Unequal Synthesis and Differential Degradation of α and β Spectrin during Murine Erythroid Differentiation

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Abstract. Murine erythroleukemia (MEL) cells represent a valuable system to study the biogenesis of the cytoskeleton during erythroid differentiation. When attached to fibronectin-coated dishes MEL cells induce, upon addition of DMSO, a 7-d differentiation process during which they enucleate and reach the reticulocyte stage (Patel, V. P., and H. F. Lodish. 1987. *J. Cell Biol.* 105:3105–3118.); they accumulate band 3, spectrin, and ankyrin in amounts equivalent to those found in mature red blood cells. To follow the biosynthesis of spectrin during differentiation, membranes and cytoskeletal proteins of cells metabolically labeled with [^{35}S]methionine were solubilized by SDS and α and β spectrins were recovered by specific immunoadsorption. In both uninduced and 3-d induced cells, the relative synthesis of α/β spectrin is ∼1:3. In uninduced MEL cells newly synthesized α and β spectrins are degraded with a similar half-life of ∼10 h. In contrast, in 3-d differentiated MEL cells newly made β spectrin is much more unstable than α spectrin; the half-lives of α and β spectrin chains are ∼22 and 8 h, respectively. Thus, accumulation of equal amounts of α and β spectrin is caused by unequal synthesis and unequal degradation. As judged by Northern blot analyses, the level of actin mRNA is relatively constant throughout the 7-d differentiation period. α and β spectrin mRNAs are barely detectable in uninduced cells, increase during the first 4 d of induction, and remain constant thereafter. In contrast, band 3 mRNA is first detectable on day 4 of differentiation. Thus, most of the spectrin that accumulates in enucleating reticulocytes is synthesized during the last few days of erythropoiesis, concomitant with the onset of band 3 synthesis. To determine whether this was occurring in normal mouse erythropoiesis, we analyzed the rate of appearance of labeled membrane proteins in mature erythrocytes after a single injection of [^{35}S]methionine. Our results show that most of the spectrin and band 3 in mature erythrocytes is synthesized during the last days of bone marrow erythropoiesis, and that, in the marrow, band 3 and protein 4.1 are synthesized at a somewhat later stage of development than are α and β spectrin, ankyrin, and actin.

THE bone marrow is the site of mammalian red blood cell differentiation. In vivo studies on membrane protein biosynthesis during erythropoiesis are few, yet pronounced molecular changes do take place in the plasma membranes of developing red blood cells throughout the several day differentiation period (Skutelsky and Farquhar, 1976; Geiduschek and Singer, 1979). The most dramatic processes in membrane remodeling occur upon maturation of orthochromatic erythroblasts to reticulocytes, accompanied by the extrusion of the cell nucleus.

Though poorly understood, these developmental changes must ultimately be explained with respect to the physicochemical properties of the erythrocyte plasma membrane and its interaction with the submembrane skeleton. These interactions restrict the lateral mobility of erythrocyte integral membrane proteins (Peters et al., 1974; Elgsaeter and Branton, 1974; Nigg and Cherry, 1980; Golan and Veach, 1980;

Fowler and Branton, 1977). The membrane-skeleton determines cell shape, membrane deformability, and strength (Lux, 1979; Bennett, 1985; Marchesi, 1985). The major component of the subcortical protein network, spectrin, accounts for ∼30% of the membrane protein, and is composed of two nonidentical polypeptides, α (M_r ∼ 240 kD) and β (M_r ∼ 220 kD) (Speicher, 1986), which self associate to form (αβ)-heterodimers and (αβ)$_2$-tetramers (Ungewickell and Gratzer, 1978). Spectrin is linked to the plasma membrane via ankyrin (M_r ∼ 210 kD), a protein that binds both to the cytoplasmic tail of the integral membrane protein band 3 (M_r ∼ 100 kD) and to the β subunit of spectrin (Luna et al., 1979; Yu and Goodman, 1979; Bennett and Stenbuck, 1979, 1980a, 1980b). Protein 4.1 enhances the interaction between the distal termini of spectrin tetramers and short F-actin helices forming a two-dimensional cytoskeletal network (Ungewickell et al., 1979; Cohen and Foley, 1980;

Fowler and Taylor, 1980; Correas et al., 1986). The assembly of the spectrin–actin scaffolding may be influenced by adducin (Gardner and Bennett, 1987).

In vivo, spectrin synthesis is initiated at early stages of murine erythroid differentiation (Chang et al., 1976) and there is a progressive increase of membrane-associated spectrin in late erythroblasts (Geiduschek and Singer, 1979). During enucleation, spectrin, band 3, and ankyrin of the orthochromatic erythroblast entirely remain with the incipient reticulocyte and are segregated away from the ejected nucleus (Geiduschek and Singer, 1979; Patel and Lodish, 1987).

Detailed studies of the synthesis and degradation of α and β spectrin during murine erythroid differentiation may shed some light onto these developmental processes. Murine erythroleukemia (MEL)[1] cells represent a valuable in vitro model system for red blood cell differentiation. These cells are transformed by the Friend virus complex and grow continuously in suspension culture. DMSO or a number of other chemicals induce a differentiation program that closely resembles erythroid differentiation in vivo (Friend et al., 1971; reviewed in Marks and Rifkind, 1978). However, after 4 d of induction in suspension these cells stagnate at a late erythroblast stage; the level of erythrocyte-specific anion exchange protein band 3, for example, never reaches that of mature red blood cells (Patel and Lodish, 1987).

The extracellular matrix protein fibronectin promotes terminal differentiation of MEL cells (Patel and Lodish, 1987). After 7 d of differentiation on this matrix >50% of the cells have enucleated forming reticulocytes. Proteins band 3, spectrin, and ankyrin are present in amounts comparable to those of mature erythrocytes. Additionally, these cells lose the fibronectin receptor and detach from the matrix a process akin to transfer of reticulocytes from the bone marrow to the circulation.

We have begun a study of the biogenesis of the erythrocyte membrane in differentiating MEL cells. Our principal result is that at 3 d of differentiation, β spectrin is synthesized in an about threefold excess over α spectrin. Both spectrin subunits are degraded with first-order kinetics; β spectrin is more unstable ($t_{1/2} \sim 8$ h) than α spectrin ($t_{1/2} \sim 22$ h). Thus, accumulation of equal amounts of the two subunits is caused both by differential synthesis and decay, and most of the spectrin that accumulates in reticulocytes is synthesized during the last few days of erythropoiesis. Accumulation of band 3 mRNA and synthesis of band 3 protein begins ~ 2 d after that of α and β spectrin. In contrast to spectrin, band 3 is stable, and may act to stabilize the spectrin cytoskeleton. Our results differ in many respects from those in avian erythroid cells (reviewed by Lazarides, 1987).

Materials and Methods

Materials

Materials were purchased from the following vendors: BCA protein assay reagent from Pierce Chemical Company (Rockford, IL); Biotrans nylon membrane from ICN Radiochemicals (Irvine, CA); Freund's complete and incomplete adjuvant, Histopaque-1119, Histopaque 1077, and Protein A-sepharose CL-4B from Sigma Chemical Co. (St. Louis, MO); FBS, FBS (dialyzed), DME, and Hanks' Balanced Salt Solution (HBSS) from Gibco

(Grand Island, NY); DME without methionine and sodium bicarbonate from Hazleton Research Products Inc. (Lenexa, KS); BSA for induction medium: Bovuminar Cohn Fraction V from Armour Pharmaceutical Co. (Tarrytown, NY); Imferon from Dow Chemical Company (Cincinnati, OH); outdated human plasma from American Red Cross Blood Services, NE Region (Boston, MA); IgGsorb (fixed Staphylococcus aureus bacteria) from The Enzyme Center Inc. (Malden, MA); Gelatin sepharose 4B from Pharmacia Fine Chemicals (Piscataway, NJ); L-[^{35}S]methionine ($\sim$1,200 Ci/mmol), uridine 5′ [α-^{32}P]triphosphate ($\sim$410 Ci/mmol), [^{125}I]-sodium iodide ($\sim$100 mCi/ml) and ^{125}I-labeled protein A (35 µCi/µg protein A) from Amersham Corp. (Arlington Heights, IL); T7 polymerase from United States Biochemical Corp. (Cleveland, OH); RQ1DNase, pGEM4, pSp64, and Sp6 polymerase from Promega Biotec. (Madison, WI); BSA for in vitro transcription from International Biotechnologies Inc. (IBI) (New Haven, CT); DMSO from Aldrich Chemical Co. (Milwaukee, WI); nitrocellulose paper, BA 83, from Schleicher & Schuell, Inc. (Keene, NH); and prestained mol wt standards (mol wt range 14,000–200,000) from Bethesda Research Laboratories (Gaithersburg, MD). All other reagents were of reagent grade.

Plasmids. sp64γ-actin originated from Enoch et al. (1986), plasmids Spα33-1.5 and Spβ6 encoding sequences of the human α and β spectrin cDNAs were a generous gift from Dr. B. G. Forget, Department of Hematology, Yale University, New Haven, CT. The cDNA encoding the murine band 3 cDNA (Kopito and Lodish, 1985a) subcloned in the vector pSp64 was kindly provided by Dr. A. M. Garcia of this laboratory.

The following reagents were generously provided: rabbit anti–pig fodrin by Dr. J. R. Glenney, The Salk Institute, San Diego, CA; human protein 4.1 and human actin by Dr. A. Husain, Department of Cellular and Developmental Biology, Harvard University, Cambridge, MA; MEL cell subclone PC3-A21 and rabbit anti–mouse protein 4.1 polyclonal antiserum by Dr. V. P. Patel, this laboratory; mouse 3T3 fibroblasts by Dr. M. Shia, this laboratory.

New Zealand female white rabbits, 8–20-wk-old female CD-1 mice were purchased from Charles River Biotechnical Services Inc., Wilmington, MA.

