Synthesis and Assembly of Membrane Skeletal Proteins in Mammalian Red Cell Precursors

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Abstract. The synthesis of membrane skeletal proteins in avian nucleated red cells has been the subject of extensive investigation, whereas little is known about skeletal protein synthesis in bone marrow erythroblasts and peripheral blood reticulocytes in mammals. To address this question, we have isolated nucleated red cell precursors and reticulocytes from spleens and from the peripheral blood, respectively, of rats with phenylhydrazine-induced hemolytic anemia and pulse-labeled them with [^{35}S]methionine. Pulse-labeling of nucleated red cell precursors shows that the newly synthesized α- and β-spectrins are present in the cytosol, with a severalfold excess of α-spectrin over β-spectrin. However, in the membrane-skeletal fraction, newly synthesized α- and β-spectrins are assembled in stoichiometric amounts, suggesting that the association of α-spectrin with the membrane skeleton may be rate-limited by the amount of β-spectrin syn-

thesized, as has been shown recently in avian erythroid cells (Blikstad, I., W. J. Nelson, R. T. Moon, and E. Lazarides, 1983. *Cell*, 32:1081–1091). Pulse-chase experiments in the rat nucleated red cell precursors show that the newly synthesized α- and β-spectrin of the cytosol turn over coordinately and extremely rapidly. In contrast, in the membrane-skeletal fraction, the newly synthesized polypeptides of spectrin are stable. In contrast to nucleated erythroid cells, in reticulocytes the synthesis of α- and β-spectrins is markedly diminished compared with the synthesis and assembly of proteins comigrating with bands 2.1 and 4.1 on SDS gels. Thus, in nucleated red cell precursors, the newly synthesized spectrin may be attached to the plasma membrane before proteins 2.1 and 4.1 are completely synthesized and incorporated in the membrane.

T HE erythrocyte membrane skeleton is a two-dimensional network lying in apposition to the cytoplasmic face of the plasma membrane. The major component of the membrane skeleton is spectrin, a protein composed of two nonidentical polypeptides, α-spectrin (mol wt 240,000) and β-spectrin (mol wt 220,000). In addition to interacting with actin and protein 4.1, spectrin binds to extrinsic membrane protein ankyrin. The association of ankyrin with the transmembrane anion transporter (2, 12) and of protein 4.1 with the transmembrane protein glycophorin (1), serves to anchor these and other skeleton proteins to the membrane.

Although the molecular organization of the mammalian erythrocyte plasma membrane is well understood, comparatively very little work has been done towards the understanding of the regulation of assembly of this membrane–cytoskeleton complex, which occurs during development of erythroid precursors. Recently, this issue was investigated by Lazarides and his co-workers in chicken embryonic erythroid cells (3, 18). Based on their observations, the same authors postulated that the availability of the anion transporter limits the extent of newly synthesized ankyrin, and, in turn, spectrin that assembles onto the membrane skeleton. Upon binding to the membrane receptor, spectrin and ankyrin are rendered

resistant to catabolism, whereas the soluble polypeptides are catabolized. More recent work from the same laboratory (22) suggest that in avian erythrocytes, the membrane skeleton is gradually stabilized by the assembly of protein 4.1

Previous in vivo pulse-labeling studies in mice (6) and rabbits (13) have shown that the synthesis of membrane proteins of the mature erythrocyte is asynchronous. During erythropoiesis, synthesis of spectrin and actin polypeptides is completed before that of band 3, and the synthesis of proteins 4.1 and 4.2 finishes last. Both in vitro and in vivo experiments in mouse suggested that reticulocytes synthesize hemoglobin and proteins 4.1 and 4.2 but none of the polypeptides 1, 2, or 3. In contrast, recently Bodine et al. (4) have reported spectrin synthesis in mouse reticulocytes. One possible explanation for this discrepancy is that the spectrin synthesis observed by Bodine et al. (4) is due to the contaminating nucleated erythroid cells which were present in significant numbers in their heterogenous population of cells used for the experiment.

It has been shown earlier that the development of the mouse erythroblast to a mature erythrocyte is accompanied by changes in the composition and properties of the plasma membranes of cells (9). An important stage of this develop-

ment occurs during the enucleation of the late erythroblast to produce the incipient reticulocyte, when all of the spectrin of the former cell is sequestered to the membrane of the reticulocyte. Thus, in the mouse erythroid series, an essential stage in concentrating spectrin occurs during erythroblast enucleation, whereas no enucleation occurs during avian erythroid cell differentiation. Mammalian and avian erythroid cell differentiation may therefore involve different strategies to achieve the appropriate accumulation of spectrin in the terminal mature erythrocyte.

We have examined the synthesis of α- and β-spectrin and proteins 2.1 and 4.1 in rat nucleated red cell precursors and reticulocytes representing two different stages of development, and their assembly onto the membrane skeleton. We show that the synthesis and assembly of both α- and β-spectrin polypeptides is completed before that of proteins 2.1 and 4.1.

Materials and Methods

Treatment of Animals

Male or female "Seh" rats (bred at St. Elizabeth's Hospital of Boston) weighing 300–500 g were used in these studies. Anemia was induced in these rats by intraperitoneal injections of 1% aqueous phenylhydrazine (11), typically 1.5 ml on days 0 and 1, and 1.0 ml on days 3 and 5. 2 d after the last phenylhydrazine administration, blood and spleens were collected. Blood was collected in heparinized tubes by cardiac puncture using a heparinized syringe. The spleens were removed and placed in an ice-cold Hank's balanced salt solution without Ca^{2+} and Mg^{2+} (M.A. Bioproducts, Walkersville, MD).

