Volume 330, number 2, 186–190 FEBS 12965 September 1993

The expression of the abnormal human red cell anion transporter from South-East Asian ovalocytes (band 3 SAO) in *Xenopus* oocytes

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Received 18 July 1993

South-East Asian ovalocytosis (SAO) is caused by the heterozygous presence of a variant form of the human erythrocyte anion transporter (band 3; AE1). The expression of band 3 SAO has been studied in *Xenopus* oocytes. Band 3 SAO is not functional as an anion transporter but is inserted stably into the plasma membrane of oocytes. Band 3 SAO translocation to the cell surface does not require co-expression of normal band 3. Co-expression of glycophorin A (GPA) increases the rate of translocation of band 3 SAO to the oocyte membrane but is not essential for this process. We suggest that the increased tendency of band 3 SAO to form oligomers may facilitate its translocation to the cell surface.

Hereditary ovalocytosis; Band 3; Glycophorin A; Anion transporter; *Xenopus* oocyte

1. INTRODUCTION

South-East Asian hereditary ovalocytosis (SAO) is common in some areas of Melanesia where malaria is endemic and is caused by the heterozygous presence of an abnormal band 3 [1]. The amino acid sequence of band 3 SAO has recently been determined from the cDNA sequence [2–5] and shown to contain a deletion of nine amino acid residues corresponding to part of the first transmembrane domain of normal band 3. SAO erythrocytes are more rigid than normal cells and show increased resistance to malarial parasite invasion. These red cells exhibit about half the anion transport activity of normal red cells which suggests that the mutant band 3 is defective for anion transport [6]. Band 3 SAO does not bind stilbene disulphonates [6], which inhibit band 3-mediated anion transport, and it lacks the polylactosaminyl oligosaccharide found on normal band 3 [7]. These findings suggest that the structure of the whole membrane domain of band 3 SAO is disrupted.

Normal band 3 associates in the membrane to form a mixture of dimers and tetramers. The tetramers are thought to interact preferentially with the cytoskeleton [8]. Band 3 SAO has a greater tendency to form tetramers relative to dimers than normal band 3 [7]. This could, at least in part, account for the increased rigidity of ovalocytic cells.

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Abbreviations: BADS, 4-benzamido-4′-aminostilbene-2,2′-disulphonate; DIDS, 4,4′-diisothiocyanato-2,2′-stilbene disulphonate; DNDS, 4,4′-dinitrostilbene-2,2′-disulphonate; GPA, glycophorin A; SAO, South-East Asian hereditary ovalocytosis.

Expression of normal band 3 in *Xenopus* oocytes induces chloride transport in the cells that is inhibitable with 4,4′-dinitrostilbene-2,2′-disulphonate (DNDS). In contrast, injection of band 3 SAO cRNA into oocytes does not induce any stilbene-disulphonate sensitive chloride transport activity [9]. However, in these experiments it was not established whether the absence of Cl^- transport activity resulted from the band 3 SAO not being translocated to the cell surface and therefore not being expressed there, or from the band 3 SAO protein being intrinsically inactive for anion transport. It could also be that co-expression of normal band 3 is required for the translocation of band 3 SAO to the plasma membrane.

In this work, we have examined whether band 3 SAO, expressed from the cRNA in oocytes, is translocated to the plasma membrane of the oocyte. We have also examined whether, like normal band 3, the movement of band 3 SAO to the cell surface is facilitated by GPA.

2. MATERIALS AND METHODS

The cDNA clones encoding human band 3 (pBSXG1·b3) and glycophorin A (pBSXG·GPA) have been described previously [10]. The cDNA coding for band 3 SAO [5] was constructed from pBSXG1·b3 by oligonucleotide-directed mutagenesis (Amersham kit). Amino acid residues 400–408 of the band 3 coding sequence were deleted using the oligonucleotide 5′-CTGAGTGACATCACAGATGTCATCTTCATCTACTTTG-3′ to give pBSXG1·SAO.

Protocols for synthesis of cRNA, oocyte isolation and cRNA injection, ^{35}S-amino acid labelling, immunoprecipitation of band 3, chymotrypsin treatment of oocytes and chloride influx assays in oocytes have all been described [10]. The anti-band 3 monoclonal antibodies used were BRIC 155, which is directed against the cytoplasmic C-terminus of band 3 [11] and BRIC 6, which is directed against an extracellular epitope in the membrane domain of band 3 (supplied by

 Published by Elsevier Science Publishers B.V.