Methods

Cell Culture. The MEL cell subclone PC3-1 originates from PC3-A21, described by Patel and Lodish (1987). The cells were subcloned by limited dilution, frozen at 1°C per minute and stored under liquid nitrogen (Volloch and Housman, 1982). Cells were grown in DME with 13% decomplemented FBS at 37°C, 5% CO_2, 95% air, and were maintained for no longer than 3 wk after thawing. Cells were induced on fibronectin-coated dishes (Patel and Lodish, 1987). In brief, cells were preincubated for 12 h first in DME containing 2.5% BSA, 0.9 mM Imferon and then in DME supplemented with 5% BSA, 1.8 mM Imferon (induction medium). Bacteriological petri dishes were coated with 50 µg/ml of fibronectin in PBS. After adhesion to the matrix, cells were incubated with either induction medium (uninduced control cells) or induction medium containing 1.8% DMSO (induced cells). Fibronectin was purified from outdated human plasma by the method of Engvall and Ruoslahti (1977) with modifications (Patel and Lodish, 1984).

Metabolic Labeling of Cells. MEL cells grown on 35-mm fibronectin-coated dishes ($\sim$1 × 10⁶ cells/dish) were washed gently with methionine-free induction medium (met-free DME). Cells were depleted of internal methionine in 1 ml met-free DME for 10 min at 37°C in a CO_2 incubator. The medium was discarded and 0.5 ml met-free DME supplemented with 100 µCi/ml [^{35}S]methionine was added. After a 30-min incubation at 37°C, the cells were rinsed twice with induction medium and chased with induction medium or induction medium containing 1.8% DMSO, respectively, for up to 48 h. Pulse-chase experiments were performed with duplicate or triplicate samples per time point. Each sample included all cells from a single dish.

Under these labeling conditions the incorporation of [^{35}S]methionine was linear for at least 1 h, ruling out the possibility that low concentrations of methionine might affect the absolute rate of protein synthesis.

Extraction of Membrane-Cytoskeletal Proteins

Procedure 1. All steps were carried out at 0–4°C unless stated otherwise. Cells were washed twice with HBSS containing 20 mM Hepes, pH 7.5 (HBSS-H). Cells ($\sim$1 × 10⁷/ml) were lysed in ice-cold detergent solution (5 mM Tris-HCl, pH 7.5, 5 mM EDTA, 1% NP-40, 10 mM 2-mercaptoethanol, 1 mM PMSF, 0.1 mM each N-tosyl-L-phenylalanine chloromethyl ketone [TPCK] and Nα-p-tosyl-L-lysine chloromethyl ketone [TLCK]).

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1. *Abbreviations used in this paper*: HBSS-H, Hanks' balanced salt solution containing 20 mM Hepes, pH 7.5; MEL, murine erythroleukemia.

The extract was kept on ice for 20 min. Soluble proteins were separated from cytoskeletal and nuclear components by centrifugation for 15 min at 12,000 *g*. The pellet was reextracted with a buffer containing 8 M urea, 5 mM Tris–HCl, pH 7.5, 5 mM EDTA, 0.2% SDS, 10 mM 2-mercaptoethanol, 1 mM PMSF, 0.1 mM each TPCK and TLCK at 10^7 cell equivalents/ml. The extract was kept on ice for 30 min with intermittent vortexing. After a 15-min centrifugation in an Eppendorf microfuge, the resulting supernatant was combined with the soluble detergent extract.

Procedure 2. Cells were washed twice with HBSS-H, lysed in 2× gel sample buffer (gel sample buffer: 62.5 mM Tris, pH 6.8, 2% SDS, 10% glycerol, 5% 2-mercaptoethanol; Laemmli, 1970) at ∼1 × 10^7 cells/ml, and immediately boiled for 3 min. The samples were allowed to cool to room temperature and subsequently were mixed with an equal volume of water to dilute the SDS concentration to 2%. In some cases the extracts were sonicated twice for 30 s each (sonicator model No. W225; Heat Systems-Ultrasonics, Inc., Farmingdale, NY), and insoluble material was separated by centrifugation for 10 min at 12,000 *g*.

Acid precipitable counts were determined for each sample by TCA precipitation. Protein concentration was measured using the BCA protein assay reagent with BSA as a standard.

Radioactive Labeling of Experimental Animals

The mouse was briefly anesthetized with ether, and given a single intraperitoneal injection of 2 mCi L-[^{35}S]methionine in an equal volume of PBS prewarmed to 37°C. 6 h after injection the initially large amount of soluble free [^{35}S]methionine in the serum was either metabolized, incorporated into TCA-precipitable plasma protein, or excreted. The decline of radioactivity in the serum was consistent with earlier observations (Chang et al., 1976). Blood samples were taken over a period of 4 d and a final sample at 10 d. Mice were anesthetized with ether and bled by cutting off a small segment of the tail. About 0.1 ml of blood was collected into a 10-fold volume of ice-cold HBSS-H, supplemented with 0.001% heparin as anticoagulant. The cells were washed three times with HBSS-H, and resuspended in 1 ml of the same buffer. To free the erythrocytes of contaminating mononuclear cells and granulocytes, Histopaque 1077 and 1119 were used according to the manufacturer's instructions.

Successive bleeding and sampling of ∼0.1 ml of blood did not induce anemia. Reticulocyte numbers were monitored by staining a small blood cell sample with New Methylene Blue solution. The percentage of reticulocytes in the total cell population remained constant and did not exceed the level of untreated control animals during the course of the experiment.

Preparation of Erythrocyte Proteins

All solutions contained protease inhibitors (1 mM PMSF, 0.1 mM each TPCK and TLCK) and all operations were carried out at 4°C unless stated otherwise. After density gradient centrifugation, mouse erythrocytes were washed twice with HBSS-H and lysed in 20 vol of lysis buffer containing 5 mM sodium phosphate, 1 mM EDTA, pH 7.4 (Dodge et al., 1963). The membranes were collected by centrifugation at 18,000 rpm for 10 min (SS34 rotor; Sorvall Instruments Div., Newton, CT) and washed three to four times with 20 vol of lysis buffer until white. One-half of the material was resuspended to its original packed red blood cell volume in 5 mM sodium phosphate, pH 7.4, and mixed with an equal volume of 2 × gel sample buffer (Laemmli, 1970) for analysis by SDS-PAGE. The other half of the ghost preparation was depleted of α and β spectrin and actin by a low ionic strength extraction slightly different from that of Bennett and Branton (1977). The pellet was resuspended in 6 vol 0.5 mM sodium phosphate, 0.2 mM EDTA, pH 7.5, and incubated at 37°C for 30 min. After centrifugation (30 min at 19,000 rpm; SS34 rotor; Sorvall Instruments Div.) the membrane vesicle pellet was resuspended to its original volume of packed erythrocytes with 5 mM sodium phosphate, pH 7.4. The supernatant, or low ionic strength extract, and the resuspended vesicular fraction were frozen at −20°C and later analyzed by SDS-PAGE.

Iodination of Erythrocyte Membranes

Mouse erythrocyte membranes from ∼5 × 10^7 red blood cells were washed 3 times with HBSS-H and then iodinated after the lactoperoxidase–glucose-oxidase method (Sefton et al., 1973). The membranes were resuspended in 1.5 ml HBSS-H and labeled for 15 min on ice with intermittent shaking using 1 mCi ^{125}I-sodium iodide. To drive the reaction to completion, a 10-fold volume of HBSS-H supplemented with 2 mM sodium iodide was added, and the membranes were washed 3 times in 15 ml of the above buffer.

Antibody Production

Polyclonal antibodies against mouse α and β spectrin as well as band 3 were raised in rabbits. The animals were bled before immunization and the sera were used as preimmune control sera. SDS gel-purified protein was used as immunogen. Erythrocyte membrane vesicles (for band 3) and low ionic strength extracts (for α and β spectrin) were prepared as described above. The gels were stained for a few minutes with Coomassie Brilliant Blue and the bands of interest were excised. The gel slices were destained completely in 25% isopropanol, 10% acetic acid and washed extensively with PBS. They were then homogenized in a small volume of PBS by several passages through hypodermic needles of increasing gauge sizes. The homogenized gel slices containing ∼200 μg of protein were emulsified with an equal volume of Freund's complete adjuvant and injected subcutaneously into multiple sites of the rabbit. The animals were boosted every 3 wk with the same amount of immunogen mixed with Freund's incomplete adjuvant and bled 8 d after each boost, starting after the third injection. The IgG fractions from preimmune and immune sera were purified by Protein A-sepharose CL-4B affinity chromatography.

Immunoprecipitation

Samples of the combined detergent and urea extract prepared as described above were adjusted to a final concentration of 1% SDS and denatured for 2 min at 100°C. After the samples were at room temperature the SDS concentration was diluted to 0.1% or lower by addition of buffer A: 10 mM Tris–HCl, pH 7.5, 5 mM EDTA, 150 mM NaCl, 0.5% deoxycholate, 1% NP-40, 2 mg/ml BSA, 1 mM PMSF, 0.1 mM each TPCK and TLCK. To preclear the solution (∼1 ml) of nonspecific protein A binding material, 50 μl of a 10% (vol/vol) suspension of fixed *Staphylococcus aureus* bacteria (IgGsorb) were added. After a 30-min incubation at 4°C with constant rotation the bacteria were pelleted by centrifugation for 30 s in an Eppendorf microfuge. Specific antibodies were then added and incubated for 8 h at 4°C with constant agitation. Thereafter, 200 μl of the 10% *Staphylococcus aureus* bacteria suspension were added to the extract and the samples rotated for another 2 h at 4°C. The bacteria-antibody-antigen complex was then recovered by a 30-s centrifugation in an Eppendorf microfuge. To remove nonspecifically bound proteins the complex was washed in sequence with buffers A–D (buffer B: 10 mM Tris–HCl, pH 7.5, 5 mM EDTA, 1 M NaCl, 0.5% Deoxycholate, 1% NP-40; buffer C: 10 mM Tris–HCl, pH 7.5, 0.15 M NaCl, 0.1% NP-40; buffer D: 10 mM Tris–HCl, pH 7.5, 2 mM EDTA). The final pellet was suspended in 50 μl 1 × gel sample buffer, heated for 4 min at 95°C, and sedimented in an Eppendorf microfuge. The supernatant was either stored at −20°C or directly loaded onto polyacrylamide gels.

