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Synthesis of spectrin in avian erythroid cells: Association of nascent polypeptide chains with the cytoskeleton

(immunoprecipitation/puromycin/two-dimensional gel electrophoresis)

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ABSTRACT The site of synthesis of spectrin was investigated in erythroid cells from 10-day chicken embryos. After various periods of [^{35}S]methionine incorporation the cells were lysed in a Triton X-100 (TX-100)-containing buffer and were separated into a TX-100-soluble and -insoluble (cytoskeletal) fraction. Analysis of these two fractions by two-dimensional gel electrophoresis after a short pulse-labeling period reveals that α-spectrin nascent polypeptides are present predominantly in the TX-100-insoluble fraction. These polypeptides can be immunoprecipitated with α-spectrin antisera and the [^{35}S]methionine incorporated into them during a short pulse can be chased into mature α-spectrin molecules. The α-spectrin nascent polypeptide chains are released quantitatively from the TX-100 cytoskeleton by treatment of lysed cells with puromycin, suggesting that they themselves are not associated with the cytoskeleton. A small fraction of the newly synthesized mature α-spectrin molecules is rapidly incorporated into the cytoskeleton, as shown by the fact that they are not released by the puromycin treatment; the rest are recovered in the soluble fraction. These results suggest that α-spectrin is synthesized in association with the cytoskeleton during chicken erythropoiesis and assembles onto the membrane–cytoskeleton posttranslationally.

The shape and structural integrity of the mammalian erythrocyte plasma membrane is maintained by a subcortical cytoskeletal network. The major component of this network is spectrin, a protein composed of two nonidentical polypeptides, α-spectrin (M_r 240,000) and β-spectrin (M_r 220,000). The formation of this network requires the self-association of the two spectrin subunits to form an $(\alpha, \beta)_2$ tetramer and the binding of the tetramers to actin oligomers. Spectrin also restricts the mobility of certain transmembrane polypeptides. Association of spectrin with transmembrane polypeptides is mediated by the protein ankyrin, one site of which binds to β-spectrin and another, to a fraction of the anion transporters (band 3) (for a review, see ref. 1).

Although the molecular organization of the mammalian erythrocyte plasma membrane is well understood, little is known about the site of synthesis and assembly of the membrane–cytoskeleton complex which occurs during erythropoiesis. In mammalian erythroid precursor cells, the membrane component of this complex, the anion transporter, is inserted cotranslationally in the endoplasmic reticulum membrane before being transported to the cell surface (2, 3). However, the site of synthesis of spectrin, which plays a key role in the assembly of the membrane-associated cytoskeleton, is unknown.

To investigate the site of synthesis and assembly of the membrane-associated cytoskeleton in detail we have chosen chicken embryo erythroid cells as a model system. Avian erythrocytes are nucleated and possess few organelles. They contain a submembranous spectrin–actin network, proteins analogous to mammalian ankyrin, and the anion transporter (see *Discussion*), a circumferential bundle of microtubules, known as the marginal band, as well as a vimentin intermediate filament system (4, 5). Treatment of erythroid cells from 10-day chicken embryos with nonionic detergents leaves an insoluble cytoskeleton composed of the nucleus, the spectrin–actin network, and intermediate filaments (4). We show here that α-spectrin nascent polypeptide chains are associated with the cytoskeleton. However, the nascent chains can be released from this complex with puromycin, indicating that they themselves are not directly bound to the cytoskeleton. Shortly after completion of synthesis, α- and β-spectrin are incorporated into the cytoskeleton. Although most of the β-spectrin molecules synthesized are recovered in association with α-spectrin in the cytoskeleton, a substantial fraction of α-spectrin is not incorporated and is recovered in a soluble form in the cytoplasm. These results suggest that α-spectrin is synthesized on polysomes associated with the cytoskeleton and that the regulation of assembly of α-spectrin onto the membrane occurs posttranslationally.