Isolation of Rat Nucleated Red Cell Precursors

The spleens of phenylhydrazine anemic rats were placed in an ice-cold Hank's balanced salt solution without Ca^{2+} and Mg^{2+}. Cell suspensions were obtained by disrupting the tissue with tweezers and passing the fragments through a 50-ml syringe several times. After filtering through a Polyethylene mesh (spectramesh) of pore size 202 μm (Spectrum Medical Industries, Inc., Los Angeles, CA) cells were collected by centrifugation at 1,000 g for 5 min, washed once by recentrifugation, and resuspended in the same solution. This cell suspension was sedimented through Ficoll-Hypaque (Pharmacia Fine Chemicals, Piscataway, NJ) to remove lymphocytes. The pellet from the Ficoll-Hypaque gradient containing 50–55% nucleated red cell precursors was then subjected to a discontinuous Percoll gradient (Percoll, Pharmacia Fine Chemicals) consisting of 45%, 65%, 70%, 77%, and 90% percoll in Ca^{2+}-Mg^{2+}-free Hank's balanced salt solution. 1.0 ml of cell suspension was layered over 30 ml gradient and centrifuged at 5,000 g for 20 min at 4°C. After centrifugation, aliquots of cells at each interface (labeled fractions I–V from the top to the bottom of the gradient) were removed and washed with Hank's solution. Nucleated red cells were identified by staining with benzidine-hematoxylin.

Isolation of Reticulocytes

Reticulocytes were isolated from the peripheral blood of phenylhydrazine anemic rats. Blood was centrifuged at 1,000 g for 5 min, the buffy coat was removed and the cells were washed three times in Hank's balanced salt solution without Ca^{2+} and Mg^{2+}. 4 ml of this cell suspension was then mixed with 18 ml 75% percoll (Pharmacia Fine Chemicals) in Hank's solutions and centrifuged in a fixed angle rotor at 45,000 g for 30 min at 4°C. After the run, 2-ml fractions were collected from the top of the gradient and diluted with Hank's solution. The cells were harvested by centrifugation and reticulocytes were identified by staining with New Methylene Blue (J. T. Baker Chemical Co., Phillipsburg, NJ). The fraction containing at least 95–97% reticulocytes was always used in the present studies.

Labeling of Cells with [35S]Methionine

Rat nucleated red cell precursors and reticulocytes were washed twice in NCTC 109 medium (M.A. Bioproducts), resuspended at a 10% (vol/vol) concentration in the medium containing 10% fetal calf serum prewarmed to 37°C, and incubated at 37°C for 15 min. They were then labeled for differ-

ent lengths of time (5–120 min) with [35S]methionine (300 μCi/ml; 1,000 Ci/mmol; New England Nuclear, Boston, MA). For the pulse-chase experiment, further incorporation of [35S]methionine was stopped by the addition of unlabeled methionine (0.4 mM) and the incubation was then continued for different time periods (0–120 min). At the end of the labeling period, 10 vol of 155 mM choline chloride, 5 mM Hepes, pH 7.1, were added and the cells were harvested by centrifugation.

Isolation of Plasma Membranes

[35S]Methionine-labeled nucleated red cell precursors and reticulocytes were treated with diisopropyl fluorophosphate (Sigma Chemical Co., St. Louis, MO) before isolating plasma membranes. Plasma membranes were isolated by using the procedure of Chan (5). The cells were suspended in hypotonic buffer (10 mM Tris HCl, pH 7.5, 10 mM KCl, and 1.5 mM $MgCl_2$) and then disrupted with 40 strokes of a tight-fitting Dounce homogenizer. An appropriate volume of 2 M sucrose was added immediately to the homogenate to restore isotonicity. The homogenate was then layered over a sucrose-step gradient (3 vol 28% (wt/vol) sucrose, 1 vol 50% (wt/vol) sucrose; sucrose solutions made in 140 mM NaCl, 2.4 m $MgCl_2$, and 5 mM Tris-HCl, pH 7.4) and centrifuged in a SW41 rotor (Beckman Instruments, Inc., Fullerton, CA) at 117,000 g for 40 min. The membrane fraction, at the 28%/50% sucrose interface and the soluble fraction on top of the sucrose gradient were collected. The membrane fraction was diluted with 20 mM Tris-HCl, pH 7.4, and centrifuged at 15,000 g for 10 min. The resulting membrane pellet was washed once more before it was solubilized in SDS sample buffer.

Characterization of Membrane Preparation

For protein determinations, the method of Lowry et al. (17) was used. The RNA and DNA contents of the membrane preparations were measured by means of the orcinol and the diphenylamine techniques, respectively (21).

The specific activity of cytochrome oxidase in the whole cell homogenate, all fractions of the sucrose step gradient, and the isolated membranes was assayed by the method of Cooperstein and Lazarow (7).

