REASSOCIATION OF MEMBRANE GLYCOPROTEIN WITH INTACT ERYTHROCYTES[1]

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SUMMARY: Purified membrane glycoprotein was found to specifically bind to erythrocyte surfaces. (1) The glycoprotein showed over 80-fold greater binding to erythrocytes than did bovine serum albumin; (2) the binding of the glycoprotein was not significantly inhibited by the presence of other proteins even when added in 175-fold greater concentration; (3) the glycoprotein's binding showed both time and concentration dependence. Radiolabeled glycoprotein was re-isolated after binding to erythrocytes and, after sodium dodecyl sulfate-polyacrylamide gel electrophoresis, over 64% of the label was recovered from the glycoprotein bands. The glycoprotein preparation would bind to erythrocyte surfaces to the extent of 6-7% of that naturally occurring in the membranes.

Because of recent speculation on the structure of plasma membranes (1), their fluidlike character (2) and the intermolecular forces involved in maintaining membrane integrity (1, 3), we sought to determine if isolated protein components would reassociate with the membrane. If such reassociation should occur, it would be particularly relevant when one considers membrane self-assembly and the possibility of tagging living membranes or modifying the antigenic nature of the membrane.

Using the method of Marchesi and Andrews, we isolated the major glycoprotein component from human erythrocyte membranes. The preparation was 75% pure, contaminated only by other minor glycoprotein components. Here we report the binding of this protein to intact erythrocytes and the related rate, competition and specificity experiments.

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METHODS AND MATERIALS

Materials. Small quantities of erythrocytes were obtained by collecting freshly drawn human blood into citrate-dextrose anticoagulant. When larger quantities of blood were required freshly drawn polycythemic blood was obtained from Holy Cross Hospital, Salt Lake City, Utah. Erythrocyte membranes were prepared by osmotic lysis (4).

Diiodosalicylic acid (Aldrich Chemical, Milwaukee, Wis.) was recrystallized from methanol. The diiodosalicylic acid was then titrated with LiOH and the resulting lithium diiodosalicylate was recrystallized from water.

Gel electrophoresis. The procedures and apparatus used in the electrophoretic experiments have been described (5). Gels were stained for protein with Coomassie blue (6); or for glycoprotein by a periodic acid-Schiff's reagent (PAS) method, modified from that of Zacharius et al. (7). We took steps to remove SDS from the gels prior to staining. The gels were kept under reducing conditions after staining.

Glycoprotein preparation. Glycoprotein was obtained from erythrocyte membranes by the procedure of Marchesi and Andrews (8); however, phenol partitioning was repeated twice. Phosphocellulose column chromatography yielded no further improvement in the purity of the preparation. The glycoprotein preparation was radiolabeled with ^{125}I by the chloramine-T method (9). Protein concentrations were determined by the method of Lowry et al. (10).

Gel solubilization and counting methods. Polyacrylamide gels for liquid scintillation counting were frozen and sliced into 3 mm sections. The protein in each slice was solubilized by digestion with 15 ml of Protosol-Omnifluor (New England Nuclear, Boston, Mass.) solution for 48 hrs at 37°C. Vials were counted in a Packard Tricarb liquid scintillation counter. Polyacrylamide gels for γ-emission counting were sliced into 5 mm sections and counted directly.

Erythrocyte binding of glycoprotein. The binding studies were carried out at room temperature. Erythrocytes were washed twice and resuspended in 310 milliosmolar phosphate buffer, pH 7.4 (310 buffer). In the competition experiments one of several pure proteins in 310 buffer were added, and the volume adjusted with additional buffer. The solution was gently mixed and the radiolabeled glycoprotein added. The tube was again

gently mixed and the first sample removed. At desired time intervals, 0.10 ml of the remaining reaction mixture was pipetted into 1.0 ml of 310 buffer. The cells were quickly filtered on Whatman GF/C glass-fiber filters which were previously equilibrated with 2.0 ml of 1% BSA in 310 buffer. The collected cells were then washed with 5 ml of 310 buffer.

Mycoplasma binding experiments. It was not possible to recover Acholeplasma laidlawii[1] cells on these filters; instead, a centrifugation washing method was used. At desired time intervals, 0.10 ml of the reaction mixture was layered on top of a 5 to 10% sucrose gradient, buffered with 0.02 M Tris-HCl in 0.9% NaCl, pH 7.8. The cells were centrifuged through the gradient in a swinging bucket rotor at 25,000 x g for 30 min. The sucrose was decanted and the lip of the inverted tube dried. The pelleted cells were then resuspended in a small amount of buffered saline and transferred to vials for counting. The resuspended cells and filters were then counted for γ-emission.

RESULTS

Gel electrophoresis. After sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Fig. 1), the glycoproteins recovered from erythrocyte membranes had a pattern and apparent molecular weight very similar to those reported by Fairbanks et al. (6). The major glycoprotein species (Fairbanks et al. refer to this species as PAS 1) had a molecular weight of 83,000. The glycoproteins amount to approximately 2% of the total membrane protein. From densitometer tracings of the gels, PAS 1 was estimated to be 1.6% of the total protein.

