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THE N-TERMINAL 11 AMINO ACIDS OF HUMAN ERYTHROCYTE BAND 3 ARE CRITICAL FOR ALDOLASE BINDING AND PROTEIN PHOSPHORYLATION: IMPLICATIONS FOR BAND 3 FUNCTION

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ABSTRACT

The 911 amino acid band 3 (*SLC4A1*) is the major intrinsic membrane protein of red cells and is the principal Cl^-/HCO_3^- exchanger. The N-terminal cytoplasmic domain of band 3 anchors the spectrin-based membrane skeleton to the lipid bilayer through its interaction with ankyrin and also binds glycolytic enzymes and hemoglobin. We identified a son of consanguineous marriage with severe anemia in association with marked deficiency of band 3 (12±4% of normal). Direct nucleotide sequencing of *SLC4A1* gene demonstrated a single base substitution (T->C) at position +2 in the donor splice site of intron 2 resulting in the generation of a novel mutant protein. Biochemical characterization of the mutant protein showed that it lacked the first 11 N-terminal amino acids (Band 3 "Neapolis"). The expression of the mutant protein resulted in the complete absence of membrane bound aldolase and the mutant band 3 could not be tyrosine phosphorylated. The ability of the malarial parasite, *P. falciparum*, to invade these red cells was significantly decreased. The identification of a novel band 3 mutant and its structural and functional characterization enabled us to identify pivotal roles for the 11 N-terminal amino acids in several protein functions and, in turn, in red cell physiology.

INTRODUCTION

The 911 amino acid human erythroid AE1 (eAE1) Cl^-/HCO_3^- exchanger *SLC4A1* (commonly referred to as band 3) is the major integral membrane protein of the red cells and serves to increase the total CO_2 carrying capacity of the blood.[1, 2] *SLC4A1* gene is transcribed in red cell precursors under the control of an erythroid-specific promoter upstream of exon 1.[3] AE1 is also expressed at high levels in type A intercalated cells of the collecting duct (kAE1). The renal transcripts arise from a distinct promoter located within intron 3 of *SLC4A1*[3, 4] and the resultant kAE1 transcript encodes a protein that lacks the N-terminal 65 amino acids present in the protein expressed in erythroid cells.[5]

Band 3 is characterized by three distinct structural and functional domains: *i*) the membrane spanning domain, which traverses the bilayer and functions as a chloride/bicarbonate exchanger*; ii*) the short C-terminal cytoplasmic domain that binds carbonic anhydrase II, and *iii*) the N-terminal cytoplasmic domain that interacts with a number of proteins including ankyrin, protein 4.2, protein 4.1, glycolytic enzymes (e.g., aldolase and glyceraldeyde-3-phosphate dehydrogenase (GAPDH)), hemoglobin, hemichromes and different protein tyrosine kinases.[1,2,6-13] Furthermore, band 3 has recently been shown to assemble a macromolecular protein complex of integral proteins including the Rh complex (Rh associated glycoprotein, Rh polypeptides, CD47 and glycophorin B) and glycophorin A.[14]

The majority of the human *SLC4A1* mutations reported to date[2, 7] result in autosomal-dominant forms of hereditary spherocytosis (HS) as a consequence of moderate decrease in membrane content of eAE1 protein. Importantly, in all these cases of HS mutant polypeptide is not assembled into the membrane.[2, 7] As such, the consequence of the assembly of mutant band 3 into the membrane on its function in red cells has not been defined.

The availability of knock-out animal models enabled the identification of the effect of complete deficiency of eAE1 on red cell physiology. Band 3 null red cells have been described in a natural strain of cattle and in two strains of knock-out mice.[15-17] Marked membrane loss leading to spherocytic morphology is a feature of red cells in these animals. In addition to the absence of band 3, the mouse red cells also exhibit complete deficiency of glycophorin A.[15,18,19] In mice, the absence of band 3 leads to a severe life threatening hemolytic anemia. So far, only one case of complete deficiency of band 3 in humans has been described. In this case, both the erythrocytic and kidney band 3 were absent resulting in life-threatening neonatal anemia and distal renal tubular acidosis.[20]

Here, we report a novel variant of eAE1, called "band 3 Neapolis", in which N-terminal 11 amino acids are truncated. In the homozygous state, red cell membranes contain only the mutant protein albeit at a much lower level. Detailed biochemical and functional characterization of these red cells has enabled us to obtain novel insights into the function of the N-terminal domain of band 3.

MATERIALS AND METHODS

Studies on SLCA1 gene and band 3 cDNA

Blood samples were drawn from normal volunteers, the proband and his parents after obtaining informed consent in accordance with the Declaration of Helsinki. DNA and total reticulocyte RNA were isolated as previously described.[21] Sequencing of band 3 genomic DNA and cDNA was performed as previously reported.[21,22]

Antibodies and immunoblotting

Monoclonal antibodies to band 3, alpha and beta spectrin, GPA and GPC were obtained from Sigma (Sigma Chemical Company, St. Louis, USA). Polyclonal antibodies to aldolase, GAPDH and CD47 were provided by Santa Cruz Biotechnologies (Santa Cruz, CA, USA). A monoclonal antibody (CDB3) directed against the N-terminus of band 3 waspreviously described.[23] Immunoblotting was performed according to the protocols reported previously.[24,25]

PCR analysis

Reticulocytes RNA was reverse transcribed to obtain random-primed cDNA using MMLV-RT (Gibco-Invitrogen). The PCR conditions and the primers employed for amplification of cDNA were as follows: 5'- aacgagtgggaacgtagctg-3' (forward); 5'- cttcatattcctcctgctccag-3' (reverse); 18-30 cycles with each cycle consisting of 95°C

for 30 sec, 55°C for 1 min, 68°C for 3 min. In order to use the same amount of cDNA in each reaction, GAPDH amplification was performed first for normalization purposes as previously reported.[26] All reaction products were run on 2% TBE-agarose gel stained with ethidium bromide. Normally spliced band 3 PCR product (exon1-exon2-exon3) was 216 bp-long, while mutant band 3 products were 132bps (exon1-exon3) and 341 bps (exon1-exon2-intron2-exon3).

