Biochimica et Biophysica Acta, 508 (1978) 571—576

BBA Report

BBA 71336

ERYTHROCYTE MEMBRANE PROTEINS

SEQUENTIAL ACCUMULATION IN THE MEMBRANE DURING RETICULOCYTE MATURATION

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(Received November 30th, 1977)

Summary

Reticulocytes of increasing maturity were separated by dextran gradient centrifugation. The accumulation in the membrane of the anion transport protein and other erythrocyte membrane proteins was studied during reticulocyte maturation by separating reticulocytes after incubation with [^{35}S]-methionine. The incorporation of the reticulocyte membrane proteins was shown to be sequential, the anion transport protein being inserted at a very early stage in the cells' maturation.

Earlier studies on the synthesis of the erythrocyte membrane proteins have utilised reticulocyte preparations containing cells of widely differing maturity [1—4]. The reticulocyte matures over a period of two days to give rise to the erythrocyte, during which time it passes through well defined stages of increasing maturity [5,6]. Previous work has indicated that most, if not all, of the erythrocyte membrane proteins are synthesised by the reticulocyte [7]. However, experiments on reticulocyte membrane protein synthesis using mixed populations of cells have not been able to clearly define the reticulocyte stage at which the major intrinsic protein of the rabbit erythrocyte membrane (band 3 on sodium dodecyl sulphate polyacrylamide gel electrophoresis) appears in the membrane. This protein is well characterised and has been shown to be responsible for anion transport in the erythrocyte [8]. We have studied the incorporation of this protein and other extrinsic erythrocyte membrane proteins into the membrane during reticulocyte maturation using reticulocytes separated into different age groups.

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Reticulocytes were prepared, washed and equilibrated in phosphate-buffered saline as previously described [7]. The reticulocyte content (typically 75—85%) of the cell preparations was estimated by supravital staining with New Methylene Blue. 2 ml of packed cells were resuspended in an equal volume of the homologous plasma and 100 μg each of penicillin G and streptomycin sulphate were added. The cell suspension was then incubated for 2 h at 37°C in the presence of 5 mM glucose, 12 μM ferrous ammonium sulphate [4] and 100 μCi of [^{35}S]methionine. The [^{35}S]methionine was prepared from carrier free $^{35}SO_4$ (The Radiochemical Centre, Amersham, Bucks., U.K.) by the method of Bretscher [9] and had an approximate specific activity of 8 Ci/mmol. After incubation the cells were washed four times in phosphate-buffered saline. The packed, washed cells were then applied to a 7 ml gradient of 15—20% (w/v) Dextran 110 (Pharmacia Ltd., Uppsala, Sweden) made up in phosphate-buffered saline containing 1% homologous rabbit plasma. The reticulocytes were then separated into age groups by centrifugation at the bench for 90 s (1800 $g \cdot$ min). Five or six fractions were collected and numbered from the top of the gradient. After washing the cells from each fraction with phosphate-buffered saline, samples were stained with New Methylene Blue and smeared on microscope slides for analysis. The remaining cells in each fraction were then lysed in hypotonic buffer [10] and the lysate was clarified by centrifugation at 40 000 $\times g$ for 20 min. The specific radioactivity of the haemoglobin in the lysate supernatant was calculated by scintillation counting and measurement of absorbance at 415 nm and was also used as an indication of the maturity of the cells in each fraction.

Table I shows the distribution of the four, histologically defined groups of reticulocyte (stages I—IV in order of increasing maturity and decreasing amount of intracellular reticulum [6]) obtained in fractions of cells separated in a dextran gradient. Fig. 1 clearly shows that the cells were separated in the gradient according to their age. This conclusion was confirmed by the experiment shown in Fig. 2a. This figure gives the results of two experiments in which the specific radioactivity of haemoglobin was calculated for the cells in the fractions obtained after a dextran gradient separation of reticulocytes which had been previously incubated with [^{35}S]methionine. As would be

TABLE I

THE RETICULOCYTE COMPOSITION OF FRACTIONS FROM A DEXTRAN GRADIENT SEPARATION OF RETICULOCYTE-RICH RABBIT BLOOD

Samples from fractions obtained after dextran gradient centrifugation were stained with New Methylene Blue and smeared on slides for microscopical analysis. Reticulocytes of varying maturity were identified as described by Seip [6]. No significant contamination by nucleated cells was found in any fraction, the remaining contaminant cells being erythrocytes.

