Decreased Content of Protein 4.2 in Ankyrin-Deficient Normoblastosis (nb/nb) Mouse Red Blood Cells: Evidence for Ankyrin Enhancement of Protein 4.2 Membrane Binding

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Hemizygous normoblastosis (nb/nb) mice, whose red blood cell (RBC) membranes are nearly completely deficient in full-length 210-kD ankyrin, were used to study interactions between ankyrin and protein 4.2 (P4.2). Although it is unclear whether or not these proteins interact in the membrane, both ankyrin and P4.2 bind to the cytoplasmic domain of band 3 (cdb3). In addition to the complete deficiency of full-length ankyrin, nb/nb RBC membranes are also partially spectrin deficient, resulting in morphologically spherocytic and mechanically fragile cells. A new finding was that nb/nb RBC membranes are severely (~73%) P4.2 deficient compared with wild type (+/+ or high reticulocyte mouse RBC membranes. Metabolic labeling of nb/nb reticulocytes showed active P4.2 synthesis at levels comparable with high reticulocyte controls suggesting that the nb/nb P4.2 deficiency was not the result of defective P4.2 synthesis. Reconstitution of nb/nb inside-out vesicles (IOVs) with human RBC ankyrin restored ankyrin levels to ~80% of +/+ IOV levels and increased binding of exogenously added human RBC P4.2 by ~60%. These results suggest that ankyrin is required for normal associations of P4.2 with the RBC membrane.

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Materials and Methods

Animals: Wild type (+/+ ) and homozygous normoblastosis (nb/nb) mice were obtained as a gift from Dr Jane Barker (The Jackson Laboratory, Bar Harbor, ME). RBCs from nb/nb mice have been characterized and reported elsewhere. nb/nb RBCs are almost completely deficient in full-length 210-kD ankyrin because of a defect in the Ank-1 gene on mouse chromosome 8. Instead, nb/nb RBC membranes contain small amounts of a truncated 150-kD form of ankyrin. The near complete absence of 210-kD ankyrin in nb/nb RBCs gave us the opportunity to test whether ankyrin deficiency would affect normal membrane associations of P4.2.

Preparation of RBC ghosts and membrane vesicles. RBC ghosts were prepared from freshly drawn (intraorbital sinus) blood anticoagulated in heparin by the hypotonic lysis procedure of Dodge et al. with some modifications; ghost membranes were incubated in 0.1 mM L, Tris/HCl, pH 8.0, at 37°C for 30 minutes to induce membrane vesiculation.

Preparation of antisera. Polyclonal rabbit antibodies against human RBC ankyrin, protein 4.1, and P4.2 were prepared as described previously. The antibodies were affinity purified using Affigel 10 (Bio-Rad Laboratories, Richmond, CA) coupled to the appropriate purified protein, according to the manufacturer's instructions. Polyclonal rabbit antihuman RBC ankyrin-(Ank-1) antibodies were a gift from the laboratory of Dr S.E. Lux (Boston, MA).

Gel electrophoresis and immunoblotting. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using the discontinuous buffer system of Laemmli, or when ankyrin was to be quantitated, the continuous buffer system of Fairbanks et al. The proteins were stained with Coomasie brilliant blue, and were quantitated by densitometry using a Biomed Soft Laser scanning densitometer (Biomed Instruments, Fullerton, CA) with appropriate software. For Western immunoblots, the proteins were immediately transferred to nitrocellulose according to the procedure of Towbin et al. and the immunoblots developed with anti-ankyrin or anti-P4.2 antibodies, as indicated, followed by alkaline phosphatase (AP)-conjugated second antibody and AP substrate (Bio-Rad Labo-
SDS-PAGE ANALYSIS OF MOUSE GHOST MEMBRANE PROTEINS

