HETEROGENEITY OF THE SIALOGLYCOPROTEINS OF THE NORMAL HUMAN ERYTHROCYTE MEMBRANE

Thomas J. Mueller, Alan W. Dow and Martin Morrison

Laboratory of Biochemistry
St. Jude Children's Research Hospital
Memphis, Tennessee 38101

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SUMMARY: The sialoglycoproteins of the human erythrocyte membrane can be separated into at least eight periodic acid Schiff-positive components using sodium dodecyl sulfate gel electrophoresis in the discontinuous buffer system of Laemmli. All eight components can also be labeled by mild periodate oxidation followed by reduction with NaB^3H_4. Periodic acid Schiff 2 can be resolved into at least three distinct components. Two sialoglycoproteins, with apparent molecular weights of 35,000 and 27,000 do not appear to be labeled by lactoperoxidase-catalyzed iodination at the external membrane surface.

INTRODUCTION

The sialoglycoproteins of the human erythrocyte membrane have been among the most widely studied membrane proteins. In the early work on this important membrane component, it was assumed that the bulk of the sialoglycoprotein was essentially a single chemical entity (1,2). With the advent of SDS[1] gel electrophoresis, the sialoglycoproteins of the erythrocyte membrane were separated into three components which were detected on gels by periodic acid-Schiff reagent and designated PAS 1, 2 and 3 (3). Further work showed that two less intensely staining components, PAS 4 (4,5) and PAS 2′ (6), were also present, and that under appropriate conditions, PAS 1 could be converted to PAS 2 indicating a monomer-dimer relationship between these components (2,7,8). However, if electrophoresis was performed under conditions where PAS 1 would not monomerize to PAS 2, lactoperoxidase labeling clearly indicated that, although PAS 1 was labeled on the intact cell, PAS 2 was not (6). These results suggested that PAS 2 might be a heterogeneous component.

Because of the complexity of the relationship between these molecules, we have continued to investigate the methods of separating the sialoglycoproteins and report here the

[1] Abbreviations: SDS –sodium dodecyl sulfate; PAS –periodic acid-Schiff; DTT –dithiothreitol; PBS –phosphate buffered saline; DMSO –dimethylsulfoxide; PPO –2,5-diphenyloxazole.

use of the discontinuous buffer system described by Laemmli (9). This system gives increased resolution of the erythrocyte glycoproteins which are labeled by the galactose oxidase procedure (10).

METHODS

The preparation of hemoglobin-free stroma from washed human erythrocytes, and lactoperoxidase-catalyzed iodination of intact erythrocytes have been previously described (6). Sialoglycoproteins were extracted using the chloroform-methanol procedure (11) as described by Hamaguchi and Cleve (12) as previously reported (6).

In order to label the sialoglycoproteins, the sialic acid residues were oxidized with periodate (13,14) as described by Steck and Dawson (5). Five ml of packed stroma were diluted with 5 ml of phosphate-buffered saline (PBS) and incubated with 10 ml of 2 mM $NaIO_4$ in 0.1M sodium acetate buffer (pH 5) for 10 min at 0°C in the dark. The mixture was diluted with an equal volume of cold 5 mM sodium arsenite in 50 mM Na_2HPO_4, centrifuged at 27,000 x g for 15 min, washed once with sodium arsenite – Na_2HPO_4, and once with PBS. The membranes were diluted to 10 ml with PBS and labeled by incubating for 20 min at room temperature with 3 mCi of NaB^3H_4 (Amersham-Searle; 271 mCi/mmol) in 30 µl 0.01 N NaOH. The membranes were washed four times with 20 mOsm phosphate buffer pH 7.4. Controls omitting the periodate were also performed under identical conditions. No changes were observed in the staining profiles following SDS gel electrophresis, indicating that protein degradation (5) did not occur.

SDS polyacrylamide gel electrophoresis using a continuous phosphate buffer (pH 7.2 –7.4) system was performed in 12 X 0.5 cm cylindrical gels as previously described (6). Samples were prepared by boiling for 3 min with at least an equal volume of sample buffer containing 2% SDS, 2% mercaptoethanol and 8 M Urea.

Discontinuous SDS gel electrophoresis was performed as described by Laemmli (9) using either cylindrical or slab gels. Cylindrical gels consisted of a running gel 10 cm in length and a 1 cm stacking gel of 3.5% acrylamide concentration. Slab gels were electrophoresed on a Bio Rad Model 220 Dual Vertical Slab apparatus. The slabs were 1.5 mm in thickness and consisted of a 10 cm running gel and a 3.5% stacking gel. Electrophoresis was conducted at 20 volts (constant voltage) until the tracking dye reached the bottom of the gel. Samples were prepared for electrophoresis by boiling for 5 min with at least an equal volume of sample buffer containing 50% glycerol, 1.25% SDS, 5% DTT, 0.06 M Tris-Cl pH 6.8 and 0.0025% bromphenol blue.

