The cytoskeletal binding domain of band 3 is required for multiprotein complex formation and retention during erythropoiesis

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ABSTRACT

Band 3 is the most abundant protein in the erythrocyte membrane and forms the core of a major multiprotein complex. The absence of band 3 in human erythrocytes has only been reported once, in the homozygous band 3 Coimbra patient. We used *in vitro* culture of erythroblasts derived from this patient, and separately short hairpin RNA-mediated depletion of band 3, to investigate the development of a band 3-deficient erythrocyte membrane and to specifically assess the stability and retention of band 3 dependent proteins in the absence of this core protein during terminal erythroid differentiation. Further, using lentiviral transduction of N-terminally green fluorescent protein-tagged band 3, we demonstrated the ability to restore expression of band 3 to normal levels and to rescue secondary deficiencies of key proteins including glycophorin A, protein 4.2, CD47 and Rh proteins arising from the absence of band 3 in this patient. By transducing band 3-deficient erythroblasts from this patient with band 3 mutants with absent or impaired ability to associate with the cytoskeleton we also demonstrated the importance of cytoskeletal connectivity for retention both of band 3 and of its associated dependent proteins within the reticulocyte membrane during the process of erythroblast enucleation.

Introduction

The bicarbonate/chloride exchanger band 3 is the most abundant membrane protein in the erythrocyte membrane where, alongside its transport function, it performs a critical role in maintaining red cell structural integrity. Band 3 is an important component of at least two major multiprotein complexes, the ankyrin and junctional membrane complexes that serve as sites of vertical association between the plasma membrane and the underlying spectrin-based cytoskeleton.[1,2] The N-terminal domain (1-359 amino acids) is responsible for the association with the cytoskeleton, possessing binding sites for ankyrin,[3] protein 4.2,[4] adducin[2] and protein 4.1[5] in addition to glycolytic enzymes[6,7] and hemoglobin.[8] The remaining C-terminal membrane domain (B3mem; 360-911 amino acids) possesses 12-14 transmembrane helices and performs a key role in regulating erythrocyte gas exchange by exchanging chloride and bicarbonate ions across the plasma membrane. The B3mem domain alone traffics efficiently to the plasma membrane when expressed in multiple cell types[9-12] but is not expressed endogenously in this form *in vivo*. Band 3 is also expressed as an N-terminally truncated isoform (kB3) in the alpha intercalated cells of the kidney where it is required for acid secretion.[13,14] The 65 amino acid kB3 N-terminal truncation removes the ankyrin binding site[15] but retains the ability to bind protein 4.2.[11]

Specific mutations in the band 3 gene result in the erythrocyte diseases hereditary spherocytosis (HS)[16] or hereditary stomatocytosis and/or the kidney disease dominant distal renal tubular acidosis.[14,17,18] HS is a form of hemolytic anemia characterized by the presence of spherocytic erythrocytes which are smaller and more fragile than normal red blood cells. Mutations in band 3 account for 10-20% of the cases of HS, are heterogeneously distributed throughout the protein structure and usually result in decreased expression of band 3.[19] The extent of this reduction and the mechanism by which it occurs varies according to the mutation but is believed to include mRNA and protein instability, or intracellular retention.[20,21]

Although band 3 null models have been artificially generated in both cattle[22] and mice,[23] the naturally occurring complete absence of band 3 in humans has only been reported in one individual with the homozygous band 3 Coimbra mutation.[24] Band 3 Coimbra is a V488M mutation in the fourth transmembrane helix of band 3 which in the heterozygous state results in typical mild HS and a partial reduction in band 3 expression (20±2%). The homozygous patient has severe HS and distal renal tubular acidosis, with almost complete absence of band 3 protein in erythrocytes.[1] Absence of band 3 causes secondary erythrocyte membrane protein deficiencies, including the loss of protein 4.2 and severe reduction of glycophorin A and the Rh sub-complex proteins, which led to the proposal that band 3 forms the core of a large macrocomplex.[1] Whilst some progress has been made in our understanding of the assembly of erythrocyte membrane subcomplexes (band 3 and protein 4.2; Rh and RhAG) during erythropoiesis,[25,26] many details relating to the timing, hierarchy of protein-protein interactions and the role of cytoskeletal attachment and remodeling during membrane biogenesis remain to be determined. The *in vitro* culture of the band 3 Coimbra patient's cells presents a unique opportunity to study membrane protein complex assembly during erythroid membrane biogenesis in the absence of band 3, uncover the basis of secondary protein loss in this most definitive of HS cases and hence probe its role as a hub for establishing and maintaining interactions with dependent proteins.

 doi:10.3324/haematol.2014.114538
The online version of this article has a Supplementary Appendix.
Manuscript received on July 23, 2014. Manuscript accepted on October 21, 2014.
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Methods

Antibodies

The antibodies used in this study are listed in the *Online Supplementary Methods*.

Flow cytometry and fluorescence assisted cell sorting

Flow cytometry was performed using $1x10^5$ cells stained for 30 min at 37°C with 5 μg/mL Hoechst33342 (Sigma). Cells were labeled with extracellular primary antibodies for 30 min at 4°C and labeled with rat APC-conjugated anti-mouse IgG_1 (Biolegend). Data were collected using a MacsQuant VYB cytometer and processed using FlowJo Version 7.2.5. For fluorescence assisted cell sorting (FACS), cultures were stained with Hoechst33342 (5 μg/mL) for 30 min at 37°C then sorted using a BD Influx Cell Sorter; reticulocytes (Hoechst negative) and extruded nuclei [Hoechst positive, forward scatter (FSC) low]. Green fluorescent protein (GFP) band 3-positive reticulocytes were obtained by sorting GFP-positive Hoechst-negative populations.

Erythroblast culture

Peripheral blood mononuclear cells were isolated from platelet apheresis blood waste (NHSBT, Bristol, UK) from healthy donors and the homozygous V488M patient with written informed consent for research use in accordance with the Declaration of Helsinki and approval from the Local Research Ethics Committee (REC 12/SW/0199). The culture systems used have been reported previously[27,28] and are outlined in the *Online Supplementary Methods*.

Lentiviral constructs

XLG3-GFP vector was modified to include SalI and MluI enzyme sites downstream of GFP. The cDNA for band 3, band 3 membrane domain and kidney band 3 was amplified by polymerase chain reaction from respective BSXG1 plasmids[12,14] using primers incorporating SalI and MluI and ligated into the modified XLG3-GFP vector. pLKO.1 short hairpin (sh) RNA plasmids were designed by the Broad Institute and purchased from Open Biosystems.

Lentiviral transduction of erythroblasts

For XLG3-GFPB3, XLG3-GFPB3mem or XLG3-GFPkB3 rescue experiments, concentrated virus was added to erythroblasts on day 9 with 8 μg/mL polybrene (Sigma). After 24 h, the cells were washed with phosphate-buffered saline and resuspended in expansion medium (*Online Supplementary Methods*). For knockdown experiments $CD34^+$ cell-derived erythroblasts were transduced on day 3 with concentrated virus in the presence of 8 μg/mL polybrene. After 24 h, the cells were washed with phosphate-buffered saline and resuspended in expansion medium containing puromycin (1 μg/mL) for at least 72 h but removed during differentiation.