Immunoprecipitation

[35S]Methionine-labeled cells were treated with diisopropyl fluorophosphate before lysing them in 4 vol of lysis-buffer containing 150 mM NaCl, 10 mM Tris-HCl, 7.2, 5 mM $MgCl_2$, 2 mM EDTA, 0.25 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 1% Triton X-100, and separated into soluble and insoluble fractions. The insoluble fraction contained membrane skeletons, cytoskeletons, and the nuclei. The latter were removed by low speed centrifugation at 800 g for 5 min. The resulting suspension of skeletal residues is referred to as the membrane-skeletal fraction (see Discussion). Solid urea and 2-mercaptoethanol were then added to both the soluble and insoluble skeletal fractions to give a final concentration of 9.5 M and 2.5%, respectively, before performing immunoprecipitations according to the method of Lingappa et al. (16) as modified by Levine and Willard (15). Samples of soluble and membrane-skeletal fractions were diluted 10 times with 130 mM NaCl, 10 mM Tris-HCl, pH 7.5, 5 mM EDTA, and 1% NP40. Affinity-purified anti-human spectrin antibodies were than added and incubated with the antigen for 16 h at 0°C. These antibodies cross-react equally with α- and β-spectrin as has been tested by immunoblotting experiments. Thereafter, a 10% (wt/vol) suspension of fixed staphylococcus aureus bacteria (Pansorbin, Calbiochem-Behring Corp., San Diego, CA) was added to 10% (vol/vol), and the samples were incubated for 2 h at 0°C. The *Staphylococcus aureus* bacteria–antibody–antigen complex was pelleted by centrifugation and washed twice in 130 mM NaCl, 20 mM Tris-HCl, pH 7.5, 5 mM EDTA, 0.1% SDS, and 1% NP40. The final pellet was resuspended in 70 λ SDS sample buffer and boiled for 2 min. The bacteria was pelleted and the supernatant was directly loaded on 3.5–12% polyacrylamide gradient gels.

SDS-Polyacrylamide Gel Electrophoresis

Proteins were separated by SDS-polyacrylamide gel electrophoresis according to the buffer systems of Laemmli (14) and Fairbanks et al. (8). The gels were processed for fluorography with En3Hance (New England Nuclear), dried, and exposed to Kodak XRP-1 x-ray film. The autoradiograms were scanned at 570 nm with a Quick-Scann (Helena Laboratories, Beaumont, TX), and the area under each peak was integrated.

Results

Isolation of Cells

The results of differential counts performed before and after the separation of cells on discontinuous Percoll gradient are presented in Table I. Fraction number IV containing ∼75% of nucleated erythroid cells (benzidine positive) and a minimal contamination of 0–2% by white cells (benzidine negative) was always used in the present studies.

Purity of Isolated Membranes from Rat Nucleated Red Cells and Reticulocytes

The isolation technique of Chan (5) for chicken embryonic erythroid cell membranes was used here for the isolation of membranes from rat nucleated red cell precursors and reticulocytes. This procedure gave pure and undegraded membranes. Negligible amounts of RNA and DNA were detected in the membrane samples: the percentage (wt/wt) of RNA to membrane proteins from nucleated red cell precursors and reticulocytes was 2.4% and 4.0%, respectively; the percentage (wt/wt) of DNA to membrane proteins from nucleated red cell precursors was 3.0%.

The presence of mitochondria was monitored by assaying for a mitochondria-specific enzyme, cytochrome oxidase in the whole cell homogenate, the isolated membranes, and all fractions of the sucrose step gradient. The results showed that essentially all of the cytochrome oxidase activity present in the whole cell homogenate was recovered in the pellet of the sucrose step gradient, with <1% activity present in the membranes from both nucleated red cell precursors and reticulocytes. These results are consistent with the results of Chan (5) for chicken embryonic erythroid cell membranes.

Synthesis and Assembly of Spectrin

Nucleated red cell precursors were isolated from spleens of phenylhydrazine anemic rats and reticulocytes were isolated from the peripheral blood of these animals as described in Materials and Methods. Both types of cells were pulse labeled with [^{35}S]methionine before isolating plasma membranes which were then examined on SDS polyacrylamide gels. Fluorographs of these gels showed that the nucleated red cell precursors synthesize both the α- and β-spectrins, which are assembled on the membrane in an approximately equimolar amount (Fig. 1).

In contrast, reticulocytes do not seem to contain any newly synthesized α- or β-spectrin in the membranes (Fig. 2). The radiolabeled band in the β, 2.1 region was identified as band 2.1 by its mobility on 5.6% Fairbanks gels.

Distribution of Newly Synthesized Spectrins in the Soluble and Membrane-Skeletal Fractions

Rat nucleated red cell precursors and reticulocytes were labeled with [^{35}S]methionine at 37°C for different periods of time. At the end of each labeling period, an equal number of cells for each time point was lysed in a buffer containing Triton X-100 and separated into an insoluble membrane-skeletal fraction and a soluble fraction by a low-speed centrifugation. The insoluble fraction was resuspended in the Triton X-100 containing buffer to the same volume as the soluble fraction, and equal volumes of the two fractions were immunoprecipitated with affinity-purified anti-spectrin antibodies. The resulting immunoprecipitates were analyzed on SDS polyacrylamide gels (Fig. 3).