The cell samples extracted with 2 × gel sample buffer were diluted to a final SDS concentration of 0.1% or lower by addition of buffer A. Proteins were immunoprecipitated as described above except that preclearing with IgGsorb was omitted.

For immunoadsorption of α and β spectrin, we combined the two antisera, since after the dilution of the SDS concentration to 0.1% some reassociation occurred between α and β spectrin (data not shown; see Calvert et al., 1980; Yoshino and Marchesi, 1984).

The amount of antibody required to quantitatively immunoadsorb a fixed amount of erythrocyte spectrin or band 3 was determined by titration (data not shown). Several samples containing comparable amounts of iodinated mouse erythrocyte ghost membranes, solubilized by boiling in SDS, were immunoprecipitated with increasing amounts of specific antibodies. The immunoadsorbed proteins were analyzed by SDS-PAGE and fluorography and the antibody concentration necessary to immunoprecipitate maximal amounts of antigen was determined. To ensure that all the antigen was immunoadsorbed, the supernatants of the *Staphylococcus aureus*-antibody-antigen complex were reimmunoprecipitated with additional specific antibodies and analyzed by SDS-PAGE and fluorography. As expected, the supernatants of the samples that yielded maximal amounts of antigen after the first immunoadsorption did not contain any additional immunoprecipitable material. The immunoadsorption conditions were quantitative. For immunoprecipitation of MEL cell proteins, a threefold excess of antibody over the amount determined to quantitatively immunoadsorb equivalent amounts of erythrocyte proteins was used. Preimmune IgG was used at the same concentration as were α and β spectrin specific antibodies.

SDS-PAGE and Fluorography

Immunoprecipitated proteins and membrane protein preparations were analyzed by SDS-PAGE (Laemmli, 1970). The acrylamide/*N,N*-methylene

Immunoblot Analysis

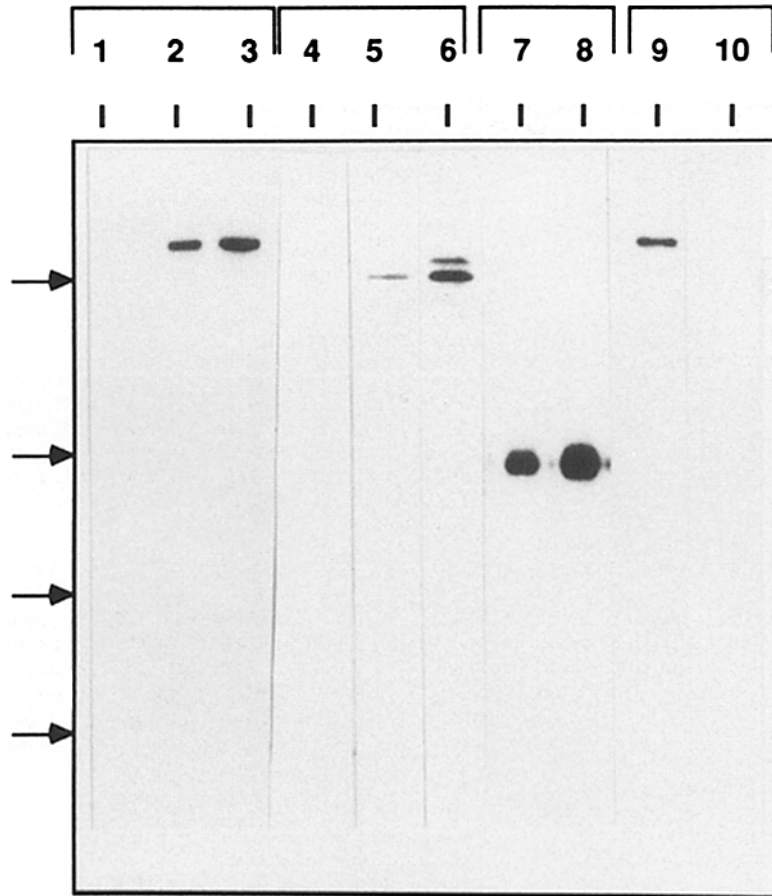Antibody : α spectrin   β spectrin   band 3   fodrin

Figure 1. Specificity of antibodies. Mouse erythrocytes, 3-d induced MEL cells, and mouse 3T3 fibroblasts were solubilized in SDS sample buffer as described in Materials and Methods. The extracts were submitted to SDS-PAGE and analyzed by immunoblotting. Rabbit anti-mouse α spectrin (lanes *1-3*), β spectrin (lanes *4-6*), and band 3 (lanes *7* and *8*) antibodies, and rabbit anti-pig fodrin antibodies (lanes *9* and *10*) were used at a 100-fold dilution and detected with ^{125}I-labeled protein A followed by autoradiography. The following cell extracts were immunoblotted: lanes *1* and *9*, 20 μg solubilized 3T3 cell protein; lane *4*, 40 μg 3T3 cell protein; lanes *2*, *5*, and *10*, 0.24 μg mouse erythrocyte proteins; lane *7*, 24 ng mouse erythrocyte protein; lanes *3* and *8*, 20 μg protein from 3-d induced MEL cells; lane *6*, 40 μg protein from induced MEL cells. The arrows indicate the apparent molecular mass of standard proteins: myosin (H chain), 199 kD; phosphorylase B, 104 kD; BSA, 66 kD; ovalbumin, 42 kD.

bisacrylamide ratio was modified. The slab gels (1.5 mm) were composed of a separating gel of 8% acrylamide and 0.1% *N,N'*-methylene bisacrylamide and a stacking gel of 3.75% acrylamide and 0.1% *N,N'*-methylene bisacrylamide. The gels were stained with 0.05% Coomassie Brilliant Blue in 25% isopropanol, 10% acetic acid. For fluorography the procedure of Skinner and Griswold (1983) was followed. Films were exposed at −70°C.

Immunoblot Analysis

Erythrocyte and MEL cell proteins were transferred from SDS–polyacrylamide gels onto sheets of nitrocellulose paper for detection with specific antisera (Towbin et al., 1979). The antisera were used at a 100-fold dilution and reacted with 0.1 μCi/ml ^{125}I-labeled protein A. The radioactivity was visualized by autoradiography.

Data Analysis

To determine the relative amount of each protein the peak height of an individual protein band was determined by densitometry (LKB Bromma 2202 Ultroscan Laser Densitometer, 2220 Recording Integrator, LKB-Produkter AB; LKB Instruments Inc., Bromma, Sweden). For the in vivo labeling experiment each lane of both the gels and the autoradiographs was scanned in at least three different positions.

RNA Analysis

MEL cells were recovered from fibronectin-coated dishes and washed three times with HBSS-H. RNA was prepared by a modification of the procedure

described by Chirgwin et al. (1979). Equal amounts of total cellular RNA were separated on a 1% agarose gel containing 6% formaldehyde (Maniatis et al., 1982). The RNA was transferred to a nylon filter, incubated for 2 h at 80°C in vacuo, and hybridized with cRNAs transcribed in vitro (Melton et al., 1984). The cDNAs were subcloned into the recombinant plasmid pGEM4 and the in vitro transcription was performed after the procedure developed by Melton et al. (1984). The hybridization was carried out at 60°C and the filters were washed twice for 20 min each, first in 2 × SSC (SSC = 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0), 0.1% SDS at 60°C, and then in 0.2 × SSC, 0.1% SDS at 70°C.

Results

Specificity of α Spectrin, β Spectrin, and Band 3 Antibodies

To determine the specificity of each of the three antisera both for the immunogen purified from mouse red blood cell membranes as well as for protein present in MEL cells, we performed immunoblot analysis. Among total erythrocyte membrane proteins each antiserum recognized only the immunogen (Fig. 1, lanes *2*, *5*, and *7*). The sera were equally specific when used to probe total extracts from 3-d induced MEL cells (Fig. 1, lanes *3*, *6*, and *8*). The β spectrin–specific
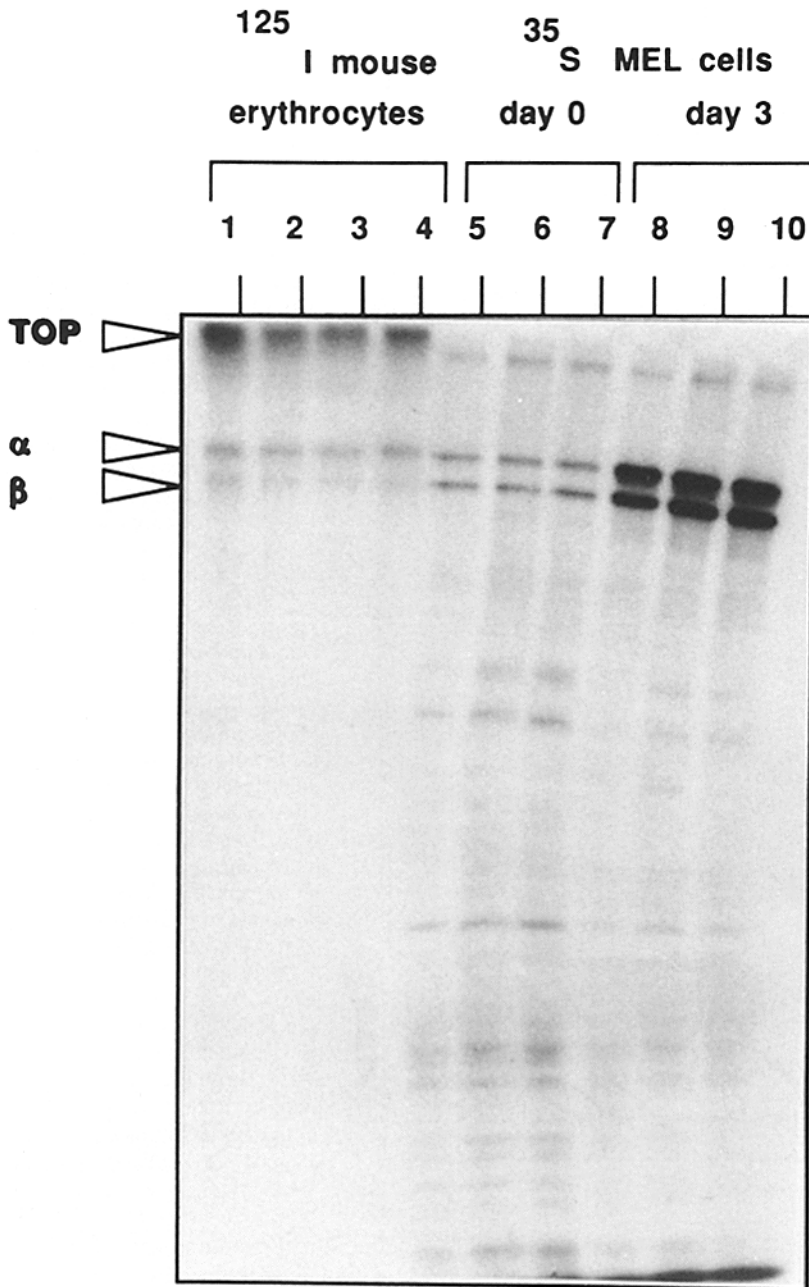
Spectrin Immunoprecipitates

