Complementation studies with co-expressed fragments of human red cell band 3 (AE1): the assembly of the anion-transport domain in *Xenopus* oocytes and a cell-free translation system

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We examined the assembly of the membrane domain of the human red cell anion transporter (band 3; AE1) by co-expression of recombinant N- and C-terminal fragments in *Xenopus* oocytes and in cell-free translation with canine pancreatic microsomes. Co-immunoprecipitation was performed in non-denaturing detergent solutions using antibodies directed against the N- and C-termini of the membrane domain. Eleven of the twelve fragments were expressed stably in oocytes in the presence or absence of their respective partners. However, the fragment containing from putative span nine to the C-terminus could be detected in oocytes only when co-expressed with its complementary partner containing the first eight spans. Co-expression of pairs of fragments divided in the first, second, third and fourth exofacial loops and in the fourth cytoplasmic loop resulted in a concentration-dependent association, but a pair of fragments divided in the

sixth cytoplasmic loop did not co-immunoprecipitate. When two complementary fragments were translated separately in the cell-free system and the purified microsomes were then mixed, co-immunoprecipitation was observed only if the membranes were first fused using polyethylene glycol. This shows that co-immunoprecipitation results from specific interactions within the membrane and is not an artefact of detergent solubilization or immunoprecipitation. We demonstrate that band 3 assembly can occur within the membrane after translation, insertion and initial folding of the individual fragments have been completed. We conclude that most band 3 fragments contain the necessary information to fold in the membrane and adopt a structure that is sufficiently similar to the native protein that it permits correct assembly with its complementary partner.

INTRODUCTION

The human erythrocyte anion transporter (band 3; AE1) is a multi-functional integral membrane protein that comprises two domains that are structurally and functionally distinct (reviewed in [1–4]). The 43 kDa N-terminal cytoplasmic domain (residues 1–359) is involved in a number of functions unrelated to anion transport, including the binding of the red cell cytoskeleton via ankyrin [5]. The 52 kDa C-terminal membrane domain (residues 360–911) is responsible for the anion-transport function and remains fully functional either when translated from a cDNA clone encoding the isolated membrane domain [6,7], or after proteolytic removal of the cytoplasmic domain [8,9]. The primary sequences of the red cell anion transporters of mammals, birds and fish are highly conserved (reviewed in [10]), with the highest level of homology in the putative transmembrane spans. The anion-transport domain contains a substantial proportion of α -helical structure [11] and may traverse the membrane either 12 or 14 times [12,13]. The membrane domain of human band 3 has been reconstituted with lipids to form two-dimensional crystals which have yielded a structural model [14,15] (reviewed in [12]). Although the resolution (20 Å) was insufficient to permit the visualization of individual features of secondary structure, this study indicated the presence of three sub-domains within the band 3 monomer, and the cross-sectional area of the molecule in the plane of the membrane was estimated to be sufficient to permit the packing of up to 14 α -helical transmembrane 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 separated within the third or fourth extracellular loops [16] and the fourth or sixth cytoplasmic loops [17], although none of the individual fragments were functional. This, combined with evidence from other re-assembly studies *in vitro* (e.g. [18,19]) and *in vivo* (e.g. [20–24]), makes it increasingly clear that the specific association of discrete subdomains may be a general property of polytopic integral membrane proteins. These observations support the ‘two-stage mode’ of membrane protein assembly [25] in which the folding of the nascent polypeptide chain to form the transmembrane segments and their subsequent assembly are effectively uncoupled.

In this paper, we examine the ability of pairs of co-expressed fragments of band 3 to associate after expression in the *Xenopus* oocyte and rabbit reticulocyte lysate cell-free translation systems. We use two non-denaturing detergents which are known to maintain the native structure of the band 3 membrane domain [26] to solubilize intact oocytes or purified microsomal membranes, followed by co-immunoprecipitation of the fragments, and show that most pairs of complementary fragments of band 3 are able to co-assemble in membranes.

MATERIALS AND METHODS

Construction of band 3 fragments and preparation of cRNAs

The cDNA clones encoding human red cell band 3 (pBSXG1.B3), the membrane domain of band 3 (pBSXG1.B3mem) and glycoporphin A (pBSXG.GPA) have been described previously [7]. They contain the respective protein coding region flanked by the

Abbreviations used: c.m.c., critical micellar concentration; AP, antiprotease; C₁₂E₈, octaethylene glycol monododecyl ether; DDM, dodecyl maltoside; GPA, glycoporphin A; H₂DIDS, 4,4'-di-isothiocyanato-2,2'-dihydrostilbene disulphonate; IP, immunoprecipitation; PEG, polyethylene glycol.

