Journal of Supramolecular Structure 4:481 (441)–486 (446) (1976)

SUBSTRATE HETEROGENEITY OF COMPONENT a OF THE HUMAN ERYTHROCYTE MEMBRANE

Allen D. Roses

Duke University Medical Center, Durham, North Carolina

Component a of the erythrocyte membrane is a specific substrate for endogenous protein kinase activity and its phosphorylation is significantly decreased under assay conditions in myotonic muscular dystrophy (Roses, A.D., and Appel, S.H., J. Membr. Biol. 20:51–58 (1975)). We have demonstrated substrate heterogeneity of two fractions of component a separated by concanavalin A (Con-A) sepharose chromatography. The fraction of component a that is retarded by Con A and eluted with α-methyl-D-glucoside does not accept the transfer of phosphate from [γ-^{32}P] ATP as a substrate for endogenous protein kinase activity. The nonretarded fraction contains > 90% of the radioactive label. These experiments also confirm the carbohydrate heterogeneity of component a (Findley, J. B. C., J. Biol. Chem. 249: 4398 (1974).

INTRODUCTION

The muscular dystrophies comprise a large number of inherited diseases with physiological, clinical, and biochemical evidence that suggests a membrane defect expressed in many tissues throughout the body (1). Our investigations have concentrated on myotonic muscular dystrophy (MyD) and have used erythrocyte ghosts as a model for studying possible membrane abnormalities (2, 3). Erythrocytes (RBC) are easily accessible, have no known functional abnormality in MyD, and do not suffer from the difficulties of diseased muscle tissues including the presence of atrophy, fibrous tissue, and possible denervation, as well as the small amount of tissue available at biopsy.

Both biophysical and biochemical techniques have defined abnormalities of the RBC membrane in this disorder. Morphological studies with scanning electron microscopy have demonstrated an increased number of cup-shaped (stomatocytes) erythrocytes compared to similarly treated and fixed controls (4). These results, however, appear to be nonspecific for several inherited diseases.

Electron spin resonance spectroscopy has substantiated the presence of membrane differences in MyD erythrocytes. Increased membrane fluidity is most apparent near the surface of the MyD membrane while polarity differences are approximately constant at various depths within the membrane (5).

The morphological and biophysical experiments were initiated following the demonstration of a decrease in endogenous protein kinase activity in aged, frozen ghosts (2). With improved techniques of ghost preparation, phosphorylation in fresh MyD membranes was noted to be decreased in protein band III (3). This region of the gel

includes several species and the purpose of these experiments was to characterize the substrate being phosphorylated. We were also interested in whether the decrease in phosphorylation of MyD was due to differences in the substrate, enzyme, or physical state of the membrane secondary to lipid, or to other differences.

METHODS

Erythrocyte ghosts were prepared from freshly drawn heparinized blood as previously described (3, 6). All electrophoretic analyses and endogenous protein kinase assays were performed as previously described (2, 3). The major sialoglycoprotein preparations were performed according to the methods of Marchesi and Andrew (7) and Hamaguchi and Cleve (8).

Concanavalin A (Con A) sepharose was purchased from Pharmacia Fine Chemicals, Inc. Ghosts were solubilized for affinity chromatography in a 1% deoxycholate (DOC) solution containing 0.3 mM $CaCl_2$. All procedures were performed at 4°C. The Con A sepharose columns were washed with 5 bed volumes of this solution and the eluates collected. The column was then washed with 5 bed volumes of 100 mM α-methyl-D-glucoside in the 1% DOC solution and the eluates collected. Portions of the pooled eluates were treated with 2%-acetic acid and centrifuged at 27,000 × g for 10 min in a refrigerated Sorvall centrifuge. The pellets were resuspended in 95% ethanol and recentrifuged three times. The remaining pellets were solubilized in 1% sodium dodecyl sulfate (SDS), 6 mM mercaptoethanol, and 5 mM ethylene diaminetetraacetic acid (EDTA) for SDS-polyacrylamide gel filtration. Staining and counting procedures were as previously described (2, 9). Triton X-100 solubilization and Con A affinity chromatography as described by Findley (14) gave identical results.

