Loss of the transferrin receptor during the maturation of sheep reticulocytes *in vitro*

An immunological approach

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Sheep reticulocyte-specific antiserum absorbed with mature sheep red cells has been used to isolate and identify reticulocyte-specific plasma-membrane proteins and to monitor their loss during incubation *in vitro*. Specific precipitation of labelled plasma-membrane proteins is obtained when detergent-solubilized extracts of ^{125}I -labelled reticulocyte plasma membranes are incubated with this antiserum and *Staphylococcus aureus*, but not when mature-cell plasma membranes are treated similarly. During maturation of reticulocytes *in vitro* (up to 4 days at 37°C), there is a marked decrease in the immunoprecipitable material. The anti-reticulocyte-specific antibodies have been identified as anti-(transferrin receptor) antibodies. By using these antibodies as a probe, the transferrin receptor has been shown to have a subunit molecular weight of 93 000. The data are consistent with reported molecular weights of this receptor and with the proposal that the receptor may exist as a dimer, since [^{125}I]iodotyrosyl-peptide maps of the 93 000- and 186 000-mol.wt. components isolated are shown to be identical. Evidence is presented for the transmembrane nature of the receptor and for the presence of different binding sites for transferrin and these antibodies on the receptor.

It is known that a number of activities associated with immature red blood cells disappear or decrease by the time the cell matures into the erythrocyte (Rubinstein *et al.*, 1956; Walsh *et al.*, 1949; Borsook *et al.*, 1952; London *et al.*, 1950; Gavosto & Rechenman, 1954; Gasko & Danon, 1972). A number of these functions such as the loss of ability to bind transferrin (Jandl *et al.*, 1959; Jandl & Katz, 1963; Kornfeld, 1969; Hemmaphardh & Morgan, 1974; Ecarot-Charrier *et al.*, 1980) and transport amino acids (Winter & Christensen, 1964, 1965; Antonioli & Christensen, 1969; Wise, 1976; Benderoff *et al.*, 1978a,b) are associated with functional changes in the plasma membrane itself. In fact, there is known to be a decrease of plasma-membrane area during maturation (Lowenstein, 1959; Ganzoni *et al.*, 1969). The maturing red cell provides an experimental model to monitor membrane maturation and mechanisms for selective excision of plasma-membrane proteins, since some functions are completely lost, whereas others, although diminished, such as $(\text{Na}^+ + \text{K}^+)\text{-dependent ATPase}$, are retained

to a significant extent to maturity. At the reticulocyte stage, few membrane proteins are synthesized (Lodish & Small, 1975; Koch *et al.*, 1975). Thus the possibility of membrane replacement by new synthesis is reduced and the problem of studying maturation is simplified. Moreover, maturation can be monitored *in vitro* (Benderoff *et al.*, 1978a,b; Blostein *et al.*, 1981), thus providing an easily accessible model for study.

It was reasoned that antibodies prepared against reticulocytes and used in conjunction with *Staphylococcus A* protein might provide a means of identifying reticulocyte-specific plasma-membrane proteins which disappear on maturation.

Previous studies by Schulman & Nelson (1969) have shown that guinea-pig antibodies can be raised against rabbit reticulocytes which do not cross-react with mature cells. In their report, the nature of the reticulocyte-specific surface antigen was not identified. In a preliminary study we have reported that rabbit antiserum against sheep reticulocytes can be used to isolate reticulocyte-specific membrane proteins (Pan & Johnstone, 1980, 1981). In the present work, we report that rabbit antibodies prepared against sheep reticulocytes can be used to monitor changes in protein composition of the reticulocyte

Abbreviations used: SDS, sodium dodecyl sulphate; FITC, fluorescein isothiocyanate; IgG, immunoglobulin G.

plasma membrane during maturation. These studies also identify the transferrin receptor as a major antigenic component on the sheep reticulocyte surface.

Materials and methods

Isolation of reticulocytes

Reticulocyte production in sheep was induced by phlebotomy as described (Benderoff *et al.*, 1978*a,b*). Cells from whole blood were washed three times with iso-osmotic NaCl and the washed cells were suspended to approx. 80% cytocrit and centrifuged at 2000 *g* for 1 h. The top 25% layer of the cells was removed, transferred to 15 ml centrifuge tubes and re-centrifuged as above. The top 10% layer, which usually contains 80–100% reticulocytes, was carefully collected. At each stage of centrifugation, buffy coats of white cells were removed before using the red-cell layer. A reticulocyte count of about 80% was used in most experiments. Reticulocytes were incubated *in vitro* as used previously by Benderoff *et al.* (1978*a,b*). Mature red cells were obtained from the bottom 25% layer after the first centrifugation or from normal, unbled sheep.