Binding kinetics. The glycoprotein binding showed typical absorption kinetics (Fig. 2). After 3 to 4 hours, the membrane was essentially saturated as no more was taken up. Half-saturation occurred at about 1 hour. In several experiments, the maximum amount of glycoprotein bound averaged 2.0 μg per mg membrane protein. This represents an addition of 6 to 7% glycoprotein to the membrane during binding.

The early phase of glycoprotein binding to erythrocytes was found to be first order with respect to binding sites on the membrane (Fig. 3). The following relation was used:

$$-\ln (C_\infty - C_t) = kt + \ln (C_\infty - C_0)$$

[1] A. laidlawii was formerly called Mycoplasma laidlawii (11).

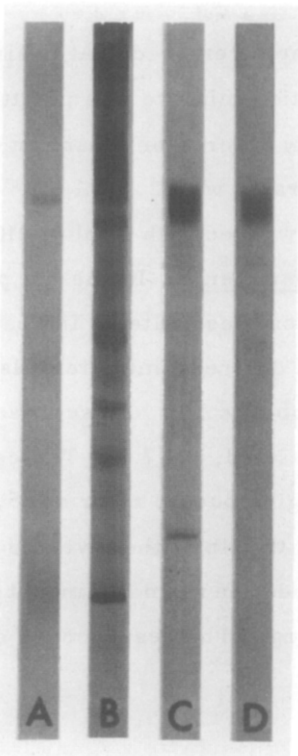
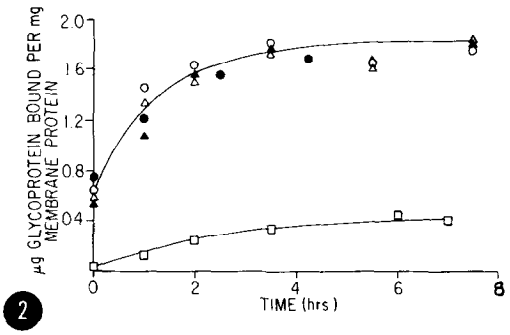


Fig. 1. SDS-polyacrylamide gel electrophoresis of whole erythrocyte membranes and the isolated ^{125}I labeled glycoprotein preparation. Gels A and B were duplicate electrophoragrams of whole membranes. A was stained for glycoprotein and B for total protein, 100 μg protein was loaded on each gel. Duplicate gels of the radiolabeled glycoprotein preparation C were stained for total protein and D for glycoprotein.

where C_∞, k, and t are the saturation concentration, rate constant, and time respectively. A saturation value of 2.0 μg bound per mg membrane protein was assumed. The first order rate constant was 0.03 μg glycoprotein per mg membrane protein per min.

Binding of bovine serum albumin. Bovine serum albumin (BSA) was radiolabeled with iodine as above and binding studies were carried out. The BSA binding did not increase with time and was insignificant compared to the glycoprotein bound (Fig 1).

Specificity studies. Competition experiments were conducted with the competing protein present at 35-fold greater concentration than the glycoprotein (Fig 2). No significant inhibition was found for any of the proteins at this concentration. It is also important to note that the protein competitors did not affect the shape of the curves. A similar lack of inhi-



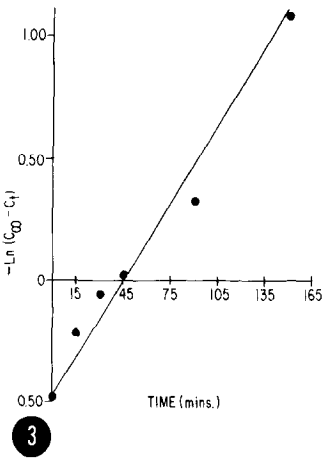


Fig. 2. Kinetics of binding of the glycoprotein to erythrocytes and A. laidlawii cells. The erythrocyte binding assay mixture contained at final concentration 8.2 μg/ml radiolabeled glycoprotein, specific activity, 1.08 x 10^5 cpm/min, and 2.4 x 10^9 erythrocytes per ml in a total volume of 3.5 ml of 310 buffer. No competing protein present, Δ - Δ. BSA present at a final concentration of 0.29 mg/ml (35-fold greater than the glycoprotein), 0 - 0. Ovomucoid present at 0.29 mg/ml, ▲ - ▲. Myoglobin present at 0.29 mg/ml, ● - ●. The uptake of glycoprotein by myoplasma cells, □ - □. The mycoplasma binding assay mixture contained the same concentration of glycoprotein as above, and 1.50 x 10^{11}.