To perform real-time RT-PCR on reticulocyte cDNA, we synthesized the following primers: **SLC4A1/e1AF**, 5'-agggaccctgaggctcgtgagcag-3'; **SLC4A1/e1BF**, 5'-ttcaccaagggaccctgaggctcg-3'; **SLC4A1/e2-3R**, 5'-tgtcttcataatcatcctgcagct-3'; **SLC4A1/e1-3F**, 5'-ctgaggctcgtgagcaggatgatt-3'; **SLC4A1/e4R**, 5'-tctgtggctgttgcctcggtgtcg-3'; **SLC4A1/i2F**, 5'-cacagcccagcctcagcggccact-3'; **SLC4A1/i2R**, 5'-actgctgatgccagggaacacccg-3'. Combinations of these primers were used to set up five different reaction mixtures: i) **SLC4A1/e1AF** and **SLC4A1/e2-3R** that will generate a product of 113 bps derived by the correct joining of exons 1-2-3, since the reverse primer can only anneal to the junction 2-3; ii) **SLC4A1/e1BF** and **SLC4A1/e2-3R** that will generate a product of 120 bps with a different forward primer but correct joining of exons 1-2-3; iii) **SLC4A1/e1-3F** and **SLC4A1/e4R** that will generate a product of 132 bps derived by the skipping of exon 2 and the aberrant joining of exons 1-3-4, since the forward primer can only anneal to the junction 1-3 and the reverse primer is located in exon 4; iv) **SLC4A1/i2F** and **SLC4A1/e4R** that will generate a product of 147 bps derived by the retention of intron 2 and the aberrant joining of exon2-intron2-exon3-exon4,

since the forward primer can only anneal to intron 2 and the reverse primer is located in exon 4; v) **SLC4A1/e1BF** and **SLC4A1/i2R** that will generate a product of 129 bps derived by the retention of intron 2 and the aberrant joining of exon1-exon2-intron2, since the forward primer is placed on exon 1 and the reverse primer will anneal to intron 2. The use of these five sets (i to v) enabled us to identify the normal allele (i and ii), and the mutant alleles, skipping of exon 2 (iii) and the retention of intron 2 (iv and v). All PCR reactions were carried out using a BioRad i Cycler with the following program: 95°C 30 sec, 63°C 2min, 72°C 1min. SYBR green was used to monitor DNA synthesis. We assayed control cDNA as well as cDNA from the parents and the proband and normalized all values assuming the wild-type allele in control as 100%.

Red cell membrane protein analysis

Red cells ghosts were prepared according to the procedure of Dodge et al.,[27] except that 5 mM phenylmethylsulfonyl fluoride was added during the lysis step. Protein concentrations were determined by the procedure of Lowry et al.,[28] using a sodium carbonate solution containing 3% SDS and bovine serum albumin as a standard. Membrane proteins were analysed by SDS-PAGE.[29,30] The gels were stained with Coomassie blue G. Erythrocyte membrane proteins were quantified by scanning of SDS-PAGE gels and through analysis of immunoblots as previously described.[31,32] Band 3 was also quantified by labelling membranes with eosine

maleimide followed by protein solubilization and fluorescence measurement (522 nm, excitation; 550 nm, emission).[33]

Band 3 purification

Band 3 monoclonal antibody (Sigma) directed against the 300 N-terminal amino acids was cross-linked to protein A-agarose essentially as described.[34] Band 3 was solubilized by suspending the membranes in 1% (w/v) Triton X-100, 5 mM phosphate, pH 8, for 1h. After centrifugation at 30000 *x g* for 30 min, the supernatant was diluted 10-fold and applied directly to the immunoaffinity column, previously equilibrated with 0.1% Triton X-100, 5 mM phosphate. The column was washed with 10 column volumes of 0.1% Triton X-100, 5 mM phosphate and band 3 was eluted with 0.1 M glycine-HCl. 300 μl fractions were collected and the proteins composition of each of the collected fractions was analysed by SDS-PAGE and Western blotting as previously reported.[25] Fractions containing the pure band 3 protein were pooled and used for mass spectrometry analysis.

The N-terminal band 3 peptide of the control, heterozygous parents and homozygous proband was prepared by digesting the membranes with trypsin (15 μg/ml) in 5 mM phosphate pH 8, 0.14 M NaCl for 2 hours at 4 °C; the reaction was stopped by the addition of trypsin inhibitor. Digests were then centrifuged at 30000 *x g* for 30 min and the supernatant containing the N-terminal peptide was purified by affinity chromatography. Fractions containing the highest concentration of the

peptide were analyzed by Western blotting to compare the apparent molecular weights of N-terminal peptides of normal and mutant band 3.

MALDI-TOF Mass SpectrometryAanalysis

Bands from SDS-PAGE were excised from the gel, triturated and washed with water. Proteins in situ in the gel were reduced, S-alkylated with iodoacetamide and digested with endoprotease AspN, as previously reported.[35] On the basis of the primary structure of the protein, this protease was selected to be optimal for obtaining N-terminal sequence information on band 3. Digested aliquots were subjected to a desalting/concentration step using μZipTipC18 (Millipore Corp., Bedford MA, USA) before MALDI-TOF mass spectrometry analysis. Peptide mixtures were loaded on the instrument target, using the dried droplet technique and α-cyano-4-hydroxycinnamic as matrix, and analysed by using a Voyager-DE PRO mass spectrometer (Applied Biosystems, Framingham MA, USA). Spectra were acquired in the reflectron mode. Assignment of the recorded mass values to individual peptides was performed on the basis of their molecular mass and protease specificity.

Quantitation of anion transport

Washed erythrocytes were subjected to assays of unidirectional disodium [^{35}S] sulfate (Du Pont, Hertfordshire, UK) uptake in the absence or in the presence of 10 μM of the anion transport inhibitor di-isothiocyano-dihydrostilbene disulphonate (Sigma), as previously describe.[36] Each flux study using proband's red cells was

performed in parallel using red cells from the parents and from unrelated healthy subjects. The sulfate flux was expressed for a constant number of cells (1 x 10^{13} cells), in order to appropriately compare flux values for samples with variable MCV values. The within and in between-run CVs for these measurements were <5%.