(A) Protein composition of +/+ (lane 1) and nb/nb (lane 2) mouse RBC ghost membranes. Twenty micrograms each of solubilized membrane protein were applied to a 9% acrylamide gel and the proteins were separated by Laemmli SDS-PAGE and stained with Coomassie brilliant blue. Nomenclature of the polypeptide bands is according to Steck. This analysis was conducted on 8 nb/nb mice from four different litters with similar results and a representative gel is shown. (B) Protein composition of anemic high-reticulocyte mouse RBCs. SDS-PAGE analysis was as in (A). nb/nb (lane 1), β-thalassemic (Thal, lane 2), and acetylphenylhydrazine-treated (Ph, lane 3) mouse RBC ghost membranes. Numbers under figure indicate percent reticulocytes. (C) Western immunoblot of RBC ghost membranes. Human (H, lane 1), +/+ mouse (lane 2), nb/nb mouse (lane 3), β-thalassemic mouse (Thal, lane 4), and acetylphenylhydrazine-treated mouse (Ph, lane 5). Conditions for SDS-PAGE were the same as those indicated in (A), after which the proteins were electrophoretically transferred to nitrocellulose and developed with affinity purified anti-P4.2 IgG. Numbers under figure indicate percent reticulocytes. The position of P4.2 is indicated by an arrow.

Reticulocyte metabolic labeling. Intraorbital blood from nb/nb and β-thalassemic mice was collected, the RBCs pelleted by centrifugation and the Buffy coat carefully removed by aspiration. RBCs were washed twice in 330 mOsm saline and separated from white blood cells (WBCs) using Ficoll-Paque (Pharmacia, Piscataway, NJ). WBC-free RBC pellets were collected by centrifugation and washed in 330 mOsm saline. Absence of WBCs was confirmed in stained smears. Reticulocyte counts were performed using an automated reticulocyte counter (Sysmex Model R-1000; Toa, Kobe, Japan) and verified by manual determinations of new methylene blue–stained blood films. Equivalent amounts of reticulocytes (1 x 10⁷) were taken for metabolic labeling. RBCs were washed once in RPMI-1640 cell culture media (GIBCO-BRL, Gaithersburg, MD) and resuspended in 5 mL of RPMI-1640 containing 10% fetal calf serum. The mixture was incubated at 37°C for 15 minutes followed by addition of 500 μCi ³⁵S-methionine (NEG-009A; New England Nuclear, Boston, MA). Labeling proceeded for 2 hours and aliquots were taken at timed intervals. Labeling was halted by washing the RBCs in ice-cold 330 mOsm saline and collecting the RBCs by centrifugation. After 4 more washings, the RBC pellets were resuspended in 0.5 mL 330 mOsm saline containing 0.025% saponin, 0.5 mmol/L EGTA, and 0.5 mmol/L phenylmethylsulfonyl fluoride (PMSF). The RBCs were lysed by vortexing. The lysates were centrifuged at 40,000g for 30 minutes at 4°C and the supernatant was collected and mixed with an equal volume of phosphate-buffered saline (PBS). P4.2 was immunoprecipitated from 0.2 mL of the
supernatant-PBS mixture with 20 μL of affinity-purified polyclonal antihuman RBC P4.2 IgG in a 0.4 mL reaction volume containing 0.03% SDS and 0.44 mmol/L PMSF for 18 hours at 4°C, followed by addition of 50 μL of 10% (wt/vol) formalin-fixed Staphylococcus aureus cells (SACs; Sigma Chemical Co, St Louis, MO) for 30 minutes at 4°C. SACs were collected in a microfuge and washed three times with PBS containing 0.05% NP-40 (Pierce Laboratories, Rockford, IL). Washed SAC pellet was resuspended in 50 μL of 2% SDS, 1% β-mercaptoethanol, vortexed to resuspend the pellet, boiled for 5 minutes and microfuged to pellet the SAC. Twenty-five-micro-liter aliquots of the supernatant were counted for radioactivity in 5 mL aqueous compatible liquid scintillation cocktail.

