Structural model for the organization of the transmembrane spans of the human red-cell anion exchanger (band 3; AE1)

Jonathan D. GROVES and Michael J. A. TANNER¹

Department of Biochemistry, School of Medical Sciences, University of Bristol, Bristol BS8 1TD, U.K.

We have examined the functional co-assembly of non-complementary pairs of N- and C-terminal polypeptide fragments of the anion transport domain (b3mem) of human red-cell band 3. cDNA clones encoding non-contiguous pairs of fragments with one transmembrane (TM) region omitted, or overlapping pairs of fragments with between one and ten TM regions duplicated, were co-expressed in *Xenopus* oocytes and a cell-free translation system. Stilbene disulphonate-sensitive chloride uptake assays in oocytes revealed that the omission of any single TM region of b3mem except spans 6 and 7 caused a complete loss of functional expression. In contrast, co-expressed pairs of fragments overlapping a single TM region 5, 6, 7, 8, 9–10 or 11–12 retained a high level of functionality, whereas fragments overlapping the clusters of TM regions 2–5, 4–5, 5–8 and 8–10 also mediated some stilbene disulphonate-sensitive uptake. The co-assembly of N- or C-terminal fragments with intact band 3, b3mem or other fragments was examined by co-immunoprecipitation in non-

denaturing detergent solutions by using monoclonal antibodies against the termini of b3mem. All the fragments, except for TM spans 13–14, co-immunoprecipitated with b3mem. The medium-sized N-terminal fragments comprising spans 1–6, 1–7 or 1–8 co-immunoprecipitated particularly strongly with the C-terminal fragments containing spans 8–14 or 9–14. The fragments comprising spans 1–4 or 1–12 co-immunoprecipitated less extensively than the other N-terminal fragments with either b3mem or C-terminal fragments. There is sufficient flexibility in the structure of b3mem to allow the inclusion of at least one duplicated TM span without a loss of function. We propose a working model for the organization of TM spans of dimeric band 3 based on current evidence.

Key words: erythrocyte, fragments, membrane, membrane protein structure, transport.

INTRODUCTION

The human erythrocyte anion exchanger (band 3, AE1) is a 95 kDa integral membrane protein that comprises two structurally and functionally distinct domains (reviewed in [1–4]). The N-terminal domain (residues 1–359) is located in the cytosol and is involved in functions unrelated to anion transport [5]. The C-terminal membrane domain (b3mem; residues 360–911) is both necessary and sufficient for the anion transport function either after proteolytic removal of the cytoplasmic domain [6,7] or when translated from a cDNA clone encoding the isolated membrane domain [8,9]. Current models for the transmembrane (TM) organization of this membrane protein are based on hydropathy analysis and experiments with vectorial labelling, proteolysis, antibody binding and N-glycosylation insertion mutagenesis (reviewed in [3,4,10]): these indicate that the membrane domain of band 3 might traverse the membrane up to 14 times, although the topology of the region containing the putative TM spans 9–12 remains unclear [11–14]. Two-dimensional crystallography has yielded a low-resolution (20 Å) structural model of the dimeric membrane domain [15] (reviewed in [4]) that indicates that the cross-sectional area of the molecule is sufficient to permit the packing of up to fourteen α -helical TM spans.

Previously [16,17,23], we have co-expressed pairs of complementary N- and C-terminal fragments of b3mem in which all the residues of the membrane domain were uniquely represented. We showed that pairs of fragments divided in any of six different

putative loop locations could reassemble into the 4,4'-dinitro-2,2'-stilbene disulphonate (DNDS)-sensitive anion exchanger in the plasma membrane of *Xenopus* oocytes. Similarly, we demonstrated that six three-fragment combinations, two four-fragment combinations and, unexpectedly, a non-complementary pair of fragments lacking putative TM spans 6 and 7 generated DNDS-sensitive chloride uptake [18]. By using non-denaturing detergents that are known to maintain the native structure of b3mem [19] followed by co-immunoprecipitation, we showed that at least five of these complementary pairs of fragments were able to co-assemble specifically in oocyte and microsomal membranes [20].

The amino acid sequences of a large number of multispanning membrane proteins that act as transporters, channels or receptors have now been determined. The results of hydropathy analysis and topological studies can lead to a two-dimensional folding model but this yields relatively little insight into the structural features responsible for the particular biological activity of the molecule. To gain an understanding of the structure–function relationship in these multispanning proteins requires information on the relative organization of the membrane-spanning segments; this would greatly facilitate the construction of a three-dimensional structural model by using molecular modelling methods.

In the present study we co-expressed a variety of non-complementary pairs of fragments that did not contain the entire sequence of the membrane domain, and overlapping pairs of fragments in which between one and ten TM spans were

Abbreviations used: DNDS, 4,4'-dinitro-2,2'-stilbene disulphonate; EC, extracellular; GPA, glycophorin A; IP, immunoprecipitation; TM, transmembrane.

¹ To whom correspondence should be addressed (e-mail m.tanner@bristol.ac.uk).

represented twice. We have examined the ability of co-expressed pairs of non-complementary fragments to generate DNDS-sensitive chloride uptake in *Xenopus* oocytes and to co-assemble in microsomal membranes by using co-immunoprecipitation in non-denaturing detergent solutions. We suggest a partial structural model for the organization of the TM spans in the band 3 dimer based on the combined study of the functional reassembly and co-immunoprecipitation of non-complementary fragments. This should provide a useful working model for designing further experiments to elucidate the detailed packing arrangement of the membrane-spanning segments of band 3.

MATERIALS AND METHODS

Materials

The following is a list of products and (in parentheses) their suppliers: T7 mMessage mMachine[™] *in vitro* transcription kits

Figure 1 Topology of the membrane domain of band 3 showing truncation positions

Diagrammatic representation of the TM topology of the membrane domain of band 3 (b3mem) according to the traditional 14-span model [2,29,30]. The arrows indicate the approximate positions at which pairs of N- and C-terminal fragments were truncated in the putative intracellular (IC) or EC loops. The number of each putative TM span is indicated at its left. The region containing spans 9–12 is of unconfirmed topology. Abbreviations: cyt, cytoplasm; mem, membrane.

(Ambion); rabbit reticulocyte lysate and canine pancreatic microsomes (Promega); L-[³⁵S]methionine and Na³⁶Cl solution (Amersham); QIAprep plasmid mini-prep kit (Qiagen); restriction enzymes, Expand[™] proof-reading PCR system and T4 DNA ligase (Boehringer Mannheim); oligonucleotide synthesis by Professor L. Hall (University of Bristol, Bristol, U.K.); other chemicals (Sigma, BDH or Fluka).