MATERIALS AND METHODS

Preparation and Labeling of Cells. Chicken embryo erythroid cells were isolated from 10-day embryos. Blood was collected from the main veins in Earle's balanced salt solution. The resulting cell suspension was filtered through four layers of cheesecloth and the erythroid cells were collected by centrifugation. The cells were resuspended in 155 mM choline chloride/5 mM Hepes, pH 7.1, at 0°C and were recentrifuged. After each centrifugation the buffy coat was removed. This was repeated four times. The erythroid cells were then washed twice in methionine-free minimal essential medium and resuspended at a 10% (vol/vol) concentration in methionine-free minimal essential medium containing 10% dialyzed calf serum prewarmed to 37°C. The cells were incubated at 37°C for 15 min prior to the addition of [^{35}S]methionine (200–400 μCi/ml, 1,200–1,400 Ci/mmol; 1 Ci = 3.7×10^{10} Bq; Amersham); they were then labeled for different lengths of time (3–5 min). For the pulse-chase experiment, further incorporation of [^{35}S]methionine was stopped by the addition of unlabeled methionine (0.4 mM) and the incubation then was continued for different time periods (5–120 min). At the end of the labeling period 10 vol of 155 mM choline chloride/5 mM Hepes, pH 7.1, were added at 0°C and the cells were harvested by centrifugation. The cells were then lysed in 4 vol of lysis buffer containing 150 mM NaCl, 10 mM Tris·HCl (pH 7.2), 5 mM $MgCl_2$, 2 mM EGTA, 0.25 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 1% Triton X-100 (TX-100). To investigate the effect of the release of the nascent polypeptide chains, the lysates to be treated

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Abbreviation: TX-100, Triton X-100.

with puromycin were incubated for 3 min at 37°C in the presence of 2 mM puromycin. All lysates were then centrifuged for 6 min in an Eppendorf centrifuge. The supernatants were removed (TX-100-soluble fraction) and the pellets were resuspended with the same buffer to the original volume (cytoskeletal fraction). Solid urea and 2-mercaptoethanol were then added to the soluble and cytoskeletal fractions to give final concentrations of 9.5 M and 2.5%, respectively. The samples were stored at −70°C.

Immunoprecipitation. Antibodies raised against electrophoretically purified chicken erythrocyte α-spectrin have been characterized elsewhere (6, 7). Samples of soluble and cytoskeletal fractions prepared as described above were diluted 10 times with 130 mM NaCl/10 mM Tris·HCl, pH 7.5/5 mM EDTA/1% Nonidet P-40 prior to the addition of antibodies. A 1:500–1:1,000 dilution of the α-spectrin antisera was used for the immunoprecipitation of α-spectrin according to the method of Lingappa *et al.* (8) as modified by Levine and Willard (9).

Gel Electrophoresis. Proteins were separated by one-dimensional $NaDodSO_4$/10% polyacrylamide gel electrophoresis based on the system of Laemmli (10) as modified and described previously (11). Two-dimensional isoelectrofocusing was performed according to the method of O'Farrell (12) with the modifications described previously (11), except that Nonidet P-40 was omitted from the gels and samples, and the first dimension was loaded directly onto the second dimension without equilibration in sample buffer. $NaDodSO_4$/12.5% polyacrylamide gels were used for separation in the second dimension. The gels were treated with either EN^{3}HANCE (New England Nuclear) or Autofluor (National Diagnostics, Somerville, NJ), dried, and exposed to Kodak XAR-5 x-ray film. For the immunoprecipitations intensifying screens were used.

RESULTS

Association of α-Spectrin Nascent Polypeptides with the Membrane–Cytoskeleton. Chicken erythroid cells from 10-day embryos were isolated as described in *Materials and Methods.* Protein synthesis was followed by the incorporation of [^{35}S]me-

