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Research Article

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Differentiation-associated Switches in Protein 4.1 Expression

Synthesis of Multiple Structural Isoforms during Normal Human Erythropoiesis

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Abstract

Erythroid differentiation is accompanied by dramatic alterations in morphology and membrane mechanical properties resulting, in large part, from reorganization of the membrane skeletal protein network. The 80-kD protein 4.1 is an important organizational component of this membrane skeleton. Recently, it has been recognized that multiple structural isoforms of 4.1 are encoded by a single gene via alternative pre-mRNA splicing, and that an upstream ATG can be spliced in and used for translation of high molecular weight 4.1. We are exploring the hypothesis that differentiation-associated switches in protein 4.1 structure play an important role in membrane reorganization.

To study changes in 4.1 gene expression during normal human differentiation, we analyzed 4.1 protein and mRNA structure at various developmental stages. Using immunofluorescence microscopy, we observed high molecular weight 4.1 isoforms in preproerythroblasts producing punctate, predominantly cytoplasmic staining with a perinuclear area of intense fluorescence, while mature red cells expressed very little high molecular weight 4.1. Isoforms containing an alternatively expressed 102-nucleotide exon near the COOH terminus were abundant in both preproerythroblasts and mature cells but produced a punctate distribution of fluorescence over the entire preproerythroblast and intense membrane-associated fluorescence in the erythrocyte. Characterization of RNA by polymerase chain reaction and nuclease protection assays revealed a differentiation-associated switch in pre-mRNA splicing in the spectrin-actin binding domain. Since this domain plays a critical role in regulating membrane material properties, we speculate that this switch may be crucial to reorganization of the skeletal network during erythropoiesis. We conclude that 4.1 isoforms are differentially expressed and differentially localized during erythropoiesis, and that this isoform family is likely to have diverse functions during terminal differentiation. (*J. Clin. Invest.* 1993. 91:329–338.) Key words: red blood cell • erythropoiesis • protein 4.1 • alternative splicing • differentiation

Introduction

During terminal differentiation, erythroid progenitors undergo multiple structural changes involving cytoplasmic organelles, skeletal and cytoskeletal proteins, and cell surface components. Although specific aspects of erythroid maturation and remodeling (such as transferrin receptor down regulation and asynchronous synthesis of membrane proteins) have been studied, many unanswered questions remain (1–6). We have previously reported that a dramatic change in membrane mechanical properties occurs during the final stages of erythroid development, the 72-h period during which the reticulocyte matures into a red cell. (7) We found that young, immature reticulocyte membranes were markedly less deformable and mechanically more unstable compared to membranes of mature red cells. Since membrane skeletal protein organization regulates the membrane properties of deformability and stability (8–10), we interpreted these data as signifying a marked reorganization of the membrane skeleton during reticulocyte maturation.

The erythroid membrane skeleton is a latticework composed of spectrin, actin, ankyrin, tropomyosin, adducin, and proteins 4.1 and 4.9, which underlies the lipid bilayer and is associated with it through protein–protein and protein–lipid interactions. Together, the proteins comprising this lattice play a crucial role in supporting the bilayer and in determining the membrane mechanical properties of deformability and stability (8–10). The 80-kD erythrocyte protein 4.1 is an important organizational component of the membrane skeleton because it participates in horizontal associations within the skeletal network by facilitating spectrin–actin interactions and it participates in vertical associations between the network and the lipid bilayer by binding to the cytoplasmic domain of the integral proteins, glycophorin C and band 3 (11–16). Recently, it has been recognized that multiple structural isoforms of protein 4.1 are encoded by a single gene via alternative pre-mRNA splicing (17–20). Included in this isoform family are proteins that vary from one another in the functionally important spectrin-actin binding domain, as well as higher molecular weight species with extended NH_2 termini that initiate translation within an alternatively spliced upstream exon. We are exploring the hypothesis that differentiation-associated switches in protein 4.1 structure play an important role in the membrane reorganization that accompanies erythroid terminal differentiation.

To study changes in 4.1 gene expression during normal human differentiation, we analyzed 4.1 protein and mRNA structure at various developmental stages. Using immunofluorescence microscopy, we observed high molecular weight 4.1 isoforms in preproerythroblasts producing punctate, cytoplasmic staining with a perinuclear area of intense fluorescence, while mature red cells expressed very little high molecular weight 4.1. Isoforms containing an alternatively expressed 102-nucleotide (nt) exon near the COOH terminus were abundant in both preproerythroblasts and mature cells but produced a punctate distribution of fluorescence over the entire preproerythroblast and intense membrane-associated fluorescence in the erythrocyte. Characterization of RNA by polymerase chain

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reaction and nuclease protection assays revealed a differentiation-associated switch in pre-mRNA splicing in the spectrin-actin binding domain. Since this domain plays a critical role in regulating membrane material properties, we speculate that this switch may be crucial to reorganization of the skeletal network during erythropoiesis. We conclude that 4.1 isoforms are differentially expressed and differentially localized during erythropoiesis, and that this isoform family is likely to have diverse functions during terminal differentiation.

Methods

Antibodies. Polyclonal monospecific antibodies to synthetic peptides corresponding to alternatively expressed regions of protein 4.1 were generated by immunizing rabbits with peptides coupled to bovine thyroglobulin. Antibodies were affinity purified using Affi Gel 10 (Bio-Rad Laboratories, Richmond, CA). Rabbit polyclonal antibodies to α-spectrin were generously provided by Dr. Schrier, Stanford University, (Stanford, CA).

Monoclonal antibodies used for the preproerythroblast purification procedure included the following: OKla (anti-human HLA-DR), OKT3 (anti-human T cells), and OKB-Calla (anti-human CALLA antigen) (Ortho Diagnostic Systems, Roissy, France); HPCA (anti-CD34) (Becton Dickinson Immunocytometry Sys., Mountain View, CA); B1 (anti-B cells) (Coulter Immunology, Hialeah, FL); CH3 (anti-colony forming unit–granulocyte macrophage [CFU-GM][1] and granulocytes), CS4 (anti-glycophorin A), and CK26 (specific for lymphoid and monocytic cells) have been described previously (21) and were generously provided by Dr. Berthier (INSERM U217, Grenoble, France); K5 (anti-human HLA-DR) was a gift from Dr. Fermand (INSERM U108, Hôpital St. Louis, Paris, France).

