Assembly of Protein 4.1 during Chicken Erythroid Differentiation

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Abstract. Protein 4.1 is a peripheral membrane protein that strengthens the actin-spectrin based membrane skeleton of the red blood cell and also serves to attach this structure to the plasma membrane. In avian erythrocytes it exists as a family of closely related polypeptides that are differentially expressed during erythropoiesis. We have analyzed the synthesis and assembly onto the membrane skeleton of protein 4.1 and in this paper we show that its assembly is extremely rapid and highly efficient since >95% of the molecules synthesized are assembled in <1 min. The remaining minor fraction of unassembled protein 4.1 differs kinetically and is either degraded or assembled with slower kinetics. All protein 4.1 variants exhibit a similar kinetic behavior irrespective of the stage of erythroid differentiation. Thus, the amount and the variants ratio of protein 4.1 assembled are determined largely at the transcriptional or at the translational level and not posttranslationally. During erythroid terminal differentiation the molar amounts of protein 4.1 and spectrin assembled change. In postmitotic cells, as compared with proliferative cells, far more protein 4.1 than spectrin is assembled onto the membrane-skeleton. This modulation may permit the assembly of an initially flexible membrane skeleton in mitotic erythroid cells. As cells become postmitotic and undergo the final steps of maturation the membrane skeleton may be gradually stabilized by the assembly of protein 4.1.

A PROTEIN network is attached to the cytoplasmic surface of the erythrocyte plasma membrane which influences cell shape and deformability and the distribution of integral membrane proteins. This network, the membrane skeleton, is based on actin oligomers bound and cross-linked by spectrin tetramers (for reviews see references 2, 6, and 20). Protein 4.1 forms a ternary complex with spectrin and actin and thereby strongly enhances and stabilizes their association (11, 12, 29). It may also confer Ca^{2+}-sensitivity on the actin-spectrin interaction (12). Anchorage of the network to the bilayer is mediated by ankyrin, which provides a link between spectrin and an integral membrane protein, the anion transporter (reviewed in references 2, 6, and 20). In addition, protein 4.1 functions in the membrane attachment of the actin-spectrin network: It binds with high affinity to the cytoplasmic domain of glycophorin (1) and associates with phosphatidylserine-containing liposomes (26). Protein 4.1 may also form a complex with the cytoplasmic domain of the anion transporter (24). Since the binding to the anion transporter appears to exclude a concurrent interaction of protein 4.1 with spectrin-actin complexes, switching between its membrane binding sites may be a means to reversibly alter the attachment of the membrane skeleton to the plasma membrane.

Protein 4.1, spectrin, ankyrin, and the anion transporter also have been identified in chicken erythrocytes (3, 9, 13–16, 23, 25, 30). The chicken system offers the advantage that embryonic erythroid cells can be obtained easily. This greatly facilitates studies of the biogenesis of the membrane skeleton during erythropoiesis. Chicken protein 4.1 is composed of a family of closely related polypeptides of a lower molecular weight group (77K, 87K, 100K, and 115K) and a higher molecular weight group (145K, 150K, 160K, and 175K) (13, 14). The higher molecular weight variants are present in considerably lesser amounts than the lower molecular weight variants. Individual protein 4.1 variants were shown to be differentially expressed during maturation of erythroid cells (14). In proliferative erythroid cells the lower molecular weight variants of both groups are present. As the cells mature and become postmitotic the upper molecular weight variants of both groups gradually accumulate. This appearance of new variants during terminal differentiation is observed in both the primitive erythroid cell series, which is present in early embryos, and the definitive cell series, which appears later during embryogenesis and persists throughout life.

In this study we have analyzed the synthesis and assembly of protein 4.1 onto the membrane skeleton. We demonstrate that the assembly process is very rapid and efficient since only a minor fraction of the molecules synthesized is not assembled. The synthesis and assembly kinetics of the different protein 4.1 variants are very similar. However, during erythroid terminal differentiation the amount of protein 4.1 syn-

thesized and assembled varies relative to the amount of spectrin assembled. In proliferative cells the molar amount of protein 4.1 assembled is substantially less than the amount of spectrin assembled. As cells become postmitotic and express the larger protein 4.1 variants of each group the molar ratio of protein 4.1 and spectrin assembled changes gradually until more protein 4.1 is assembled than spectrin. This regulation of assembly may ensure a relatively flexible membrane skeleton in proliferative cells but lead to a stabilization of this structure during the final stages of erythroid terminal differentiation.

Materials and Methods

Preparation of Cells

Circulating chicken embryo erythroid cells were isolated from fertilized eggs after different times of incubation. The embryos were removed from the eggs and bled into minimal essential medium (MEM,[1] Gibco, Grand Island, NY). The diluted blood was filtered through glass wool, washed five times in MEM or MEM minus methionine (for labeling with methionine), and any buffy coat was removed. All steps were performed at room temperature with medium prewarmed to 37°C.

