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

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Introduction

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. Elliptocytosis, resulting from protein 4.1R gene mutations, lack not only 4.1R but also glycophorin C, which links the cytoskeleton and bilayer. In HS resulting from ankyrin-1 mutations, band 3, Rh-associated antigen, and glycophorin A are deficient. The current study was undertaken to explore whether aberrant protein sorting, during enucleation, creates these membrane-spanning protein deficiencies. We found that although glycophorin C sorts to reticulocytes normally, it distributes to nuclei in 4.1R-deficient HE cells. Further, glycophorin A and Rh-associated antigen, 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. (Blood. 2010;116(2):267-269)

Methods

Antibodies

Rabbit antibodies specific for mouse GPC, band 3, and RhAG were generated in our laboratory. Anti-GPC was labeled with Alexa Fluor 555 (Invitrogen–Molecular Probes) according to the manufacturer’s instructions. Other antibodies were obtained from commercial sources detailed in “Immunofluorescence microscopy.”

Mice

Ankyrin-1–deficient nb/nb mice,8 provided by Dr Luanne Peters (The Jackson Laboratory), and protein 4.1R knockout mice1 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) and stained with Syto-17 (1μM; Invitrogen) and fluorescein isothiocyanate–conjugated TER 119 (0.25 μg/106 cells; eBioscience) or Alexa Fluor 555–labeled anti-GPC antibody (1 μg/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)–coated coverslips with 3% paraformaldehyde for 5 minutes at room temperature. The cells were then blocked for 1 hour at 4°C with TER 119 (0.25 μg/106 cells; eBioscience) or Alexa Fluor 555–labeled anti-GPC antibody (1 μg/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)–coated coverslips with 3% paraformaldehyde for 5 minutes at room temperature. The cells were then blocked for 1 hour at 4°C in 0% albumin-phosphate–buffered saline at room temperature and double stained overnight at 4°C with TER 119 (1:50; BD PharMingen) and either rabbit
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. By 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 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, because these 3 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 because these 2 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 GPA, 4.1R, actin, and spectrin (recently reviewed in Mohandas and Gallagher15). 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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**Figure 2. Analysis of band 3, RhAG, and GPA sorting during enucleation of WT and nb/nb erythroblasts.** 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 Fluor 488–conjugated donkey anti–rat IgG (green) or rabbit anti–mouse band 3 and Alexa Fluor 594–labeled goat anti–rabbit IgG (red; B); or 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 4′,6-diamidino-2-phenylindole staining (DAPI; 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. The number of enucleating erythroblasts examined under each staining condition was 6 or more. Of note, during extrusion the nucleus transiently deforms, and a portion of it is visualized within the nascent reticulocyte, as evidenced in these images.


**Authorship**

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

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

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