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5'- and 3'-non-coding region of *Xenopus* β -globin. The cDNA constructs encoding six pairs of complementary N- and C-terminal band 3 fragments (Figure 1) have been described previously [16,17]. Briefly, cDNA constructs were prepared in which band 3 was truncated within the fourth intracellular loop (between amino acid residues 695 and 696) [17] to generate an N-terminal fragment, designated b3(1:8), that contains the cytoplasmic domain and first eight transmembrane spans, and a C-terminal fragment, designated (9:14) that contains the final six putative transmembrane spans, according to the 14-span model of band 3 [2,3,13]. A pair of fragments, designated b3(1:12) and (13:14), were prepared similarly by truncation within the sixth intracellular loop (between amino acids 824 and 825) [17]. Band 3 fragments were also prepared containing truncations within the first (between amino acids 432 and 433), second (between amino acids 481 and 482), third (between amino acids 553 and 554) or fourth (between amino acids 639 and 640) exofacial loop; in each of these cases the C-terminal fragments (2:14), (4:14) etc. were preceded by the cleavable leader sequence of glycophorin A (GPA) to ensure that the first span inserted with the correct transmembrane polarity [16]. A control cDNA clone (pSPGT1) encoding the human glucose transporter Glut1 was supplied by Professor G. W. Gould (University of Glasgow, U.K.).

Cell-free translation and *Xenopus* oocyte expression

Procedures of *in vitro* transcription of cDNA constructs, cell-free translation in the rabbit reticulocyte lysate system with canine pancreatic microsomes, isolation of oocytes, microinjection of cRNAs and [35 S]amino acid labelling of oocyte expressed proteins were as described previously [7].

Non-denaturing detergent immunoprecipitations from *Xenopus* oocytes

Groups of ten oocytes were disrupted in immunoprecipitation (IP) buffer [50 mM Tris/HCl, pH 7.4, 190 mM NaCl, 6 mM EDTA containing an antiprotease cocktail (AP mixture) of 2 mM PMSF, 50 μ g/ml leupeptin, 50 μ g/ml antipain and 20 μ g/ml pepstatin A] without detergent (50–100 μ l), divided into either two or three equal portions (20–50 μ l), depending on the particular experiment, and solubilized by addition of either: (i) 450–480 μ l of 2.5% (w/v) Triton X-100 (Sigma) in IP buffer; (ii) 450–480 μ l of 2% (w/v) octaethylene glycol monododecyl ether ($C_{12}E_8$; Fluka) in IP buffer; or (iii) 50 μ l of 2% (w/v) SDS/1 mM EDTA containing the antiprotease mixture followed by 400–430 μ l of 2.5% (w/v) Triton X-100 in IP buffer. The total volume in each case was 500 μ l. Extracts were incubated for 2 min at 20 °C and then centrifuged for 2–5 min at 12000 g in a microfuge. The supernatant was transferred to another tube and centrifuged again to remove all traces of particulate matter. The supernatant was divided and immunoprecipitated using monoclonal antibodies BRIC170 (against the N-terminal end of the band 3 membrane domain) obtained from Professor D. Anstee (International Blood Group Reference Laboratory, Bristol, U.K.) or BRIC155 (against the C-terminus of band 3 [27]). The procedure was similar to that used in [7], except that Protein A-Affigel (Bio-Rad, Hemel Hempstead, U.K.) was re-suspended in IP buffer containing the above detergents and samples were washed six times in 1 ml of wash buffer [50 mM Tris/HCl (pH 7.4), 150 mM NaCl, 5 mM EDTA and 10% concentration of AP mixture] containing either: (i) 0.1% (w/v) Triton X-100; (ii) 0.1% (w/v) $C_{12}E_8$; or (iii) 0.02% (w/v) SDS/0.1% (w/v) Triton X-100.

Non-denaturing detergent immunoprecipitations from cell-free translations

Microsomal membranes were purified from the cell-free translation (12.5 μ l or 25 μ l) by centrifugation through 100 μ l 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) in an Airfuge (Beckman Instruments) at 30 lb/in 2 (1 lb/in 2 = 6.9 kPa) for 15 min at 4 °C. Membrane pellets were solubilized in 400 μ l of IP buffer in the presence of either 2% (w/v) Triton X-100 or 2% (w/v) $C_{12}E_8$. Samples were divided and immunoprecipitated as above using BRIC170 or BRIC155, and then washed six times in 1 ml of wash buffer containing either 0.1% (w/v) Triton X-100 or 0.1% (w/v) $C_{12}E_8$.