Band 3 and membrane-domain fragment cDNA constructs

The cDNA clones encoding intact band 3 (pBSXG1.b3), the membrane domain of band 3 (pBSXG1.b3mem; residues 361–911) glycophorin A (pBSXG.GPA), and the construction of cDNA species encoding N- and C-terminal fragments of band 3 and the band 3 membrane domain (b3mem) has been detailed previously [9,16,18,23]. These constructs contain the respective protein-coding region flanked by the 5' and 3' non-coding region of *Xenopus* β -globin. Putative TM spans are described in terms of the 14-span model (Figure 1). Fragments were truncated in each of the loops that extend from the membrane by more than six residues, i.e. not the loops between TM spans 2–3, 9–10, 11–12 and 13–14 (Table 1). In brief, the N-terminal fragments of b3mem were prepared by the incorporation of a translation termination codon in putative loop positions in the coding sequence and the deletion of the region of the cDNA on the 3' side of this termination codon; these fragments were designated bm(1:12), bm(1:10), bm(1:8), and so on. N-terminal fragments of intact band 3 (which include the cytoplasmic domain) were prepared similarly; these were designated b3(1:12), b3(1:8), and so on. The C-terminal fragments were prepared by insertion of an N-terminal methionine codon and deletion of the region of the band 3 cDNA immediately upstream of this protein initiator codon; these fragments were designated (2:14), (4:14), (5:14), and so on. In some experiments, a version of the C-terminal fragment cDNA species (6:14) and (*8:14) was used that encoded the 19-residue cleavable signal sequence of glycophorin A (GPA) preceding the N-terminal residue of the fragment (constructs described in [17]). These fragments behave in a fashion functionally identical with those of the normal fragments [23]; the occasions on which they have been used are indicated in the Figure legends with the designations gp(6:14) and gp(*8:14).

Table 1 N- and C-terminal fragments of b3mem

The nomenclature and amino acid composition of each of the truncated fragments used in this paper are shown. The putative intracellular (IC) or EC loop location of each truncation site is shown in Figure 1. The site between Glu-755 and Val-756 is located in the spans 9–12 region, which has unconfirmed topology. An asterisk indicates the inclusion of the sequence Val-640 to Arg-646 (which contains the endogenous N-glycosylation site at Asn-642) in fragments truncated within EC loop 4. Intact band 3 and b3mem comprise residues M¹–V⁹¹¹ and M G³⁶¹–V⁹¹¹ respectively. N-terminal fragments b3(1:6), b3(1:8) and b3(1:12) initiate at residue M¹ rather than M G³⁶¹.

Truncated in loop	N-terminal fragments		C-terminal fragments	
	Fragment name	Amino acid residues	Fragment name	Amino acid residues
EC1	bm(1)	M G ³⁶¹ –R ⁴³²	(2:14)	MG N ⁴³³ –V ⁹¹¹
EC2	bm(1:3)	M G ³⁶¹ –T ⁴⁸¹	(4:14)	MG N ⁴⁸² –V ⁹¹¹
IC2	bm(1:4)	M G ³⁶¹ –S ⁵¹⁷	(5:14)	MG R ⁵¹⁸ –V ⁹¹¹
EC3	bm(1:5)	M G ³⁶¹ –Y ⁵⁵³	(6:14)	MG N ⁵⁵⁴ –V ⁹¹¹
IC3	bm(1:6)	M G ³⁶¹ –P ⁵⁹⁸	(7:14)	M G ⁵⁹⁹ –V ⁹¹¹
EC4	bm(1:7)	M G ³⁶¹ –K ⁶³⁹	(*8:14)	M V ⁶⁴⁰ –V ⁹¹¹
EC4	bm(1:7*)	M G ³⁶¹ –R ⁶⁴⁶	(8:14)	MG W ⁶⁴⁷ –V ⁹¹¹
IC4	bm(1:8)	M G ³⁶¹ –K ⁶⁹⁵	(9:14)	M ⁶⁹⁶ –V ⁹¹¹
IC5	bm(1:10)	M G ³⁶¹ –E ⁷⁵⁵	(11:14)	M V ⁷⁵⁶ –V ⁹¹¹
IC6	bm(1:12)	M G ³⁶¹ –Y ⁸²⁴	(13:14)	M V ⁸²⁵ –V ⁹¹¹

Transcription *in vitro* and expression in *Xenopus* oocytes

The T7 mMessage mMachine and T7 Megascript kits were used to prepare m⁷GpppG-capped cRNA species by the transcription *in vitro* of HindIII-linearized plasmid DNA. Procedures for the isolation of oocytes, microinjection of capped cRNA species and chloride uptake assays in the presence and absence of 2 mM DNDS were as detailed previously [9,18].

Cell-free translation and co-immunoprecipitation

Cell-free translation was performed in the rabbit reticulocyte lysate system with canine pancreatic microsomes and L-[³⁵S]-methionine for 60 min at 30 °C with the use of capped cRNA species. Microsomal membranes were purified from the cell-free translation by centrifugation through 130 µl of a neutral cushion [350 mM sucrose/150 mM KCl/5 mM dithiothreitol/3 mM MgCl₂/50 mM Hepes/K⁺ (pH 7.5)] or alkaline cushion [250 mM sucrose/100 mM sodium carbonate (pH 11.5)] at 70 000 rev./min (g_{av} = 189 000) for 20 min at 4 °C in a TLA100 ultracentrifuge (Beckman Instruments). Membrane pellets were solubilized in 400 µl of immunoprecipitation buffer (IP buffer) in the presence of either 2% or 0.5% (w/v) Triton X-100 (detailed in the various experiments) and immunoprecipitated with the monoclonal antibodies BRIC170 (directed against the N-terminus of b3mem) or BRIC155 (directed against the C-terminus of b3mem) [21], as described previously [20]. Immunoprecipitated proteins were washed six times in 1 ml of wash buffer containing 0.1% (w/v) Triton X-100. Samples of translated or immunoprecipitated proteins were separated by SDS/PAGE [22]. The relative yields of co-translated and co-immunoprecipitated proteins were determined by scanning densitometry of fluorographs using Image-master 1d scanner software (Pharmacia-LKB), as detailed previously [20]. Results were normalized to take account of the different numbers of methionine residues in each fragment (see Tables 2 and 3, column 1).

RESULTS

Functional co-expression of non-complementary pairs of band 3 fragments in oocytes

We co-injected *Xenopus* oocytes with non-complementary pairs of N- and C-terminal fragments encoding (1) pairs of non-contiguous fragments in which one TM span was not represented in either the N- or C-terminal fragment, and (2) pairs of overlapping fragments in which between one and ten of the TM spans were present in both of the fragments. Human red-cell GPA cRNA was also co-injected routinely because this single-spanning integral protein has been shown to facilitate the oocyte cell-surface expression of band 3 and some complementary pairs of fragments [9,16,17]. After 48 h of expression, mean chloride uptake was measured in the presence and the absence of the band 3 inhibitor DNDS (Figure 2). In *t* tests, probability values of $P < 0.001$ and in some cases $P < 0.01$ (marked *** and ** respectively in Figure 2) indicated significant levels of DNDS-sensitive chloride uptake, whereas probability values of $P < 0.1$ (marked * in Figure 2) probably reflected only small unavoidable systematic differences in the handling of the parallel groups of oocytes at the levels of replication used.

Non-contiguous fragments

Oocytes were co-injected with cRNA species encoding pairs of fragments in which the N-terminal fragment was truncated one loop before the initiation point of its C-terminal partner, i.e. a

single TM region was absent. Co-expression of the fragments bm(1:7)+(9:14), bm(1:8)+(11:14) (Figure 2a) or bm(1:10)+(13:14) (Figure 2b) yielded little or no DNDS-sensitive chloride uptake into oocytes, as observed when these fragments were expressed individually [16]. This indicates that each of the regions containing spans 8, 9–10 and 11–12 was necessary for the generation of a functional molecule. In contrast, co-expression of fragments bm(1:5)+(7:14), lacking the span 6 region, and bm(1:6)+(*8:14), lacking the span 7 region, mediated a significant amount of DNDS-sensitive chloride uptake ($P < 0.001$ in *t* tests) (Figure 2a), although at a lower level than was observed with the contiguous pairs of fragments bm(1:5)+(6:14), bm(1:6)+(7:14) and bm(1:8)+(9:14). These results agreed with our previous observation that bm(1:5)+(*8:14), which lacks the entire region including spans 6 and 7, can support a modest level of DNDS-sensitive chloride uptake [18]. Co-expression of fragments bm(1:7)+(8:14), lacking residues 640–646, mediated a high level of DNDS-sensitive chloride uptake. This result showed that the central portion of the fourth extracellular (EC) loop, which included the endogenous N-glycosylation site at Asn-642, was not essential for function.