Cells. Preproerythroblasts were obtained from normal human bone marrow by negative selection using an avidin-biotin immune rosetting technique as previously described (21). In brief, after informed consent, bone marrow was obtained at the time of hip replacement surgery and the low-density mononuclear cells collected by Ficoll-Hypaque (1.077 g/cm^3) (Eurobio, Paris, France) separation. Plastic adherent cells were removed by incubation in 75 cm^2 flasks for 1 h at 37°C. The nonadherent mononuclear cells (0.5–3×10^8) were depleted of mature granulocytes by pelleting out granulocytes which had phagocytozed sheep red blood cells coupled to avidin. The remaining cells (4×10^7 cells/ml) were then incubated with an excess of the following monoclonal antibodies: OKla, CALLA, CS4, B1, K5, HPCA, CH3, CK26, NKH-1, Leu 4, and Leu 5b, antibodies to lineage-specific antigens on CFU-GM, myeloid precursors, T and B cells, macrophages, monocytes, red cells, and platelets. The antibody-coated cells were removed by centrifugation after rosetting with biotin-conjugated goat anti-mouse IgM and IgG and sheep red blood cells conjugated to avidin. The nonrosetting cells were recovered and characterized by immunophenotyping, electron microscopy, and by their ability to form colonies on methylcellulose colony assays. On the basis of this characterization, the purified population was 80–90% erythroid and the distribution within the purified erythroid population was 15% colony forming unit–erythroid, 75% preproerythroblasts, and 10% proerythroblast.

Pure populations of differentiated erythroblasts (> 90% purity) were obtained by plucking individual erythroid colonies derived from primitive normal marrow burst forming unit–erythroid (BFU-E) grown 5, 8, 12, and 14 d in methylcellulose colony assays in the presence of erythropoietin and leukocyte-conditioned medium as previously described (21). Cells were washed twice and incubated overnight in the presence of erythropoietin to eliminate plastic-adherent macrophages. Although there was heterogeneity in the maturation stage of cells even within a single colony, most were late erythroblasts (polychromatophilic or acidophilic) after 12–14 d of culture.

MOLT-4 cell cultures were maintained in RPMI 1640 medium with 10% FCS.

Immunofluorescence microscopy. For immunofluorescence microscopy, cells were layered or cytocentrifuged onto poly-L-lysine-coated slides, fixed and permeabilized in acetone in dry ice, and then incubated sequentially in primary antibody and in FITC-conjugated goat anti-rabbit IgG (Cappel Laboratories, Cohranville, PA). For DNA staining, the cells were incubated in propidium iodide (Molecular Probes, Inc., Eugene, OR) at 0.25 μg/ml for 1 min and then washed with PBS, pH 7.5.

Western blot analysis. Cells were solubilized in 100 mM Tris pH 6.8, 200 mM DTT, 4% SDS, 0.2% bromophenol blue, and 20% glycerol, then heated at 100°C for 5 min. Proteins were then separated using a discontinuous system described by Laemmli (22) on slab gels composed of a 8.5% polyacrylamide separating gel and a 3.5% polyacrylamide stacking gel. After separation, proteins were electrophoretically transferred from the polyacrylamide SDS gels to nitrocellulose membrane as previously described by Burnette (23). Unbound reactive sites were blocked by incubation in 5% milk, 0.02% Tween and 20 mM Tris pH 7.5 for 1 h at room temperature. The membrane was then incubated in affinity-purified polyclonal rabbit antibodies in 0.5% milk, 0.02% Tween, and 20 mM Tris pH 7.5 for 1 h at RT and washed three times in 0.5% milk, 0.02% Tween and 20 mM Tris. This was followed by incubation in goat anti-rabbit IgG conjugated to horseradish peroxidase, in 0.5% milk, 0.02% Tween, and 20 mM Tris for 1 h at RT. The membrane was washed three times as above and incubated for 1 min at RT in enhanced chemiluminescence horseradish peroxidase detection reagents (Amersham Corp., Arlington Heights, IL). Finally, the membrane was exposed to x-ray film to detect fluorescence.

Amersham Rainbow protein molecular weight markers were used to obtain approximate molecular masses (±10%) by graphing relative mobility versus the log of molecular mass.

RNA isolation. Reticulocyte mRNA was isolated as previously described from blood of patients with sickle cell anemia undergoing exchange transfusion (24). RNA from preproerythroblasts and BFU-E-derived erythroblasts was prepared using the method of Laski and colleagues (25).

PCR amplification of protein 4.1 RNA. Total reticulocyte RNA was transcribed into single stranded DNA at 37°C for 120 minutes in a 50-μl reaction (2 μg RNA, 100 ng random hexanucleotide primer, 100 U murine Moloney lev virus reverse transcriptase, 10 U RNasin Promega, Madison, WI) in 40 mM KCl, 50 mM Tris-HCl (pH 8.3), 8 mM $MgCl_2$, and 0.5 mM dNTPs. 10 μl of cDNA was amplified in a 100-μl PCR reaction containing Taq polymerase buffer (50 mM KCl, 10 mM Tris-HCl, 1.5 mM $MgCl_2$, 0.1% gelatin), 5 U of Taq polymerase (Cetus Corp., Berkeley, CA), dNTPs to a final concentration of 0.2 mM, and 50 pmol of sense- and antisense-strand oligonucleotides. The six sets of oligonucleotides used are described in Table I.

Thirty cycles of amplification were performed using an automated thermal cycler (Perkin-Elmer Cetus Instruments, Norwalk, CT) under the following conditions: denaturation for 30 s at 94°C, renealing for 30 s at 50–55°C, extension for 1 min and 45 s at 72°C. The amplification products were analyzed by 5% PAGE.

Nuclease protection assays. To quantitate mRNA of various protein 4.1 isoforms at different stages of erythroid maturation, mRNA levels were measured using a nuclease protection assay. For these studies, a fragment of DNA containing the exon of interest was inserted into Bluescript II SK± (Stratagene, La Jolla, CA). After sequencing to determine the orientation of the fragment within the plasmid, the DNA was linearized by digestion with AvaII. Antisense RNA probes were synthesized by incubation of 0.2–1.0 μg linearized plasmid template DNA in a total volume of 20 μl containing 5× transcription buffer (Promega), 100 mM DTT, 20 U RNasin (Promega), 2.5 mM each of ATP, GTP, and CTP, 50 μCi α[^{32}P]rUTP, 5–10 U T7 RNA polymerase (Promega), and diethyl pyro-carbonate-treated water. The mixture was incubated for 60 min at 15°C and then incubated for 15 min at 37°

1. *Abbreviations used in this paper:* BFU-E, burst forming unit-erythroid; CFU-GM, colony forming unit–granulocyte macrophage; MEL, mouse erythroleukemia (cells); nt, nucleotide; PCR, polymerase chain reaction.

