

Hereditary spherocytosis and hereditary elliptocytosis: aberrant protein sorting during erythroblast enucleation

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ABSTRACT

During erythroblast enucleation, membrane proteins distribute between extruded nuclei and reticulocytes. In Hereditary Spherocytosis (HS) and Hereditary Elliptocytosis (HE), deficiencies of membrane proteins, in addition to those encoded by the mutant gene, occur. Elliptocytes, resulting from protein 4.1R gene mutations, lack not only 4.1R but also glycophorin C (GPC), which links the cytoskeleton and bilayer. In HS resulting from ankyrin-1 mutations, band 3, Rh-associated antigen (RhAG) and glycophorin A (GPA) are deficient. The current study was undertaken to explore whether aberrant protein sorting, during enucleation, creates these membrane-spanning protein deficiencies. We found that while GPC sorts to reticulocytes normally, it distributes to nuclei in 4.1R deficient HE cells. Further, GPA and RhAG, which normally partition predominantly to reticulocytes, distribute to both nuclei and reticulocytes in an ankyrin-1 deficient murine model of HS. We conclude that aberrant protein sorting is one mechanistic basis for protein deficiencies in HE and HS.

INTRODUCTION

During erythroblast enucleation, plasma membrane and cytoskeletal proteins dynamically reorganize while the nucleus, surrounded by plasma membrane, separates from the nascent reticulocyte. A key aspect of this process is the partitioning of erythroblast proteins to extruded nuclei and/or nascent reticulocytes. Hence, protein sorting during enucleation plays a crucial role in determining the protein content of reticulocyte membranes and cytoskeleton. Others have shown that cytoskeletal actin, spectrin and protein 4.1 partition to reticulocytes¹, while we have discovered that one molecular mechanism regulating membrane-spanning protein sorting to reticulocytes is their degree of connectivity to the cytoskeleton.²

In Hereditary Spherocytosis (HS) and Hereditary Elliptocytosis (HE), as well as in murine models of these disorders, deficiencies of red cell membrane proteins, in addition to those encoded by the mutant gene, are well described. Elliptocytic erythrocytes, resulting from protein 4.1R gene mutations^{3,4}, lack not only protein 4.1R but also the membrane-spanning protein glycophorin C (GPC)^{5,6}, a 4.1R binding partner with a key role in linking the cytoskeleton to the bilayer. In HS resulting from ankyrin-1 gene mutations⁷⁻⁹, deficiencies of band 3, Rh-associated antigen (RhAG) and glycophorin A (GPA) have been documented.^{10,11} Similarly, in HS resulting from band 3 gene mutations, members of the band 3 macromolecular complex are decreased.¹²⁻¹⁴

Various mechanisms, either singly or in combination, could produce the protein deficiencies observed in HS and HE. Specifically, proteins might not be normally assembled on the erythroblast membrane; sorting during enucleation might be perturbed; or proteins might be intracellularly degraded or released in exosomes during reticulocyte maturation. The current study explores whether aberrant protein sorting during enucleation creates some of the specific protein deficiencies.

METHODS

Antibodies

Rabbit antibodies specific for mouse GPC, band 3, and RhAG were generated in our laboratory.⁶ Anti-GPC was labeled with Alexa Fluor 555 (Invitrogen-Molecular Probes, Carlsbad, CA) according to manufacturer's instructions. Other antibodies were obtained from commercial sources detailed below.

Mice

Ankyrin-1 deficient *nb/nb* mice⁸, provided by Dr. Luanne Peters (The Jackson Laboratory, Bar Harbor, ME), and protein 4.1R knockout mice⁴ were maintained in the New York Blood Center animal facility. The Institutional Animal Care and Use Committee of the New York Blood Center approved all protocols.