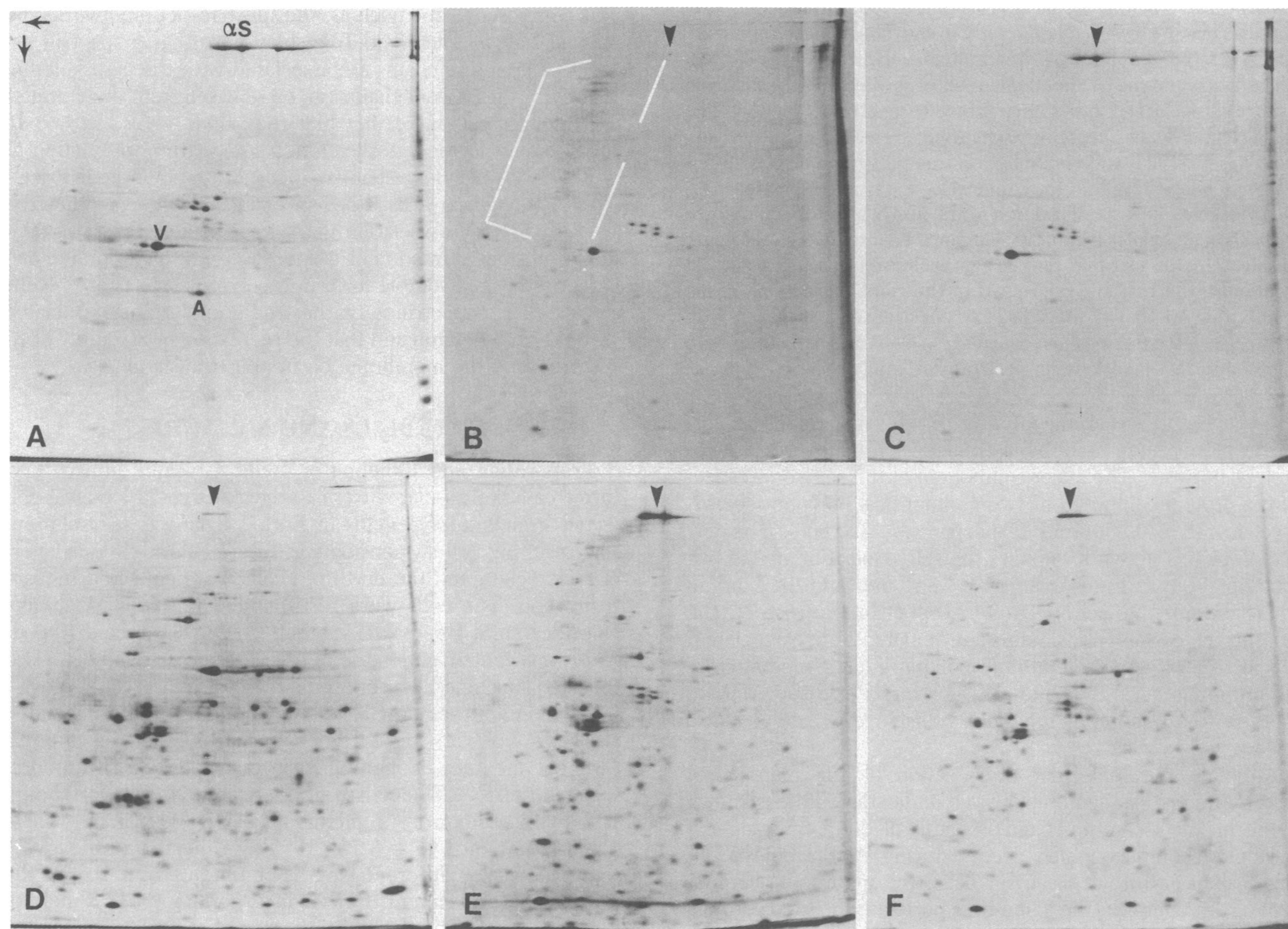

FIG. 1. Two-dimensional gel electrophoresis of TX-100 cytoskeletal and soluble fractions of chicken embryo erythroid cells pulse-labeled with [^{35}S]methionine and chased with unlabeled methionine. The cells were prepared as described in *Materials and Methods*. After a labeling period of 3 min with [^{35}S]methionine (400 μCi/ml), unlabeled methionine was added (0.4 mM). After different periods of time (0–120 min) an equal amount of cells (50 μl of packed cells roughly corresponding to 2.5×10^8 cells) was harvested, lysed in 200 μl of lysis buffer, and separated by centrifugation into cytoskeletal and soluble fractions. The cytoskeletal fractions were resuspended to the original volume (250 μl), and solid urea and 2-mercaptoethanol were added to all samples. An equal volume of each urea-containing sample (150 μl) was analyzed by two-dimensional gel electrophoresis. This volume contained 3×10^5 cpm from the cytoskeletal fractions and 2.7×10^6 cpm from the soluble fraction. The gels were dried and exposed to x-ray film for 4 days. The different gels show Coomassie brilliant blue staining of proteins from cytoskeletal (*A*) and soluble fractions (*D*) and autoradiograms of cytoskeletal (*B* and *C*) and soluble fractions (*E* and *F*), chased for 0 min (*B* and *E*) and 60 min (*C* and *F*), respectively. In *A* the arrows in the upper left corner mark the directions of electrophoresis. Isoelectrofocusing was from right (basic) to left (acidic) and $NaDodSO_4$ gel electrophoresis was from top to bottom. The region of the autoradiogram where the α-spectrin nascent polypeptides focus is marked with a white bracket (B). α-Spectrin (αS) is marked with an arrowhead (▼). A, actin; V, vimentin.

thionine into whole cells incubated at 37°C. After various labeling periods the cells were lysed in a TX-100-containing buffer and the lysate was centrifuged, which resulted in a soluble fraction and an insoluble cytoskeletal fraction. Fig. 1 *A* and *D* show the two-dimensional gels of these two fractions stained with Coomassie brilliant blue. Within the isoelectric point range examined here (pH 4.0–6.5), the cytoskeletal fraction has a relatively simple protein composition consisting of actin, vimentin, spectrin, and nuclear proteins (Fig. 1*A*). α- and β-tubulin are not present in the cytoskeletal fraction because the marginal band of microtubules is solubilized under the lysis conditions used here (4, 5). It should be noted that under the conditions of two-dimensional electrophoresis employed here, α-spectrin focuses into the gel but β-spectrin does not (4). The majority of α-spectrin was found in the cytoskeletal