Table I. Oligonucleotides Used for PCR Amplification

	Sense strand	Antisense strand
I.	CAAAACAGACCCATCTTTGGATCTTCATTC (525-554)*	TGTAAAATTCCAAGGGACACCACGAACCTG (1037-1008)
II.	GAAGAAAGCCCTCAATC (744-760)	TGTAAAATTCCAAGGGACACCACGAACCTG (1037-1008)
III.	CCATTTGGGATAACGC (948-964)	ACTTTTTGGCATTCTC (1365-1350)
IV.	GAAGAGAAGGTCATGGAACTGCATAAGTCA (1278-1307)	GGGCCGAGGACTTCGGTCTGCCGAATCGACAGC (1862-1830)
V.	GCTGTCGATTCGGCAGACCGAAGTCCTCGGCCC (1830-1862)	TCCTGTGGGGATTTGCCCATTGATGTTAAG (2324-2295)
VI.	CTTAACATCAATGGGCAAAT (2295-2314)	AATACGTGTCTCTGAATCC (2594-2575)

* Numbers in parenthesis indicate the location within the protein 4.1 sequence as published by Conboy et al. (20).

after addition of 3 μg RNase-free DNase. The sample was then electrophoresed on a 4% acrylamide/7 M urea gel and the probe eluted from the excised band by 60-min incubation at 50°C in 0.5 M sodium acetate (pH 5.0) and 1 mM EDTA. These RNA probes were then hybridized to RNA prepared from preproerythroblasts, BFU-E–derived erythroblasts, reticulocytes, and MOLT-4 cells. For hybridization 1×10^5 cpm of probe and 2–5 μg RNA were ethanol precipitated together and then incubated overnight at 50°C in 10 μl of 3 vol formamide with 1 vol 4× hybridization buffer (1.6 M NaCl, 80 mM Tris, pH 7.0, 4 mM EDTA and 0.4% SDS). The samples were then digested for 30 min at RT in 75 μg ribonuclease A and 150 U ribonuclease T_1 in 300 mM NaCl, 10 mM Tris, pH 7.5, and 5 mM EDTA. After an incubation of 15 min at 37°C in 50 μg proteinase K and 10% SDS, the protected fragments were phenol extracted, ethanol precipitated, and analyzed on a 4% polyacrylamide/7 M urea gel.

Results

Protein 4.1 was initially characterized in mature red cells as an 80-kD protein composed of four structural domains: an NH_2-terminal 30-kD glycophorin binding domain, a 16-kD domain, an 8-kD spectrin-actin binding domain, and a COOH-terminal 22–24 kD domain (Fig. 1) (26). However, recent studies show that at least seven distinct peptide sequences within the 80-kD 4.1 protein can be variably expressed by alternative splicing of the 4.1 pre-mRNA (20). In addition, a fifth domain of 209 aa, which we refer to as the NH_2-terminal extension, can be encoded in a population of mRNAs that splice in an upstream ATG around nt 100 (19, 20). Using immunofluorescence microscopy with antipeptide antibodies, we examined the expression during terminal erythroid differentiation of isoforms containing this NH_2-terminal extension. For a detailed characterization of the differentiation-associated alternative splicing events controlling structure of the 80-kD 4.1 polypeptide, we examined RNA species by reverse transcriptase/polymerase chain reaction (PCR) and quantitated mRNA isoforms by nuclease protection assay.

Expression of isoforms with NH_2-terminal extension. For these studies, we prepared affinity-purified antibodies to two synthetic peptides: a 20-amino acid peptide that is unique for the amino terminal extension (residues 96–115); and a 34-amino acid peptide from the alternatively expressed 102-nt exon in the 22–24 kD carboxy-terminal region (Fig. 1). The presence or absence of the alternatively expressed protein sequences during erythroid differentiation was initially evaluated by performing immunofluorescence microscopy on mature red cells and erythroid progenitors isolated from human bone marrow (Fig. 2 *A*). Very immature erythroid precursors in the preproerythroblast stage of maturation were obtained by negative selection using an avidin-biotin immune rosetting technique. When these cell populations were examined with antibody to the 34-amino acid insert within the 22–24-kD domain (anti 24-2), a punctate distribution of fluorescence was present in the preproerythroblast, while intense membrane-associated fluorescence was observed in mature red cells (Fig. 3). When preproerythroblasts were probed with antibody to the isoform characterized by an elongated amino terminus (anti N-1) there was punctate, predominantly cytoplasmic, fluorescence with an area of intense fluorescence apparently adjacent to the nucleus (Fig. 3). However, no fluorescence was observed in mature red cells examined with this antibody. Thus, isoforms con-

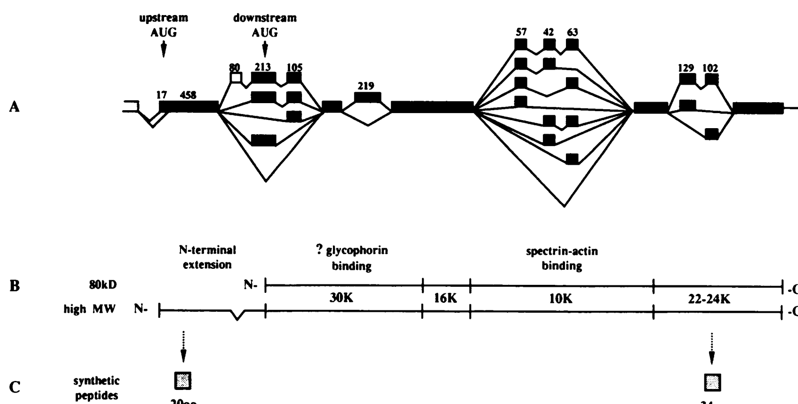

Figure 1. Schematic diagrams of protein 4.1 mRNA, protein and synthetic peptide structure. (*A*) Alternative splicing map of mRNA depicting the multiple splicing possibilities among the 4.1 alternative exons. □, noncoding exon; ■, alternative coding exon; ■, constitutive exon. (*B*) Protein 4.1 structure depicting the high molecular weight form produced by initiation of translation at the upstream AUG and the 80-kD form produced by initiation of translation at the downstream AUG. The chymotryptic fragments of 4.1 include the 30-kD glycophorin binding domain, the 16-kD polypeptide, the 10-kD spectrin-actin binding domain, and the 22–24-kD polypeptide. (*C*) Synthetic peptides of 20 amino acids from the NH_2-terminal extension and 34 amino acids from the 102-nt exon within the 22–24-kD domain used as immunogens for antipeptide antibody production.