Overlapping fragments

Oocytes were injected with cRNA species encoding various pairs of overlapping fragments: each N-terminal fragment was truncated at least one loop downstream of the initiation point of its C-terminal partner, i.e. one or more TM regions were duplicated. Co-expression of the fragments bm(1:5)+(5:14), bm(1:6)+(6:14), bm(1:7)+(7:14) (Figure 2a), bm(1:8)+(*8:14) (Figures 2a and 2c), bm(1:10)+(9:14) or bm(1:12)+(11:14) (Figure 2b), each of which overlapped by a single TM region, mediated a high level of DNDS-sensitive chloride uptake ($P < 0.001$ in *t*-tests), similar to that observed with the corresponding contiguous pairs of fragments. This showed that the duplication of any single TM region between spans 5 and 12 inclusive did not affect the ability of these fragments to co-assemble into the functional anion transporter. In contrast, fragments bm(1:3)+(2:14), which overlapped in the span 2–3 region, did not show any DNDS-sensitive chloride uptake, similarly to bm(1)+(2:14) and bm(1:3)+(4:14) [17,23].

A significant amount of DNDS-sensitive chloride uptake was measured after co-expression of the fragments bm(1:5)+(2:14), bm(1:5)+(4:14), bm(1:10)+(*8:14) or bm(1:8)+(5:14) ($P < 0.001$ in *t* tests) as well as bm(1:12)+(5:14) ($P < 0.01$ in *t* tests) (Figures 2b and 2c), which overlapped by two or more TM spans. However, a lower level of chloride uptake was observed in all these samples than with pairs of fragments that overlapped only by a single TM region. Co-expression of all the other combinations of fragments resulted in little or no DNDS-sensitive chloride uptake (either not significant or $P < 0.1$ in *t* tests) under the conditions of the influx assay (Figures 2b and 2c). Although overlapping of the TM span 2–5, 4–5, 5–8 or *8–10 regions and perhaps the entire span 5–12 region did not prevent functional co-assembly, in most cases the presence of clusters of several duplicated spans resulted in a loss of most of the anion transport function.

Co-immunoprecipitation of N-terminal fragments with b3mem or intact band 3

In previous studies we used co-immunoprecipitation in non-denaturing detergent solutions to show that at least five different complementary pairs of band 3 fragments are able to co-assemble in microsomal membranes [20]. Furthermore, we confirmed that

Figure 2 Chloride uptake into oocytes expressing non-complementary or overlapping pairs of B3mem fragments

Oocytes were co-injected with pairs of B3mem fragment cRNA species at equimolar concentrations as indicated below, together with 2.5 ng of GPA cRNA. In **(a)** and **(b)** the amount of each cRNA injected into each oocyte was: 15.0 ng of b3mem, 4.9 ng of bm(1:3), 6.6 ng of bm(1:5), 7.6 ng of bm(1:6), 8.6 ng of bm(1:7), 9.9 ng of bm(1:8), 11.3 ng of bm(1:10), 12.9 ng of bm(1:12), 14.2 ng of (2:14), 13.0 ng of (4:14), 12.2 ng of (5:14), 11.3 ng of (6:14), 10.3 ng of (7:14), 9.3 ng of (*8:14), 8.0 ng of (9:14), 6.6 ng of (11:14) and 4.9 ng of (13:14). In **(c)** the amount

these interactions were specific under the conditions of the assay and were not an artifact of the co-immunoprecipitation procedure [20]. We have now used this approach to investigate the interaction of N-terminal fragments with the intact protein or membrane domain of band 3. In a first experiment (Table 2, Expt. 1), cRNA encoding the N-terminal band 3 fragment b3(1:12) or b3(1:8) was co-translated with b3mem cRNA in the rabbit reticulocyte lysate cell-free translation system with canine pancreatic microsomes. Translations were performed with cRNA species at two different relative molar proportions: either b3(1:12) or b3(1:8) at a molar equivalent of 180 ng of band 3 cRNA was co-translated with 30 ng of b3mem cRNA in a 12.5 μ l translation mixture, i.e. at a 4-fold molar excess of fragment over b3mem; alternatively, the fragment cRNA species at a molar equivalent of 45 ng of band 3 cRNA was co-translated with 120 ng of b3mem cRNA, i.e. at a 4-fold molar excess of b3mem over fragment (Table 2, column 2). Microsomal membranes were purified from the translation mixture by ultracentrifugation through a neutral sucrose cushion and then solubilized in IP buffer containing 2% (w/v) Triton X-100. Samples were divided and immunoprecipitated, either with the monoclonal antibody BRIC155 (directed against an epitope at the C-terminus of b3mem that was absent from the N-terminal fragments) or BRIC170 (against an epitope at the N-terminus of b3mem that was also present in N-terminal fragments) and Protein A-agarose. After SDS/PAGE and fluorography, the relative yields of both the fragment and b3mem polypeptides were quantified by scanning densitometry. Samples containing excess b3(1:12) or b3(1:8) cRNA yielded an approx. 2-fold excess of fragment polypeptides in translation mixtures (Table 2, column 3) and BRIC170 immunoprecipitates (Table 2, column 4); samples containing excess b3mem cRNA produced a 5–9-fold excess of b3mem polypeptide (Table 2, columns 3 and 4). This indicated that the complete b3mem polypeptide was produced at twice the rate of band 3, b3(1:12) and b3(1:8) and suggested that at least a 4-fold molar excess of N-terminal band 3 fragment cRNA over b3mem cRNA was needed for co-immunoprecipitation studies under these conditions. Control co-translations of intact band 3 with b3mem cRNA species showed that the BRIC170 and BRIC155 immunoprecipitated intact band 3 and b3mem similarly and confirmed that the action of the antibodies was not affected by the presence or absence of the cytoplasmic domain. The amounts of b3(1:12) or b3(1:8) detected in the BRIC155 immunoprecipitations (Table 2, column 5) were considerably lower than those observed in the corresponding BRIC170 immunoprecipitations (Table 2, column 4), because the BRIC155 samples contained only co-immunoprecipitated fragment polypeptides that were in association with b3mem. The amount of co-immunoprecipitated fragment was higher in the translations containing the excess fragment cRNA. Significantly, at both the cRNA ratios tested, the BRIC155 co-immunoprecipitation of b3(1:8) with b3mem was approx. 3–4-fold that of b3(1:12).