In nucleated red cell precursors, approximately stoichiometric amounts of α- and β-spectrin are recovered in the membrane-skeletal fraction, as shown by Coomassie Brilliant Blue staining (Fig. 3 a) as well as by fluorography (Fig. 3 b), suggesting that the two polypeptides of spectrin incorporate at the same rate into the skeletal fraction. The soluble fraction, however, contains an excess of newly synthesized α-spectrin over newly synthesized β-spectrin (Fig. 3 d). The quantitation of newly synthesized α- and β-spectrin in the insoluble membrane-skeletal fraction and the soluble fraction is shown in Fig. 4. The results show that only about 10–15% of the newly synthesized α-spectrin is incorporated in the skeletal fraction while the rest is recovered in the soluble fraction. In contrast, most of the β-spectrin synthesized is incorporated in the skeletal fraction, suggesting that the association of α-spectrin with the membrane is rate-limited by the amount of β-spectrin synthesized.

In contrast to nucleated red cell precursors, reticulocytes contain only small amounts of newly synthesized α- and β-spectrin in the membrane-skeletal fraction (Fig. 3 f), suggesting that the synthesis of the bulk of both polypeptides of spectrin is completed before the reticulocyte stage. This result is in agreement with the previous work of Chang et al. (6) who showed that the synthesis of spectrin by mouse erythroid cells ceases before enucleation of the erythroblast.

Turnover of α- and β-Spectrin

The results presented above show that in rat nucleated red

Table I. Differential Counts Performed before and after the Separation of Phenylhydrazine-treated Rat Spleen Cells on Percoll Gradient

Sample	Benzidine positive		Benzidine Negative
	Nucleated	Enucleated	
	%	%	%
Spleen cells	47	31	22
Pellet from Ficoll-Hypaque	55.5	28	16.5
Fractions from Percoll gradient			
I	16	6	78
II	32	10	58
III	55.5	15.5	29
IV	74.8	23.2	2.0
V	40	60	0.0

Coomassie Stain

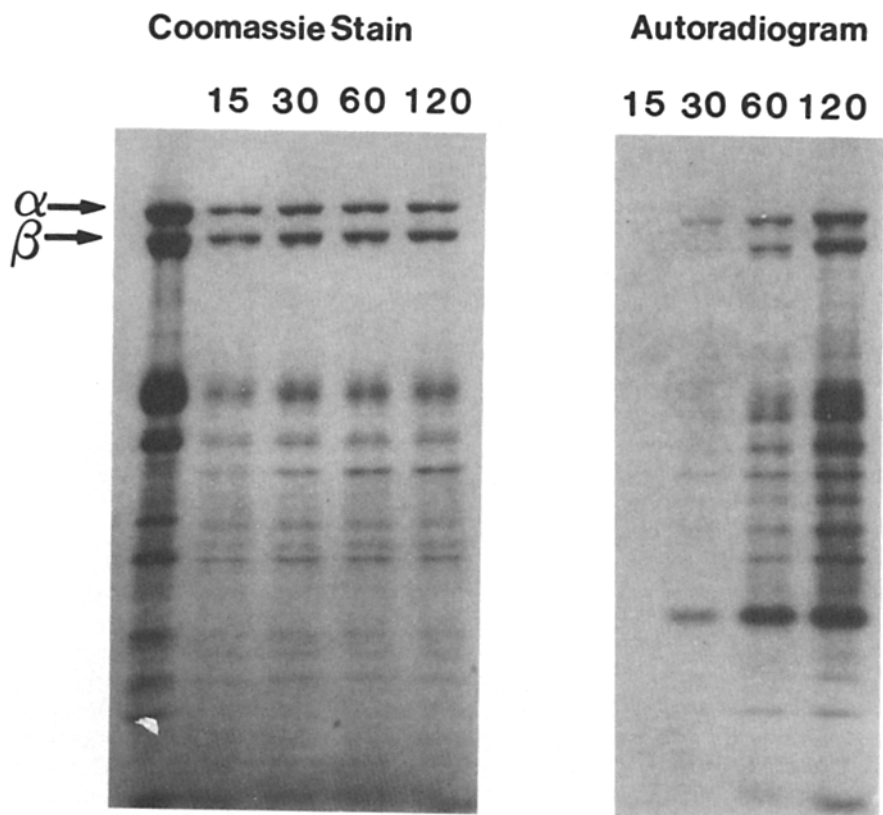15 30 60 120

Autoradiogram

15 30 60 120

α →
β →

Figure 1. SDS-PAGE of plasma membranes isolated from rat nucleated red cell precursors and the corresponding autoradiogram. Nucleated red cell precursors from spleens of phenylhydrazine anemic rats were labeled with [^{35}S]methionine for the times indicated (15–120 min) before isolating plasma membranes. Aliquots of each membrane sample were separated on a 3.5–12% polyacrylamide gel according to the gel system of Laemmli (14). The gel was processed for fluorography by soaking in En^{3}Hance, and was exposed to x-ray film for 4 d. The lane on the extreme left shows Coomassie-stained membrane proteins from rat erythrocytes.

