Functional reassembly of the anion transport domain of human red cell band 3 (AE1) from multiple and non-complementary fragments

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Received 21 June 1998

Abstract **We constructed cDNA clones encoding N-terminal, C-terminal and internal polypeptide fragments of the human red cell anion exchanger (band 3; AE1). The internal fragments comprised between one and seven putative transmembrane spans with two or more spans deleted from both termini of the membrane domain of band 3. Sets of three, four or five complementary fragments, which together represented the complete amino acid sequence of the membrane domain, were co-expressed in *Xenopus* oocytes. Stilbene disulphonate-sensitive chloride uptake assays revealed that all six of the three-fragment combinations and two of the four-fragment combinations reassembled functionally in vivo. Unexpectedly, co-expression of a non-complementary pair of fragments comprising the first five and last seven putative transmembrane spans (i.e. entirely lacking spans six and seven) was also found to be sufficient to generate stilbene disulphonate-sensitive chloride uptake.**

Key words: Anion exchanger; Band 3 protein; Chloride transport; Heterologous expression; Membrane protein fragment; *Xenopus* oocyte

1. Introduction

The human red cell anion exchanger (band 3; AE1) is a multifunctional integral membrane protein that is expressed in erythrocytes and the intercalating cells of the distal tubule of the kidney. Band 3 comprises two distinct structural and functional domains [1–3]: the 43 kDa N-terminal cytoplasmic domain (aa residues 1–359) is involved in functions unrelated to anion transport, including binding to the red cell cytoskeleton [4], while the 52 kDa C-terminal membrane domain (aa residues 360–911) is both necessary and sufficient to mediate the chloride-bicarbonate exchange function [5–7]. The membrane domain traverses the bilayer up to 14 times in current topology models [8] and contains a high proportion of α-helical structure [9]. Two-dimensional crystallography has yielded a low resolution (20 Å) structural model of the dimeric membrane domain [10] (reviewed by [3]), which indicates that the cross-sectional area of the monomer in the plane of the membrane is sufficient to allow the packing of up to fourteen α-helical spans.

In previous work, we have shown that functionally active band 3 can be expressed in the plasma membrane of *Xenopus* oocytes from pairs of complementary fragments truncated within the fourth or sixth putative cytoplasmic loops [11] and the third or fourth exofacial loops [12], although none of the fragments was individually functional. In addition, many (although not all) of the fragments were expressed stably in oocytes, so it is likely that they form tightly folded structures which are not degraded by the quality control systems of the cell. These results suggest that the different regions of the membrane domain fold independently into native-like structures, since it is unlikely that major unfolding and refolding events occur during the assembly of the fragments [11]. Similar phenomena have been observed with other proteins, for example bacteriorhodopsin [13], lactose permease [14] and opsin [15], and it is likely to be a general property of polytopic membrane proteins. These observations are consistent with the 'two-stage model' for the folding and assembly of multispanning membrane proteins [16], in which the folding and insertion of individual α-helical spans and their subsequent assembly to form the final protein are considered as conceptually distinct steps.

In this paper, we examine the ability of three or more contiguous band 3 fragments to insert into membranes, associate, translocate to the cell surface and mediate stilbene disulphonate-sensitive anion transport in *Xenopus* oocytes. We demonstrate functional co-expression in eight different combinations of three or four complementary band 3 fragments. In addition, we show that two transmembrane spans are not essential for stilbene disulphonate-sensitive chloride uptake.

2. Materials and methods

2.1. Construction of band 3 fragments

The cDNA clones encoding human red cell band 3 (pBSXG1.B3), the membrane domain of band 3 (pBSXG1.B3mem) and glycophorin A (pBSXG.GPA) have been described previously [7]. These plasmids contain the respective protein coding region flanked by the 5′- and 3′-non-coding region of *Xenopus* β-globin. The construction from pBSXG1.B3 of cDNAs encoding pairs of complementary N- and C-terminal band 3 fragments have been detailed previously [11,12] and the resulting fragment polypeptides are represented as cartoons in Fig. 1 of [17], according to the 14 span topology model of band 3 [8]. The N-terminal fragments b3(1:5), $b3(1:7)_a$, b3(1:8) and b3(1:12), and the C-terminal fragments (6:14), $(8:14)_a$, (9:14) and (13:14), which are truncated at sites within the third exofacial, fourth exofacial, fourth cytoplasmic and sixth cytoplasmic loops respectively, were all used directly in this study.