In a further investigation, b3(1:12), b3(1:8) or b3(1:6) cRNA at a molar equivalent of 180 ng of band 3 cRNA was co-translated with 30 ng of b3mem cRNA in a 12.5 μ l translation mixture, i.e. at a 4-fold molar excess over b3mem cRNA, then immunoprecipitated with BRIC170 or BRIC155 (results not shown). As above, the fragment b3(1:12) was co-immuno-

precipitated with b3mem at least 3-fold less than b3(1:8) or b3(1:6) despite the immunoprecipitation of similar quantities of b3mem from the various reactions. Two further results were obtained in this experiment. First, a parallel set of samples containing 30 ng of GPA cRNA per 12.5 μ l mixture, in addition to the N-terminal fragment and b3mem cRNA species, were co-translated. These assays gave results that were essentially identical with those for the samples without GPA and indicated that GPA had no detectable effect on the association of these proteins in the cell-free system. Secondly, we confirmed that the N-terminal fragments could not be detected in BRIC155 immunoprecipitates of samples containing fragment cRNA species in the absence of b3mem cRNA.

A further co-translation experiment was performed (Table 2, Expt. 2) in which cRNA species encoding the N-terminal fragments bm(1:12) and bm(1:8) (lacking the residues of the cytoplasmic domain) were co-translated with the cRNA encoding the intact band 3 polypeptide. The ratio of polypeptides in the translation mixtures (Table 2, column 3) and BRIC170 immunoprecipitates (Table 2, column 4) reflected the 4–5-fold excess N-terminal fragment and band 3 cRNA species added to each translation mix (Table 2, column 2). The BRIC155 co-immunoprecipitations showed approx. 2-fold lower co-immunoprecipitation of bm(1:12) than bm(1:8) with intact band 3, as observed above (Expt. 1).

The cytoplasmic domain of band 3 might form homooligomers of higher order than the dimer by extramembraneous interactions [5]. To validate the co-immunoprecipitation results above, we performed a series of co-translations with N-terminal b3mem fragments and b3mem (Figures 3a and 3b; Table 2, Expt. 3), which both lacked the cytoplasmic domain of band 3. The bm(1:12), bm(1:10), bm(1:8), bm(1:7) or bm(1:6) cRNA species were co-translated with b3mem cRNA at a 4-fold molar excess of the fragment cRNA (Table 2, column 2) with L-[³⁵S]methionine to label the protein (Figure 3a, lanes 1–3 and 7–9). Because fragments bm(1:5) and bm(1:4) contained only two methionine residues, co-translations of these fragments with b3mem were performed with L-[³H]leucine, a more highly represented residue in these fragments (Figure 3a, lanes 13 and 14). To maximize the opportunity for interactions to occur between polypeptides within the microsomes, all the cRNA concentrations were 4-fold those used in the previous experiments, as detailed in the legend to Figure 3. A parallel series of control translations was performed in the presence of fragment cRNA only (Figure 3a, lanes 4–6, 10–12 and 15–16). Results showed that the fragment polypeptides were translated at an approx. 3–7-fold molar excess over the b3mem polypeptide (Table 2, column 3). After solubilization in IP buffer containing 0.5% (w/v) Triton X-100, co-immunoprecipitations were performed with BRIC155 (Figure 3b) and quantified (Table 2, column 4). Co-immunoprecipitated N-terminal fragments were detected in all the BRIC155 co-immunoprecipitates of samples containing both fragment and b3mem; in contrast, N-terminal fragment polypeptides were not detected in BRIC155 immunoprecipitations of samples in the absence of b3mem. The proportion of expressed b3mem that was associated with bm(1:12) or with bm(1:4) was lower than that observed with the N-terminal fragments bm(1:10), bm(1:8), bm(1:7), bm(1:6) or bm(1:5).

of each cRNA injected was half that in (a) and (b). Chloride influx (over 60 min) was measured 24 h after injection with groups of 19–25 oocytes (a), 14–22 oocytes (b) or 17–23 oocytes (c), either in Barths saline (hatched columns) or in Barths saline containing 2 mM DNDS [shaded columns]. The DNDS-sensitive chloride influx is derived from the difference between the mean values in the presence and the absence of DNDS. The bar indicates the S.E.M. of the chloride influx in each case. ***Highly significant differences ($P < 0.001$ in t tests) between (1) the chloride influx in DNDS-treated and untreated oocytes and (2) the DNDS-sensitive influx into parallel sets of injected and uninjected oocytes.

Table 2 Expression and association of N- or C-terminal fragments with intact band 3 in microsomal membranes

N-terminal (N-frag) (Expts. 1–4) or C-terminal (C-frag) (Expts. 4 and 5) band 3 fragments were co-translated with intact band 3 (Expt. 2) or b3mem (Expts. 1 and 3–5) in the rabbit reticulocyte lysate cell-free system. The molar ratio of cRNA species added (column 2) is the amount of N-frag or C-frag cRNA divided by the amount of band 3 or b3mem cRNA in the co-translation mixture. The molar ratio of polypeptides translated (column 3) is the molar amount of N-frag or C-frag divided by the molar amount of band 3 or b3mem in the translation mixture (Expts. 1–3) or purified microsomal membrane pellet (Expts. 4–5). After solubilization with IP buffer containing Triton X-100, radiolabelled proteins were separated by SDS/PAGE and fluorographs were quantified by scanning densitometry after allowing for the differing amounts of radioactivity in each fragment (column 1). The molar association ratio of the fragments with band 3 or b3mem was determined by co-immunoprecipitation with BRIC170 (against the N-terminus; column 4) or BRIC155 (against the C-terminus; column 5). Fragments (6:14) and (*8:14) were provided by constructs gp(6:14) and gp(*8:14). Abbreviation: n.d., not determined.

Experiment 1

Constructs		No. Met or Leu residues		Molar ratio cRNAs added, N-frag/b3mem	Molar ratio, polypeptides translated, N-frag/b3mem	Molar association ratio, N-frag/b3mem	
N-frag	b3mem	N-frag	b3mem			BRIC170	BRIC155
Band 3	b3mem	24 Met	17 Met	4.0	2.53	1.87	1.72
b3(1:12)	b3mem	22 Met	17 Met	4.0	2.94	1.74	0.06
b3(1:8)	b3mem	16 Met	17 Met	4.0	2.70	2.74	0.22
Band 3	b3mem	24 Met	17 Met	0.25	0.12	0.11	0.17
b3(1:12)	b3mem	22 Met	17 Met	0.25	0.22	0.20	0.013
b3(1:8)	b3mem	16 Met	17 Met	0.25	0.21	0.17	0.037

Experiment 2

Constructs		No. Met or Leu residues		Molar ratio cRNAs added, N-frag/band 3	Molar ratio, polypeptides translated, N-frag/band 3	Molar association ratio, N-frag/band 3	
N-frag	b3mem	N-frag	band 3			BRIC170	BRIC155
b3mem	band 3	17 Met	24 Met	4.2	4.8	5.9	n.d.
bm(1:12)	band 3	22 Met	24 Met	4.8	2.4	3.7	0.15
bm(1:8)	band 3	16 Met	24 Met	4.8	4.3	5.6	0.31

Experiment 3

Constructs		No. Met or Leu residues		Molar ratio cRNAs added, N-frag/b3mem	Molar ratio, polypeptides translated, N-frag/b3mem	Molar association ratio, N-frag/b3mem	
N-frag	b3mem	N-frag	b3mem			BRIC170	BRIC155
bm(1:12)	b3mem	15 Met	17 Met	4.0	4.9	n.d.	0.29
bm(1:10)	b3mem	13 Met	17 Met	4.0	3.4	n.d.	0.49
bm(1:8)	b3mem	9 Met	17 Met	4.0	5.7	n.d.	0.53
bm(1:7)	b3mem	7 Met	17 Met	4.0	4.4	n.d.	0.56
bm(1:6)	b3mem	6 Met	17 Met	4.0	6.4	n.d.	0.72
bm(1:5)	b3mem	29 Leu	82 Leu	4.0	7.6	n.d.	0.99
bm(1:4)	b3mem	25 Leu	82 Leu	5.3	4.1	n.d.	0.42