Fraction number	% Total cells applied to gradient	% Reticulocytes	Reticulocyte stages (% of each type)			
			I	II	III	IV
1	6	95	56	23	18	3
2	17	88	17	53	23	7
3	14	78	9	36	36	19
4	20	73	5	37	39	19
5	43	70	2	20	45	33

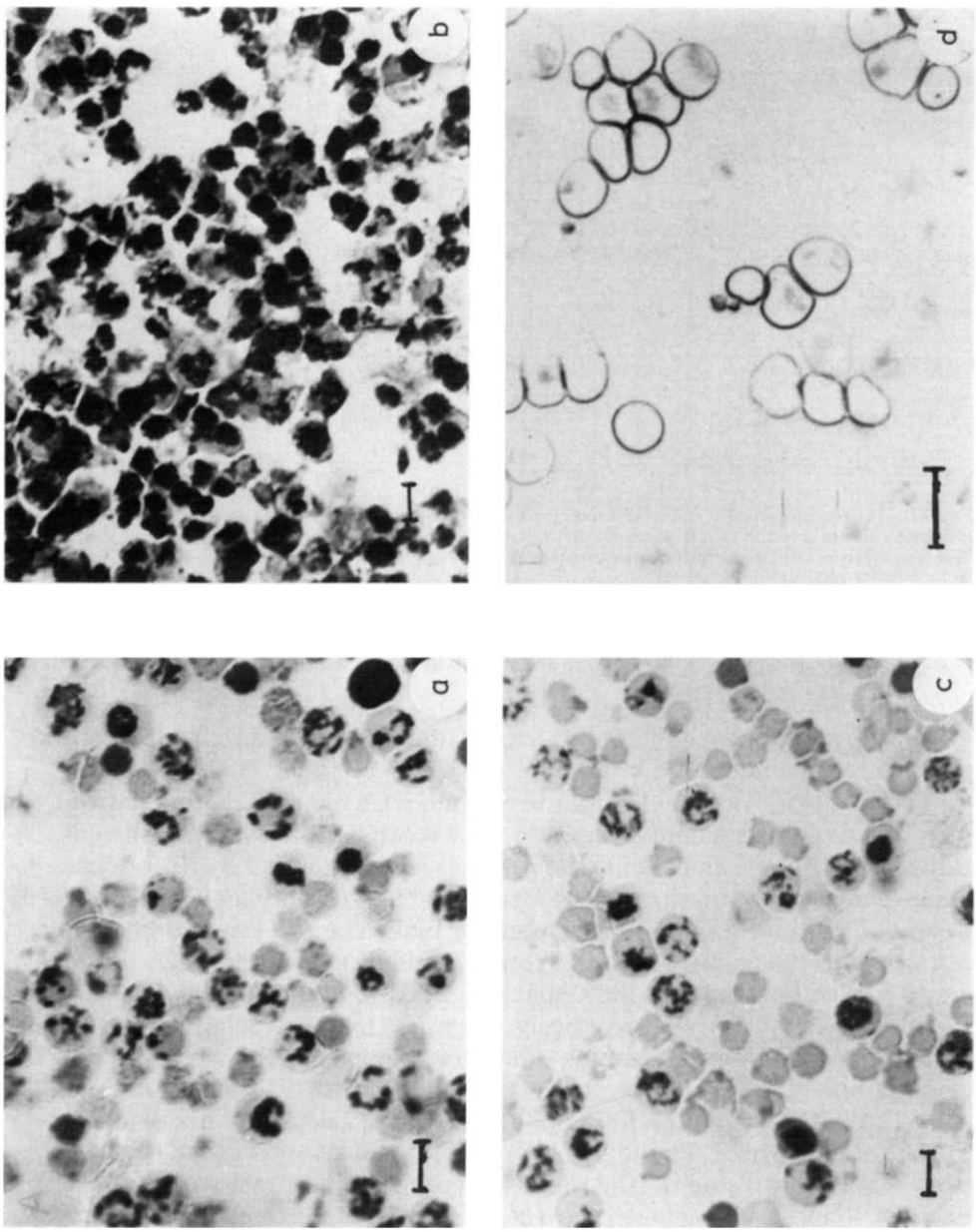

Fig. 1. Dextran gradient separation of reticulocyte stages. Red cells obtained from an anaemic rabbit containing 75% reticulocytes were fractionated by dextran gradient centrifugation as described in the text. A, Cell sample before fractionation; B, cells collected from the top of the gradient (fraction 1); C, cells collected in fraction 4; D, cells collected in fraction 6 (bottom of gradient). Scale bar is 10 μm in each case. Cells were smeared on microscope slides and stained with New Methylene Blue.

