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# FURTHER STUDIES ON THE RABBIT ERYTHROID CELL PLASMA MEMBRANE TRANSFERRIN RECEPTOR

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SUMMARY: Studies reported here indicate that a major cell surface glycoprotein of the rabbit bone marrow erythroid cell may be involved in binding transferrin. The glycoprotein has an apparent molecular weight of 18,000. It is suggested that bone marrow erythroid cells may provide an invaluable source of the red cell membrane transferrin receptor for future studies.

The nature of the reticulocyte plasma membrane transferrin receptor is still much disputed, with molecular weight estimations for this protein ranging from 35,000 (1) and 36,000 (2) to 230,000 (3-6). Several different sources of haemoglobin synthesising cells as well as many different methods of identification of the receptor have been used. However, a fundamental problem which has hindered all these studies has been the difficulty in obtaining large enough amounts of the receptor to allow in depth characterisation of the protein. The present study reports the finding of a major cell surface glycoprotein of the rabbit bone marrow erythroid cell which appears to bind transferrin. The protein is present in the membrane in large enough amounts to allow its preparation and further characterisation.

## MATERIALS AND METHODS

Mature New Zealand White rabbits were made anaemic by five daily subcutaneous injections of 6mg of phenylhydrazine hydrochloride in isotonic NaCl/kg of body weight. On the seventh day animals were sacrificed and bone marrow cells were prepared in isotonic NaCl suspension by the method described by Light and Tanner (7). Bone marrow preparations

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were shown to contain 90% erythroid cells by staining by the May-Grunwald/Geimsa method (8). Plasma membranes of bone marrow erythroid cells were prepared as previously described (7) and were solubilised in Triton X-100 by incubation in 5 vols. of 56mM sodium borate pH 8.0 containing 0.5% (v/v) Triton X-100 (9) for 30min at 37°C. Insoluble material was removed by centrifugation at 40,000g for 20min and the clear supernatant was reserved for affinity chromatography.

Affinity chromatography of bone marrow erythroid cell plasma membrane proteins in Triton X-100 solution was carried out on transferrin/Sepharose 4B (Pharmacia Ltd., Uppsala, Sweden) gels, prepared by the method previously described (2), as follows. The clear Triton X-100 supernatant derived from solubilised bone marrow erythroid cell plasma membranes was applied to a column (2.5 x 1.0cm) containing 2ml of transferrin liganded Sepharose 4B equilibrated in 54mM sodium borate pH 7.4 containing 0.5% (v/v) Triton X-100. The column effluent was monitored at 280nm and the gel was washed exhaustively in the borate/Triton X-100 buffer until no further protein was eluted. Non-specifically bound protein was then washed from the column with the borate/Triton X-100 buffer containing 0.3M NaCl. Specifically bound proteins were then eluted with a 5mg/ml rabbit transferrin solution (Miles Laboratories Ltd., Stoke Poges, Bucks., U.K.). Proteins in the eluate were then labelled with $^{14}$C-N-ethyl-maleimide ($^{14}$C-MalNEt; 2mCi/mmole, The Radiochemical Centre, Amersham, Bucks., U.K.) by the method previously described (2) in order to break down any transferrin/membrane protein complexes. After the $^{14}$C-MalNEt treatment the proteins were separated by gel filtration in the 54mM borate buffer pH 7.4 containing 0.5% (v/v) Triton X-100 on a Sephadex G-100 column (80 x 2.5cm). The column effluent was scanned at 280nm and analysed for radioactivity by sampling each 2ml fraction and scintillation counting as previously described (2).

