Vol. 94, No. 3, 1980 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
June 16, 1980 Pages 861-866

IDENTIFICATION OF THE BINDING SITE FOR TRANSFERRIN IN HUMAN RETICULOCYTES

M. Steiner

Division of Hematologic Research, The Memorial Hospital
Pawtucket, R.I. and Brown University, Providence, R.I. 02860

Received April 26,1980

SUMMARY. The receptor site for transferrin was investigated in normal human reticulocytes by the use of photoactive 4-fluoro-3-nitrophenyl azide which was conjugated to chromatographically pure human transferrin saturated with iron. The photoprecursor-bearing protein was further treated with fluorescein isothiocyanate. The nonactivated transferrin conjugate was fully competitive with respect to binding characteristics with normal transferrin. The aryl nitrene-containing photoactivated transferrin-reticulocyte receptor complex was isolated by sodium dodecyl sulfate polyacrylamide gel electrophoresis. Separated proteins and polypeptides were eluted from the gels and analyzed for fluorescence. A fluorescent band of 123,000 daltons was identified as possible transferrin-receptor complex. The molecular weight of the membrane receptor was estimated to be 43,000. This corresponds to the approximate weight of one of the major red cell glycopeptides.

INTRODUCTION

The receptor site for the transferrin molecule on erythroid precursor cells has been under intensive investigation over the past few years. Identification of the membrane polypeptide carrying the binding site for this plasma protein has been attempted by a variety of methods. The one which has been most commonly used is based on the isolation of the solubilized receptor-transferrin complex (1-7). So far no clear consensus on its size has emerged even when the receptor-bearing cell was from the same species. A somewhat different approach was pursued by Witt and Woodworth (8) who searched for and identified a reticulocyte-specific protein exposed on the external surface of the plasma membrane. Bifunctional crosslinking agents have been used by Nunes et al. (9) and a photoanalogue study of conalbumin in chicken embryo red cells has been reported (10).

In this report I describe the utilization of a photoanalogue of human transferrin to establish a covalently bonded complex of membrane polypeptide(s)

0006-291X/80/110861-06$01.00/0

with the transferrin molecule. To clearly identify the complex, fluorescent-conjugated transferrin was used as the ligand. This method proved to be sufficiently sensitive to demonstrate directly the membrane constituent with binding properties for transferrin.

METHODS AND MATERIALS. Reticulocyte-rich blood, not exceeding 5 - 7 ml, was obtained from consenting patients with congenital hemolytic anemias or recovering from megaloblastic anemias. Reticulocytes were concentrated by standard methods. In each of 3 consecutive steps the cells were washed with 10 mM phosphate buffer containing 0.145 M NaCl, pH 7.4[1] (PBS) and concentrated by aspirating the top 1/3 of the packed cell volume and discarding the lower portion. This procedure increased the fraction of reticulocytes to between 30 - 50%.

Human transferrin was obtained from Sigma Chemical Co. (St. Louis, Mo). It was dialyzed against several changes of 0.05 M Tris, pH 8.0 and then purified by chromatography on DEAE-Sephadex A-50 using a Tris gradient ranging from 0.05 - 0.2 M, pH 8.0. The transferrin peak was then further purified by gel filtration through a column of Sephadex G-100. To the chromatographically pure transferrin was added iron as $Fe(ClO_4)_2$ in the presence of 20 mM sodium bicarbonate and 1 mM nitriloacetate.

A photoactive derivative of transferrin, 4-azido-2-nitrophenyl (-NAP)-transferrin was prepared by reacting 1 vol of a 4% solution of 4-fluoro-3-nitrophenyl azide (11) dissolved in ethanol with 10 vol of 12.5 mM sodium borate buffer, pH 9.8 containing 10 mg of chromatographically pure human transferrin per ml. The reaction mixture, protected from light, was stirred at 37°C for 90 min. The mixture was then filtered and the filtrate dialyzed against several changes of 10 mM ammonium bicarbonate solution for 2 - 3 days. After filtration the protein was freeze-dried.

The number of mol of NAP groups per mol of transferrin was determined by measurement of the optical absorbance at 460 nm and of the protein concentration by the method of Lowry et al. (12). A molar extinction coefficient of 4,800 at 460 nm was used for estimating NAP groups (11). The ratio in three batches of human transferrin labeled in this manner was 2.2, 1.9 and 1.7. This ratio is based on a molecular weight of human transferrin of 80,000.

NAP-transferrin was then conjugated with fluorescein isothiocyanate (FITC). For this reaction 4.8 mg of NAP-transferrin was dissolved in 0.5 ml of 0.05 M carbonate-bicarbonate buffer, pH 9.5 containing 0.145 M NaCl. FITC, 0.1 mg, was added and the mixture incubated in the dark for 60 min at room temperature. Unreacted FITC was separated by gel filtration through a column of Sephadex G-25. PBS was used to elute the FITC-conjugated NAP-transferrin (F-NAP-transferrin). The brightest fluorescing fractions were pooled and the mol ratio FITC/transferrin determined. The optical absorbance of FITC was measured at 495 nm ($E^{1\%} = 2,400$). The ratio was usually in the range of 1.1 - 1.5.