Proteins were visualized in gels by staining with Coomassie Brilliant Blue. Glycoproteins were detected using a modification of the periodic acid-Schiff base procedure described by Glossman and Neville (16). Dansylated protein markers were prepared as described by Talbot and Yphantis (15).

Fluorographic detection of radioactive components was conducted at -70°C using Kodak RP Royal X-Omat film (RP/R-54) following impregnation of the gels with PPO in DMSO as described by Bonner and Laskey (17). Gels were vacuum dried prior to fluorography.

RESULTS AND DISCUSSION

A typical PAS staining profile of the isolated sialoglycoproteins following SDS gel electrophoresis using a continuous phosphate buffer system is shown in Fig. 1 – gel A. Three major PAS-positive components can be seen, PAS 1, 2 and 3. In addition, a less intensely staining band, PAS 2', can also be detected.

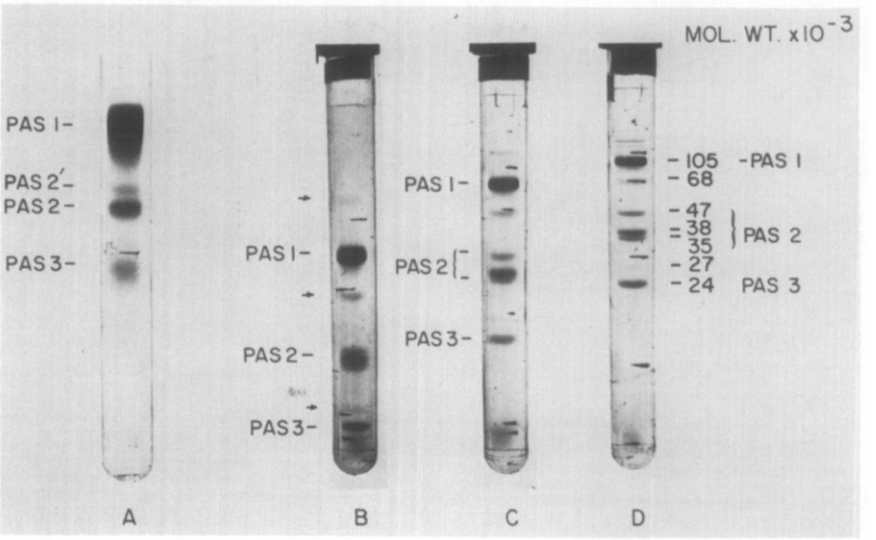

Figure 1:
A comparison of the electrophoretic separation of normal human erythrocyte sialoglycoproteins using a continuous phosphate buffer system (gel A) and the discontinuous buffer system of Laemmli (9; gels B,C, and D). The percentage of acrylamide in each SDS gel is as follows: gels A,C – 10%; gel B – 7.5%; gel D – 12.5%. The stab marks in gels B,C, and D indicate the position of the dansylated protein markers (from the top of the gel): B-galactosidase (130,000), bovine serum albumin (68,000), carbonic anhydrase (29,000), and cytochrome *c* (12,500). The bottom stab mark in gels C and D indicates the position of the tracking dye. In gel B, the tracking dye coincides with the position of dansylated cytochrome *c*. The arrows adjacent to gel B indicate the position of additional components detected with the periodic acid-Schiff staining procedure.

The PAS-staining profiles of the isolated sialoglycoproteins following electrophoresis on SDS gels of varying acrylamide concentrations using the discontinuous buffer system of Laemmli are shown in gels B–D of Fig. 1. On gel B (7.5%), at least three PAS-positive components can be detected, in addition to PAS 1, 2 and 3. The PAS 2 region of gel B also appears to consist of more than one component. This heterogeneity of PAS 2 is further accentuated on gels of higher acrylamide concentration. As seen in gels C (10%) and D (12.5%), this region appears to consist of at least three distinct components.