N-terminal fragments lacking the cytoplasmic domain of band 3 (amino acid residues 1–359) were prepared by polymerase chain reaction (PCR) mutagenesis of pBSXG1.B3mem, using the same protocol and oligonucleotide primers as were used to prepare the entire

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Abbreviations: DNDS, 4,4′-dinitrostilbene-2,2′-disulphonate; GPA, glycophorin A; PCR, polymerase chain reaction; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis

PII: S0014-5793(98)00909-0

N-terminal fragments [11,12]. After amplification, the cDNAs were digested with *Bst*EII, recircularised and cloned in *Escherichia coli* strain TG2. The new constructs, designated bm(1:5), bm(1:7)$_a$, bm(1:8) and bm(1:12), encoded an initiator methionine followed respectively by aa residues 360–553, 360–639, 360–695 or 360–824 of band 3 (Fig. 1).

cDNAs encoding six internal fragment polypeptides of band 3 (Fig. 1) were prepared by the same PCR and *Bst*EII digestion procedure as the N-terminal fragments described above, but using the C-terminal fragment constructs pBSXG1.(6:14), pBSXG1.(8:14)$_a$ [12] or pBSXG1.(9:14) [11] as templates for amplification. For example, the cDNA encoding the fragment designated (6:8), which comprises putative spans six, seven and eight (aa residues 554–695), was prepared by PCR amplification of pBSXG1.(6:14) using the antisense primer that generated b3(1:8) and bm(1:8) and a sense primer that annealed to the cDNA sequence immediately upstream of the C-terminus of band 3. Constructs encoding the other internal fragments, designated (6:7)$_a$, (6:12), (8)$_a$, (8:12)$_a$ and (9:12), were prepared likewise.

The cDNA coding sequences of all the new constructs were verified using either a Du Pont Genesis 2000 automated sequencer or manually by double stranded dideoxy sequencing with Sequenase (U.S. Biochemical Corp.).

2.2. Expression in Xenopus oocytes and cell-free translation

The T7 mMessage mMachine kit (Ambion, Austin, TX) was used to prepare capped cRNAs by in vitro transcription of cDNA constructs. Procedures for the isolation of oocytes, microinjection of cRNAs and chloride influx assays were performed as described previously [7], except that chloride uptake was measured in groups of 18–25 oocytes in either the presence and absence of 2 mM dinitrostilbene-2,2′-disulphonate (DNDS). Cell-free translations were performed using capped cRNA in the rabbit reticulocyte lysate system (Promega) with canine pancreatic microsomes. The competitive *N*-glycosylation acceptor peptide benzoyl-Asn-Leu-Thr-*N*-methylamide (BzNLT) was included in some reaction mixtures (30 μM final concentration), as described previously [18]. Microsomes were isolated in 7×20 mm tubes by centrifugation through 120 μl of a neutral cushion (350 mM sucrose, 150 mM KCl, 5 mM dithiothreitol, 3 mM $MgCl_2$, 50 mM HEPES-K$^+$, pH 7.5) or alkaline cushion (250 mM sucrose, 100 mM sodium carbonate, pH 11.5) at 70000 rpm for 20 min at 4°C in a TLA100 ultracentrifuge (Beckman Instruments). The yields of each fragment were quantified on Laemmli [19] or tricine [20] SDS-PAGE gels by fluorography using BioMax MR film (Kodak) and Imagemaster 1d scanner software (Pharmacia-LKB) as detailed previously [17].

3. Results and discussion

3.1. Preparation and characterisation of internal fragments of band 3

We used PCR mutagenesis to prepare six cDNA constructs encoding internal polypeptide fragments of band 3 which have at least five putative transmembrane spans deleted from the N-terminus and at least two spans deleted from the C-terminus (Fig. 1). The internal fragment designated (9:12) was prepared by C-terminal truncation of the cDNA coding sequence of the C-terminal fragment (9:14) [11], which has its N-terminus located in a putative cytoplasmic loop of band 3. The five internal fragments designated (6:7)$_a$, (6:8) and (6:12), (8)$_a$ and (8:12)$_a$ were prepared by C-terminal truncation of the cDNA coding sequence of the C-terminal fragments (6:14) and (8:14)$_a$ [12]. These polypeptides have their N-terminus located in a putative exofacial loop. The cleavable signal sequence of glycophorin A (GPA) was appended to the N-terminus of these fragments [12] to assist in the translocation of the N-terminus into the lumen of the endoplasmic reticulum.