fraction, whereas only small amounts were detected in the soluble fraction after Coomassie brilliant blue staining (Fig. 1*D*). However, after a pulse-labeling period of 3 min with [^{35}S]methionine most of the newly synthesized α-spectrin was present in the soluble fraction (Fig. 1*E*, arrow) and only a relatively small amount was present in the cytoskeletal fraction (Fig. 1*B*). A detailed examination of the autoradiograms reveals a series of polypeptides with an electrophoretic mobility between that of α-spectrin (M_r 240,000) and vimentin (M_r 52,000) in the cytoskeletal fraction (Fig. 1*B*, white brackets). The relative amount of these polypeptides in the soluble fraction varies between different preparations (compare Figs. 1*E* and 3*B*; see *Discussion*).

To rule out the possibility that these polypeptides were degradation products of α-spectrin, we performed a pulse-chase experiment. During the chase period these polypeptides gradually disappeared, with the lowest molecular weight polypeptides disappearing first. After a 20-min chase they were all no longer visible. Fig. 1*C* shows an autoradiogram of the cytoskeletal fraction after a chase period of 60 min. As can be seen, the disappearance of these polypeptides was followed by a concomitant increase in the amount of radioactivity in the mature α-spectrin molecule in the cytoskeletal fraction (Fig. 1*C*). To further examine the possibility that these polypeptides were nascent polypeptide chains of α-spectrin, we performed the chase with unlabeled methionine in the presence of cycloheximide (0.36 mM). If these polypeptides were indeed nascent chains of α-spectrin they would not be chased into mature α-spectrin molecules in the presence of cycloheximide. These polypeptides remained in the cytoskeletal fraction even after a chase period of 60 min in the presence of cycloheximide with no increase of radioactivity in the mature α-spectrin molecule (data not shown), suggesting that they are indeed nascent polypeptides. The presence of mature α-spectrin molecules in the cytoskeletal fraction even after a 3-min labeling period suggests that a subset of the newly synthesized α-spectrin molecules has assembled onto the membrane–cytoskeleton complex shortly after completion of synthesis. The association of α-spectrin nascent polypeptide chains with the cytoskeleton does not appear to be due to nonspecific entrapment during lysis, because similar results have also been obtained when the cells were lysed in a hypotonic buffer (data not shown). This is supported also by the observation that a very high molecular weight polypeptide was found only in the soluble fraction (Fig. 1 *D* and *F*).