RESULTS

For the purpose of clarity we define Band III as the total Coomassie blue staining band that migrates in SDS-polyacrylamide gels with an apparent molecular weight of 90,000–100,000 (6). Band III is not a single species but is known to represent the area of migration of several polypeptides including Na^+K^+ ATPase (10, 11) and component a (12–14). We have referred to component a as the minor glycoprotein described by Bretscher (12) that comigrates at mol wt 90,000–100,000 and can be extracted by using 36 mM–50 mM Lis, as described by Steck and Yu (15).

Several preliminary studies were performed to differentiate component a from the major sialoglycoprotein of the erythrocyte. We attempted to isolate the major sialoglycoprotein by the method of Marchesi and Andrew (7) and were unable to obtain a preparation that was completely clean of Coomassie blue stainable material at 90,000–100,000 mol wt area. The major sialoglycoprotein migrated anomalously on SDS-polyacrylamide gels with an apparent mol wt of 75,000–80,000 as demonstrated by PAS staining. When this procedure was utilized following phosphorylation of the erythrocyte with [γ-^{32}P] ATP, the radioactivity was located only in the 90,000–100,000 mol wt region of the gels.

In order to assure that component a and not the sialoglycoprotein was the site of phosphorylation, we used the procedure of Hamaguchi and Cleve (8) to extract the major

sialoglycoprotein, designated glycoprotein I in their paper. Coomassie blue staining revealed no stain in the 90,000–100,000 mol wt region and no phosphorylation was demonstrated on the gels at either the 90,000–100,000 mol wt area or at the 75,000–80,000 mol wt area of migration of glycoprotein I.

Futher studies have demonstrated that component a is not a single species of glycoprotein. Ghosts were solubilized with 1% deoxycholate as described in Methods and the material was applied to sepharose derivatives of Con A. The material retained on the Con A column and eluted with α-methyl-D-glucoside is a fraction of the Band III or component a minor glycoproteins. This behavior has also been reported recently by Findley (14) and is apparently due to heterogeneity of the carbohydrate moiety within the component a glycoproteins (Fig. 1).

Evidence suggesting that the protein of component a is not a single species results from the Con A separation of ^{32}P-labeled component a. The procedure was identical to previous experiments except that endogenous protein kinase assays (3) were performed before solubilization in 1% DOC. The phosphorylated substrate of the endogenous protein kinase reaction is not retained by the Con A affinity chromatography procedure and can be demonstrated in the comigrating protein peak that is not retained by the insolubilized lectin (Table I; Fig. 1). We reproducibly obtained less than 1% total radioactivity and less than 10% of the phosphorylation of component a in the Con A-retained fraction in 12 experiments. Differences in the phosphorylation of MyD component a were again demonstrated in the unretarded fraction (3).

DISCUSSION

Our results confirm the suggestion of carbohydrate heterogeneity of component a (14). In addition, we have demonstrated that component a is heterogeneous with respect to its ability to accept ^{32}P as a substrate for endogenous protein kinase activity. There are several possible explanations for this result.

Component a may be a diverse group of glycoproteins that not only have carbohydrate heterogeneity but also have differing apoprotein moieties. Indeed, there are no data to support the assumption that component a apoprotein is uniform. Component a has been demonstrated to contain 8% carbohydrate and to migrate as a broad peak in SDS-polyacrylamide gels (3, 6, 14, 16). No additional structural or functional data are available concerning the homogeneity of the apoprotein portion of component a. We suggest that component a may contain at least two (and probably more) species of apoprotein that are involved in separate reactions within the membrane and probably subserve various functions.

The recent analysis by Ho and Guidotti (6) of the amino acid and carbohydrate composition of component a was determined from protein purified on the basis of elution from sepharose 4B columns and from polyacrylamide gels. Both steps were run in the presence of SDS and an apparent 100,000 dalton protein was analyzed. This procedure does not account for possible differences in apoprotein (or carbohydrate) heterogeneity and represents the amino acid composition of at least two similar glycoproteins, one of which acts of an acceptor of ^{32}P from [γ-^{32}P] ATP and the Con A retarded molecular species.