Experiment 4

Constructs		No. Met or Leu residues		Molar ratio cRNAs added, C-frag/b3mem	Molar ratio, polypeptides translated, C-frag/b3mem	Molar association ratio, C-frag/b3mem	
C-frag	b3mem	C-frag	b3mem			BRIC170	BRIC155
(5:14)	b3mem	16 Met	17 Met	2.0	4.7	2.1	n.d.
(6:14)	b3mem	15 Met	17 Met	2.0	4.6	2.4	n.d.
(7:14)	b3mem	12 Met	17 Met	2.0	8.7	2.2	n.d.
(*8:14)	b3mem	10 Met	17 Met	2.0	9.0	2.4	n.d.
(9:14)	b3mem	8 Met	17 Met	2.0	7.6	1.7	n.d.
(11:14)	b3mem	5 Met	17 Met	2.5	12.1	0.97	n.d.
(13:14)	b3mem	3 Met	17 Met	2.5	9.8	0.27	n.d.

Experiment 5

Constructs		No. Met or Leu residues		Molar ratio cRNAs added, C-frag/b3mem	Molar ratio, polypeptides translated, C-frag/b3mem	Molar association ratio, C-frag/b3mem	
C-frag	b3mem	C-frag	b3mem			BRIC170	BRIC155
(9:14)	b3mem	34 Leu	82 Leu	1.0	27	3.9	n.d.
(11:14)	b3mem	27 Leu	82 Leu	1.2	22	1.04	n.d.
(13:14)	b3mem	13 Leu	82 Leu	1.25	9.1	0.21	n.d.

Column no...

1

2

3

4

5

Co-immunoprecipitation of C-terminal fragments with b3mem

We investigated the interaction of C-terminal fragments with the entire membrane domain of band 3 by using the same co-immunoprecipitation approach. cRNA encoding the C-terminal fragment (5:14), (6:14), (7:14), (*8:14), (9:14), (11:14) or (13:14) was co-translated with b3mem cRNA at concentrations detailed in the legend to Figure 3, with L-[³⁵S]methionine to label the translation products (Figure 3c, lanes 1–4 and 9–12; Table 2, Expt. 4). In a second experiment, the fragments (9:14), (11:14) and (13:14) were each co-translated with b3mem by using L-[³H]leucine (gel results not shown; Table 2, Expt. 5). Results showed that in both experiments the C-terminal fragment polypeptides were translated at a more than 4-fold molar excess over b3mem (Table 2, column 3). All the C-terminal fragments except (13:14) were co-immunoprecipitated strongly with b3mem by using BRIC170 (reactive against an N-terminal epitope that was absent from the C-terminal fragments) (Figure 3d, lanes 1–4 and 9–12). Control translations of C-terminal fragments alone (Figure 3c, lanes 5–8 and 13–16) were processed in parallel with the co-translations and no C-terminal fragment polypeptides were detected in the BRIC170 immunoprecipitations of these samples (Figure 3d, lanes 5–8 and 13–16); this confirmed that co-immunoprecipitation of the C-terminal fragments was dependent on the co-translated b3mem. Although C-terminal fragment polypeptides ran as less tightly defined bands on SDS/PAGE than N-terminal fragments owing to their greater susceptibility to limited proteolysis during cell-free translation [20], each of the fragments (5:14), (6:14), (7:14), (*8:14) and (9:14) were co-immunoprecipitated with b3mem at a molar ratio of greater than unity. This phenomenon has been reported previously in co-immunoprecipitations of b3(1) or b3(1:3) with its complementary C-terminal partner by using BRIC155 [20] and might reflect the formation of heterotrimers or higher oligomers between two or more C-terminal fragments and a b3mem molecule. Although the fragment (13:14) was poorly radiolabelled with either methionine or leucine, the level of co-immunoprecipitation of this fragment with b3mem was similar to the background level, as observed previously when (13:14) was co-translated with fragment b3(1:12) [20].

Co-immunoprecipitation of non-complementary N- and C-terminal fragments

The C-terminal fragments (6:14), (*8:14), (9:14) and (11:14) were each co-translated with the N-terminal fragment bm(1:12), bm(1:10), bm(1:8), bm(1:7), bm(1:6), bm(1:5) or bm(1:4) in four parallel series of reactions. The relative molar concentrations of the cRNA species (Table 3, column 2) were selected to give an approx. 4-fold molar excess of the N-terminal over the C-terminal polypeptide fragments in purified microsomal membranes (Table 3, column 3). The expressed fragment molecules were integrated into the membrane, as determined by ultracentrifugation of the microsomes through an alkaline sucrose cushion (results not shown). The relative molar ratios of the translated N- and C-terminal fragment polypeptides were determined in the microsomal membranes (Figures 4a–4d, lanes 1–8; Table 3, column 3), which were co-immunoprecipitated with BRIC155 (Figures 4a–4d, lanes 9–16; Table 3, column 4). The molar association ratio of the co-immunoprecipitated N-terminal fragment (which lacked the BRIC155 epitope) with immunoprecipitated C-terminal fragment (which contains the epitope) was determined; the results are summarized in Figure 4(e). The fragments bm(1:10)+(6:14) and bm(1:8)+(*8:14) ran at the same position on SDS/PAGE gels and therefore the relative yields of the two polypeptides could not be quantified.

Each of the C-terminal fragments (6:14), (*8:14), (9:14) and (11:14) co-immunoprecipitated with N-terminal fragments to some extent. The fragments bm(1:6), bm(1:7) and bm(1:8) co-immunoprecipitated particularly strongly with the C-terminal fragments (*8:14) or (9:14), and to a moderate extent with (6:14). This suggested that these combinations of fragments interacted most strongly, perhaps across an extensive molecular interface involving several TM spans. Alternatively, these C-terminal fragments might have associated with more than one N-terminal fragment molecule, e.g. across interfaces within and between b3mem monomers. In contrast, bm(1:4) and bm(1:12) co-immunoprecipitated at much lower levels; these were broadly independent of the co-translated C-terminal fragment. Fragments bm(1:5) and bm(1:10) co-immunoprecipitated to an intermediate level. The C-terminal fragment (11:14) co-immunoprecipitated with all N-terminal fragments less strongly than the other C-terminal fragments, suggesting that TM spans of importance for interaction with the N-terminal fragments might not have been represented in this polypeptide.

DISCUSSION

In previous papers [16,17,23] we have shown that six different pairs of complementary N- and C-terminal fragments of the integral membrane domain of band 3 (b3mem; residues 360–911 of band 3) are able to co-assemble functionally in *Xenopus* oocytes. In those experiments, all the residues of b3mem were represented once in either the N- or C-terminal fragments. With the use of the same co-expression approach, we demonstrated that six three-fragment combinations, two four-fragment combinations and, unexpectedly, a pair of fragments lacking putative TM spans 6 and 7 also generated DNDS-sensitive chloride uptake [18]. We have shown that contiguous pairs of band 3 fragments divided in the first, second, third or fourth exofacial loops or the fourth cytoplasmic loop co-associate in a specific and concentration-dependent manner [20]. This result was obtained by the solubilization of membranes containing these pairs of fragments in the non-ionic detergent Triton X-100, which does not denature band 3 [19], and then co-immunoprecipitation with the anti-(band 3) monoclonal antibodies BRIC155 (directed against the C-terminus of band 3) or BRIC170 (directed against the N-terminus of b3mem) and Protein A–agarose.

