# Temporal Synthesis of Band 3 Oligomers During Terminal Maturation of Mouse Erythroblasts. Dimers and Tetramers Exist in the Membrane as Preformed Stable Species

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**Band 3, the anion transport protein of the erythrocyte membrane, exists in the membrane as a mixture of dimers (B3D) and tetramers (B3T). The dimers are not linked to the skeleton and constitute the free mobile band 3 fraction. The tetramers are linked to the skeleton by their interaction with ankyrin. In this report we have examined the temporal synthesis and assembly of band 3 oligomers into the plasma membrane during red cell maturation. The oligomeric state of newly synthesized band 3 in early and late erythroblasts was analyzed by size-exclusion high-pressure liquid chromatography of band 3 extracts derived by mild extraction of plasma membranes with the nonionic detergent $C_{12}E_8$ (octaethylene glycol n-dodecyl monoether). This analysis revealed that at the early erythroblast stage, the newly synthesized band 3 is present predominantly as tetramers, whereas at the late stages of erythroid maturation, it is present exclusively as dimers. To examine whether the dimers and tetramers exist in the membrane as preformed stable species or whether they are interconvertible, the fate of band 3 species synthesized during erythroblast maturation was examined by pulse-chase analysis. We showed that the newly synthesized band 3 dimers and tetramers are stable and that there is no interconversion between these species in erythroblast membranes. Pulse-chase analysis followed by cellular fractionation showed that, in early erythroblasts, the newly synthesized band 3 tetramers are initially present in the microsomal fraction and later incorporated stably into the plasma membrane fraction. In contrast, in late erythroblasts the newly synthesized band 3 dimers move rapidly to the plasma membrane fraction but then recycle between the plasma membrane and microsomal fractions. Fluorescence photobleaching recovery studies showed that significant fractions of B3T and B3D are laterally mobile in early and late erythroblast plasma membranes, respectively, suggesting that many B3T-ankyrin complexes are unattached to the membrane skeleton in early erythroblasts and that the membrane skeleton has yet to become tightly organized in late erythroblasts. We postulate that in early erythroblasts, band 3 tetramers are transported through microsomes and stably incorporated into the plasma membrane. However, when ankyrin synthesis is downregulated in late erythroblasts, it appears that B3D are rapidly transported to the plasma membrane but then recycled between the plasma membrane and microsomal compartments. These observations may suggest novel roles for membrane skeletal proteins in stabilizing integral membrane protein oligomers at the plasma membrane and in regulating the endocytosis of such proteins.**



BAND 3, THE ANION EXCHANGER, is the most abundant integral membrane protein of the mammalian red blood cell (RBC). The protein consists of two domains with distinct structure and function. The C-terminal 56-kD membrane domain consists of multiple hydrophobic segments that traverse the plasma membrane 12 to 14 times. This domain of band 3 mediates the electroneutral exchange of bicarbonate for chloride.[1] The 41-kD N-terminal domain has a net negative charge, projects into the cytoplasm, and anchors the membrane skeleton to the membrane through its interactions with ankyrin,[2] protein 4.1,[3] and protein 4.2.[4] This domain also binds hemoglobin and several glycolytic enzymes.[5]

Band 3 is thought to play a pivotal role in the assembly of the membrane skeleton in developing erythroblasts. In vitro evidence indicates that the proper assembly of membrane skeleton components into a stable supramolecular complex at the plasma membrane requires prelocalization of band 3 within the plasma membrane. Pulse-labeling and pulse-chase studies of avian[6-8] and mammalian[9-12] erythroid precursors reveal that spectrin and ankyrin are synthesized before band 3 in developing erythroblasts but are assembled into a stable membrane skeleton only after the synthesis of band 3 is initiated. However, recent findings of normal membrane skeleton assembly in RBCs lacking band 3 challenge this interpretation of the in vitro studies.[13,14]

Band 3 is self-associated and it exists in the red cell membrane as a mixture of dimers (B3D) and tetramers (B3T). The dimers are not tightly associated with the skeleton and constitute the free mobile band 3 fraction that can be removed from the membrane by mild extraction.[15] The tetramers are tightly associated with ankyrin with the stoichiometry of four band 3 copies to one ankyrin.[15] Recent studies using sedimentation equilibrium analysis have shown that purified band 3 tetramers also bind to band 4.1 protein in solution. One band 3 tetramer can bind up to 4 (or even up to 8) band 4.1 molecules.[16] However, in an earlier study from our laboratory[15] using high-pressure liquid chromatography (HPLC) fractionation of crude band 3 extracts, we detected protein 4.1 only in the band 3 dimer fraction and not in the tetramer fraction. Thus, at this stage it is not clear which species of band 3 associates with protein 4.1 in situ.

Recent evidence suggests that the transport of band 3 to the

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plasma membrane is facilitated by at least two chaperon-like proteins. The first potential chaperon is glycophorin A, whose coexpression with band 3 in oocytes leads to accelerated band 3 expression in the oocyte membrane.[17] The second possible chaperon is ankyrin, as suggested by recent studies in mouse erythroleukemia (MEL) and embryonic kidney 293 cell lines.[18] These studies indicate that the band 3–ankyrin complex is detected in the pre-Golgi compartment, suggesting that the formation of this complex may facilitate band 3 delivery to the plasma membrane. Using *nb/nb* mice as an ankyrin-deficient red cell model, recent studies from our laboratory have shown that in severely ankyrin-deficient erythrocyte membranes, band 3 is present exclusively as band 3 dimers. These results suggest that ankyrin is required for the formation of stable band 3 tetramers.[19] However, it is not known whether ankyrin binds to preformed band 3 tetramers or whether ankyrin can link two preformed band 3 dimers into a stable ankyrin–band 3 tetramer complex.

Here, we have examined the temporal relationship between the synthesis of band 3 dimers and tetramers during terminal maturation of mouse erythroid cells. We have also studied the rates of turnover of band 3 oligomers in situ in early (proerythroblasts and basophilic normoblasts) and late (polychromatophilic/orthochromatophilic normoblasts and reticulocytes) stages of erythroblast maturation. We find that the synthesis of band 3 tetramers precedes that of dimers and that the newly synthesized dimers and tetramers do not undergo interconversion during the terminal maturation of erythroblasts. Pulse-chase analysis indicates that, while newly synthesized band 3 tetramers are initially present in the microsomal fraction of early erythroblasts and later incorporated stably into the plasma membrane fraction, newly synthesized band 3 dimers appear rapidly in the plasma membrane fraction of late erythroblasts and later recycle between plasma membrane and microsomal compartments. Fluorescence photobleaching recovery (FPR) studies show that ankyrin-linked band 3 tetramers are laterally mobile in early erythroblast plasma membranes and that band 3 dimers are laterally mobile in late erythroblast membranes.