Immunological procedures and purification of an IgG fraction

A 1 ml portion of a washed 1% suspension of sheep reticulocytes or mature red cells was injected subcutaneously into rabbits with complete Freund's adjuvant, followed by five to six weekly intravenous injections with the same number of cells. At 7–8 weeks after the first injection, antiserum was collected and inactivated at 56°C for 30 min. The antiserum against sheep reticulocytes was exhaustively absorbed with mature sheep red cells and the absorbed antiserum designated 'antiserum I'. To purify anti-reticulocyte surface-membrane antibodies, antiserum I was incubated for 24 h at 0°C with well-washed reticulocytes. The cells were pelleted, washed four times with iso-osmotic NaCl, and resuspended to obtain a 10% suspension in iso-osmotic NaCl. An equal volume of 0.2 M-glycine, pH 2.3, in iso-osmotic NaCl was added at 0°C. After 5 min, the suspension was neutralized with phosphate-buffered saline (a minimal volume of 0.5 M-phosphate in 0.15 M-NaCl), pH 7.4, and immediately centrifuged at 12000 *g* for 10 min. The supernatant was passed through a protein A–Sepharose-4B affinity column. The column was washed extensively with phosphate-buffered saline, pH 7.4, to remove unadsorbed materials. IgG bound to the protein A–Sepharose-4B column was eluted with 0.1 M-glycine-buffered saline 0.15 M-NaCl, pH 3.0 (Ey *et al.*, 1978). This IgG fraction represents a purified IgG fraction which agglutinates only reticulocytes. Rabbit antiserum against mature red blood cells was used directly after

heat inactivation at 56°C without further purification. Rabbit anti-(bovine albumin) and goat anti-(rabbit γ -globulin) antiserum were purchased from Grand Island Biological Co. (New York, NY, U.S.A.).

Transferrin purification

Transferrin was purified from sheep plasma as described by Morgan (1964*a,b*) and Morgan *et al.* (1978), with the modification that ⁵⁹Fe-labelled proteins of plasma which precipitated between 50–75% saturation of (NH₄)₂SO₄ were isolated and dialysed before gel filtration on Sephadex G-150.

Purification of membrane proteins with transferrin and immunoaffinity columns

The transferrin and the immunoaffinity columns were prepared by coupling 2–5 mg of the respective proteins (purified transferrin or IgG) to 1 g of CNBr-activated Sepharose-4B (Axen *et al.*, 1967). Reticulocyte or mature-red-cell membranes were prepared by using the procedure described by Dodge *et al.* (1963). The membrane proteins (1 mg/ml) were solubilized with a solution containing 1% Triton X-100, 10 mM-Tris/HCl, pH 7.4, and 1% Aprotinin (Sigma), with constant stirring at 0°C for 1 h. The solution was diluted with phosphate-buffered saline, pH 7.4, to give 0.25% Triton, then centrifuged either at 30900 *g* for 2 h or at 100000 *g* for 1 h. The supernatant solution was applied to the immunoaffinity column and the column was washed with 30 bed-vol. of phosphate-buffered saline, pH 7.4, containing 0.25% Triton, followed by elution with 0.25% Triton X-100 in 0.1 M-glycine-buffered saline at pH 2.3 (Ey *et al.*, 1978).

To isolate the transferrin receptor with the transferrin affinity column, the supernatant solution was adjusted to pH 5 as suggested by Ecarot-Charrier *et al.* (1980) with 0.1 M-sodium citrate-buffered saline containing 0.25% Triton X-100 and applied to the immobilized-transferrin–Sepharose-4B column. The column was washed with 30 bed-vol. of the same Triton/citrate-buffered saline and eluted with 0.1 M-glycine buffered saline, pH 2.3, containing 0.25% Triton X-100. The eluates from the immunoaffinity column or the transferrin column were neutralized and dialysed at 4°C against water for at least 24 h with two or three changes of water. The samples then were freeze-dried and used for electrophoresis.