Immunoprecipitation of α-Spectrin Nascent Polypeptides. To examine further whether, after a short pulse with [^{35}S]methionine, the α-spectrin-related polypeptides were indeed α-spectrin nascent chains, we analyzed samples the same as those above by immunoprecipitation with α-spectrin antibodies. Fig. 2 shows the resulting autoradiograms of immunoprecipitates of cytoskeletal (Fig. 2*A*) and soluble (Fig. 2*C*) fractions from a pulse-chase experiment and the corresponding Coomassie brilliant blue-stained gels (Fig. 2 *B* and *D*, respectively). Newly synthesized α-spectrin was immunoprecipitated by the α-spectrin antisera in both the cytoskeletal and soluble fractions. In the cytoskeletal fraction several polypeptides with a molecular weight lower than that of α-spectrin (marked with a black bracket in Fig. 2*A*) were also immunoprecipitated by the α-spectrin antibodies. During the chase period they gradually disappeared with a concomitant relative increase of radioactivity in the mature α-spectrin molecule, indicating that they represent nascent polypeptides of α-spectrin. The preimmune serum did not precipitate either α- or β-spectrin or any of the nascent polypeptide chains (data not shown; see ref. 7). However, several other polypeptides (marked with black dots in Fig. 2*A*) were immunoprecipitated by the preimmune serum and thus represent nonspecific reactants with the α-spectrin antisera (data not shown). Coomassie brilliant blue staining of the immunoprecipitates (Fig. 2*B*) shows that undegraded α- and β-spectrin were immunoprecipitated in the cytoskeletal fraction in all time points of the pulse-chase experiment, further demonstrating that they are not proteolyzed during sample preparation. The soluble fraction contains [^{35}S]methionine-labeled mature α-spectrin molecules but very little of the nascent chains. In the cytoskeletal fraction, β-spectrin was coprecipitated with α-spectrin, as shown by both [^{35}S]methionine labeling of newly synthesized molecules (Fig. 2*A*) and by Coomassie brilliant blue staining (Fig. 2*B*). This is not due to crossreactivity of the antibodies with β-spectrin but is due to the fact that α- and β-spectrin form (or remain as) a complex under these conditions of sample preparation for immunoprecipitation (7). At present we do not know whether β-spectrin nascent polypeptide chains also coimmunoprecipitate with the α-spectrin antibodies. Thus,

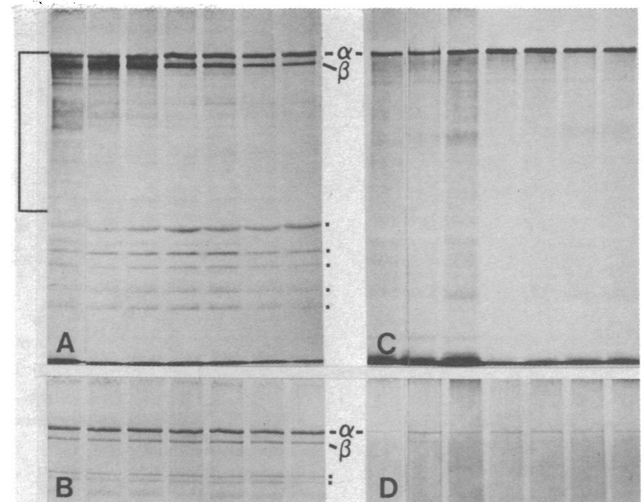

FIG. 2. Immunoprecipitations with α-spectrin antibodies. Chicken embryo erythroid cells were labeled with [^{35}S]methionine and chased for different lengths of time as described in the legend to Fig. 1. Equal volumes (100 μl) of each cytoskeletal fraction (2×10^5 cpm) and soluble fraction (1.7×10^6 cpm) were diluted 1:10 and were immunoprecipitated with α-spectrin antibodies. The immunoprecipitated proteins were separated on $NaDodSO_4$/10% polyacrylamide gels. The gels were incubated in EN^{3}HANCE, dried, and exposed to x-ray film with an intensifying screen for 72 hr (*A*) or 24 hr (*C*). Autoradiograms are shown of immunoprecipitates from cytoskeletal fraction (*A*) and soluble fraction (*C*). The spectrin region of the corresponding Coomassie brilliant blue staining is displayed underneath the cytoskeletal (*B*) and soluble fractions (*D*). The lanes from left to right are chase periods of 0, 6, 10, 20, 30, 60, and 120 min, respectively. The polypeptides immunoprecipitated with the preimmune serum are marked with a black dot (●). The region of the nascent chains in *A* is marked by a black bracket. α, α-spectrin; β, β-spectrin.

