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THE DISSIMILAR NATURE OF TWO FORMS OF THE MAJOR HUMAN ERYTHROCYTE MEMBRANE GLYCOPROTEIN

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SUMMARY

Human red blood cell membranes solubilized in sodium dodecylsulfate contain two major and one minor glycoprotein species. The major species demonstrate a dimer–monomer relationship that becomes apparent when the solubilization step is carried out at 100 °C. Treatment of the membranes of intact cells with trypsin yields two proteolyzed forms of the dimer and one proteolyzed form of the monomer. Results of chymotrypsin treatment are similar. A model is presented indicating that the glycoprotein may exist in two forms in situ. One (Type A) is tightly bound, not dissociated by sodium dodecylsulfate at room temperature, and extremely sensitive to trypsin. The other (Type B) is loosely bound, easily dissociated by sodium dodecylsulfate at room temperature, and highly resistant to trypsin. It is possible that the Type B form may slowly depolymerize and reform while in the intact membrane.

INTRODUCTION

A change in lectin-induced agglutinability is a major characteristic demonstrated when normal cells are transformed into cancer cells. Although the mechanism of agglutination remains undetermined, it is known that lectin binding involves interaction with the carbohydrate portion of cell membrane glycoproteins [1]. However, the relationship of binding to agglutination is still unclear. Some investigators have reported a difference in the amount of lectin bound by cells that do or do not agglutinate [2, 3]. Other workers suggest that the amount of lectins bound remains the same [4–7]. They then postulate a difference in the topological distribution of glycoprotein agglutination sites as a mechanism for explaining the difference in agglutinability [8]. There is evidence that the mobility of lectin-binding sites is required for agglutination [9–12].

Changes in the distribution of glycoprotein sites in the erythrocyte membrane which resemble those found in some types of transformed cells may be induced by a variety of means. Intramembranous protein-containing particles were shown to be aggregated in low pH, low ionic strength buffer [13]. Topographical changes, which are also pH dependent, have been shown to affect sialic acid containing colloidal

iron-binding components such as the major human erythrocyte membrane glycoprotein (glycophorin) [14]. Anti-spectrin antibodies directed at the interior of the membrane surface were shown to alter the distribution of colloidal iron-binding sites on the exterior [15]. Non-reversible aggregation of glycoprotein containing sites was induced by trypsin or phospholipase C [16].

The structure of human erythrocyte membrane glycophorin has recently been elucidated and a model for its organization in the intact membrane proposed [17–19]. In this paper evidence will be presented indicating that glycophorin exists in more than one form in the intact membrane. Furthermore, it will be shown that these forms respond differently to dissociation in sodium dodecylsulfate and to the action of proteolytic enzymes. The possibility will be discussed that a change in the distribution of the different species significantly alters the behavior of the cell membrane.

METHODS

Outdated human blood was donated by the laboratory of the Ivinson Memorial Hospital, Laramie, Wyo. Red cells were separated by sedimentation and were washed twice in 5 mM Na_2HPO_4–0.9 % NaCl, pH 7.5 (isotonic buffer) before use.

Trypsin or chymotrypsin (Worthington) was dissolved in the isotonic buffer immediately before use. All proteolytic digestions were carried out at room temperature on samples containing approximately $1 \cdot 10^9$–$2 \cdot 10^9$ cells/ml, as calculated by assuming $5.7 \cdot 10^{-10}$ mg of protein/ghost [20]. Trypsin digestion was stopped by the addition of trypsin inhibitor (lyophilized jack bean trypsin inhibitor, Worthington) which had been dissolved in the isotonic buffer immediately before use. Samples were then centrifuged and washed twice with isotonic buffer. Chymotrypsin activity was retarded by lowering the temperature of the samples to 5 °C followed by immediate centrifugation and washing.

Red cell ghosts were prepared by washing cells three times with at least 20 vol. of 5 mM Na_2HPO_4, pH 7.8 at 5 °C. Centrifugations were for 5 min each at 16 000 rev./min at 5 °C on a Beckman J-21 centrifuge using the JA-20 rotor.