Phosphorylation of band 3

[γ^{32}P]ATP was purchased from Amersham. Anti-P-Tyr antibody was purchased from ICN Biotechnology (Irvine, CA). Syk and Lyn were isolated from human red cells as previously described.[11] Erythrocyte stimulation by diamide, cell membranes preparation and the sequential phosphorylation of band 3 catalyzed *in vitro* by Syk and Lyn tyrosine kinases were performed as previously described. [11, 37]

Plasmodium falciparum invasion of red cells

Plasmodium falciparum invasion of red cells was measured in the first and second cycle of parasite growth essentially as reported in.[38]

RESULTS

A new SLC4A1 mutation, "band 3 Neapolis", causes aberrant mRNA splicing

A 21-month-old child, the second son of a consanguineous marriage, was referred for the evaluation of a life-threatening non-immune hemolytic anaemia. The patient was transfusion-dependent prior to undergoing splenectomy in 1999. At the present time no major clinical complications or thromboembolic events have been noted. The liver is enlarged and the red blood cell indices are: RBC $3.0x10^9$/mL, Hb 90g/L, Htc 27%; MCV 91 fL, MCH 31 pg, MCHC 34 g/dl; reticulocytes 10%. The blood smear shows a wide range of abnormal red cell morphologies, including stomatocytes, spherocytes and microcytes. Since a mild form of autosomal dominant HS due to band 3 deficiency was noted in both parents, the proband was analyzed for a possible *SLC4A1* homozygous mutation. Direct nucleotide sequence of *SLC4A1* gene showed a single base substitution (T->C) at position +2 in the donor splice site of intron 2 (Fig. 1A). The observed mutation abolishes a BspMI restriction site. The novel variant is designated as "Band 3 Neapolis".

RT-PCR analysis of reticulocyte mRNA was performed using primers located in *SLC4A1* exons 1 and 3. A 215 bps product was obtained in the control while two amplified fragments of 340 bps and 132 bps were noted in the patient (Fig. 1B). Sequencing analysis revealed that the 340 bps resulted from the retention of intron 2, while the 132 bps fragment is the result of skipping of exon 2 (Fig. 1C). All three amplified fragments (340, 215 and 132 bps) were noted in the parents (Fig. 1B).

Quantitative evaluation of the various transcripts using real time PCR of reticulocyte RNA showed that in the proband, as expected, no normal mRNA was present and each of the two mutant mRNAs was present at 11-13% of normal levels. In the heterozygous parents 52% of the message was of wild type with 6% each of the two mutant transcripts. The retention of intron 2 leads to a premature termination of translation after the 19th triplet, while skipping of exon 2 results in the loss of the normal translation start site for erythroid band 3 (Fig. 1D). Thus, the first alternative splicing event can only generate a very truncated form of altered erythroid band 3, while the second splicing event will generate a mutant form of band 3 with an altered N-terminus. Due to the location of the mutation, kidney band 3 expression will be unaffected and, consistent with this expectation, no defect in the distal urinary acidification in the homozygous child was noted (data not shown).

Band 3 Neapolis gene encodes a protein lacking the 11 N-terminal amino acids

As the aberant splicing pattern of mutant gene suggested the generation of an altered protein in the red cells of the proband, we biochemically characterized band 3 from the proband's red cells. Sodium-dodecyl-sulphate polyacrylamide gel electrophoresis (SDS-PAGE)/immunoblotting experiments showed that the mutant band 3 migrated slightly faster than the normal protein suggesting a decreased molecular weight and that the band 3 content of the membrane was significantly reduced (data not shown). In order to substantiate the alteredmobility of the mutant band 3, isolated red cell membranes were digested with trypsin for 2 hours and the

supernatant of the digests was passed over a column of protein A Sepharose cross-linked to a monoclonal antibody raised against the first 300 amino acids of band 3 to obtain purified N-terminal fragments. Western blot analysis of the various purified fragments clearly showed that the apparent molecular weight of the N-terminus of mutant band 3 was smaller than that of the normal (Fig. 2A). Interestingly, the red cell membranes from the heterozygous parents showed only the normal N-terminal fragment of the protein, implying an apparent lack of assembly of the mutant band 3 in these membranes (Fig. 2A).

Although the DNA analysis precisely identified the mutation in the band 3 Neapolis gene, it did not provide definitive information on the nature of the truncated protein product. While the normal translation initiation ATG is deleted due to skipping of exon 2, there are at least three different ATG triplets in exon 3, which could function as the starting codon for the synthesis of mutant band 3 (Fig. 2B). The first two ATGs are located at codons 11 and 12 and their use should result in the lack of either 10 or 11 amino acids at the N-terminus, respectively, while the use of the third ATG located at codon 31 would result in the loss of 30 amino acids at the N-terminus (Fig. 2B).

To characterize the N-terminus of the mutant protein, normal and mutant band 3 were purified to homogeneity by immunoaffinity techniques, reduced, alkylated and digested with endoprotease AspN. The resulting digests were subjected to peptide mapping analysis by MALDI-TOF mass spectrometry. As shown in Fig. 2C, the spectra obtained from normal band 3 showed distinct peaks at *m/z* 3335.3031,

3351.3044 and 3367.3101 that correspond to the expected peptide (10-37) and its oxidized products. While these signals are absent in the spectra of mutant protein, unique signals at *m/z* 1484.6521, 1696.8653, 3131.2913 and 3147.2927 were noted instead. On the basis of their molecular mass and protease specificity, these peaks can be assigned to a protein with a modified N-terminus and, in particular, to peptides (12-22), (12-24), (12-37) and oxidized (12-37), all bearing acetylated Met12 as the N-terminal residue. These findings enabled us to definitively demonstrate that the truncated mutant band 3 is devoid of the first eleven amino acids and that it is generated through the use of the second ATG in exon 3. Interestingly, the resulting N-terminal residue Met12 is acetylated, as is the case for normal Met1. A definitive confirmation for the absence of 11 N-terminal amino acids in the mutant band 3 was obtained by Western blot analysis using a monoclonal antibody directed against this region. As shown in Fig. 2D, while Western blot analysis readily detected normal band 3 in membranes of red cells from normals and the parents, it failed to detect mutant band 3 in membranes of red cells from the proband.

Red cells of the homozygous band 3 Neapolis contain reduced amount of the truncated protein and show altered membrane function

Following the detailed biochemical characterization of the mutant band 3, we investigated the effect of this genetic alteration on the various red cell membrane and cellular functions. Band 3 content of red cell membranes of the proband and parents was determined by three different methodologies, namely SDS-PAGE analysis,

immunoblotting analysis and eosine maleimide labeling of band 3 in intact red cells. Band 3 content of the membranes proteins assayed by SDS-PAGE or immunoblotting analysis was normalized to protein 4.1 and glycophorin C (GPC) contents of the membrane since the GPC complex (GPC, protein 4.1 and p55) is thought to be distinct and independent on the band 3 complex. Band 3 and protein 4.2 content of red cell membranes of the proband was 12±4% of normal, while that of red cells from the heterozygous parents was 82±6% of normal (Figs. 3A, 3B and data not shown). Eosine maleimide labeling showed that the band 3 content of proband red cells was 13±3% of normal. Glycophorin A and CD47 content of the membrane were also markedly reduced to 11±5% and 42±10% of normal, respectively (Fig. 3C). The membrane content of α-spectrin, β-spectrin and ankyrin was also reduced but to a much lesser extent, 79±3%, 81±4.% and 75±6.% of normal, respectively (Fig. 3A). A marked reduction in the membrane content of GAPDH (corresponding to band 6) was also noted (Fig. 3A).