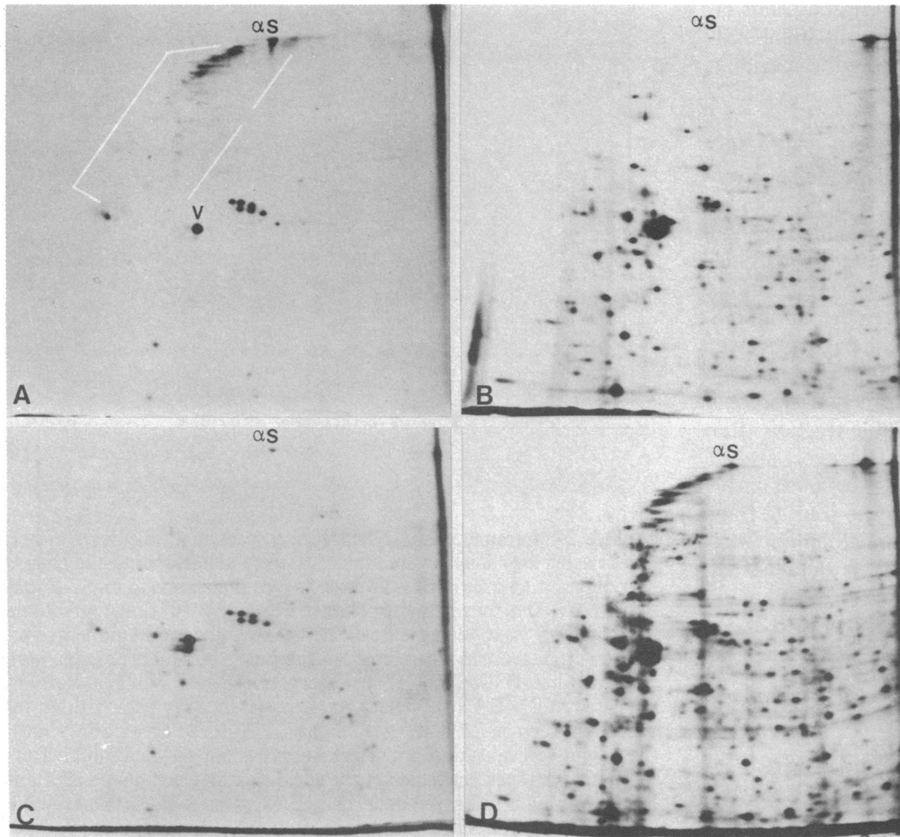

FIG. 3. Two-dimensional gel electrophoresis of cytoskeletal and soluble fractions of cell lysates treated with puromycin. An equal number of cells (1.2×10^8) were labeled with [^{35}S]methionine (200 μCi/ml) for 5 min. The cells were lysed in 100 μl and the lysates were incubated at 37°C for 3 min with or without 2 mM puromycin and then were separated into a cytoskeletal and soluble fraction by centrifugation. Equal amounts of each sample (100 μl) were analyzed by two-dimensional gel electrophoresis. The gels were incubated in Autofluor, dried, and exposed to x-ray film for 6 days. The autoradiograms are cytoskeletal (*A*) and soluble fractions (*B*) without puromycin and cytoskeletal (*C*) and soluble (*D*) fractions treated with puromycin. The nascent polypeptides of α-spectrin (αS) are marked in *A* with a white bracket. V, vimentin.

we cannot exclude the possibility that the α-spectrin nascent polypeptide chains observed in Fig. 2*A* also contain β-spectrin nascent polypeptides. When the autoradiogram in Fig. 2*A* was underexposed, it was evident that mature β-spectrin coprecipitated with α-spectrin even after a short (3 min) pulse-labeling period (leftmost lane, Fig. 2*A*). Collectively, these results suggest that the α-spectrin is synthesized on the cytoskeletal fraction, and that after completion of synthesis a fraction of the molecules is bound to the cytoskeleton in association with β-spectrin.