Coomassie Stain

30 60 120

Autoradiogram

30 60 120
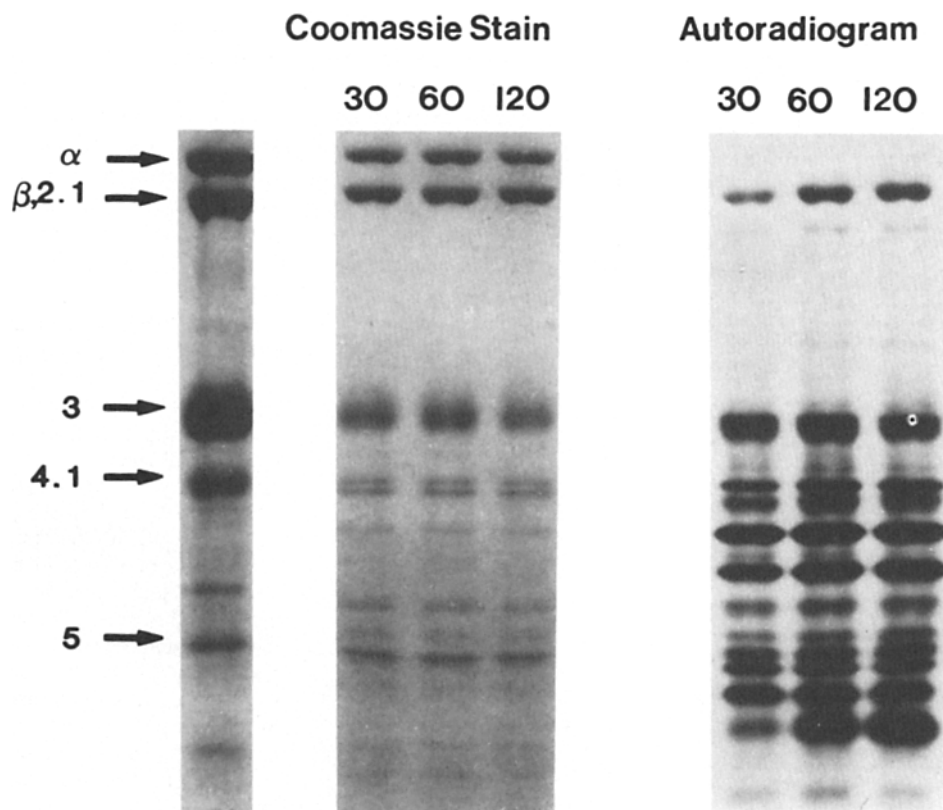

α →
β,2.1 →

3 →
4.1 →

5 →

Figure 2. SDS-PAGE of plasma membranes isolated from rat reticulocytes and the corresponding autoradiogram. Reticulocytes isolated from the peripheral blood of phenylhydrazine anemic rats were labeled with [^{35}S]methionine for 30, 60, and 120 min before isolating plasma membranes. The membrane samples were examined on a 3.5–12% polyacrylamide gel according to Laemmli (14). The gel was processed for fluorography and was exposed to x-ray film for 7 d. Note the absence of spectrin and the presence of a radiolabeled band in the β, 2.1 region in the autoradiogram.
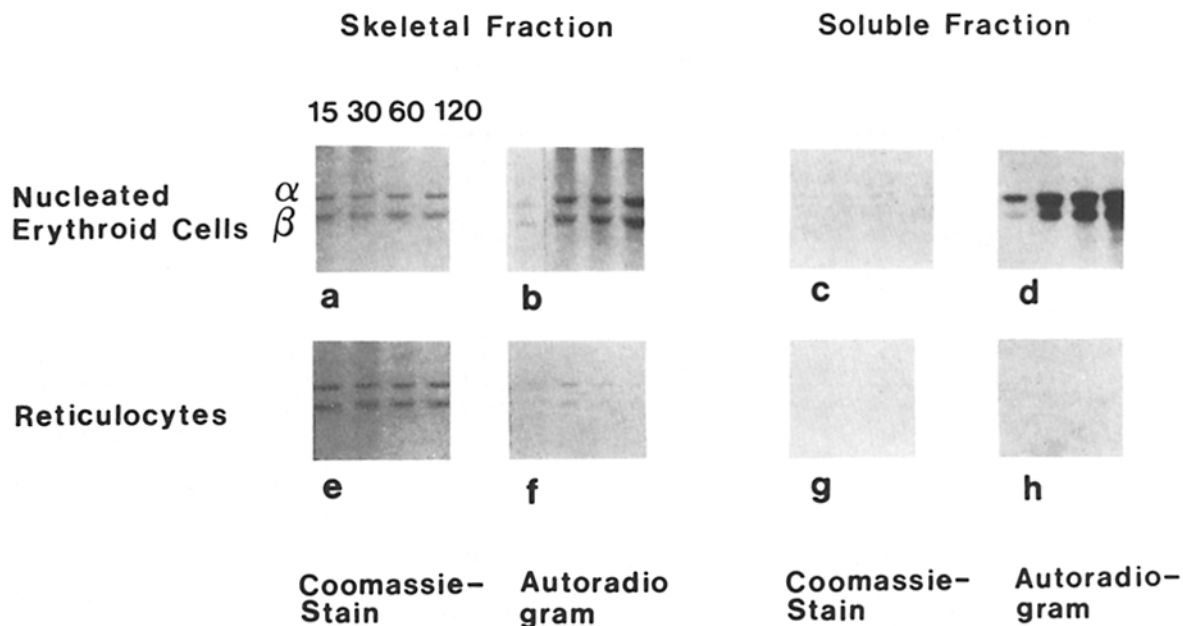
Nucleated Erythroid Cells α β