We examined the biosynthesis of the internal fragments in the rabbit reticulocyte lysate cell-free translation system with canine pancreatic microsomes. SDS-PAGE and fluorography were used to resolve fragments (6:12) and (8:12)$_a$ (12%

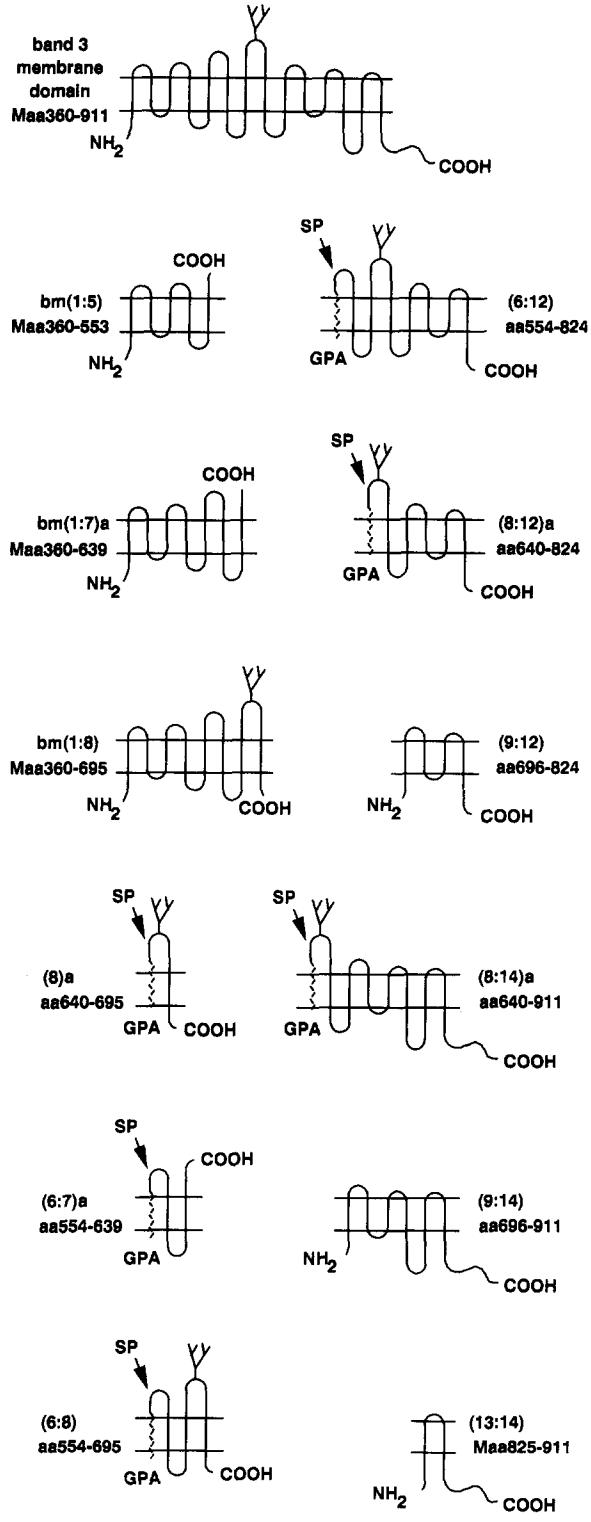

Fig. 1. Structure of band 3 fragments. The nomenclature and truncation sites of the fragments used in this paper are shown in cartoon form, together with their putative transmembrane topology and the epitopes of the antibodies BRIC170 and BRIC155. The single *N*-glycan chain of band 3 is shown in the fourth exofacial loop. The dashed zigzag line represents the GPA signal sequence which precedes those fragments that have their N-terminus in an exofacial loop. The arrow indicates the cleavage site of the GPA signal sequence by signal peptidase (SP). Cartoons of intact band 3 and other band 3 fragments discussed are shown in Fig. 1 of [17].