Figure 2. Quantitative immunoadsorption of α and β spectrin from mouse erythrocyte membranes and undifferentiated and differentiated MEL cell proteins extracted with NP-40 and 8 M urea. ^{125}I-labeled erythrocyte ghosts and uninduced and 3-d differentiated MEL cells grown on fibronectin-coated dishes and labeled for 30 min with [^{35}S]methionine were first extracted with NP-40 and subsequently with urea (Procedure 1 in Materials and Methods). The combined detergent-urea extracts were denatured with SDS and processed for α and β spectrin immunoadsorption, electrophoresis, and fluorography, as described in Materials and Methods. Lanes *1–10*, α and β spectrin immunoadsorptions of extracts of the following: lanes *1* and *2*, 1.5 μg ^{125}I-labeled erythrocyte ghosts; lanes *3* and *4*, as lanes *1* and *2* with addition of 20 μg unlabeled protein from undifferentiated (lane *3*) and 3-d differentiated MEL cells (lane *4*); lane *5*, 20 μg [^{35}S]methionine-labeled proteins of uninduced MEL cells grown on fibronectin-coated dishes; lanes *6* and *7*, as lane *5*, with addition of 1.5 μg of unlabeled erythrocyte membrane proteins; lane *8*, 20 μg protein from [^{35}S]methionine-labeled MEL cells differentiated for 3 d on fibronectin-coated dishes; lanes *9* and *10*, as in lane *8*, with addition of 1.5 μg unlabeled erythrocyte membrane proteins.

antiserum recognized an additional polypeptide band, migrating between the α and β spectrin subunits, in only induced, not uninduced MEL cells (data not shown; see Fig. 2, lanes *8–10* and Fig. 3, lanes *10* and *11*). The antibody directed against the β spectrin chain recognized mouse red blood cell β spectrin as well as MEL cell β spectrin chains. Note that the amount of erythrocyte membrane protein loaded in Fig. 1, lane *5*, was ∼160-fold lower than the amount of MEL cell protein applied to lane *6*.

Since MEL cells coexpress both spectrin and the nonerythroid analog of spectrin, fodrin (Glenney and Glen-

ney, 1984), we wanted to make sure that the spectrin antisera did not cross react with mouse fodrin. In contrast to the antiserum directed against fodrin (Fig. 1, lane *9*) neither the α nor the β spectrin–specific antibodies recognized fodrin in SDS-extracted mouse 3T3 fibroblasts (Fig. 1, lanes *1* and *4*). The specificity of the fodrin antiserum was demonstrated by showing that it did not react with α and β spectrin in red blood cell membrane proteins (Fig. 1, lane *10*). The antibodies directed against murine erythrocyte band 3 reacted with equal specificity to the immunogen from erythrocyte or MEL cell extracts (lanes *7* and *8*). No preimmune serum

Spectrin Immunoprecipitates

^{125}I mouse erythrocytes

^{35}S MEL cells day 0   day 3

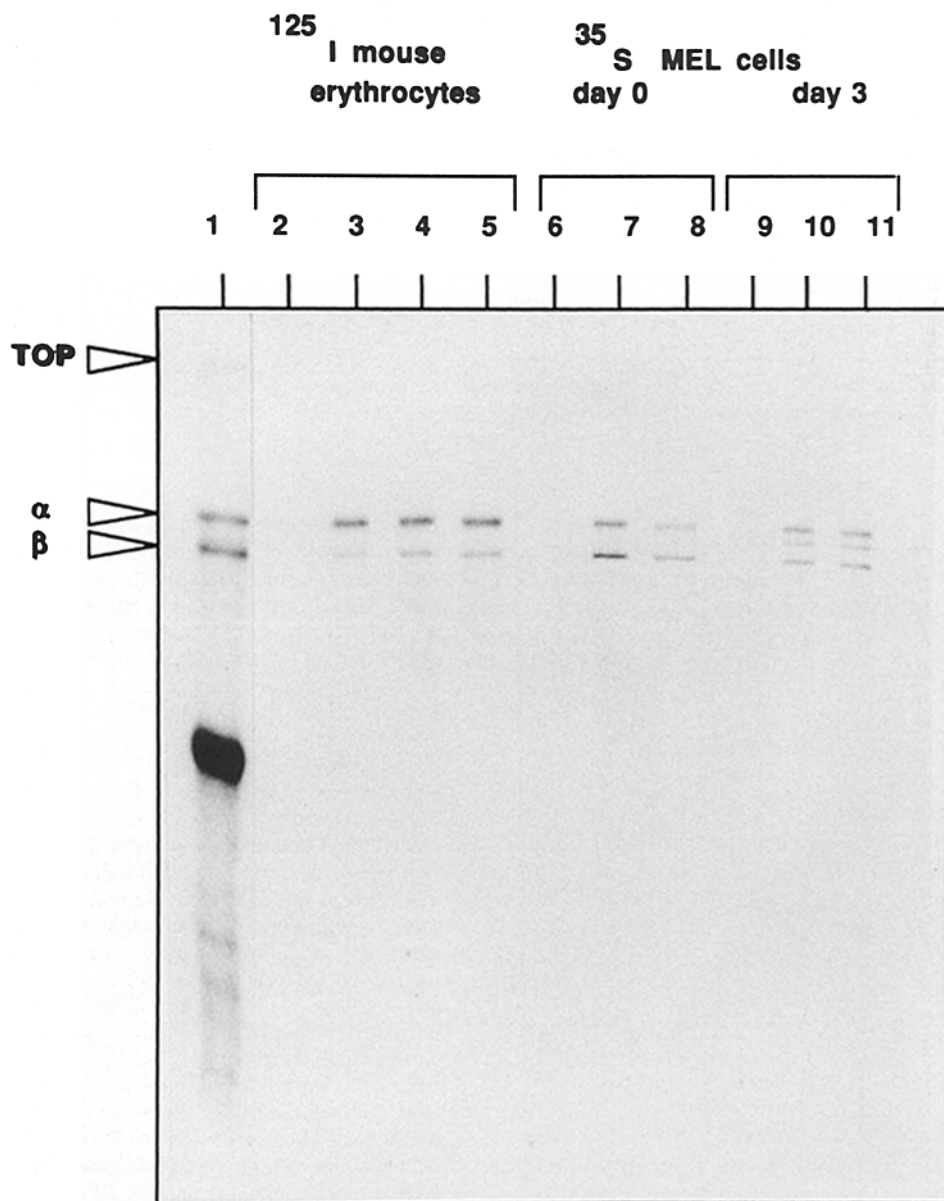1   2   3   4   5   6   7   8   9   10   11

TOP ▷

α ▷
β ▷

Figure 3. Quantitative immunoadsorption of α and β spectrin from mouse erythrocyte membranes and undifferentiated and differentiated MEL cell proteins after solubilization in gel sample buffer. ^{125}I-labeled erythrocyte ghosts and uninduced and 3-d differentiated MEL cells grown on fibronectin-coated dishes labeled for 30 min with [^{35}S]methionine were extracted with a solution of 4% SDS (Procedure 2 in Materials and Methods), boiled for 3 min, and processed for α and β spectrin immunoadsorption, electrophoresis, and fluorography, as described in Materials and Methods. Lane *1*, 120 ng ^{125}I-labeled erythrocyte ghosts. Lanes *3-5, 7, 8, 10*, and *11*, α and β spectrin immunoadsorptions of extracts of the following: lane *3*, 120 ng ^{125}I-labeled erythrocyte ghosts; lanes *4* and *5*, as lane *3*, with addition of 50 µg unlabeled protein from undifferentiated (lane *4*) and 3-d differentiated (lane *5*) MEL cells; lane *7*, 50 µg [^{35}S]-methionine-labeled proteins from uninduced MEL cells grown on fibronectin-coated dishes; lane *8*, as lane *7*, with addition of 10 µg of unlabeled erythrocyte membrane proteins; lane *10*, 50 µg protein from [^{35}S]-methionine-labeled MEL cells differentiated for 3 d on fibronectin-coated dishes; lane *11*, as in lane *10*, with addition of 10 µg unlabeled erythrocyte membrane proteins. Lanes *2, 6*, and *9*, preimmune control immunoadsorptions of the following: lane *2*, as in lane *3*; lane *6*, as in lane *7*; lane *9*, as in lane 10.

recognized any protein from either mouse erythrocyte, MEL cell, or 3T3 fibroblast extracts (data not shown).

Unequal Synthesis of α and β Spectrin during MEL Cell Differentiation

To determine the relative amounts of α and β spectrin synthesized by uninduced and 3-d induced MEL cells, we immunoprecipitated α and β spectrin from extracts of metabolically [^{35}S]methionine-labeled cells. An essential control is immunoprecipitation of erythrocyte spectrins.

In mammalian erythrocytes, both spectrin chains are present in equimolar amounts. As determined by Pfeffer et al. (1986), the molar percentages of tyrosine and methionine in each murine spectrin subunit are comparable (tyrosines: α subunit 3, β subunit 3.2; methioinines: α subunit 1.7, β subunit 1.4). Thus, labeling of cells with either ^{125}I-sodium iodide in vitro or [^{35}S]methionine in vivo results in incorporation of about equal amounts of the radioisotope in α and β spectrin polypeptides.