15 30 60 120

a   b   c   d

Reticulocytes

e   f   g   h

Coomassie- Stain   Autoradio gram   Coomassie- Stain   Autoradio- gram

Figure 3. Immunoprecipitation of α, β-spectrin from soluble and membrane-skeletal fractions of rat nucleated erythroid cells and reticulocytes labeled with [^{35}S]methionine. Rat nucleated red cells and reticulocytes were labeled with [^{35}S]methionine for the times indicated (15–120 min), lysed in a buffer containing Triton X-100, and separated into a skeletal (*SK*) and a soluble (*S*) fraction. Aliquots of each sample were immunoprecipitated with affinity-purified anti–spectrin antibodies and the immunoprecipitates were separated on a 3.5–12% polyacrylamide gel. The gel was processed for fluorography and was exposed to x-ray film for 6 d.

cell precursors, newly synthesized α- and β-spectrins are present in the soluble fraction with about seven-fold excess of α-spectrin over β-spectrin, and that both subunits are associated in equimolar amounts in the membrane-skeletal fraction. In order to examine the stability of newly synthesized α- and β-spectrin polypeptides in the membrane-skeletal and soluble fractions, we performed a pulse-chase experiment. Nucleated erythroid cells isolated from hemopoietic spleens of rats were labeled with [^{35}S]methionine for 15 min and chased for various lengths of time in a medium containing unlabeled methionine. Equal numbers of cells for each time point were lysed in a buffer containing Triton

X-100 and separated into insoluble and soluble fractions. Equal volumes of these two fractions were then immunoprecipitated with anti–spectrin antibodies as described in Materials and Methods. As shown in Figs. 5 and 6, the total amount of radioactivity incorporation in both α- and β-spectrin increases for at least 30 min after the beginning of the chase and then reaches a plateau which remains constant up to 120 min of chase. However, the amount of radioactivity in the soluble fraction of α- and β-spectrin is greatest after 5 min of chase and subsequently declines, suggesting that both polypeptides turnover coordinately and extremely rapidly.
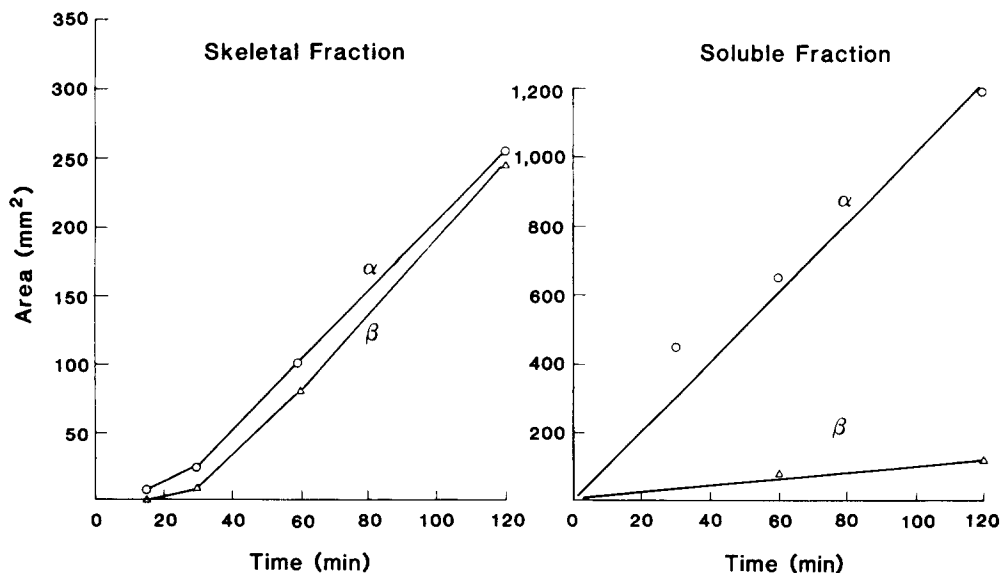

Figure 4. Synthesis and assembly of α- and β-spectrin in rat nucleated red cell precursors. Skeletal and soluble fractions of [^{35}S]methionine-labeled nucleated erythroid precursors were immunoprecipitated as described in Fig. 3. The resulting autoradiograms were scanned and the area under each peak was integrated. The area on the left-hand axis represents the relative area. The results show that α-spectrin is synthesized in excess of β-spectrin, yet both subunits assemble in equimolar amounts on the membrane.

Coomasie Stain Autoradiogram

0 5 10 30 60 120

Skeletal α
Fraction β

A   B

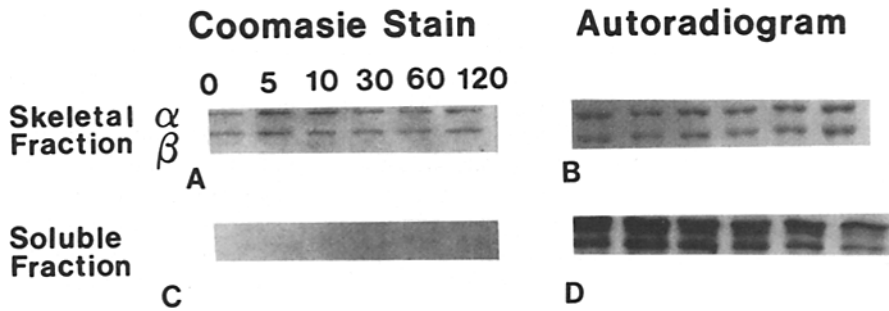Soluble
Fraction

C   D

Figure 5. Immunoprecipitation of spectrin from rat nucleated red cell precursors labeled with [35S]methionine and chased with unlabeled methionine. Rat nucleated red cell precursors were labeled with [35S]methionine for 15 min. Thereafter, unlabeled methionine was added to equal amounts of cells and incubation was continued for different time periods (0–120 min). The cells were lysed in lysis buffer and separated into soluble and insoluble membrane-skeletal fractions, and urea and 2-mercaptoethanol were added to each sample. Equal volumes of each sample were immunoprecipitated for spectrin and the immunoprecipitates were separated on a 3.5–12% polyacrylamide gradient gel. En³Hance-impregnated gel was exposed to x-ray film for 6 d.

