RESEARCH COMMUNICATION

Band 3 HT, a human red-cell variant associated with acanthocytosis and increased anion transport, carries the mutation Pro-868 → Leu in the membrane domain of band 3

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1. We have studied band 3 HT, a human red-cell band 3 variant with increased M_r, which is associated with abnormal red-cell shape (acanthocytosis) and increased anion-transport activity. 2. We have shown that the increased M_r does not result from the presence of the band 3 Memphis mutation, and that the variant band 3 is covalently labelled by 4,4′-di-isothiocyanato-1,2-diphenylethane-2,2′-disulphonic acid (H_2DIDS) less readily than normal. 3. cDNA cloning studies show that band 3 HT results from the mutation Pro-868 → Leu, and the possible significance of the mutation in the altered anion-transport activity and cytoskeleton binding properties of band 3 HT is discussed.

INTRODUCTION

The red-cell anion transporter (band 3) is a multifunctional protein which carries out red-cell anion exchange and also has a role in the attachment of the skeleton and other peripheral proteins to the membrane (recently reviewed by Alper, 1991; Tanner, 1993). These functions are broadly associated with the two domains of the protein: the N-terminal 40 kDa domain, which is involved in the binding of cytoplasmic proteins, and the glycosylated C-terminal 55 kDa domain, which carries out anion transport.

Acanthocytosis describes a condition in which red blood cells have irregular spiculated projections or 'spines'. Kay et al. (1988) reported studies on a family with acanthocytosis and showed that the affected family members had an unusual form of red-cell band 3 that migrated slower than normal on SDS/PAGE, and that the red cells of the acanthocytic individuals had markedly increased anion-transport activity resulting from a increase in $V_{max.}$ compared with that of control red cells. The red cells of the parents of these individuals also showed increased anion transport, but the $V_{max.}$ was not increased to the same degree as in the affected children, suggesting that the children were homozygous for the abnormality which was inherited in an autosomal recessive fashion (Kay, 1992). We describe this variant band 3 with increased transport activity and increased M_r as band 3 HT (High Transport). Peptide-mapping studies suggested that band 3 HT had an alteration in the membrane domain, and spin-labelling experiments indicated that the band 3 in the abnormal cells had an increased rotational correlation time (Kay et al., 1988). In addition, ankyrin-rebinding studies to membranes from the band 3 HT-containing red cells suggested they had a decreased number of high-affinity ankyrin-binding sites. However, despite all these changes the red-cell survival of these individuals is normal, so that the condition appears to be benign (Kay et al., 1988; Kay, 1992).

We have carried out further studies on the red cells of a band 3 HT individual. In this paper we show that the condition results from the presence of a single amino acid change in band 3 (Pro-868 → Leu) located in the putative last membrane-spanning segment of the protein. We also show that the variant band 3 shows differences from normal in its covalent labelling by 4,4′-di-isothiocyanato-1,2-diphenylethane-2,2′-disulphonic acid (H_2DIDS).

MATERIALS AND METHODS

Materials

The red cells studied were from one of the affected siblings (sibling 2) with band 3 HT described by Kay et al. (1988) and Bosman and Kay (1990). Red cells were treated with chymotrypsin and membranes were prepared and separated by SDS/PAGE as described by Spring et al. (1992). Immunoblotting was done as described by Wainwright et al. (1989). Mouse monoclonal antibodies BRIC 4 and BRIC 10 (against glycophorin C), BRIC 163 (against glycophorin A) and BRIC 170 were gifts from Dr. D. J. Anstee (International Blood Group Reference Laboratory, Bristol). A rabbit anti-peptide serum against residues 477/492 of the human glucose transporter (GLUT1) was a gift from Dr. S. A. Baldwin (University of Leeds).

Labelling of red cells with H_2DIDS

Red cells (20% haematocrit) were labelled with [^{3}H]H_2DIDS (200 mCi/mmol; HSC Research and Development, Toronto, Canada) and digested with chymotrypsin as described by Hsu and Morrison (1985). The specific radioactivity of the N-terminal 60 kDa band 3 chymotryptic fragment was determined after SDS/PAGE and fluorography as described by Spring et al. (1992).

Abbreviations used: H_2DIDS, 4,4′-di-isothiocyanato-1,2-diphenylethane-2,2′-disulphonic acid; DIDS, 4,4′-di-isothiocyanatostilbene-2,2′-disulphonic acid.