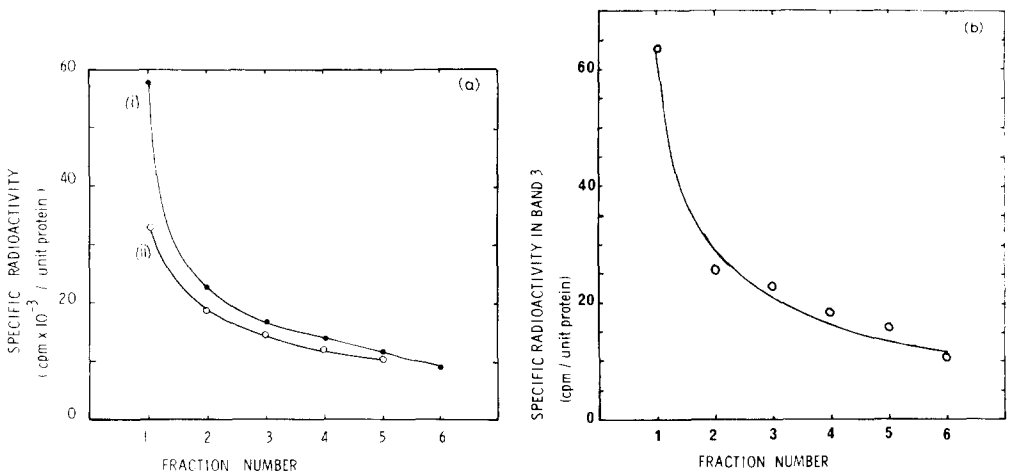

Fig. 2. a. Incorporation of [^{35}S]methionine into haemoglobin in reticulocytes separated into age groups by sedimentation in a dextran gradient. The results of two experiments are shown, (i) and (ii). b. Accumulation of the anion transport protein in the reticulocyte plasma membrane during maturation. [^{35}S]Methionine-labelled membrane proteins from reticulocytes separated into age groups by dextran gradient centrifugation were separated by SDS polyacrylamide gel electrophoresis and stained with Coomassie Brilliant Blue. The specific radioactivity in band 3 was estimated by excision of the protein band from the gels and scintillation counting. The protein in band 3 was estimated from scans of the stained gel at 550 nm before excision and scintillation counting of band 3.

expected, the specific activity of haemoglobin decreases throughout the fractions to reach a minimum in the fraction which contains the oldest cells.

The 'ghosts' prepared from the reticulocytes in each fraction derived from the dextran gradient separation were solubilised in sodium dodecyl sulphate (SDS) and the membrane proteins were separated by SDS polyacrylamide gel electrophoresis in gels containing 8% (w/v) acrylamide as previously described [7] and stained with Coomassie Blue. The [^{35}S]methionine incorporated into the anion transport protein (band 3) and bands 4, 4A, R1, 5 and 6 (which have subunit molecular weights of 90 000, 75 000, 72 000, 54 000, 39 000, 36 000, respectively, see ref. 7 for nomenclature) was estimated by densitometric scanning of autoradiographs of the same gels after drying.

The [^{35}S]methionine found in each protein, calculated as a percentage of the total [^{35}S]methionine incorporated into all six proteins in each fraction, is shown in Table II. The relative incorporation of radioactivity into bands 3, 4 and 4A is highest in the most immature cell fraction and decreases in the fractions of increasing maturity. In contrast, the incorporation into bands R1 and 5 increases with increasing maturity of the cells. The relative incorporation into band 6 is highest in the cell fractions of intermediate maturity, but is lower in the very early and late reticulocyte containing fractions. The same trends were found for each of these proteins when the data was calculated on the basis of specific radioactivity in each band (Table II). We have also confirmed the data for band 3 by direct escision of the protein from gels and subsequent scintillation counting (Fig. 2b).