Size Fractionation of Cells by Centrifugal Elutriation

Erythroid cells from 9-d-old embryos were resuspended in MEM (9×10^8 cells in 10 ml MEM). They were loaded with a flow rate of 16 ml/min into a Beckman JE-6B elutriator rotor (Beckman Instruments Inc., Palo Alto, CA) spinning at 2,860 rpm and containing cold Hank's buffered saline solution minus Mg^{2+}, Ca^{2+} supplemented with 0.25% bovine serum albumin. The cells were sequentially eluted with flow rates of 20, 30, 36, and 46 ml/min (fractions A to D, respectively). Fractions A and B contained definitive cells, fractions C and D contained primitive cells as has been shown previously (14). After elutriation the cells were pelleted, washed once in MEM, and labeled with ^{14}C-amino acids.

Labeling of Cells

For continuous labeling with [^{35}S]methionine the pelleted cells were resuspended in 10 vol prewarmed MEM minus methionine and 0.1 to 1 mCi [^{35}S]-methionine (specific activity, 7,800 Ci/mmol; Amersham Corp., Arlington Heights, IL) was added. Cells were incubated at 37°C for 2 h, pelleted in a clinical centrifuge, and washed once with cold MEM and fractionated (see below). For pulse-chase labeling pelleted cells were resuspended in 4 vol prewarmed MEM-methionine containing 1.5 mCi [^{35}S]methionine. They were then pulse labeled for 3 min at 37°C. The cells were pelleted for 1 min in a clinical centrifuge, resuspended in 20 vol MEM supplemented with 0.5 mM L-methionine, and incubated at 37°C (chase). In some experiments cells were chased in the presence of a protein synthesis inhibitor (50 μM emetine). Samples were taken immediately after the resuspension of the cells in chase medium (pulse sample) and at different times thereafter (chase samples). The cells were pelleted for 1 min in the cold and fractionated. For continuous labeling with amino acids the pelleted cells were resuspended in 50 vol Earle's balanced salt solution (Gibco) supplemented with 1% MEM, 1% nonessential amino acids (Gibco), and from 50 to 150 μCi of a L-^{14}C-amino acid mixture (specific activity, 55 mCi [maton carbon, New England Nuclear, Boston, MA]). Cells were incubated at 37°C for 4 h, pelleted thereafter, washed once in cold MEM, and then fractionated.

Cell Fractionation

Pelleted erythroid cells were resuspended in 20 vol 5 mM Hepes, pH 7.3, 5 mM $MgCl_2$, 5 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM aprotinin (Boehringer Mannheim Biochemicals, Indianapolis, IN), 0.1 mM leupeptin (Boehringer Mannheim Biochemicals), and lysed on ice for from 3 to 5 min. The structures were pelleted in a clinical centrifuge in the cold and washed once. Both supernatants were combined (hypotonically soluble extract). The structures were either dissolved in SDS sample buffer (125 mM Tris, pH 6.8, 1% SDS, 25 mM dithiothreitol), and the DNA was sheared by brief sonication. Alternatively they were extracted with 10 vol 10 M urea in one-half of the lysis buffer for 15 min at 4°C. The suspension was centrifuged for 5 min in an Eppendorf centrifuge in the cold, and the supernatant, which quantitatively contained protein 4.1 and spectrin, was removed.

Immunoprecipitation, SDS PAGE, and Quantitation

For immunoprecipitation, extracts were diluted with from 10 to 20 vol of 100 mM Tris, pH 9.0, 200 mM NaCl, 5 mM EDTA, 5 mM EGTA, 5 mM dithiothreitol, 1% Triton X-100, 0.1% SDS, 1% sodium deoxycholate, 1 mM aprotinin, 1 mM phenylmethylsulfonyl fluoride, 0.1 mM leupeptin. Chicken erythrocyte protein 4.1 antiserum (13) or β-spectrin antiserum (22) was added, and the immunoprecipitation was as described (28). Supernatants of the precipitation were reprecipitated with the same antiserum to ensure a quantitative precipitation. Precipitated proteins were eluted by heating in SDS sample buffer and loaded onto 12.5% SDS polyacrylamide gels (25). After electrophoresis gels were stained with Coomassie Brilliant Blue, destained, and prepared for fluorography (7, 17). Fluorograms were scanned with a Quick-Scan (Helena Laboratories, Beaumont, TX), and the area under the peaks was integrated or cut out and weighed.