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Anion-transport assays

Anion-transport experiments measuring $[^{35}S]SO_4^{2-}$ influx were done at 6% haematocrit by using an equal number of cells of each type (as determined on a cell counter), and 4,4′-di-isothiocyanatostilbene-2,2′-disulphonic acid (DIDS) titration of sulphate transport was carried out as described previously (Schofield et al., 1992a; Spring et al., 1992).

Preparation of band 3 cDNA and DNA sequencing

Total RNA was isolated from peripheral blood, and cDNA was prepared and amplified by the PCR as described by Schofield et al. (1992b). The red-cell band 3 cDNA was amplified in four overlapping sections by using the pairs of oligonucleotide primers listed below [nucleotide (nt) numbering based on the band 3 cDNA sequence reported by Tanner et al. (1988)]:

S15 (nt −141 to −120) and AS17 (nt 1336 to 1354)

S19 (nt 1171 to 1190) and AS4 (nt 2037 to 2053)

S12 (nt 2017 to 2035) and AS13 (nt 2684 to 2701)

S13 (nt 2566 to 2582) and AS27 (nt 2754 to 2771)

The four PCR-amplified band 3 fragments were sequenced directly on both strands by using a Cycle Sequencing kit (U.S. Biochemicals, Cleveland, OH, U.S.A.). The S12–AS13 product containing the band 3 HT mutation was also cloned into Bluescript phagemid (Stratagene Cloning Systems, San Diego, CA, U.S.A.) and sequenced on both strands by using double-stranded templates and Sequenase (U.S. Biochemicals).

RESULTS AND DISCUSSION

The higher M_r of band 3 HT is not due to the presence of the band 3 Memphis mutation

We have studied the red cells of one of the affected siblings described by Kay et al. (1988), thought to be homozygous for the band 3 HT variant (Kay, 1992). Kay et al. (1988) showed that the band 3 in these cells migrates more slowly than normal band 3 on SDS/PAGE. We confirmed that the lower mobility of band 3 HT was not due to the common band 3 Memphis mutation (Lys-56 → Glu; Yannoukakos et al., 1991), which causes a decrease in the SDS/PAGE mobility of intact band 3 and fragments of band 3 containing the N-terminal region (Mueller and Morrison, 1977). The red cells were treated with chymotrypsin and the membranes separated by SDS/PAGE and immunoblotted with a monoclonal antibody reactive with the N-terminal cytoplasmic domain (BRIC 170). The results in Figure 1 show that there was no difference in the mobility of the N-terminal 60 kDa chymotryptic fragment of normal and HT band 3, confirming the absence of the Memphis mutation and suggesting that the alteration was located on the C-terminal side of the chymotryptic cleavage site (Tyr-553; Tanner et al., 1988). No differences from normal were found in immunoblots of the variant cell membranes by using antibodies against glycophorin A (BRIC 163), glycophorin C (BRIC 4 and BRIC 10) and the glucose transporter.

Effects of stilbenedisulphonates

We examined the effect of the anion-transport inhibitor DIDS on sulphate transport into band 3 HT cells and normal red cells. Figure 2 shows the titration with DIDS of the anion-transport activity of equal numbers of either band 3 HT cells or control cells. Without inhibitor, the variant red cells had an anion-transport activity approx. 1.5 times that of the control cells, consistent with the increased $V_{max.}$ for anion transport found by Kay et al. (1988). The extrapolated intercept with the DIDS concentration was the same as for normal cells, showing that the number of reversible or irreversible DIDS-binding sites to band 3 is the same in the two cell types. The different slopes of the two

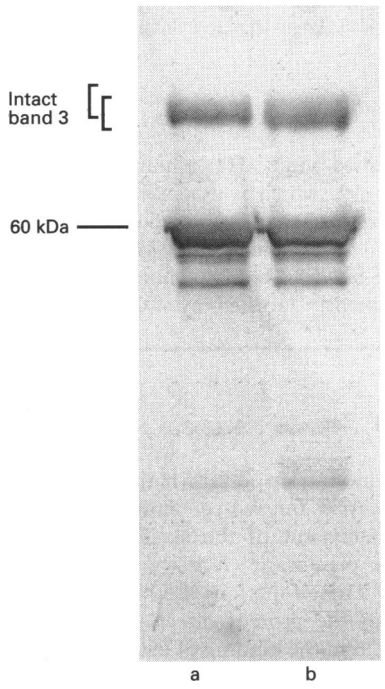

Figure 1 Immunoblotting of control and variant band 3

Intact band 3 HT (**a**) and control (**b**) red cells were treated with chymotrypsin, and membranes were prepared and separated by SDS/PAGE. The samples were immunoblotted with BRIC 170. The positions of the N-terminal 60 kDa fragment and residual undigested intact band 3 are marked.