To 0.1 ml F-NAP-transferrin was added an equal volume of reticulocyte suspension in a quartz glass tube. The tube was slowly rotated at 4°C about its long axis spreading a film of the reaction mixture over the inside of

[1] Abbreviations: NAP: 4-azido-2-nitrophenyl-; FITC: fluorescein isothiocyanate; FITC-conjugated NAP-transferrin: F-NAP-transferrin. PBS: 10 mM phosphate buffer containing 0.145 M NaCl, pH 7.4.

the tube. It was irradiated for 60 min by a long wavelength ultraviolet light source (366 nm) placed at a distance of 7 cm from the reaction tube. Control samples containing F-transferrin or nonconjugated transferrin were processed identically.

The reaction products of the photolabeling experiments were analyzed by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis. The gels were prepared according to Fairbanks et al. (13) and calibrated with β-galactosidase, reduced and alkylated fibrinogen, lactic dehydrogenase, transferrin, pepsin and cytochrome c. The samples containing about 1 mg protein/ml were prepared for electrophoresis by adding 1% SDS, 1 mM EDTA, 10 mM Tris-HCl, pH 8.0, 40 mM dithiothreitol, bromophenol blue (tracker dye) and about 5% sucrose. Between 20 - 50 µg protein were applied per gel. The gels were stained with Coomassie blue according to Fairbanks et al. (13) and scanned at 580 nm. Replicate gels were fixed with a solution containing 25% isopropyl alcohol and 10% acetic acid and were then cut into 2 mm sections. Individual gel slices were cut up into small pieces and were placed on 1 - 1.5 cm high spacer gels. The cylindrical tubes had small bags made of dialysis tubing fastened to their lower end which were filled with a small amount of electrode buffer. The spacer gel, consisting of 1.5% acrylamide with 0.375% bisacrylamide was prepared according to Maizel (14). The electrophoretic elution was completed within 60 - 90 min. The protein eluted into the electrode buffer, 5 mM Tris-glycine, pH 8.2 was dialyzed against PBS and its fluorescence measured with a Perkin-Elmer fluorospectrophotometer (MPF-44A) using excitation light of wavelength 493 nm. Emission was checked at 516 nm.

RESULTS AND DISCUSSION

The photoactive fluorescent transferrin, F-NAP-transferrin, was tested for its structural and functional characteristics. The iron-binding capacity was found to be unchanged from the nonconjugated protein. Functionally, the binding of F-NAP-transferrin to reticulocytes could be completely suppressed by normal transferrin. The latter, at a concentration 10 times in excess of F-NAP-transferrin, was able to reduce the binding of the photoactive fluorescent transferrin to $< 5\%$ of the amount ligated in the absence of the normal, nonconjugated protein. These findings suggested that conjugation of transferrin with NAP and FITC did not interfere with its principal biological characteristics. SDS polyacrylamide gel electrophoresis showed perfect identity between labeled and free transferrin. Immunoelectrophoresis demonstrated an essentially unchanged antigenic activity but indicated a small charge difference compared to unconjugated transferrin. F-NAP-transferrin migrated slightly ahead of normal transferrin.

Photolysis of the NAP-transferrin-reticulocyte complex, resulting in the formation of an aryl nitrene at the binding site gave rise to a covalent-

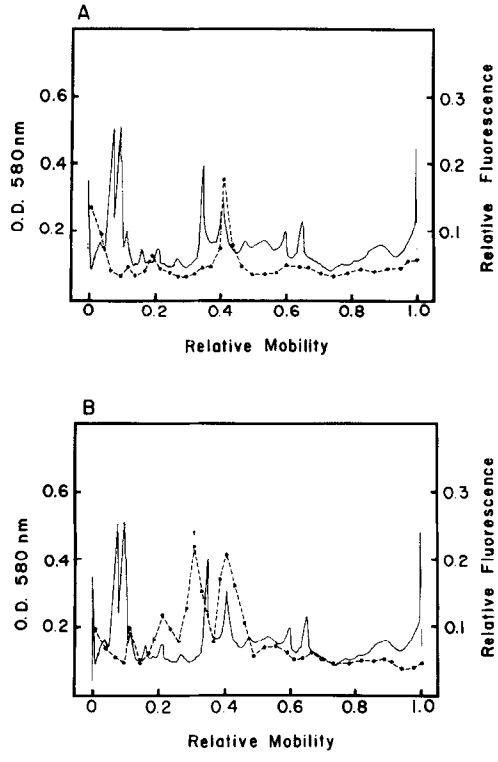

Figure 1

Electrophoretic pattern of human reticulocyte membrane polypeptides derived from F-transferrin-(A) or F-NAP-transferrin-(B) treated cells. After photoactivation reticulocytes were washed twice with PBS and resuspended in 5 mM Tris-HCl buffer, pH 7.1. After lysis of the cells, the ghosts were sedimented by centrifugation, washed once and then applied to SDS polyacrylamide gels. The polypeptide pattern was developed by migration from the right (cathode) towards the left (anode). The solid line indicates the absorbance of the Coomassie-blue stained gels scanned at 580 nm. The solid points connected by the interrupted line indicate the relative fluorescence (FITC) eluted from individual 2 mm wide slices of an identical fixed but unstained gel.