The consequences of the marked deficiency of band 3 on red cell morphology were significant. Stomatocytes and spherocytes were the dominant morphological feature revealed by scanning electron microscopy of mutant red cells (Fig. 3D). In addition there were also a number of microspherocytes and membrane protrusions similar to those reported in band 3 null mouse red cells.[15, 16, 39] Osmotic gradient ektacytometry [40] confirmed a marked increase in osmotic fragility of the red cells as a conseqeunce of a significant reduction in membrane surface area of mutant red cells (data not shown).

Using 4,4'-di-isothiocyano-dihydrostilbene-2,2-disulphonate (DIDS), a specific inhibitor of the anion transporter, we evaluated the anion transport activity of red cells with mutant band 3. The DIDS sensitive sulfate influx was reduced to 10% of normal in red cells from the proband and to 78 % of normal in the heterozygous parents (Fig. 3E and data not shown). As the extent of reduction in anion transport activity was proportional to the extent of decrease in membrane content of band 3, this finding implies that the mutant band 3 retained its ability to transport anions.

The 11 N-terminal amino acids of band 3 are critical for the binding of glycolytic enzymes and for the in vivo protein phosphorylation

The cytoplasmic domain of band 3 is the anchoring site for several red cell glycolytic enzymes such as aldolase, GAPDH and phosphofructokinase and is also a substrate for the protein tyrosine kinase, Syk. [11, 37] Several NMR studies provided important insights into the structure and function of the 11 N-amino terminal amino acids in solution. The structure of this region is inferred to form a loop consisting of residues 4 through 9 in which the phenolic ring of Y8 is sandwiched between the methyl groups of L4 and M12. [41, 42] Phosphorylation of tyrosine 8 destabilizes the intermolecular protein-protein association, particularly between band 3 and glycolytic enzymes. [43]

Since the naturally occurring band 3 Neapolis assembled on to the red cell membrane lacks these N-terminal 11 amino acids, we explored the altered function of this domain in the context of intact red cell membrane. The association of aldolase

with band 3 in the membranes of red cells from the proband and from normals was assessed. In contrast to the strong association of aldolase with intact normal membranes, no aldolase could be detected in association with membranes of red cells from the proband (Fig. 4A). This lack of association was not due to absence of aldolase in red cells, since the total aldolase content of cells was similar in red cells from normals and from proband (data not shown). This finding implies that N-terminal 11 amino acids are critical for association of aldolase with band 3 in intact red cell membranes.

We next explored whether Syk tyrosine kinase could recognize the truncated band 3 as a substrate *in vitro* by assessing the ability of Syk purified from normal erythrocytes to phosphorylate band 3. As shown in Fig. 4B, in spite of the absence of one of two phosphorylation sites (Tyr8), the mutant protein was phosphorylated *in vitro* by the exogenously added purified enzyme (lane 2). The extent of Syk-phosphorylation, determined using equivalent amounts of normal and mutant band 3, showed that the phosphorylation of the mutant protein was approximately 50% of normal (data not shown), consistent with the presence of a single phosphorylation site in the mutant protein at Tyr21. Furthermore, we were able to document that the presence of the single phosphotyrosine docking site at Tyr 21 is sufficient for the interaction of band 3 with the SH2 domain of Lyn and subsequent phosphorylation of the truncated band 3 (Fig. 4B, lanes 3,6). In order to establish whether these *in vitro* phosphorylation events can be recapitulated in intact cells, we stimulated red cells from proband and normals with diamide to induce tyrosine phosphorylation of band

3. [37] As shown in Fig. 4C, while band 3 was tyrosine phosphorylated in normal red cells, no phosphorylation could be detected in proband's red cells. These findings imply an absolute requirement for Tyr8 for band 3 phosphorylation *in vivo* in intact membranes.

Malaria parasite life cycle is altered in band 3 Neapolis erythrocytes

Previous studies using various peptides corresponding to the extra-cellular loops of band 3 have suggested an important role for this surface protein in red cell invasion by *Plasmodium falciparum*.[44] In order to further substantiate a requirement for band 3 in parasite invasion, we assayed the ability of two different strains of *Plasmodium falciparum* to invade red cells of the proband with markedly reduced membrane content of band 3. As shown in Table I, invasion and maturation of the parasites were markedly inhibited in red cells of the proband. The decreased invasion and maturation could be seen at 24 hours (early stage during first cycle of invasion), at 48 hours (late stage during first cycle of invasion) and at 72 hours (early stage during second cycle of invasion). The ability of parasites to invade red cells of the parents was not significantly different from that of normal red cells (Table 1). These findings establish an important role for band 3 in red cell invasion by *Plasmodium falciparum*.

DISCUSSION

In the present study, we have identified and characterized a new variant of human red cell AE1 protein, designated band 3 Neapolis that provides novel insights into the role of the 11 N-terminal amino acids in regulating protein function. A single base substitution (T->C) at position +2 in the donor splice site of intron 2 was identified as the mutation responsible for band 3 "Neapolis". The mutation causes altered splicing of AE1 gene with the consequent generation of two different mature band 3 mRNAs, one that includes intron 2 and the other that skips exon 2. Both transcripts are produced, since the GU to GC mutation does not completely abrogate its ability to function as a donor splice site. In fact, a minority of genes (approximately 1%) use the GC donor splice site instead of the canonical GU donor splice site.[45] Only the shorter mRNA that skips exon 2 is translated into a protein that is assembled into the red cell membrane. Mass spectrometric analysis showed that the membrane assembled mutant band 3 lacked the first 11 N-terminal amino acids due to elimination of the normal start site in exon 2 and the use of an alternate start site in exon 3. Importantly, the red cell membrane content of the mutant band 3 is dramatically decreased to the 12% of normal. As such, the red cells of the proband exhibit both a qualitative defect and a quantitative deficiency of band 3. These band 3 defects thus account for the severe transfusion-dependent hemolytic anemia noted in the proband prior to splenectomy. Following splenectomy, the proband became transfusion independent with a moderately compensated hemolytic anemia. The

quantitative deficiency of band 3 can account for the observed reduction in anion transport and loss of red cell membrane surface area. As a result of the location of the mutation, only the synthesis of red cell band 3 protein was affected while the synthesis of the kidney form of band 3 was uncompromised, accounting for the normal renal function noted in the proband.