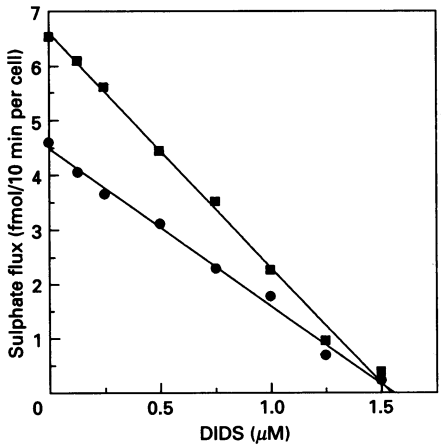

Figure 2 DIDS titration of anion transport in control (●) and band 3 HT (■) red cells

Details are given in the Materials and methods section.

lines in Figure 2 do not necessarily imply that there is a difference in the affinity of reversible or irreversible binding of DIDS to the variant and normal band 3, since in this experiment the concentration of band 3 sites (approx. 1 μM) is much higher than the K_i for either reversible or irreversible binding of DIDS (0.03–0.04 μM), and DIDS stoichiometrically binds band 3 until inhibition is almost complete (see Schofield et al., 1992a).

DIDS and H_2DIDS bind band 3 with very high affinity both in their fast initial binding as reversible inhibitors and in their slower subsequent covalent reaction with the protein. We compared the covalent labelling by [^{3}H]H_2DIDS of the variant and normal band 3. Red cells were labelled with 2 μM or 5 μM [^{3}H]H_2DIDS and treated with chymotrypsin. The specific radioactivity of the N-terminal 60 kDa chymotrypsin fragment of the normal and band 3 HT was estimated from scans of the autoradiographs and the protein-stained SDS gels. When 5 μM [^{3}H]H_2DIDS was used, the specific radioactivity in the band 3 HT was found to be 0.42, 0.43 and 0.51 (three determinations) of that in the band 3 of normal cells. With 2 μM [^{3}H]H_2DIDS the specific radioactivity in the band 3 HT was further decreased to 0.38 of that of normal band 3. Thus band 3 HT is covalently labelled by H_2DIDS much less readily than normal band 3. Figure 2 shows that DIDS still binds and inhibits band 3 HT with high affinity, but we cannot assess whether there is any quantitative change in the affinity of binding of the inhibitor to band 3 HT. The less ready covalent reaction of H_2DIDS with the variant protein probably results from a decrease in the reactivity of the lysine group which covalently binds to the inhibitor (Lys-539 or Lys-542; Bartel et al., 1989; Garcia and Lodish, 1989).

Band 3 Memphis variant 2 also shows an altered covalent reactivity with H_2DIDS (Hsu and Morrison, 1985; Spring et al., 1992). This variant band 3 was covalently labelled more readily than normal band 3, but there was no difference in the binding of the inhibitor or in the anion-transport activity of the variant protein. The increased labelling of band 3 Memphis variant 2 probably results from increased reactivity of the H_2DIDS-binding lysine residue. The mutations in this and the HT variant probably induce subtle changes in the structure of the protein which influence the pK of this lysine residue or its orientation relative to the bound inhibitor.

Identification of the amino acid change in band 3 HT

cDNA was prepared from mRNA obtained from the blood of the band 3 HT individual. The entire band 3 coding region was amplified by using PCR in four overlapping sections from this cDNA, and the DNA sequence was determined either directly from the PCR products or after cloning the PCR products. The band 3 HT cDNA differed from normal only in the substitution of T for C at nucleotide 2603, which results in the alteration of Pro-868 of normal band 3 to Leu in band 3 HT. This change was found on direct sequencing of the PCR products from two separate amplification experiments starting from the cDNA, as well as in five independent cDNA clones derived from the PCR products. Pro-868 is conserved in the known erythroid band 3 (AE1) and non-erythroid band 3-related protein (AE2 and AE3) sequences (reviewed by Wood, 1992). The cDNA sequence of the rest of the coding region of band 3 HT was the same as for normal band 3 (with Lys-56, as is found in the most common band 3 form; Kaul et al., 1983; Lux et al., 1989).