Results

Separation of Assembled and Unassembled Protein 4.1

To analyze the assembly of protein 4.1 onto the membrane skeleton a cell fractionation method was required that allowed for a separation of assembled and unassembled polypeptides. In previous studies of spectrin and ankyrin, molecules solubilized during cell lysis with buffers containing nonionic detergents were operationally defined as unassembled, and the insoluble fraction was designated as the membrane skeleton (5, 21). During pulse-chase experiments unassembled spectrin and ankyrin polypeptides turned over, but polypeptides assembled onto the membrane skeleton were stable (5, 21). Our initial studies with protein 4.1 using this fractionation procedure, however, showed some variability among different experiments with regard to both the amount of protein 4.1 rendered soluble and its metabolic stability. More detailed investigations indicated that protein 4.1 could be solubilized from chicken erythroid ghosts (i.e., the membrane skeleton) by nonionic detergents (e.g., Triton X-100, Nonidet P-40, Tween 20), high salt concentration (2 M NaCl), or mechanical forces. Since the amount extracted was dependent on the length of the extraction time and additional protein 4.1 was solubilized if the extractions were repeated (data not shown), no distinct subpopulations of protein 4.1 were obtained under these conditions. In contrast, hypotonic lysis of chicken red blood cells reproducibly solubilized a bona fide subpopulation of protein 4.1, as indicated by the facts that two extractions were exhaustive and the remaining material could not be removed when the extractions were repeated (data not shown). Under these conditions the stable association of the insoluble protein 4.1 with the membrane skeleton was not detectably decreased by reducing agents (dithiothreitol), chelators (EDTA and EGTA), the final protein concentration in the extraction buffer, the ratio of buffer and to extracted cells, or the length of the extraction time. Extracted protein 4.1 was soluble by the criteria that it could not be pelleted by centrifugation at 150,000 *g* (30 min) and it ran as monomers on glycerol gradients (data not shown).

We next examined the metabolic behavior of the hypotonically soluble and insoluble protein 4.1 subpopulations during a pulse-chase experiment. Erythroid cells from 15-d-old embryos were pulse-labeled with [^{35}S]methionine for 3 min, pelleted, and resuspended in medium containing an excess of unlabeled methionine. After the pulse and after different times of chase aliquots were removed and hypotonically lysed, and

[1] *Abbreviation used in this paper:* MEM, minimum essential medium.

the residual structures were solubilized. The hypotonically soluble and the membrane skeletal extracts were analyzed for protein 4.1 by immunoprecipitation. To obtain a sufficient signal the hypotonically soluble protein 4.1 was precipitated from 10 times as many cells as the insoluble protein 4.1. A fluorogram from such an experiment is shown in Fig. 1. As has been shown (14), the predominant variants synthesized at this time were the 100K and 115K polypeptides. Smaller amounts of the 87K polypeptide were also synthesized. The higher molecular weight variants were synthesized in small amounts and only the 160K and 175K polypeptides were visible at this exposure. The maximum amount of total (unassembled plus assembled) protein 4.1 synthesized was reached after only 5 to 10 min of chase. After this time the amount of protein 4.1 in the unextracted fractions remained constant during the 4 h chase period. The stability of protein 4.1 in the unextracted fraction indicated that these molecules were assembled onto the membrane skeleton. In contrast hypotonically soluble, unassembled protein 4.1 turned over (Fig. 1, lanes *7–12*).

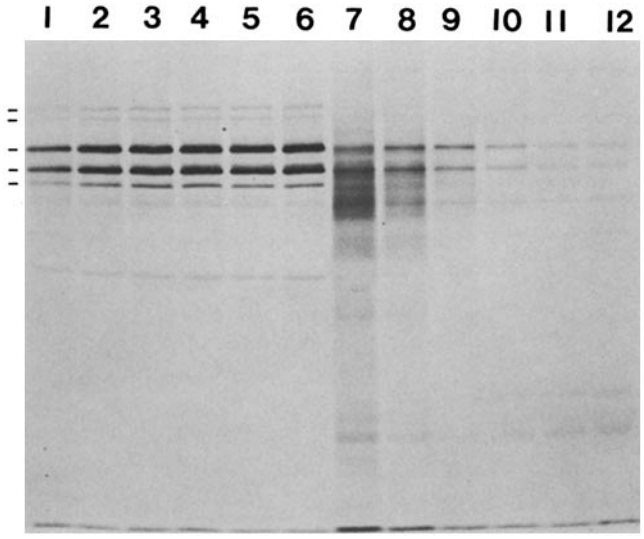

Figure 1. Metabolic behavior of assembled and unassembled protein 4.1 in erythroid cells from 15-d-old embryos. Cells were pulse-chase labeled and fractionated in a hypotonically soluble (unassembled) fraction (lanes *7–12*) and the corresponding insoluble (membrane skeletal) fraction (lanes *1–6*) at different times as described in the text. Protein 4.1 was precipitated from both fractions using hypotonic extracts from 10 times as many cells as the membrane skeletal fraction. The immunoprecipitates were separated on 12.5% SDS polyacrylamide gels and visualized by fluorography. Lanes *1* and *7* pulse. Chase of: 5 min (lanes *2* and *8*), 10 min (lanes *3* and *9*), 30 min (lanes *4* and *10*), 75 min (lanes *5* and *11*), and 180 min (lanes *6* and *12*). Bars designate protein 4.1 variants: 87K, 100K, 115K, 160K, and 175K.