Fig. 3. First order plot of the glycoprotein binding to erythrocytes. The assay mixture is the same as in Fig. 2 except that the erythrocyte cell count was 2.32 x 10^9 cells/ml.

bition also resulted when BSA was added in 175-fold greater concentration. Glycoprotein bound to A. laidlawii cells to an appreciable extent, but the rate was slower and the maximum binding was significantly less than with erythrocytes (Fig 2, Table 1). Thus the binding was at least moderately specific for cell type. The maximum amount of glycoprotein bound per erythrocyte was 6-7% of the glycoprotein naturally occurring in the membranes

Glycoprotein recovery experiments. In order to demonstrate that the glycoprotein was bound in an undegraded form, erythrocytes carrying bound radiolabeled glycoprotein were lysed and the membranes prepared for electrophoresis as usual. The gels were stained for protein and glycoprotein, then sliced and counted for γ-emission. By summation of counts in the glycoprotein bands, the recovery of radiolabel from bound glycoprotein totaled over 64% of the maximum number of counts recoverable

TABLE 1. Specificity of Glycoprotein Binding.

Radiolabeled protein	Cells	Maximum amount of protein bound (μg bound per mg membrane protein)
erythrocyte glycoprotein[a]	erythrocytes	1.8-2.1
BSA[b]	erythrocytes	0.025
erythrocyte glycoprotein[a]	A. laidlawii	0.41
erythrocyte glycoprotein[a]	none	N.S.
BSA[b]	none	N.S.

[a] final protein conc. 8.3 μg/ml
[b] final protein conc. 21.4 μg/ml

(Fig 4). Note, also, that in comparing the bound glycoprotein to the unbound glycoprotein, that the ratios of activities in the various PAS positive bands have not changed.

Erythrocyte membranes were washed until white, and, in duplicate experiments, 1×10^{10} cells yielded 0.417 gm membrane protein. For A. laidlawii cells, again in duplicate experiments, 1×10^{11} cells yielded 0.281 mg membrane protein.

DISCUSSION

At this point we can only speculate about the mechanism by which the glycoprotein binds to membranes. We found that the binding of the glycoprotein to membranes reached a saturation level and displayed first order kinetics with respect to binding sites available, thus indicating a limited number of association sites on the membrane. In order to broaden the importance of our findings, the ability of other isolated membrane proteins to bind to membranes should be investigated.

The antigens found on membranes at various stages in the cell cycle have been implicated in regulation of cellular function (12). In the future, by binding glycoproteins which have specified antigenicity to cell surfaces, it may be possible to regulate cellular function. Further work is needed to determine what bond interactions are involved in binding the glycoprotein to the membrane. Such interactions may have important implications for membrane self-assembly.

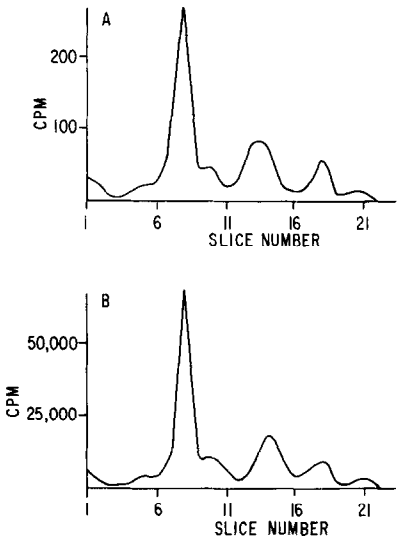


Fig. 4. Recovery of radiolabeled glycoprotein bound to erythrocytes. A. Electrophoresis of erythrocyte membranes carrying radiolabeled glycoprotein, 3020 cpm were loaded onto the gel. B. Electrophoresis of the radiolabeled glycoprotein preparation previous to binding. In this case, 382,500 cpm were loaded onto the gel. The activity was determined by γ-emission.

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REFERENCES

1. Vanderkooi, G., and Green, D. E., Bioscience, 21, 409 (1971).
2. Frye, L. D., and Edidin, M., J. Cell. Sci., 7, 319 (1970).
3. Singer, S. J., and Nicolson, G. L., Am. J. Path., 65, 427 (1971).
4. Dodge, J. T., Mitchell, C. D., and Hanahan, D. J., Arch. Biochem. Biophys., 100, 119 (1963).
5. Yarrison, G., Young, D. W., and Choules, G. L., J. Bact., 110, 494 (1971).
6. Fairbanks, G., Steck, T. L., and Wallach, D. F. H., Biochemistry, 10, 2606 (1971).
7. Zacharius, R. M., Zell, T. E., Morrison, J. H., and Woodlock, J. J., Anal. Biochem., 30, 148 (1969).
8. Marchesi, V. T., and Andrews, E. P., Science, 174, 1247 (1971).
9. Hunter, W. in Handbook of Experimental Immunology, Weir, B. M., ed p. 608. F. A. Davis and Co., Phila., 1967.
10. Lowry, O. H., Rosenburgh, N. J., Farr, A. L., and Randall, R. J., J. Biol. Chem., 193, 265 (1951).
11. Edward, D. G. and Freundt, E. A., J. Gen. Microbiology, 62, 1-2 (1970).
12. Fox, T. O., Sheppard, J. R., and Burger, M. M., Proc. Natl. Acad. Sci. U. S., 68, 244 (1971).