Purification and radiolabeling of ankyrin and P4.2. Ankyrin and P4.2 were purified from human RBCs, according to the method of Bennett and Stenbuck15 for ankyrin and the method of Korsgren and Cohen1 for P4.2. For the P4.2 membrane-binding studies, the purified protein was radioiodinated using Bolton Hunter reagent (New England Nuclear, Boston, MA).16

Preparation of ankyrin-reconstituted nb/nb IOVs. IOV membranes were prepared from nb/nb mouse ghost membranes, as described above. The membranes were reconstituted with human RBC ankyrin (666 μg ankyrin/mg IOV), as described.15 Ankyrin reconstitution resulted in the repletion of ~80% of +/-IOV ankyrin levels determined by SDS-PAGE and densitometry. The content of band 3 in nb/nb IOVs before and after ankyrin-reconstitution was within 8% of each other.

P4.2 binding to nb/nb IOVs. Binding of 125I-P4.2 to nb/nb IOVs (with or without ankyrin reconstitution) was measured according to the method of Korsgren and Cohen,7 with some modifications. Siliconized tubes were used and 125I-P4.2 was precentrifuged at 40,000g for 30 minutes at 4°C immediately before use. Under these conditions, nonspecific binding of 125I-P4.2 to IOVs was less than 20% as assayed with heat denatured 125I-P4.2. IOV protein was determined by the bicinchoninic acid method (Pierce Laboratories, Rockford, IL) after solubilizing in 1% SDS. 125I-P4.2 (0 to 150 μg/mL) was incubated with 20 μg nb/nb IOVs in 120 mmol/L KCl, 5 mmol/L sodium phosphate, pH 8.0, 0.5 mmol/L EGTA, 0.5 mmol/L DTT, 0.02% sodium azide (reassociation buffer) in a total volume of 0.25 mL for 18 hours at 4°C. For ankyrin-reconstituted nb/nb IOVs, protein determinations were made after ankyrin reconstitution. IOVs were collected by centrifugation in a Beckman TLA 100.3 rotor at 50,000g for 30 minutes at 4°C in a Beckman Model TLA tabletop ultracentrifuge (Beckman Industries, Palo Alto, CA). IOVs were washed once in reassociation buffer and 125I counted. Data were fitted using Enzfitter software version 1.04 (Biosoft, Cambridge, UK) using a one-site ligand-binding equation.

RESULTS

nb/nb RBC membrane protein analysis. As previously reported by Lux et al17 and confirmed by Bodine et al,8 nb/nb RBC membranes are spectrin deficient (Fig 1A); our
analysis showed a 40% spectrin deficiency that is comparable with the 50% deficiency previously reported. In contrast with human RBC membranes, mouse RBC membranes do not contain appreciable amounts of band 6 (glyceraldehyde-3-phosphate dehydrogenase), which may be due to the fact that the extreme N-terminal domain of band 3 that contains the band 6 binding site is poorly conserved in mouse band 3. A new finding was the nb/nb membranes were severely P4.2 deficient along with the appearance of several lower molecular-weight bands migrating between P4.2 and actin (Fig 1A). The P4.2 deficiency ranged from 42% (expressed as a ratio of P4.2 to band 3) to 73% (expressed as a ratio of P4.2 to protein 4.1) of wild-type +/- mouse levels (Table 1). It is likely that the latter value is a more accurate representation of the actual P4.2 deficiency since loss of band 3 by membrane vesiculation that occurs in nb/nb RBCs would result in an underestimation of the P4.2 deficiency. P4.2 content of high reticulocyte mouse controls was normal when expressed as a ratio to band 3 (Table 1).

Because nb/nb mice are anemic and have a 35% reticulocytes (earlier studies reported 60% reticulocytes for young adult nb/nb mice), it was possible that the P4.2 deficiency was an artifact of anemia and reticulocytosis. To test this, we measured RBC membrane protein content in other anemic high reticulocyte mouse controls, including beta-thalassemic mice (27% reticulocytes) and acetylphenylhydrazine-treated mice (>50% reticulocytes). As shown in Fig 1B, only the nb/nb RBCs are P4.2 deficient, although several of the new protein bands migrating below P4.2 in nb/nb RBCs are also found in the anemic controls, suggesting that some of these additional bands may be derived from proteolysis associated with high reticulocytosis. Immunologic analysis using polyclonal antihuman RBC P4.2 IgG confirmed the P4.2 deficiency in nb/nb RBC membranes (Fig 1C) and also showed that the P4.2 deficiency was not a result of accelerated P4.2 degradation in vitro during membrane isolation because lower molecular-weight P4.2 bands were not detected in either nb/nb or any of the other high reticulocyte mouse controls.