Detection of Nascent Protein 4.1 Polypeptide Chains

From the pulse-labeled hypotonic extract (Fig. 1, lane *7*) many polypeptides were precipitated that formed a smear on the gel in the molecular weight region below the major protein 4.1 variants (115K and 100K). These polypeptides were less apparent after longer pulse-labeling times (data not shown) and disappeared during a short (10 min) chase (Fig. 1, lanes *7–9*). However, they remained stable for >1 h if elongation of polypeptide synthesis was blocked with 50 μM emetine (data not shown). We conclude that these polypeptides are protein 4.1 nascent chains. After longer exposure times polypeptides with the same characteristics also could be detected below the minor protein 4.1 variants (160K and 170K). They were much less abundant than the lower molecular weight nascent chains, as were the mature 160K and 170K polypeptides, suggesting that the larger molecular weight cluster of nascent chains are precursors only for the larger protein 4.1 variants. Generation of the lower molecular weight protein 4.1 variants from the higher molecular weight variants or even larger polypeptides by posttranslational proteolytic cleavage therefore appears unlikely. The nascent protein 4.1 polypeptides were recovered quantitatively in the soluble fraction not only after hypotonic lysis but also after detergent lysis of cells in an isotonic buffer. In this respect they differ in their extraction properties from α-spectrin nascent chains which were found mostly in the cytoskeletal fraction (4). The structural basis for this difference remains to be investigated.

Protein 4.1 Is Assembled onto the Membrane Skeleton Immediately after Its Synthesis

To determine the kinetics of assembly and degradation of protein 4.1, the data from the experiment shown in Fig. 1 were quantitated (Table I*A*). Interestingly, immediately after

Table I. Kinetic Analysis of Protein 4.1 Synthesis and Assembly

A. Erythroid cells from 15-d-old Embryos						
Subcellular fraction (pulse then chase [*min*])		5	10	30	75	180
Unassembled membrane skeleton (amount of protein 4.1 [*arbitrary units*])	606 13,856	636 25,789	667 32,037	321 33,390	168 28,682	222 32,337
Unassembled membrane skeleton (*% total protein 4.1*)	4.2 95.8	2.4 97.6	2.0 98.0	1.0 99.0	0.6 99.4	0.7 99.3
B. Erythroid cells from 5-d-old Embryos						
Subcellular fraction (pulse then chase [*min*])		6	11	30	74	180
Unassembled membrane skeleton (amount of protein 4.1 [*arbitrary units*])	94.3 3,149	105.4 5,668	82.9 5,777	29.5 6,616	— 4,776	— 4,228
Unassembled membrane skeleton (*% total protein 4.1*)	2.9 97.1	1.8 98.2	1.4 98.6	0.4 99.6	0 100	0 100

(*A*) For erythroid cells from 15-d-old embryos, the fluorogram shown in Fig. 1 was scanned, and the peak areas were integrated as described in Materials and Methods. The values obtained for the individual protein 4.1 polypeptides were added. Nascent protein 4.1 polypeptide chains were not considered. (*B*) A similar fluorogram for erythroid cells from 5-d-old embryos was quantitated for the data presented.

the pulse only ~4% of the mature 4.1 polypeptides were found in the unassembled fraction. About 96% of protein 4.1 was already assembled after this short time (5 min including incubation, centrifugation, and resuspension of cells). The amount of assembled protein 4.1 increased for the first 5 min by almost the same factor as during the pulse; then assembly of labeled molecules slowed to reach a plateau at ~10 min. We attribute this increase in assembled material after the end of the pulse-labeling to the assembly of polypeptide chains that were completed only during the chase period. The small amount of unassembled protein 4.1 present after the pulse was by far insufficient to explain the assembly during the initial chase period, demonstrating that the vast majority of protein 4.1 is assembled extremely rapidly after completion of synthesis. The half-life of a newly synthesized precursor molecule must be <1 min since 96% of the newly synthesized molecules is assembled after 5 min of continuous labeling.