## MATERIALS AND METHODS

*Isolation of cells.* Erythroid cells were obtained from spleens of mice infected with the anemia-inducing strain of Friend virus (FVA) as described previously.[20] Briefly, Balb/c mice were injected through the tail vein with approximately $10^4$ spleen focus-forming units of FVA. After 2 weeks, spleens were obtained from the mice, and early erythroblasts (proerythroblasts and basophilic normoblasts) were isolated by unit gravity sedimentation over a linear gradient of 1% to 2% deionized bovine serum albumin (BSA).[11]

To obtain late erythroblasts, early erythroblasts were cultured in vitro for 40 to 44 hours in 5% $CO_2$/air at a density of $1 \times 10^6$ cells per mL.[11] The cell culture medium consisted of Iscove's modified Dulbecco's medium (IMDM; GIBCO-BRL, Gaithersburg, MD) containing 30% fetal calf serum (FCS; Hyclone, Logan, UT), 0.1% deionized BSA, 0.1 mmol/L thioglycerol, penicillin/streptomycin, and 0.2 U/mL erythropoietin (generously provided by Ortho Biotech, Raritan, NJ). Cellular morphology was assessed by staining with Wright Giemsa and benzidine-hematoxylin. After 44 to 48 hours of culture, a majority of cells were at the polychromatophilic/orthochromatic normoblast stage (late erythroblasts).

*Metabolic labeling of cells.* Both early and late erythroblasts ($1 \times 10^8$ cells for each time point) were washed twice in methionine-free Dulbecco's modified minimum essential medium (DMEM; GIBCO-BRL) and resuspended in 10 mL of the medium containing 20% FCS prewarmed to 37°C for 15 minutes. They were then incubated for different lengths of time with 300 μCi [$^{35}$S]methionine (1,000 Ci/mmol; ICN Biomedicals, Irvine, CA). For the pulse-chase experiment, further incorporation of [$^{35}$S]methionine was stopped by the addition of unlabeled methionine (0.8 mmol/L), and the incubation was then continued for different time periods. At the end of the labeling period, 10 volumes of 155 mmol/L choline chloride and 5 mmol/L HEPES, pH 7.1, were added, and the cells were obtained by centrifugation and washed once with ice-cold saline buffer (150 mmol/L NaCl, 5 mmol/L KCl, 1 mmol/L $MgCl_2$, and 1 mmol/L $CaCl_2$).

*Cell fractionation.* [$^{35}$S]methionine-labeled early and late erythroblasts were lysed in a buffer containing 5 mmol/L Tris-HCl, pH 8.0, 5 mmol/L $MgCl_2$, 2 mmol/L EDTA, 200 mmol/L tosyl-L-lysine chloromethyl ketone (TLCK), 200 mmol/L tosylamide-2-phenyl-ethyl chloromethyl ketone (TPCK), and 2 mmol/L phenylmethylsulfonyl fluoride (PMSF) and homogenized with a Dounce homogenizer (Wheaton Scientific, Millville, NJ). The homogenate was made isotonic by adding appropriate volumes of 5 mol/L NaCl and then ultracentrifuged at 100,000*g* for 2 hours. The resulting pellet was resuspended in 1 mL of concanavalin A (Con A) loading buffer (20 mmol/L Tris-HCl, pH 7.4, 300 mmol/L NaCl, 1 mmol/L $CaCl_2$, 1 mmol/L $MgCl_2$, 1 mmol/L $MnCl_2$) and loaded onto a column made of Con A lectin (Sigma, St Louis, MO) preequilibrated in this buffer. The eluate containing microsomal vesicles was collected. Vesicles made from plasma membranes bound to the column and were eluted with 2 mL of the Con A loading buffer containing 300 mmol/L α-methyl mannoside.[18]

*Spectrin extraction from late erythroblast plasma membranes.* Spectrin was extracted from [$^{35}$S]methionin-labeled late erythroblast membranes by incubation in equal volumes of a low ionic strength buffer containing 0.1 mmol/L $NaPO_4$, 0.1 mmol/L EDTA, 0.1 mmol/L PMSF, 0.1 mmol/L N-α-p-tosyl-L-lysine chloromethyl ketone HCl, and 0.1 mmol/L dithiothreitol, pH 8.0, at 37°C for 20 minutes. Spectrin-depleted membranes were obtained by centrifugation at 150,000*g* for 35 minutes at 4°C.

*Size exclusion HPLC of band 3 extracted from erythroblast membranes by the nonionic detergent octaethylene glycol n-dodecyl monoether ($C_{12}E_8$).* Plasma membranes prepared from early and late erythroblasts were dissolved in 5 volumes (usually 50 μL of membranes were dissolved in 250 μL) of 0.5% $C_{12}E_8$ in hypotonic buffer (5 mmol/L NaPi, pH 7.4) at 4°C. After centrifugation at 150,000*g* for 30 minutes, the supernatants were analyzed by size exclusion HPLC using a TSK-4000 $SW_{XL}$ column (7.8 × 300 mm; Tosohaas, Tokyo, Japan), as described previously.[21] The standard elution buffer contained 0.01% $C_{12}E_8$, 100 mmol/L NaCl, 5 mmol/L NaPi, pH 7.0.

Protein in HPLC fractions was concentrated by centrifugation of 0.5 to 2.0 mL aliquots in a 10,000 molecular weight cut-off ultracentrifugation unit (Millipore, Lexington, MA) at 5,000 rpm for 15 to 20 minutes. The concentrated supernatant was removed, dissolved in sodium dodecyl sulfate (SDS) and analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) according to Laemmli.[22]

*Immunoprecipitation.* Band 3 was immunoprecipitated from the HPLC peaks (B3T and B3D) and from the microsomal and plasma membrane fractions obtained after Con A column treatment. The samples were suspended in immunoprecipitation buffer (IPB) containing 10 mmol/L Tris, pH 7.5, 1% NP-40, 0.5 % sodium deoxycholate, 150 mmol/L NaCl, 5 mmol/L EDTA, 2 mg/mL BSA, 200 mmol/L TLCK, 200 mmol/L TPCK, and 2 mmol/L PMSF. The resulting extracts were precleared by mixing 50 μL of protein A–sepharose CL-4B (Pharmacia LKB Biotechnology Inc, Piscataway, NJ; 50 mg of beads/mL of IPB) for 1 hour at 4°C and centrifuged to remove sepharose beads. The supernatants were then immunoprecipitated with rabbit anti-mouse band 3 IgG, and the samples were incubated overnight at 4°C with gentle shaking. Thereafter, 100 μL of protein A–sepharose CL-4B was

added and the samples incubated for another 3 hours at 4°C with gentle shaking. The immunoprecipitates were washed successively with buffer 1 (IPB containing 0.1% SDS); buffer 2 (IPB without BSA and NP-40); and buffer 3 (10 mmol/L Tris, 1 mmol/L EDTA). The final pellet was resuspended in 70 µL of SDS sample buffer and boiled for 2 minutes. Beads were removed by centrifugation and the supernatants were directly loaded on 10% SDS-polyacrylamide Laemmli[22] gels.