The mechanism responsible for the marked deficiency of mutant band 3 is not completely clear. Quantitative PCR showed a marked decrement in the number of mutant transcripts and a strong correlation between the amount of mutant mRNA and the membrane content of truncated protein. Thus, either a lower rate of transcription of the mutant gene or an intrinsic instablity of the aberrant mRNA is likely to account for marked deficiency of the mutant protein in red cells. It is interesting, in this context, that red cells of heterozygous parents do not express detectable level of band 3 Neapolis protein, in spite of the fact they synthesize measurable amounts of altered mRNA. Similar findings have been reported for other eAE1 mutations in the heterozygous state. [46, 47]

A major advantage of our analysis compared to earlier studies on red cells with quantitative deficiencies of band 3 is that for the first time we are able to assess the function of the N-terminus of band 3 in the context of an intact membrane. Our *in vivo* demonstration that the 11 N-terminal amino acids form the major binding site of aldolase and that other glycolitic enzymes (for example GAPDH) employ different band 3 binding domains validates that this domain of band 3 is critical for recruiting

aldolase to the red cell membrane. These findings lend strong support to studies using normal red cells. [48]

A second significant finding is the validation of a critical role for the 11 N-terminal amino acids in the sequential Tyr-phosphorylation of band 3 triggered by diamide stimulation.[11, 37] The observation that the truncated band 3 can be phosphorylated by Syk and thereby promote the recruitment of Lyn tyrosine kinase *in vitro* but not *in vivo* has major structural and functional implications. Our finding provide strong evidence to support a critical role for Tyr8 and its neighboring amino acids in the overall control of the reversible band 3 phosphorylation in intact membranes. The observation also implies that the structural alterations involving both band 3 and the overall membrane architecture play a key role in phosphorylation of band 3 *in vivo*.

The finding that the red cells of the proband are resistant to malaria infection supports a pivotal role for band 3 in parasite invasion and maturation. As band 3 has been identified to be an important receptor for the merozoite surface protein MSP1, it is likely that deficiency of band 3 is responsible for the reduced invasion. However, we cannot rule out the potential contribution of an altered metabolic and/or redox state of red cells in defective invasion and maturation of the parasites in band 3 deficient red cells.

In addition to marked deficiency of band 3, red cells from the proband also exhibit deficiencies in protein 4.2 (10 % of normal), glycophorin A (11 % of normal) and CD47 (47 % of normal). These findings are similar to those previously reported

for the only case of complete band 3 deficiency in humans, band 3 Coimbra [20] in which there is complete deficiency of protein 4.2 and CD47, while the membrane content of glycophorin A is 29 % of normal. [14] As the major interacting site for CD47 in mature human red cells is protein 4.2, [49] the presence of 10% of protein 4.2 could account for the significant difference in CD47 of the proband's red cells compared to band 3 Coimbra erythrocytes. The presence of similar amount of band 3 and protein 4.2 in membranes of red cells of the proband implies that the membrane content of protein 4.2 is exclusively determined by its interaction with band 3 and that the 11 N-terminal amino acids of band 3 are not involved in the binding of protein 4.2. [50] Furthermore, the presence of significant amounts of ankyrin in band 3 Neapolis membranes implies that the N-terminal 11 amino acids of band 3 are not required for band 3-ankyrin interaction. [12]

Despite the marked deficiency of band 3, spectrin, ankyrin and protein 4.1, the content of red cells from proband is very similar to normal and the membrane skeletal architecture also appears to be normal. These findings further support the notion that band 3 is not essential for the stable biogenesis of membrane skeleton. [15-17]

In summary, we identified a novel mutant band 3 (band 3 Neapolis) that lacks the first 11 N-terminal amino acids in association with hereditary spherocytosis. Detailed studies have enabled us to show that the mutant band 3 fails to bind aldolase but does bind other glycolitic enzymes and that the 11 N-terminal amino acids are critical for tyrosine phosphorylation of membrane associated band 3. Furthermore, we established a key role for band 3 in malarial parasite invasion of human red cells.

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FIGURE LEGENDS

Figure 1. Genetic characterization of band 3 Neapolis mutation

A) Genomic analysis. Sequence of PCR-amplified genomic DNA of proband band 3. The sequence shown encompasses the donor splice site of intron 2. Note that the second nucleotide of intron 2 was changed from T to C. **B) RT-PCR analysis.** Reticulocytes cDNAs were PCR amplified using primers localized in exons 1 and 3. cDNA of a normal subject generated a 215-bps band. Two bands of 340-bps and 132-bps were noted in the cDNA of the proband. In the heterozygous parent (the mother) all three amplified products were seen. A similar pattern was also seen using cDNA from the father (data not shown). **C) Sequencing of PCR products.** The two abnormal amplified products obtained in the proband (panel 1B, 340-bps and 132-bps), were sequenced. The 340 bp fragment corresponds to mRNA in which intron 2 is retained and the 132 bp fragement corresponds to mRNA in which exon 2 is skipped. **D) Schematic diagram of the different patterns of splicing.** In contrast to normal splicing shown in the top panel, two aberrant splicing patterns are seen in Band 3 Neapolis. Retaining of intron 2 leads to a premature termination of translation after the 19th triplet (a) and skipping of exon 2 leads to a loss of the normal translation start site for erythroid AE1 protein (b).

Figure 2. Band 3 Neapolis protein analysis

A) Immunoblotting of tryptic fragments of band 3. Red cell membranes were digested by trypsin and band 3 in the supernatant fraction was purified using an immunoaffinity colum. The fraction eluted with 1M glycine, pH 3.0 was analyzed by immunoblotting using antibodies directed against the band 3. The top panel shows the results from a short exposure of film while the bottom panel represents a longer exposure. **B)** The potential ATG start sites in Exon 3. **C) Mass spectrometric analysis of normal and mutant band 3.** Each of the derived signals was characterized on the basis of its molecular mass and protease specificity. Asterisks indicate peptides generated from endoprotease AspN autoproteolysis. **D) Immunoblotting of band 3.** Equivalent amounts of red cell membranes were analyzed by immunoblotting using monoclonal antibodies directed against N-terminal amino acids (clone CDB3). Note complete absence of the band 3 epitope in red cell membranes of the proband.