Electrophoresis

The Laemmli (1970) method was used for electrophoresis. The freeze-dried proteins were dissolved in 2% SDS/10% (v/v) glycerol/10% (v/v) mercaptoethanol/20 mM-Tris/HCl, pH 7.4, and heated for 5 min at 100°C before electrophoresis.

Membrane surface iodination

The iodination of the outside surface of the intact cell and the cytoplasmic surface of the ghost were carried out as described by Reichstein & Blostein (1973, 1975). Isolated plasma membranes were iodinated in a similar way and dissolved in 1% Triton X-100/1% aprotinin/154 mM-NaCl/20 mM-Tris, pH 7.4 at 0°C, and centrifuged at 30 900 *g* for 2 h or at 100 000 *g* for 1 h. The pellet obtained was discarded and the supernatant was subjected to immunoprecipitation (see below). To monitor the changes in the plasma membranes during maturation of the cell *in vitro*, portions containing equal numbers of cells were taken at the intervals given in the Figures. Membranes were prepared from the cells on the day the sample was taken and kept frozen until all samples in the series had been taken. Then the membranes from all the samples were iodinated as described above. Determinations of the total ^{125}I incorporated showed that all samples prepared on a particular day contained the same amount of radioactivity within $\approx 5\%$.

Immunoprecipitation of membrane proteins

A 100–200 μl sample of the supernatant (30–40 μg of protein/100 μl) obtained from the iodinated, dissolved membranes, was incubated for 1 h at 37°C with reticulocyte specific antiserum or with non-immune serum. Subsequently 200 μl of a 10% suspension of formaldehyde-fixed *Staphylococcus aureus* (Cowan strain) was added and the incubation continued for another 1 h at 37°C. The immune complex was centrifuged and washed three times with 0.25% Triton X-100/10 mM-Tris/HCl (pH 7.4)/5 mM EDTA in 0.154 M-NaCl (Kessler, 1975). To measure total ^{125}I -labelled protein precipitated, the washed pellet was counted for radioactivity. The antigen–antibody complex was dissociated from the bacterial cell surface by heating the washed pellet at 100°C for 3 min in 2% SDS/10% mercaptoethanol/154 mM-NaCl/20 mM-Tris HCl, pH 7.4. After centrifugation to remove the staphylococci the supernatant was electrophoresed on SDS/6% (w/v)-polyacrylamide gels using the Laemmli (1970) procedure, sliced into 2 mm sections and counted for radioactivity in a Packard γ -counter.

In the above procedure, 6 μl of preabsorbed antiserum I was usually sufficient to precipitate the maximum amount of ^{125}I -labelled proteins from 33 μg of membrane protein contained in 100 μl solubilizing medium, and 200 μl of a 10% suspension of *Staphylococcus aureus* was found sufficient to precipitate out the maximum amount of ^{125}I -labelled proteins contained in the 100 μl of membrane extract. By using (1) mature cell membranes with reticulocyte-specific antiserum or (2) reticulocyte membranes with non-immune serum or (3) reticulo-

cyte membranes with reticulocyte preabsorbed immune serum, identical levels of ^{125}I -labelled proteins were found associated with the *Staphylococcus aureus* pellets. This non-specific radioactivity was $\approx 25\%$ of that found with reticulocyte membranes and reticulocyte-specific antiserum and was not observed after SDS/polyacrylamide-gel electrophoresis.

Immunoprecipitation of transferrin

To 0.2 ml of antiserum I or anti-(mature red blood cell) antiserum was added 0.01 ml of [^{59}Fe]transferrin (0.5 mg/ml) and the mixture incubated at 4°C for 24 h. Then 0.2 ml of goat antiserum against rabbit IgG was added and incubation continued for another 48 h at 4°C. The aggregates formed were centrifuged down at 30 900 *g* for 10 min, washed, and counted for radioactivity.