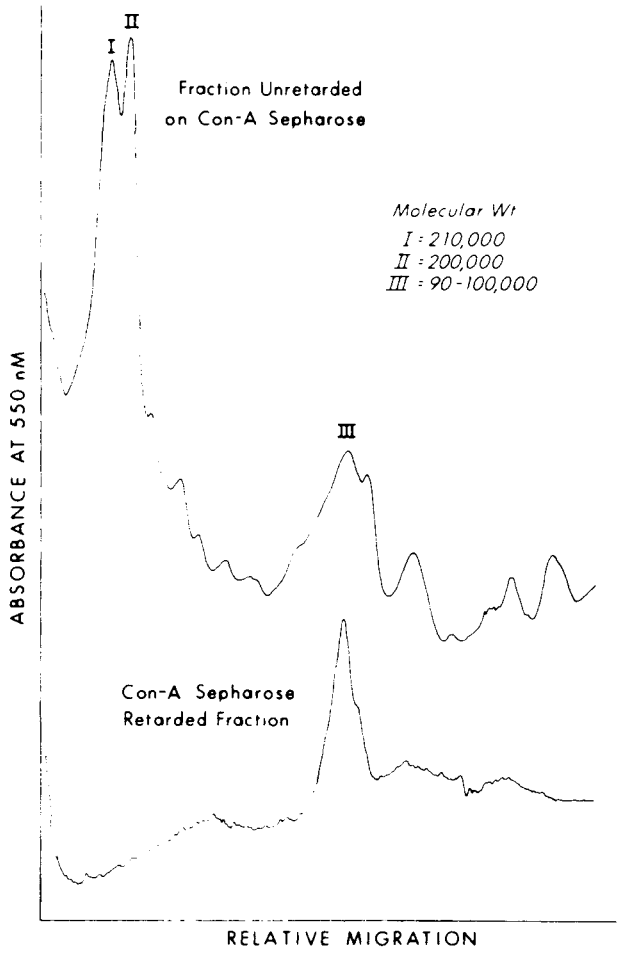

Fig. 1. Concanavalin A sepharose separation of erythrocyte ghost proteins. Top: fraction unretarded by Con A (contains the radioactivity in labeled experiments, Table I). Bottom: fraction retarded by Con A and eluted with α-methyl-D-glucoside.

Molecular weight estimations from SDS-polyacrylamide gels assume that the solubilized protein is monomeric and that the amount of SDS (gram per gram) bound to the protein is the same as water-soluble proteins. It is further assumed that the hydrodynamic size of the glycoprotein and its molecular weight are identical to water-soluble proteins that are true standards for molecular weight estimation. None of these criteria have been established for component a and it is quite possible that binding of detergents may be anomalous and that the hydrodynamic behavior may be affected by the presence of carbohydrate. This behavior has recently been demonstrated with respect to the major sialoglycoprotein (17). Component a migrates as a wide band at an apparent molecular

TABLE I. Phosphorylation of Protein Bands Illustrated in Fig. 1

Area of Gel	Unretarded by Con A (cpm[1])	Con A retarded (cpm)
Band I	1,910	0[2]
Band II	6,204	0[2]
Band III	4,343	284

Erythrocyte ghosts were phosphorylated under assay conditions with [γ-^{32}P] ATP (3). Ghosts were solubilized with 1% DOC and applied to Con A sepharose columns as described in Methods. The fractions were collected and run on 6% SDS-polyacrylamide gels, stained, cut, solubilized, and counted (3, 9). All experiments (n = 12) were performed in duplicate. Data are from one typical experiment.

[1] Counts per minute above gel blank.

[2] No stainable peaks but area of gel analyzed. No counts on gel except as shown.

weight of 90,000–100,000, but may indeed be a collection of several glycoproteins of quite different apoprotein structure.