*Endoglycosidase H digestion.* Early and late erythroblasts were metabolically labeled with [$^{35}$S]methionine for 30 minutes and then chased with cold methionine for 0 or 4 hours. Cells were then lysed in an NP-40 buffer (10 mmol/L Tris, pH 7.5, 1% NP-40, 0.5% sodium deoxycholate, 150 mmol/L NaCl, 5 mmol/L EDTA, 2 mg/mL BSA, 200 mmol/L TLCK, 200 mmol/L TPCK, and 2 mmol/L PMSF), centrifuged at low speed to remove nuclei, and the resulting extracts were immunoprecipitated with anti–band 3 antibody as described above. Half of the immunoprecipitated material was incubated with 0.1 volume of endoglycosidase H (30 µg/mL) at 37°C for 16 to 18 hours, as described.[23]

*FPR.* Band 3 lateral mobility was measured in plasma membranes of early and late erythroblasts in culture. Briefly, 100 µL containing a 20% suspension of erythroblasts in 140 mmol/L KCl, 15 mmol/L $NaPO_4$, 10 mmol/L glucose, pH 7.4, was incubated with 40 µL of eosin-5-maleimide (Molecular Probes, Eugene, OR), 0.25 mg/mL, for 15 minutes at room temperature. Fluorescence SDS-polyacrylamide gel scanning of membranes from identically labeled human erythroblasts has shown that greater than 90% of the eosin fluorescence comigrates with band 3, and that the band 3–deficient erythroleukemia cell line K562 does not label upon incubation with eosin-5-maleimide (unpublished observations, May 1989). FPR was used to measure the lateral mobility of eosin-labeled band 3 in membranes of early and late erythroblasts at 37°C.[24]

## RESULTS

*Newly synthesized band 3 is tetrameric in early erythroblasts and dimeric in late erythroblasts.* To elucidate the role of band 3 oligomerization in the biogenesis of the erythrocyte membrane skeleton during terminal maturation of mouse erythroid cells, we examined the temporal synthesis and assembly of band 3 dimers and tetramers. Erythroblasts at the early and late stages of maturation were isolated from the spleens of mice infected with FVA. Both early and late erythroblasts were metabolically labeled with [$^{35}$S]methionine for 60 minutes before isolating plasma membranes, which were then subjected to a mild extraction of band 3 with the nonionic detergent $C_{12}E_8$. The $C_{12}E_8$ extract was analyzed by size-exclusion HPLC to determine the oligomeric states of band 3 (Fig 1). Using this analysis in mature mouse erythrocytes, we previously showed[15] that the $C_{12}E_8$ extract contains only B3D, whereas the tetrameric species remains associated with the $C_{12}E_8$-insoluble membrane skeleton. In striking contrast, the extracts of early erythroblasts contained predominantly B3T with only a trace amount of B3D (Fig 1A). Autoradiograms showed that the tetrameric peak containing band 3 and ankyrin was radiolabeled, suggesting that in early erythroblasts the newly synthesized band 3 exists predominantly as tetramer. Because the spectrin-based skeleton is not yet assembled on the membrane of early erythroblasts, a mild extraction of such membranes with $C_{12}E_8$ releases band 3 tetramers, which are inextractable from mature cells under these conditions. In contrast, in late erythroblasts mild extraction of the membranes with $C_{12}E_8$ released only B3D (Fig 1B), whereas B3T could be released only when spectrin was removed before $C_{12}E_8$ extraction (Fig 1C). Densitometric scans of gels corresponding to the tetramer fraction showed that the band 3-to-ankyrin stoichiometry was similar to that in mature red cells, ie, four copies of band 3 for each copy of ankyrin. These results suggest that at the late stages of erythroid maturation when the skeleton has been nearly completely formed, band 3 extracted under mild conditions is mainly dimeric whereas the tetrameric band 3 remains associated with the spectrin-based skeleton. Furthermore, autoradiograms show that the radiolabeled band 3 is detected only in the B3D peak and not the B3T peak. Thus, at the early erythroblast stage, the newly synthesized band 3 is present predominantly as tetramers, whereas at the late stages of erythroid maturation it is present exclusively as dimers.

*Band 3 dimers and tetramers do not undergo interconversion during erythroid maturation.* The above results show that band 3 dimers and tetramers are synthesized at distinct stages of erythroid development. However, whether they exist as preformed stable species or are interconvertible is not known. To address this question, we used pulse-chase analysis to examine the fate of newly synthesized band 3 species during terminal maturation of erythroblasts. We took advantage of the fact that, at a given stage of erythroid maturation, the newly synthesized band 3 exists predominantly as either dimers or tetramers.

Early and late erythroblasts were metabolically labeled with [$^{35}$S]methionine, which predominantly labels band 3 tetramers at the early stage and dimers at the late stage of maturation. The cells were then chased with unlabeled methionine; early erythroblasts were chased for 48 hours, during which time the cells matured to the late erythroblast stage, while late erythroblasts were chased for 2 or 16 hours. Thereafter, plasma membranes were isolated from both the unchased and chased cells and were subjected to mild extraction of band 3 with the detergent $C_{12}E_8$. The resulting pellets and supernatants were then either subjected to size-exclusion HPLC or immunoprecipitated with anti–band 3 antibodies. Using this approach, we first examined the stability of band 3 tetramers in early erythroblasts. The $C_{12}E_8$ supernatants of chased and unchased erythroblast membranes were subjected to HPLC analysis. As shown in Fig 2A, before the chase HPLC analysis yielded a predominant peak containing band 3 tetramers, whereas after the 48-hour chase band 3 was eluted predominantly as dimers. Autoradiograms showed that the tetramers isolated before the chase were radiolabeled whereas the dimers isolated after the chase were unlabeled. These results suggest that radiolabeled tetramers did not dissociate into dimers during the 48-hour chase period. Furthermore, the $C_{12}E_8$ pellets and supernatants of chased and unchased erythroblast membranes were immunoprecipitated with anti–band 3 antibodies. As shown in Fig 2B, before the chase when the cells were at the early erythroblast stage, the radiolabeled band 3 was present exclusively in the supernatant as tetramers, whereas after the 48-hour chase when the cells had matured to the late erythroblast stage, practically all of the radiolabeled band 3 was recovered in the pellet that normally contained skeletal-associated band 3 tetramers. On the other hand, in the supernatant that typically contained dimers at this stage, virtually no radiolabeled band 3 was detected, suggesting that the tetramers synthesized in early erythroblasts were stable and that they did not convert into dimers during maturation.