Some immunoprecipitations were carried out using other non-denaturing detergents. Microsomal membranes were solubilized in IP buffer containing 0.5% (w/v) dodecyl maltoside (DDM; Sigma), 0.5% (w/v) dodecyl phosphocholine (CDN isotopes, Quebec, Canada), 0.5% (w/v) lauroyl L- α -lysophosphatidyl choline (Sigma), 0.5% (w/v) myristoyl L- α -lysophosphatidyl choline (Sigma), 1.5% (w/v) octyl-D-glucopyranoside (Fluka), 0.7% (w/v) nonyl-D-glucopyranoside (Fluka) or 0.5% N,N-dimethyl dodecylamine-N-oxide (Fluka). In each case, samples were washed six times in IP buffer containing the same detergent at 0.1% (w/v) in excess of the reported critical micellar concentration (c.m.c.) [28].

Polyethylene glycol (PEG)-mediated fusion of canine pancreatic microsomes

Cell-free translations of complementary pairs of band 3 fragments were performed either as a single co-translation (25 μ l) or as two separate translations (12.5 μ l of each) which were subsequently pooled. Microsomal membranes were purified by Airfuge centrifugation (as above) and the pellets were treated with 10 μ l of 50% PEG 4000 in PBS (Boehringer) or 20 mM Hepes/KOH, pH 7.5/50 mM KCl/250 mM sucrose (HKS buffer) [7] for 30 min at 37 °C. Subsequently, HKS buffer (10 μ l) was added to all tubes, the pellet was resuspended by pipetting up and down, and the membranes were centrifuged through a neutral sucrose cushion, solubilized and immunoprecipitated as above.

Analysis of cell-free translations and immunoprecipitations

Samples were analysed by SDS/PAGE [29] using either linear or gradient gels (4–20% acrylamide; Bio-Rad Laboratories) and treated with Amplify (Amersham) and fluorographed (–80 °C) using either X-AR or BioMax MR film (Kodak). The yield of each fragment in the cell-free translation mixtures, purified microsomes or immunoprecipitations was quantified by scanning densitometry. Exposures were made in the linear sensitivity range of the film and densitometry values were normalized to allow for the different numbers of methionine and/or cysteine residues in each of the band 3 fragments.

RESULTS

Procedure for quantitative analysis of co-expressed fragments

We examined the association of six pairs of complementary truncated fragments of band 3 using two membrane protein expression systems: *Xenopus* oocytes and cell-free translation in the rabbit reticulocyte lysate system with microsomal membranes.

Figure 1 Structure of band 3 fragments

The nomenclature and truncation sites of the fragments used in this paper are shown, together with their putative transmembrane topology and the epitopes of the antibodies BRIC170 and BRIC155. The N-glycan chain is shown in the fourth exofacial loop. The broken zigzag line represents the GPA signal sequence which precedes each C-terminal fragment that has its N-terminus in an exofacial loop. The arrow indicates the cleavage site of the GPA signal sequence by signal peptidase (SP).

After solubilization of the membranes in a non-ionic detergent which does not denature band 3 [26], samples were immunoprecipitated with monoclonal antibodies BRIC170 or BRIC155 which recognize epitopes at the N- and C-terminal ends of the band 3 membrane domain respectively (Figure 1). In the case of oocytes, the detergent micelle concentration [28] was always at least 2-fold higher than the estimated number of protein molecules in the sample, to ensure complete solubilization and to prevent inherently separable protein molecules from becoming trapped in the same detergent micelle. The ability of pairs of radiolabelled polypeptide fragments to associate and hence to co-immunoprecipitate under these non-denaturing conditions was determined by SDS/PAGE and fluorography.

To quantify the expression of each polypeptide, scanning densitometry was performed on fluorographs exposed within the linear range of the film. Densitometry values were adjusted to take account of the respective numbers of methionine and cysteine residues in each fragment. The yield of each fragment was compared from samples in which it was expressed alone or together with its complementary partner. We allowed for the difference in the efficiency with which the two antibodies BRIC155 (class IgG_{2b}) and BRIC170 (class IgG₁) recovered cell-free translation and oocyte-expressed protein during immunoprecipitation: five control samples of oocytes expressing intact band 3 were solubilized in Triton X-100, divided into two equal portions, immunoprecipitated and the SDS/PAGE gels were fluorographed and scanned. The results showed that band 3 was immunoprecipitated by BRIC155 3–4-fold more efficiently than by BRIC170 (results not shown), probably because of the additional step required during the immunoprecipitation when using this class IgG₁ antibody. We determined the relative molar amounts of N- and C-terminal fragment polypeptides that were expressed and compared these with the relative molar amounts of N-terminal and C-terminal fragment cRNAs added to the expression system. This showed that some constructs were