Results

Band 3 Coimbra is expressed at low levels during erythropoiesis but is not present at the plasma membrane

Although band 3 is absent from the membranes of band 3 Coimbra erythrocytes, it is unknown whether this mutant protein is actually expressed in erythroblasts during terminal differentiation. To determine this, erythroblasts derived from peripheral blood mononuclear cells of the band 3

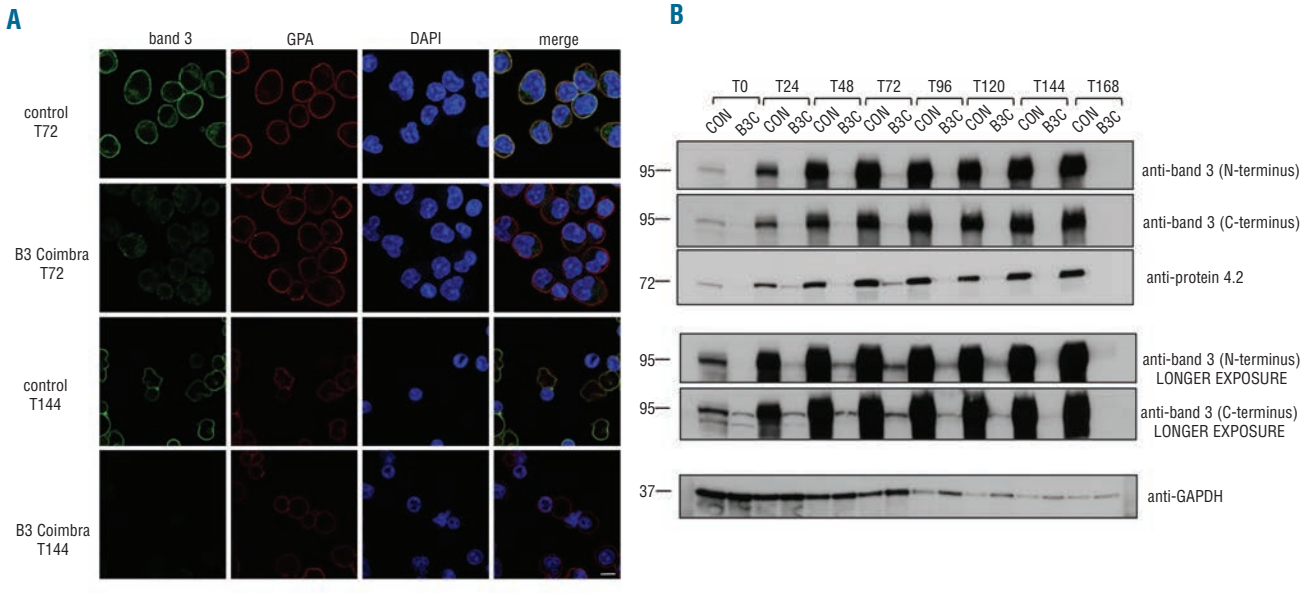


Figure 1. The V488M band 3 mutant (band 3 Coimbra) is expressed at very low levels during erythropoiesis but does not traffic to the plasma membrane. (A) Erythroblasts derived from healthy donors and the homozygous band 3 Coimbra patient's cultures at indicated timepoints were fixed and labeled with a monoclonal antibody for band 3 (BRIC170) and a rabbit polyclonal to glycophorin (GPA), stained with DAPI and imaged using confocal microscopy. This confirms that the band 3 Coimbra protein is weakly expressed in the patient's erythroblasts and did not localize to the plasma membrane unlike normal band 3. By 144 h the band 3 Coimbra expression was not detectable by immunofluorescence imaging. Images were acquired using a Leica SP5 AOBS confocal laser scanning microscope with a 100x oil-immersion objective (N.A 1.4). Scale bar represents 5 μm. (B) Differentiating erythroblasts (5×10^5) derived from the peripheral blood of a healthy donor control or the band 3 Coimbra patient were removed from culture at 24 h intervals; cells were lysed and the proteins separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and immunoblotted with monoclonal antibodies to band 3 N-terminus (BRIC170), protein 4.2 (BRIC273) and rabbit polyclonal antibodies to band 3 C-terminus and GAPDH.

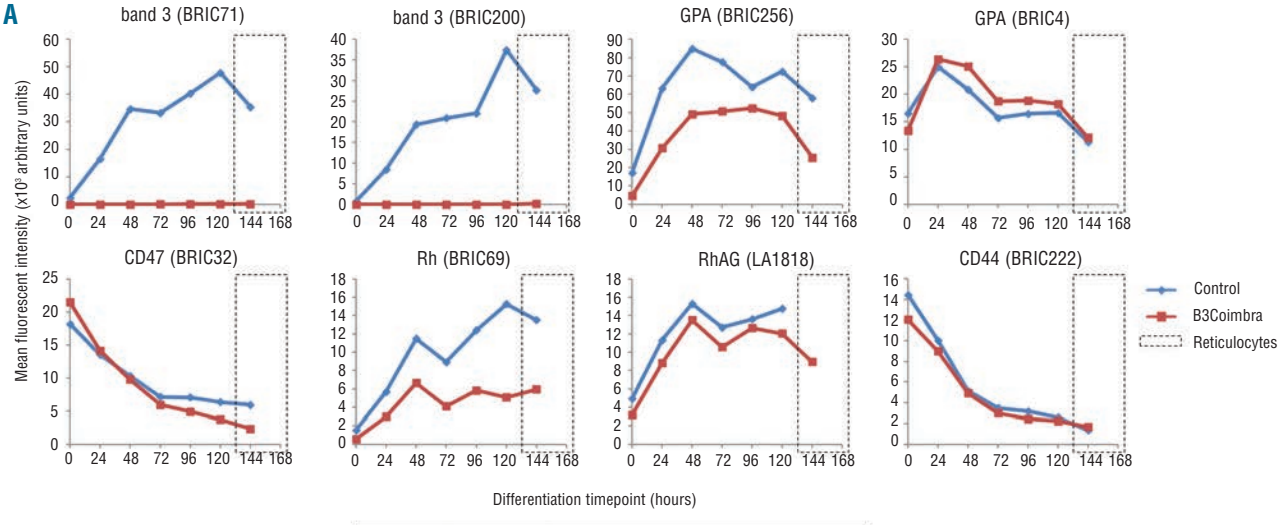
Coimbra patient were expanded and differentiated in parallel to those derived from healthy donors. Figure 1 shows that only low levels of band 3 V488M mutant were synthesized during erythropoiesis as judged by immunoblotting (<1%); this protein has a barely detectable, diffuse intracellular localization. Surface expression of band 3, as assessed by flow cytometry, was less than 1% at all stages of erythropoiesis (Figure 2A). Expression levels of the band 3 binding protein 4.2 mimic the relative expression of band 3 mutant from the onset of its expression, consistent with the known early dependence of protein 4.2 on band 3.[25,29]

Secondary protein loss due to the absence of band 3 occurs early during erythropoiesis

To determine the stage of secondary protein loss in the absence of band 3, surface expression of major erythrocyte membrane proteins was monitored throughout terminal differentiation in erythroblast cultures from both the band 3 Coimbra patient and healthy donors (Figure 2A). The expression of the band 3 chaperone protein glycophorin A, which in healthy donors closely mimics that of band 3, was reduced throughout terminal differentiation of erythroblasts derived from the band 3 Coimbra patient with a reticulocyte expression of 38.6% compared to control. The Wrb antigen, a dual epitope formed at the interface of band 3 and glycophorin A was largely absent, its expression being 3.1% in reticulocytes (Figure 2B).