Immunofluorescence microscopy

Freshly harvested 4.1R null and wild type (WT) bone marrow cells were suspended in RPMI with 20% fetal calf serum (Invitrogen, Carlsbad, CA) and stained with Syto-17 (1 μ M; Invitrogen) and FITC-conjugated TER 119 (0.25 μ g/10⁶ cells; eBioscience, San Diego, CA) or Alexa Fluor 555-labeled anti-GPC antibody (1 μ g/10⁶ cells) for 45 minutes at 37 C°. After washing, the cells were imaged. *nb/nb* and WT bone marrow cells were fixed on Cell Tak (BD Biosciences, San Jose, CA) –coated coverslips with 3% paraformaldehyde for 5 minutes at room temperature. The cells were then blocked for 1 hour in 1% albumin-phosphate buffered saline (PBS) at room temperature and double stained overnight at 4 C° with TER 119 (1:50; BD Pharmingen, San Diego, CA) and either rabbit anti-mouse band 3, rabbit anti-GPC or rabbit anti-RhAG (1:100). After washing with 0.1% albumin-PBS, the cells were labeled with Alexa Fluor 594-conjugated goat anti-rabbit IgG (1:100; Invitrogen) and Alexa Fluor 488-conjugated donkey anti-rat IgG (1:500; Invitrogen) for 1 hour at room temperature. After washing, the slides were mounted with Vecta Shield (Vector Labs, Burlingame, CA).

RESULTS AND DISCUSSION

To explore whether aberrant protein sorting might be responsible for deficiencies of membrane proteins in HE, we examined sorting of GPC during enucleation of normal and protein 4.1R null erythroblasts. Protein 4.1R knockout mice have fragmented red cells, which lack GPC, thus phenotypically mimicking human HE. Using immunofluorescent microscopy, we first analyzed GPC sorting in enucleating erythroblasts from WT bone marrow. We found that GPC partitioned almost exclusively to nascent reticulocytes, with little or no GPC observed in plasma membranes of extruding nuclei (Figure 1). Strikingly, in 4.1R null erythroblasts, GPC distributed exclusively to nuclei (Figure 1). These data unequivocally establish that GPC deficiency in 4.1R null erythrocytes is attributable, in large part, to markedly abnormal protein partitioning during enucleation. Hence, our findings provide a novel, molecular explanation for the underlying basis of specific membrane protein deficiencies observed in 4.1R-deficient HE.

To determine whether aberrant sorting was specific for 4.1R-associated proteins, we studied the behavior of GPA, a membrane-spanning molecule that is not part of the 4.1R complex. GPA was an ideal control, since in prior studies we showed that GPA sorts almost exclusively to reticulocytes during normal erythroblast enucleation.² In 4.1R null cells, GPA partitioning was not perturbed; it sorted to nascent reticulocytes in both WT and 4.1R null enucleating erythroblasts (Figure 1). Collectively, these findings provide clear evidence that cytoskeletal protein 4.1R is critical for normal sorting of GPC to reticulocytes. Moreover, they further strengthen the concept that cytoskeletal attachments are an important factor in regulating transmembrane protein sorting to reticulocytes.

We next asked whether aberrant sorting is also mechanistically responsible for creating deficiencies of specific membrane proteins in mature erythrocytes in HS. To address this, we employed ankyrin-1 deficient *nb/nb* mice, a faithful model of human HS secondary to mutations

in the gene encoding ankyrin-1. We first analyzed GPC, a membrane-spanning molecule that is not part of the ankyrin-1 complex. GPC partitioning was unperturbed in *nb/nb* cells; it sorted to nascent reticulocytes in both WT and *nb/nb* enucleating erythroblasts (Figure 2A). We then focused on the sorting patterns of band 3, RhAG, and GPA, since these three proteins are deficient in *nb/nb* erythrocytes and in human HS secondary to ankyrin-1 mutations. In WT enucleating erythroblasts, both band 3 and RhAG distributed predominantly to reticulocytes (Figure 2B,C), similar to GPA. In marked contrast, in ankyrin-1 deficient *nb/nb* enucleating erythroblasts, band 3, GPA, and RhAG sorted to both expelled nuclei and reticulocytes (Figure 2B,C). These data clearly demonstrate that one mechanism producing deficiencies of band 3, RhAG and GPA in mature *nb/nb* red cells is their abnormal sorting during nuclear extrusion.