Western blot analysis using antihuman RBC ankyrin antibodies (anti-ank-1) confirmed the absence of 210-kD full-length ankyrin in nb/nb RBC membranes, which was unique to nb/nb RBCs, not associated with anemia or high reticulocytosis, and confirmed the presence of small amounts of a truncated form of ankyrin in these membranes (Fig 1D). To further rule out reticulocytosis as a cause of nb/nb P4.2 deficiency, reticulocytosis was induced in +/- mice by repeated phlebotomies. The presence of about 20% reticulocytes had no effect on the amount of, or degradation of membrane P4.2 (data not shown). Moreover, there was a marked decrease in the P4.2 content of nb/nb compared with +/- RBCs, that was not accompanied by P4.2 degradation (Fig 1E). Taken together, these results strongly suggest that the severe P4.2 deficiency of nb/nb RBC membranes is unrelated to anemia or reticulocytosis and is not generated as an artifact during RBC membrane preparation and SDS-PAGE analysis.

**Synthesis of P4.2 in nb/nb reticulocytes.** P4.2 synthesis was actively ongoing in nb/nb reticulocytes, and was even more active than in reticulocytes from anemic beta-thalassemic mice (Fig 2). Morphologic analysis of nb/nb and beta-thalassemic reticulocytes showed that both contained early-stage stress reticulocytes, suggesting that reticulocyte age was similar (not shown). To exclude the possibility that P4.2 immunoprecipitation is sensitive to different levels of P4.2 present in thenb/nb and beta-thalassemic reticulocytes, we performed an immunologic titration of radiolabeled P4.2 over a 400-fold concentration range. Recovery of protein over this range was 88% ± 2%, indicating that the different levels of P4.2 present in the mouse reticulocytes were not contributing to artifactual differences in the measured synthetic rates (not shown).

**Binding of P4.2 to ankyrin-reconstituted nb/nb membranes.** Reconstitution of nb/nb IOVs with human RBC ankyrin resulted in the restoration of about 80% of +/- IOV ankyrin levels (Fig 3A). Ankyrin-reconstituted nb/nb IOVs had increased binding of 125I-P4.2 compared with ankyrin-deficient nb/nb IOVs (Fig 3B). Because IOV protein quantitation was assayed after ankyrin reconstitution, the actual amount of P4.2-binding augmentation by ankyrin (expressed per milligram IOV) is likely to be higher than represented. For these experiments, radiolabeled P4.2 was purified from human RBCs by the original method of Korsgren and Cohen using potassium iodide as the chaotropic salt during gel filtration. Radiolabeled P4.2 prepared by the alternative procedure of Korsgren and Cohen did not give reproducible binding in our assays.

**DISCUSSION**

Mice homozygous for the normoblastosis (nb/nb) mutation have RBCs almost completely deficient in full-length 210-kD ankyrin and instead have a small amount of a truncated ankyrin species. The ankyrin deficiency results in a secondary spectrin deficiency and fragile spherocytic RBCs subject to shortened survival. We find that the nb/nb RBC membranes are also about 73% deficient in P4.2 as determined by SDS-PAGE analysis and verified by Western blotting using polyclonal antihuman RBC P4.2 antibodies. Control studies showed that the P4.2 deficiency was not caused by anemia, reticulocytosis, or in vitro protein degradation during membrane preparation and SDS-PAGE analysis.