Immunofluorescence and double staining of reticulocytes

Fluorescent antibodies were prepared as described by (The & Feltkamp, 1970). For the double-staining methods (Methylene Blue and FITC–antibody), 100 μl of a 1% reticulocyte suspension in phosphate-buffered saline, pH 7.4, was incubated with 10 μl of FITC–antibody (500 $\mu\text{g}/\text{ml}$) at 0°C for 90 min. The cells were washed twice with phosphate-buffered saline, pH 7.4. The cell pellet was resuspended with phosphate-buffered saline to give a 1.0% cell suspension and a drop of the suspension was placed on a polylysine-coated glass coverslip. The cells were allowed to sediment for a few minutes and the excess fluid in the drop was removed carefully. A drop of new Methylene Blue solution was then added to the coverslip. After 5 min the excess Methylene Blue solution was removed and the coverslip rinsed gently with phosphate-buffered saline, pH 7.4. Fluorescence-stained cells were viewed under a Zeiss microscope. Indirect immunofluorescence was also carried out by using FITC-labelled protein A and antiserum I.

Peptide mapping

The method described by Elder *et al.* (1977) was used, except that the electrophoresis buffer (v/v) was butanol/pyridine/acetic acid/water (2:1:1:36, by vol.) as used by J. P. Julien and W. E. Mushynski (personal communication).

Materials

Na^{125}I and [^{59}Fe]ferrous citrate were purchased from Frosst Co., Montreal, Quebec, Canada. Lactoperoxidase, glucose oxidase and Aprotinin were purchased from Sigma, St. Louis, MO, U.S.A. Fluorescein-conjugated protein A was a product of Pharmacia Fine Chemicals, Pharmacia, Dorval, Quebec, Canada. Tissue-culture media and calf

serum were obtained from Grand Island Biological Co., Burlington, Ontario, Canada. The culture of *Staphylococcus aureus* (Cowan strain) was obtained from Dr. D. Thomson, Montreal General Hospital.

Results

Immunization of rabbits with sheep reticulocytes results in the production of an antiserum directed against the reticulocyte cell surface. Antiserum from 12 different rabbits gave similar results. The conclusion that the antiserum is directed against the reticulocyte plasma membrane, is shown by (i) immunofluorescence and (ii) immunoprecipitation studies.

Indirect immunofluorescence

Reticulocytes and mature red cells were incubated with antiserum I, washed and incubated with FITC-labelled protein A. Immunofluorescence was observed only with cells which had reticulum (Fig. 1). No fluorescence was seen when reticulocytes were incubated with (1) non-immune rabbit serum, (2) when mature cells were incubated with anti-reticulocyte serum, or (3) when reticulocytes were incubated with reticulocyte preabsorbed antiserum.

Immunoprecipitation

Solubilized, iodinated reticulocyte plasma-membrane proteins were treated with antiserum I and *Staphylococcus aureus* as described in the Materials and methods section. After SDS/polyacrylamide-gel electrophoresis (Fig. 2), the data show two clear iodinated components in the 85–95 kDa region. An additional broad peak is frequently detected in the 200 kDa region, but its level of radioactivity is generally much lower than the activity in the 95 kDa region. No labelled components were obtained from mature-red-cell membranes after incubation with anti-reticulocyte serum, nor from reticulocyte membranes treated with either nonimmune serum or immune serum preabsorbed with reticulocytes.

To ascertain that the ^{125}I -labelled peptides detected originate from the reticulocyte plasma membrane and not from cytosolic components which adhere to the membrane during isolation, the labelling with ^{125}I and lactoperoxidase was conducted with intact reticulocytes and mature cells followed by isolation of membranes and immunoprecipitation. This procedure results in labelling of surface components only (Reichstein & Blostein, 1973, 1975). Before immunoprecipitation, the total radioactivity in the ^{125}I -labelled sample used was similar ($\pm 5\%$) with both reticulocyte and mature cells, whereas four times more radioactivity was found in the immunoprecipitate from labelled reticulocytes than from the controls (mature cells pretreated with antiserum).

Fig. 1. Binding of FITC-labelled protein A to reticulocytes in presence of antiserum

A mixture of reticulocytes and erythrocytes was incubated with immune serum or control serum for 90 min at 0°C and then washed with phosphate-buffered saline at 4°C . The washed suspensions were stained with FITC-labelled protein A and Methylene Blue as described in the Materials and methods section. (a) FITC-protein A fluorescence in the presence of immune serum I; (b) Methylene Blue staining.

These data show that the anti-reticulocyte serum reacts specifically with components on the reticulocyte cell surface but not with those on the surface of the mature sheep red cell.