Figure 3. Studies on erythrocytes of the homozygous band 3 Neapolis

A) SDS-PAGE analysis of red cell membrane proteins. Membrane proteins of red cells were separated by SDS-PAGE [41] and the gel stained by Coomassie blue G. **B) Immunoblotting of Band 3.** The membrane content of band 3 was determined by immunoblotting using an anti-band 3 monoclonal antibody. Equivalent amount of membrane proteins were loaded based on the glycophorin C content. **C) Immunoblotting of Glycophorins A and C and CD47.** Red cell membrane content of GPA, GPC and CD47 was evaluated by immunoblotting using monospecific antibodies. **D) Scanning electron micrograph of red cells of the proband. E) Quantitation of anion transport. DIDS-sensitive** unidirectional disodium [^{35}S] sulfate uptake in red cells of normal and proband red cells.

Figure 4. Band 3 Neapolis functions: binding of glycolytic enzymes and protein phosphorylation

A) Aldolase binding to normal and mutant band 3. Red cell membrane proteins from a normal subject and the proband were separated by SDS-PAGE, transferred to a nitrocellulose paper and analyzed by immunoblotting using an anti-aldolase antibody. **B) Sequential phosphorylation of band 3 *in vitro***. Red cell membrane proteins were subjected to various treatments as outlined. Specifically, ghosts were incubated with Syk (lane 2) or with Lyn (lane 3) or without the enzymes (lane 1). Phosphate incorporation was evaluated on the basis of ^{32}P signal (determined by exposing of dried gels to X-ray films). In lanes 4, 5 and 6, membranes that were previously treated with Syk in the presence of unlabelled phosphate were incubated with Syk (lane 5) or with Lyn (lane 6) in the presence of labeled phosphate. Lane 4 is a control without addition of either Syk or Lyn. **C) Phosphorylation of band 3 *in vivo***. Red blood cells were incubated in the absence or presence of diamide. Cell membranes were isolated, solubilized, and submitted to SDS-PAGE followed by transfer to nitrocellulose. The upper part of blot shows immunostaining of band 3 with anti–phosphotyrosine antibody. The filter was stripped, and reprobed with anti–band 3 antibody (bottom panel). **Nor** – control membranes; **Prob** – proband membranes.

Table 1. Plasmodium falciparum invasion of Band 3 "Neapolis" red cells

	Parasitemia (%)		
	24 hrs (Invasion) (%)	**48 hrs (Maturation) (%)**	**72 hrs (Reinvasion) (%)**
Control	**5.80 ± 0.71 (100)**	**3.65 ± 1.63 (100)**	**5.20 ± 1.27 (100)**
Mother	**4.35 ± 1.48 (75)**	**2.55 ± 1.34 (70)**	**4.20 ± 0.42 (81)**
Proband	**0.75 ± 0.07 (13)**	**0.20 ± 0.00 (5.5)**	**0.10 ± 0.00 (1.9)**

Cultures were inoculated by the addition of purified schizont-stage parasitized normal red cells (purity > 95 %) to test red cells suspended in growth medium (hematocrit: 0.5%). Two strains of parasite (Palo alto and FCR3) were used in each case. After 24 hours (ring-stage of first cycle), 48 hours (trophozoite-stage of the first cycle) and 72 hours (ring-stage of second cycle) of culture, slides were prepared, stained with Diff-Quik parasite stain and stage-dependent parasitemia assessed by microscopic inspection. Values are expressed as percent of parasitized red cells during the course of the experiment. In brackets, growth is normalized assuming 100 % growth for the control.

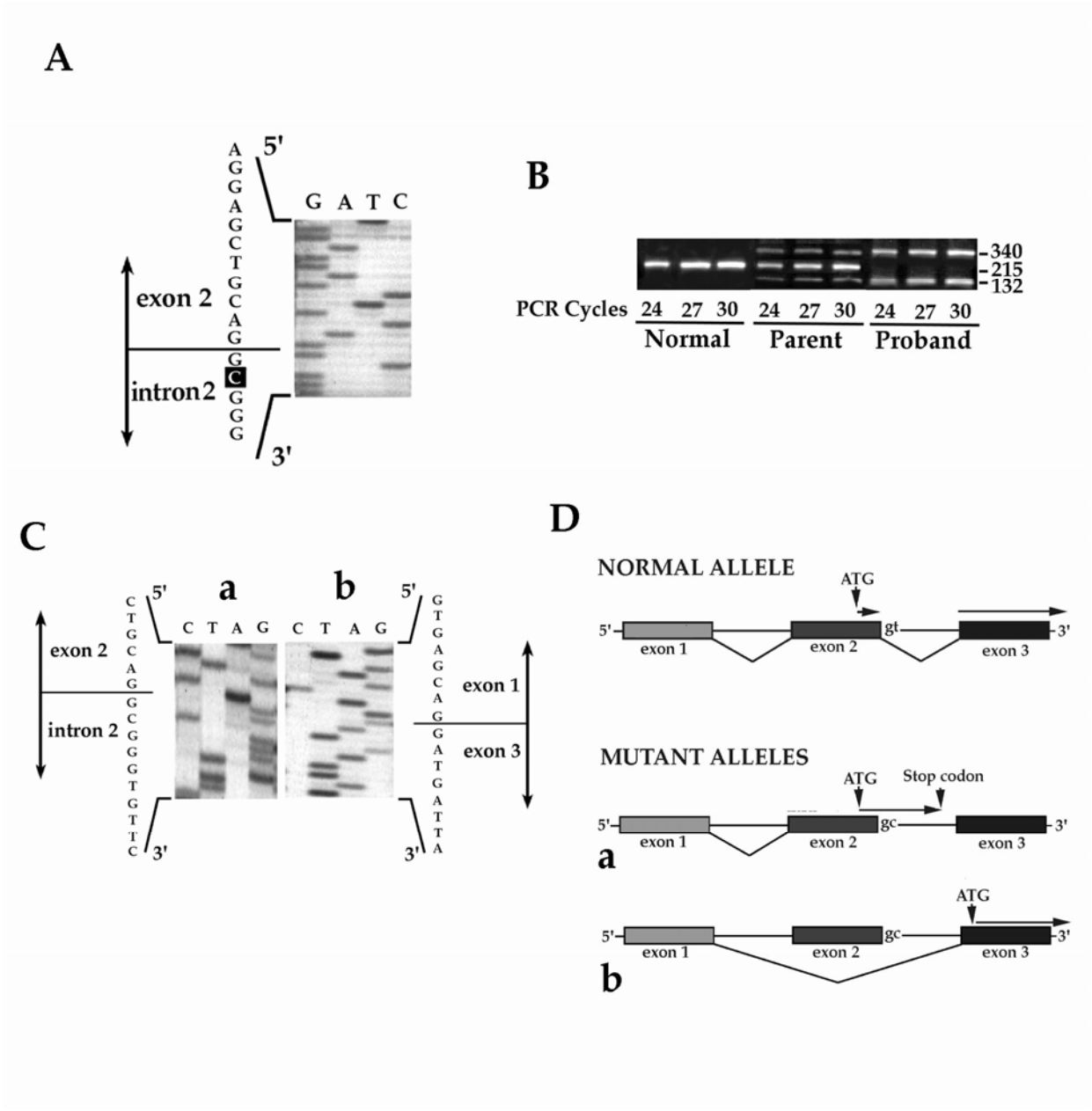
A
5'
G A T C
exon 2
intron 2
3'
B
340
215
132
PCR Cycles 24 27 30 24 27 30 24 27 30
Normal
Parent
Proband
C
a
b
5'
C T A G
exon 2
intron 2
exon 1
exon 3
3'
D
NORMAL ALLELE
ATG
exon 1
exon 2
exon 3
MUTANT ALLELES
Stop codon
a
b