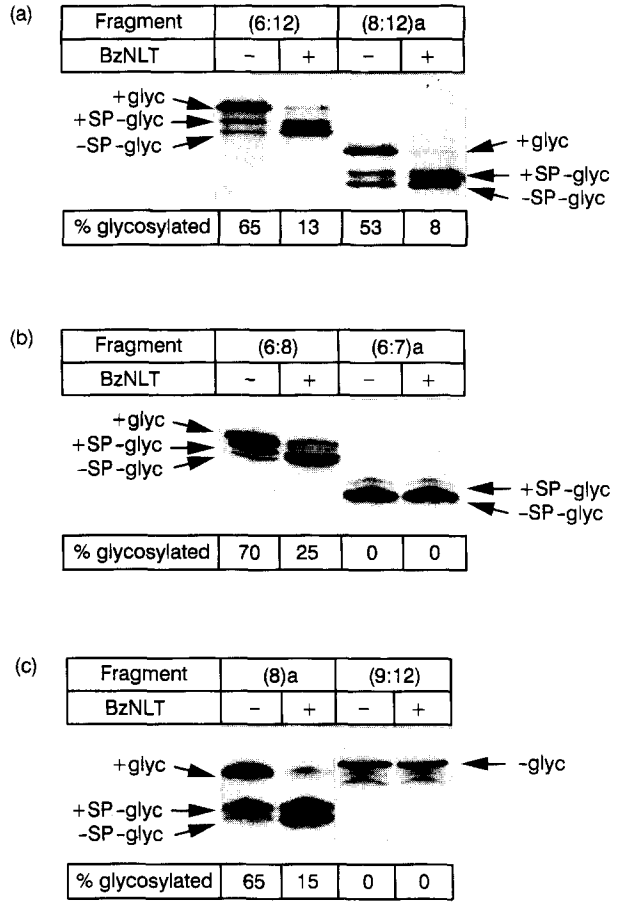

Fig. 2. Biosynthesis of internal fragments of band 3 in the cell-free system. Band 3 fragment cRNAs were translated in the rabbit reticulocyte lysate cell-free translation system in the presence of canine pancreatic microsomes, as described in [7]. The amount of cRNA translated (per 6.25 μl reaction) was: 44 ng of $(6:7)_a$; 55 ng of (6:8); 79 ng of (6:12); 39 ng of $(8)_a$; 63 ng of $(8:12)_a$. In each case, parallel reactions were performed in the presence and absence of the *N*-glycosylation acceptor tripeptide (BzNLT), as described in [18]. Microsomal membranes were purified by ultracentrifugation of translation mixtures (3 μl) through a neutral sucrose cushion (120 μl) and then analysed by SDS-PAGE on either: (a) 12% Laemmli or (b,c) 12% tricine gels, as detailed in Section 2. Translation products with or without signal peptide (±SP) and core *N*-glycosylation (±glyc) have been identified. The apparent molecular weights of the SP cleaved, unglycosylated fragments were (approximately): 29 kDa (6:12); 21 kDa $(8:12)_a$; 16 kDa (6:8); 10 kDa $(6:7)_a$; 7 kDa $(8)_a$; 15 kDa (9:12).

Laemmli gels; Fig. 2a) and fragments (6:8), $(6:7)_a$, $(8)_a$ and (9:12) (12% tricine gels; Fig. 2b,c). Parallel reactions were performed in the presence and absence of the *N*-glycosylation acceptor tripeptide BzNLT (Fig. 2) and in the absence of microsomes (data not shown), to facilitate identification of translation products which had been subjected to signal peptide cleavage (±SP) and core *N*-glycosylation (±glyc). The various polypeptides were *N*-glycosylated between 53 and 70% in the absence of BzNLT, and this decreased to 8–25% in the presence of this competitive inhibitor (Fig. 2). The total amount of polypeptide translated in the presence of BzNLT was ca. 90% of that in its absence. The cleavage of the signal peptide was less straightforward to quantify because the cleaved and uncleaved glycosylated products were not clearly resolved. However, quantifications of the two unglycosylated products (in the presence of BzNLT) indicated that the signal peptide was cleaved in >50% of these polypeptides. The apparent molecular weight of each unglycosylated fragments was consistent with its calculated value (see legend to Fig. 2). When samples of each translation mixture were ultracentrifuged through an alkaline sucrose cushion, each of the fragments were pelleted with the microsomal membranes (data not shown). These results confirm that all six internal fragments, including the one and two span polypeptides, insert into the endoplasmic reticulum membrane as an integral polypeptide.