Next, using a similar approach, we examined the stability of band 3 dimers in late erythroblasts. Following a 60-minute

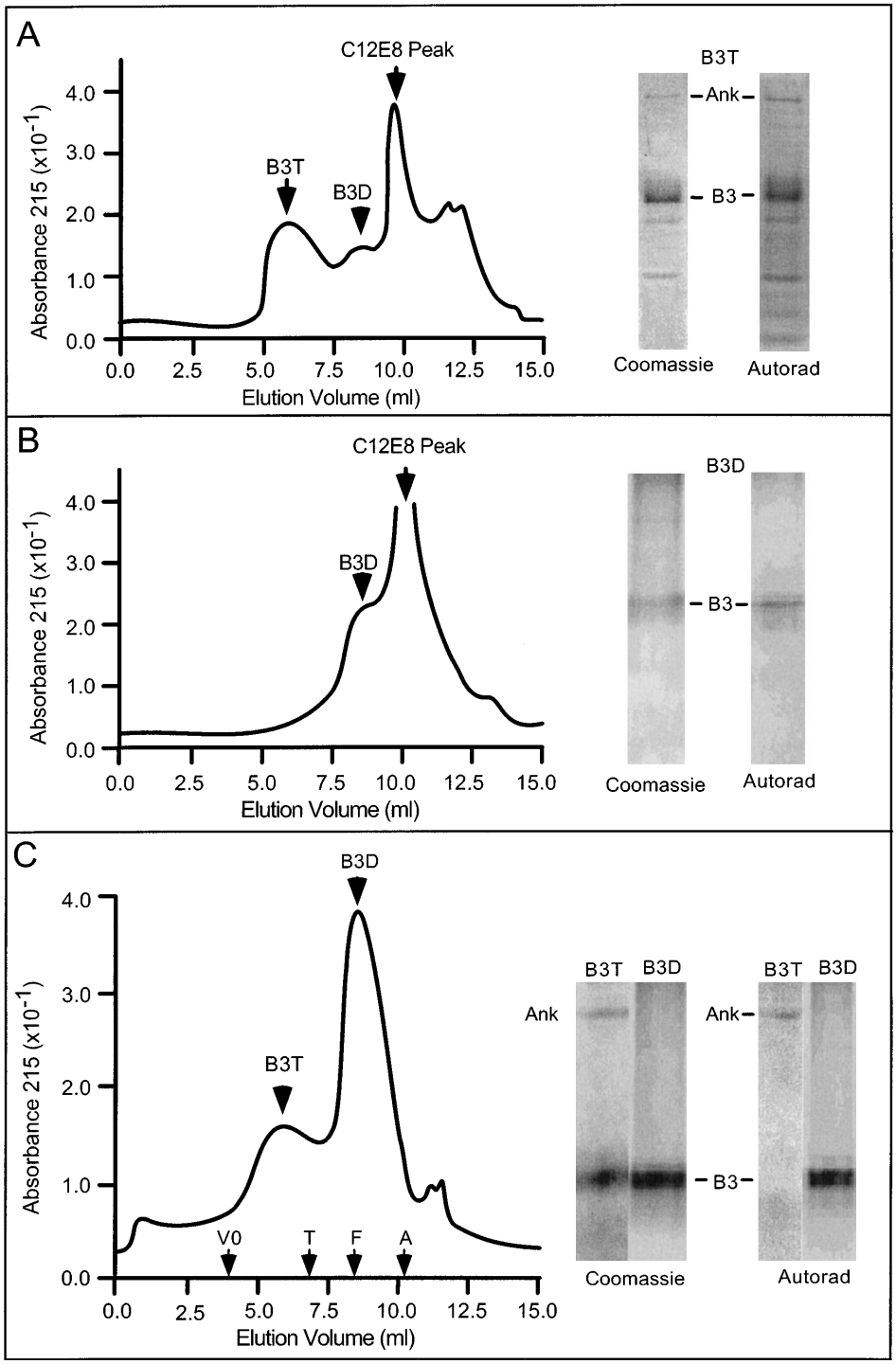


**Fig 1. Size-exclusion HPLC of $C_{12}E_8$ extracts from (A) early erythroblast membranes, (B) late erythroblast membranes, and (C) late erythroblast membranes after spectrin extraction. Plasma membranes isolated from [$^{35}$S]methionine-labeled early and late erythroblasts were dissolved in 0.5% $C_{12}E_8$ in hypotonic buffer at 4°C. Samples were centrifuged at 150,000*g* for 30 minutes and the supernatants were analyzed by size-exclusion HPLC using a TSK-4000 $SW_{XL}$ column. At the bottom of the HPLC profile are shown the elution positions for the standard proteins: T, thyroglobulin; F, ferritin; and A, aldolase. The void volume ($V_0$) was determined from the elution position of blue dextran 2000 (average molecular weight 2 × $10^6$ daltons). The tetramer (B3T) and dimer (B3D) peaks were examined by SDS-PAGE followed by autoradiography. Newly synthesized band 3 exists as tetramer in early erythroblasts and as dimer in late erythroblasts.**

labeling, late erythroblasts were chased for 0, 2, or 16 hours. HPLC analysis showed that band 3 was present as dimers both before and after the chase (Fig 3A). Immunoprecipitation of band 3 from the $C_{12}E_8$ pellets and supernatants isolated from the plasma membranes of unchased and chased erythroblasts showed that the amount of radiolabeled band 3 dimers in the supernatant remained the same after the chase (Fig 3B). Moreover, no radiolabeled band 3 was detected in the pellet that typically contained tetramers in late erythroblasts, suggesting that band 3 dimers that had been labeled before the chase were stable and were not able to convert into tetramers during the chase period. Quantitation of radiolabeled band 3 confirmed that the newly synthesized dimers in late erythroblasts did not turn over during the 16 hours of chase (Fig 3C).