The surface expression of Rh proteins and RhAG increased within the first 48 h of cell differentiation, although the total surface level was reduced compared to that of controls. However, no further increase in Rh protein expression occurred in the band 3 Coimbra patient's erythroblasts after 48 h. This resulted in an accumulating relative deficiency as terminal differentiation progressed, culminating in a relative expression level of 39% for Rh in reticulocytes (compared to 33% in erythrocytes[1]). Unexpectedly surface RhAG expression, which was reduced to 15% in band 3 Coimbra erythrocytes, was not dramatically reduced relative to the control expression during erythropoiesis, and this protein was expressed at an average of 65% of healthy donor levels in reticulocytes. The surface expression of CD47, another component of the Rh sub-complex that is expressed prior to terminal differentiation, was progressively reduced relative to control expression, manifesting approximately 48 h into terminal differentiation. Surface levels on reticulocytes were 34% of wild-type levels (compared to 15% in erythrocytes[1]). CD44 expression levels in erythroblasts from the patient and healthy donors were closely matched prior to enucleation,

A



B

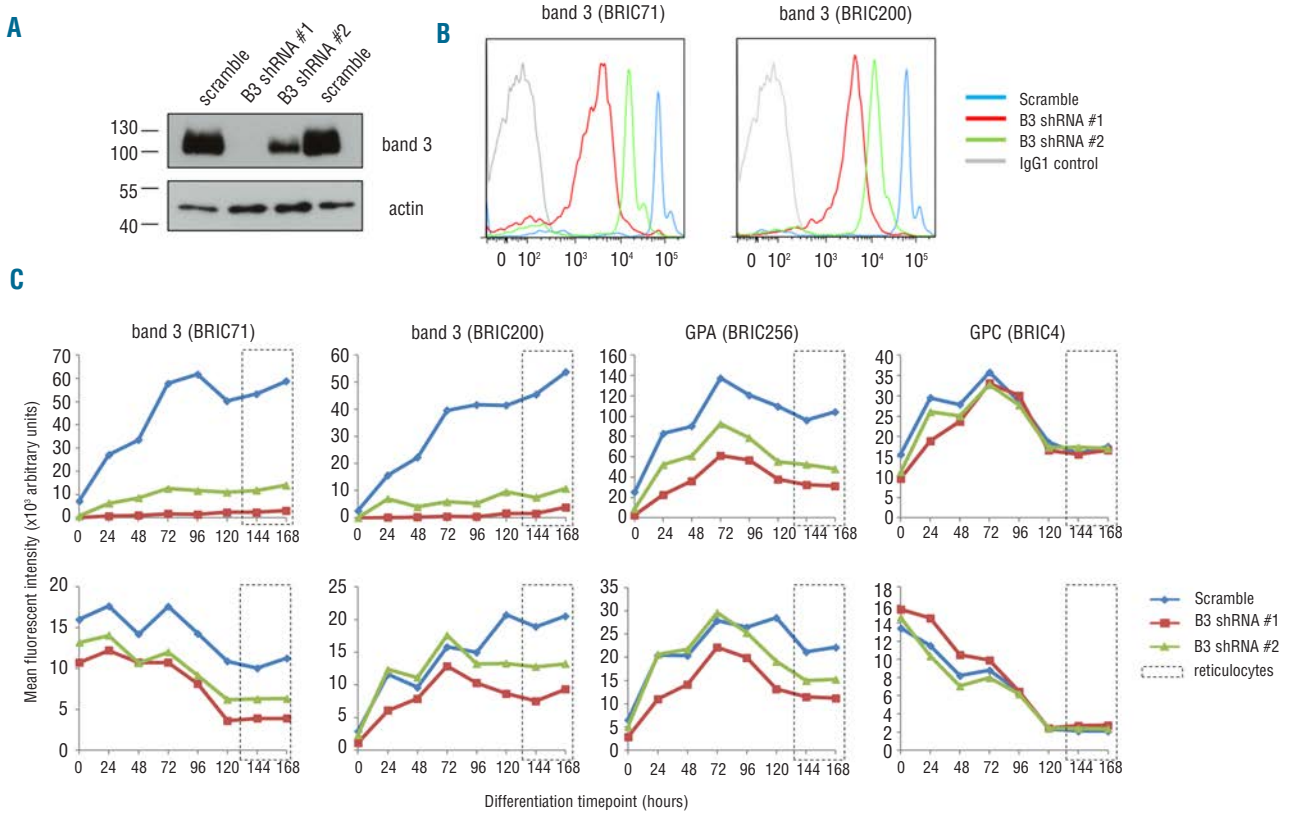
Protein	Antibody	B3Coimbra Mean Surface Expression (% of control)
band 3	BRIC71	0.3 ± 0.1
band 3	BRIC200	0.2 ± 0.2
Wrb	BRIC13	3.1± 0.3
GPA	BRIC256	38.6 ± 3.4
CD47	BRIC32	33.6 ± 2.9
Rh	BRIC69	39.2 ± 2.4
RhAG	LA1818	65.4 ± 10.1
CD44	BRIC222	133.7 ± 6.6
GPC	BRIC4	94.9 ± 6.3

Figure 2. Reduced surface expression of known band 3 associated proteins in the absence of band 3 is evident throughout erythropoiesis. (A) Surface expression of indicated proteins was monitored on differentiating erythroblasts from healthy donor control and the band 3 Coimbra patient's cultures at 24 h intervals using the indicated monoclonal antibodies as detailed in *Online Supplementary Methods*. The dashed box highlights the switch from gating on erythroblasts to reticulocytes after enucleation. Note that antibody-induced agglutination prevented collection of T144 control cells within this specific experiment, but the average RhAG expression of band 3 Coimbra reticulocytes relative to control across additional cultures is shown in Figure 2B. (B) Table showing mean band 3 Coimbra reticulocyte surface expression of indicated proteins relative to expression in a healthy donor control. Means (± standard error) are derived from three independent cultures using average mean fluorescent intensities of reticulocyte populations from multiple timepoints where possible. Representative histograms used to derive the reticulocyte data are shown in *Online Supplementary Figure S1*.

but increased to 134% in the patient's reticulocytes relative to control cells, consistent with erythrocyte levels.[1] Glycophorin C, which is not dependent upon band 3 for its expression or stability, is unaltered in its expression relative to control throughout erythropoiesis.

Short hairpin RNA-mediated depletion of band 3 recapitulates the patient's phenotypes

To confirm that the secondary deficiencies observed in band 3 Coimbra erythroblasts and reticulocytes are due specifically to the deficiency in band 3, shRNA were used to deplete expression of band 3 in erythroblasts. Figure 3A-B shows western blots and flow cytometry data illustrating band 3 expression levels for reticulocytes differentiated from erythroblasts expressing two independent band 3 shRNA (95% and 75% band 3 knockdowns, respectively) compared with a non-targeting shRNA control (scramble). Expression of band 3-dependent erythrocyte proteins, including glycophorin A and Rh complex components, was heavily reduced, recapitulating the alterations observed in band 3 Coimbra erythroblasts throughout differentiation (Figure 3C-D).