In the present study we used these two techniques in combination with other recent experimental evidence to propose a working structural model for the organization of the TM segments of b3mem. First, we examined the functional co-expression of pairs of non-contiguous N- and C-terminal fragments (in which one TM span was not represented in either fragment) and in pairs of overlapping fragments (in which between one and ten of the TM spans were present in both fragments) in *Xenopus* oocytes. Secondly, we used co-immunoprecipitation to examine the ability of fragments to co-assemble either with the entire membrane domain or with other non-contiguous or overlapping band 3 fragments. We have found that: (1) most combinations of N- and C-terminal fragments contain sufficient structural information to co-assemble and to form hetero-oligomers with intact b3mem and (2) that some combinations of co-expressed non-complementary fragments can mediate DNDS-sensitive chloride uptake.

Plasticity in the structure of functional band 3

Co-expression of the overlapping N- and C-terminal fragments bm(1:5)+(5:14), bm(1:6)+(6:14), bm(1:7)+(7:14), bm(1:8)+(*8:14), bm(1:10)+(9:14) or bm(1:12)+(11:14) resulted in

Figure 3 For legend see facing page.

Table 3 Quantification of the association of non-contiguous and overlapping pairs of N- and C-terminal fragments

N-terminal (N-frag) and C-terminal (C-frag) fragment cRNA species were co-translated in the rabbit reticulocyte lysate cell-free system. The molar ratio of cRNA species added (column 2), the molar ratio of expressed polypeptides in the purified microsomal membrane pellet (column 3) and the molar association ratio of N-frag to C-frag by using BRIC155 co-immunoprecipitation (column 4) was determined as described in the caption to Table 2. Fragments (6:14) and (*8:14) were provided by constructs gp(6:14) and gp(*8:14). Abbreviation: n.d., not determined.

Constructs		Number of Met residues		Molar ratio, cRNA species added, N-frag/C-frag	Molar ratio, polypeptides translated, N-frag/C-frag	Molar association ratio,
N-frag	C-frag	N-frag	C-frag			BRIC155, N-frag/C-frag
bm(1:12)	(6:14)	15	15	12	2.7	0.36
bm(1:10)	(6:14)	13	15	12	n.d.	n.d.
bm(1:8)	(6:14)	9	15	12	4.3	0.52
bm(1:7)	(6:14)	7	15	12	3.8	0.57
bm(1:6)	(6:14)	6	15	12	5.0	0.71
bm(1:5)	(6:14)	2	15	12	4.6	0.58
bm(1:4)	(6:14)	2	15	12	2.2	0.31
bm(1:12)	(*8:14)	15	10	16	4.5	0.46
bm(1:10)	(*8:14)	13	10	16	3.0	0.54
bm(1:8)	(*8:14)	9	10	16	n.d.	n.d.
bm(1:7)	(*8:14)	7	10	16	4.4	1.02
bm(1:6)	(*8:14)	6	10	16	5.7	1.02
bm(1:5)	(*8:14)	2	10	16	4.6	0.70
bm(1:4)	(*8:14)	2	10	16	2.8	0.43
bm(1:12)	(9:14)	15	8	16	2.6	0.44
bm(1:10)	(9:14)	13	8	16	3.4	0.60
bm(1:8)	(9:14)	9	8	16	7.6	0.68
bm(1:7)	(9:14)	7	8	16	7.9	0.93
bm(1:6)	(9:14)	6	8	16	8.0	1.03
bm(1:5)	(9:14)	2	8	16	7.0	0.67
bm(1:4)	(9:14)	2	8	16	4.0	0.45
bm(1:12)	(11:14)	15	5	12	4.8	0.32
bm(1:10)	(11:14)	13	5	12	3.9	0.33
bm(1:8)	(11:14)	9	5	12	6.8	0.30
bm(1:7)	(11:14)	7	5	12	8.4	0.37
bm(1:6)	(11:14)	6	5	12	5.4	0.48
bm(1:5)	(11:14)	2	5	12	6.0	0.50
bm(1:4)	(11:14)	2	5	12	3.5	0.20
Column no....		1		2	3	4

highly significant levels of DNDS-sensitive chloride uptake in oocytes (Figure 2), similar to the complementary pairs of fragments bm(1:5)+(6:14), bm(1:6)+(7:14), bm(1:7)+(*8:14), bm(1:8)+(9:14) and bm(1:10)+(11:14) or intact b3mem. In addition, the overlapping fragments bm(1:5)+(4:14), bm(1:10)+(*8:14) and to a smaller extent bm(1:5)+(2:14) and bm(1:8)+(5:14) also showed significant functional activity. It is

inconceivable that all of TM spans 5–12 are located at the periphery of the b3mem dimer, where the duplicated span might project into the lipid bilayer. Consequently, this indicates that there is sufficient flexibility within the folded structure of band 3 to allow the displacement of one or more TM spans from the centre to the periphery of the co-assembled fragments. The third and fourth exofacial loops and perhaps the fourth and sixth

Figure 3 Co-immunoprecipitation of N- or C-terminal b3mem fragments with intact b3mem

(a, b) N-terminal fragment cRNA species at a molar equivalent of 480 ng/ μ l b3mem cRNA were co-translated with 120 ng/ μ l b3mem cRNA in the rabbit reticulocyte lysate cell-free system (1.2 μ l of cRNA per 15 μ l of mixture) with L-[³⁵S]methionine (lanes 1–12) or L-[³H]leucine (lanes 13–16) to radiolabel the translation products. The amount of each cRNA (in 15 μ l translation mixtures) was: 497 ng of bm(1:12), 434 ng of bm(1:10), 380 ng of bm(1:8), 330 ng of bm(1:7), 293 ng of bm(1:6), 252 ng of bm(1:5) and 250 ng of bm(1:4). (c, d) C-terminal fragment cRNA species at a molar equivalent of 300 ng/ μ l b3mem cRNA for (11:14) or (13:14) and 240 ng/ μ l b3mem cRNA for other C-terminal fragments were co-translated with 120 ng/ μ l b3mem cRNA as above, with L-[³⁵S]methionine as radiolabel. The amount of each cRNA (in 15 μ l translation mixtures) was: 175 ng of (5:14), 172 ng of (6:14), 148 ng of (7:14), 143 ng of (*8:14), 115 ng of (9:14), 118 ng of (11:14) and 89 ng of (13:14). Microsomes were purified from all samples through a neutral sucrose cushion by ultracentrifugation, solubilized with IP buffer containing 0.5% (w/v) Triton X-100 and then immunoprecipitated as detailed in the Materials and methods section. Samples of microsomal membranes (a, c), BRIC155 co-immunoprecipitations (b) and BRIC170 co-immunoprecipitations (d) were analysed by SDS/PAGE on 10% (w/v) Laemmli gels [(a, b), lanes 1–3], 12% (w/v) Laemmli gels [(a, b), lanes 7–12; (c, d), lanes 1–8] or 15% (w/v) Laemmli gels [(a, b), lanes 13–16; (c, d), lanes 9–16], followed by fluorography. The position of b3mem has been aligned across all the lanes of each panel and is indicated (bm). The two bands observed result from the presence and absence of the N-glycan chain because of the non-quantitative nature of N-glycan addition in the cell-free translation system. Co-immunoprecipitated proteins were quantified by scanning densitometry as detailed in the Materials and methods section. The total amount of bm (glycosylated plus non-glycosylated bands) was used in the quantification calculation. The diffuse higher-molecular-mass material in some gel samples contained aggregates of the hydrophobic membrane fragments under study. This material was omitted when quantification of the gels was performed. The ability of fragments to co-assemble with b3mem was determined from the co-immunoprecipitation association ratio, which is defined as the molar amount of N- or C-terminal fragment (i.e. not containing the antibody epitope) divided by the molar amount of b3mem polypeptide (i.e. containing the antibody epitope) in the co-immunoprecipitation. The results are presented quantitatively in Table 3. Fragments (6:14) and (*8:14) were provided by constructs gp(6:14) and gp(*8:14). The positions of molecular mass markers are indicated (in kDa) at the left.