Dr. D.J. Anstee, International Blood Group Reference Laboratory, Bristol, UK).

3. RESULTS

The 9 amino acid residue deletion in band 3 SAO has been suggested to cause the protein to mis-fold in the membrane so that it does not support any anion transport activity [6]. In this work we examined whether the inability of band 3 SAO cRNA to induce DNDS-sensitive chloride transport activity in *Xenopus* oocytes [9] might be due to an inability of this protein to be translocated to the oocyte plasma membrane. The expression of normal band 3 at the cell surface of oocytes is facilitated by the co-expression of glycophorin A (GPA) [10] and we investigated whether this effect is also observed with band 3 SAO. The cRNAs coding for normal and/or band 3 SAO were injected into oocytes, with or without GPA cRNA (1.5 ng each/oocyte). The presence of both normal and band 3 SAO at the cell surface was studied using two approaches.

In a first experiment, groups of intact oocytes were treated with the monoclonal antibody BRIC 6 (which recognizes an extracellular epitope on band 3). Band 3 was then immunoprecipitated from oocytes which were solubilized in buffer containing Triton X-100 as the only detergent. More band 3 was immunoprecipitated from oocytes in which both normal band 3 and GPA were co-expressed than those expressing band 3 alone (Fig. 1, lanes A and B), as observed previously [10]. However, band 3 SAO was not immunoprecipitated in either the presence or absence of GPA (Fig. 1, lanes C and D). To confirm that similar amounts of both normal and band 3 SAO had been synthesized by the oocytes, the homogenates were also immunoprecipitated with the monoclonal antibody BRIC 155 (which recognizes the cytoplasmic COOH-terminus of band 3) (Fig. 1, lanes E–H). These results suggested either that band 3 SAO is not translocated to the plasma membrane of oocytes or that BRIC 6 is not able to bind to the variant band 3 in the plasma membrane of oocytes.

To resolve this question, a second experiment was performed in which the expression of both normal band 3 and band 3 SAO in the plasma membrane of oocytes was assessed by an independent method. Band 3 in red cells is cleaved by chymotrypsin at its extracellular surface to yield an N-terminal 60 kDa and a C-terminal 35-kDa fragment [12] and chymotrypsin treatment has been used previously to probe for normal band 3 expressed at the surface of oocytes [10]. Groups of 15 oocytes were injected with normal band 3 cRNA, band 3 SAO cRNA and GPA cRNA in different combinations. Oocytes were pulse-labelled with ^{35}S-labelled amino acids and then subjected to a chase with unlabelled amino acids for 48 h. Some of the groups of oocytes were treated with chymotrypsin for 1 h at 4°C. Both the untreated and the chymotrypsin-treated

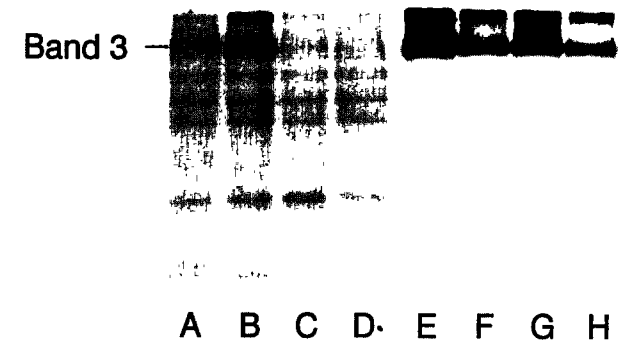

Fig. 1. Immunoprecipitation of oocyte surface band 3 with BRIC 6. Groups of 10 oocytes were injected with either 1.5 ng/oocyte of band 3 cRNA (lanes A,B,E and F) or 1.5 ng/oocyte of band 3 SAO cRNA (lanes C,D,G and H) Some were co-injected with 1.5 ng/oocyte GPA cRNA (lanes B,D,F and H). Oocytes were pulse-labelled with ^{35}S-labelled amino acids and chased with unlabelled amino acids for 48 h. The intact cells were treated with the monoclonal antibody BRIC 6 (which is directed against an extracellular epitope on band 3), washed free from antibody and homogenized. Surface band 3 was assessed in one half of the homogenate by immunoprecipitation of bound BRIC 6 (lanes A–D) as detailed in reference [10]. To confirm that band 3 had been expressed equally in all four sets of injected oocytes, BRIC 155 (reactive with the cytoplasmic COOH terminus of band 3) was added to the second half of the homogenate and total band 3 was immunoprecipitated (lanes E–H). Each lane represents immunoprecipitated protein from the equivalent of 5 oocytes, separated on 10% SDS-PAGE.