We have estimated the turnover of unassembled protein 4.1 from the 10-min time point onwards since the amount of labeled material increased during the first 10 min of chase. Such a calculation yields a half-life of 15 to 20 min, considerably longer than the half-life calculated above for a presumed soluble assembly precursor. Therefore, two populations of newly synthesized protein 4.1 with different kinetic behavior exist: a population that is assembled either cotranslationally or posttranslationally with a very short half-life; and a population with a much longer half-life that may be either assembled differently or remain unassembled and be degraded.

Quantitation of the individual protein 4.1 variants shows no significant difference in their synthesis, assembly, or degradation kinetics (data not shown). In addition, the ratio of the different 4.1 polypeptides remains constant during the chase time in both the assembled and unassembled fraction. Only the 4.1 polypeptides precipitated from the hypotonic extract after the short pulses (Fig. 1, lane *7*) appear to have a different ratio. We attribute this to the nascent chains present that contribute to the amount of the mature 100K variant. This conclusion is supported by experiments with amino acid analogues. After incubation of cells with the valine analogue L-threo-α-amino-3-chlorobutyric acid and the arginine analogue canavanine the level of unassembled protein 4.1 is increased considerably, probably because assembly is reduced. As a consequence nascent chains are much less prominent. Under these conditions the same ratio of protein 4.1 polypeptides is obtained in all unassembled and assembled fractions (data not shown).

Since there is a change in the protein 4.1 variants expressed during erythropoiesis (14), it was important to analyze also the assembly kinetics of the "early" protein 4.1 variants, which can be done best using erythroid cells from 5-d-old embryos. The variants synthesized at this time are primarily the 87K polypeptide, less of the 77K polypeptide, and very little of the 100K polypeptide and the corresponding higher molecular weight variants (145K, 150K, 160K) in the same ratio as the polypeptides of the lower molecular weight group (see also Fig. 2, lane *1*). It can be seen from Table I *B* that the maximum amount of labeled total protein 4.1 was reached after ~5 to 10 min of chase. The half-life of the unassembled polypeptides was ~15 min, slightly shorter than in 15-d erythroid cells. About 97% of mature protein 4.1 was assembled after the pulse-labeling, whereas the assembly reached a plateau after ~6 min of chase. No change in the ratio of different protein 4.1 polypeptides was detected during the experiment. We conclude that there is no major difference in the assembly kinetics of protein 4.1 in 5- and 15-d erythroid cells.

Protein 4.1 Assembly onto the Membrane Skeleton Increases Relative to β-Spectrin Assembly during Erythroid Terminal Differentiation

Aside from a determination of the kinetics, our understanding of protein 4.1 assembly also requires knowledge of the amount of protein 4.1 assembled relative to the molecules it interacts with. We therefore determined the ratio of protein 4.1 and β-spectrin assembled onto the membrane skeleton in erythroid cells from 5- and 14-d-old chicken embryos. Erythroid cells were labeled with a ^{14}C-amino acid mixture for 4 h, hypotonically lysed, and the cytoskeletal fractions were solubilized with urea. From these fractions protein 4.1 and β-spectrin were quantitatively immunoprecipitated and separated by SDS PAGE. The corresponding fluorographs are shown in Fig. 2. The different protein 4.1 variants synthesized in erythroid cells from 5- and 14-d-old embryos are seen in this figure (Fig. 2, lanes *1* and *3*). The fluorograms were scanned and the peak areas were determined. These values and the ratios of protein 4.1 to β-spectrin are given in Table II. The ratios of protein 4.1 to β-spectrin assembled onto the membrane skeleton clearly were different in erythroid cells from 5- and 14-d-old embryos. Proliferative primitive cells (5 d) assembled (and therefore synthesized) about five to six times less protein 4.1 relative to β-spectrin than did postmitotic definitive cells (14 d). The interpretation of this result is somewhat complicated since the cells analyzed belong to two different erythroid cell series (8). Differences in the relative amounts of protein 4.1 assembled therefore may be due to inherent properties of each cell series. Alternatively, they may reflect a true differentiation-specific phenomenon if erythroid cells of both series assemble more protein 4.1 relative to β-spectrin as they terminally differentiate.