Undifferentiated (Fig. 2, lanes 5–7) and 3-d differentiated (lanes 8–10) MEL cells induced on fibronectin-coated dishes were labeled for 30 min with [^{35}S]methionine. After NP-40 and urea extraction, SDS denaturation of membrane and cytoskeletal proteins, and dilution of the SDS by a nonionic detergent, immunoreactive α and β spectrins were recovered by specific immunoadsorption and analyzed by SDS-gel electrophoresis. In uninduced MEL cells the ratio of [^{35}S]-methionine in α/β spectrin was 0.98 (Fig. 2, lane 5; Table I), and in induced cells it was 1.13 (Fig. 2, lane 8; Table I). However, we could not be sure that our antisera were immunoadsorbing α and β spectrins equally, even though the

Lanes	Apparent ratio of α/β spectrin	Normalized ratio of α/β spectrin
1 and 2	2.95	1
3	3.16	1.07
4	3.4	1.15
5	0.98	0.33
6 and 7	0.99	0.33
8	1.13	0.38
9 and 10	0.90	0.31

α and β spectrin (Fig. 2, lanes *1-10*) isolated by immunoadsorption as described in the legend of Fig. 2 were quantitated by densitometric scanning. The polypeptide migrating in between α and β spectrin was not included in the quantitation. The resulting apparent ratios of α/β spectrin in immunoprecipitations of mouse red blood cell ghosts, uninduced, and 3-d induced MEL cells were normalized to the ratio of α and β spectrin determined in immunoadsorptions of ^{125}I-labeled erythrocyte ghosts (ratio = 2.95; Fig. 2, lanes *1* and *2*). Lanes *1-10*, as in Fig. 2.

Lanes	Apparent ratio of α/β spectrin	Normalized ratio of α/β spectrin
3	2.78	1
4	2.3	0.82
5	2.4	0.86
7	0.79	0.28
8	0.83	0.30
10	1.00	0.36
12	1.07	0.38

α and β spectrin (Fig. 3, lanes *3-5, 7, 8, 10, 11*) isolated by specific immunoadsorption as described in the legend of Fig. 3 were quantitated by densitometric scanning. The polypeptide migrating in between both spectrin subunits was not included in the quantitation. The resulting apparent ratio of α/β spectrin was normalized to the ratio of α/β subunits in the immunoprecipitate of ^{125}I-labeled erythrocyte ghosts (ratio = 2.78; Fig. 3, lane *3*). Lanes *3-5, 7, 8, 10, 11*, as in Fig. 3.

amount of each antiserum used was optimal for recovery of both [^{35}S]methionine-labeled spectrin subunits. As expected, we observed that in total ghost extracts and in low ionic strength extracts (data not shown), both spectrin subunits were labeled equally with ^{125}I-sodium iodide by lactoperoxidase (Fig. 3, lane *1*; see Fig. 8, lane *1*). Spectrin immunoadsorption, however, yielded 2.95-fold more ^{125}I-labeled α than β spectrin (Fig. 2, lanes *1* and *2*).

To determine whether the two spectrin subunits were differentially immunoadsorbed, we mixed radiolabeled erythrocyte ghosts with unlabeled MEL cell membranes and unlabeled ghosts with ^{35}S-labeled MEL cells, extracted the mixtures with NP-40 and urea, and subsequently isolated α and β spectrin subunits by specific immunoadsorption. This procedure is equivalent to mixing ^{125}I-labeled ghosts and ^{35}S-labeled MEL cells, and counting ^{125}I and ^{35}S radioactivity in the α and β spectrin immunoprecipitates. Lanes *3* and *4* of Fig. 2 and Table I show that addition of unlabeled MEL cell membranes has no significant effect on the recovery of ^{125}I-labeled α and β spectrin chains, confirming our preliminary results that we are using appropriate amounts of antisera. Importantly, the recovery of ^{35}S-labeled α and β spectrins from uninduced (Fig. 2, lanes *6* and *7*) and 3-d induced (lanes *9* and *10*) MEL cells was unaffected by addition of unlabeled erythrocyte ghosts (Table I). Thus, we were able to use the recovery of α and β spectrins in lanes *1* and *2* of Fig. 2 to standardize the immunoprecipitation of ^{35}S-labeled MEL cell extracts. As shown in Table I, the normalized ratio of α/β spectrin synthesized in uninduced MEL cells is 0.33 (apparent ratio of ^{35}S-labeled α/β spectrin in uninduced MEL cells [0.98] divided by their apparent ratio in iodinated ghosts [2.95]) and in 3-d induced cells is 0.38 (1.13:2.95).

Since we could not rule out the possibility that some spectrin was aggregated at the top of the separating gel depicted in Fig. 2 and thus was excluded from our quantitation we devised a method to entirely solubilize all radioactively labeled proteins.

Erythrocyte ghosts labeled or not with ^{125}I-sodium iodide and uninduced and 3-d differentiated MEL cells labeled or not with [^{35}S]methionine were boiled immediately after addition of a solution of 4% SDS to the cell pellet. After the

dilution of the SDS with nonionic detergent, α and β spectrin were recovered by specific immunoadsorption. With this procedure, no aggregation of labeled proteins occurred at the top of the separating gel (Fig. 3). Immunoadsorption with preimmune sera did not precipitate any protein (Fig. 3, lanes *2, 6,* and *9*). Using saturating amounts of antibody, spectrin immunoprecipitates from ^{125}I-labeled erythrocyte ghosts contained 2.8-fold more α than β radioactivity, whether (Fig. 3, lanes *4* and *5*) or not (lane *3*) unlabeled MEL cells were added before dissolution in SDS. The original ^{125}I-ghosts contained, as expected, equal amounts of ^{125}I-radioactivity in α and β spectrin (Fig. 3, lane *1*). In ^{35}S-labeled uninduced MEL cells, a ratio of ^{35}S-radioactivity in α and β spectrin was ∼0.8:1.0, whether (lane *8*) or not (lane *7*) cold red cell ghosts were added. The corresponding ratio with ^{35}S-labeled 3-d induced cells was 1:1, again, whether or not unlabeled ghosts were added (lanes *10* and *11*). These data, summarized in Table II, confirm the results of Table I. The ratio of α to β spectrin synthesized in uninduced MEL cells (Fig. 3, lane *7*) is 0.28 (0.79:2.78) and in induced cells (Fig. 3, lane *10*) is 0.36 (1.0:2.78). We conclude that both uninduced and induced MEL cells synthesize about three times more β than α spectrin.

Differential Degradation of α and β Spectrin Subunits

Pulse–chase experiments were conducted to determine the stability of newly synthesized α and β spectrin chains in 3-d induced and uninduced MEL cells. Cells were biosynthetically labeled for 30 min with [^{35}S]methionine and subsequently chased for up to 48 h. Total cellular extracts were prepared by a combination of NP-40 and urea solutions, and immunoadsorbed with the spectrin antisera as described above.

In uninduced cells, which contain a significantly lower amount of spectrin than do mature red blood cells (Eisen et al., 1977; Pfeffer and Redman, 1981; Glenney and Glenney, 1984; Pfeffer et al., 1986; Patel and Lodish, 1987), newly made α and β spectrin subunits are both degraded with first-order kinetics and with the same half-life, ∼10 h (Figs. 4 *B* and 5 *B*).

A
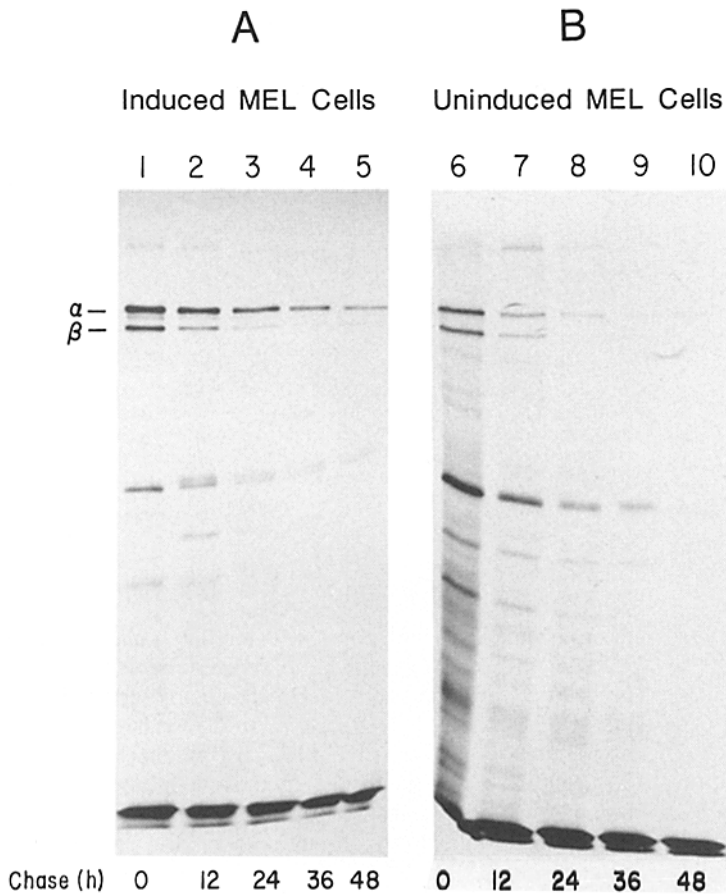### Induced MEL Cells

B
Uninduced MEL Cells

Figure 4. Differential degradation of α and β spectrin. MEL cells induced on fibronectin-coated dishes for 3 d (*A*) or uninduced cells (*B*) were labeled for 30 min with [^{35}S]methionine and chased for 0 (lanes *1* and *6*), 12 (lanes *2* and *7*), 24 (lanes *3* and *8*), 36 (lanes *4* and *9*), and 48 h (lanes *5* and *10*). Cells were extracted with NP-40 and urea and extracts were processed for α and β spectrin immunoadsorption, electrophoresis, and fluorography, as described in Materials and Methods. Samples in *A* and *B* were processed at the same time. The additional bands of molecular mass lower than spectrin were not present in other experiments that yielded the same ratio of α/β spectrin and similar half-lives for both subunits, and therefore do not seem to affect the results.