All chemicals for electrophoresis were purchased from Eastman Kodak Co. Samples for electrophoresis were solubilized for 15 min at room temperature in a solution containing 2 % sodium dodecylsulfate–8 % sucrose–pyronin Y tracking dye. Electrophoresis was carried out on gels 5.6 % in acrylamide and 0.1 % in sodium dodecylsulfate. Approximately 100 μg of protein was applied to each gel. Samples for the boiling experiments were solubilized in 3 % sodium dodecylsulfate and heated to 100 °C for varying lengths of time. Sucrose and pyronin Y tracking dye were added and the samples were electrophoresed as usual. All gels were subjected to 4 mA of current, and a typical run required 2.5 h. After electrophoresis, all gels were stained by a modification of the periodate–Schiff procedure of Fairbanks et al. [20]. Sodium dodecylsulfate was removed from the gels by washing in 25 % isopropyl alcohol–7 % acetic acid for 8 h, 10 % isopropyl alcohol–7 % acetic acid for 6 h, and 7 % acetic acid for 2 h. They were then stained by washing in the following solutions: 0.5 % periodic acid for 2 h, deionized water for 5 min, 0.5 % sodium arsenite for 50 min, 0.1 % sodium arsenite for 40 min, 7 % acetic acid for 10 min, Schiff's reagent for 6 h, and 0.1 % sodium metabisulfite–0.01 M HCl for 4 h. Gels were scanned at 550 nm on an Isco gel scanner equipped with a Linear Instruments Corp. integrating recorder.

Peak areas calculated from the recorder were the same as those obtained by cutting out and weighing the peaks.

RESULTS AND DISCUSSION

Human erythrocyte membranes solubilized in sodium dodecylsulfate contain two major and one minor glycoprotein species (Fig. 1). The predominant electrophoretic band (Band 1) represents about 60% of the staining material, and has apparent molecular weight of 50 000–60 000 [20, 21]. The second band (Band 2) has about 30 % of the staining material and has apparent molecular weight of 25 000–30 000. Recently, data from light scattering photometry has yielded approximate molecular weights of 59 000 for the Band 1 material and 29 500 for the Band 2 material [22]. The minor band (Band 3) accounts for the remaining 10 % of the staining material. No change in this distribution is seen even after the solubilized membranes remain in sodium dodecylsulfate for 24 h at room temperature (Fig. 1).

A major alteration of the normal distribution is caused by carrying out the solubilization step at 100 °C. Fig. 2 shows the scans of gels containing the glyco-

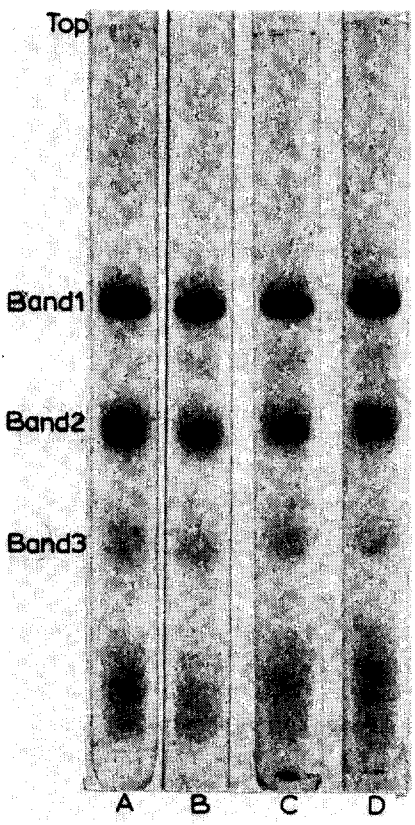

Fig. 1. Normal distribution of erythrocyte glycoproteins on polyacrylamide gels. Samples were solubilized in sodium dodecylsulfate at room temperature for 15 min (A), 1 h (B), 12 h (C), or 24 h (D). Electrophoresis and staining were performed as described in Methods.

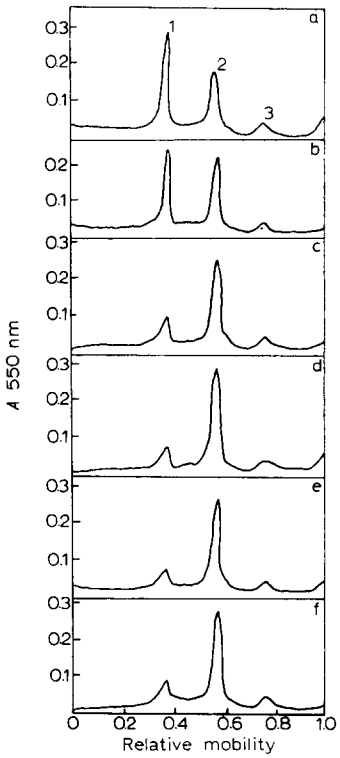

Fig. 2. Change in glycoprotein distribution when solubilization in sodium dodecylsulfate was carried out at 100 °C. Samples were solubilized in boiling sodium dodecylsulfate for either 1 min (B), 3 min (C), 10 min (D), 30 min (E), or 60 min (F). Gel A contains unboiled control. Electrophoresis and staining were as described in Methods. Gels were scanned on an Isco gel scanner at 550 nm.