Release of Cytoskeleton-Associated α-Spectrin Nascent Polypeptides by Puromycin. To analyze the nature of the association of the α-spectrin nascent polypeptide chains with the cytoskeleton, a lysate from cells labeled with [^{35}S]methionine for 5 min was treated with puromycin. Fig. 3 shows autoradiograms of the cytoskeletal and the soluble fractions of a lysate after treatment with 2 mM puromycin for 3 min prior to separation of the two fractions by centrifugation (Fig. 3 *C* and *D*, respectively). Fig. 3 *A* and *B* show autoradiograms of two-dimensional gels of an identical preparation of the cytoskeletal and soluble fractions, respectively, except that the lysate was not treated with puromycin. The result of the puromycin treatment clearly shows that α-spectrin nascent polypeptide chains are released into the soluble fraction (compare Fig. 3 *A* and *C* and Fig. 3 *B* and *D*). This shows that the nascent polypeptide chains are not themselves directly bound to the cytoskeleton. On the other hand, the subset of mature newly synthesized α-spectrin molecules that are associated with the cytoskeleton is resistant to treatment with puromycin and is retained in the cytoskeletal fraction (Fig. 2*C*).

DISCUSSION

In this study we have shown that α-spectrin is synthesized in association with the cytoskeleton in chicken embryo erythroid cells and that the mature molecule either is incorporated into the cytoskeleton in association with β-spectrin or is released into the cytoplasm. Elsewhere we show in more detail that in chicken embryo erythrocytes α-spectrin is synthesized in a severalfold excess over β-spectrin and that most of the β-spectrin synthesized is incorporated into the membrane–cytoskeleton complex (13). Here we have concentrated on the characterization of the site of synthesis of α-spectrin because we could unambiguously identify its nascent polypeptide chains by two-dimensional gel electrophoresis. That the synthesis of α-spectrin occurs in association with the cytoskeleton is shown by the presence of α-spectrin nascent polypeptide chains in the insoluble cytoskeletal fraction after cell lysis in the presence of TX-100. Evidence that these are indeed α-spectrin nascent polypeptides stems from the observation that they can be immunoprecipitated with α-spectrin antibodies and that the [^{35}S]methionine incorporated into these polypeptides can be chased into full-sized α-spectrin molecules after a short pulse-labeling period. Furthermore, this process is inhibited by the presence of cycloheximide during the chase period. The time needed for all of the polypeptides to be chased to mature α-spectrin is $\approx$10–20 min, which corresponds to the time it would take to synthesize a molecule the size of spectrin, assuming a rate of synthesis of about five amino acids per sec at 37°C (14).

A small fraction of α-spectrin nascent polypeptide chains can also be detected by two-dimensional gel electrophoresis in the soluble fraction after cell lysis in the presence of TX-100 (Fig. 1*E*). This might be due either to their release from the cytoskeleton during cell lysis and handling of the extracts or to incomplete centrifugation of all the cytoskeletal proteins. The former can arise from polysome degradation during the preparation of the cytoskeletal fraction, in particular, because the lysis conditions employed here have not been optimized for polysome stabilization. However, the nascent polypeptides are still associated with the cytoskeleton after washing of the cytoskeletal fraction once with lysis buffer (not shown).