Figure 4 For continuation of Figure and for legend see facing page.

Figure 4 Association of pairs of b3mem fragments in microsomal membranes

(a–d) The N- and C-terminal fragment cRNA species indicated were co-translated in the rabbit reticulocyte lysate cell-free system (1.2 μ l of cRNA per 15 μ l of mixture) at a molar equivalent of 480 ng/ μ l b3mem cRNA for N-terminal fragments, 30 ng/ μ l b3mem cRNA for (6:14) or (11:14), and 40 ng/ μ l b3mem cRNA for (*8:14) or (9:14). The amount of each cRNA (in 15 μ l translation mixtures) was: 497 ng of bm(1:12), 434 ng of bm(1:10), 380 ng of bm(1:8), 330 ng of bm(1:7), 293 ng of bm(1:6), 252 ng of bm(1:5) or 218 ng of bm(1:4) and 38 ng of (6:14), 24 ng of (*8:14), 19 ng of (9:14) or 21 ng of (11:14). Microsomes were purified through a neutral sucrose cushion, solubilized with Triton IP buffer and immunoprecipitated with BRIC155 as described in the legend to Figure 3. Samples were analysed by SDS/PAGE on 12% (w/v) Laemmli gels [(a–c), lanes 1–3 and 9–11] or 15% (w/v) Laemmli gels (other lanes), followed by fluorography. The position of the C-terminal fragment in each panel has been aligned across all the lanes and is indicated with an arrow at the right. The two bands observed for (6:14) and (*8:14) result from the presence and the absence of the N-glycan chain because of the non-quantitative nature of N-glycan addition in the cell-free translation system. The total amount of each polypeptide (glycosylated plus non-glycosylated bands) was used in the quantification calculations. The diffuse higher-molecular-mass material in some gel samples contained aggregates of the hydrophobic membrane fragments under study. This material was omitted when quantification of the gels was performed. (e) Co-immunoprecipitated proteins were quantified by scanning densitometry as detailed in the Materials and methods section. The results of quantification by scanning densitometry are presented in Table 3. The co-immunoprecipitation association ratio is defined as the molar amount of N-terminal fragment (i.e. not containing the BRIC155 epitope) divided by the molar amount of C-terminal fragment (i.e. containing the BRIC155 epitope). The fragments bm(1:10) + (6:14) and bm(1:8) + (*8:14) ran at a co-incident position on SDS/PAGE gels; the relative yields of the two polypeptides could therefore not be quantified. Fragments (6:14) and (*8:14) were provided by constructs gp(6:14) and gp(*8:14). The positions of molecular mass markers are indicated (in kDa) at the left.

cytoplasmic loops are of sufficient length to permit the adjacent TM spans considerable lateral freedom within the plane of the bilayer. The co-expression of other pairs of fragments containing several overlapping TM spans did not support significant levels of chloride uptake, presumably because the global structure of the protein was disrupted too extensively.

Co-expression of fragments bm(1:5) + (5:14) or bm(1:5) + (4:14) resulted in functional co-assembly, whereas bm(1:4) + (5:14) has been confirmed previously to be inactive [23]. This result suggests that the redundant spans 4 and/or 5 are derived from the C-terminal rather than the N-terminal fragment; the suggestion that spans 4–5 interact more intimately with spans 1–3 than with spans 6–14 concurs with the proposal that bm(1:5) corresponds to a tightly folded structural subdomain of band 3, in which the assembly of spans 2–3 depends on interactions with spans 1, 4 and 5 [23]. In contrast, the co-expression of fragments bm(1:3) + (2:14) did not mediate any DNDS-sensitive chloride uptake, similarly to bm(1) + (2:14) and bm(1:3) + (4:14) [17,23]. The inability of overlapping fragments divided in exofacial loops 1 and 2 to complement confirms the essential role of polypeptide chain integrity in the span 1–5 region and/or the limited lateral mobility of the TM spans adjacent to these short loops.

Interactions between fragments and intact band 3

The oligomeric nature of band 3 in the red-cell membrane is well established [1] and the two-dimensional crystal structure of b3mem indicates that the fundamental structural unit is the dimer [15]. The basic functional unit might be either the monomer within a structural dimer or the obligate dimer; the anion-exchange pathway might be located within the dimer interface or within each individual monomer.

We used the co-immunoprecipitation of N- or C-terminal fragments with the intact membrane domain as a probe for interactions across the band 3 dimer interface. Each series of co-immunoprecipitations was performed with a monoclonal antibody that recognized a unique epitope in the intact protein that was not present in the co-translated fragment. The relative molar amount of co-immunoprecipitated fragment provided a semi-quantitative measure of the strength of the interaction between the co-translated polypeptides. Table 2 indicates that the strongest interactions were between b3mem and the N-terminal fragments bm(1:5) or bm(1:6) and between b3mem and the C-terminal fragments (5:14) to (9:14) inclusive. In the conditions of an excess of co-translated fragment over b3mem that were used in these experiments, the presence of any of TM regions 5, 6, 7, 8 and/or 9–10 in either the N- or C-terminal fragment polypeptide did not interfere substantially with the ability of the fragment to co-assemble with b3mem. This concurs with results (described above) for the functional co-expression in oocytes of fragments that overlap by a single TM span.

The interaction between b3mem and the N-terminal fragment bm(1:4) was weaker than that with bm(1:5). This suggests that span 5 might contribute either directly or indirectly to the formation of the interface between bm(1:5) and b3mem. Because bm(1:5) and (9:14) are the smallest N- and C-terminal fragments that are consonant with formation of the strongest interactions with b3mem, we conclude that spans 6, 7 and/or 8 are unlikely to be involved in heterodimer formation with b3mem. Unexpectedly, the 12-span N-terminal fragments bm(1:12) or b3(1:12) consistently co-immunoprecipitated less extensively with b3mem or intact band 3 than bm(1:5), bm(1:6), bm(1:7), bm(1:8) or b3(1:8); the 10-span fragment bm(1:10) showed slightly decreased co-immunoprecipitation, but not to the same