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<th>Table 1. Mouse RBC Membrane Protein Content</th>
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High reticulocyte controls include beta-thalassemic (27% reticulocytes) and acetylphenylhydrazine-treated (>50% reticulocytes) mice. nb/nb mice had 38% reticulocytes. RBC ghost membranes were prepared by hypotonic hemolysis. Membrane proteins were separated by SDS-PAGE on 9% acrylamide gels using the Laemmli discontinuous buffer system and stained with Coomassie brilliant blue followed by laser densitometry to quantitate the amount of protein in the stained bands. Results are the mean ± 1 SD of the number of experiments shown in the parentheses. The probability of the null hypothesis was calculated using the Student's t-test (two-tailed, parametric distribution).
The presence of \( \approx 25\% \) of P4.2 in \( nb/nb \) membranes despite near complete absence of 210-kD ankyrin suggests several possibilities: (1) the absence of 210-kD ankyrin reduces high-affinity P4.2 binding to cdb3, but allows partial low-affinity binding; (2) the 150-kD truncated ankyrin is capable of providing partial enhancement of P4.2 binding to cdb3; (3) P4.2 binds to membrane sites other than cdb3. This possibility is consistent with earlier in vitro findings of Korsgren and Cohen\(^\text{1} \) who reported that proteolytic elimination of most of the high-affinity band 3 binding sites in P4.2-stripped IOVs decreased, but did not eliminate P4.2 membrane binding.

Metabolic labeling studies showed that \( nb/nb \) reticulocytes were actively synthesizing P4.2 at rates comparable with \( \beta \)-thalassemic high-reticulocyte mouse controls. Morphologic analysis of \( rtlhl? \) and \( \& \)-thalassemic reticulocytes showed that both populations contained young stress reticulocytes, making it less likely that differences in reticulocyte matura-

Fig 2. Mouse reticulocyte P4.2 synthesis. Reticulocytes were isolated from \( nb/nb \) (■) or \( \beta \)-thalassemic (●) mice and equivalent amounts were metabolically labeled with \( ^{35} \text{S}-\text{methionine}. \) P4.2 was immunoprecipitated from RBC lysates using affinity-purified anti-P4.2 IgG and formalin-fixed SACS. Results given as the incorporation of \( ^{35} \text{S}-\text{methionine} \) (counts per minute) into P4.2 per \( 1 \times 10^7 \) reticulocytes.

Fig 3. (A) SDS-PAGE analysis (Fairbanks buffer system\(^\text{17} \)) of purified human RBC ankyrin (lane 1), \( nb/nb \) IOVs (lane 2), and \( nb/nb \) IOVs reconstituted with human RBC ankyrin (lane 3). The position of 210-kD ankyrin and band 3 is indicated. (B) Binding of \( ^{125} \text{I}-\text{human RBC P4.2} \) to \( nb/nb \) IOVs with (■, □), or without (●, ○) reconstitution with human RBC ankyrin. Curves shown include data from two different binding experiments (■, ○ and □, □) and were fitted using Enzfitter curve fitting software using a ligand-binding [one-site] equation (Bio-soft, Cambridge, UK).
tion were a factor in these comparisons. Although we did not determine the accumulation of newly synthesized P4.2 into β-thalassemic reticulocyte membranes, in other studies in which we compared nb/nb and wild-type (+/+ ) reticulocyte membranes, we found that accumulation of newly synthesized P4.2 into nb/nb membranes was 13-fold lower than in +/+ membranes (not shown). One may argue that part of the difference in P4.2 membrane assembly in +/+ and nb/nb reticulocytes may reflect the maturational stage of the P4.2 binding site. Evidence against this is the fact that band 3 is synthesized early in erythroid development, whereas P4.2 is synthesized in the latter stages of erythroid development making it less likely that the P4.2 binding site would be significantly different between nb/nb and +/+ reticulocytes. Thus, we conclude that the difference in P4.2 binding is more likely caused by the deficiency of 210-kD ankyrin in nb/nb membranes. In either event, these results argue that the mechanism underlying P4.2 deficiency in nb/nb RBCs is unlikely to be decreased P4.2 synthesis.