## RESULTS

Fig.1 shows a typical trace obtained in an affinity chromatography experiment to prepare transferrin binding moieties from Triton X-100 solubilised rabbit bone marrow erythroid cell plasma membranes. The break-through peak of unbound protein (I) was followed by a smaller peak (II) of non-specifically bound protein which was eluted with 54mM borate buffer pH 7.4 containing 0.5% Triton X-100 and 0.3M NaCl. The third peak (III) was eluted with two bed volumes of a 5mg/ml rabbit transferrin solution. The protein in this peak was treated with $^{14}$C-MalNEt to block sulphydryl groups in the eluted proteins and thus to break down any transferrin/membrane protein complexes (2). The incubation mixture was then analysed by gel filtration. A typical gel filtration elution profile is shown in Fig.2. As can be seen from the trace of absorption at 280nm two peaks were obtained. The higher molecular weight component corresponded to the transferrin used in the elution from the affinity

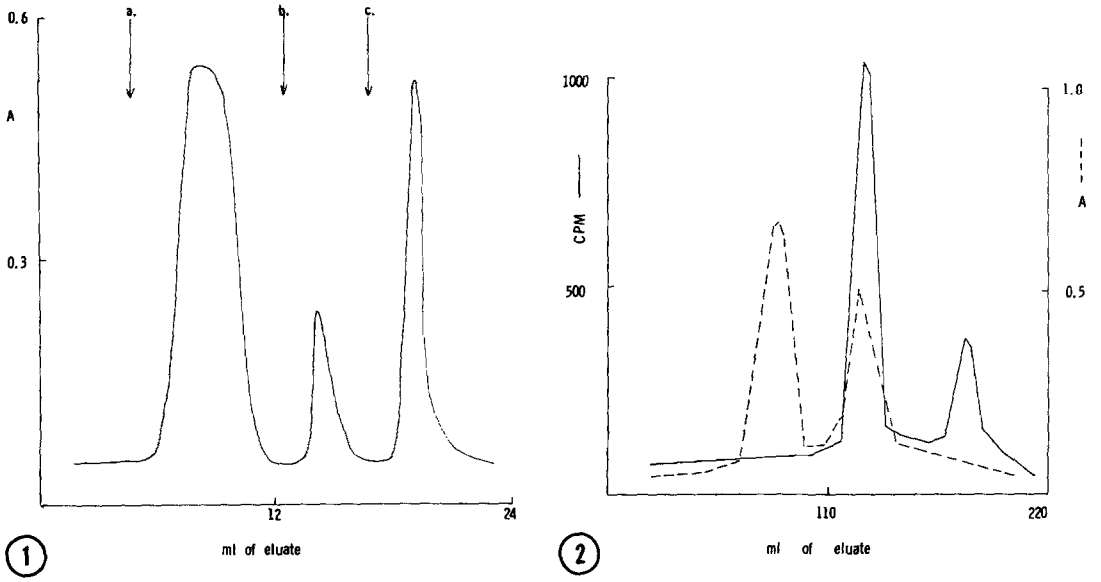


Figure 1. Affinity chromatography preparation of transferrin binding component(s) from rabbit bone marrow erythroid cells. (a) Application of sample, (b) Elution with 0.3M NaCl in 56mM borate buffer containing 0.5% (v/v) Triton X-100, (c) Elution with 5mg/ml rabbit transferrin in the same buffer.

Figure 2. Gel filtration of $^{14}$C-MalNEt labelled eluate from affinity chromatography of bone marrow erythroid cell plasma membrane extract.

column and was not labelled with $^{14}$C-MalNEt, whilst the lower molecular weight protein had an apparent molecular weight of 18,000 - 20,000 and was labelled with $^{14}$C-MalNEt. A third peak of radioactivity which did not correspond to a peak of absorption at 280nm was observed at the included volume and was probably unreacted $^{14}$C-MalNEt.

## DISCUSSION

The results of the affinity chromatography preparation of transferrin binding proteins from bone marrow erythroid cell plasma membranes show that only one component was eluted with transferrin. This protein was labelled with $^{14}$C-MalNEt and had an observed molecular weight of 18,000 - 20,000 on gel filtration after labelling with $^{14}$C-MalNEt. The protein

appeared to correspond to the major protein staining bone marrow erythroid cell plasma membrane component (band G - see ref.7 for nomenclature). In a previous report, this protein was shown to constitute over 40% of the total Coomassie Blue staining material of the plasma membrane of bone marrow erythroid cells and to be a cell surface glycoprotein (7).