To distinguish between these two possibilities postmitotic (mature) cells of the primitive series and proliferative defini-

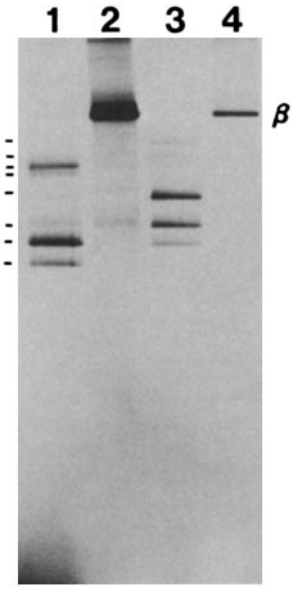

Figure 2. Analysis of protein 4.1 and β-spectrin assembled onto the membrane skeleton in erythroid cells from 5- and 14-d-old embryos. The cells were continuously labeled for 4 h with a ^{14}C-amino acid mixture, fractionated, and protein 4.1 or β-spectrin was immunoprecipitated from aliquots of the cytoskeletal fractions as described. The polypeptides were separated on 12.5% SDS polyacrylamide gels and visualized by fluorography. Lanes *1* and *2*; 5-d erythroid cells; lanes *3* and *4*, 14-d erythroid cells. Protein 4.1 variants are designated by bars: 77K, 87K, 100K, 115K, 145K, 150K, 160K, and 175K. β, β-spectrin.

Table II. Quantitative Analysis of the Amounts of Protein 4.1 and β-Spectrin Assembled During Different Stages of Erythropoiesis

	Relative amounts		Ratio of relative amounts of protein 4.1/β-spectrin	Molar ratio of protein 4.1/β-spectrin
	Protein 4.1	β-Spectrin		
	(*arbitrary units*)			
A. Erythroid cells				
Incubation time (*d*)	415	1,127	0.33	0.83
5	160	291	1.82	3.64
14				
B. Primitive and definitive erythroid cells,* from 9-d-old embryos				
Fraction				
A	116	343	0.34	0.86
B	95	131	0.72	1.58
C	153	185	0.83	1.83
D	515	386	1.33	2.66

Appropriate exposures of the fluorograms shown in Figs. 2 and 3 were scanned, and the peak areas were measured by weighing out the recording paper. The sum of all protein 4.1 variants in each fraction is given. Molar ratios were calculated using the apparent molecular weights of the different protein 4.1 variants expressed at different stages of erythroid development.

* Separated by centrifugal elutriation.

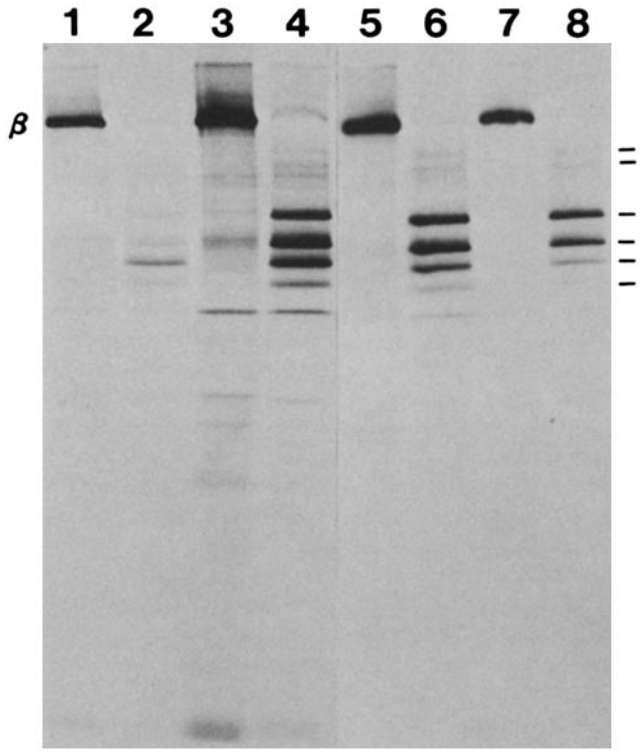

Figure 3. Protein 4.1 and β-spectrin assembled onto the membrane skeleton in primitive and definitive erythroid cells from 9-d-old embryos. Erythroid cells from embryos of 9 d of incubation were separated by centrifugal elutriation as described in Materials and Methods. Fractions were eluted with flow rates of 20 (A, lanes *1* and *2*), 30 (B, lanes *3* and *4*), 36 (C, lanes *5* and *6*), and 40 (D, lanes *7* and *8*) ml/min. Cells were labeled for 4 h with ^{14}C-amino acids, the cytoskeletal fractions were isolated, and protein 4.1 and β-spectrin were immunoprecipitated after solubilization of these fractions. The precipitated polypeptides were separated on 12.5% SDS polyacrylamide gels and visualized by fluorography. Bars designate protein 4.1 variants: 77K, 87K, 100K, 115K, 160K, 175K. β, β-spectrin.