oocytes were then incubated with BRIC 6 overnight at 4°C to allow the antibody to react with surface band 3. After removal of excess antibody by washing in Barths saline, groups of ten BRIC 6-treated oocytes were homogenized in Triton X-100 immunoprecipitation buffer (with no SDS present) and each homogenate was divided into three equal portions. The surface band 3 to which the BRIC 6 had bound during the earlier incubation with the intact oocytes was immunoprecipitated from one of the portions by addition of protein A-Sepharose (Fig. 2a). The total band 3 present in the oocyte homogenates was immunoprecipitated from each of the remaining portions by addition of either BRIC 6 (Fig. 2b) or BRIC 155 (Fig. 2c), followed by protein A-Sepharose.

When groups of intact oocytes were treated with BRIC 6 (without prior chymotrypsin digestion) and surface band 3 was subsequently immunoprecipitated, normal band 3 but not band 3 SAO was detected (Fig. 2a). These results are similar to those in Fig. 1. When immunoprecipitation of surface band 3 from chymotrypsin-treated intact oocytes was attempted using BRIC 6, neither normal nor band 3 SAO were detected (Fig. 2a). This suggests that the BRIC 6 epitope of normal band 3 is destroyed by digestion of intact oocytes with chymotrypsin and confirms that the cleavage of surface band 3 was complete. The BRIC 6 epitope on band 3 is lost when red cells are treated with chymotrypsin (Dr. D.J. Anstee, personal communication).

Fig. 2. Immunoprecipitation of normal and South-East Asian ovalocytic band 3 from chymotrypsin-treated oocytes. Groups of 10 oocytes were injected with 1.5 ng of the cRNAs corresponding to normal band 3 (B3), ovalocytic band 3 (SAO) and GPA (A) in different combinations as indicated. Cells were pulse-labelled with ^{35}S-labelled amino acids and chased with unlabelled amino acids for 48 h. The intact oocytes were incubated for 1 h at 4°C with or without chymotrypsin, and then treated with BRIC 6 for 18 h at 4°C. All oocytes were homogenized and equal portions of the homogenate were incubated with either (a) no further antibody; (b) BRIC 6 or (c) BRIC 155. Band 3 was then immunoprecipitated from all the samples using Protein A–Sepharose CL-4B. Each lane represents immunoprecipitated protein from the equivalent of 3–4 oocytes, separated on 11% SDS-PAGE. The faint bands which are also visible in the GPA-injected oocyte samples are due to low level non-specific precipitation of other proteins. The position of the COOH-terminal 35-kDa band 3 fragment is indicated. The proportion of band 3 cleaved by chymotrypsin was determined by scanning densitometry of the SDS-PAGE fluorographs. Expressed protein was estimated from the areas under the peaks. The 35-kDa band 3 fragment was assumed to contain 60% of the radioactivity incorporated into intact band 3, based on methionine and cysteine content. Values are percentage of total band 3 (i.e. intact + fragment) which was found as the 35-kDa fragment. Chloride influx (60 min) was measured 24 h after injection of cRNAs. The mean DNDS-sensitive chloride influx was determined for each of the cRNA mixtures from the difference between the means of groups of 10–12 oocytes which were not treated with chymotrypsin and were assayed with and without 400 μM DNDS treatment.