Disappearance of reticulocyte membrane proteins during maturation in vitro

If the peptides identified above are associated with proteins whose function will be lost on reticulocyte

Fig. 2. Immunoprecipitation of iodinated membrane proteins

Isolated membranes from reticulocytes and mature cells were iodinated, dissolved, and treated with antiserum and *Staph. aureus* as described in the Materials and methods section. Before the addition of serum the dissolved reticulocyte membrane sample contained $33\mu\text{g}$ of protein and a total radioactivity of 2.5×10^5 c.p.m. in $100\mu\text{l}$. After treatment with immune serum I and staphylococci, the immunocomplex was centrifuged, washed, then dissociated with 2% SDS in 0.154 M-NaCl at 100°C (see the Materials and methods section). The eluate, containing 3500 c.p.m., was applied to an SDS/polyacrylamide disc gel, electrophoresed, sliced into 0.2 cm sections and counted for radioactivity. A total of 1800 c.p.m. were recovered after counting all gel fractions. This represents a recovery of 75% of the specifically labelled protein in the SDS eluate. (Total c.p.m. = 3500; non-specific c.p.m. = 1100, see below.) A second portion of labelled reticulocyte membrane proteins was treated with non-immune serum or with reticulocyte preabsorbed antiserum I. After dissociation with 2% SDS in saline, the total radioactivity recovered (1130 c.p.m. and 1100 c.p.m. respectively) was applied to the gel (non-specific labelling). No radioactivity above background was obtained with the latter two samples after electrophoresis. By using a portion of labelled membrane proteins ($37\mu\text{g}$ and 1.8×10^5 c.p.m. in $100\mu\text{l}$) from mature cells, treatment with immune serum I precipitated out 970 c.p.m. The SDS eluates obtained from this immunocomplex showed no detectable radioactivity above background after electrophoresis. The reason for this high radioactivity in the non-immune precipitate is not known. ●, Reticulocytes + immune serum; ○, reticulocytes + non-immune serum; ×, mature cells.

Fig. 3. Diminished labelling of the reticulocyte-specific surface proteins with time in culture

Equal numbers of cells were injected into a series of culture flasks. At intervals, the flasks were removed and the cells centrifuged, washed, and the plasma membranes isolated and stored frozen. When the last sample had been taken, all the membrane samples were iodinated with ^{125}I . The iodinated membranes were washed and then disaggregated as described in the Materials and methods section. To $600\mu\text{l}$ of dissolved membranes (containing 100–120 μg of protein), antiserum I was added, followed by a 10% suspension of *Staph. aureus* (see the Materials and methods section). The pellet containing the immunocomplex was washed three times and then counted for radioactivity. The counts are expressed as c.p.m. per μg of membrane protein. After labelling the membranes and before precipitation with antiserum, the total c.p.m. values for each sample were: zero time, 5×10^5 ; 1 day, 4.3×10^5 ; 4 days, 4.4×10^5 . A representative experiment is shown from three similar experiments. □, Mature cells; ■, reticulocytes.

followed *in vitro* as described before (Benderoff *et al.*, 1978a,b).

The results (Fig. 3) show that the total amount of ^{125}I -labelled immunoprecipitable material decreases with time in culture. After 72 h there is little difference in the level of radioactivity isolated from reticulocyte membranes and from mature-cell membranes. If SDS eluates from the *Staphylococcus* A complex are subjected to SDS/polyacrylamide-gel electrophoresis, the labelling pattern obtained is similar to that seen in Fig. 2, and by 72 h the level of radioactivity approaches that seen with the mature cells.

Two lines of evidence argue against the possibility that the loss of iodinated peptides is due to

maturation, it may be possible to demonstrate that there is a reduction in these peptides during incubation *in vitro*. Reticulocyte maturation was

either cell lysis or non-specific changes in the sites available for iodination. (1) There is no significant increase in haemoglobin in cell-free supernatants of the medium indicating the absence of cell lysis during incubation. (2) The total ^{125}I incorporated into the plasma-membrane proteins stays constant during the culture periods.

To ascertain functional integrity of the cells during incubation *in vitro*, cellular K^+ was measured. The cellular K^+ level remains relatively constant $\approx 100\text{ mmol/litre}$ of cell water (see also Benderoff *et al.*, 1978*a,b*) (results not given) characteristic of a high- K^+ sheep (Evans & King, 1955).