protein from membranes solubilized in sodium dodecylsulfate at 100 °C for varying lengths of time. After 1 min a noticeable decrease in Band 1 is seen accompanied by an increase in Band 2. It has been suggested that the carbohydrate content of these two species might be similar [22, 23], allowing the possibility that an equimolar amount of them could be stained with equal intensity by the periodate–Schiff procedure. Thus, a decrease in Band 1 and an increase in Band 2 indicate a shift in glycoprotein and not a different response to the stain. After 3 min of boiling in sodium dodecylsulfate the change in the gel pattern has become quite drastic with Band 1 having been reduced to only 20 % of the staining material while Band 2 has increased to nearly 80 % (discounting the amount in Band 3). These data are in close agreement with those previously reported [24]. The amount of material lost from Band 1 is in all cases identical to the amount gained by Band 2. Thus, a condition exists where there is no net loss of glycoprotein. It has been suggested that the decrease in Band 1 and increase in Band 2 is due to depolymerization of the dimeric Band 1 form of the glycoprotein into the monomeric Band 2 form [24]. Peptide mapping of isolated pure Band 1 material and Band 2 material indicates that the peptides released from both molecules are the same, showing that the two species are probably the same glycoproteins

(Slutzky, G. M. and Ji, T. H., unpublished). Since there is little or no decrease in Band 1 or increase in Band 2 found during sodium dodecylsulfate solubilization at room temperature, it is very likely that they exist in both forms in the intact membrane. Differential labeling of the two species by lactoperoxidase has recently shown that the two glycoproteins are independent entities and not artifacts of the isolation and separation procedures [25]. It has also been recently shown that isolated Band 1 material labeled with ^{125}I electrophoresed in the Band 2 position after treatment at 100 °C. Under the conditions employed in their investigation, Tuech and Morrison [26] were able to demonstrate aggregation of ^{125}I-labeled Band 2 material back to the Band 1 position.

Trypsinization of the intact red blood cell mainly removes one peptide fragment of 16 000 molecular weight [17, 27, 28]. Removal of a particle of this size would significantly increase the mobility of the molecule on electrophoretic gels. If the glycoproteins were dimers, two types of product would be expected under mild trypsinization. One of higher molecular weight would have a fragment removed from only one subunit. The other type of product would have fragments removed from both subunits and would be of lower molecular weight. If the glycoproteins were all monomers, only one type of product would be present. A mixture of monomers and dimers would give a mixture of products.

