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Studies on the Brush Border Membrane of Mouse Duodenum

III. Iodination of Membrane Proteins

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Summary. Microvillus membranes were iodinated from luminally administered lactoperoxidase, H_2O_2 and ^{125}I before and after neuraminidase treatment. Membranes were isolated, solubilized in sodium dodecyl sulphate buffer and electrophoresed on gels. Gels were stained for protein, and then sliced for liquid scintillation counting. When membranes were not treated with neuraminidase, the nonpermeating iodination probe attached only to a band containing protein of 150,000 daltons approximate molecular weight. This size class of protein may reside on the luminal side of the brush border membrane as opposed to the serosol side. Qualifications of this statement are discussed with reference to the location of tyrosyl residues and to the possibilities of masking molecules. Membranes treated from the luminal side with neuraminidase to remove possibly masking carbohydrate and then iodinated, appeared to contain an additional protein of estimated molecular weight 220,000 daltons which was accessible to ^{125}I. Thus, a 220,000 dalton protein may also be on the luminal side of the membrane. An explanation is attempted for the discrepancy between the very few proteins labelled and the many proteins involved in terminal digestion and transport which would all be expected to be available to luminally administered iodination probe. Membranes were isolated, exposed to iodination and then solubilized for electrophoresis. Nearly all proteins were labelled, which indicated that there is an asymmetric distribution of proteins in the plane of the membrane. The two smallest molecular weight polypeptides which were not iodinated were proposed to be so disposed in the membrane that they were inaccessible to the probe, from either side.

In attempts to probe the surface structure of cell membranes, considerable use has been made of reagents which covalently attach labels to the surface of cells. Theoretically, it should be possible to locate surface-situated molecules, so long as the cell membrane is impermeable to the reagent.

Reagents which have been employed in such analyses are formylmethionyl sulfone methyl phosphate (Bretscher, 1971), sulphanilic acid diazonium salts (Bender, Garan & Berg, 1971), and diiodosulphanilic acid (Sears, Reed & Helmkamp, 1971). However, because of its more ready applicability to cell surface, most work seems to have been done on the

iodination of surface molecules using the lactoperoxidase-hydrogen peroxide system of Phillips and Morrison (1971). These workers have apparently identified a protein which resides on the outer surface of the human erythrocyte. The IgM of the cell surface of spleen lymphocytes was shown to be on the outside of the plasma membrane by Vitetta, Baur and Uhr (1971). Also using the lactoperoxidase technique, Marchalonis, Cone and Santer (1971) probed the accessible proteins of the lymphocyte. Tsai, Huang and Canellakis (1973) extended this iodination method to identify the membrane surface proteins of HeLa cells.

In the present studies it was hoped to identify luminal surface proteins of the microvillus membrane of mouse duodenum, using the iodination techniques of Phillips and Morrison (1971).

Materials and Methods

Lactoperoxidase was purchased from Calbiochem, U.S.A. Carrier-free $Na^{125}I$ (80–120 mCi/ml) was obtained from The Radiochemical Centre, Amersham, U.K.

Methods

Iodination of Membrane Proteins in situ. Duodenal segments of batches of 20 mice were excised, and immediately placed in ice-cold normal saline, buffered to pH 7.4 with phosphate. They were then gently irrigated to remove ingesta, and separated into two groups. Both ends of each segment were ligatured. One group was used immediately for iodination. The other group was treated with neuraminidase to attempt to remove the carbohydrate coat from the luminal surface of the brush border membranes.

Neuraminidase Treatment. The solution for the digestion consisted of sodium acetate, 0.05 M, pH 5.5, 1 unit of neuraminidase (*Vibrio cholerae*) per 0.2 ml, NaCl (9 mg/ml) and $CaCl_2$ (1 mg/ml). 0.2 ml of this solution was injected into each ligatured duodenal segment, and the reaction allowed to proceed for 15 min at 37 °C, segments being bathed in phosphate-buffered saline. Ligatures were then removed and segments washed through with ice-cold phosphate-buffered saline. New ligatures were placed on the segments, in preparation for iodination *in situ*.

Details of Iodination Procedure. Both the untreated and neuraminidase-treated ligatured segments were injected with 0.1-ml portions of the isotope-containing solution. The solution consisted of 0.05 ml enzyme solution (12.5 μg lactoperoxidase), 0.1 ml $Na^{125}I$ solution (150 μCi) and 0.83 ml phosphate-buffered saline. Just prior to the injections, 0.02 ml of 9 mM hydrogen peroxide was mixed into the solution to initiate the iodination. Reaction was allowed to proceed for 15 min at 30 °C with segments immersed in buffered saline. Ligatures were then removed and each segment was irrigated with saline to remove unbound isotope and unreacted oxidant. Several such washes were given, each time samples being monitored for counts. Washing was continued until the counts per minute of ^{125}I had decreased to about 10% above background.