D

Protein	Antibody	B3 shRNA #1 Mean Surface Expression (% of scramble)	B3 shRNA #2 Mean Surface Expression (% of scramble)
band 3	BRIC71	4.3 ± 0.3	23.2 ± 0.4
band 3	BRIC200	4.1 ± 0.6	19.9 ± 1.0
Wrb	BRIC13	7.7 ± 0.5	31.6 ± 4.4
GPA	BRIC256	30.6 ± 1.4	48.8 ± 1.6
CD47	BRIC32	33.1 ± 1.5	61.5 ± 1.5
Rh	BRIC69	35.7 ± 3.6	61.3 ± 3.7
RhAG	LA1818	46.9 ± 2.6	69.2 ± 2.4
CD44	BRIC222	116.5 ± 6.9	118.2 ± 3.4
GPC	BRIC4	93.8 ± 1.2	99.9 ± 2.0

Figure 3. shRNA mediated knockdown of band 3 recapitulates secondary protein deficiencies observed in band 3 Coimbra erythroid cultures. (A) 2.5×10⁵ FACS sorted reticulocytes derived from erythroblasts transduced with shRNA targeting band 3 or a non-targeting scramble control were lysed, then the proteins were separated by sodium dodecyl sulfate - polyacrylamide gel electrophoresis and immunoblotted with antibodies to band 3 (BRIC170) and actin.(B) Flow cytometry histograms illustrating efficiency of band 3 knockdown in reticulocytes expressing two independent shRNA. (C) Surface expression of indicated proteins was monitored on differentiating erythroblasts expressing two independent band 3 shRNA and a non-targeting control at 24 h intervals using the indicated monoclonal antibodies as detailed in the *Online Supplementary Methods*. The dashed box highlights the point at which gating was switched from erythroblasts to reticulocytes after enucleation. (D) The table shows the mean surface expression of indicated proteins on reticulocytes derived from band 3 knockdown erythroid cultures relative to the non-targeting scramble control. Means (± standard error) for each shRNA are derived from two independent cultures using average mean fluorescent intensities of reticulocyte populations from three consecutive timepoints.

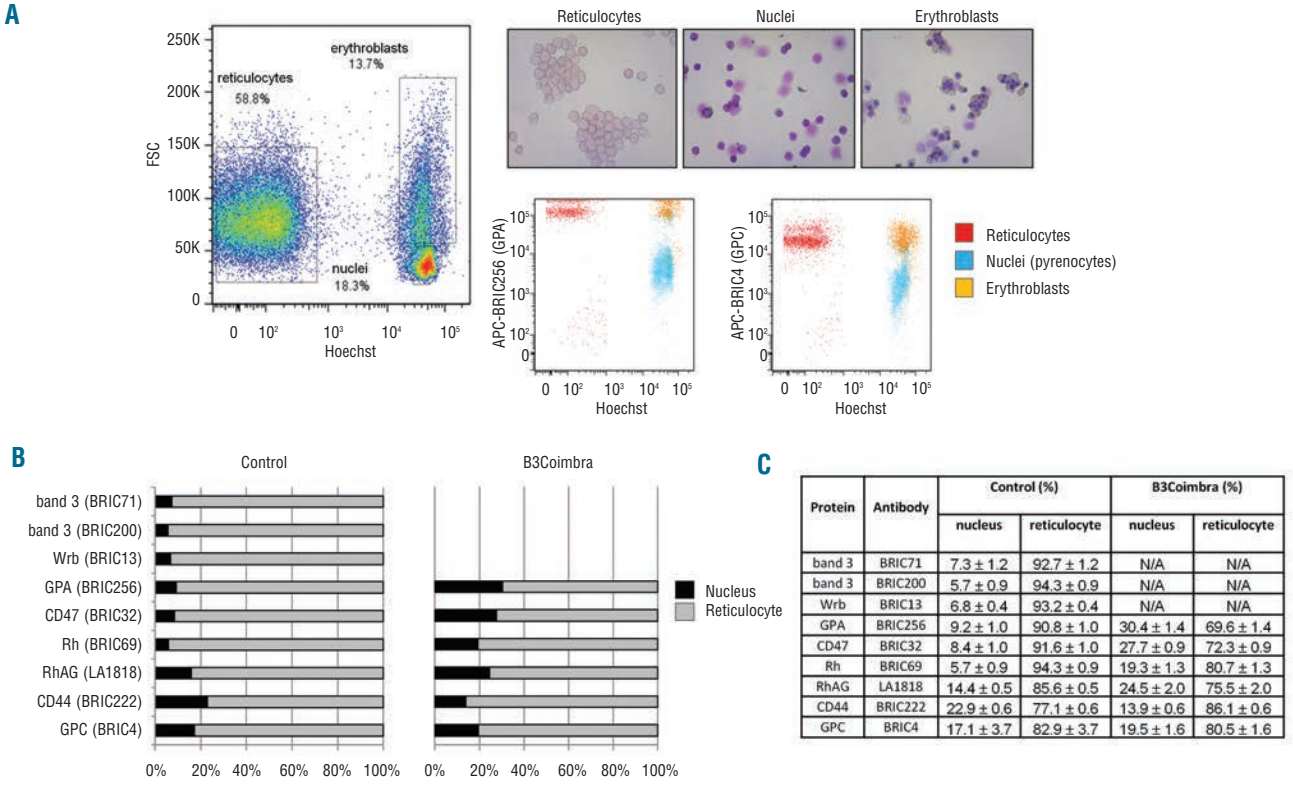
Membrane protein sorting during enucleation is disrupted in the absence of band 3

Since the absence of band 3 has an impact on multiple membrane proteins, including glycophorin A which has previously been used successfully to obtain separate reticulocyte and extruded nuclei populations,[28] cells were instead stained with Hoechst and then separated based on FSC gates as indicated in Figure 4A. Reticulocyte and extruded nuclei (pyrenocyte) populations obtained using this strategy were sorted by FACS for verification of population purity and found to be comparable to populations sorted using glycophorin A/Hoechst or glycophorin C/Hoechst in healthy donors.

By double labeling cell populations with Hoechst together with antibodies recognizing extracellular epitopes of proteins of interest, ratios were derived for the partitioning of specific proteins between the reticulocyte and pyrenocyte plasma membrane during enucleation. Figure 4B,C illustrates that in addition to the decrease in surface expression of proteins, including glycophorin A, CD47 and Rh and RhAG, prior to enucleation of band 3 Coimbra erythroblasts, there is also a decrease in retention of the residual pool of these proteins within the reticulocyte plasma membrane during erythroblast enucleation. Thus mis-sorting during enucleation due to the absence of band 3 contributes to the overall reduction in expression relative to control; however, the proportion of protein loss during enucleation for the majority of dependent proteins is minor compared to the loss due to altered plasma membrane stability prior to enucleation. No effect on partitioning was observed for glycophorin C, which resides in the junctional complex and is not known to be dependent on band 3. The retention of CD44 within the reticulocyte membrane was increased in the absence of band 3, demonstrating that the increased relative expression observed in band 3-deficient erythrocytes and reticulocytes occurs during enucleation.

Exogenous expression of green fluorescent protein-tagged wild-type band 3 in homozygous band 3 Coimbra erythroblasts can completely restore surface expression of key erythrocyte membrane proteins

To determine whether secondary protein deficiencies resulting from the absence of plasma membrane band 3 during erythropoiesis could be rescued, erythroblasts from the band 3 Coimbra patient were lentivirally transduced with a plasmid expressing GFP-tagged band 3 (GFPB3). An N-terminal GFP tag did not affect delivery to the plasma mem-



Protein	Antibody	Control (%)		B3Coimbra (%)	
		nucleus	reticulocyte	nucleus	reticulocyte
band 3	BRIC71	7.3 ± 1.2	92.7 ± 1.2	N/A	N/A
band 3	BRIC200	5.7 ± 0.9	94.3 ± 0.9	N/A	N/A
Wrb	BRIC13	6.8 ± 0.4	93.2 ± 0.4	N/A	N/A
GPA	BRIC256	9.2 ± 1.0	90.8 ± 1.0	30.4 ± 1.4	69.6 ± 1.4
CD47	BRIC32	8.4 ± 1.0	91.6 ± 1.0	27.7 ± 0.9	72.3 ± 0.9
Rh	BRIC69	5.7 ± 0.9	94.3 ± 0.9	19.3 ± 1.3	80.7 ± 1.3
RhAG	LA1818	14.4 ± 0.5	85.6 ± 0.5	24.5 ± 2.0	75.5 ± 2.0
CD44	BRIC222	22.9 ± 0.6	77.1 ± 0.6	13.9 ± 0.6	86.1 ± 0.6
GPC	BRIC4	17.1 ± 3.7	82.9 ± 3.7	19.5 ± 1.6	80.5 ± 1.6