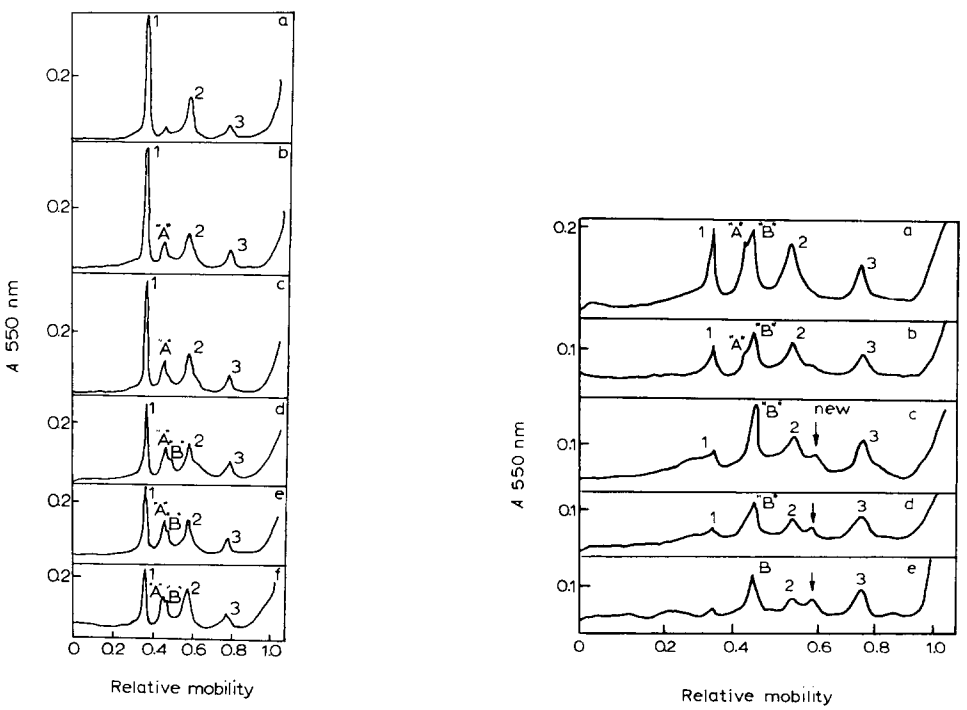

Fig. 3. (a) Scans of gels with glycoproteins from mildly trypsinized cells showing fine structure of the new intermediate band. Gels were scanned at 550 nm. Gel A is an untreated control. The others were treated with 0.5 μg/ml of trypsin at room temperature for 1 h (B), 2.5 h (C), 5 h (D) 6.25 h (E) or 7.5 h (F). (b) Scans of gels with glycoproteins from harshly trypsinized cells showing fine structure of the new intermediate bands and the new trypsinized Band 2 band. Samples were treated with 10.0 μg/ml of trypsin for the same time periods as those in (a).

Trypsin has a precise step-wise effect on the number and intensity of the glycoprotein bands. Gel scans (Figs 3a and 3b) reveal the following sequence of events. After treatment with 0.5 μg/ml of trypsin, Band 1 begins to decrease in intensity as a new band appears between Band 1 and Band 2. At first this band appears in the "A" position (closer to Band 1), but after 2.5 h of exposure a shoulder appears in the "B" position (closer to Band 2). By 7.5 h of exposure it is apparent that the intermediate band is a doublet. At the higher trypsin concentration (10 μg/ml) the intensity of the "A" peak diminishes until by 5 h of exposure all the intermediate is in the "B" position (Fig. 3b). Band 2 is not affected by trypsin until 2.5 h of exposure. At this time a new peak begins to appear that migrates faster than Band 2. By 7.5 h this new peak (trypsinized Band 2) has nearly the same intensity as the remaining intact Band 2. The minor glycoprotein, Band 3, is not affected by exposure to trypsin under the conditions employed in this investigation.

Band 2 is not affected by 0.5 μg/ml of trypsin (Fig. 4a) during 7.5 h of exposure to the enzyme. Band 1, however, is proteolyzed to yield two closely related bands of

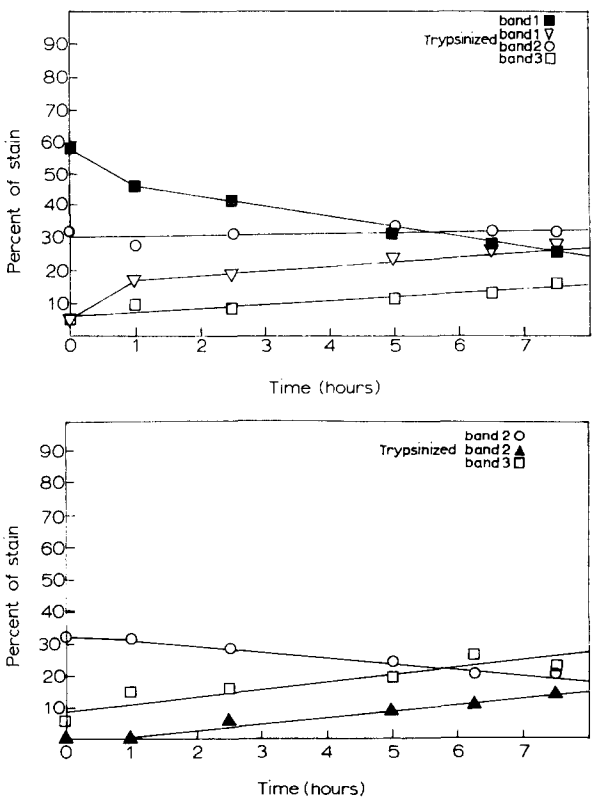

Fig. 4. (a) Kinetics of mild trypsinization. The areas under the glycoprotein peaks (from Fig. 3a) were integrated and plotted as percentages of the total periodate–Schiff's stain positive glycoprotein material from each gel. (b) Kinetics of harsh trypsinization (appearance of trypsinized Band 2). The areas under the peaks from Fig. 3b were integrated and plotted as percentages of the total Schiff's staining material. The apparent increase in the Band 3 line is due to Band 3 remaining constant while the total amount of staining material is decreasing.

lower molecular weight. When this intermediate is all in the "B" position, it undergoes no further proteolysis. At longer exposure to the higher trypsin concentration, Band 2 is proteolyzed to yield a new band of lower molecular weight (Fig. 4b).