*Newly synthesized band 3 dimers are transported more rapidly to the plasma membrane than are band 3 tetramers, and*

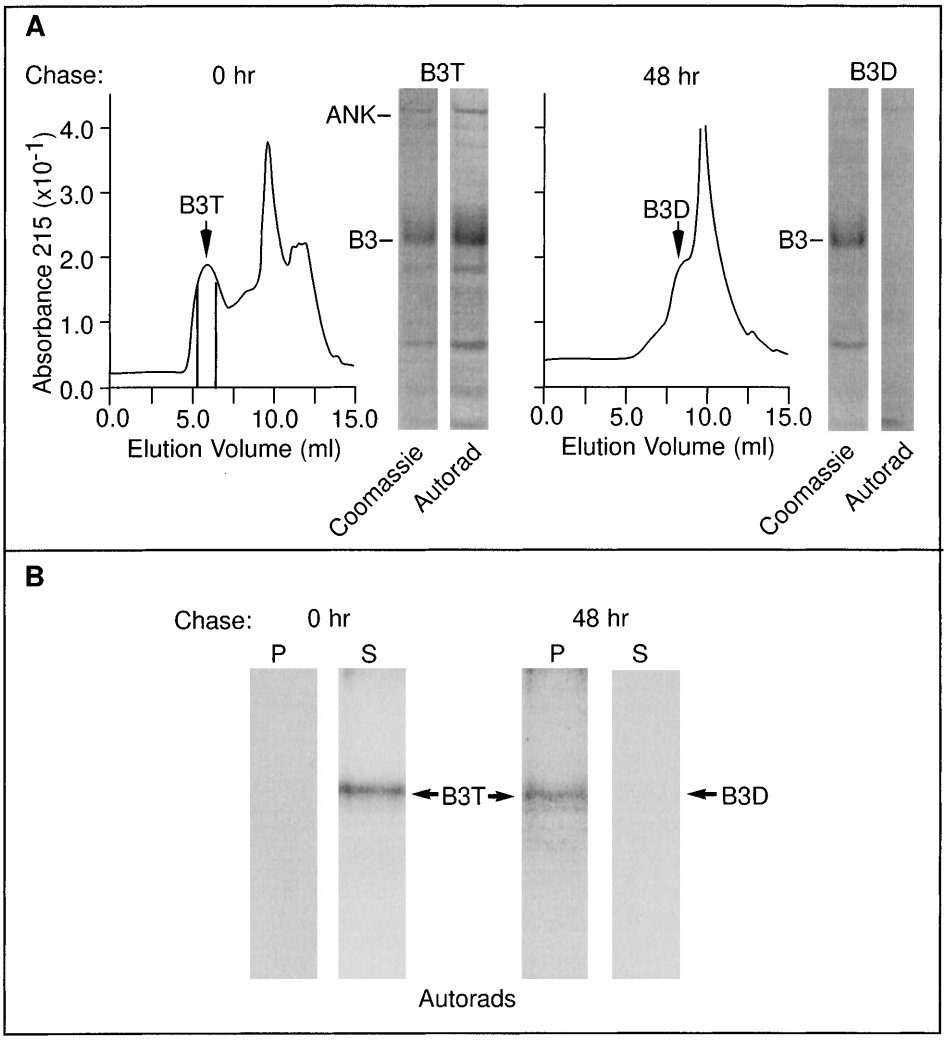


**Fig 2. Pulse-chase analysis of metabolically labeled early erythroblasts. Early erythroblasts were labeled with [$^{35}$S]methionine for 60 minutes and then chased with unlabeled methionine for 48 hours. Plasma membranes isolated from unchased and chased cells were extracted with $C_{12}E_8$. (A) The $C_{12}E_8$ extracts were subjected to size exclusion HPLC. (B) $C_{12}E_8$-insoluble pellets (P) and -soluble supernatants (S) were immunoprecipitated with anti–band 3 antibodies. The immunoprecipitates were analyzed by SDS-PAGE and the gels were processed for fluorography. Band 3 tetramers synthesized before the chase were stable and did not convert into dimers during the 48-hour chase period.**

*are recycled between plasma membrane and microsomal compartments.* To determine the efficiency of band 3 transport to the plasma membrane, the rate of turnover of band 3 was measured in the microsomes and plasma membranes of early and late erythroblasts. Early and late erythroblasts were metabolically labeled with [$^{35}$S]methionine for 30 minutes followed by a chase with unlabeled methionine for different periods of time. Thereafter, cells were lysed to obtain microsomes and plasma membranes as described in Materials and Methods. Band 3 was then immunoprecipitated from these fractions using anti–band 3 antibody.

As shown in Fig 4, in early erythroblasts newly synthesized band 3 appeared initially in the microsomal fraction and later in the plasma membrane fraction. In contrast, in late erythroblasts newly synthesized band 3 moved rapidly to the plasma membrane fraction and only later appeared in the microsomal fraction. These results are consistent with the hypotheses that (1) band 3 tetramers are transported more slowly to the plasma membrane than are band 3 dimers; (2) band 3 tetramers remain stably localized in the plasma membrane after insertion; and (3) band 3 dimers recycle between plasma membrane and microsomal compartments after rapid insertion into the plasma membrane. These results were confirmed by Endoglycosidase H (Endo H) digestion of band 3 immunoprecipitated from [$^{35}$S]methionine-labeled early and late erythroblasts. Susceptibility to Endo H reflects an absence of processing in the Golgi and retention of the protein in the ER.[25-27] Early and late erythroblasts were labeled for 30 minutes and chased for 0 or 4 hours followed by band 3 immunoprecipitation. Half of the immunoprecipitated sample was treated with Endo H. As shown in Fig 5, [$^{35}$S]methionine-labeled band 3 immunoprecipitated from early erythroblasts was sensitive to Endo H if the cells were lysed immediately following the pulse (Fig 5, lanes 1 and 2), but the protein became Endo H–resistant after a 4-hour chase (Fig 5, lanes 3 and 4) indicating its exit from the ER or the early Golgi compartment. However, in late erythroblasts, a significant fraction of band 3 was sensitive to Endo H after 4 hours of chase reflecting its movement from the plasma membrane back to the ER/Golgi. To determine the oligomeric state of the band 3 species that was retained in the microsomes of late erythroblasts, size exclusion HPLC analysis was used; $C_{12}E_8$ extracts of microsomes isolated from [$^{35}$S]methionine-labeled late erythroblasts were analyzed by HPLC. This analysis revealed the