Figure 4. Reticulocyte retention of band 3 associated proteins during erythroblast enucleation is reduced in the absence of band 3. (A) The gating strategy for separation of reticulocytes, erythroblasts and extruded nuclei using forward scatter and fluorescent intensity of DNA (Hoechst) was validated by FACS sorting of the indicated populations. Representative cytospins from sorted populations are shown together with Hoechst/antibody dual labeling for glycophorin A (GPA) and glycophorin C (GPC) illustrating the validity of this gating approach compared to a previously published method. (B) Graphical representation of protein partitioning profiles between reticulocytes and nuclei of indicated proteins from healthy donor and the band 3 Coimbra patient's cultures. (C) The table shows protein partitioning values for the indicated proteins. Means (± standard error) are derived from three independent cultures using average mean fluorescent intensities of reticulocyte and nuclei populations from multiple timepoints where possible.

brane in K562 cells.[30] When expressed in normal erythroblasts we observed the expected plasma membrane localization and accumulation (*data not shown*). *Online Supplementary Figure S2A* shows flow cytometry data demonstrating surface expression of GFPB3 in band 3 Coimbra reticulocytes. The transduction efficiency achieved using the lentiviral GFPB3 vector was up to 97%. The rescue of band 3 surface expression in the patient's reticulocytes varied between transductions and within the GFP-positive population, displaying an average of 54% (BRIC71) or 41% (BRIC200) of healthy donor reticulocyte band 3 levels (*Online Supplementary Figure S2A*). The patient's reticulocytes expressing the highest levels of the GFPB3 vector exhibited the same level of band 3 as healthy donor reticulocytes (*Online Supplementary Figure S2A*, right panel), illustrating for the first time the complete rescue of band 3 expression within a population of cells from a band 3 null patient. Importantly, introduction of GFPB3 into the patient's erythroblasts rescued the expression of glycophorin A, CD47, Rh and RhAG in reticulocytes. The degree of rescue correlated directly with the level of expression of the GFPB3 in these cells (*Online Supplementary Figure S2B* and Figure 5B).

V488M band 3 surface expression is not rescued by association with wild-type band 3

To determine whether the introduction of GFPB3 rescued surface expression of the band 3 Coimbra protein (V488M band 3), surface immunoprecipitations using the extracellular monoclonal band 3 antibody BRIC6 were performed on sorted GFP-positive populations of transduced band 3 Coimbra orthochromatic erythroblasts. *Online Supplementary Figure S2C* shows that BRIC6 immunoprecip-