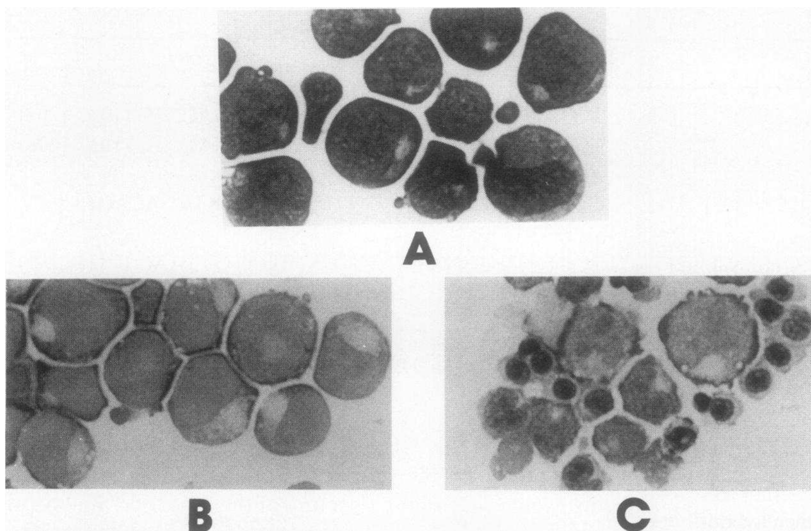

Figure 2. Micrographs of erythroid progenitors. Very immature erythroid precursors in the preproerythroblast stage of maturation obtained from normal human marrow by negative selection using avidin-biotin immune rosetting (*A*). Erythroblasts derived from normal marrow BFU-E cultured for 8 d (*B*) and 12 d (*C*). Cells were stained with Wright Giemsa.

taining the 34-amino acid insert were present in both preproerythroblasts and mature red cells, while isoforms containing the amino terminal extension were abundant in preproerythroblasts but not in mature red cells. Moreover, the cellular localization of the anti N-1 staining isoforms appeared different from that of the anti 24-2 staining isoforms.

To characterize 4.1 isoform expression in greater detail, erythroblasts at multiple time points during erythropoiesis were examined by immunofluorescence microscopy and Western blot analysis. BFU-E–derived erythroblasts, cultured for 5 and 8 d and probed with antibody 24-2, demonstrated staining similar to that observed in the preproerythroblasts with a diffuse punctate pattern that was predominantly cytoplasmic (Fig. 4 *b* and *c*). To determine whether the absence of protein 4.1 on the plasma membrane of these erythroblasts could be caused by lack of membrane assembly of spectrin, the cells were probed with an antibody to α-spectrin. As shown in Fig. 4 *h* and *i*, plasma membrane associated fluorescence was already present in erythroblasts cultured for 5 d implying that by day 5, spectrin was assembled on the plasma membrane and suggesting that the lack of membrane assembly of 4.1 isoforms could not be attributed to the absence of spectrin. With antibody N-1 staining, BFU-E derived erythroblasts cultured for 5 d contained a body of intense fluorescence similar to that observed in the preproerythroblasts (Figs. 3 *b* and 4 *e*). To study the relationship of this fluorescent body to the nucleus, nuclear staining with propidium iodide was performed. As shown in Fig. 4 *d*, the region of intense anti N-1 staining clearly localized to the cytoplasm. To determine whether there was a differentiation-associated change in expression of isoforms containing the amino-terminal extension, 8-d erythroblasts were probed with anti N-1. In contrast to preproerythroblasts and 5-d erythroblasts, the 8-d progenitors did not contain any cytoplasmic region of intense fluorescence (Fig. 4 *f*). These immunofluorescent data clearly show that protein 4.1 isoforms are differentially expressed during erythropoiesis. Furthermore, the observed differences in cellular localization of the 4.1 isoforms suggest multiple functions for this protein family during erythroid differentiation.

Immunoblotting was performed to further analyze the structure of the protein 4.1 isoforms containing the alternatively expressed peptides (Fig. 5). Four distinct bands of ~ 135 kD, ~ 124 kD, ~ 72 kD, and ~ 69 kD were detected by anti N-1 in day 8 and day 12 erythroblasts. To further characterize these bands, the erythroblast proteins were stained with antibody 24-2. This antibody detected the ~ 72-kD band, as well as an additional band of ~ 78 kD. These data suggest that the day 8 and day 12 progenitors express an ~ 72 kD isoform that splices in both the 102-nt exon (from the 22–24-kD domain) and the amino terminal extension. In addition, the differentiating erythroblasts express three isoforms that splice in the amino-terminal extension but not the 102-nt exon, as well as one isoform that splices in the 102 nt exon but not the amino terminal extension.

Analysis of 80-kD 4.1 mRNA species. To characterize alternative splicing events within the coding domain of the 80-kD protein that occur during terminal erythroid differentiation, we performed reverse transcriptase/PCR assays. Using six pairs of oligonucleotide primers that spanned the entire coding domain of the 80-kD polypeptide, we amplified cDNA from four erythroid maturational stages by PCR. The RNA for these studies was prepared from preproerythroblasts, BFU-E–derived erythroblasts cultured for 8 and 12 d and reticulocytes. cDNAs were transcribed from RNA, then amplified by polymerase chain reaction, and analyzed on polyacrylamide gels (Fig. 6). The NH_2-terminal 30-kD glycophorin binding domain includes two alternatively spliced exons, one containing 105 nucleotides and the other 219 nucleotides. In reticulocytes, both exons are spliced in to the majority of 4.1 mRNAs (20). In this experiment, oligonucleotide primers flanking the 105 exon amplified a single PCR product representing the +105 phenotype from cDNA of all four developmental stages. Similarly, the PCR products amplified from all four stages represented the +219 phenotype, indicating that no differentiation-associated splicing differences occur in the protein 4.1 30-kD domain. No alternative splicing has been previously documented in the 16-kD domain, and as anticipated, PCR products of 585 nucleotides were amplified from the cDNAs of all four maturational stages. However, a major differentiation-associated change in splicing of the spectrin-actin binding domain was observed. Within this domain, the 63-nucleotide exon was deleted in both preproerythroblast mRNA and BFU-E-derived erythro-