Figure 2 Association in oocytes of band 3 fragments truncated in the fourth cytoplasmic loop

Oocytes were co-injected with an equimolar quantity of b3(1:8) and/or (9:14) cRNA, together with 1.5 ng per oocyte of GPA cRNA. The band 3-derived cRNAs injected were (per oocyte): 3.75 ng of b3(1:8) (lanes 1, 5, 9 and 12); 1.5 ng of (9:14) (lanes 4, 6, 7 and 10); 3.75 ng of b3(1:8) + 1.5 ng of (9:14) (lanes 2, 3, 8 and 11). Injected oocytes were radiolabelled with ³⁵S-labelled amino acids for 38 h and then chased with unlabelled amino acids for a further 5 h. Groups of ten oocytes were solubilized either with SDS followed by Triton X-100 (lanes 1–6) or with Triton X-100 only (lanes 7–12) and then immunoprecipitated either with BRIC170 (lanes 3–5 and 7–9) or with BRIC155 (lanes 1–2, 6 and 10–12) as detailed in the Materials and methods section. Each lane represents the immunoprecipitated protein from the equivalent of 3–4 oocytes, separated by SDS/PAGE on 4–20% Laemmli gradient gels (Bio-Rad).

expressed more readily than others from the same molar quantity of cRNA (see below and Tables 1 and 2). Likewise, we compared the relative molar expression of each fragment alone and in the presence of its complementary partner. To quantify the co-immunoprecipitation of fragments in non-denaturing detergents, we determined the relative molar association. This is defined as the amount of co-immunoprecipitated fragment (i.e. not possessing the antibody epitope) divided by the molar amount of directly immunoprecipitated fragment.

Association of pairs of complementary band 3 fragments in *Xenopus* oocytes

We examined the association in *Xenopus* oocytes of two pairs of complementary fragments separated in putative cytoplasmically orientated loops and four pairs of fragments separated in putative exofacially orientated loops (Figure 1), with loops defined according to the 14-span topology model [2,3]. In previous work, we have shown that four of these combinations, truncated in the fourth or sixth cytoplasmic loops [17] and the third or fourth exofacial loops [16] can re-assemble to form a functional anion transporter at the oocyte cell surface. In this study, groups of ten *Xenopus* oocytes were microinjected with equimolar quantities of band 3 fragment cRNAs, either individually or as a complementary pair. The cRNA encoding GPA was also co-injected as the interaction of this single-span membrane protein subtly affects the structure-function of band 3 [7,30,31] and band 3 fragments [17]. After incubation for 48 h in the presence of L-[³⁵S]amino acids, oocytes were solubilized either with one of the non-ionic detergents, Triton X-100 (Triton) or C₁₂E₈, or with the denaturing detergent SDS. In the latter case, the initial solubilization in SDS was followed by addition of Triton (SDS/Triton) to allow immunoprecipitation.

Table 1 Expression and association of complementary pairs of band 3 fragments in *Xenopus* oocytes

Band 3 fragments were expressed in oocytes either individually ('alone') or as a complementary pair ('with partner') by microinjection of equimolar quantities of cRNA. Oocytes were solubilized in IP buffer containing SDS/Triton (Figures 1 and 3), Triton (Figures 1–3) and/or $C_{12}E_8$ (results not shown). N-terminal fragments (N-frag) were immunoprecipitated with BRIC170 and C-terminal fragments (C-frag) with BRIC155. Radiolabelled proteins were separated by SDS/PAGE and fluorographs were scanned by densitometry. The expressed protein was quantified from the areas under the peaks after allowing for the different amounts of radioactivity incorporated into the various fragments. The immunoprecipitation yield of each fragment was compared in the presence and absence of its complementary partner and is presented as a relative molar ratio in each case (columns 1–4). The ability of pairs of fragments to associate is presented as the ratio of the molar amount of co-immunoprecipitated fragment (i.e. not containing the antibody epitope) divided by the molar amount of immunoprecipitated fragment (i.e. containing the antibody epitope) using either BRIC155 (against the C-terminal, column 5) or BRIC170 (against the N-terminal, column 6). (0), none detectable.