tive erythroid cells had to be analyzed. A mixed population of both cell series is present in the circulation between 5 and 15 d of incubation. However, there are more definitive than primitive cells already in 9-d-old embryos, and after about the twelfth day of incubation the primitive cells constitute only a very minor fraction of all circulating erythroid cells (8). As primitive and definitive cells differ in their size we separated them by velocity sedimentation (14, 19) from a mixed population of erythroid cells derived from 9-d-old embryos. The cells were size-fractionated by centrifugal elutriation (14), and the fractions obtained were labeled for 4 h with a ^{14}C-amino acid mixture, fractionated, and analyzed for protein 4.1 and β-spectrin as described above. Fig. 3 shows a fluorogram with protein 4.1 and β-spectrin immunoprecipitated from all fractions. The two fractions eluting first (A, B; Fig. 3, lanes *1* and *2*, *3* and *4*) contained definitive cells at different stages of differentiation (14). The two fractions eluting thereafter (C, D; Fig. 3, lanes *5* and *6*, *7* and *8*) contained two sets of primitive erythroid cells (15). The relative amounts of protein 4.1 and β-spectrin as well as the ratio of both proteins were determined for all fractions and are given in Table II. The mitotic definitive cells (Table II*B*, fraction A; Fig. 3, lanes *1* and *2*) assemble protein 4.1 and β-spectrin onto the membrane skeleton in a ratio similar to the mitotic primitive erythroid cells (Fig. 2, lanes *1* and *2*; Table II*A*). As the definitive cells become postmitotic, indicated by the set of protein 4.1 variants expressed (Fig. 3, lanes *3* and *4*), relatively more protein 4.1 is assembled (Table II*B*, fraction B). In the earlier postmitotic primitive cells (Fig. 3, lanes *5* and *6*) that express slightly more of the larger protein 4.1 variants than the definitive cells of fraction B, the ratio of protein 4.1 to β-spectrin assembled is slightly higher (Table II*B*, fraction C). As maturation of primitive cells proceeds (Fig. 3, lanes *7* and *8*; Table II*B*, fraction D) even more protein 4.1 relative to β-spectrin is assembled, though the ratio of protein 4.1/β-spectrin is not as high as that obtained with 14-d definitive erythroid cells (Table II*A*). Protein 4.1 and β-spectrin may only be assembled in such a ratio in primitive cells at even later stages of embryogenesis.

Table II also gives the molar ratios in which protein 4.1 and β-spectrin are assembled that were calculated using the apparent molecular weights of the individual polypeptides. Therefore, the values obtained can be regarded only as approximations. They indicate that protein 4.1 is assembled in less than equimolar amounts as compared with β-spectrin in proliferative erythroid cells. However, the molar ratio of protein 4.1 to β-spectrin assembled is considerably larger than unity in postmitotic cells. Since protein biosynthetic activity decreases during erythroid differentiation, the large ratios obtained with erythroid cells from older embryos do not necessarily indicate that the total amount of protein 4.1 assembled throughout differentiation is larger than the amount of β-spectrin assembled. This is evidenced by the fact that the molar ratio of protein 4.1 to β-spectrin in adult erythrocytes is one (6; data not shown). The data presented indicate that the molar ratio of protein 4.1 to β-spectrin that is assembled onto the membrane-skeleton changes gradually as a function of the differentiation of both erythroid cell

series. Since we analyzed only cell populations we cannot exclude the possibility that a rapid change of this ratio occurs in individual cells as they become postmitotic. Asynchrony of the population may result in the overall appearance of a gradual alteration.

Discussion

In this study we have sought to analyze the mechanism of assembly of protein 4.1 and the mechanism by which the ratio of the variants to each other and to spectrin is achieved in erythroid cells during chicken embryo development. A prerequisite was to define extraction conditions for chicken erythroid cells that allowed for a separation of assembled and unassembled protein 4.1 molecules. A readily soluble protein 4.1 subpopulation could be obtained that was unassembled based on its extraction properties and its metabolic instability. The corresponding insoluble population, which was metabolically stable, was regarded as assembled onto the membrane skeleton. This designation is based on the available evidence which indicates that all or at least most of steady state (i.e., metabolically stable) protein 4.1 is associated with the membrane skeleton (13, 14).

The kinetic studies demonstrated that newly synthesized protein 4.1 is assembled extremely rapidly so that we cannot discriminate if it occurs co- or posttranslationally. Thus, during or shortly after synthesis a distinction must be made between protein 4.1 polypeptides which give rise to two subpopulations with different kinetic behavior and which channel the protein 4.1 polypeptides into either of two distinct pathways. One subpopulation assembles rapidly with a <1-min half-life of a presumed precursor molecule. The other subpopulation forms a readily soluble pool and exhibits a much slower turnover rate ($t_{1/2}$, 15 to 20 min). The mechanism or mechanisms by which newly synthesized protein 4.1 polypeptides are segregated into the two different pathways are not known at present. However it is apparent from the kinetic evidence presented that after its synthesis protein 4.1 does not simply enter a homogeneous soluble pool from which molecules are removed with a certain probability by either of two competing pathways, self-assembly or degradation. Instead a more sophisticated mechanism(s) appears to exist that probably requires the action of additional proteins.