Chymotrypsin has a similar effect on the glycoproteins (Fig. 5). At low concentrations an intermediate is formed of molecules of molecular weight intermediate between Band 1 and Band 2. At higher concentrations another intermediate is formed between Band 2 and Band 3. These intermediates are probably not identical to the trypsin intermediates, as trypsin and chymotrypsin attack different peptide bonds. The effect of chymotrypsin on the glycoproteins is not as severe as that of trypsin, as Band 1 is never totally removed as it is at very high trypsin concentrations. The effects of solubilization, proteolysis, and ^{125}I labeling on the major glycoproteins are compared in Table I.

Trypsin removes a glycopeptide fragment from the portion of the glycoprotein exposed on the outside of the cell surface [17, 27, 29]. It is possible that in one dimeric form [24, 26] (Type A) this glycopeptide region is strained into a conformation more accessible to trypsin cleavage than in the less strained Type B form (Fig. 6a).

Trypsin in low concentration would in a short time remove a fragment from one subunit of the Type A dimer. The remaining molecule would then electrophorese as the "A" intermediate. After longer exposure the fragment would be removed from

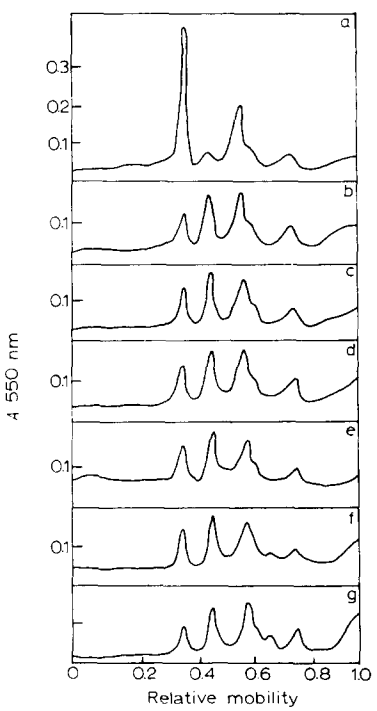

Fig. 5. Scans of gels of samples treated with chymotrypsin. Gel A is an untreated control. The others were treated for 30 min at room temperature with 5 μg/ml (B), 10 μg/ml (C), 25 μg/ml (D), 50 μg/ml (E), 100 μg/ml (F), or 250 μg/ml (G) of chymotrypsin. Proteolysis was inhibited by lowering the temperature to 5 °C and diluting with ice-cold buffer. Samples were then washed twice with cold buffer. Electrophoresis and staining were as in Methods. Gels were scanned at 550 nm.

TABLE I

COMPARISON OF REACTIVITIES OF TYPE A AND TYPE B FORM OF THE MAJOR HUMAN ERYTHROCYTE GLYCOPROTEIN

Experimental approach	Type A		Type B	
	Response	Position on gel	Response	Position on gel
Solubilization in sodium dodecyl-sulfate				
Room temperature	Intact dimer	Band 1	Some monomers	Band 1, Band 2
100 °C	Some monomers	Band 1, Band 2	Monomers	Band 2
Trypsin treatment				
0.5 μg/ml	Sensitive	Intermediates A and B	Resistant	Band 1, Band 2
10 μg/ml	Sensitive	Intermediate B	Some sensitive	Band 2, trypsinized Band 2
Chymotrypsin treatment				
<25 μg/ml	Sensitive	Band 1 and intermediate	Resistant	Band 1, Band 2
>25 μg/ml	Sensitive	Band 1 and intermediate	Some sensitive	Band 2, and below Band 2
^{125}I labeling by lactoperoxidase				
	Sensitive (1.0)*	Band 1	Sensitive (1.7)*	Band 2

* Radioactivity/glycoprotein calculated from Fig. 3 of Tuech and Morrison [26].

the second subunit. This species of even lower molecular weight would then electrophorese in the intermediate "B" position. The intermediate doublet containing bands at both the A position and the B position seen on gels at some exposure times eventually gives way to only the band in the B position, which persists at longer exposure times. The Type B form is not cleaved by 0.5 μg/ml of trypsin during 7.5 h of exposure. After 2.5 h of exposure to 10 μg/ml of trypsin a fragment is finally removed from the Type B molecule, leaving the trypsinized Band 2 molecules behind (Fig. 6b). The accessibility of the Type B form at this time may be due to changes in the membrane induced by trypsinization of other membrane components.