Identification of the surface antigen which is lost on maturation

With a purified IgG fraction coupled to fluorescein, it was established that the IgG fraction reacted only with reticulocytes as seen in Fig. 1 with the immune serum I and FITC-protein A. In addition, with time in culture there was decreased binding of the ^{125}I -labelled purified IgG fraction to the reticulocyte, by 72 h only 20% of the initial binding being obtained.

With an immunoaffinity column prepared from the purified IgG fraction, the data in Fig. 4 show that three-molecular-weight species from a Triton X-100 extract of reticulocyte plasma membranes, but not mature-cell membranes, are retained by the immobilized IgG column. That the immunoaffinity column reacts specifically is shown by the fact that no detectable proteins from the reticulocyte membranes were retained by the anti-(bovine albumin) affinity column.

Identification of the peptides retained by the immunoaffinity column

(a) [^{125}I]Iodotyrosyl-peptide mapping. To determine whether the peptides isolated from reticulocyte membrane were related structurally, their iodotyrosyl peptide maps were compared. As shown in Figs. 5(a) and 5(b), the 186 kDa and 93 kDa species have very similar peptide maps, suggesting that the 186 kDa species is a dimer of the 93 kDa component. The 78 kDa is entirely different. It appears, however, that the 78 kDa peptide has a map similar to sheep transferrin (Figs. 5c and 5d), thus identifying it as transferrin itself.

(b) *Studies with transferrin and immunoaffinity columns.* To determine if the 93 kDa and the 180 kDa are components of the transferrin receptor, solubilized membrane proteins were passed through the transferrin-Sepharose 4B affinity column and through the immunoaffinity column. The peptides isolated after passage through the transferrin affinity column are shown in Fig. 6(a) and those from the immunoaffinity column in Fig. 6(b). It may be seen

Fig. 4. Reticulocyte membrane proteins isolated by the purified-IgG-Sepharose-4B column

Solubilized membrane proteins from mature red blood cells or from reticulocytes were applied to the column. After washing with 0.25% Triton X-100/phosphate-buffered saline, pH 7.4, the column was eluted with glycine buffer, pH 2.3, as described in the Materials and methods section. The eluates were dialysed, freeze-dried and redissolved in 2% SDS/10% mercaptoethanol, followed by electrophoresis in SDS/polyacrylamide gel. Staining was with Coomassie Blue. (a) Solubilized reticulocyte membrane proteins; (b) solubilized mature-red-cell membrane proteins.

that the same protein profile is obtained with both columns. In conjunction with the data on the peptide maps, the data show that both transferrin and the transferrin receptor are isolated from the reticulocyte membranes.

Verification that immobilized transferrin and the immunoaffinity procedure isolate the same peptides

If the two affinity procedures retain the same peptides, prepassage of the solubilized membrane

Fig. 5. [^{125}I]Iodotyrosyl-peptide mapping

The areas corresponding to 186 kDa (a), 93 kDa (b), 78 kDa (c) and sheep transferrin (d) were cut from Coomassie Blue-stained gels and were iodinated with Na^{125}I . The iodinated peptides were treated with trypsin, the digests eluted from the gels, and electrophoresed and chromatographed on thin-layer cellulose plates using Polygram CEL 300 (Macherey, Nagel and Co., Düren, Germany). Autoradiography on Kodak XR 1 film was used to reveal the radiolabelled areas.

proteins through one column should remove all protein species capable of binding to the other column. The data in Figs. 6(c) and 6(d) verify this prediction. No detectable membrane proteins are retained by the transferrin column after prepassage through the immunoaffinity column and vice versa. These observations confirm that the proteins retained are transferrin and the transferrin receptor.

Identification of the purified IgG as anti-(transferrin receptor) antibody

Since transferrin has a high affinity of its receptor (Morgan, 1974), co-isolation of transferrin and transferrin receptor by the purified IgG immuno-

affinity column could result from anti-transferrin or anti-(transferrin receptor) antibody or both. Two types of studies show the antibody to be directed against the receptor.

(a) *Studies with [^{59}Fe]transferrin.* Incubation of [^{59}Fe]transferrin with either antiserum I or rabbit anti-(mature-cell) serum followed by addition of goat anti-(rabbit IgG) serum precipitated equal but only small amounts (5%) of [^{59}Fe]transferrin.