Although the deficiency of GPC in HE red cells secondary to 4.1R mutations, and the deficiencies of band 3, GPA and RhAG in HS red cells secondary to ankyrin-1 mutations, are well documented, the concept that these deficiencies might be caused by mechanisms active at the time of enucleation had not been entertained. Our findings focus attention, for the first time, on this stage of erythropoiesis in HS and HE. Further, they raise the possibility that reticulocytes in HS and HE may differ from normal reticulocytes in their biophysical properties of membrane cohesion or membrane deformability, since these two crucial properties are regulated by vertical linkages between specific membrane-spanning proteins embedded in the lipid bilayer and cytoskeletal proteins, such as the interactions of band 3, ankyrin-1 and spectrin or interactions of GPC, 4.1R, actin and spectrin. (recently reviewed¹⁵) If HS and HE reticulocytes are abnormal then membrane loss of surface area may be exacerbated at this stage rather than occurring only in mature cells. Support for such a thesis can be inferred from an earlier study showing that surface area loss is a distinct feature of HS reticulocytes.¹⁶ Future studies will determine the relative contributions of sorting abnormalities and other mechanisms for the observed protein deficiencies in HS and HE.

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AUTHORSHIP

Contributions: X.A. and J.A.C. designed research, analyzed data and wrote the manuscript. M.S., K.C. and J.V. performed research and analyzed data. N.M. analyzed data and edited the manuscript.

Conflict of interest disclosure: The authors declare no competing financial interests.

REFERENCES

1. Koury S, Koury M, Bondurant M. Cytoskeletal distribution and function during the maturation and enucleation of mammalian erythroblasts. *J Cell Biol.* 1989;109(6 Pt 1):3005-3013.
2. Lee JC, Gimm JA, Lo AJ, et al. Mechanism of protein sorting during erythroblast enucleation: role of cytoskeletal connectivity. *Blood.* 2004;103(5):1912-1919.
3. Conboy JG, Chasis JA, Winardi R, Tchernia G, Kan YW, Mohandas N. An isoform-specific mutation in the protein 4.1 gene results in hereditary elliptocytosis and complete deficiency of protein 4.1 in erythrocytes but not in nonerythroid cells. *J Clin Invest.* 1993;91(1):77-82.
4. Shi ZT, Afzal V, Collier B, et al. Protein 4.1R-deficient mice are viable but have erythroid membrane skeleton abnormalities. *J Clin Invest.* 1999;103(3):331-340.
5. Jeremy KP, Plummer ZE, Head DJ, et al. 4.1R-deficient human red blood cells have altered phosphatidylserine exposure pathways and are deficient in CD44 and CD47 glycoproteins. *Haematologica.* 2009;94(10):1354-1361.
6. Salomao M, Zhang X, Yang Y, et al. Protein 4.1R-dependent multiprotein complex: new insights into the structural organization of the red blood cell membrane. *Proc Natl Acad Sci U S A.* 2008;105(23):8026-8031.