The data suggest that the rate of accumulation of bands 3, 4 and 4A into the red cell plasma membrane is highest in type I reticulocytes (these

TABLE II

INCORPORATION OF [^{35}S]METHIONINE INTO RETICULOCYTE PLASMA MEMBRANE PROTEINS

The incorporation of [^{35}S]methionine into each of the six proteins investigated was calculated by the method described in the text for the cells in five fractions obtained after dextran gradient centrifugation. The figures in brackets represent the specific radioactivity (arbitary units) of each protein in each fraction. The protein content of each band was estimated from scans of the stained gels at 550 nm prior to radioautography [7].

Fraction number	% [^{35}S]Methionine incorporated					
	Band number					
	3	4	4A	R1	5	6
1	20 (0.33)	20 (0.28)	31 (0.20)	9 (0.21)	14 (0.12)	6 (0.03)
2	15 (0.23)	20 (0.25)	22 (0.17)	20 (0.22)	13 (0.15)	10 (0.04)
3	8 (0.11)	16 (0.21)	19 (0.15)	24 (0.36)	22 (0.26)	11 (0.06)
4	7 (0.10)	16 (0.22)	17 (0.13)	26 (0.53)	22 (0.45)	12 (0.12)
5	5 (0.05)	10 (0.20)	11 (0.12)	42 (0.57)	28 (0.48)	5 (0.03)

cells only constitute about 10% of the total circulating reticulocyte population in these highly anaemic animals). As the cells mature, the rate of accumulation of these proteins decreases with a concomitant increase in the incorporation of bands R1, 5 and 6 into the membrane. Even at a very late reticulocyte stage (type IV) the accumulation of bands R1 and 5 continues but in these cells the incorporation of band 6 into the membrane is approaching completion. These results suggest that the reticulocyte membrane proteins are sequentially incorporated into the plasma membrane. It seems likely that the initiation of the synthesis of bands 3, 4 and 4A occurs in the bone marrow during the formation of the reticulocyte [7].

In these experiments we studied the overall accumulation of the proteins in the reticulocyte membrane. It is possible that there are intermediate stages between the de novo synthesis of the proteins and their final appearance in the membrane. Indeed, Lodish and his group [1,2] have shown that band R1 (their band B2) and band 6 (glyceraldehyde-6-phosphate dehydrogenase, their band E) are synthesised on cytoplasmic ribosomes, released into the cytoplasm and then bound to specific sites at the cytoplasmic surface of the membrane. A similar pathway might be expected for the other extrinsic proteins.

Lodish and Small [2] showed that two proteins (bands R1 and 6) were the major products of membrane protein synthesis in unfractionated reticulocyte preparations. Their data also showed that bands co-migrating with 3, 4, 4A and 5 were synthesised in reticulocytes, albeit at a lower level. This might be expected since unfractionated reticulocyte populations contain relatively few of the most immature reticulocytes which are actively synthesising bands 3, 4 and 4A. It is interesting that the data of these workers also shows that [^{35}S]-methionine is incorporated into bands 4A, R1 and 5 in very small amounts by normal rabbit red cells. Since normal blood contains 1—2% of late reticulocytes, this observation is consistent with our suggestion that the synthesis of bands R1 and 5 continues into the late reticulocyte stage. The continued accumulation of band R1 even in the stage IV reticulocyte is somewhat surprising as this is a reticulocyte specific protein [7] which is not found associated with the membrane of the mature erythrocyte. It is not clear

whether this protein is lost from the cells on maturation or merely is no longer associated with the membrane in the erythrocyte.

Yu and Steck [11] have shown that human erythrocyte glyceraldehyde-3-phosphate dehydrogenase (band 6) is associated with the transmembrane protein, band 3 at the intracellular surface of the membrane. Aldolase (comigrating with band 5) appears to be similarly associated with band 3 [15]. Although there is little direct evidence that the other cytoplasmic extrinsic membrane proteins of the erythrocyte are bound to the membrane by specific associations with intrinsic membrane proteins such as band 3 (and possibly the sialo-glycoprotein in the case of the human red blood cell), there is some indirect evidence that this may be the case [12—14]. If this is so, it would provide a rationale for the observed sequential accumulation of membrane proteins in the reticulocyte. It might be expected that the intrinsic protein (band 3) should be inserted in the plasma membrane of the early reticulocyte as an initial step in order to provide the necessary binding sites at the cytoplasmic face of the membrane for glyceraldehyde-6-phosphate dehydrogenase and other extrinsic membrane proteins.

NDL was the recipient of a Medical Research Council studentship, and the work was supported in part by a grant from the Wellcome Trust.

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