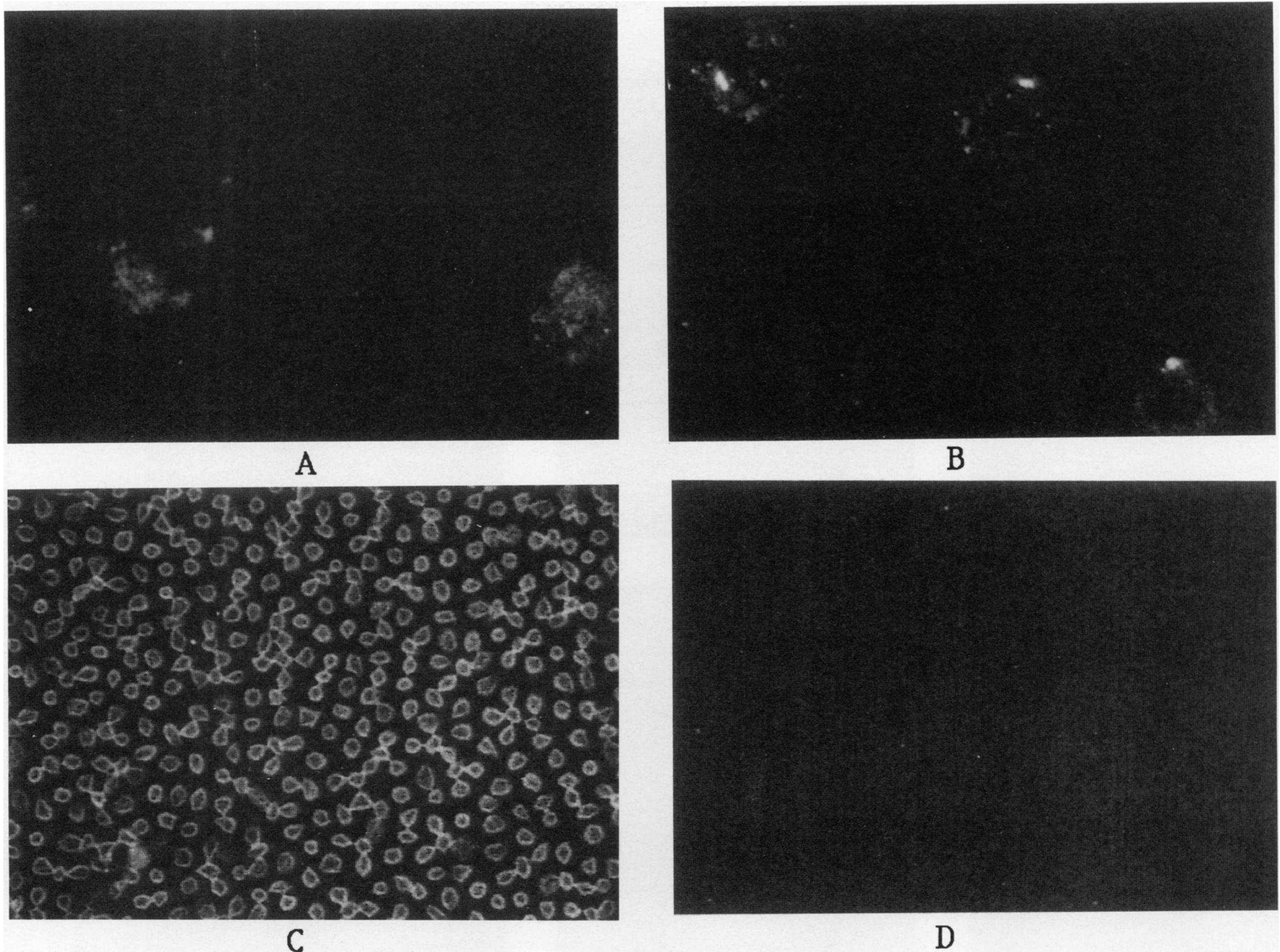

Figure 3. Immunofluorescent micrographs of preproerythroblasts and mature red cells probed with antipeptide antibodies. Preproerythroblasts examined with anti 24-2 (*A*) showed a punctate fluorescent pattern while mature red cells (*C*) exhibited intense membrane-associated fluorescence. Preproerythroblasts probed with anti N-1 (*B*) contained an intensely fluorescent body not present in mature red cells (*D*), which had no fluorescent signal. Thus, spliced variants of protein 4.1 were differentially expressed and differentially localized during erythropoiesis.

blasts cultured for 8 d. Messenger RNA from BFU-E-derived erythroblasts cultured for 12 d expressed both the +63 and −63 species, while reticulocyte RNA contained only the +63 species. To complete the analysis of the 80-kD polypeptide, we amplified a region containing two alternatively-spliced exons within the carboxy-terminal coding domain using one set of oligonucleotide primers. Messenger RNA from all four maturational stages generated a similar set of three bands: a 300-nucleotide fragment containing both the 129- and 102-nt exons, a 198-bp band deleting the 102-nt exon, and a 171-bp band deleting the 129-nt exon. These results indicate that no differentiation-associated splicing changes occur in the carboxy-terminal domain of protein 4.1.

To analyze in greater detail the differentiation-associated splicing switch involving the spectrin-actin binding domain, we quantitated the percentage of +63 and −63 mRNA species using nuclease protection assays. For these studies, a fragment of DNA containing the 63-bp exon was inserted into Bluescript plasmid and antisense RNA probes synthesized. In this assay system, antisense mRNA containing the 63-nucleotide exon will generate a single large fragment upon hybridization to mRNA containing this exon (+63 phenotype). mRNA that lacks the 63-nucleotide exon will generate two smaller protected fragments, as seen in the MOLT-4 lymphoblast RNA control (−63 phenotype). In preproerythroblasts ~ 99% of the protected fragment was −63, in BFU-E–derived erythroblasts about 50% was −63 and 50% was +63, and in reticulocytes > 99% was +63 (Fig. 7). These data imply that the splicing switch from the −63 species to the +63 species occurs late in erythropoiesis as the cells differentiate into polychromatophilic and acidophilic erythroblasts. From the PCR and nuclease protection assays, we conclude that during erythroid differentiation, the splicing pattern of the glycophorin binding domain is invariant, while that of the spectrin-actin binding domain varies.

To characterize the expression of protein 4.1 isoforms containing the spectrin-actin binding domain encoded by the 63-nucleotide exon, immunoblotting was performed. Protein from equivalent numbers of 12-, 13-, and 14-day BFU-E–derived erythroblasts was probed with an antipeptide antibody to the 21 amino acids encoded by the 63-nucleotide exon. As shown in Fig. 8, the expression of an isoform containing the spectrin-actin binding domain increased as the erythroid progenitors progressed along the differentiation pathway.