3.2. *Functional co-expression of three or more complementary band 3 fragments*

Groups of oocytes were co-injected with various cRNAs encoding sets of three or four complementary band 3 fragments, which together directed the contiguous expression of all 911 amino acid residues of band 3. The N- and C-terminus of each fragment was located within a putative loop of band 3, at one of four different sites that have permitted re-assembly of the functional transporter from co-expressed pairs of complementary fragments [11,12]. Each cRNA was present in the injection mixture at a concentration that was the molar equivalent of 15 ng intact band 3 cRNA/oocyte. The cRNA encoding GPA was also co-injected at 4.5 ng/oocyte, since GPA enhances the cell-surface expression of intact band 3 [7] and some complementary pairs of co-expressed band 3 fragments [11,12] in oocytes. After 24 h incubation at 18°C to allow expression, the chloride uptake was measured in groups of 6–15 oocytes over a 1 h period in the presence and absence of the band 3 inhibitor 4,4′-dinitro-2,2′-stilbene disulphonate (DNDS) (data not shown). Under these conditions, we detected a relatively low and slightly significant level of DNDS-sensitive chloride uptake (1–3 nmol Cl^-/oocyte; $P<0.1$ in *t*-tests) into four sets of oocytes co-expressing three contiguous fragments: b3(1:5)+$(6:7)_a$+$(8:14)_a$, b3(1:5)+(6:12)+(13:14)a, b3(1:7)$_a$+$(8)_a$+(9:14) and b3(1:7)$_a$+$(8:12)_a$+(13:14), and into one set of oocytes co-expressing four contiguous fragments b3(1:5)+$(6:7)_a$+$(8:12)_a$+(13:14). In contrast, no DNDS-sensitive chloride uptake was detected in oocytes co-injected with the cRNAs encoding b3(1:8)+(9:12)+(13:14), under these conditions.

In subsequent experiments (Fig. 3), we modified several parameters in an attempt to increase the concentration of polypeptides in the oocytes and hence to assist the assembly and cell surface expression of the functional protein. First, a lower concentration of GPA cRNA (1.5 ng/oocyte) was co-injected, to reduce the level of competition for biosynthesis from GPA. In a previous study, we have shown that submolar quantities of GPA are sufficient to enhance cell-surface expression of intact band 3 significantly [21]. Second, cDNA constructs were prepared encoding three N-terminal membrane domain fragments (Fig. 1) which lack the cytoplasmic domain (aa residues 1–359) of band 3. These new constructs, designated bm(1:5), bm(1:7)$_a$ and bm(1:8), were expressed instead of the intact band 3 fragments b3(1:5), b3(1:7)$_a$ and b3(1:8). This obviated biosynthesis of the cytoplasmic domain which is not required for expression of band 3-mediated anion transport in oocytes [6,7]. Third, the period of time between injection of the cRNA mixtures and the assay of chloride uptake was increased from 24 h to 48 h to allow more time for biosynthesis, assembly and translocation to the plasma