Figure 1

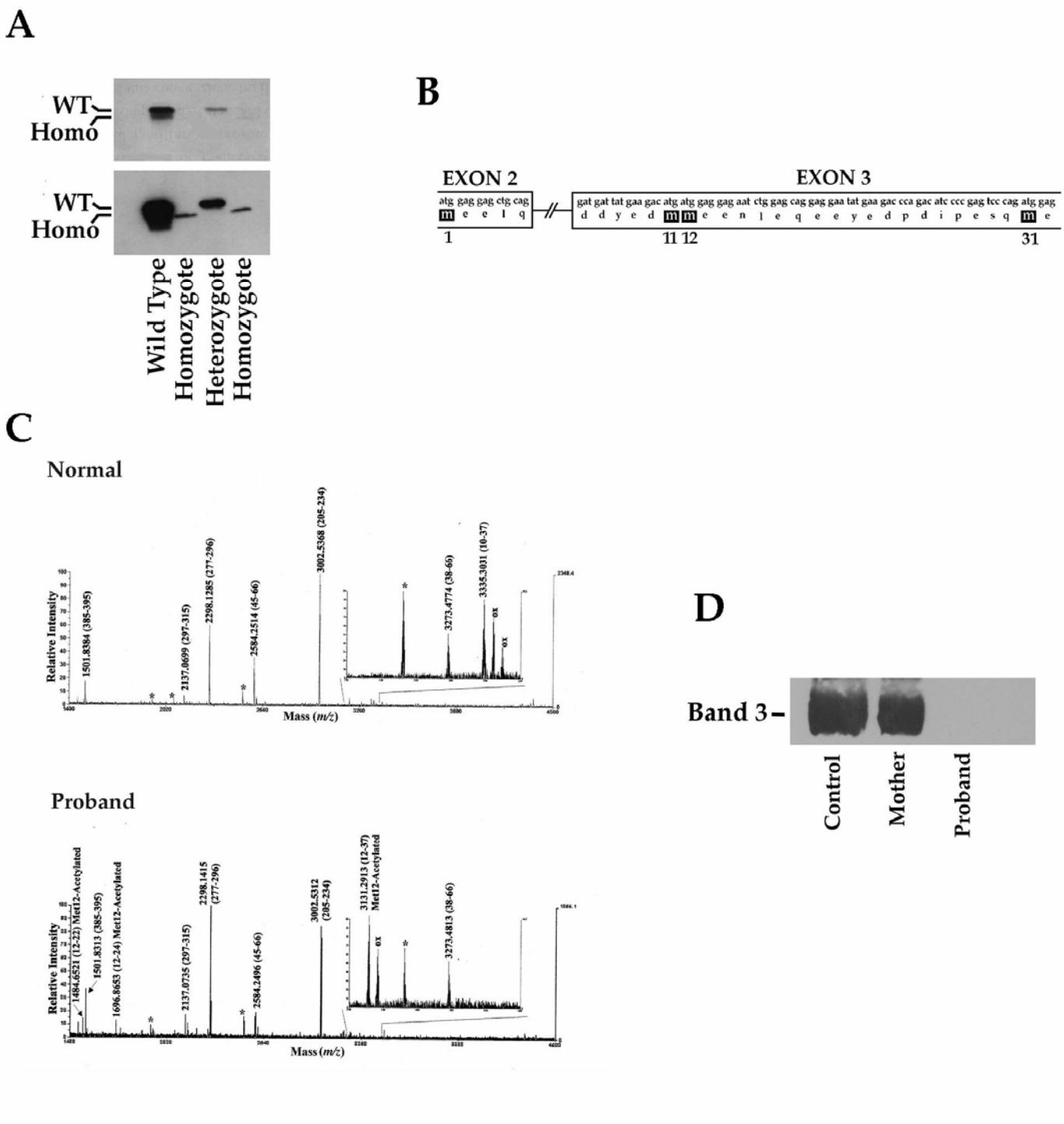
A
WT
Homo
WT
Homo
Wild Type
Homozygote
Heterozygote
Homozygote
B
EXON 2
EXON 3
atg gag gag ctg cag
gat gat tat gaa gac atg atg gag gag aat ctg gag cag gag gaa tat gaa gac cca gac atc ccc gag tcc cag atg gag
1
11 12
31
C
Normal
Relative Intensity
Mass (m/z)
1501.8384 (385-395)
2137.0699 (297-315)
2298.1285 (277-296)
2584.2514 (45-66)
3002.5368 (205-234)
3273.4774 (38-66)
3335.3031 (10-37)
Proband
Relative Intensity
Mass (m/z)
1484.6521 (12-22) Met12-Acetylated
1501.8313 (385-395)
1696.8653 (12-24) Met12-Acetylated
2137.0735 (297-315)
2298.1415 (277-296)
2584.2496 (45-66)
3002.5312 (205-234)
3131.2913 (12-37) Met12-Acetylated
3273.4813 (38-66)
D
Band 3
Control
Mother
Proband