Synthesis of Proteins 2.1 and 4.1

To examine the synthesis of proteins 2.1 and 4.1 with respect to α- and β-spectrin, rat nucleated red cell precursors and reticulocytes were pulse-labeled with [35S]methionine for 60 min before isolating plasma membranes which were then examined on SDS-polyacrylamide gels according to the buffer system of Fairbanks et al. (8). As can be seen in Fig. 7, although the assembly of spectrin (and therefore synthesis) is ceased in these reticulocytes newly synthesized proteins in the bands 2.1 and 4.1 region are being incorporated in the membrane both in nucleated red cell precursors and reticulocytes. The bands labeled 2.1 and 4.1 are the protein bands comigrating with proteins 2.1 and 4.1 on SDS gels and the immunoblots. Currently, work is in progress in our laboratory to identify these proteins by immunoprecipitation using polyclonal affinity-purified anti–2.1 and anti–4.1 antibodies, and also to determine their turnover rates in the soluble fractions and the insoluble membrane skeletal fractions.

Discussion

The results presented in this paper show that rat nucleated red cell precursors actively synthesize spectrin and that the synthesis of both polypeptides of spectrin is completed before the reticulocyte matures into erythrocyte. At the reticu-

locyte stage, the synthesis of spectrin α- and β-chains is markedly decreased (Figs. 3 and 7).

In rat nucleated red cell precursors, both α- and β-spectrin are detected exclusively in the Triton X-100–insoluble fraction as shown by Coomassie-stained gels. However, continuous labeling of cells with [35S]methionine followed by immunoprecipitation of spectrin, revealed that newly synthesized α- and β-spectrin are partitioned into both the Triton X-100–soluble and –insoluble fractions. In addition, the newly synthesized α-spectrin that is present in the soluble fraction is in large excess relative to β-spectrin. In contrast, the membrane-skeletal fraction contains equimolar amounts of spectrin α- and β-chain polypeptides. It should be pointed out, that the fraction referred to as membrane skeletal fraction contained both the membrane skeletons and cytoskeletons. However, similar immunoprecipitations of Triton extracts of plasma membranes (rather than whole cells) revealed a stoichiometric assembly of the newly synthesized spectrin α- and β-chains.

To investigate the ratio of newly synthesized α- and β-spectrin, we labeled nucleated red cell precursors for different time periods with [35S]methionine, lysed the cells in presence of Triton X-100 and immunoprecipitated α, β-spectrin from both the membrane-skeletal fraction and the soluble fraction, with specific antibodies. Quantification of α- and
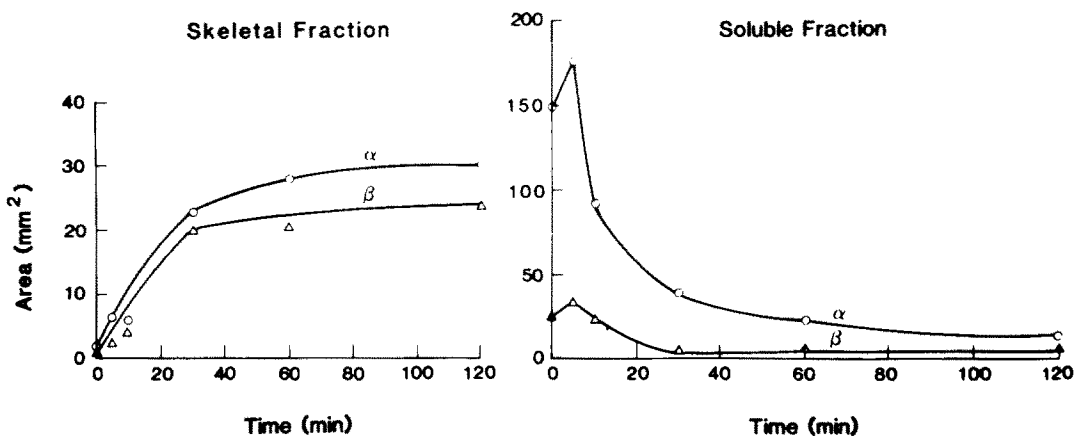
Figure 6. Turnover of spectrin. The autoradiograms of the same pulse-chase experiment described in Fig. 5 were scanned, and the area under each spectrin peak was integrated. The figure shows that the newly synthesized α- and β-spectrin are assembled in equimolar amounts in the membrane skeleton and are stable, whereas the soluble fraction contains an excess of α-spectrin over β-spectrin, both of which are rapidly degraded during incubation.