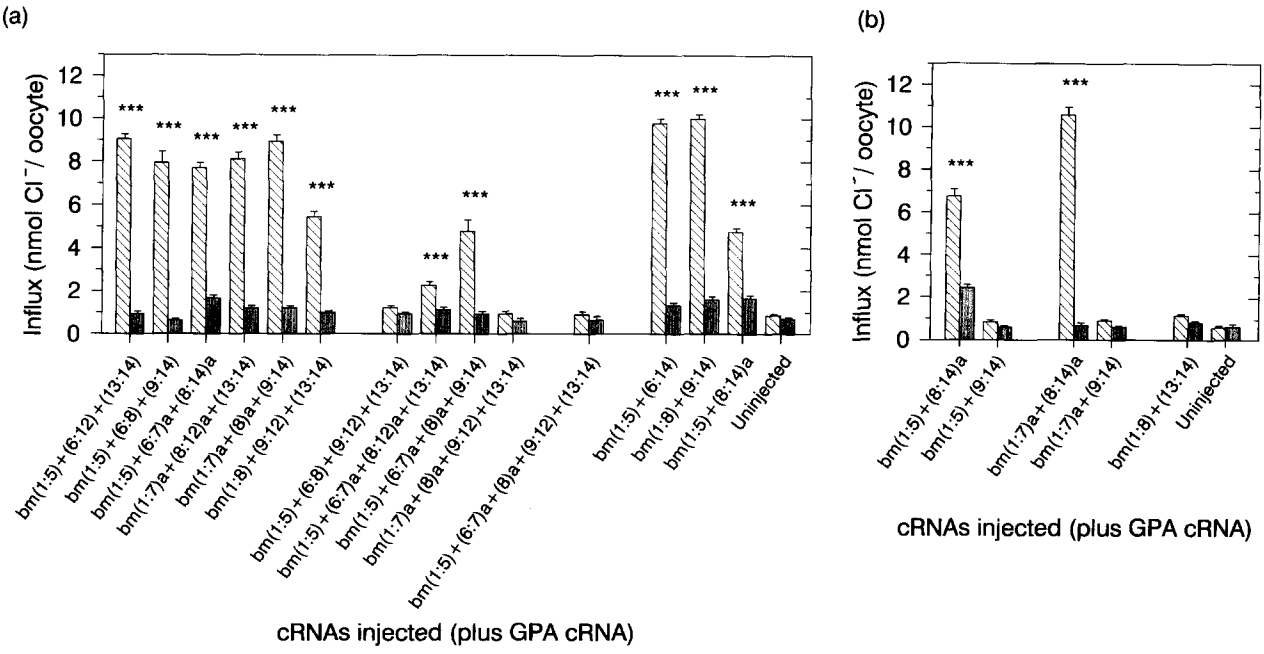

Fig. 3. Chloride influx into oocytes expressing various combinations of band 3 fragments. Oocytes were co-injected with the molar equivalent of 15 ng intact band 3 cRNA of each of the band 3 fragment cRNAs indicated, together with 1.5 ng of GPA cRNA. The amount of each cRNA injected was (per oocyte): 6.0 ng of bm(1:5); 7.9 ng of bm(1:7)$_a$; 9.1 ng of bm(1:8); 5.1 ng of (6:7)$_a$; 6.3 ng of (6:8); 9.1 ng of (6:12); 4.4 ng of (8)$_a$; 7.2 ng of (8:12)$_a$; 5.4 ng of (9:12); 11.0 ng of (6:14); 9.1 ng of (8:14)$_a$; 7.4 ng of (9:14); and 2.3 ng of (13:14). Chloride influx (over 60 min) was measured 48 h after injection using groups of 18–25 oocytes, either in Barth's saline (crosshatched) or in Barth's saline containing 2 mM DNDS (solid fill). The DNDS-sensitive chloride influx may be derived from the difference between the mean values in the presence and absence of DNDS. Key: the bar indicates the standard error of the chloride influx in each case. Samples marked with *** show highly significant differences ($P < 0.001$ in t-tests) when comparing (i) the chloride influx in DNDS-treated and untreated oocytes, and (ii) the DNDS-sensitive influx into parallel sets of injected and uninjected oocytes.

membrane. To distinguish between specific and non-specific DNDS-sensitive chloride influx, the mean and standard error of the DNDS-sensitive chloride uptake was calculated for each sample and these values were compared in t-tests with those of a control set of uninjected oocytes.

Fig. 3a shows the chloride influx data obtained under these conditions. We co-expressed each of the possible combinations of three, four and five contiguous band 3 fragments, divided in these four loops. All six combinations of three fragments (Fig. 3a, left side) showed a very high level of DNDS-sensitive chloride influx into oocytes ($P \ll 0.001$ in t-tests). Five of these sets of fragments, bm(1:5)+(6:12)+(13:14), bm(1:5)+(6:8)+(9:14), bm(1:5)+(6:7)$_a$+(8:14), bm(1:7)$_a$+(8:12)$_a$+(13:14) and bm(1:7)$_a$+(8)$_a$+(9:14), generated a chloride uptake level that was similar to that observed with intact band 3 [7,21] and with the complementary pairs of fragments bm(1:5)+(6:14) and bm(1:8)+(9:14) (Fig. 3a, right side) under these experimental conditions.