Constructs	N-frag	C-frag	Detergent	Ratio of immunoprecipitated polypeptides				Polypeptide association ratio	
				N-frag (alone)		C-frag (alone)		BRIC155	BRIC170
				N-frag (with partner)	C-frag (with partner)	N-frag (alone)	N-frag (with partner)	N-frag	C-frag
b3(1)	(2:14)	Triton X-100	1.3		1.1	5.6	4.7	0.4	> 0.1
b3(1:3)	(4:14)	Triton X-100	0.3		2.0	0.6	4.0	1.2	0.3
b3(1:5)	(6:14)	Triton X-100	1.6		0.9	8.2	4.6	0.8	0.1
b3(1:7) _a	(8:14) _a	Triton X-100	0.4		0.5	1.7	2.0	0.3	> 0.1
b3(1:8)	(9:14)	SDS/Triton	0.4		(0)	> 50	6.0	(0)	(0)
b3(1:8)	(9:14)	Triton X-100	0.9		(0)	> 50	4.0	0.9	0.2
b3(1:8)	(9:14)	$C_{12}E_8$	1.1		(0)	> 50	4.5	0.9	0.1
b3(1:12)	(13:14)	SDS/Triton	0.9		1.0	0.4	0.4	(0)	(0)
b3(1:12)	(13:14)	Triton X-100	0.8		0.9	0.6	0.7	(0)	(0)
b3(1:12)	(13:14)	$C_{12}E_8$	1.2		0.9	0.8	0.7	(0)	(0)
Column no....				1	2	3	4	5	6

Figure 2 shows the results of immunoprecipitations from oocytes of the fragments truncated in the fourth cytoplasmic loop (the 8 + 6 span combination) using SDS/Triton (lanes 1–6) and Triton only (lanes 7–12). Co-injection of both fragment cRNAs resulted in the immunoprecipitation of the N-terminal fragment b3(1:8) (see Materials and methods section for an explanation of the nomenclature used for the fragments) using BRIC170 (lanes 3 and 8) and the C-terminal fragment (9:14) using BRIC155 (lanes 2 and 11) and little or none of the N- and C-terminal fragments was detected in control immunoprecipitations using BRIC155 (lanes 1 and 12) and BRIC170 (lanes 4 and 7) respectively. While the expression of b3(1:8) was similar in the presence and absence of (9:14) (lanes 3 and 8, cf. lanes 5 and 9), it was not possible to detect any expression of (9:14) using BRIC155 in the absence of b3(1:8), even on fluorographs that were exposed for five times longer (lanes 6 and 10). The samples using $C_{12}E_8$ (gel results not shown) were virtually identical with those using Triton, although immunoprecipitation of oocyte proteins using Triton generally gave a lower level of non-specific bands. Quantification of these results (Table 1) showed that the separate injection of equimolar quantities of b3(1:8) and (9:14) cRNAs into different oocytes resulted in a greater than 50-fold higher expression of b3(1:8) than of (9:14). In contrast, co-expression of b3(1:8) and (9:14) in the same oocytes resulted only in a 4–6-fold molar excess of b3(1:8) over (9:14). Since these immunoprecipitations were derived from homogenized whole oocytes, we conclude that (9:14) is degraded rapidly in the absence of b3(1:8). Co-expression of b3(1:8) and (9:14) resulted in co-immunoprecipitation in Triton using either BRIC155 (lane 11) or BRIC170 (lane 8). In the case of the BRIC155 immunoprecipitation of (9:14), an approximately equal stoichiometric quantity of b3(1:8) was co-immunoprecipitated. Since a 4–6-fold excess of b3(1:8) was present in these cells, we infer that almost all the (9:14) molecules were associated with their complementary

partners and that assembly with b3(1:8) is required for the stable expression of (9:14) in oocytes.

Figure 3 shows the results of Triton co-immunoprecipitation of the fragments truncated in the first exofacial loop (Figure 3a, 1 + 13 span combination), second exofacial loop (Figure 3b, 3 + 11 span combination), third exofacial loop (Figure 3c, 5 + 9 span combination) and fourth exofacial loop (Figure 3d, 7 + 7 span combination). The nomenclature of the fragments [e.g. b3(1), (2:14) etc.] is similar to that used for Figure 2. In each case, fragments were expressed from equimolar quantities of cRNA either individually (lanes 1 and 2 and 5 and 6) or as a complementary pair (lanes 3 and 4). Equal portions of each sample were immunoprecipitated with either BRIC155 (lanes 1, 3 and 5) or BRIC170 (lanes 2, 4 and 6). Quantification by scanning (Table 1) showed that all eight fragments were immunoprecipitated at broadly similar levels in the presence or absence of their co-expressed partners: the greatest differences were observed with b3(1:7)_a + (8:14)_a, where co-expression increased the yield of both fragments 2-fold, and b3(1:3) + (4:14), where co-expression increased the yield of b3(1:3) by 4-fold. In comparison with the effect of b3(1:8) on the level of expression of (9:14), discussed above, these differences were small. Nevertheless, our data suggest that several fragments may gain some increased resistance to degradation by the oocyte in the presence of their complementary partner. Co-injection of equimolar quantities of cRNA resulted in a 2–5-fold molar excess of the N-terminal fragment polypeptide in each case, similarly to b3(1:8) + (9:14).