7. Costa FF, Agre P, Watkins PC, et al. Linkage of dominant hereditary spherocytosis to the gene for the erythrocyte membrane-skeleton protein ankyrin. *N Engl J Med.* 1990;323(15):1046-1050.
8. Peters LL, Birkenmeier CS, Bronson RT, et al. Purkinje cell degeneration associated with erythroid ankyrin deficiency in nb/nb mice. *J Cell Biol.* 1991;114(6):1233-1241.
9. White RA, Birkenmeier CS, Lux SE, Barker JE. Ankyrin and the hemolytic anemia mutation, *nb*, map to mouse chromosome 8: presence of the *nb* allele is associated with a truncated erythrocyte ankyrin. *Proc Natl Acad Sci U S A.* 1990;87(8):3117-3121.
10. Nicolas V, Le Van Kim C, Gane P, et al. Rh-RhAG/ankyrin-R, a new interaction site between the membrane bilayer and the red cell skeleton, is impaired by Rh(null)-associated mutation. *J Biol Chem.* 2003;278(28):25526-25533.
11. Yi SJ, Liu SC, Derick LH, et al. Red cell membranes of ankyrin-deficient nb/nb mice lack band 3 tetramers but contain normal membrane skeletons. *Biochemistry.* 1997;36(31):9596-9604.
12. Bruce LJ, Beckmann R, Ribeiro ML, et al. A band 3-based macrocomplex of integral and peripheral proteins in the RBC membrane. *Blood.* 2003;101(10):4180-4188.
13. Hassoun H, Hanada T, Lutchman M, et al. Complete deficiency of glycophorin A in red blood cells from mice with targeted inactivation of the band 3 (AE1) gene. *Blood.* 1998;91(6):2146-2151.
14. Peters LL, Shivdasani RA, Liu SC, et al. Anion exchanger 1 (band 3) is required to prevent erythrocyte membrane surface loss but not to form the membrane skeleton. *Cell.* 1996;86(6):917-927.
15. Mohandas N, Gallagher PG. Red cell membrane: past, present, and future. *Blood.* 2008;112(10):3939-3948.
16. Da Costa L, Mohandas N, Sorette M, Grange MJ, Tchernia G, Cynober T. Temporal differences in membrane loss lead to distinct reticulocyte features in hereditary spherocytosis and in immune hemolytic anemia. *Blood.* 2001;98(10):2894-2899.

FIGURE LEGENDS

Figure 1. Analysis of GPC sorting during enucleation of WT and 4.1R null erythroblasts. Differential Interference Contrast (DIC) and immunofluorescent micrographs of WT and 4.1R null enucleating erythroblasts, including nascent reticulocyte (R) and extruding nucleus (N), probed with FITC-conjugated TER 119, specific for glycophorin A (*green*) or Alexa Fluor 555-labeled rabbit anti-mouse GPC antibody (*red*). Nuclei were identified by Syto-17 staining (*blue*). Number of enucleating erythroblasts examined under each staining condition ≥ 6 . The images were observed using a Zeiss LSM META 510 Confocal microscope (Carl Zeiss Microimaging Inc., Thronwood, NY) with an APOCHROMAT 63x/1.4 Oil DIC objective and

acquired using Zeiss Laser Scanning Microscope LSM 510, Version 3.2 SP2 software with a Zeiss AxioCam HRm Rev. 2/3.3V camera. The images were processed using Adobe Photoshop (Adobe Systems Inc., San Jose, CA).

Figure 2. Analysis of band 3, RhAG, and GPA sorting during enucleation of WT and *nb/nb* erythroblasts. Differential Interference Contrast (DIC) and immunofluorescent micrographs of WT and *nb/nb* enucleating erythroblasts, including nascent reticulocyte (R) and extruding nucleus (N); probed with rabbit anti-mouse GPC and Alexa Fluor 594-labeled goat anti-rabbit IgG (*red*) (A); probed with rat anti-mouse TER 119 and Alexa 488-conjugated donkey anti-rat IgG (*green*) or rabbit anti-mouse band 3 and Alexa Fluor 594-labeled goat anti-rabbit IgG (*red*) (B); probed with rat anti-mouse TER 119 and Alexa 488-conjugated donkey anti-rat IgG (*green*) or rabbit anti-mouse RhAG and Alexa Fluor 594-labeled goat anti-rabbit IgG (*red*) (C). Nuclei were identified by DAPI staining (*blue*). Dashed lines outline the spherical portion of extruding nuclei in the red and green images in which there is no fluorescent labeling of the nucleus. Number of enucleating erythroblasts examined under each staining condition ≥ 6 . Of note, during extrusion the nucleus transiently deforms and a portion of it is visualized within the nascent reticulocyte, as evidenced in these images.