Puromycin, which causes premature termination of the nascent polypeptide chains, releases all of the α-spectrin nascent chains from the cytoskeleton into the soluble fraction. This indicates that the nascent chains are not directly assembled onto the membrane–cytoskeleton and that they do not mediate the attachment of the polysomes to the cytoskeleton. However, the mature newly synthesized α-spectrin molecules that are present in the cytoskeletal fraction are resistant to release by puromycin. From the experiments presented here the association of α-spectrin with the membrane occurs within 3 min, providing further evidence that the mechanism of assembly of α-spectrin on the membrane is posttranslational, rather than cotranslational. This is different from that which is the case for the synthesis of secretory and integral membrane proteins, which are cotranslationally inserted into the membrane. In this case, attachment of the polysomes to the membrane appears to involve the nascent chains of the polypeptide (15, 16). In the case of the transmembrane mammalian erythrocyte anion transporter, onto which β-spectrin is anchored through ankyrin (1), it has been shown that this protein is synthesized on the rough endoplasmic reticulum where it is cotranslationally inserted and assembled into the endoplasmic reticulum membrane in the orientation that it is found in the plasma membrane; the protein is subsequently transported to the plasma membrane after a considerable lag period (2, 3). Chicken erythrocytes contain also a protein analogous to the mammalian erythrocyte anion transporter (6, 17, 18) and it would be of interest to determine in the future how the transport of the anion transporter to the plasma membrane and its interaction with ankyrin and, in turn, β-spectrin is coordinated with the synthesis and assembly of ankyrin and α- and β-spectrin onto the membrane (see below).

From the immunoprecipitation data presented here it is evident that newly synthesized cytoskeleton-associated α-spectrin is immunoprecipitated with mature β-spectrin even after short pulse-labeling periods. Because the insertion of, at least, α-spectrin into the cytoskeleton appears to be posttranslational, this suggests that β-spectrin also associates with the cytoskeleton shortly after completion of the nascent chains. However, unlike newly synthesized β-spectrin, most of which is recovered in the cytoskeletal fraction (13), a considerable amount of α-spectrin is also recovered in the supernatant. This observation, in conjunction with the observation that α-spectrin is synthesized in excess of β-spectrin in chicken embryo erythroid cells (13), suggests that a fraction of α-spectrin does not associate with β-spectrin on the membrane and is released into the cytoplasm.

We have not investigated in detail as yet the site of synthesis of other protein components of the erythrocyte subcortical membrane cytoskeleton. From indirect evidence presented elsewhere (13)—namely, that most of the mature β-spectrin molecules synthesized after a short labeling period are present in the cytoskeletal fraction—we may assume that β-spectrin is also synthesized in association with the cytoskeleton. Avian erythrocytes also contain a phosphoprotein, termed goblin (19, 20), which appears to be analogous to mammalian ankyrin on the basis that it has solubility properties similar to mammalian erythrocyte ankyrin (4) and that it is serologically crossreactive with antibodies to human erythrocyte ankyrin (7, 13). Goblin can also be resolved by two-dimensional isoelectric focusing/NaDodSO$_4$ gel electrophoresis (4) and preliminary evidence suggests that goblin nascent polypeptide chains can also be identified in association with the cytoskeletal fraction. Similarly to the α-spectrin nascent polypeptides, the goblin nascent polypeptides can be pulse-chased to the mature goblin molecule (unpublished data). Whether β-spectrin and goblin also associate posttranslationally remains to be determined. The site of synthesis of actin is also presently unclear. From Figs. 1*B* and 3*A* it appears that after short labeling periods, actin is present almost exclusively in the TX-100-soluble fraction. In other systems it has been suggested that the majority of the newly synthesized actin mRNA associates with the cytoskeleton (21). Whether this is also the case in the chicken embryo erythroid cells remains to be determined.

The mechanism of the association of the α-spectrin polysomes with the cytoskeleton also remains to be established. The puromycin data suggest that the association is through some component of the messenger ribonucleoprotein–ribosome complex rather than the nascent polypeptides. A number of reports have suggested that in cells grown in tissue culture a substantial fraction of all mRNA species remains in association with the cytoskeletal framework after a gentle lysis with TX-100 (22–25), but the molecular details of such an association remain to be determined. The association of α-spectrin nascent polypeptide chains with the cytoskeleton and the rapid incorporation of mature α-spectrin molecules into the cytoskeleton suggest that the distribution of α-spectrin mRNA is polarized and in close proximity to the site of assembly of α-spectrin on the membrane–cytoskeleton. Because the chicken erythroid cell membrane–cytoskeleton is relatively simple in polypeptide composition, it provides an ideal system in which the nature of the association of α-spectrin polyribosomes with the cytoskeleton can be elucidated.

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