Another possibility for the phosphorylation of a defined fraction of component a is the effect the carbohydrate moiety may have on the conformation of component a with respect to its relationship to the protein kinase enzyme. Rubin et al. have demonstrated and we have confirmed that the protein kinase activity is located on the inner surface of the membrane (18). Carbohydrates have been repeatedly demonstrated on the outer surface available to the external environment of the erythrocyte (19–21). It is possible that the carbohydrate could affect the molecular disposition, but the nature of the intrinsic portion of the molecule is more likely to determine its relationship to the enzyme.

The separation of a nonphosphorylated fraction of component a by means of Con A-sepharose columns does not provide the necessary purification of substrate with respect to our MyD studies, since the specific substrate is not retained on the affinity chromatography column. The protein heterogeneity of this group of glycoproteins is, however, germane to the interpretation of theories already suggested in the literature. Any quantitation of ion transport sites or reactions of substrates within this protein band must be more specific than previously assumed (16, 22–24). The functional significance of component a may be more complicated and varied than previously suspected. Determination of the specific amounts and relationships of the various glycoproteins making up component a are prerequisites for understanding their functions.

ACKNOWLEDGMENTS

This work was supported in part by grant RG 923-A-2 from the National Multiple Sclerosis Society and by grant 5-36 from the National Foundation March of Dimes. The author wishes to thank Mrs. Karen Case for secretarial assistance and Mr. Michael Herbstreith for expert technical assistance.

REFERENCES

1. Roses, A. D., and Appel, S. H., Lancet II, 1400 (1974).
2. Roses, A. D., and Appel, S. H., Proc. Natl. Acad. Sci. U.S.A. 70:1855 (1975).
3. Roses, A. D., and Appel, S. H., J. Membr. Biol. 20:51–58 (1975).
4. Miller, S. E., Roses, A. D., and Appel, S. H., Arch. Neurol. In press (1975).
5. Butterfield, D. A., Roses, A. D., Cooper, M. L., Appel, S. H., and Chestnut, D. B., Biochemistry 13:5078 (1974).
6. Fairbanks, G., Steck, T. L., and Wallach, D. F. H., Biochemistry 10:2606 (1971).
7. Marchesi, V. T., and Andrews, E. P., Science 174:1207 (1971).
8. Hamaguchi, H., and Cleve, H., Biochim. Biophys. Acta 278:271 (1972).
9. Roses, A. D., and Appel, S. H., J. Biol. Chem. 248:1408 (1973).
10. Avruch, J., and Fairbanks, G., Proc. Natl. Acad. Sci. U.S.A. 69:1216 (1972).
11. Knauf, P. A., Proverbio, F., Hoffman, J. F., J. Gen. Physiol. 63:324 (1974).
12. Guidotti, G., Annu. Rev. Biochem. 41:731 (1972).
13. Bretscher, M. S., J. Mol. Biol. 59:351 (1971).
14. Findley, J. B. C., J. Biol. Chem. 249:4398 (1974).
15. Steck, T. L., and Yu, J., J. Supramol. Struct. 1:220 (1973).
16. Ho, M. K., and Guidotti, G., J. Biol. Chem. 250:675 (1975).
17. Grefrath, S. P., and Reynolds, J. A., Proc. Natl. Acad. Sci. U.S.A. 71:3913 (1974).
18. Rubin, C. S., Rosenfeld, R. D., and Rosen, O. M., Proc. Natl. Acad. Sci. U.S.A. 70:3735 (1973).
19. Bretscher, M. S., J. Mol. BIol. 58:775 (1971).
20. Berg, H. C., Biochim. Biophys. Acta. 183:65 (1969).
21. Phillips, D. R., and Morrison, M., Fed. Eur. Biochem. Soc. Lett. 18:95 (1971).
22. Bretscher, M. S., Science 181:622 (1973).
23. Cabantchik, Z. I., and Rothstein, A., J. Membr. Biol. 15:207 (1974).
24. Cabantchik, Z. I., and Rothstein, A., J. Membr. Biol. 15:227 (1974).