ly bonded complex which could be identified by SDS polyacrylamide gel electrophoresis. A comparison of control, i.e. F-transferrin, and F-NAP-transferrin (Fig. 1) clearly demonstrated the difference in the eluted polypeptides analyzed for fluorescence. Incubation of reticulocytes with the photoprecursor-carrying transferrin gave rise to a fluorescent peak with R_f 0.29 corresponding to a polypeptide of 123,000 daltons. There were in addition peaks corresponding to peptides of 80,000 and 160,000 daltons. These undoubtedly present single and crosslinked transferrin. They were also seen

to a lesser extent with F-transferrin but with the latter as the ligand, no membrane component of R_f 0.29 was observed (Fig. 1A).

These findings give evidence of a single membrane component with a molecular weight of about 43,000 daltons involved in the binding of transferrin. I cannot rule out the existence of other membrane polypeptides with ligand activity for this plasma protein. However, their relative abundance compared to that of the 43,000 dalton component appears to be significantly lower. In comparison to other methods which have been used to identify membrane components with receptor qualities for transferrin, the one reported here, similar to that of Witt and Woodworth (10), provides a more direct approach. One can assume that it is less susceptible to some of the difficulties which are observed with the use of solubilized membrane protein-ligand complexes, particularly their tendency to form aggregates and the uncertainty of their characterization by chromatography with detergent solutions (15). This, however, is not to say that photolabeling studies of the type described in this report are not without disadvantages. Even though the conjugated protein behaves for all intents and purposes identical to unlabeled transferrin, photolysis may produce free radicals which could alter the reactivity of potential ligand sites. Also, the 2 NAP groups attached to transferrin may not be in appropriate positions for crosslinkage to all types of transferrin receptor. Nevertheless, with these limitations, the results of this study indicate the identity of at least one membrane polypeptide in human reticulocytes with transferrin-binding activity. It is interesting to note that the experiments which utilized either crosslinking agents (9) or NAP-conjugates of conalbumin (10) or of transferrin as in this study, all yielded fairly low molecular weights for the receptor of the iron binding protein. The molecular weight of approximately 43,000 calculated for the receptor in human reticulocytes corresponds to one of the major glycopeptides of normal red cells. The fact that this polypeptide has carbohydrate attached to it even in mature red cells does not rule it out as a potential receptor

for transferrin. Experiments to be reported elsewhere have shown that hydrolytic cleavage of only one type of monosaccharide from reticulocyte membranes abolishes their transferrin-binding activity. Thus complete loss of the carbohydrate moiety of the responsible membrane polypeptide does not seem to be a prerequisite for this change.

ACKNOWLEDGEMENTS. This work was supported by USPHS grant AM-17327.

REFERENCES

1. Garrett, N.E., Garrett, R.J., and Archdeacon, J.W. (1973) Biochem. Biophys. Res. Commun. 52, 466-474.
2. Speyer, B.E., and Fielding, J. (1974) Biochim. Biophys. Acta 332, 192-200.
3. van Bockxmeer, F., Hemmaplardh, D., and Morgan, E.H. (1975) Proteins of Iron Storage and Transport in Biochemistry and Medicine (Crichton, R.R., ed.) Proc. EMBO Workshop Conf., p 111-119.
4. Sly, D.A., Grolich, D., and Bezkorovainy, A. (1975) Proteins of Iron Storage and Transport in Biochemistry and Medicine (Crichton, R.R., ed.) Proc. EMBO Workshop Conf., p 141-145.
5. Leibman, A., and Aisen, P. (1977) Biochemistry 16, 1268-1272.
6. van der Heul, C., Kroos, M.J., and van Eijk, H.G. (1978) Biochim, Biophys. Acta 511, 430-441.
7. Light, N.D. (1977) Biochim. Biophys. Acta 495, 46-57.
8. Witt, D.P., and Woodworth, R.C. (1978) Biochemistry 17, 3912-3917.
9. Nunez, M.T., Fischer, S., Glass, J., and Lavidor, L. (1977) Biochim. Biophys. Acta 490, 87-93.
10. Witt, D.P., and Woodworth, R.C. (1975) In Proteins of Iron Storage and Transport in Biochemistry and Medicine (Crichton, R.R., ed.) Proc. EMBO Workshop Conf., p 133-140.
11. Fleet, G.W.J., Knowles, J.R., and Porter, R.R. (1972) Biochem. J. 128, 499-508.
12. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275.
13. Fairbanks, G., Steck, T.L., and Wallach, D.F.H. (1971) Biochemistry 10, 2606-2616.
14. Maizel, J.V. (1969) in Fundamental Techniques in Virology (Habel, K. and Salzman, N.P., eds) Academic Press, New York p 334-362.
15. Tanford, C., Nozaki, Y., Reynolds, J.A., and Makino, S. (1976) Biochemistry 13, 2369-2376.