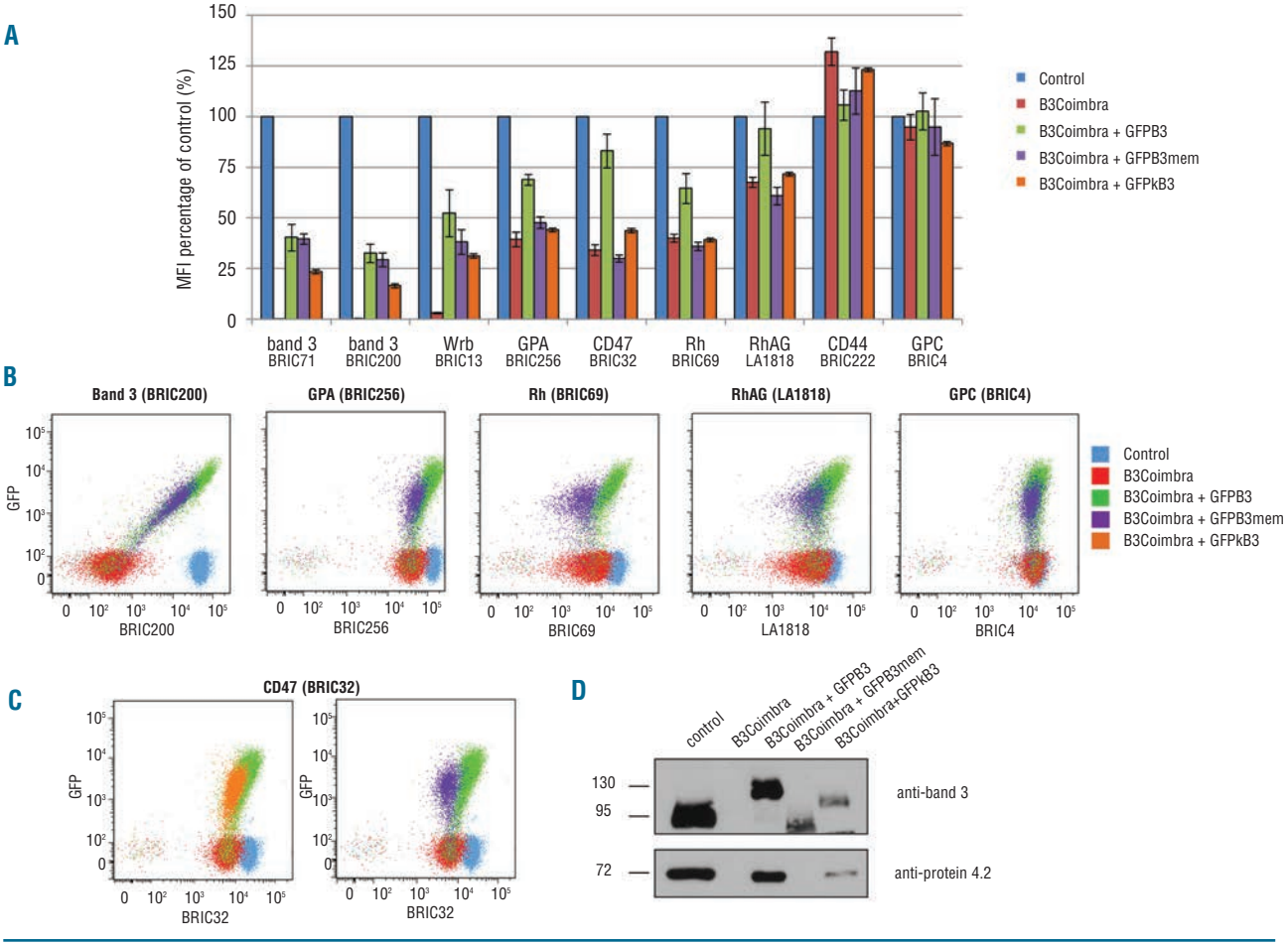


Figure 5. The N-terminal cytoskeletal binding domain of band 3 is required for rescue of band 3-associated protein expression in band 3 Coimbra reticulocytes. (A) Bar chart illustrating reticulocyte surface expression level of indicated proteins relative to healthy donor control for untransduced band 3 Coimbra patient's reticulocytes and GFP-positive patient's reticulocytes derived from erythroblasts rescued with GFP tagged band 3, band 3 membrane domain and kidney band 3. Means (± standard error) are derived from three independent cultures using average mean fluorescent intensities (MFI) of reticulocyte populations from multiple timepoints where possible. For GFPkB3 rescue experiments, data are derived from two independent cultures. Values used are provided as a table in *Online Supplementary Figure S1*. (B) Dot plots illustrating the correlation between reticulocyte expression of GFP-tagged band 3 (GFP intensity) and expression of band 3-associated proteins (x axis with indicated antibodies) in healthy donor reticulocytes (blue), untransduced band 3 Coimbra patient's reticulocytes (red), patient's cells rescued with full length band 3 (green) or with band 3 membrane domain only (purple). Cells were fixed with 1% paraformaldehyde + 0.0075% glutaraldehyde prior to labeling to reduce antibody-induced cell clustering and gated using Hoechst to exclude nucleated cells. (C) Dot plots showing rescue of CD47 in band 3 Coimbra reticulocytes by expression of GFP-tagged band 3 (right and left panels), a partial rescue with GFPkB3 (left panel) but not GFP-tagged band 3 membrane domain (right panel). (D) Reticulocytes were FACS-sorted based on Hoechst negativity and GFP positivity where appropriate. Cells (2.5×10^5) were lysed, and then the proteins separated by sodium dodecylsulfate - polyacrylamide gel electrophoresis and immunoblotted with monoclonal antibodies to band 3 (BRIC170) and protein 4.2 (BRIC273). Note the absence of protein 4.2 in untransduced band 3 Coimbra cells, rescue of protein 4.2 expression with GFPB3 but not GFPB3mem and a partial rescue with GFPkB3.

itates the ~135kDa GFPB3 from the surface of these cells but fails to precipitate any 95kDa V488M mutant band 3 protein, indicating that the V488M mutant cannot be rescued by heterodimerization with wild-type protein. GFP-tagged V488M band 3 could not be immunoprecipitated by BRIC6 when transduced into healthy donor erythroblasts (*data not shown*). These data indicate that expression of the band 3 in heterozygous V488M is accounted for by expression of protein from the wild-type allele alone and not by wild-type rescue of the mutant protein.

The N-terminal cytoplasmic domain is required for multiprotein complex stabilization

Band 3 forms vertical linkages between the plasma membrane and the cytoskeleton through protein associations mediated via its cytoplasmic N-terminus. However, the membrane domain of band 3 is also a significant site of interaction in itself which could alone potentially rescue secondary protein deficiencies upon reintroduction to band 3 null erythroblasts. To explore the hypothesis that band 3 association with the cytoskeleton is required for the stability of band 3 interacting/dependent proteins during erythropoiesis and enucleation, erythroblasts from the band 3 Coimbra patient were transduced with lentivirus expressing GFPB3, band 3 membrane domain (GFPB3mem; lacking the N-terminus) or GFP-tagged kidney band 3 (GFPkB3), which binds protein 4.2 but not ankyrin. Figure 5A shows that expression of key proteins reduced in band 3 Coimbra reticulocytes which are normally associated with band 3 in the ankyrin/band 3 tetramer based multiprotein complex were significantly rescued by expression of GFPB3. In contrast, with the exception of a small increase in glycophorin A, reticulocytes expressing GFPB3mem exhibited no increase in surface expression of these proteins compared to expression levels in untransduced reticulocytes from the patient. This was true even when samples were matched for GFP intensity to account for the differential expression and retention of band 3 after enucleation. The expression level of GFPB3 within these cells correlated with the degree of rescue of secondary protein deficiencies, as illustrated in Figure 5B, which demonstrates a positive correlation between GFP intensity and surface expression of glycophorin A and Rh in particular. Expression of GFPkB3 resulted in a partial rescue of CD47 (Figure 5C), which correlates with the partial rescue of protein 4.2 observed using GFPkB3 compared to the high level of rescue achieved by GFPB3 and the complete absence of protein 4.2 expression in band 3 Coimbra reticulocytes expressing GFPB3mem (Figure 5D). Rh protein expression was also rescued by expression of GFPB3 in the reticulocytes of the band 3 Coimbra patient, but not by the introduction of either GFPB3mem or GFPkB3. Interestingly, rescued expression of RhAG was only observed in the patient's reticulocytes expressing the highest levels of full length GFPB3 (Figure 5B) and was not rescued by expression of either GFPB3mem or GFPkB3.

To explore the expression of GFPB3 and the N-terminally truncated mutant proteins in detail, surface protein expression profiles were acquired throughout differentiation for the patient's erythroblasts transduced with GFPB3, GFPB3mem and GFPkB3 alongside the profiles of untransduced erythroblasts from the patient and healthy donors' erythroblasts. Figure 6A shows that expression of GFPB3 resulted in a partial rescue of band 3 associated proteins including CD47, Rh and RhAG, which was not observed with expression of GFPB3mem. Rescue of CD47 expression by GFPB3 was evident from an early stage of differentiation, consistent with the previously reported establishment of dependence of this protein on protein 4.2 in basophilic erythroblasts[25] but for other proteins was most evident following the transition from orthochromatic erythroblasts to reticulocytes coincident with the point of enucleation. Interestingly, this figure also demonstrates the observed consistently higher expression of GFPB3mem compared to GFPB3. Despite this high expression, which peaked at the late polychromatic/early orthochromatic stages, no rescue of secondary protein deficiencies occurring in the band 3 Coimbra patient was observed using GFPB3mem. In fact, the expression of the majority of these proteins was reduced relative to the expression in untransduced erythroblasts from the patient. This was accompanied by a higher level of cell death (*unpublished observation*), which may indicate that GFPB3mem expression at such a high level is toxic to erythroblasts. Importantly, transduction of band 3 Coimbra erythroblasts with the cytoskeleton binding compromised GFPkB3 (which was expressed at a lower level) also failed to rescue expression of the majority band 3 dependent proteins without reducing protein expression relative to that in the patient's untransduced erythroblasts illustrating that it is inability to bind the cytoskeleton that accounts for the absence of rescue of band 3 associated protein expression by GFPB3mem. These data were confirmed by a separate experiment in which GFPB3mem was expressed at a lower level and no gross reduction in surface protein expression levels relative to that in the patient's untransduced erythroblasts was observed (*Online Supplementary Figure S2*).

Cytoskeletal binding via the N-terminus is important but not absolutely required for reticulocyte membrane retention of band 3

Cytoskeletal attachment has long been proposed as the predominant factor governing retention of intrinsic membrane proteins during enucleation; however, empirical evidence in support of this presumption, particularly in humans, is lacking. By expressing a truncated band 3 without capacity to bind the cytoskeleton in the absence of full length, wild-type protein, transduced band 3 Coimbra erythroblasts provide a model that enables the assessment not only of the importance of this attachment for retention of band 3 itself but also the retention of proteins for which band 3 provides an indirect cytoskeletal linkage.

