler, I. and Crane, R. K. (1962) Biochim. Biophys. Acta 59, 78–82.
[2] Alvarado, F. and Crane, R. K. (1962) Biochim. Biophys. Acta 56, 170–172.
[3] Hopfer, U., Nelson, K., Perrotto, J. and Isselbacher, K. J. (1973) J. Biol. Chem. 248, 25–32.
[4] Tannenbaum, C., Toggenburger, G., Kessler, M., Rothstein, A. and Semenza, G. (1977) J. Supramol. Struct. 6, 519–533.
[5] Toggenburger, G., Kessler, M., Rothstein, A., Semenza, G. and Tannenbaum, C. (1978) J. Membr. Biol. 40, 269–290.
[6] Lemaire, J. and Maestracci, D. (1978) Can. J. Physiol. Pharmacol. 56, 760–770.
[7] Smith, M. W., Ferguson, D. R. and Burton, K. A. (1975) Biochem. J. 147, 617–619.
[8] Crane, R. K., Malathi, P. and Preiser, R. (1976) Biochem. Biophys. Res. Commun. 71, 1010–1016.
[9] Stirling, C. E., (1967) J. Cell Biol. 35, 605–618.
[10] Schmitz, J., Preiser, H., Maestracci, D., Ghosh, B. K., Cerda, J. J. and Crane, R. K. (1973) Biochim. Biophys. Acta 323, 98–112.
[11] Kessler, M., Acuto, O. Storelli, C., Murer, H., Müller, M. and Semenza, G. (1978) Biochim. Biophys. Acta 506, 136–154.
[12] Bradford, M. M. (1976) Anal. Biochem. 72, 248–251.
[13] Fleischer, S. and Fleischer B. (1967) Methods Enzymol. 10, 406–433.
[14] Tilney, L. G. and Mooseker, M. (1971) Proc. Natl. Acad. Sci. USA, 68, 2611–2615.
[15] Rodriguez Boulan, E., Sabatini, D. D., Pereyra, B. N. and Kriebich, G. (1978) J. Cell Biol. 78, 894–909.
[16] Richards, M. H. and Gardner, C. R. (1978) Biochim. Biophys. Acta 543, 508–522.
[17] Kinne, R. (1976) Curr. Top. Membr. Trans. 8, 209–267.
[18] Glossmann, H. and Neville, D. M. (1973) Biochim. Biophys. Acta 323, 408–414.
[19] Thomas, L. and Kinne, R. (1972) FEBS Lett. 25, 242–244.