On day 3 of induction, when spectrin expression in MEL cells is maximal (Eisen et al., 1977; Pfeffer and Redman, 1981), newly synthesized α spectrin is degraded with an approximate $t_{1/2}$ of 22 h (Figs. 4 *A* and 5 *A*). In contrast, the $t_{1/2}$ for β spectrin is ∼8 h. Both subunits are degraded with first-order kinetics. This finding of differential turnover of the two spectrin subunits was confirmed in a number of experiments, each carried out in duplicate or triplicate.

Stability of Band 3

In uninduced cells we can not detect synthesis or presence of band 3 by immunoblot analysis or immunoadsorption with specific antibodies (data not shown). On day 3 of differentiation, pulse–chase experiments with labeled MEL cells reveal that newly synthesized band 3 is stable in contrast to α and β spectrin (Fig. 5 *A*).
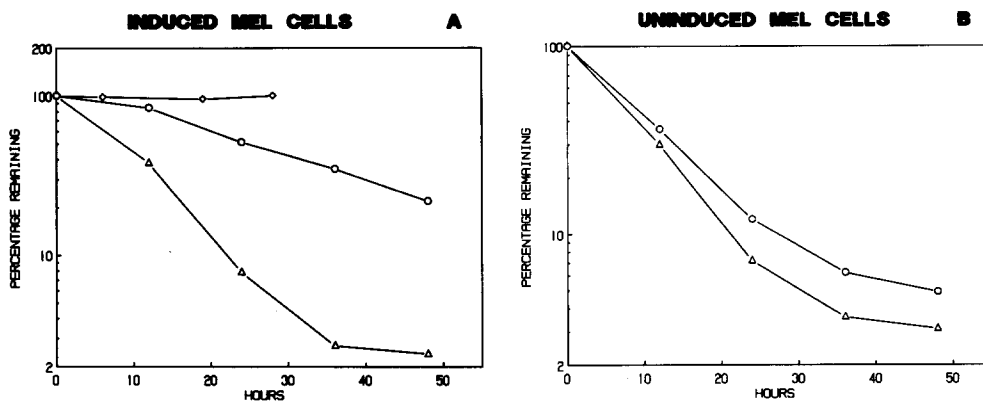