Figure 6B shows enucleation protein partitioning ratios for GFPB3, GFPB3mem and GFPkB3 in band 3 Coimbra cells. Importantly, GFPB3 partitioned with a similar ratio (5% loss with the nucleus) to endogenous band 3 in healthy donors (5-8%). In contrast, 38% of the non-cytoskeletal binding GFPB3mem domain and a lower 27% of GFPkB3 were lost with the plasma membrane surrounding the nucleus during enucleation (Figure 6C). Gross surface expression of band 3 within the extruded nuclei population is shown in Figure 6D, which illustrates that while average band 3 surface expression across the population of nuclei extruded from erythroblasts was lower for the patient's cells rescued with GFPB3 than for the truncated mutants, a proportion of these nuclei contained much higher levels of band 3. We hypothesize that these nuclei derived from erythroblasts with the highest GFPB3 expression. On this basis alongside its increased mobility, it is likely that the level of protein expressed in erythroblasts prior to enucleation may contribute partly to the increased partitioning of GFPB3mem to the nucleus compared to that of GFPkB3, which is similarly

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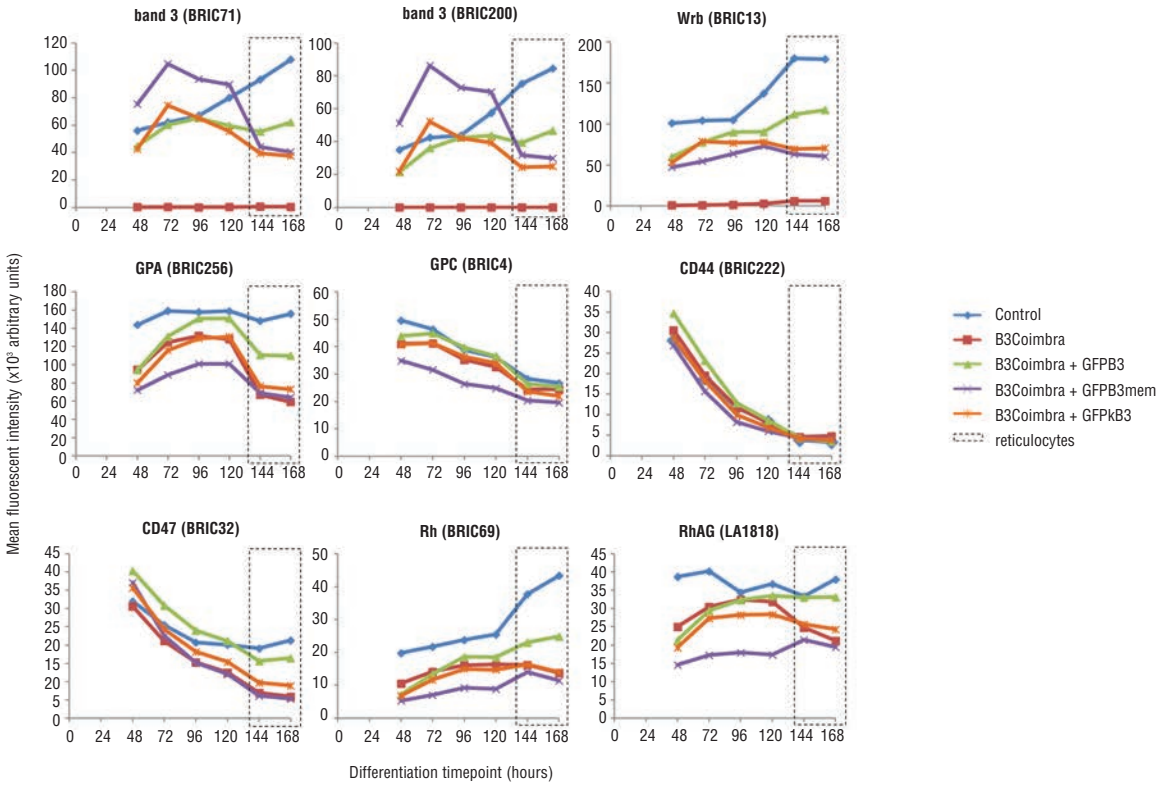
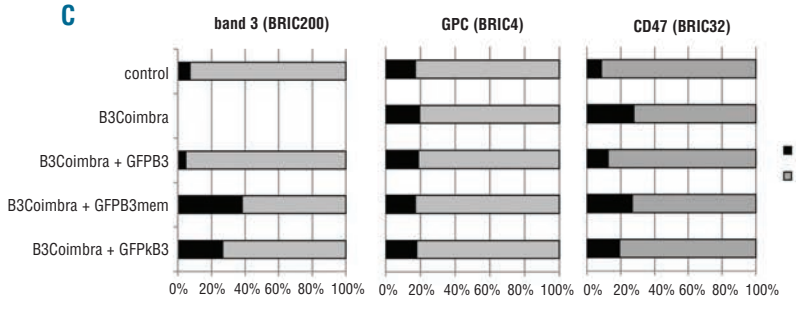


B

Protein	Antibody	Control (%)		B3Coimbra (%)		B3Coimbra + B3GFP (%)		B3Coimbra + B3memGFP (%)		B3Coimbra + kB3GFP (%)	
		nucleus	reticulocyte	nucleus	reticulocyte	nucleus	reticulocyte	nucleus	reticulocyte	nucleus	reticulocyte
GPC	BRIC4	17.1 ± 3.7	82.9 ± 3.7	19.5 ± 1.6	80.5 ± 1.6	18.8 ± 2.7	81.2 ± 2.7	17.0 ± 1.8	83.0 ± 1.8	17.7 ± 2.0	82.3 ± 2.0
Wrb	BRIC13	6.8 ± 0.4	93.2 ± 0.4	N/A	N/A	4.9 ± 2.3*	95.1 ± 2.3	27.4 ± 0.9*	72.6 ± 0.9	21.7 ± 5.0	78.3 ± 5.0
CD47	BRIC32	8.4 ± 1.0	91.6 ± 1.0	27.7 ± 0.9*	72.3 ± 0.9	12.5± 2.8*	87.5 ± 2.8	26.9 ± 1.6	73.1 ± 1.6	19.4 ± 0.7	80.6 ± 0.7
Rh	BRIC69	5.7 ± 0.9	94.3 ± 0.9	19.3 ± 1.3*	80.7 ± 1.3	11.9 ± 1.8*	88.1 ± 1.8	21.5 ± 1.5	78.5 ± 1.5	20.2 ± 1.4	79.8 ± 1.4
Band 3	BRIC71	7.3 ± 1.2	92.7 ± 1.2	N/A	N/A	4.8± 1.3*	95.2 ± 1.3	38.3 ± 4.6*	61.7 ± 4.6	26.6 ± 3.6	73.4 ± 3.6
Band 3	BRIC200	5.7 ± 0.9	94.3 ± 0.9	N/A	N/A	5.2 ± 1.8*	94.8 ± 1.8	35.9 ± 6.1*	64.1 ± 6.1	22.8± 5.2	77.2 ± 5.2
CD44	BRIC222	22.9 ± 0.6	77.1 ± 0.6	13.9 ± 0.6*	86.1 ± 0.6	17.7 ± 0.5*	82.3 ± 0.5	14.4 ± 0.8	85.6 ± 0.8	16.2 ± 1.0	83.8 ± 1.0
GPA	BRIC256	9.2 ± 1.0	90.8 ± 1.0	30.4 ± 1.4*	69.6 ± 1.4	14.3± 3.2*	85.7 ± 3.2	31.6 ± 2.8	68.4 ± 2.8	27.0 ± 2.0	73.0 ± 2.0
RhAG	LA1818	15.9 ± 1.8	84.1 ± 1.8	24.5 ± 2.0*	75.5 ± 2.0	18.4 ± 2.6	81.6 ± 2.6	23.1 ± 2.5	76.9 ± 2.5	23.5 ± 0.5	76.5 ± 0.5