Isolation of Membrane, Solubilization and Gel Electrophoresis. Brush border membranes were isolated from the segments, solubilized and electrophoresed on 7% acrylamide gels as outlined in an earlier paper (Billington & Nayudu, 1975).

Iodination of Membranes After Isolation. Membranes were isolated from groups of 20 mice (Billington & Nayudu, 1975) and suspended in 5 mM EDTA solution at 4 °C. 12.5 μg of lactoperoxidase and 150 μCi of $Na^{125}I$, both in 20 μl of phosphate-buffered saline, were added to the membrane suspension so that the total volume was 1.0 ml. Following the addition of 20 μl of hydrogen peroxide solution, the reaction was allowed to continue, with agitation, for 15 min at 30 °C.

Reaction was stopped by a rapid reduction in temperature to 4 °C, and the membranes were pelleted by centrifugation at $400 \times g_{av}$ for 5 min at 4 °C. The EDTA supernatant was monitored for counts and membranes were resuspended in 1 ml of 5 mM EDTA at 4 °C and repelleted until the number of counts per minute in the supernatant had been reduced to about 10% above background. In this way, unreacted oxidant and unbound isotope were removed from the membrane preparation. Membranes were solubilized and subjected to electrophoresis.

Analysis of Gels for Protein and Radioactivity. Gels were stained for protein with Coomassie Brilliant Blue, and destained, before being scanned densitometrically at 500 nm (Billington & Nayudu, 1975). Gels were sliced into discs exactly 1 mm thick, then each slice was placed in a separate vial and solubilized at 50 °C overnight in a solution consisting of 0.1 ml each of 0.1 M HCl and 30% hydrogen peroxide. Vials were then counted in the presence of Instagel liquid scintillant, using the rationale developed for liquid scintillation counting of ^{125}I and ^{131}I by Bransome and Sharpe (1972).

Results

Iodination in situ

Fig. 1 demonstrates the counts per minute profile of a typical acrylamide gel on which protein from brush border membrane iodinated *in situ* had been electrophoretically fractionated. The designation 'Control' means that the membranes were iodinated without prior treatment. We note that the ^{125}I label seemed to bind only to the proteins in the sixth band from the origin, estimated to have a molecular weight of close to 150,000 daltons (Billington & Nayudu, 1975).

Fig. 2 shows the apparent effect of removing some of the masking carbohydrate from the glycoproteins of the membrane. An additional band of estimated molecular weight 220,000 daltons was able to bind ^{125}I label.

Iodination of Isolated Membranes

Fig. 3 demonstrates the result of incubating a preparation of membranes with the iodination probe. Here, nearly every size of membrane

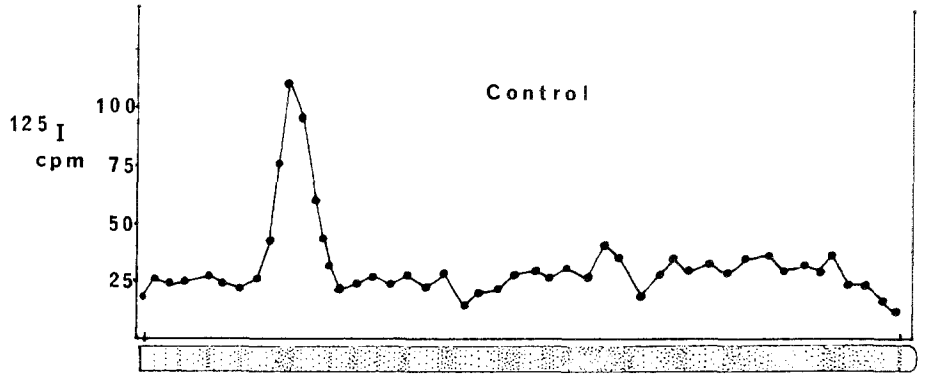

Fig. 1. Counts per minute profile of a gel on which microvillus membrane protein labelled *in situ* with an iodination probe had been electrophoretically separated. An artist's impression of the stained gel is shown below the profile

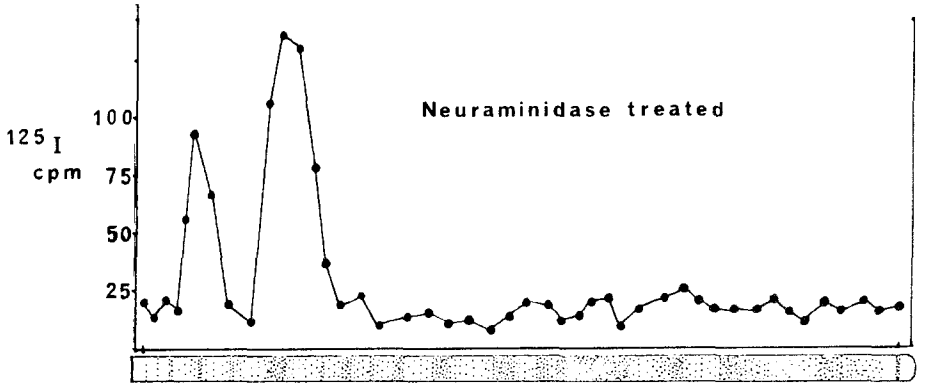

Fig. 2. Counts per minute profile of electrophoresed, charged with microvillus membrane proteins which had been treated with neuraminidase prior to being labelled *in situ* by the iodination probe. An artist's impression of the stained gel is shown below the profile