Figure 2 is a fluorograph of isolated sialoglycoproteins separated on a 12.5% slab gel using the discontinuous buffer system. Sample A is a sialoglycoprotein preparation labeled by mild periodate oxidation followed by reduction with NaB^3H_4. This procedure labels primarily sialic acid residues. All of the components which stain with PAS in gel D of Fig. 1 are labeled by this procedure. Sample B is a sialoglycoprotein preparation isolated from intact erythrocytes labeled with ^{125}I at the external membrane surface using lactoperoxidase-catalyzed iodination. The 35,000 and 27,000 molecular weight components,

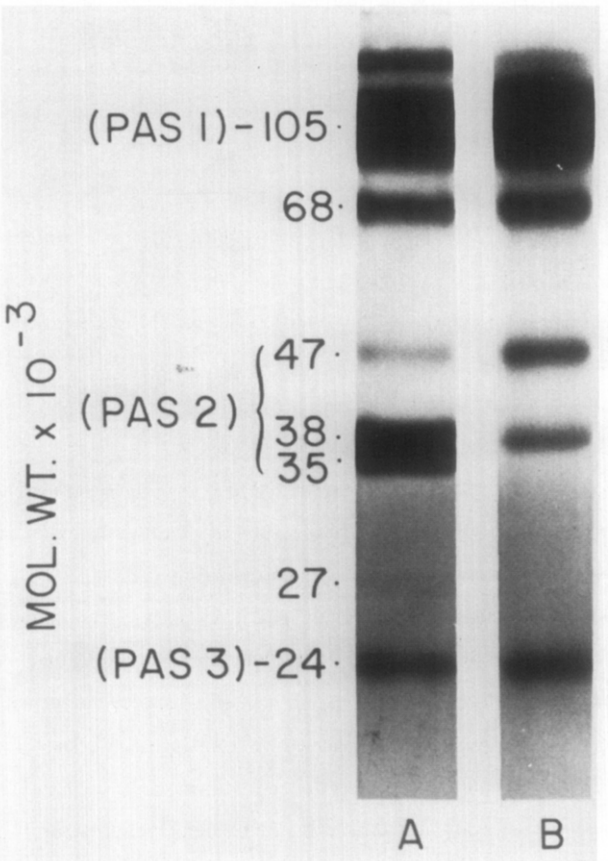

Figure 2:
A comparison of the electrophoretic separation of the sialoglycoproteins labeled by NaB^3H_4 reduction following mild periodate oxidation (A) and the sialoglycoproteins labeled by lactoperoxidase-catalyzed iodination at the external surface of intact erythrocytes (B). The labeled components were detected by fluorography as described in Methods. Electrophoresis was conducted on a 12.5% SDS acrylamide slab using the discontinuous buffer system of Laemmli (9).

which are labeled by periodate-NaB^3H_4, do not appear to be labeled by lactoperoxidase at the external membrane surface.

These results clearly indicate that the erythrocyte sialoglycoproteins are a more heterogeneous population than previously thought. The heterogeneity of the PAS 2 region is most interesting in light of the fact that PAS 1 can dissociate to a monomeric form which runs in the PAS 2 region (2,7,8). We have previously reported that PAS 1 is readily labeled by lactoperoxidase at the external membrane surface, but that PAS 2 contained no label when SDS gel electrophoresis was conducted under conditions where no interconversion of PAS 1 and 2 would occur (6). However, if electrophoresis was performed using conditions

appropriate for the interconversion of PAS 1 to PAS 2, then the PAS 2 region of the gel contained labeled components (8). These earlier results suggested that PAS 2 might be heterogenous and contain both an unlabeled component as well as a labeled monomer of PAS 1.

The results presented here indicate that either the 47,000 or 38,000 molecular weight component is the monomeric form of PAS 1, since both of these components are labeled by lactoperoxidase at the external membrane surface. Results obtained using two dimensional electrophoresis demonstrate that the 38,000 molecular weight glycoprotein is the monomeric form of PAS 1, and that the 47,000 molecular weight component is in equilibrium with the 68,000 and 24,000 molecular weight components (manuscript in preparation). In addition, the unlabeled 35,000 molecular weight glycopeptide appears to be an interconversion product of another component which also migrates in the PAS 1 region, suggesting that PAS 1 is also heterogeneous. Marchesi and coworkers have also reported the existence of two distinct molecules in PAS 1 which differ in amino acid composition (18).

The nature of the high molecular weight PAS-positive components is unclear. We consistently see one band of approximately 140,000–170,000 molecular weight. On occasion, several other minor high molecular weight bands are also observed which are PAS-positive and can be labeled with NaB^3H_4 after mild periodate oxidation. Whether these high molecular weight species are exclusively aggregation products is unclear, although other workers have also reported the presence of aggregates of the erythrocyte sialoglycoproteins (18,19).

It is clear that SDS acrylamide gel electrophoresis using the discontinuous buffer system of Laemmli (9) provides increased resolution of the sialoglycoproteins of the human erythrocyte membrane. Two of these sialoglycoproteins are not labeled by lactoperoxidase-catalyzed iodination at the external membrane surface.

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