During the process of protein 4.1 assembly no major regulation of the number of molecules assembled occurs since only a few percent of the molecules synthesized remain unassembled. The amount of protein 4.1 assembled therefore must be determined largely by the availability of protein 4.1 polypeptides. This is in contrast to the assembly of α- or β-spectrin and ankyrin, where the amount of each of these three polypeptides assembled was shown to be determined primarily at the posttranslational level (5, 21, 31). In this case it was postulated that the number of available receptor molecules (anion transporter) at the plasma membrane may limit the amount of spectrin and ankyrin which is assembled (18, 27).

Chicken protein 4.1 is encoded by a single gene in the haploid genome (Ngai, J., R. T. Moon, and E. Lazarides, manuscript in preparation). The variety observed on the polypeptide level thus must be generated during mRNA or protein synthesis or processing. In this context it should be noted that one polypeptide of the lower and one of the higher molecular weight cluster of protein 4.1 variants are expressed always together during erythroid development (e.g., the 87K and the 150K, or the 100K and the 160K polypeptides). This observation may suggest that the generation of the four protein 4.1 variant clusters during erythropoiesis is determined by one mechanism (on the RNA level) whereas the generation of a higher and a lower molecular weight variant within a given cluster occurs via a different mechanism (on the protein level). The kinetic behavior of the individual protein 4.1 variants gives no indication for a posttranslational processing involved in the generation of the variants (see also reference 14). All protein 4.1 polypeptides exhibit similar kinetics (synthesis, assembly, or degradation) irrespective of the stage of erythroid terminal differentiation. Consequently, the same ratio of protein 4.1 variants that was synthesized was assembled and maintained at the cytoskeleton or degraded. Furthermore, protein 4.1 nascent polypeptide chains of lower and higher molecular weight were found in a similar ratio as the full-length molecules. This indicates that the larger and the smaller protein 4.1 variants are synthesized separately and not generated posttranslationally from the same precursor molecule. However, this observation does not exclude limited cotranslational cleavage of the nascent polypeptide chains, which could give rise to the lower molecular weight polypeptides.

During terminal differentiation a gradual shift in the protein 4.1 variants expressed occurs in both the primitive and the definitive chicken erythroid cell series when the cells become postmitotic, resulting in an alteration of the ratio of the protein 4.1 polypeptides synthesized (14). Protein 4.1 is assembled onto the membrane skeleton in all the different ratios without any detectable kinetic difference. However, the shift in the protein 4.1 variants expressed is paralleled by a change in the amount of protein 4.1 assembled relative to spectrin. In proliferative erythroid cells substantially less protein 4.1 is assembled than spectrin on a molar basis. As the cells become postmitotic and the protein 4.1 variants synthesized change, increasingly more protein 4.1 is assembled relative to spectrin. Since protein 4.1 assembles quantitatively at all stages of erythropoiesis the ratio change could be achieved by an increase in the level of translatable protein 4.1 mRNA relative to the spectrin RNAs. In addition, it has been shown that the fraction of newly synthesized spectrin that assembles decreases during erythropoiesis (5). This may contribute also to the relative increase in protein 4.1 assembly. The shift in the ratio of protein 4.1 and spectrin assembled indicates that the assembly of the individual components of the membrane skeleton is not coupled strictly during differentiation, even though protein 4.1 and spectrin directly interact with each other on the membrane skeleton. The interaction of protein 4.1 with spectrin strongly increases the association of spectrin and actin in vitro (11, 12, 29). In vivo an association of protein 4.1 with spectrin should therefore stabilize the membrane skeleton. As the membrane skeleton is assembled during erythropoiesis less than equimolar amounts of protein 4.1 are assembled initially and a relatively flexible structure may be generated in the proliferative cells. When the cells become postmitotic and undergo terminal differentiation, protein 4.1 is assembled in more than equimolar amounts. The excess protein 4.1 may associate with previously assembled spectrin and actin, increase the overall stability of the latter two, and form additional links to the plasma membrane. It may thereby contrib-

ute to the final stabilization of the erythrocyte membrane skeleton during terminal differentiation.

We thank Shelly Diamond (California Institute of Technology) for performing the centrifugal elutriation. We also thank Dr. John Ngai (California Institute of Technology) for his valuable comments on the manuscript.

This work was supported by grants from the National Institutes of Health, National Science Foundation, and Muscular Dystrophy Association. M. Staufenbiel was also supported by Cancer Research Campaign International Fellowship awarded by the International Union Against Cancer and a Senior Investigatorship of the American Heart Association, Greater Los Angeles Affiliate.

Received for publication 25 October 1985, and in revised form 25 November 1985.

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