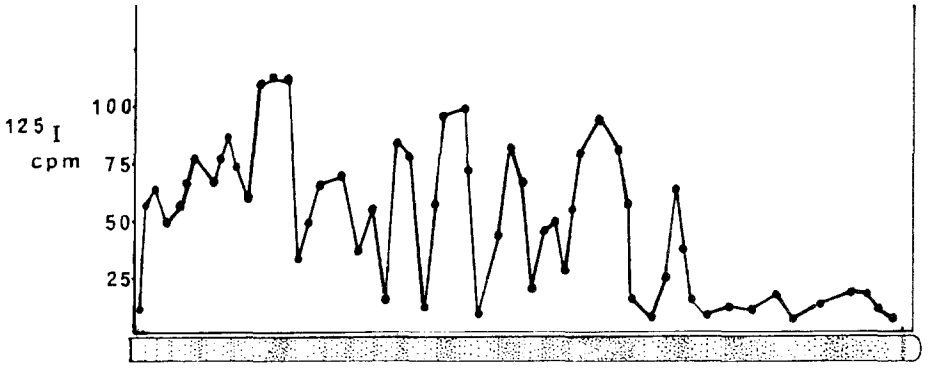

Fig. 3. Counts per minute profile of a gel with electrophoretically separated proteins from microvillus membranes, iodinated post-isolation

polypeptide was labelled. There are, however, two size classes of MW 25,000 and 16,000 daltons which were never iodinated from either luminally or bilaterally administered label.

Discussion

Microvillus membranes were exposed to luminally administered lactoperoxidase, hydrogen peroxide and ^{125}I *in situ* with and without neuraminidase treatment. Membranes were also iodinated post isolation. When membranes were not pretreated with neuraminidase the iodination probe attached only to the membrane protein of estimated molecular weight 150,000 daltons. This class of protein may thus reside in or on the surface of the microvillus membrane being exposed to the duodenal lumen.

If the iodination probe only attaches to tyrosyl residues, then it is the position of these residues which really defines the iodinatability of proteins. It is thus clear that though a particular protein may in fact be in the surface of a membrane, it will not be iodinated if the tyrosyl residues are masked from the probe.

This masking could be due to the tyrosyl residue being protected by other chains in the protein which do not contain tyrosines. Another likely occurrence is that although a protein may have a surface location it will not appear so if it does not contain tyrosyl peptides.

Several glycoprotein classes were shown to exist in the microvillus membrane (Billington & Nayudu, 1975), and it could be reasoned that their carbohydrate moieties may be preventing labelled iodide ions from binding to tyrosines in chains near the glycosylated portions of the membrane.

The difficulties associated with masking carbohydrate molecules may be overcome by the removal of substantial portions of the said complex sugar. Neuraminidase was chosen because it was thought that this was the mildest form of attack which could be used without adversely altering the remainder of the membrane. In the results obtained, sufficient carbohydrate has been removed to permit the iodide to label an additional membrane polypeptide of 220,000 daltons approximate molecular weight. This class may also have a luminal disposition in the microvillus membrane. There is the possibility that even after neuraminidase treatment, there still remains a significant amount of carbohydrate which may yet mask some truly luminally disposed protein types. Additional evidence is needed to resolve this question.

It is difficult to account for the discrepancy between the very few radioactive peaks and the many protein types involved in terminal digestion (disaccharidases and peptidases) or in transport, which would all be expected to be available to a luminally introduced iodination probe. An explanation for this apparent difference between expected and observed may be that all these luminally resident protein species have much the same molecular size and would thus not be resolved in the system used.

In the isolated membrane, more protein species were available to the probe, which may have been expected since label can come into contact with serosally as well as luminally disposed protein species. One or perhaps two classes of proteins are luminally disposed, whereas about 13 classes are apparently not thus disposed, as judged from the results obtained with this impermeant and thus exclusively vectorial probe. This seems to be very persuasive evidence for the proposition that there is an asymmetrical disposition of proteins in the lateral plane of the microvillus membrane.

The two polypeptide types which were never labelled from either luminally or bilaterally administered iodine probe, may be hypothesized to have a somewhat deep location in the membrane structure such that they are neither luminally or serosally exposed, and would not be expected to be readily accessible to the impermeant iodination probe. It was noted in an earlier paper (Billington & Nayudu, 1975) that the two polypeptides in question also took up Oil Red O stain. Since the publication of that paper, amino acid analysis has shown that the two polypeptides have tyrosine residues in their structure. Thus it is unlikely that the lack of iodination was due to a lack of tyrosines.

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