Coomassie Stain   Autoradiogram
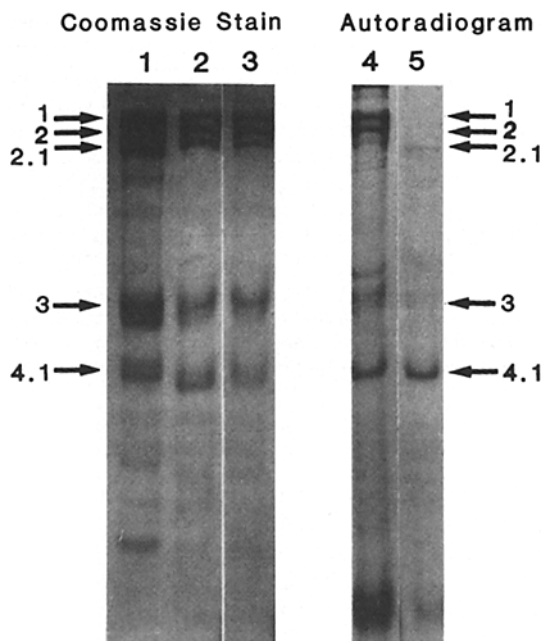   1   2   3   4   5

Figure 7. SDS-PAGE of plasma membranes isolated from rat nucleated erythroid precursors and reticulocytes. Rat nucleated red cell precursors and reticulocytes were labeled with [^{35}S]methionine for 60 min before isolating plasma membranes which were then examined on 5.6% SDS polyacrylamide gels according to the buffer system of Fairbanks et al. (8). The figure shows rat erythrocyte ghosts in lane *1*, plasma membranes from nucleated red cell precursors in lane *2* and the corresponding autoradiogram in lane *4*, plasma membranes from reticulocytes in lane *3* and the corresponding autoradiogram in lane *5*.

β-spectrin from the fluorogram (Fig. 4) showed that the ratio of newly synthesized α-spectrin to β-spectrin is ∼4.4:1 (the total amount of α-spectrin and β-spectrin synthesized at each time point was calculated from the sum of the soluble and skeletal fractions). Thus our results on the synthesis and assembly of spectrin in rat nucleated red cell precursors are similar to the results of Lazarides and co-workers in chicken embryonic erythroid cells (3), suggesting that there are some similarities between the avian and mammalian erythroid membrane skeleton biogenesis. By contrast, Friend erythroleukemia cells treated with dimethyl sulphoxide, to promote erythroid differentiation, synthesize ∼15–30% more β-spectrin that α-spectrin (19), suggesting an alternate pattern of spectrin synthesis and assembly in these cells.

Our recent studies in human reticuloctyes show that the extent of spectrin synthesis is dependent on the age of these cells as determined by their RNA contents. Early reticulocytes containing large amounts of RNA synthesize both spectrin α- and β-chains, whereas the late reticulocytes containing only small amounts of RNA do not synthesize spectrin α- or β-chains; yet the proteins 2.1 and 4.1 are still being synthesized (unpublished results). Rat reticulocytes as isolated here on continuous Percoll gradients contained a heterogeneous mixture of early and late reticulocytes. Hence, based on our observations in human reticulocytes, such heterogeneity can account for a slight variation of results as is observed here in Figures 2, 3, and 7.

In human erythrocytes, the association of spectrin with the plasma membrane is mediated by proteins 2.1 and 4.1, both

of which in turn bind to integral membrane proteins band 3 and the family of glycophorins, respectively. To determine the sequence of protein assembly in mammalian erythroid cells, we examined the synthesis and assembly of proteins 2.1 and 4.1 in relation to that of spectrin. Our results show that the synthesis and assembly of proteins 2.1 and 4.1 on the membrane is continued to the stage where spectrin is no longer being synthesized. These results are somewhat different from the reported sequence of protein assembly in chicken embryonic erythroid cells (18). In the latter system, sequential binding of ankyrin to the anion transporter and α, β-spectrin onto ankyrin results in the stabilization of the assembled complex. Since in mature mammalian erythrocytes spectrin is bound to band 2.1, which, in turn, is bound to band 3, it is not clear why protein 2.1 is still being incorporated in the membrane after spectrin synthesis is completed. Furthermore, from the previous work of Geiduschek and Singer (9) in mouse erythroid cells, it is unclear how spectrin is bound to the membrane in early red cell precursors because under the conditions of their experiments, they observed redistributions of concanavalin A receptors and hence of band 3 (since band 3 is the major concanavalin A–binding protein in the mouse as well as human erythrocyte membrane [20]) but no corresponding redistributions of spectrin. We propose two possible explanations for the results obtained by us in mammalian erythroid precursors: (a) in nucleated red cell precursors, the proteins 2.1 and 4.1 are rapidly degraded by an abundance of active proteolytic enzymes present in these cells. Thus, to replenish the lost proteins 2.1 and 4.1, these are still being synthesized in reticulocytes which contain relatively lower levels of active proteases (10); or (b) the attachment of newly synthesized spectrin to the plasma membrane is mediated by interactions other than spectrin–2.1 and spectrin–4.1, before full amounts of proteins 2.1 and 4.1 are synthesized and incorporated in the membrane and that both proteins 2.1 and 4.1 are involved in ultimate stabilization of the membrane skeleton as results suggested to be the case for protein 4.1 (22).

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