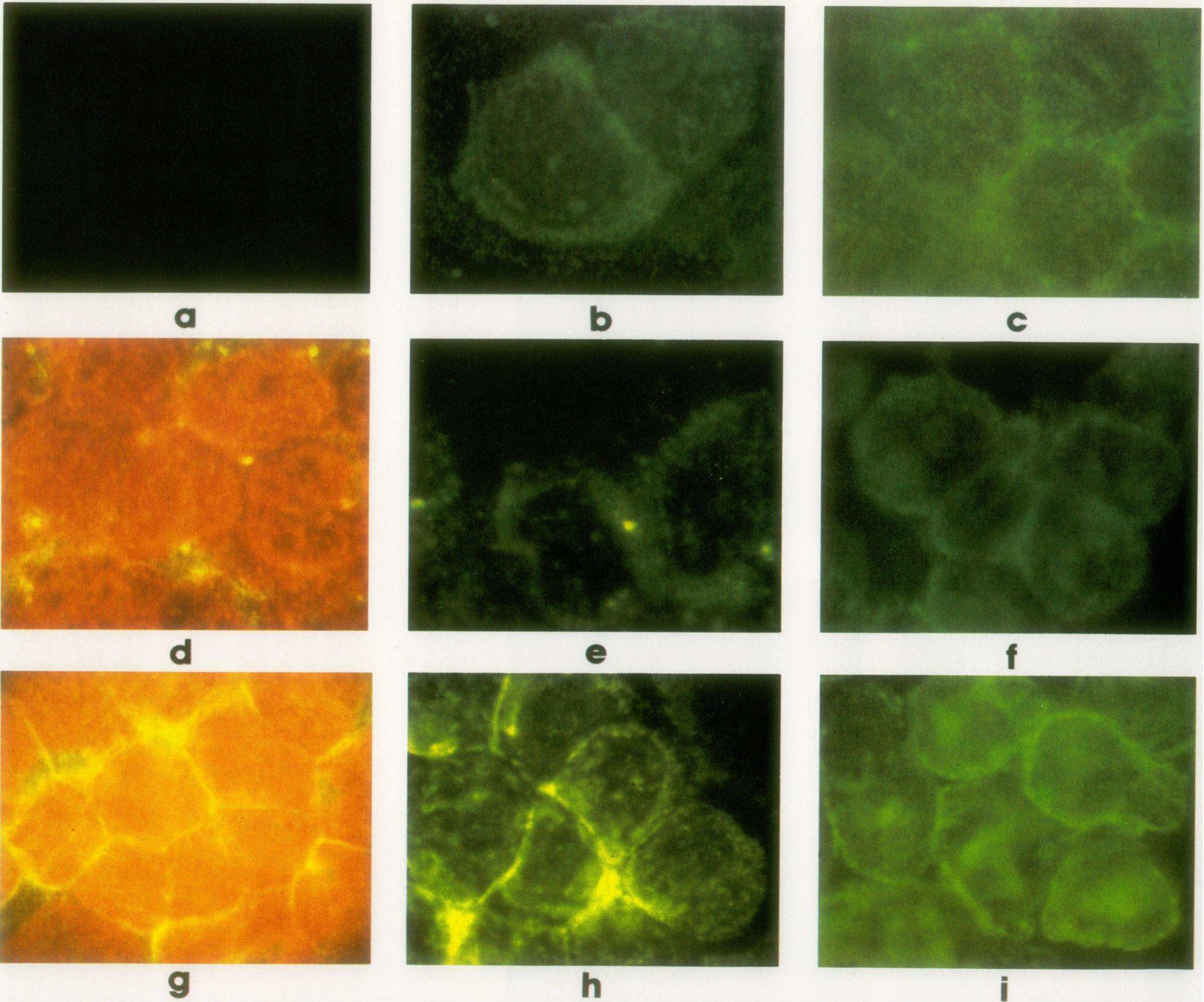

Figure 4. Immunofluorescent micrographs of erythroid progenitors probed with antipeptide antibodies. Preimmune Ig staining of BFU-E–derived erythroblasts cultured for 5 d (*a*) produced no fluorescent image. In contrast, antibody 24-2 staining of BFU-E–derived erythroblasts cultured for 5 d (*b*) and 8 d (*c*) showed a diffuse punctate pattern of fluorescence which localized predominantly to the cytoplasm. 5-d erythroblasts probed with antibody N-1 (*e*) contained a dense fluorescent body that was localized to the cytoplasm by counterstaining the nucleus with propidium iodide (*d*). This intensely staining body was not present in 8-d erythroblasts (*f*). Plasma membrane associated fluorescence was detected with anti-spectrin Ig in 5-d erythroblasts also stained with propidium iodide (*g*) and without propidium iodide (*h*) and in 8-d erythroblasts (*i*).

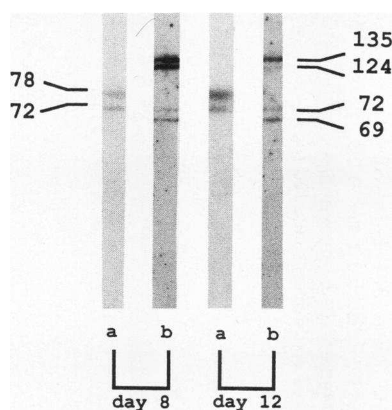

Figure 5. Western blot analysis of protein 4.1 isoforms during differentiation. Day 8 and day 12 erythroblast proteins were probed with antibody 24-2 (*a*) and antibody N-1 (*b*). Polypeptides of ~ 78 kD and ~ 72 kD reacted with anti 24-2 in both day 8 and day 12 erythroblasts, implying that two protein 4.1 isoforms expressing the 102-nt exon (from the 22–24-kD domain) are present in these progenitors. Four polypeptides of ~ 135 kD, ~ 124 kD, ~ 72 kD and ~ 69 kD reacted with anti N-1 in both day 8 and day 12 erythroblasts, implying that four isoforms containing the amino-terminal extension are expressed in day 8 and day 12 erythroblasts.

Discussion

Dramatic contrasts exist between circulating, nonadherent erythrocytes and their adherent progenitors within the bone marrow. To traverse the microcirculation, a mature erythrocyte must undergo repeated and extensive passive deformation, requiring a highly deformable membrane. In addition, the mature cell membrane must be both mechanically stable, so that the cell can resist fragmentating under normal circulatory fluid stresses, and also possess a distribution of negative charge and surface component characteristics that render it nonadherent. In contrast, the functional requirements of the plasma membrane of the committed erythroid progenitor are vastly different. These progenitors engage in cell–cell and cell–extracellular matrix adhesion through expression of receptors for matrix components, such as the fibronectin receptors, VLA-4 and VLA-5, and a sialyated macrophage receptor (27–32). In addition, the progenitor membrane facilitates endocytosis with, for example, transferrin receptor recycling (33–35), while the membrane of mature red cells does not undergo extensive receptor-mediated endocytosis.

A wealth of evidence has accumulated from studies of pathologic erythrocytes and biochemically perturbed normal red cells indicating that membrane properties of deformability and mechanical stability are regulated by skeletal protein organization (8–10). In previous studies, we observed that membranes of immature reticulocytes in the stage immediately after nuclear extrusion were tenfold less deformable and mechanically stable than mature erythroid membranes, but that during the period of reticulocyte maturation, there was a marked increase in membrane deformability and acquisition of mechanical stability (7). These observed changes in membrane properties imply extensive membrane reorganization during reticulocyte maturation. We hypothesize that changes in skeletal protein expression may play a critical role in the membrane reorganization that accompanies erythroid terminal differentiation. This process of reorganization could involve either quantitative and/or qualitative changes in skeletal protein components.