(b) *Studies with immunoaffinity columns.* As shown in Figs. 7(b) and 7(e), transferrin is not retained by the immunoaffinity column and the IgG fraction is not retained by the transferrin column. These data show that the IgG fraction obtained is not directed against transferrin itself.

Fig. 6. Identity of reticulocyte membrane proteins isolated by the purified-IgG-Sepharose-4B and transferrin-Sepharose-4B columns

(a and b) Solubilized membrane proteins from equivalent numbers of reticulocytes were applied to both the purified-IgG-Sepharose 4B and transferrin-Sepharose-4B columns. The proteins retained were eluted with glycine buffer and electrophoresed on SDS/polyacrylamide gels followed by staining with Coomassie Blue. (a) Eluates from the purified IgG immunoaffinity column; (b) eluates from the transferrin affinity column. (c) Same as (a) except that the solubilized reticulocyte membrane proteins were passed through the transferrin column before being applied to the purified-IgG column. (d) Same as (b), except the solubilized reticulocyte membrane proteins were passed through the purified IgG column before being applied to the transferrin column.

Non-identity of the transferrin binding sites and the antibody binding sites

The data show that the transferrin column does not retain the purified IgG fraction (and vice versa). However, if detergent extracts of reticulocyte membranes are first applied to the transferrin column, the purified IgG can be retained by the column together with transferrin and transferrin receptor. The reciprocal experiment with the immunoaffinity column shows that transferrin can be retained by the column only when extracts of the reticulocyte membrane proteins have been prepassed through the column (Figs. 7c and 7d). These results suggest that

Fig. 7. The purified IgG is not anti-transferrin antibody

Transferrin (1 mg/ml) and purified IgG (1 mg/ml) were passed through the purified-IgG immunoaffinity and transferrin affinity columns respectively with and without the presence of detergent extracts of the reticulocyte membrane. The columns were washed with phosphate buffer and eluted with glycine buffer, pH 2.3. The eluates were electrophoresed in SDS/polyacrylamide gels. Staining was with Coomassie Blue. (a) Transferrin marker; (b) eluate from an IgG column after application of transferrin; (c) as in (b), except a detergent extract of reticulocyte membranes was pre-passed through the column; (d) eluate from a transferrin column after application of IgG. A detergent extract of membranes was applied to the column before IgG; (e) as in (d) above without the membrane extract; and (f) IgG standard.

the binding sites for the antibodies are distinct from those for transferrin.

Transmembrane orientation of the putative transferrin receptor

To determine whether the putative receptor spans the membrane or is accessible only at the external surface, techniques were applied that have been used to determine the disposition of other red-cell membrane proteins (Reichstein & Blostein 1973, 1975).

Labelled peptides corresponding to molecular masses of 186, 93 and 78 kDa were obtained from the membranes derived from externally iodinated cells (Fig. 8a). Peptides of 93 kDa plus a weakly labelled species of 186 kDa were obtained with membranes from ghosts labelled at the cytosolic surface (Fig. 8b). The fact that the 186 kDa and 93 kDa peptides retained by the immunoaffinity column can be labelled from both surfaces suggests a transmembrane orientation for the transferrin

Fig. 8. Accessibility of the transferrin receptor at the external and cytoplasmic surface to lactoperoxidase-catalysed iodination

Reticulocytes were labelled with Na^{125}I and lactoperoxidase to tag the external-surface membrane proteins. The cytoplasmic-surface membrane proteins were labelled with lactoperoxidase incorporated into resealed ghosts. Membranes were solubilized and passed through the anti-(transferrin receptor) column. The proteins retained were eluted and electrophoresed in SDS/polyacrylamide disc gels. Gels were sliced into 0.2 cm sections and counted for radioactivity. (a) External-surface iodination; (b) cytoplasmic-surface iodination.

receptor. The relatively weak labelling at the cytosolic surface may be due to the fact that residual cytosolic components may interfere with the labelling at the cytosolic surface. Alternatively, the transferrin receptor may have fewer tyrosine residues accessible to iodination at the cytosolic surface. It is noteworthy that the 78 kDa peptide (transferrin) is not measurably labelled by iodination of the internal surface. This observation shows that the inner-surface labelling of the 93 kDa peptide is not due to spurious labelling of the external surface and supports the conclusion that the receptor spans the membrane.