All four pairs of exofacially divided fragments were co-immunoprecipitated readily using BRIC155. The 3 + 11 (Figure 3b, lane 3) and 5 + 9 (Figure 3c, lane 3) span combinations were co-immunoprecipitated very strongly, with each pair of fragments detectable at an approximately stoichiometric ratio (Table 1). Co-immunoprecipitation of the 1 + 13 (Figure 3a, lane 3) and 7 + 7 (Figure 3d, lane 3) span combinations were also observed,

Figure 3 Association in oocytes of band 3 fragments truncated in exofacial loops

Oocytes were co-injected with an equimolar quantity of one or both of the complementary pairs of band 3 fragment cRNAs at the molar equivalent of 7.5 ng of band 3 cRNA per oocyte, together with 2.5 ng per oocyte of GPA cRNA. The band 3-derived cRNAs injected were: **(a)** b3(1) (lanes 1 and 2); b3(1)+(2:14) (lanes 3 and 4); (2:14) (lanes 5 and 6). **(b)** b3(1:3) (lanes 1 and 2); b3(1:3)+(4:14) (lanes 3 and 4); (4:14) (lanes 5 and 6). **(c)** b3(1:5) (lanes 1 and 2); b3(1:5)+(6:14) (lanes 3 and 4); (6:14) (lanes 5 and 6). **(d)** b3(1:7)_a (lanes 1 and 2); b3(1:7)_a+(8:14)_a (lanes 3 and 4); (8:14)_a (lanes 5 and 6). Injected oocytes were radiolabelled with ³⁵S-labelled amino acids for 40–48 h without a subsequent chase with unlabelled amino acids, solubilized with Triton X-100 and then immunoprecipitated with BRIC155 (lanes 1, 3 and 5) or BRIC170 (lanes 2, 4 and 6) as detailed in the Materials and methods section. Each lane represents immunoprecipitated protein from the equivalent of five oocytes, separated by SDS/PAGE on 9% **(a)** or 12% **(b–d)** gels.

Figure 4 Association in oocytes of band 3 fragments truncated in the sixth cytoplasmic loop

Oocytes were co-injected with an equimolar quantity of b3(1:12) and/or (13:14) cRNA, together with 1.5 ng per oocyte of GPA cRNA. The band 3-derived cRNAs injected were (per oocyte): 4.5 ng of b3(1:12) (lanes 1, 5, 9 and 12); 1.5 ng of (13:14) (lanes 4, 6, 7 and 10); 4.5 ng of b3(1:12)+1.5 ng of (13:14) (lanes 2, 3, 8 and 11). Oocytes were radiolabelled, solubilized either with SDS/Triton X-100 (lanes 1–6) or with Triton X-100 only (lanes 7–12), immunoprecipitated with BRIC170 (lanes 3–5 and 7–9) or BRIC155 (lanes 1, 2, 6 and 10–12) and separated by SDS/PAGE exactly as in Figure 2.

although the levels were slightly lower. Co-immunoprecipitation using BRIC170 was also observed (Figure 3a–3d, lane 4), although in each case the C-terminal fragment was co-immunoprecipitated using BRIC170 at a proportionately lower efficiency than the corresponding N-terminal fragments using BRIC155 (Table 1). However, the BRIC170 results showed the same relative pattern, with clearly detectable co-immunoprecipitation of the 3+11 (Figure 3b, lane 4) and 5+9 (Figure 3c, lane 4) span combinations and faint co-immunoprecipitation of the 1+13 (Figure 3a, lane 4) and 7+7 (Figure 3d, lane 4) span combinations that was barely detectable above background.