Figure 2

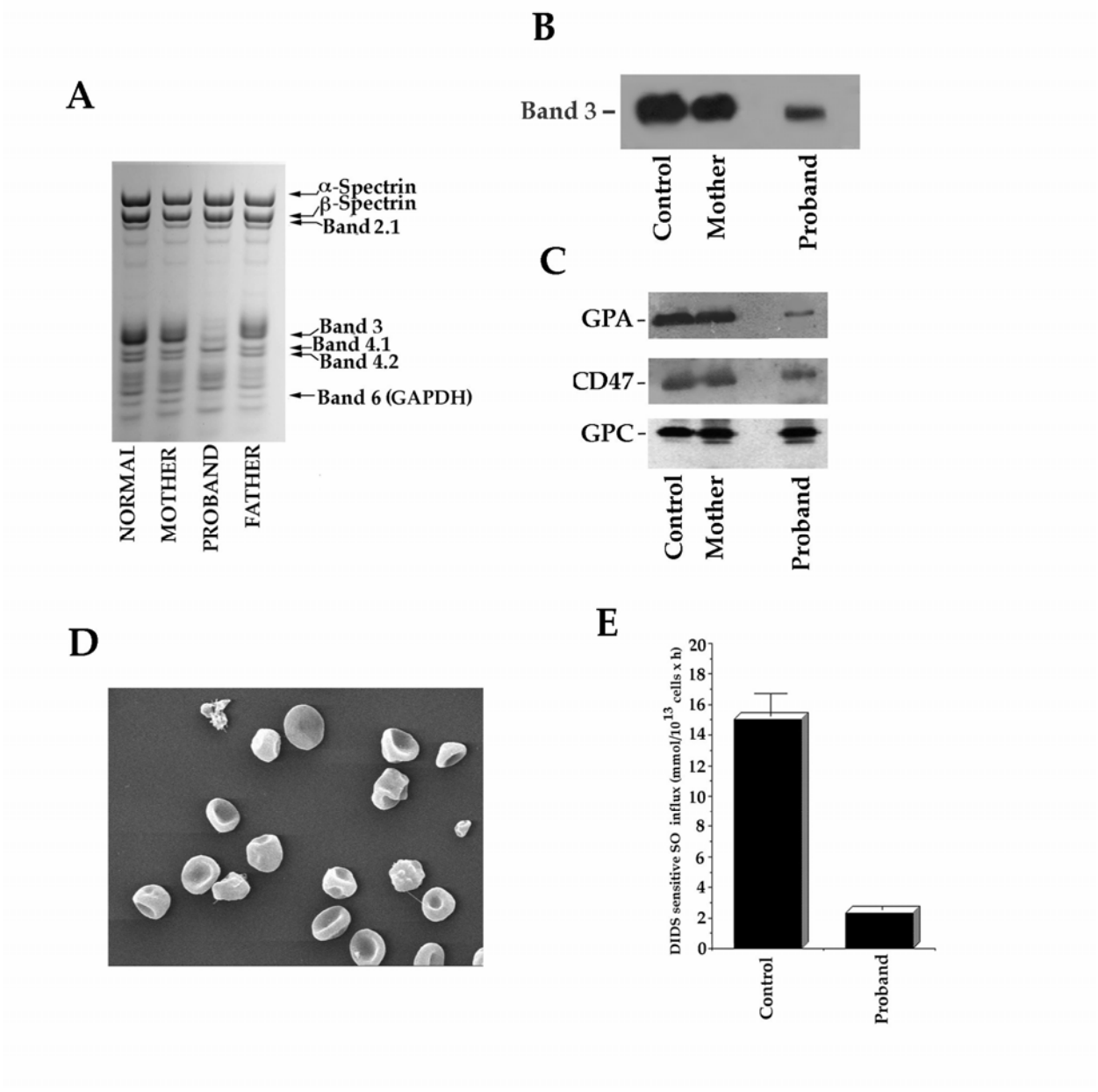
A
α-Spectrin
β-Spectrin
Band 2.1
Band 3
Band 4.1
Band 4.2
Band 6 (GAPDH)
NORMAL
MOTHER
PROBAND
FATHER
B
Band 3 –
Control
Mother
Proband
C
GPA -
CD47 -
GPC -
Control
Mother
Proband
D
E
DIDS sensitive SO influx (mmol/10^13 cells x h)
20
18
16
14
12
10
8
6
4
2
0
Control
Proband

Figure 3

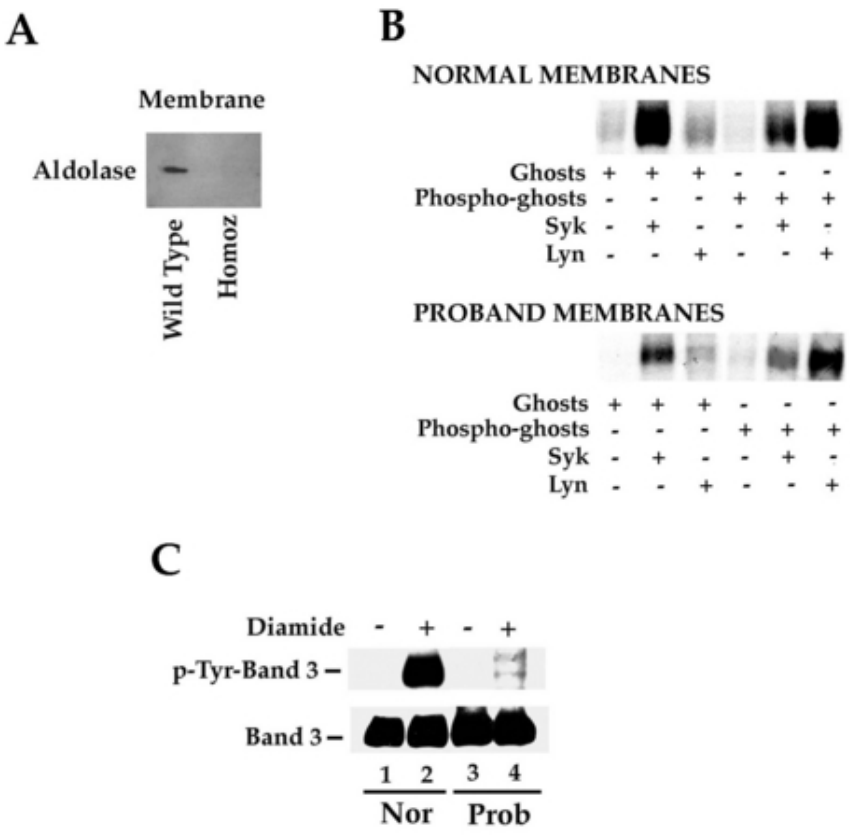
A
Membrane
Aldolase
Wild Type
Homoz
B
NORMAL MEMBRANES
Ghosts + + + - - -
Phospho-ghosts - - - + + +
Syk - + - - + -
Lyn - - + - - +
PROBAND MEMBRANES
Ghosts + + + - - -
Phospho-ghosts - - - + + +
Syk - + - - + -
Lyn - - + - - +
C
Diamide - + - +
p-Tyr-Band 3 –
Band 3 –
1 2 3 4
Nor Prob

Figure 4