Discussion

The present study shows that an antiserum against reticulocytes can be used to identify plasma

membrane peptides that are characteristic of the reticulocytes. With culture time *in vitro* during which cellular integrity is maintained, there is a diminished capacity of reticulocyte plasma-membrane components to react with reticulocyte-specific antiserum. Thus decreasing levels of ^{125}I -labelled membrane proteins are precipitable with antiserum and decreasing amounts of purified ^{125}I -labelled IgG are bound to the reticulocytes with time in culture. These observations provide a means to monitor and quantify maturation of reticulocytes at the molecular level under conditions *in vitro*. Decreased binding of antibody could be due to modification of the antigenicity of the receptor or a loss of the protein. At present, definitive data are not available to discriminate between these possibilities, but preliminary data indicate that the protein is lost from membrane, and detectable in the incubation medium. The disappearance of the reticulocyte-specific plasma-membrane peptides follows a time course similar to that seen for the loss of Na^+ -dependent glycine transport and Na^+ -independent histidine exchange (Benderoff *et al.*, 1978a,b).

By using reticulocyte-specific antibodies, the reticulocyte-specific membrane protein has been identified as the transferrin receptor by demonstrating that identical membrane proteins are retained from reticulocyte membranes by the immunoaffinity column and the immobilized transferrin column.

Two molecular-mass species isolated (186 kDa and 93 kDa) have identical peptide maps, suggesting that the 186 kDa peptide is a dimer of the 93 kDa species. Several investigators have concluded that it may be dimeric (Ecarot-Charrier *et al.*, 1980; Sullivan & Weintraub, 1978; Leibman & Aisen, 1977; Hsiang-Yen & Aisen, 1978; Trowbridge & Omary, 1981; Sutherland *et al.*, 1981; Wada *et al.*, 1979; Morgan, 1974) with a molecular mass in the range reported here. If the 186 kDa peptide is indeed a dimer of the 93 kDa component, the data would suggest that even heating at 100°C with reducing agents may be insufficient to disaggregate the receptor completely (see also Trowbridge & Omary, 1981).

The third component isolated appears to be transferrin itself (78 kDa). That transferrin is co-isolated with its receptor is not surprising, since transferrin is tightly bound to the receptor (Morgan, 1974). Control experiments show that the antibody produced is not directed against transferrin.

A comment should be made about the apparent differences in the molecular-mass estimates of the peptides shown in Fig. 2 with those in subsequent Figures. In Figs. 4–7, the molecular masses were estimated from polyacrylamide slab gels with appropriate standards. The molecular masses in Fig. 2 were estimated from slice number of polyacryl-

amide cylindrical gels, each slice representing 2 mm of distance, and compared with standards treated similarly. Furthermore, the gels in Fig. 2 contained more extraneous proteins, i.e. those eluted from staphylococci, in addition to reticulocyte proteins which may have interfered with the resolution of the molecular-mass estimates in Fig. 2.

That transferrin and receptor are co-isolated with the immunoaffinity column as well as the transferrin column suggests that: (1) the sites for antibody binding and transferrin binding are different and (2) that the receptor has more than one site for transferrin binding. Similar conclusions have been reached by others. Sutherland *et al.* (1981) have presented experimental evidence that in human leukaemic cells the binding sites for antibody and transferrin are different. Enns & Sussman (1981) have concluded that there are two binding sites for transferrin on the receptor in human placenta. These observations are in accord with our conclusions on the sheep reticulocyte system.

In conclusion, it is noteworthy that the transferrin receptor has recently been recognized by identifying it as the antigen to a monoclonal antibody made against a conspicuous cell-surface glycoprotein (Trowbridge & Omary, 1981; Sutherland *et al.*, 1981). In the present study the transferrin receptor was identified as the antigenic component when rabbit antibodies are produced against sheep reticulocytes. This holds true for antibodies produced in a relatively large number of animals. To our knowledge this is the first report that a major surface antigenic marker characteristic of the sheep reticulocyte is the receptor for transferrin. These data are consistent with the conclusion that the receptor is highly antigenic in heterologous species. In the absence of evidence to the contrary, it is tempting to speculate that if there is polymorphism of the receptor in a given species, the transferrin receptor may be a member of a class of histocompatibility antigens expressed on cell surfaces.

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