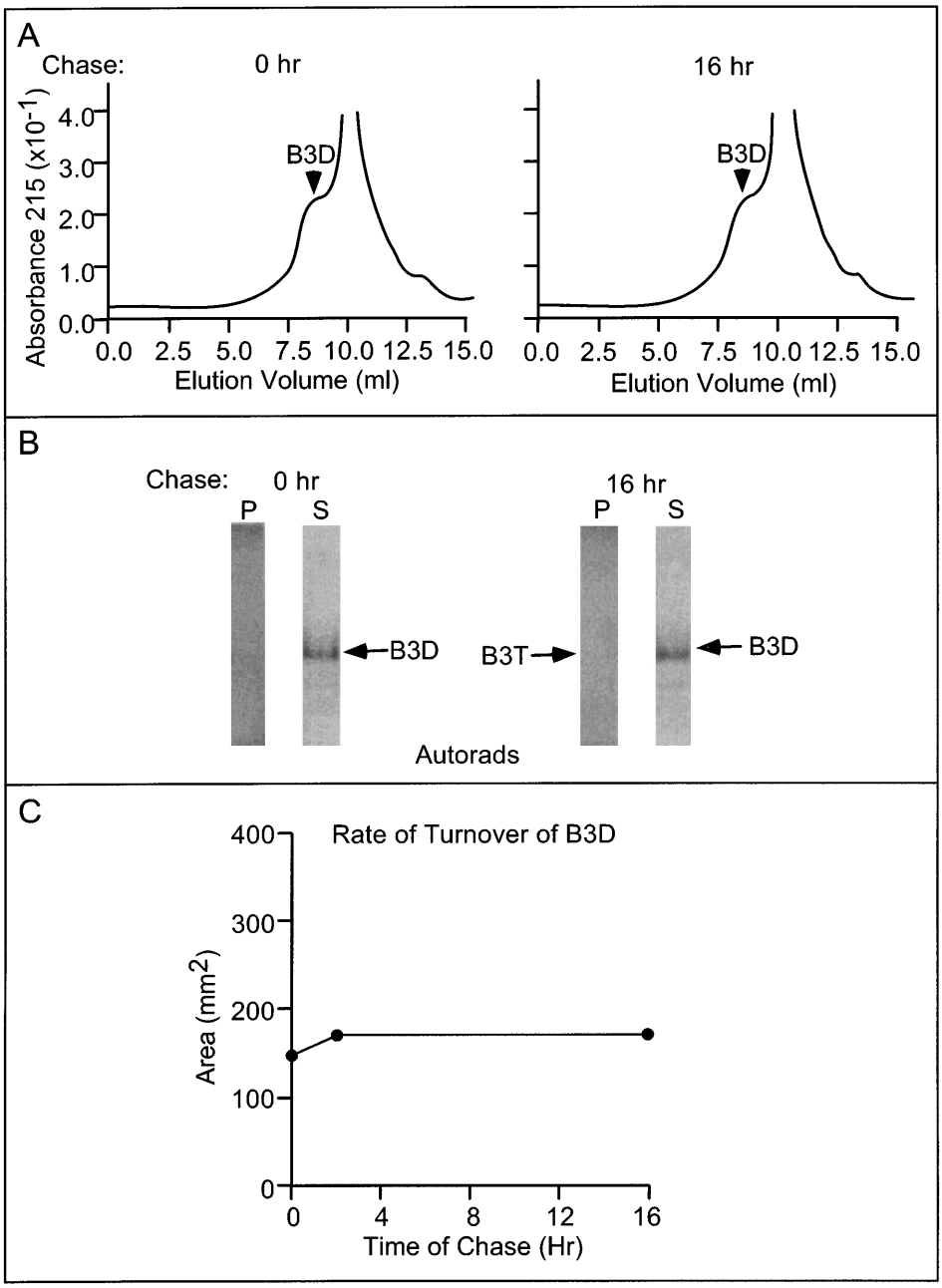


**Fig 3. Pulse-chase analysis of metabolically labeled late erythroblasts. Late erythroblasts were labeled with [$^{35}$S]methionine for 60 minutes and then chased with unlabeled methionine for 16 hours. Plasma membranes isolated from unchased and chased cells were extracted with $C_{12}E_8$. (A) The $C_{12}E_8$ extracts were subjected to size exclusion HPLC. (B) $C_{12}E_8$-insoluble pellets (P) and -soluble supernatants (S) were immunoprecipitated with anti–band 3 antibodies. The immunoprecipitates were analyzed by SDS-PAGE and the gels were processed for fluorography. (C) The autoradiograms shown in B were scanned and the area under each peak integrated (the autoradiograms for the 2-hour chase period are not shown). Band 3 dimers synthesized before the chase were stable and did not associate to form tetramers during the 16-hour chase period.**

presence of only B3D (data not shown), indicating that at the late erythroblast stage band 3 dimers accumulated in microsomes. This accumulation was likely caused by recycling of band 3 dimers between plasma membrane and microsomal compartments in the late stages of erythroid maturation when ankyrin synthesis was downregulated.

*Significant fractions of band 3 tetramers and dimers are laterally mobile in early and late erythroblast plasma membranes, respectively.* Lateral mobility measurements have been used to measure the extent of interaction between band 3 oligomers and the membrane skeleton in intact human and mouse erythrocytes. In normal mouse erythrocytes approximately 90% of the band 3 molecules are laterally immobile, suggesting the presence of tight binding and/or steric hindrance interactions between band 3 oligomers and the membrane skeleton in these cells.[19] However, no studies of band 3 mobility have been performed in developing erythroblasts. To quantify the development of binding and/or steric hindrance interactions between band 3 molecules and the membrane skeleton in developing erythroid cells, we used FPR to measure the lateral mobility of eosin-labeled band 3 molecules in the plasma membrane of early and late erythroblasts. Band 3 manifested a lateral diffusion coefficient of $(2.1 \pm 1.1) \times 10^{-10}$ cm$^2$/s and a fractional mobility of 36 ± 5% (mean ± SD; n = 9 cells) in plasma membranes of early erythroblasts, showing that a significant fraction of B3T is laterally mobile in these membranes. In late erythroblast membranes band 3 had a lateral diffusion coefficient of $(3.9 \pm 2.6) \times 10^{-10}$ cm$^2$s$^{-1}$ and a fractional mobility of