Two of the four fragment combinations, bm(1:5)+(6:7)$_a$+(8)$_a$+(9:14) and bm(1:5)+(6:7)$_a$+(8:12)$_a$+(13:14), showed a significantly greater ($P < 0.001$) level of DNDS-sensitive chloride uptake than uninjected oocytes (Fig. 3a, centre) although the levels were lower than each of the three-fragment combinations, described above. This difference may reflect the greater number of discrete polypeptides that must interact to complete the assembly of the protein. The DNDS-sensitive uptake of bm(1:5)+(6:8)+(9:12)+(13:14), bm(1:7)$_a$+(8)$_a$+(9:12)+(13:14) and the five fragment combination bm(1:5)+(6:7)$_a$+(8)$_a$+(9:12)+(13:14) were each very low and not significantly different from that of the uninjected cells. All three of these combinations contained fragment (9:12); although we have not examined the expression of this fragment individually in oocytes, the C-terminal fragment (9:14) was uniquely found to be degraded by oocytes unless b3(1:8) was co-expressed [17]. Since bm(1:8)+(9:12)+(13:14) gave the lowest chloride uptake of the three-fragment combinations, it is possible that (9:12) is particularly unstable in the absence of other transmembrane spans of band 3.

3.3. *Functional co-expression of a pair of non-complementary band 3 fragments*

In previous studies, we have shown that each of the N- or C-terminal band 3 fragments cannot mediate DNDS-sensitive chloride uptake when expressed individually [11,12]. In Fig. 3, we show the results of co-expressing four pairs of non-complementary fragments in the absence of the complementary internal fragment. Surprisingly, one of these combinations bm(1:5)+(8:14)$_a$ (which completely lacks fragment (6:7)$_a$, i.e. amino acid residues 554–639) generated a moderate but nevertheless highly significant ($P < 0.001$ in t-test) level of DNDS-sensitive chloride uptake (Fig. 3a right block and Fig. 3b). This unexpected result indicates that putative transmembrane spans six and seven and a portion of the flanking loops are not essential for band 3-mediated transport of chloride. However, co-expression of the three fragment combination bm(1:5)+(6:7)$_a$+(8:14)$_a$ generated significantly higher DNDS-sensitive chloride influx ($P < 0.001$ in t-test) than bm(1:5)+(8:14)$_a$, indicating that the wild type transporter has a higher level of functionality than the deletion assemblage. Transmembrane spans six and seven and the intercon-

necting loop contain the sites of point mutations (R589H, R589C and S613F) which are responsible for certain forms of the pathological condition distal renal tubular acidosis (dRTA) [22]. Although the heterozygous presence of both these mutants causes modified red cell sulphate transport, expression studies in oocytes have shown that the recombinant mutants retain substantial levels of chloride transport activity. In consequence, it was suggested that the intervening loop, which includes a cluster of basic residues, is involved in divalent but not monovalent anion transport [22]. The amino acid sequences of transmembrane spans six and seven are conserved across the AE family of proteins, indicating a critical role for these spans in either the structure or function of the protein. We cannot at this stage exclude the possibility that co-expression of bm(1:5)+$(8:14)_a$ generates a radically different but misassembled anion conducting structure, such as a channel.

3.4. Conclusion

This paper supports the 'two-stage' model [16] by demonstrating that each of the six internal fragments of band 3 independently integrates into microsomal membranes and is functionally co-expressed in oocytes with its complementary N- and C-terminal partners. These results suggest that in most cases assembly and translocation to the plasma membrane are concentration-dependent processes and that the level of functional expression is constrained by the biosynthetic capacity of the oocytes and the stability of the transiently unassociated fragments rather than misfolding. There are few reports of functional co-expression from more than two membrane protein fragments. The ability to dissect band 3 so extensively without disrupting its global structure will greatly facilitate future studies of the structure-function relationship.

Acknowledgements: L.W. was the recipient of a Bristol University Scholarship and Overseas Research Studentship Award. This work was supported by a grant from the Wellcome Trust.

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