The role of ankyrin in enhancing P4.2 membrane binding was further shown in experiments in which reconstitution of nb/nb IOVs with ankyrin approximately doubled P4.2 binding. Although these results clearly establish that ankyrin enhances P4.2 membrane binding, they are of limited usefulness for obtaining details about binding constants or affinities because human RBCs were the source of both the ankyrin and P4.2 used in these binding experiments. Because band 3 sequence is not highly conserved in the N-terminal cytoplasmic domain that contains binding sites for both ankyrin and P4.2, it is impossible to determine what effect interspecies variability in ankyrin, P4.2, and band 3 sequence has on P4.2 membrane binding.

Our findings differ from earlier results of Korsgren and Cohen who found no evidence for ankyrin enhancement of P4.2 binding to ankyrin-reconstituted stripped IOVs. Possible reasons for this difference could be that (1) pH 11 extraction used to strip IOVs altered or denatured cdb3 in a way that prevented ankyrin enhancement of P4.2 binding; (2) different levels of P4.2 and ankyrin were used in the binding experiments; or (3) as stated above, species differences were a factor (human v mouse IOVs).

In human RBC membranes, band 3 exists as dimers, tetramers, and higher order oligomers, the latter forms are thought to be stabilized by direct interactions with cytoskeletal proteins. Because ankyrin and P4.2 bind to distinct sites on cdb3, mechanisms by which ankyrin could enhance the binding of P4.2 to cdb3 include (1) ankyrin-induced stabilization of specific conformational or self-association states of band 3; (2) ankyrin deficiency could result in modification of the cdb3 binding site for P4.2, resulting in poorly bound protein that is susceptible to endogenous proteolysis. We did not find evidence of P4.2 proteolysis in nb/nb ankyrin-deficient membranes, suggesting that proteolysis occurs before the release of circulating RBCs or that the P4.2 degradation products are very small and avoid detection in our system. An alternative explanation for the deficiency of P4.2 in the ankyrin-deficient nb/nb RBCs is a second genetic mutation that affects P4.2 synthesis. This is unlikely because metabolically labeled nb/nb reticulocytes actively synthesize P4.2. Moreover, the mouse ankyrin gene is (Ank-1) located on chromosome 8, whereas the mouse P4.2 gene is located on chromosome 2, precluding any linkage between these genetic defects.

If ankyrin is required for normal associations of P4.2 with band 3, then why is it that ankyrin can be extracted from RBC membranes under conditions that have little effect on the removal of P4.2? One explanation could be that binding of ankyrin to band 3 promotes high-affinity P4.2 binding to some other membrane site and once P4.2 is bound, subsequent removal of ankyrin would not result in P4.2 release. This would imply that band 3 molecular interactions are important in establishing normal P4.2 binding. Alternatively, it is possible that binding of P4.2 to band 3 establishes interactions between P4.2 and other membrane components that stabilize its membrane binding in the absence of ankyrin.

We conclude from these studies that ankyrin enhances the association of P4.2 with RBC membranes. This conclusion is consistent with naturally occurring human RBC ankyrin deficiency that is associated with a secondary partial P4.2 deficiency. The P4.2 deficiency associated with ankyrin deficiency is not secondarily caused by spectrin deficiency likely because RBC P4.2 content is normal in hereditary spherocytosis RBCs with a primary spectrin deficiency in the absence of ankyrin deficiency in both humans and mice.

ACKNOWLEDGMENT

The authors are grateful to Dr Jane Barker for providing nb/nb and +/+ mice; Dr Mary Fabry for providing β-thalassemic and acetylsalicylic acid treated mice; Dr Sam Lux’s laboratory for providing anti-Ank-1 antibody; and Dr Hannah Shear and the late Dr Eugene Roth for performing mouse phlebotomies and helpful discussions.

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Decreased content of protein 4.2 in ankyrin-deficient normoblastosis (nb/nb) mouse red blood cells: evidence for ankyrin enhancement of protein 4.2 membrane binding

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