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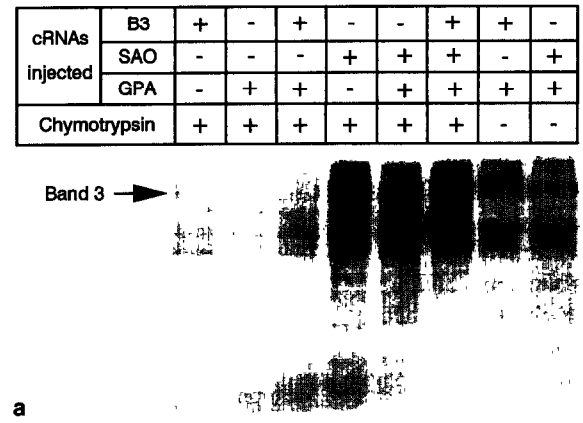
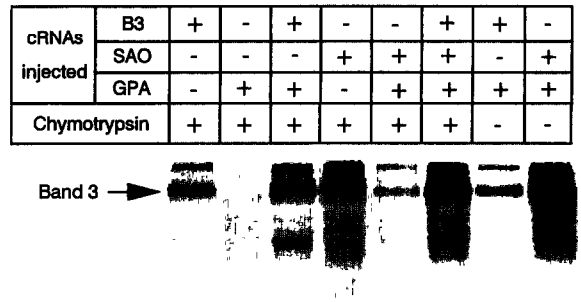


a



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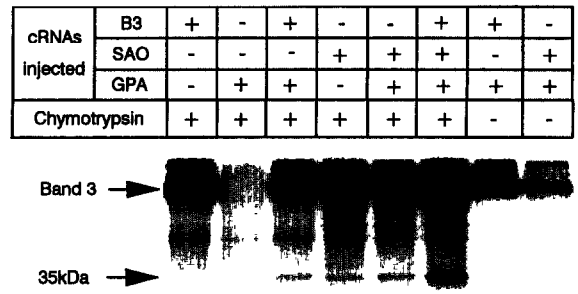

c

% band 3 cleaved by chymotrypsin	6	-	14	10	23	21
Cl^- influx (nmol/cell) (no chymotrypsin)	5.1	1.2	10	0.1	0.5	7.1

When intact oocytes that had been treated with BRIC 6 were subsequently homogenized in the presence of Triton X-100 and then immunoprecipitated using a second addition of BRIC 6, both normal band 3 and band 3 SAO were detected (Fig. 2b). Since the action of chymotrypsin on surface band 3 destroys the BRIC 6 epitope, only intact (intracellular) band 3 was immunoprecipitated from chymotrypsin-treated oocytes. It appears that BRIC 6 immunoprecipitates Triton-solubilized SAO and normal band 3 in oocyte homogenates but that the antibody binds only to normal band 3 at the surface of intact oocytes.

Both normal band 3 and band 3 SAO were detected in the immunoprecipitates from Triton-solubilized oocyte homogenates using BRIC 155 (Fig. 2c). Immunoprecipitates from the chymotrypsin-treated oocytes contained both intact (intracellular) band 3 and the 35-kDa chymotryptic fragment of band 3, which is derived from band 3 at the cell surface. Both normal band 3 and band 3 SAO were cleaved by chymotrypsin. This shows that band 3 SAO is translocated to the plasma membrane of oocytes and that this takes place without co-expression of either GPA or normal band 3. A greater proportion of band 3 SAO was cleaved by chymotrypsin in oocytes in which GPA was co-expressed than those in which band 3 SAO cRNA was expressed alone. This observation was confirmed in a second experiment (data not shown) and indicates that GPA facilitates the translocation of band 3 SAO to the cell surface, similarly to the effect of GPA on normal band 3.

When equivalent concentrations of either band 3 SAO or normal band 3 cRNAs are expressed in oocytes, a greater proportion of band 3 SAO is cleaved by chymotrypsin than in the corresponding oocytes expressing normal band 3 (Fig. 2c). This effect was observed whether or not GPA was present and suggests that band 3 SAO is translocated to the oocyte surface more readily than normal band 3.

The DNDS-sensitive chloride influx into oocytes was also measured 24 h after injection of the cRNAs using parallel sets of oocytes that had not been treated with chymotrypsin (Fig. 2c). The levels of anion transport were similar to those observed previously in each case [9]. Co-expression of normal band 3, band 3 SAO and GPA resulted in a slightly lower level of chloride transport than co-expression of normal band 3 and GPA. This could be due to a reduction in the amount of normal band 3 synthesized (due to competition between the cRNAs) when band 3 SAO and GPA cRNAs are also expressed at the surface. Alternatively, hetero-oligomers of normal band 3 and band 3 SAO may be formed, and the rate of chloride transport by normal band 3 may be reduced in these hetero-oligomers.