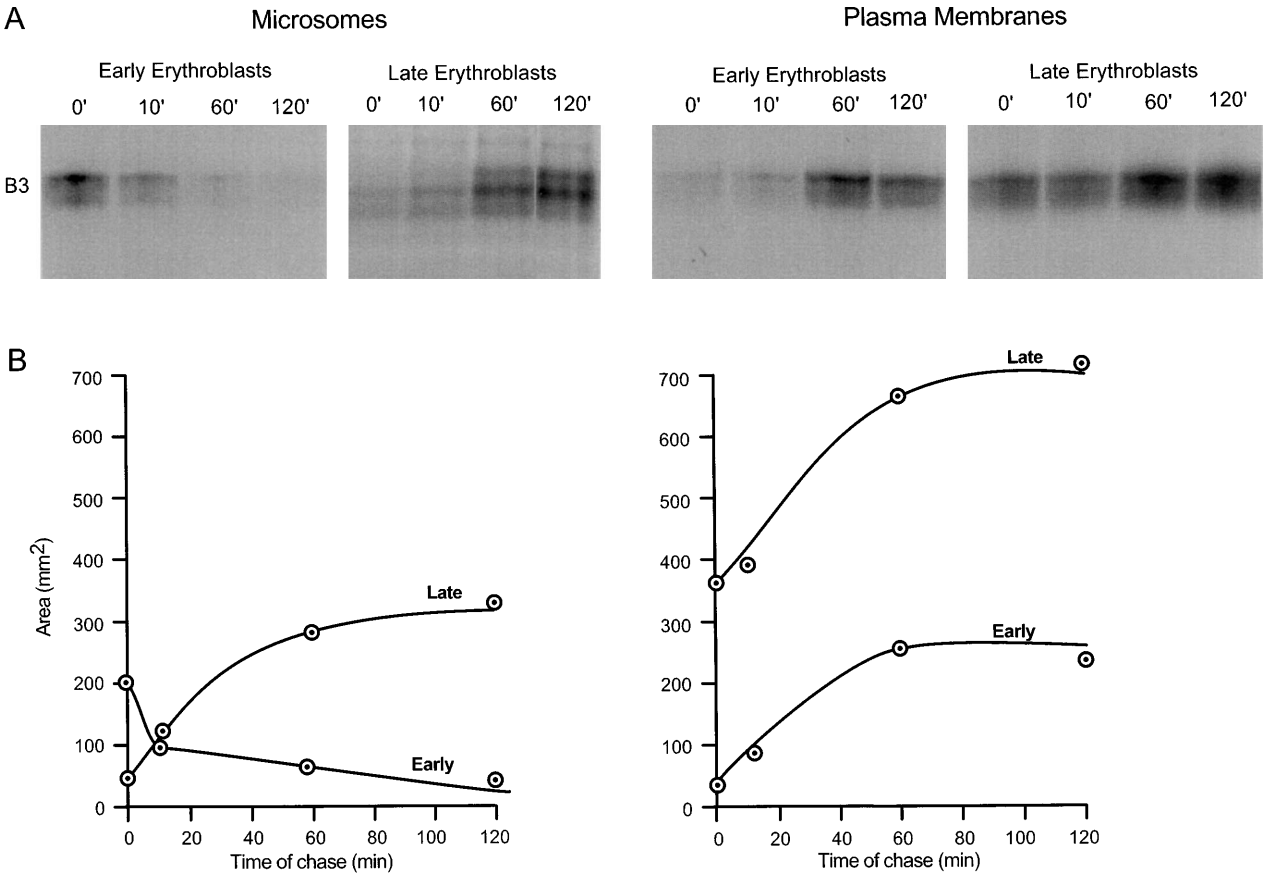


**Fig 4. Turnover of band 3 in the microsomal and plasma membrane fractions of early and late erythroblasts. (A) Microsomes and plasma membranes were isolated from erythroblasts labeled with [$^{35}$S]methionine for 30 minutes and chased for different time periods (0 to 120 minutes). Band 3 was immunoprecipitated using anti–band 3 antibodies. The immunoprecipitates were analyzed by SDS-PAGE and the gels were processed for fluorography. B3: Band 3. (B) The autoradiograms shown in (A) were scanned and the area under each peak integrated.**

29 ± 11% (n = 13 cells), suggesting that a significant fraction of B3D is laterally mobile in these membranes.

## DISCUSSION

This study addresses the role of band 3 oligomerization in the biogenesis of the erythrocyte membrane skeleton during terminal maturation of mouse erythroid cells. We have used FVA cells at the early and late stages of erythroid maturation. Although one study using normal human bone marrow cells has suggested that transformed cells may not recapitulate the sequence of protein synthesis in vivo,[28] FVA cells have been used extensively to study the biogenesis of the erythroid membrane skeleton.[11,29] In an attempt to confirm the validity of the FVA model system for the present studies, we compared the oligomerization state of newly synthesized band 3 in late FVA erythroblasts with that in explanted late erythroblasts from *nb/nb* mouse spleens. The *nb/nb* erythroblast isolation procedure was identical to that described.[13] The newly synthesized

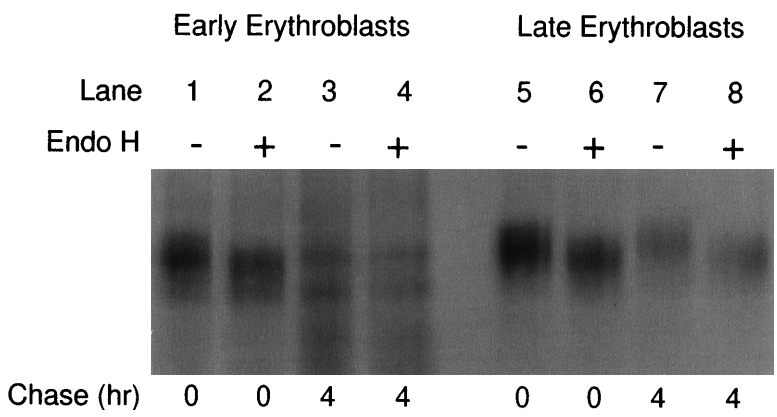


**Fig 5. Endo H digestion of immunoprecipitated band 3 from early and late erythroblasts. Early and late erythroblasts were either [$^{35}$S]methionine-labeled for 30 minutes (lanes 1, 2, 5, and 6) or labeled for 30 minutes and chased for 4 hours (lanes 3, 4, 7, and 8). NP-40 extracts were immunoprecipitated with band 3 antibodies, and half of the samples were incubated with (lanes 2, 4, 6,and 8) or without (lanes 1, 3, 5, and 7) Endo H before the separation of proteins on an SDS gel.**

band 3 was present exclusively as dimers in both FVA and *nb/nb* late erythroblasts (data not shown). Similar studies could not be performed in early *nb/nb* erythroblasts because of the lack of a sufficient number of cells.

Oligomeric states of band 3 were analyzed in the native erythroblast membrane by mild extraction of band 3 with the nonionic detergent $C_{12}E_8$ followed by size-exclusion HPLC. This analysis revealed the presence of B3T in the extracts of early erythroblast membranes and B3D in the extracts of late erythroblast and mature mouse erythrocyte membranes. B3T can, however, be extracted from the membranes of late erythroblasts and mature erythrocytes when spectrin is removed before $C_{12}E_8$ extraction. SDS-PAGE analysis shows that the tetrameric peak of early erythroblasts contains band 3 and ankyrin, whereas the dimeric peak of late erythroblasts contains only band 3. Thus, in the extracts of early erythroblast membranes the predominant band 3 species is tetramer. Because only small amounts of spectrin and ankyrin are incorporated into a highly unstable membrane skeleton of these cells,[11] mild extraction of early erythroblast membranes with $C_{12}E_8$ releases B3T, a species that is inextractable from mature cells under these conditions. In contrast, in late erythroblasts where substantial amounts of spectrin and ankyrin have already been assembled into a stable membrane skeleton,[11] mild extraction of membranes with $C_{12}E_8$ releases only B3D whereas B3T remains associated with the skeleton. HPLC analysis of band 3 extracts derived from the plasma membranes of metabolically labeled early and late erythroblasts shows that the newly synthesized band 3 exists as tetramers in early and as dimers in late erythroblasts.