C



D

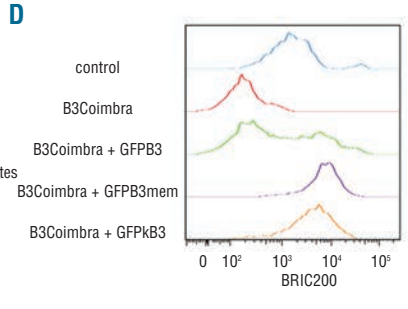


Figure 6. Efficient reticulocyte retention of band 3 and associated membrane proteins is dependent on the cytoskeleton binding N-terminal domain during erythroblast enucleation. (A) Surface expression of indicated proteins was monitored on differentiating erythroblasts from healthy donor control, band 3 Coimbra patients' cultures and GFP-positive populations from respective rescue experiments commencing 48 h aftr differentiation at 24 h intervals using monoclonal antibodies as detailed in the *Online Supplementary Methods*. The dashed box highlights the switch from gating on erythroblasts to reticulocytes after enucleation. (B) Table showing protein partitioning profiles between plasma membranes of extruded nuclei and reticulocytes for indicated proteins and cultures. Means (± standard error) are derived from three independent cultures for healthy donor, band 3 Coimbra patient and patient's cells rescued with GFPB3 or GFPB3mem and two independent cultures for patients' cells rescued with GFPkB3 using average mean fluorescent intensities of reticulocyte and nuclei populations from multiple timepoints where possible. Asterisks indicate statistically significant differences (P<0.05 Student T test) between the patient and control or between B3GFP or B3memGFP transduced and untransduced patient's cells respectively. Statistical evaluation of GFPkB3 transduced cells was not performed due to the number experiments being only two. Note that amalgamated protein partioning data for control and untransduced band 3 Coimbra nuclei and reticulocytes were initially presented in Figure 4C and are repeated as a reference for comparison here. (C) Graphical representation of protein partitioning profiles between reticulocytes and nuclei for band 3 (BRIC200), glycophorin C (BRIC4) and CD47 (BRIC32) derived from healthy donor, untransduced band 3 Coimbra patient's and transduced band 3 Coimbra patient's erythroblasts as indicated. (D) Histogram showing gross surface levels of band 3 within extruded nuclei populations of healthy donors, band 3 Coimbra and respective GFPband 3 rescue erythroid cultures.

compromised in its capacity to bind ankyrin but expressed at a lower level in orthochromatic erythroblasts. Flow cytometry performed on cytoskeleton shells of Triton X-100 extracted band 3 Coimbra erythroblasts showed complete absence of GFP signal for shells derived from cells expressing GFPB3mem, demonstrating its complete extraction, in contrast to those from erythroblasts expressing GFPB3, and validated the absence of cytoskeletal association of GFPB3mem prior to enucleation (*data not shown*).

Enucleation protein partitioning ratios obtained for band 3-dependent proteins reinforce the relevance of the capacity of band 3 to associate with the cytoskeleton, with the retention of band 3-based multiprotein complex proteins glycophorin A, Rh, and CD47 in the reticulocyte membrane rescued to wild-type levels by reintroduction of full length GFPB3 but not GFPB3mem. The increased loss of CD47 during enucleation in the absence of band 3 was partially rescued by GFPkB3, which also rescued protein 4.2 expression, but not by GFPB3mem which does not bind protein 4.2. Neither Rh nor RhAG retention was rescued by kB3 during enucleation, reinforcing the particular dependence of CD47 on the presence of protein 4.2 as observed previously.[29,31,32]

Discussion

By studying *in vitro* cultures of erythroblasts derived from the unique homozygous V488M 'band 3 null' patient and reproduced by shRNA-mediated depletion of band 3 in erythroblasts of healthy donors, we have demonstrated the importance of band 3 for stable surface expression of multiple dependent proteins (glycophorin A, CD47, Rh, protein 4.2) throughout erythropoiesis. Reductions in the relative plasma membrane expression of these proteins are conspicuous from the onset of erythropoiesis, accrue predominantly throughout the remainder of terminal differentiation and are compounded by additional loss during enucleation. Interestingly, throughout erythropoiesis, RhAG expression, while reduced relative to that in healthy donors, was maintained at significantly higher levels than those previously reported for mature band 3 Coimbra erythrocytes. In contrast, the expression of Rh, which associates closely with RhAG, was depressed to a much greater degree throughout terminal differentiation. We cannot exclude the possibility that a larger reduction in RhAG expression was partially masked by increased availability of the LA1818 epitope, although an alternative explanation is that in the absence of band 3, RhAG can be stabilized in the erythroblast plasma membrane by direct binding to the cytoskeleton or indirectly through the formation of alternative sub-complexes that may be subsequently disrupted as the reticulocyte matures. In fact, it is very likely that in the absence of band 3, further loss of band 3-dependent proteins occurs during remodeling of the reticulocyte to generate mature erythrocytes since the relative expression levels were still higher than those observed in the patient's erythrocytes.

We also show here that secondary protein loss could be efficiently rescued by lentiviral expression of a GFP-tagged full-length band 3 in the patient's erythroblasts, with a direct correlation observed between the expression of the tagged band 3 and the degree of rescue of secondary deficiencies. Band 3mem, which lacks the capacity to associate directly with the cytoskeleton, partitioned to the membrane surrounding the extruded nucleus at a much greater level than full length band 3 and was incapable of rescuing either the relative drop in expression of band 3-dependent proteins in erythroblasts or the reduced reticulocyte retention of these proteins during enucleation. Expression of kB3 in the patient's erythroblasts resulted in a partial rescue of protein 4.2 and its dependent protein CD47. Although a large proportion of this protein was also lost during enucleation compared to band 3, enhanced retention of kB3 in the reticulocyte membrane was observed compared to band 3mem. This supports a weak association of kB3 with the cytoskeleton which is likely accounted for by the retention of protein 4.2 binding.

The capacity of band 3 to associate with the cytoskeleton clearly influences the degree to which it is retained in the partitioning of membrane and associated proteins during erythroblast enucleation. However, erythrocytes are known to contain mobile pools of band 3 without apparent cytoskeletal constraint. One explanation for the presence of this pool could be detachment from the cytoskeleton after enucleation during reticulocyte remodeling. Our data show that, despite an increased loss of band 3mem relative to wild-type band 3 with the plasma membrane surrounding the nucleus, the majority of band 3 that is unable to bind the cytoskeleton is still retained in the reticulocyte membrane during enucleation suggesting that cytoskeletal association is not the sole determinant of band 3 retention in the reticulocyte membrane.

In summary, we present data here that uncover the molecular basis of secondary protein loss in a unique and definitive case of severe hereditary spherocytosis. We showed that loss of proteins associated either directly with band 3 or indirectly as part of band 3 centered multiprotein complexes can be accounted for, in the absence of band 3, by a combination of reduced surface expression in the early stages of erythropoiesis and inefficient retention within the nascent reticulocyte membrane during enucleation. This work demonstrates the role of band 3 as a hub for assembly or stabilization of proteins within the ankyrin-associated band 3 tetramer-based complex at an early stage of terminal erythroid differentiation and illustrates the importance of cytoskeletal connectivity via its N-terminus for efficient retention of associated proteins within the reticulocyte membrane during enucleation. Finally, we demonstrate the feasibility of manipulating band 3 expression in primary erythroblasts both using shRNA and by exogenous expression of GFP-tagged band 3 and mutants thereof in a unique band 3 null patient environment.

Acknowledgments

The authors wish to thank the patient and the patient's family for their generous cooperation in this study. Dr. Giles Corey is thanked for providing the original XLG3 vector. We also acknowledge Dr. Rosey Mushens for preparing the monoclonal BRIC antibodies and the assistance of Dr. Andrew Herman and the University of Bristol Faculty of Medical and Veterinary Sciences Flow Cytometry Facility for cell sorting.

The work of TJS was supported by a Wellcome Trust project grant (N. 094277) awarded to AMT. AJB was supported by a Wellcome Trust PhD studentship. This work was funded in part by NHSBT R&D and a National Institute for Health Research program grant (RP-PG-0310-1004-AMT).

Authorship and Disclosures

Information on authorship, contributions, and financial & other disclosures was provided by the authors and is available with the online version of this article at www.haematologica.org.

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