4. DISCUSSION

We have shown that band 3 SAO is expressed at the plasma membrane of oocytes in both the presence and absence of GPA. However, anion transport was not detected in parallel sets of oocytes that were injected with the same cRNAs as were used to study cell surface expression (Fig. 2c). This confirms that, when expressed in oocytes, the lack of anion transport in band 3 SAO is caused by loss of the ability of the band 3 SAO protein to transport chloride and not by the lack of movement of band 3 SAO to the cell surface.

All known individuals with hereditary ovalocytosis are heterozygous for SAO and normal band 3, and the homozygous condition is presumed to be lethal. We have found that band 3 SAO is translocated to the plasma membrane of oocytes without requiring the co-expression of normal band 3. This implies that the formation of a heterodimer between band 3 SAO and normal band 3 is not essential for band 3 SAO to move to the cell surface.

In band 3 SAO, the deletion of amino acid residues 400–408 of normal band 3, which are located at the boundary of the N-terminal cytoplasmic domain and first transmembrane helix of normal band 3, affects several properties associated with the extracellular surface of band 3. The anion transport inhibitors 4,4′-diisothiocyanato-2,2′-stilbene disulphonate (DIDS) [6], and 4-benzamido-4′-aminostilbene-2,2′-disulphonate (BADS) [7] do not bind to band 3 SAO and the formation of the polylactosaminyl oligosaccharide on the extracellular side of the mutant band 3 is prevented. We have found that the monoclonal antibody BRIC 6, (which reacts with an extracellular epitope on band 3) does not immunoprecipitate band 3 SAO expressed in the plasma membranes of intact oocytes, whereas it reacts with normal band 3 under these conditions. This demonstrates that the folded structure of cell surface band 3 SAO in the region of the BRIC 6 epitope is different from that of normal band 3 and suggests that the BRIC 6 epitope is concealed or misfolded in native band 3 SAO, even though the antibody binding site is distal from the band 3 SAO deletion in the amino acid sequence. However, when the oocyte membranes are solubilized using the detergent Triton X-100, both normal band 3 and band 3 SAO are immunoprecipitated using BRIC 6. One possible explanation for these unexpected results is that the structures of intracellular and cell surface band 3 SAO are different, and that BRIC 6 binds more strongly to the intracellular form. Alternatively, BRIC 6 may bind to band 3 SAO with a lower affinity than to normal band 3. In this case, the higher concentration of band 3 present in the oocyte homogenates may allow detection of the band 3 SAO in the immunoprecipitates.

Although the folding of some regions of the band 3 molecule are severely affected by the SAO deletion, it appears that these effects may be local, and that the global structure of band 3 SAO is similar to that of normal band 3. The circular dichroism spectra of normal and band 3 SAO are comparable, when either the intact proteins or the isolated membrane domain is used [7], and the mutant protein is expressed stably in both ovalocytic red cells and *Xenopus* oocytes. Previous attempts to separate normal band 3 and band 3 SAO by lectin or inhibitor affinity chromatography were not successful [7], which suggests that SAO and normal band 3 may be able to associate to form hetero-oligomers. We have found that band 3 SAO interacts with GPA in internal membranes of oocytes to facilitate the translocation of band 3 SAO to the plasma membrane, similarly to normal band 3 [10]. This shows that those regions of the band 3 molecule that are responsible for this GPA-dependent effect are not disrupted in band 3 SAO and provides further evidence that certain aspects of the overall structure of normal band 3 are conserved in band 3 SAO.

Band 3 from SAO erythrocytes has been shown previously to contain a higher proportion of tetramers relative to dimers than normal band 3 [7]. We have shown that a higher proportion of band 3 SAO is expressed at the surface of oocytes than normal band 3, and that this difference is independent of the presence of GPA. It is possible that the increased tendency of band 3 SAO to form oligomers encourages formation of a structure that is more readily translocated to the oocyte surface. This is consistent with the previous suggestion that oligomerization of band 3 may be required for the efficient translocation of band 3 to the cell surface.

Acknowledgements: We thank Dr. David Anstee for antibodies and Dr. Reinhardt Reithmeier for communicating results prior to publication. This work was supported by the Medical Research Council and Wellcome Trust.

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