To examine the stability of the newly synthesized band 3 oligomers in the plasma membrane, we have used pulse-chase analysis of metabolically labeled erythroid precursors. Pulse-chase of early erythroblasts for 2 days, during which time the erythroblasts concomitantly matured to late erythroblasts, shows that radiolabeled newly synthesized band 3 tetramers are stable and that they do not turn over or dissociate into dimers. Radiolabeled tetramers, which are easily extractable by $C_{12}E_8$ before the chase, become skeletal-associated and hence inextractable after the chase. Similarly, pulse-chase of late erythroblasts for up to 16 hours shows that the newly synthesized band 3 dimers are stable. Moreover, radiolabeled dimers do not convert into tetramers, as shown by their extractability by $C_{12}E_8$ both before and after the chase. These results show that band 3 dimers and tetramers exist in the membrane as preformed stable species and that they do not undergo interconversion.

Recent evidence has suggested that band 3 is delivered to the plasma membrane as a band 3–ankyrin complex, which is already formed in the ER/pre-Golgi compartment. The formation of this complex has been shown to be important for efficient processing of band 3 through the early secretory compartments of the cell.[18] Recent studies in ankyrin-deficient *nb/nb* mouse red cells have shown, however, that although ankyrin is required for the formation of stable band 3 tetramers, it is not absolutely required for delivery of band 3 to the plasma membrane.[19] These results support the notion that band 3 is transported to the plasma membrane in both ankyrin-dependent and -independent modes. Based on the temporal synthesis of band 3 oligomers, we propose that at early stages of erythroid maturation, band 3 is delivered to the plasma membrane via an ankyrin-dependent pathway in which ankyrin acts as a chaperon to transport B3T, presumably as preformed B3T-ankyrin complexes, from the ER to the plasma membrane. In contrast, at late erythroblast stages when ankyrin synthesis is downregulated, band 3 is inserted as a dimer. In the absence of ankyrin, the possibility that glycophorin A or another chaperon-like protein transports band 3 dimers to the plasma membrane remains to be investigated.

We have used a pulse-chase metabolic labeling design and Endo H digestion to study the kinetics of band 3 transport to the plasma membrane. In early erythroblasts, the newly synthesized band 3 appears initially in the ER and later exits this compartment to become stably incorporated in the plasma membrane. Because at this stage of erythroid maturation the rate of ankyrin synthesis is greater than that of band 3,[11] the kinetics of band 3 transport from ER to plasma membrane presumably represents the efficiency of formation of B3T-ankyrin complexes in the ER and of transport of these complexes to the plasma membrane. In contrast, in late erythroblasts the newly synthesized band 3 is transported rapidly to the plasma membrane and only later accumulates in the ER as well as the plasma membrane. Because at this stage of erythroid maturation ankyrin synthesis has decreased markedly while band 3 dimers are being synthesized in abundance, this process presumably represents the efficiency of transport of ''free'' B3D to the plasma membrane. Although the late appearance of band 3 in the ER could represent ''backing up'' of band 3 along its biosynthetic pathway as the band 3 level in the plasma membrane approaches saturation, we favor a model in which ''free'' band 3 dimers continue to be transported rapidly to the plasma membrane and then to recycle between the plasma membrane and ER compartments. Many other integral membrane proteins have been shown to recycle between plasma membrane and endosome compartments, including hormone and autacoid receptors,[30-35] receptors that internalize extracellular ligands or present ligands that have been processed intracellularly,[36-40] adhesion molecules,[41,42] and ion and water transporters.[43-46] In many cases the molecular mechanisms responsible for both retention of integral proteins in the plasma membrane and recycling of such proteins between plasma membrane and endosome compartments are incompletely understood. Our data suggest that stable linkages to membrane skeletal proteins could serve both to stabilize the incorporation of integral proteins in the plasma membrane and to prevent protein recycling to endosomes. With specific reference to erythroid maturation, we postulate that band 3 tetramers are required at the plasma membrane of early erythroblasts to mediate mechanical ''coupling'' between the plasma membrane and the developing membrane skeleton, but that the full complement of anion transporters is not required at the plasma membrane until the nearly mature reticulocytes are released from the bone marrow into the peripheral circulation. Only at this last stage of development is it important to localize B3D as well as B3T to the plasma membrane of the mature erythrocyte.

About 35% of band 3 molecules in the plasma membranes of early erythroblasts are capable of lateral diffusion in the plane of the membrane. This observation is consistent with the finding that much of the band 3 in these membranes is extractable by the

nonionic detergent $C_{12}E_8$. It is notable, however, that the band 3 in early erythroblast membranes consists almost entirely of B3T, which are laterally immobile in membranes of mature mouse red cells.[19] In mature red cells the major molecular mechanism that immobilizes ankyrin-linked B3T is high-affinity binding interactions between ankyrin and the β chain of spectrin.[19] In early erythroblasts, however, the spectrin skeleton is not yet assembled on the plasma membrane,[9-12] so ankyrin-linked B3T are free to diffuse laterally. The diffusion rate of the mobile fraction of band 3 molecules is similar to that of other transmembrane proteins in models of developing erythroid cells.[47]

About 30% of band 3 molecules in late erythroblast membranes are capable of lateral diffusion, consistent with the result that some of the band 3 in these membranes is extractable by $C_{12}E_8$. Unlike the extractable band 3 in early erythroblasts, which consists of B3T, the extractable band 3 in late erythroblasts consists only of B3D. Presumably, by the late erythroblast stage of development the B3T are immobilized by high-affinity binding interactions between ankyrin and spectrin. It is interesting, however, that a significant fraction of the B3D population is capable of lateral diffusion in late erythroblasts whereas the entire B3D population is laterally immobile in membranes of mature mouse red cells.[19] In mature red cells the major molecular mechanism that immobilizes B3D is steric hindrance interactions between the cytoplasmic domain of band 3 and the spectrin-based membrane skeleton.[19] In late erythroblasts, however, although the spectrin-based skeleton is assembling on the membrane, it is apparently not yet organized sufficiently to effect lateral immobilization of B3D. In summary, although a significant fraction of band 3 molecules is laterally mobile in early and late erythroblast membranes, the laterally mobile molecules represent different molecular species in the two cases. In early erythroblasts, the absence of ankyrin binding sites on spectrin allows the ankyrin-linked B3T fraction to diffuse laterally, whereas in late erythroblasts the relatively loose organization of the developing membrane skeleton allows the B3D fraction to diffuse laterally.

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