The case for the receptor being a low molecular weight membrane component is well supported by consideration of the estimated number of membrane sites of the receptor. Baker and Morgan (10) have estimated that each rabbit reticulocyte contains between 250,000 and 560,000 monovalent, cell surface transferrin binding sites. A receptor with a molecular weight of over 100,000 (3,4,5) and 500,000 molecules per reticulocyte "ghost" would constitute a major membrane protein (11). However, gel electrophoresis analyses of "ghost" preparations, using protein staining, carbohydrate staining or direct lectin binding techniques (7), show only one protein with a molecular weight greater than 100,000, the well-described cytoplasmic face membrane protein "spectrin". Furthermore, only one major membrane protein can be located at the outer cell surface by the $^{125}$I-lactoperoxidase method in intact reticulocytes (7). This is the major membrane protein of the reticulocyte and erythrocyte (7) which has a molecular weight of 93,000 and contains 8% carbohydrate (11). The protein is responsible for rapid anion transport in the cells (12) and inhibition of this process has no direct effect on either transferrin binding or iron uptake by reticulocytes (13). Previous work (7) has shown that this protein is not present in bone marrow erythroid cell plasma membranes. It seems unlikely in the face of this evidence that this protein should be involved in transferrin binding (especially as the anion transport protein is not trypsin sensitive (7,11) and the reticulocyte transferrin receptor is (14)). However, the 93,000 protein of the reticulocyte (band 3) is known to form membrane macromolecular complexes (11); it has been shown to form

dimers in Triton X-100 solution (15) and it appears to co-purify with the reticulocyte transferrin receptor under certain conditions (2). These facts indicate that the recently described 95,000 PAS staining component of Leibman and Aisen (6) (and their 176,000 component, which could be a dimer of this protein) may well correspond to the anion transport protein of the reticulocyte. As this protein is unlikely to be involved as a transferrin receptor, we must conclude that it may form complexes with the receptor in Triton X-100 solution.

A transferrin receptor with a molecular weight of 18,000 and a cell surface density of 500,000 sites would not be expected to figure as a major component on gel electrophoresis. Additionally, if the receptor is a glycoprotein with a high carbohydrate to polypeptide ratio it probably would only stain weakly or not at all with protein stains such as Coomassie Brilliant Blue (cf. human erythrocyte sialoglycoprotein ref.11). Minor, low molecular weight glycoproteins which labelled with $^{125}$I-lactoperoxidase in whole reticulocytes but do not appear on protein stained gels have been reported (7). All this data seems to indicate that the reticulocyte transferrin receptor must be a low molecular weight molecule. This is confirmed by the results presented in this paper.

The molecular weight of the protein isolated here by affinity chromatography requires explanation in the light of a recent publication (2) in which the reticulocyte transferrin receptor was shown to have a molecular weight of 36,000 in Triton X-100 solution in the presence of $^{14}$C-MalNEt. A possible explanation may be gained by examination of the maturation process of the rabbit erythroid cell. It has been shown that the change from nucleated bone marrow cell to non-nucleated circulating reticulocyte is associated with a complete changeover in plasma membrane proteins (7). The relatively large amount of the affinity chromatography prepared bone marrow erythroid cell receptor reported here compared with the very small recovery of the reticulocyte receptor previously reported (2)

and the observed decreased rates of haemoglobin synthesis and thus, presumably iron uptake in the reticulocyte when compared with the bone marrow erythroid cell (16), may be due, therefore, to the loss of most of the receptor molecules from the membrane during the changeover of plasma membrane components. Thus, only residual amounts of the protein may be present in the reticulocyte plasma membrane. Also, the suggestion that proteins may be removed from membranes by aggregation and "capping" (17) may explain why so many different molecular weights have been reported for the reticulocyte transferrin receptor. Thus, the mechanism by which the reticulocyte loses its ability to bind transferrin may involve the dimerisation and subsequent polymerisation of the receptor prior to its removal from the membrane. This possibility, together with the apparently large amounts of the receptor in the nucleated bone marrow erythroid cell plasma membrane makes a strong case for the use of the latter cells as a source of the receptor in future studies.

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