Structural and functional consequences of the 3 HT mutation

The Pro-868 → Leu mutation present in band 3 HT is located in the membrane interior near the cytoplasmic boundary of the most C-terminal of the predicted membrane-spanning segments of the protein. It is likely that Pro-868 induces a kink or other deformation of this probably helical membrane span. Secondary-structure prediction suggests that the Pro-868 → Leu substitution increases the helical tendency in this region, and this would both extend the helix and make it more rigid in this region. The mutation clearly modifies the overall structure of the membrane domain of the protein, since it affects the reactivity of Lys-539 or Lys-542 with extracellular H_2DIDS.

It is of considerable interest that the band 3 HT mutation is associated with mild acanthocytosis and a decrease in the number of ankyrin-binding sites on the membrane (Kay et al., 1988). Ankyrin binds the N-terminal cytoplasmic domain of band 3, and the binding appears to involve band 3 tetramers (Mulzer et al., 1990; Thevenin and Low, 1990). There is no evidence for the direct involvement in ankyrin binding of the extreme C-terminus of band 3 around the region of the mutation. It has been the general view that the cytoplasmic and membrane domains of band 3 are structurally independent, but recent evidence suggests they may interact in some way (reviewed by Batenjany et al., 1993). One possibility is that the mutation in the membrane domain changes the degree of association of band 3 in the membrane, or the structure of the associated complexes of band 3, so that ankyrin-binding band 3 tetramers are less readily formed. Changes in quaternary structure may also contribute to the abnormal H_2DIDS binding of band 3 HT. There is evidence that the covalent binding of DIDS to normal band 3 may be sensitive to the quaternary state of different band 3 oligomeric forms (Salhany et al., 1990).

Changes in red-cell shape and other cytoskeleton-associated properties are also found in Southeast Asian ovalocytosis (SAO), a condition which results from the heterozygous presence of band 3 with a nine-amino-acid deletion in the membrane domain (Jarolim et al., 1991; Tanner et al., 1991; Schofield et al., 1992b). The deletion in band 3 SAO is at the cytoplasmic boundary of the first transmembrane segment (a similar location to the band 3 HT mutation, but at the extreme N-terminus of the membrane domain) and also results in the loss of a proline residue. No alterations have been detected in the glycosylation of band 3 SAO, and there are conflicting reports concerning the alteration of ankyrin binding in these cells (see, e.g., Schofield et al., 1992b). The effects of the band 3 SAO mutation on red-cell properties have been interpreted in terms of an increased resistance to bending at the boundary between the cytoplasmic and membrane domains (Schofield et al., 1992b; Mohandas et al., 1992). However, alterations in the structure or size distribution of the oligomeric complexes of band 3 SAO in the membrane, with consequential effects on the cytoskeletal associations of the protein, as suggested for the band 3 HT mutation, may provide an alternative explanation. It is possible that the regions containing the two mutations (the first and last transmembrane segments) may be involved in the inter-subunit interactions which give rise to band 3 oligomers.

The increase in $V_{max.}$ for anion transport in band 3 HT is very striking. Anion translocation appears to be the rate-limiting step in the transport mechanism (Falke et al., 1985). The increased $V_{max.}$ can be interpreted in terms of a decrease in the activation energy of a structural change in the protein during the translocation step or an increase in stability of the transition-state intermediate (Krupka, 1989; Tanner, 1993). The amino acid sequence around the HT mutation may be directly involved in the region of the protein active in translocation or linked to this active region. Detailed kinetic studies on this variant band 3 may give a valuable insight into the nature of the structural intermediates involved in the translocation process. Finally, since

band 3 HT is a more efficient anion transporter than normal, why has it not undergone positive selection? It may be a new mutation. Alternatively, positive selection may have been restrained by the multifunctional nature of band 3, so that adverse effects from the abnormal cytoskeleton binding of the variant protein counter its more efficient transport properties.

We are grateful to Dr. D. J. Anstee and Dr. S. A. Baldwin for providing antibodies. L.J.B. is the recipient of a Medical Research Council Studentship. This work was supported in part by grants from the Medical Research Council, the Central Blood Laboratory Authority, NIH grants AG08444, AG08574 and AG0925, and a Veterans Administration Merit Review.

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Received 15 March 1993/23 April 1993; accepted 12 May 1993

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