In this study, we have clearly documented a qualitative change in expression of a skeletal protein during erythropoiesis by showing two differentiation-associated switches in 4.1 gene expression. We observed that isoforms containing the NH_2-terminal extension are expressed in large amounts in preproerythroblasts and not in more mature cells. This switch involves the use of a different ATG site for initiation of protein synthesis. While the early erythroblasts use both the upstream and downstream ATG, the reticulocytes appear to use the downstream ATG almost exclusively. It is interesting to note that many nonerythroid cells, such as T and B lymphocytes, as well as fibroblasts, use both the upstream and downstream ATG for initiation of translation. In this context, we have recently documented the expression of 4.1 isoforms in lymphocytes and fibroblasts of an individual with hereditary elliptocytosis whose mature red cells lack 4.1 as the result of a deletion of the exon which includes the downstream ATG (36).

Although the function of the NH_2-terminal domain is not yet well characterized, our immunofluorescent data suggest that 4.1 isoforms containing this domain do not localize to the plasma membrane, and it therefore seems unlikely that these isoforms provide structural integrity to the membrane. Marchesi and colleagues have performed immunofluorescence in lymphocytes with a recombinant fusion protein in *Escherichia coli* that expresses the NH_2-terminal extension (37). They observed interactions of this fusion protein with intranuclear structures, including centrioles and midbodies, and conclude that the protein plays a role in the mitotic process. We speculate that the intense area of fluorescence in preproerythroblasts may be localized to the centriole that is present in the perinuclear area in preproerythroblasts (38). This cellular localization also suggests a role for the high molecular weight isoforms of 4.1 in mitosis.

The second switch in 4.1 gene expression involves the functionally important spectrin-actin binding domain. Preproerythroblasts and BFU-E–derived erythroblasts cultured for 8 d express little of the 63-nt exon within this domain. However, expression of the 63-nt exon is activated during differentiation with 12-d erythroblast mRNA composed of 50% +63 species and 50% −63 species. Once the activation has occurred increasing quantities of expressed protein can be demonstrated by immunoblotting in 12-, 13-, and 14-d erythroblasts. Thus, expression of a 4.1 isoform with an intact spectrin-actin binding domain appears to be a very late event during erythropoiesis. This functional binding domain mediates the formation of the structurally important ternary complex of spectrin, actin and protein 4.1. Biochemical conditions that inhibit the formation of the ternary complex result in alterations in membrane mechanical properties. For example, red cells from certain patients with hereditary spherocytosis contain a mutant spectrin that leads to decreased spectrin-4.1 binding, and decreased membrane mechanical stability (39). Similarly, abnormal stability is observed in normal red cells treated with 2,3-diphosphoglyceric acid, a polyphosphate known to dissociate the spectrin-actin-4.1 complex (10). In addition, deletion of the spectrin-actin binding domain in protein 4.1 of mature red cells results in marked membrane mechanical instability and hereditary elliptocytosis (40). On the basis of these observations in pathologic red cells and biochemically perturbed normal cells, we believe that activation of the expression of the 63-nt exon may play a critical role in the assembly of a mechanically stable membrane during the final stage of erythropoiesis. Alternative splicing of the 63-nt exon has functional signifi-

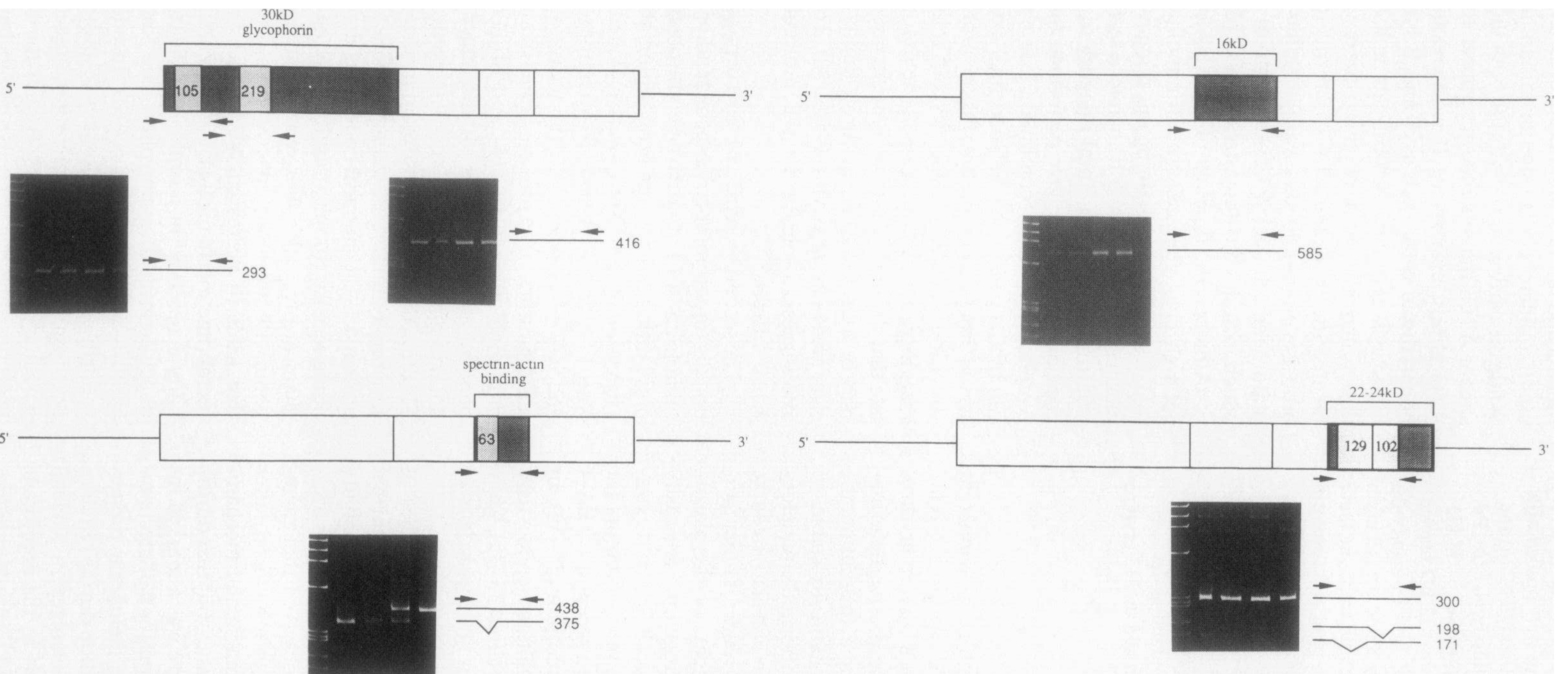

Figure 6. Reverse transcriptase/PCR analysis of 80-kD 4.1 mRNA. Structural models of 4.1 mRNA with black arrows indicating location of oligonucleotide primers are shown above 5% PAGE of amplified DNA products. Lanes (*left to right*) on the polyacrylamide gels are molecular mass markers, preproerythroblasts, BFU-E-derived erythroblasts cultured for 8 d, BFU-E-derived erythroblasts cultured for 12 d and reticulocytes. No differentiation-associated splicing differences were detected in the 30-kD, 16-kD, or 22–24-kD domains; however, a major change in splicing of the spectrin-actin 10-kD binding domain was observed. Within this domain, the 63-nucleotide exon was deleted in both preproerythroblast mRNA and BFU-E–derived erythroblasts cultured for 8 d. Messenger RNA from BFU-E–derived erythroblasts cultured for 12 d expressed both the +63 and −63 species, while reticulocyte RNA contained only the +63 species.