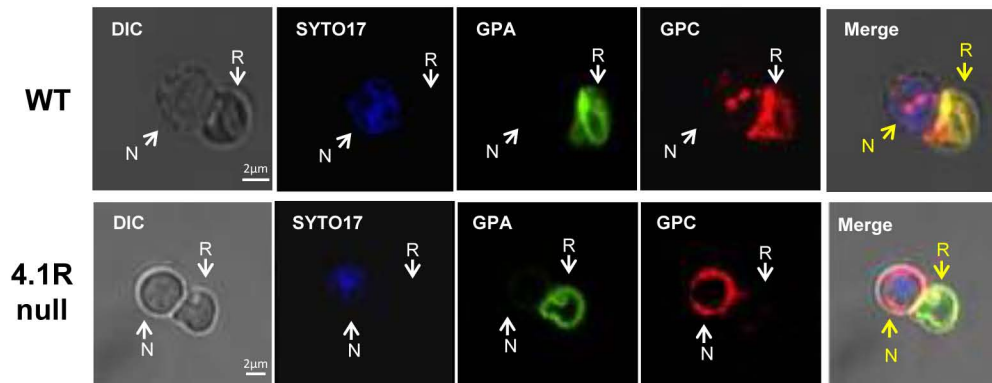


Figure 1

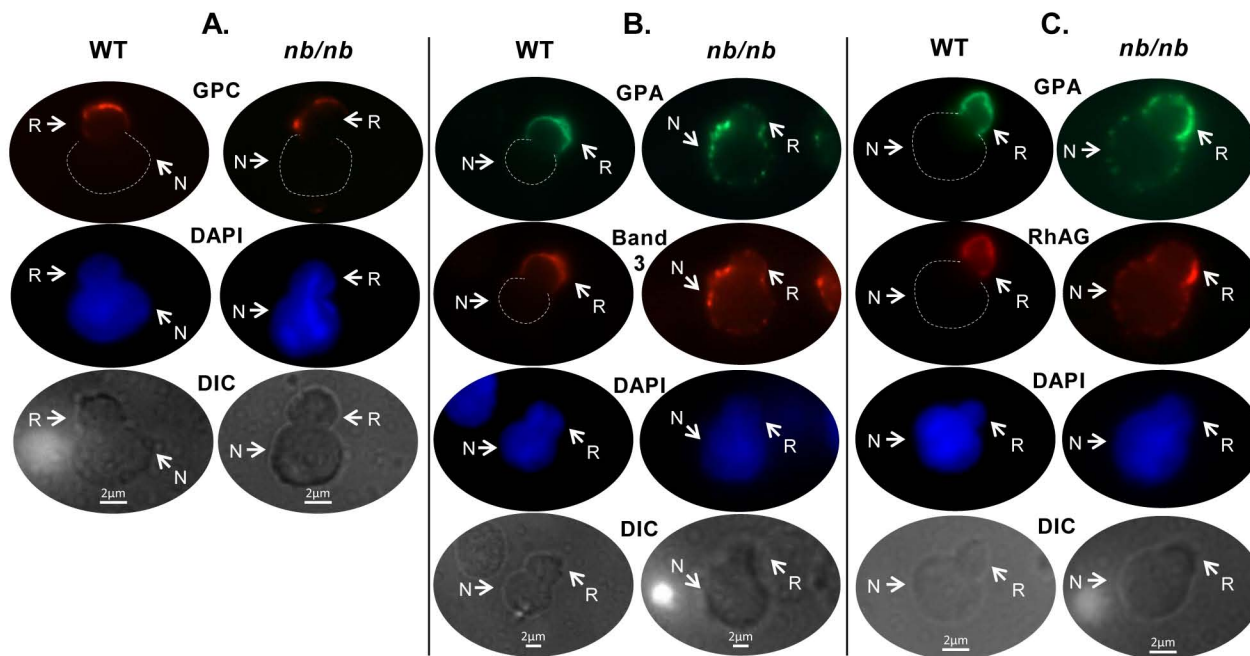


Figure 2