Figure 5. Differential stability of α and β spectrin and of band 3. 3-d differentiated (*A*) and undifferentiated (*B*) MEL cells induced on fibronectin-coated dishes were processed for α and β spectrin immunoadsorption at various times after a 30-min labeling with [^{35}S]methionine. The fluorographs presented in Fig. 4 were analyzed by densitometry. To analyze band 3 stability, MEL cells on day 3 of differentiation were labeled and extracted as in Fig. 4. The amount of band 3 purified by specific immunoadsorption 0, 6, 19, and 28 h after labeling was quantitated by densitometry (*A*). Data are expressed as percent of band 3 and α and β spectrin present at *t* = 0 h vs. time. A different experiment was used for band 3 and for spectrin, but the same results were obtained in two other repeats of this experiment. o, alpha spectrin; △, beta spectrin; ◇, band 3.

```
        A              B              C              D
      Actin        α Spectrin     β Spectrin      Band 3

    1  2  3  4    5  6  7  8    9  10  11  12    13  14  15  16
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Days of
Differentiation   0 2 4 6   0 2 4 6   0 2 4 6   0 2 4 6
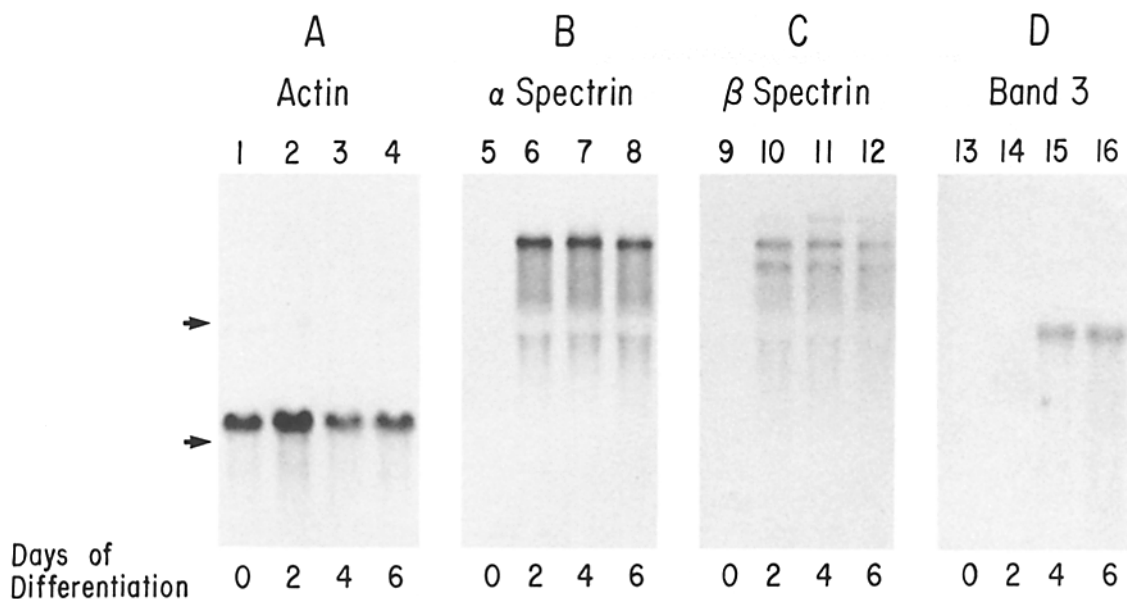
Figure 6. Northern blot analysis of total cellular RNA from MEL cells hybridized with in vitro transcribed cRNA probes for actin (*A*), α spectrin (*B*), β spectrin (*C*), and band 3 (*D*). RNA was prepared from MEL cells induced on fibronectin-coated dishes for 0 (lanes *1, 5, 9, 13*), 2 (lanes *2, 6, 10, 14*), 4 (lanes *3, 7, 11, 15*), and 6 d (lanes *4, 8, 12, 16*). In all cases, ~10 µg of total cellular RNA were loaded. The location of the 28-S and 18-S ribosomal bands as relative size markers is identified by the arrows at the left of the figure.

mRNA Levels during MEL Cell Differentiation

To examine the accumulation of mRNAs during the course of MEL cell differentiation, total cellular RNA was prepared from cells induced on fibronectin-coated dishes for 0 (uninduced), 2, 4, and 6 d and subjected to Northern blot analysis. The level of actin mRNA remained relatively constant throughout differentiation (Fig. 6 *A*). In contrast, α and β spectrin mRNAs were barely detectable in uninduced cells, but could be detected at day 2 through day 6 of differentiation (Fig. 6, *B* and *C*). Hybridization with the β spectrin-specific probe detected three distinct mRNA species (Fig. 6 *C*). The mRNA of intermediate size was predominant, and the levels of the three bands increased synchronously. Band 3 message could first be detected on day 4 of differentiation (Fig. 6 *D*).

To standardize for variabilities in amounts of RNA loaded, the relative amounts of band 3 and α spectrin and the intermediate-sized β spectrin message determined by densitometry were normalized to those of actin mRNA. As depicted in Table III the relative amounts of α and β spectrin mRNAs increased up to day 4 of differentiation and remained constant thereafter. In contrast, band 3 mRNA (Table III) was not detectable at day 2 but reached its steady state level by day 4.

Biogenesis of Erythroid Membrane Proteins during Differentiation in the Mouse

We used an indirect method to study biogenesis of erythrocyte membrane proteins during erythroid differentiation in mouse bone marrow, and compared the results with those obtained with MEL cells.

Murine red blood cell membrane proteins observed by SDS-PAGE are very similar to their human counterparts. The identity of band 3, as well as of ankyrin, α and β spectrins, and protein 4.1 on our SDS-gel system was confirmed by either immunoblot analysis or by immunoadsorption with

specific antibodies (data not shown). Human actin comigrated with mouse actin whereas purified human protein 4.1 was of slightly slower electrophoretic mobility than its murine homologue (data not shown).

To study the major erythrocyte membrane proteins we prepared ghosts, membrane vesicles, and low ionic strength extracts of ghosts from equal parts of each blood sample. This procedure enabled us to separate α and β spectrin and actin, in the low salt extract, from ankyrin and band 3, which were retained in the membrane vesicle fraction.

To study the biosynthesis of murine erythrocyte membrane proteins in vivo we used the protocol of Chang et al. (1976). One nonanemic mouse was injected with 2 mCi of [^{35}S]methionine. Erythroid cells at all stages of differentiation in the bone marrow incorporate the label. Cells labeled at a more advanced developmental stage will enter the peripheral blood at an earlier time than those cells labeled at an early stage of the differentiation process. By taking blood samples from the animal at various times after the initial injection, the appearance of radiolabeled proteins in the circulation can be followed. The earlier a radiolabeled protein is incorporated

Table III. Relative mRNA Levels for α and β Spectrin and Band 3 during MEL Cell Differentiation

Day of induction	α Spectrin/actin	β Spectrin/actin	Band 3/actin
0	0	0	0
2	43	54	0
4	100	100	100
6	100	100	100

The RNA blots presented in Fig. 6 were analyzed by densitometry. We determined the amounts of α spectrin, the intermediate-sized β spectrin, and band 3 mRNAs relative to the amount of actin mRNA. The ratios were normalized setting the maximal value to 100.
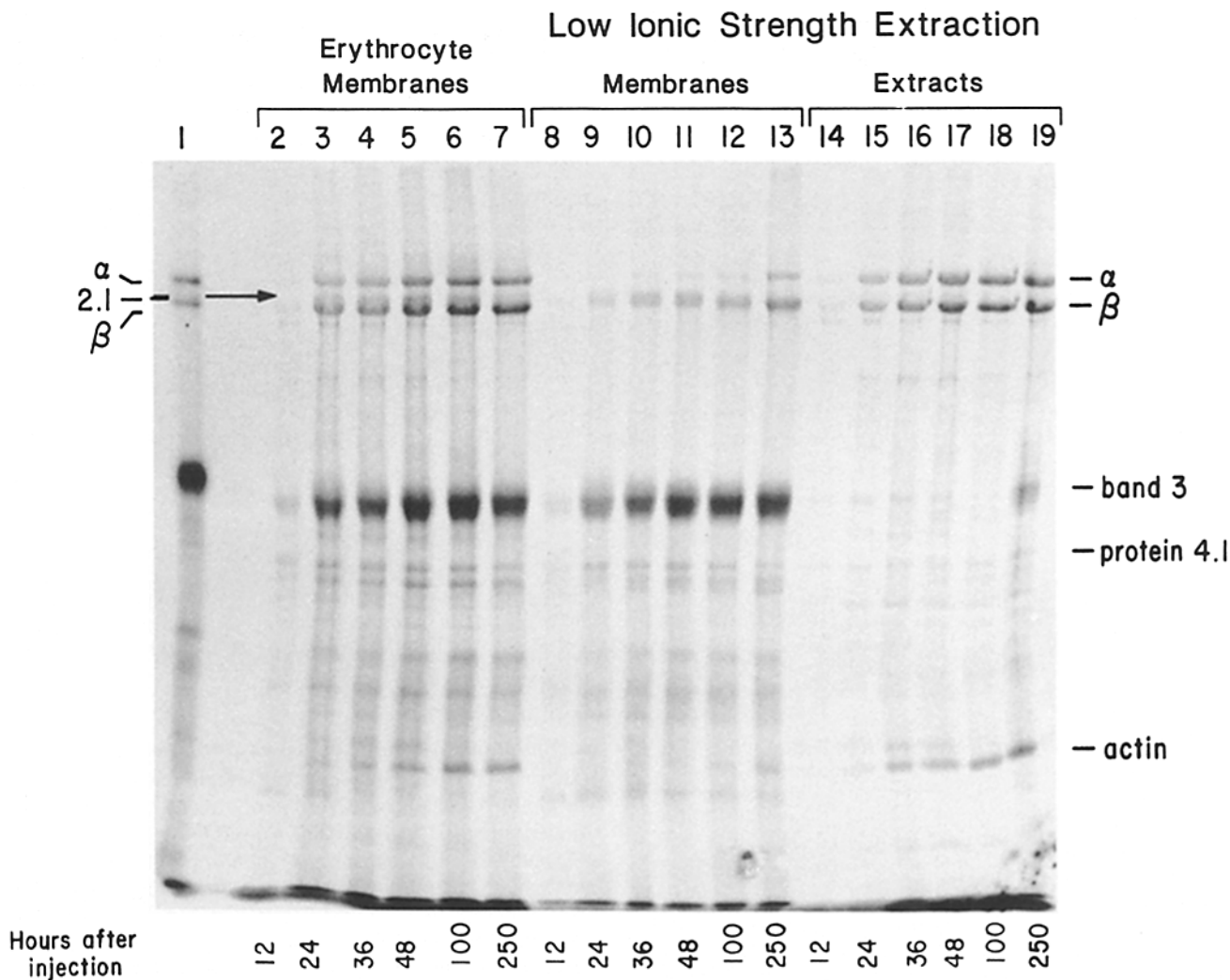
Figure 7. Time course of appearance of [^{35}S]methionine labeled erythrocyte proteins in circulating red blood cells after in vivo labeling of a mouse with 2 mCi [^{35}S]methionine. *Lane 1,* ^{125}I-labeled mouse erythrocyte ghosts used as molecular mass standard. Lanes *2–19,* proteins derived from 5 × 10^7 [^{35}S]methionine-labeled mouse erythrocytes, as follows: lanes *2–7,* erythrocyte ghosts; lanes *8–13,* membranes after incubation of ghosts in low ionic strength buffer; lanes *14–19,* corresponding low ionic strength extracts of ghosts. Protein samples were prepared and processed for SDS-PAGE and fluorography after 12 (lanes *2, 8, 14*), 24 (lanes *3, 9, 15*), 36 (lanes *4, 10, 16*), 48 (lanes *5, 11, 17*), 100 (lanes *6, 12, 18*), and 250 h (lanes *7, 13, 19*) after injection of the [^{35}S]methionine. The fluorograph was exposed for 10 d. The position of the major erythrocyte membrane proteins are indicated.

into circulating erythrocytes, the later is its accumulation during erythropoiesis.

The fluorograph shown in Fig. 7 represents a series of six erythrocyte samples taken at different times after the initial injection of [^{35}S]methionine. As expected, labeled actin and spectrin are present in low salt extracts of ghosts (Fig. 7, lanes *14–19*), while ankyrin, band 3, and most of protein 4.1 are found associated with the membrane vesicles (Fig. 7, lanes *8–13*). By only 12 h after the injection, radioactivity has been incorporated into all membrane proteins of circulating red blood cells (Fig. 7, lanes *2, 8, 14*). Incorporation reaches a maximal level after 4 d, when cells that were at very early stages of erythroid differentiation at the time of [^{35}S]methionine incorporation have entered the circulation (Fig. 7).

To determine the relative radioactivity in each individual protein, the amount of ^{35}S-labeled protein was normalized to total protein as determined by protein staining before fluorography (Fig. 8). The amount was normalized to the amount of the radiolabeled protein present at 100 h after injection. After 24 h, more than half of the maximal level of radioactivity in protein 4.1 and band 3 has been achieved (Fig. 8 *B*), whereas the two spectrin subunits, ankyrin, and actin reach the half-maximal level of specific labeling between ∼36 and 50 h (Fig. 8, *A* and *B*). As confirmed in three independent experiments, band 3 and protein 4.1 accumulate to a half-maximal level ∼12 h earlier than spectrin in circulating red blood cells and therefore are synthesized on the average 12 h later during erythroid differentiation in the bone marrow than are the two spectrin subunits.
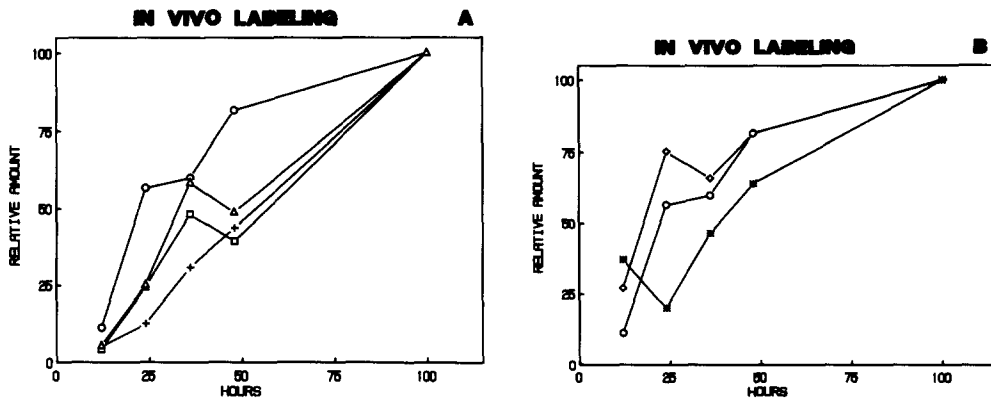
Figure 8. Increase in relative specific amounts of erythrocyte membrane proteins in 5 × 10⁷ circulating erythrocytes over time after in vivo labeling of a mouse with 2 mCi of [³⁵S]methionine. The fluorograph shown in Fig. 7 and the corresponding Coomassie Brilliant Blue–stained gel were analyzed by densitometry. The specific activities of [³⁵S]methionine-labeled α and β spectrin (Fig. 7, lanes *14–19*), band 3 (lanes *8–13*), and ankyrin (lanes *8–13; A* in this figure) as well as protein 4.1 (Fig. 7, lanes *8–13* and *14–19*) and actin (lanes *14–19; B* in this figure) were calculated as the ratio of radioactivity in the protein to the amount of the Coomassie Brilliant Blue–stained protein. These ratios were normalized setting the value of the 100-h timepoint at 100. △, alpha spectrin; □, beta spectrin; ○, band 3; +, ankyrin; ◇, protein 4.1; *, actin.

Discussion

The extracellular matrix protein fibronectin promotes terminal differentiation and enucleation of MEL cells (Patel and Lodish, 1987). During the course of differentiation, the synthesis of the membrane-cytoskeletal proteins ankyrin, spectrin, and band 3 is induced, whereas the loss of the surface fibronectin receptor results in detachment of the late erythroblast and enucleated cells from the fibronectin matrix (Patel and Lodish, 1986, 1987). As shown by Patel and Lodish (1987), uninduced cells contain only one-fifth the amount of α and β spectrin compared to an equal number of mature erythrocytes. After 4 d of differentiation on fibronectin-coated dishes, MEL cells accumulate amounts of spectrin comparable to those in red blood cells and maintain these levels through day 7 (enucleation). In contrast, the amount of band 3, relative to that in mature erythrocytes, increases from 3% in uninduced to ~50% in 4-d differentiated MEL cells and reaches maximal levels between ~80–98% after 7 d of induction (Patel and Lodish, 1987).

This pattern of protein accumulation is extended by our mRNA blot analyses (Fig. 6 and Table III). In uninduced cells α and β spectrin mRNAs are barely detectable. The mRNA levels increase through day 4 of induction and remain constant thereafter. We do not know the nature of the three transcripts detected with the β spectrin probe; one or more might code for a β spectrin–related protein. As for MEL cells induced in suspension culture (Ngai et al., 1984), actin mRNA levels are relatively constant throughout differentiation on fibronectin-coated dishes. In contrast, band 3 mRNA is first detected on day 4 of differentiation and remains at that level through day 6 (Fig. 6 and Table III), extending results of Kopito and Lodish (1985 *b*) and Fraser and Curtis (1987) on cells induced in suspension culture.

Our principal result is that both uninduced MEL cells and cells induced 3 d on a fibronectin-coated surface synthesize about three times as much β than α spectrin (Figs. 2 and 3, Tables I and II). Cells induced in suspension culture for 3 d are reported to synthesize a large excess of β spectrin relative to α spectrin (Glenney and Glenney, 1984) or a slight excess of ~15–30% more β than α spectrin (Pfeffer et al., 1986).