Figure 4 shows the results of oocyte immunoprecipitations of the fragments truncated in the sixth cytoplasmic loop (12+2 span combination) using SDS/Triton (lanes 1–6) and Triton only (lanes 7–12). Results using C₁₂E₈ (gel results not shown) were similar to those using Triton (gel results not shown). Quantification by scanning (Table 1) showed that the levels of expression of both fragments b3(1:12) and (13:14) did not alter significantly in the presence and absence of their partners, and hence that co-expression does not affect the stable expression of either fragment. Although co-expression of these fragments generates a high level of stilbene disulphonate-sensitive anion transport [17], neither b3(1:12) nor (13:14) was co-immunoprecipitated with its partner (lanes 5 and 11). This surprising result is particularly significant in the case of the BRIC155 sample, since b3(1:12) contains eight times more methionine

Figure 5 Association in microsomal membranes of band 3 fragments truncated in cytoplasmic loops

Complementary pairs of band 3 fragment cRNAs were co-translated in the rabbit reticulocyte lysate cell-free system. The amounts of each cRNA (in 25 μ l mixtures) were: (a) 220 ng of b3(1:8) + 32 ng of (9:14) (lanes 1 and 2); 220 ng of b3(1:8) + 250 ng of (9:14) (lanes 3 and 4); (b) 320 ng of b3(1:12) + 10 ng of (13:14) (lanes 5 and 6); 320 ng of b3(1:12) + 80 ng of (13:14) (lanes 7 and 8). Microsomes were purified by Airfuge centrifugation, solubilized with IP buffer containing 1% (w/v) $C_{12}E_8$ and then immunoprecipitated with either BRIC155 (lanes 2 and 6) or BRIC170 (lanes 4 and 8) as detailed in the Materials and methods section. Samples were analysed by SDS/PAGE on pre-cast 4–20% Laemmli gradient gels (Bio-Rad), followed by autoradiography.

residues than (13:14) and we would expect relatively low levels of co-immunoprecipitated b3(1:12) to be detectable.

Association of pairs of complementary band 3 fragments in cell-free translation

Co-translation experiments analogous to those performed in oocytes were also carried out using the rabbit reticulocyte lysate cell-free system and canine pancreatic microsomes. In previous studies using cell-free translation we have shown that many band 3 fragments can integrate into microsomal membranes similarly to intact band 3 [16,17]. To investigate further the insertion of the fragments into the membrane, translations containing each of the fragment molecules labelled with L-[35 S]methionine were ultracentrifuged through an alkaline sucrose cushion (0.1 M Na_2CO_3 /0.25 M sucrose, pH 11.5) which removes all peripherally attached proteins from the membrane [32]. SDS/PAGE analysis showed that each of the translated fragments was predominantly pelleted with the microsomes (results not shown). Interestingly, this experiment showed that the fragment (13:14), which includes only two very short transmembrane spans, was expressed in microsomes as an integral membrane polypeptide and that insertion was not affected by the presence or absence of co-translated b3(1:12).

Scanning of preliminary co-translation experiments in the cell-free system showed that all the larger C-terminal fragments yielded at least twice as much polypeptide from the same amount of cRNA as did their N-terminal partners (results not shown). In the case of the smaller C-terminal fragments, the over-representation was even greater: fragment (9:14) was translated at 3–5-fold excess over b3(1:8) and fragment (13:14) at 7–8-fold

Table 2 Expression and association of complementary pairs of band 3 fragments in microsomal membranes

Complementary pairs of band 3 fragments were co-translated in the rabbit reticulocyte lysate cell-free system from various concentrations of each cRNA. The relative molar ratio of cRNAs used (column 1) is the amount of N-terminal fragment (N-frag) cRNA divided by the amount of C-terminal fragment (C-frag) cRNA added to the co-translation mixture. The relative molar ratio of expressed polypeptides (column 2) is the molar amount of N-terminal fragment divided by the molar amount of C-frag polypeptide in the purified microsomal membrane pellet. After solubilization with IP buffer containing $C_{12}E_8$ (fragments divided in exofacial loops) or Triton X-100 (fragments divided in cytoplasmic loops, Figure 4), the ability of pairs of fragments to associate was determined by co-immunoprecipitation with BRIC155 (column 3) or BRIC170 (column 4), as described in the legend to Table 1. (0), none detectable; nd, not determined.