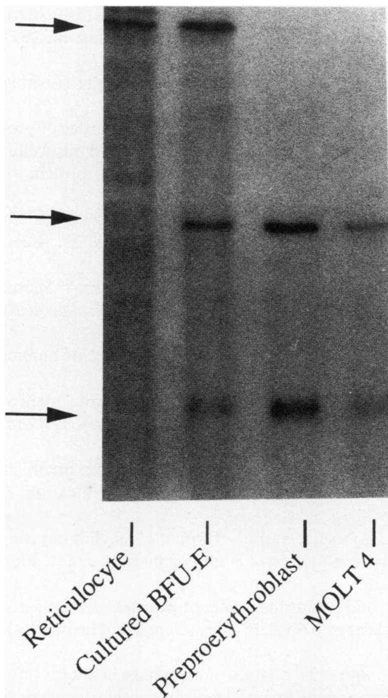

Figure 7. Ribroprobe ribonuclease protection analysis of RNA from erythroid progenitors. The position of the protected fragment in the +63 phenotype is indicated by the upper arrow, while mRNA that lacks the 63-nt exon will generate two smaller protected fragments (*middle and bottom arrows*), as seen in the MOLT-4 lymphoblast RNA control (−63 phenotype). In preproerythroblasts ~ 99% of the protected fragment was −63, in cultured BFU-E–derived erythroblasts about 50% was −63 and 50% was +63 and in reticulocytes > 99% was +63.

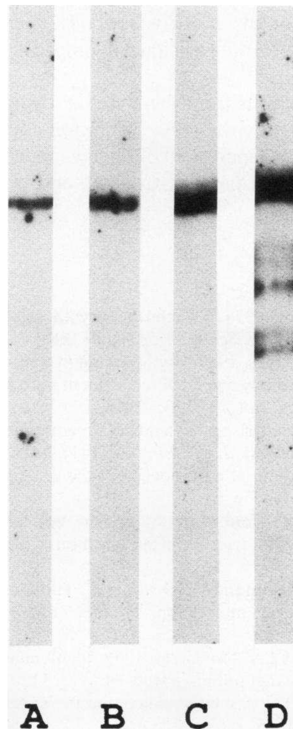

Figure 8. Western blot analysis of protein 4.1 isoforms containing the spectrin-actin binding domain. Protein from equivalent numbers of day 12 (*A*), day 13 (*B*), and day 14 (*C*) BFU-E–derived erythroblasts were probed with an antipeptide antibody to the 21 amino acids encoded by the 63-nt exon. A band migrating at a similar molecular mass as the major band in mature red cell membranes (*D*) was detected at each of the three time points. This band increased in intensity as differentiation proceded implying an increasing expression of the protein 4.1 isoform containing the spectrin-actin binding domain.

cance in that while mature red cells need a mechanically stable membrane to function normally, early progenitors that have to undergo mitosis can ill-afford a stable membrane that would be resistant to the membrane failure required for cell division.

Our data obtained at four developmental stages in normal human erythropoiesis are consistent with earlier observations made in mouse erythroleukemia (MEL) cells, in which the undifferentiated MEL cells deleted the 63-nt exon in the spectrin-actin binding doman, while MEL cells undergoing chemically-induced differentiation contained this exon (17, 20). Since induced MEL cells exhibit ultrastructural abnormalities and do not express normal amounts of either hemoglobin or membrane proteins (41), we believe that protein synthesis and membrane assembly may not be entirely analogous in induced MEL cells and normally differentiating human erythroid progenitors, and that it is crucial to study the biology of human erythropoiesis using normal human erythroid progenitors rather than transformed mouse cell lines.

Interestingly, our analysis of 80-kD 4.1 mRNA species revealed no differentiation-associated splicing switches other than that involving the spectrin-actin binding domain. These data suggest that a second, important functional domain, the glycophorin binding domain, is invariantly expressed from the preproerythroblast through the reticulocyte stage of maturation. Glycophorin C, an integral membrane protein, appears to associate with 4.1 through its cytoplasmic tail. Although no direct binding studies have yet been done to characterize this interaction and define the amino acids included in the binding site, strong indirect evidence exists, suggesting that a functionally important 4.1–glycophorin C interaction indeed occurs. Specifically, elliptocytic red cells deficient in 4.1 because of a primary mutation in the 4.1 gene are secondarily deficient in glycophorin C (14). Further, membrane skeletons prepared with Triton X-100 from 4.1-deficient cells lack glycophorin C, while skeletons prepared from 4.1-deficient cells that had been reconstituted with normal 4.1 retain glycophorin C (13, 14). In support of the functional role of this protein–protein interaction are the observations that glycophorin C–deficient red cells (Leach phenotype) have membrane instability and abnormal morphology (42). During terminal erythroid differentiation, glycophorin C is first expressed on the surface at the colony forming unit–erythrocyte stage, a maturational stage before the preproerythroblast (43). This finding suggests that the 4.1 isoform(s) anchoring glycophorin C within the lipid bilayer is also expressed at an early stage of erythropoiesis and is consistent with our observations showing the invariant expression of the glycophorin binding domain from the preproerythroblasts through the reticulocyte stage of maturation.

We believe that dynamic reorganization of the membrane skeleton occurs during terminal erythroid differentiation resulting in membranes with markedly different functional and mechanical characteristics. While previous studies have described the sequential synthesis and assembly of various skeletal proteins during erythropoiesis (1–4), our current studies show that there is also differential expression of 4.1 isoforms during erythroid maturation. Recently, other structural proteins of the erythroid membrane, such as ankyrin and protein 4.2, have also been shown to undergo alternative splicing so that they are also potential candidates for differentiation-associated switches in gene expression. Our findings that switches in protein 4.1 gene expression occur during erythropoiesis represent one potentially important mechanism for membrane skeletal reorganization. Ahead lies the challenge of defining specific functions of 4.1 isoforms during erythropoiesis and of determining the molecular mechanisms that trigger the switch in 4.1 gene expression.

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