Glycoproteins are known to migrate anomalously on polyacrylamide gels which contain sodium dodecylsulfate. In some cases, such as following neuraminidase treatment of the erythrocyte glycoproteins, removal of groups from the glycoprotein actually causes an apparent increase in molecular weight [21]. Therefore, it is very possible that a dimer with two fragments removed might electrophorese only slightly faster than one with only one fragment removed, although the latter migrates substantially faster than the intact dimer.

The results of the trypsin experiments also indicate that there is no interconversion between the molecular forms electrophoresing as Band 1 and Band 2 or trypsinized Band 1 and trypsinized Band 2 when solubilization is carried out at room tempera-

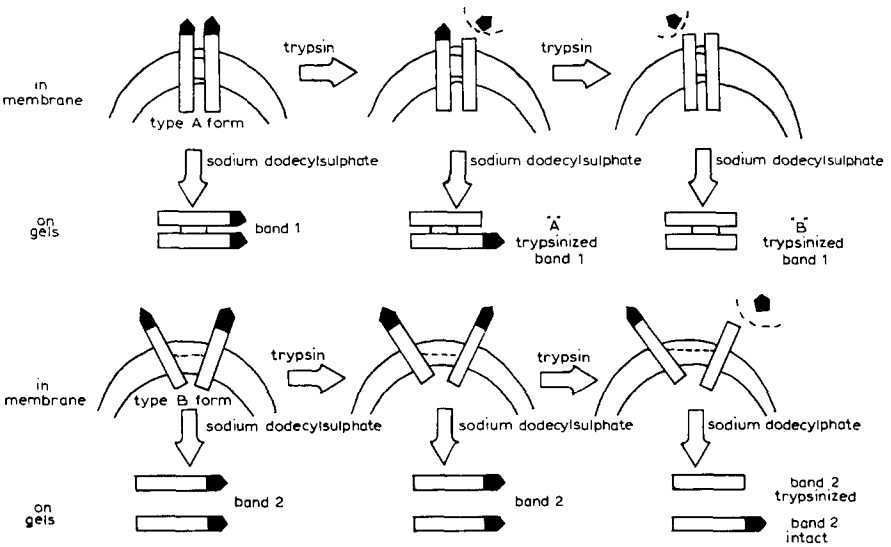

Fig. 6. (a) Model of organization of Type A molecules. The response of the Type A form to trypsin and sodium dodecylsulfate is described in Discussion. (b) Model of organization in the Type B form. The response of the Type B form to trypsin and sodium dodecylsulfate is described in Discussion. Although a dimeric form is implied by the model, evidence for a dimer is not conclusive.

ture. Any decrease in a high molecular weight band is matched by a corresponding increase in a band of lower molecular weight. We find no indication of lower molecular weight molecules aggregating into higher molecular weight forms.

Chymotrypsin produces essentially the same results as trypsin. However, the fine structure of the intermediate band is not as apparent as that produced by trypsin.

The tightly bound Type A dimeric form of the major erythrocyte glycoprotein is much more susceptible to the action of proteolytic enzymes than is the more easily dissociable Type B form. Also the Type A molecules are significantly more reactive toward bifunctional imidoester crosslinking reagents than are the Type B molecules [30, 31]. Since the forces holding the Type A dimers together require boiling in sodium dodecylsulfate to be disrupted, it is not likely that these dimers dissociate under normal conditions in the intact membrane. It is possible, however, that certain conditions favor the aggregation of free monomers into the Type B form. This would not change the number of glycoprotein sites, but could modify the cell's response to factors in the environment. Type B molecules may slowly dissociate into free monomers in the intact membrane. It would be expected that any free monomers would be even less available for proteolysis than are the Type B molecules. This might explain why even massive trypsin concentrations, 250–300 μg/ml for 1 h, fail to completely proteolyze Band 2 (Slutzky, G. M. and Ji, T. H., unpublished).

We have seen significant differences in the distribution of glycoproteins into dimers and monomers on gels containing membranes from the blood of patients with certain diseases (Slutzky, G. M. and Ji, T. H., unpublished). If it is found that the major glycoproteins of more complex cells exhibit a polymeric relationship similar to that seen on red cells, then the nature of specific sites as well as their distribution and mobility may be an important determinant of membrane behavior.

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