The observed differences among MEL cell clones may be due to culture and labeling conditions, clonal variation, or antisera used. Control immunoprecipitation of iodinated erythrocyte membrane proteins with α and β spectrin-specific antibodies yields about three times more α than β spectrin (Figs. 2 and 3, Tables I and II), despite saturating amounts of antibodies. This result corroborates the observations that spectrin antisera often have antibodies of lower reactivity directed against the β than those directed against the α subunit, reflected in a severalfold weaker signal for immunoblotted β spectrin as compared to α spectrin (Glenney and Glenney, 1984; Bodine et al., 1984; Patel and Lodish, 1987). These observations point out the difficulties in quantitating total amounts of protein after immunoadsorption with specific antibodies with different reactivities. We feel that our results are accurate since we included an internal control of ¹²⁵I-labeled erythrocyte ghosts to normalize for unequal recovery of α and β spectrin subunits during immunoprecipitation, and also since we used two independent protocols (Figs. 2 and 3) for solubilizing and immunoprecipitating cytoskeletal proteins, with the same result. We cannot, however, exclude the possibility that iodination of erythrocyte membrane proteins might affect the relative immunoprecipitation of α and β spectrin chains. To affect our results any such putative alterations would have to preferentially affect one of the two spectrin subunits in such a way that polyclonal antisera would fail to recognize it.

A second new result is that both α and β spectrin subunits are unstable, and the stability changes during differentiation. In uninduced MEL cells, the small amounts of both α and β spectrin subunits synthesized are degraded with first-order kinetics and a $t_{1/2}$ of ~10 h (Figs. 4 *B* and 5 *B*). In other experiments (not shown) spectrin chains synthesized in uninduced cells remain also unstable ($t_{1/2}$ ~ 8 h) even if the cells are subsequently induced to differentiate by DMSO. However, in 3-d induced cells (as well as in 4-d induced cells, not shown) which express spectrin at a maximal level (Eisen et al., 1977; Pfeffer and Redman, 1981; Patel and Lodish, 1987) the subunits are differentially degraded: the half-lives of α and β spectrin chains are ~22 and 8 h, respectively (Figs. 4 *A* and 5 *A*).

Since between day 4 and day 7 of differentiation the level of spectrin remains constant and comparable to that in mature erythrocytes (Patel and Lodish, 1987), and since spectrin is synthesized until late stages of MEL cell differentiation (data not shown), there ought to be continued synthesis and degradation of both subunits. We conclude, therefore, that most of the spectrin present at the time of enucleation has been synthesized during the last 2 d of differentiation.

The observation of differential degradation is consistent with unequal synthesis of α and β spectrins: a ratio of α to β spectrin synthesis of $\sim$1:3 together with an $\sim$3-fold higher turnover of β spectrin could result in accumulation of equimolar amounts of both polypeptides in mature red blood cells. It is difficult to be more precise about the rates of accumulation of α and β spectrin chains since we do not know the absolute rate of α and β polypeptide synthesis throughout differentiation.

We do not know the nature of the polypeptide of higher molecular mass than β spectrin recognized by the β spectrin–specific antibodies in only induced, not uninduced, MEL cells (Fig. 1, lane 6). It is conceivable that differentiating cells specifically express a β spectrin–related protein, or a different isoform of the β subunit not present in erythrocytes, as already suggested by Eisen et al. (1977). This protein may be an erythrocyte-related subtype of spectrin, similar to the one described in mouse neural cells (Riederer et al., 1986).

We are unable to follow the stability of spectrins synthesized by MEL cells induced on fibronectin-coated dishes for more than 5 d. The heterogeneous population of MEL cells becomes very fragile during the last 2 d of differentiation and some of the cells lyse when washed repeatedly. We presume, however, that the spectrin subunits are stabilized near the end of differentiation, at the time when band 3 accumulates to its maximal level and the cells enucleate. It is important to recall that MEL cells are transformed by virus, and that the implications of this on membrane biogenesis are not understood. In induced MEL cells spectrin has been localized not only at the plasma membrane but also near complex vacuoles that are associated with virus (Pfeffer et al., 1986). The fragility of the cells might be due to a defective membrane–skeleton assembly in MEL cells.

In vivo labeling of mice has enabled us to follow the appearance of the major membrane cytoskeletal proteins after an initial injection of [35S]methionine. Cells at all stages of development incorporate the label. Since the most mature cells in the bone marrow will enter the circulation first, their labeled proteins represent those that are made last by the erythroid cell. In contrast to earlier observations made by Chang et al. (1976) we find that all major membrane proteins are labeled in circulating erythrocytes only 12 h after the initial injection (Fig. 7). In the earlier experiments only two polypeptides, designated band 4.1 and 4.2, and traces of band 3 were labeled after 12 h. We do not understand the reasons for the slight differences in the temporal appearance of radiolabeled proteins in the circulating red blood cells. We observed coordinate accumulation of radiolabeled α and β spectrin subunits, ankyrin, and actin during several days after injection of label (Figs. 7 and 8). The half-maximal level of accumulation of α and β spectrin and actin is achieved $\sim$36 h and that of ankyrin $\sim$50 h. Band 3 and protein 4.1, however, reach the half-maximal level earlier, by 24 h, and

therefore must have been synthesized on the average at a later stage of development than α and β spectrin, actin, and ankyrin.

All major membrane proteins are synthesized until very late stages of erythroid differentiation in the bone marrow, as indicated by their appearance in erythrocyte membrane samples as early as 12 h after injection (Fig. 7). Moreover, Bodine et al. (1984) have demonstrated spectrin synthesis and assembly in a population of early reticulocytes and some nucleated erythroid cells from phenylhydrazine-treated mice, and β spectrin cDNAs have been cloned from a human reticulocyte library (Prchal et al., 1987).

These conclusions are consistent with our results on differentiating MEL cells, but this cell system allows us to look at much earlier events in differentiation than those easily accessable in vivo. During MEL cell differentiation spectrin is synthesized earlier than band 3. Due to the rapid degradation of the two spectrin chains, spectrin synthesized by cells at an early developmental stage will be degraded long before these cells enucleate and emerge as reticulocytes. The observed 12-h time difference in vivo for accumulation of 35S-radioactivity in band 3 relative to spectrins, in circulating erythrocytes (Figs. 7 and 8), is probably an underestimate of the time difference in the onset of their syntheses in the bone marrow. In MEL cells, spectrin synthesis is initiated $\sim$2 d earlier than that of band 3, but continuous synthesis and degradation of both spectrin subunits results in accumulation in the most mature cells of only those spectrin chains that were synthesized shortly before the onset of band 3 synthesis.

Hanspal and Palek (1987) reported that nucleated rat red cell precursors from spleens of phenylhydrazine-induced anemic animals synthesize $\sim$4.4 times more α than β spectrin, and that all spectrin subunits soluble in Triton X-100 are degraded within a matter of minutes. Even though these cells are normal, nontransformed precursor cells, the questionable integrity of the erythroid membrane and skeleton after treatment with phenylhydrazine, the protracted isolation procedure used for red cell precursors, and the limited viability of the cells during culture all point out the problems of this cell system. The results of Hanspal and Palek (1987), however, are in good agreement with those obtained with nucleated avian erythroid cells.

Biogenesis of membrane-skeletal proteins has been extensively studied in chicken embryo erythrocytes (Lazarides and Moon, 1984; Moon and Lazarides, 1983, 1984). In contrast to the MEL cell system (Glenney and Glenney, 1984; Pfeffer et al., 1986), avian erythroid cells synthesize 2–3 times more α than β spectrin (Blikstad et al., 1983). Integration of spectrins into the cytoskeleton renders the proteins stable, whereas the polypeptides present in the cytoplasmic pool are rapidly catabolized (Moon and Lazarides, 1984). The half-lives of unassembled (detergent soluble) forms of α and β spectrin in this system are $\sim$2 h and $\sim$15–20 min, respectively (Woods and Lazarides, 1985). The discrepancy between the results in rat and chicken erythroid cells and those obtained with MEL cells may be due to the fact that MEL cells are virus-transformed cells. Equal recovery of erythrocyte α and β spectrin by specific immunoadsorption, however, can not be assessed in any previous study, since internal controls for different reactivities of α and β spectrin antibodies were not included.

Woods et al. (1986) describe simultaneous expression of α and β spectrin, ankyrin, and protein 4.1 in avian erythroblastosis virus and S13 virus-transformed chicken erythroid progenitor cells before the initiation of band 3 expression. Asynchrony of expression of the peripheral membraneskeletal proteins and band 3 in the avian system is in agreement with our work with MEL cells. However, we never observe the extremely rapid degradation of spectrin found in avian cells and, importantly, MEL cells synthesize an ~3-fold excess of β spectrin, not α spectrin.

In preliminary studies we showed that in 3-d induced cells ~50% of newly made α and β spectrin are extracted with 1% Triton X-100; the balance remains in the putative cytoskeletal pellet (data not shown). Similarly, when labeled cells are Dounce homogenized and membranes are separated by equilibrium gradient centrifugation, about half of the newly made spectrin associates with membranes. This proportion does not change during a 48-h chase period. Since any unstable or metastable complexes between the cytoskeleton and the plasma membrane may not be detected using these procedures, we are not certain that they adequately resolve "unattached" and "cytoskeletal" spectrins. We therefore prefer not to speculate whether only unattached spectrin is degraded. Furthermore, since at least 90% of the α and β spectrin sythesized in 3- and 4-d induced cells is eventually degraded (Figs. 4 A and 5 A), any cytoskeletal spectrin must also be catabolized eventually, at least in these immature cells.

Maximal band 3 levels may determine the long term stability of spectrin. It is conceivable that band 3 might serve as an integral membrane "receptor" for the spectrin–actin network (Moon and Lazarides, 1984). Furthermore, the membrane-skeleton may be linked to the integral membrane protein glycophorin via protein 4.1 (Gahmberg et al., 1978; Sarris and Palade, 1979; Anderson and Lovrien, 1984), to the (Na⁺+K⁺)ATPase via ankyrin (Nelson and Veshnock, 1987), or to another yet unidentified membrane component. Such situation(s) may explain the linkage of spectrin–actin complexes to the membrane of uninduced MEL cells in the absence of band 3.

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