extent as bm(1:12). We have shown previously [24] that the proportion of band 3 expressed at the cell surface of *Xenopus* oocytes is concentration-dependent and hence that oligomeric associations might also be involved in making the band 3 competent for cell surface translocation. In another previous study [16], b3(1:12) exhibited GPA-dependent translocation to the plasma membrane in the absence of its complementary partner (13:14), whereas b3(1:8) displayed this property only when co-expressed with its complementary fragment (9:14). Taken together, these results suggest that spans 1–12 might be capable of homodimerization similarly to intact b3mem and in a manner that allows the translocation of this fragment to the cell surface. This competitive self-association would effectively decrease the availability of monomeric fragment to associate with b3mem (which also forms competitive homodimers), leading to the lower level of co-immunoprecipitation observed. This implies that spans 13–14 are not required for the formation of the band 3 dimer interface. Little or no co-immunoprecipitation was observed between fragment (13:14) and b3mem, which might reflect a lack of specific interaction between these polypeptides. Fragment (11:14) co-immunoprecipitated with b3mem to a much greater extent than (13:14); this implies that spans 11–12 might interact across the dimer interface with b3mem, though to a smaller extent than all the larger C-terminal fragments, which contain the entire spans 9–12 region. The super-stoichiometric co-immunoprecipitation of fragments (5:14) to (9:14) with b3mem suggests that the absence of the spans 1–4 region exposes site(s) that allow the co-association of additional molecules of the fragment with itself and/or b3mem.

Interactions between pairs of fragments

We have examined the interaction of non-complementary pairs of N- and C-terminal fragments by co-immunoprecipitation with a monoclonal antibody reactive against an epitope that is present only in the C-terminal fragment. Table 3 and Figure 4(e) show that each of the N-terminal fragments co-immunoprecipitated with all of the C-terminal fragments to some extent, indicating an extensive range of interactions occur between various TM segments of band 3. The strength with which C-terminal fragments co-immunoprecipitated the range of N-terminal fragments was broadly ordered (9:14) = (*8:14) (6:14) = b3mem (11:14).

The strongest interactions between pairs of fragments were observed between bm(1:6) or bm(1:7) and (*8:14) or (9:14), where the molar association ratio was approximately stoichiometric. Because all four combinations of fragments showed similar levels of interaction, we conclude that spans 7 and 8 do not increase the strength of associations within the band 3 monomer or across the dimer interface (see above). The presence of span 6 in bm(1:6) facilitates the interaction of this fragment with (9:14) relative to bm(1:5). In contrast, bm(1:4) was co-immunoprecipitated much less strongly with either (*8:14) or (9:14); this is similar to the co-immunoprecipitation pattern of these N-terminal fragments with b3mem. Taken together, these results suggest that span 5 might contribute (either directly or indirectly) to the interface between the fragment and b3mem, and that span 6 might assist. It has been suggested recently [25,26] that span 6 might assist in the correct TM integration of span 5.

Fragment (11:14) co-immunoprecipitated all the N-terminal fragments considerably less strongly than (*8:14) or (9:14). The decreased level of co-immunoprecipitation was particularly marked with bm(1:6) or bm(1:7). These results suggest that the spans 9–14 region might be the smallest C-terminal fragment that interacts strongly with N-terminal fragments, although we have

Figure 5 Working model of the structure of the dimeric membrane domain of band 3

A possible arrangement of TM spans in the b3mem dimer is shown in diagrammatic form. The numbers indicate the proposed locations of the various TM regions. One set of TM regions (first monomer) is delineated with solid lines and a second set (second monomer) with long broken lines. The extended short broken line indicates a likely interface between the two monomer polypeptides. The mobile region proposed by [15] from the crystal structure is suggested to be the spans 13–14 region; arbitrary positions are depicted by dotted lines.

shown (see above) that spans 11–14 co-immunoprecipitate with intact b3mem to a smaller extent. A recent study has found that the topology of spans 9–10 is affected by the proximity of the spans 11–12 region [23], supporting the view that the spans 9–12 region might form a closely interacting subdomain of b3mem.

The N-terminal fragments were co-immunoprecipitated by (6:14) to a smaller extent than with (*8:14) or (9:14). The levels of association were similar to those observed when N-terminal fragments were co-immunoprecipitated with b3mem. Although it is possible that (6:14) might be competent to form homodimers, whereas (*8:14) cannot do so, decreased co-immunoprecipitation of this fragment with b3mem was not observed, as with bm(1:12) and b3mem (discussed above). It is possible that (*8:14) and (9:14) might be able to associate with more than one N-terminal fragment (e.g. both within the monomer and across the dimer interface), whereas (6:14) might form only one of these associations.

Working model of the structure of the dimeric membrane domain of band 3

Figure 5 depicts a possible structural model for the organization of the TM spans of the band 3 dimer, based on the above and other current evidence. This model is not exclusive but is consistent with several lines of experimental results as well as with the low-resolution crystal structure of the band 3 membrane domain [15]. We suggest that the core of the protein might comprise two subdomains that contain the closely interacting TM span 1–5 region (delineated at the C-terminal end by the long and unconserved EC loop 3) and the TM span 9–12 region orientated to form an anti-parallel homodimer. The regions containing TM spans 6, 7 and 8 are thought to be positioned away from the interface between these core subdomains. The region comprising TM spans 13–14 and the C-terminus is suggested to be at the periphery of the structure and might correspond to the 'mobile element' proposed by Wang et al. [15] from the two different crystal forms obtained.

We summarize the experimental evidence consistent with this model as follows.

(1) Spans 6–8 are unlikely to be involved in the dimer interface, because the interaction between spans 1–5 and b3mem or spans 9–14 and b3mem is strong and is not increased by the presence of spans 6, 7 and/or 8 in either fragment (Table 2).

(2) Span 6 interacts with spans 9–14 and spans 1–6 interact with spans 9–10, because spans 1–6 and 9–14 are the minimal size of N- and C-terminal fragments that form the strongest interactions (Table 3).

(3) Spans 11–12 contribute to formation of the dimer interface, because b3mem forms a stronger interaction with spans 11–14 than spans 13–14 (Table 2).

(4) Spans 13–14 are unlikely to form part of the core protein dimer interface because they do not interact with either b3mem (Table 2) or spans 1–12 [16] in a detergent-stable manner.

(5) In recent studies of the topology of band 3 fragments comprising one or more TM spans, it was suggested that in the intact protein TM region 1 might interact with 2; 2 with 4; 4 with 5; 7 with 8; 8 with 9–10 and 9–10 with 11–12 [23].

(6) Spans 6–7 and EC loop 4, which contains the endogenous N-glycosylation site, are likely to be situated at the periphery because the co-expression of pairs of fragments lacking these regions can still mediate DNDS-sensitive chloride uptake in oocytes (Figure 2) [18].

(7) Span 8 is likely also to have a peripheral location because the W_r^b antigen seems to involve an interaction between GPA and Glu-658 of band 3 in EC loop 4 [27]; furthermore, the mutation Gly-701 → Asp, which is located in intracellular loop 4, makes the movement of band 3 to the cell surface absolutely dependent on the presence of GPA when expressed in oocytes [28].

Conclusion

Although band 3 is regarded as one of the better-understood multispinning membrane proteins, there is continuing uncertainty about the topology and structure of the molecule. This emphasizes the urgent need for direct, experimentally derived, structural data. The working model that we propose might act as a springboard for the design of further experiments (e.g. by using protein cross-linking) that will improve our understanding of the detailed packing arrangement of the membrane-spanning seg-

ments of band 3. To our knowledge, this is the first attempt to describe the organization of the TM spans of a polytopic protein with the use of biochemical approaches.

This work was supported by a grant from the Wellcome Trust.

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