Constructs		Ratio of cRNAs used N-frag	Ratio of translated polypeptides N-frag	Polypeptide association ratio	
				BRIC155 N-frag	BRIC170 N-frag
N-frag	C-frag	C-frag	C-frag	C-frag	C-frag
b3(1)	(2:14)	6.5	1.3	4.2	0.1
b3(1)	(2:14)	1.3	0.3	0.6	0.25
b3(1:3)	(4:14)	8.2	2.3	4.4	0.09
b3(1:3)	(4:14)	4.9	1.0	1.2	0.2
b3(1:3)	(4:14)	3.7	0.4	0.5	0.54
b3(1:5)	(6:14)	4.8	2.1	1.1	0.23
b3(1:5)	(6:14)	3.2	0.8	0.33	0.54
b3(1:5)	(6:14)	2.1	0.24	0.14	0.75
b3(1:7) _a	(8:14) _a	7.0	1.4	0.5	(0)
b3(1:7) _a	(8:14) _a	3.5	0.9	0.4	0.14
b3(1:7) _a	(8:14) _a	2.3	0.23	0.16	0.26
b3(1:8)	(9:14)	4.0	0.8	0.3	nd
b3(1:8)	(9:14)	0.25	0.1	nd	1.0
b3(1:12)	(13:14)	4.0	2.0	(0)	nd
b3(1:12)	(13:14)	0.25	0.2	nd	(0)
Column no. ...		1	2	3	4

Figure 6 Association in microsomal membranes of band 3 fragments truncated in exofacial loops

Complementary pairs of band 3 fragment cRNAs were co-translated in the cell-free system. The amounts of each cRNA (in 12.5 μ l mixtures) were: 200 ng of b3(1) + 65 ng of (2:14) (lane 1); 200 ng of b3(1:3) + 90 ng of (3:14) (lane 2); 200 ng of b3(1:5) + 90 ng of (5:14) (lane 3); 200 ng of b3(1:7)_a + 55 ng of (8:14)_a (lane 4). Microsomes were solubilized with IP buffer containing 1% (w/v) C₁₂E₈ and immunoprecipitated with BRIC155. Samples were analysed by SDS/PAGE on 12% Laemmli gels, followed by autoradiography. (a) Cell-free translation mixtures (0.5 μ l per lane); (b) BRIC155 immunoprecipitations.

excess over b3(1:12). These results contrast with those of the oocyte expression system where N-terminal fragments were expressed more efficiently. Several other control experiments were performed to confirm the suitability of the cell-free system for these studies. First, the relative proportions of the various N- and C-terminal fragments did not differ significantly when co-translated or separately translated samples were compared (results not shown). This is in contrast with the oocyte system, where several fragments, particularly (9:14), showed instability in the absence of their partners. Second, the relative quantities of two or more polypeptides in a co-translation could be controlled reproducibly by the quantity of each cRNA included in the reaction mixture. Competition at the level of translation was apparent only when very high levels of cRNA (> 1 μ g of band 3 cRNA per 25 μ l of mixture) were used (results not shown). Third, when samples were ultracentrifuged through a neutral pH sucrose cushion and then analysed by SDS/PAGE, the molar proportions of the two fragments in the membrane pellet were usually similar to those in the crude translation mixtures, indicating that both fragments had become incorporated into microsomes to the same extent. Fourth, co-expression did not appear to affect the binding of BRIC155 to its epitope, since similar yields of (9:14) and (13:14) were obtained in the presence and absence of their N-terminal partners, both in the microsomal

membranes and after immunoprecipitation (results not shown). Fifth, when an unrelated multi-spanning red cell membrane protein (the human glucose transporter Glut1) was co-translated with either the band 3 membrane domain, fragment (8:14)_a or fragment (9:14), Glut1 was not co-immunoprecipitated using BRIC155 (results not shown; see also Figure 7b). Taken together, these preliminary studies confirmed that cell-free translation was likely to be useful for co-translation and specific association studies with band 3 fragments.

To maximize the sensitivity with which associations might be detected, co-translations of complementary pairs of fragments were carried out at cRNA concentrations that would give an excess of the co-immunoprecipitating partner in each case. Figure 5 shows the result of co-expression of the 8 + 6 span combination. Co-translations containing a 4-fold molar excess of the b3(1:8) cRNA (lane 1) was solubilized with C₁₂E₈ and immunoprecipitated with BRIC155 (lane 2). Similarly, co-translations containing a 4-fold molar excess of (9:14) cRNA (lanes 3) was immunoprecipitated with BRIC170 (lanes 4). Quantification of these results (Table 2) showed substantial co-immunoprecipitation of the co-translated partner using either antibody. The membranes used for the BRIC170 immunoprecipitation contained a 10-fold excess of (9:14) and co-immunoprecipitated a stoichiometric quantity of (9:14) with b3(1:8). Similar results were obtained in a second experiment (results not shown). Additional immunoprecipitations confirmed that SDS/Triton solubilization disrupts the association of b3(1:8) and (9:14) in microsomes, and that individually expressed b3(1:8) or (9:14) was not detected in negative control immunoprecipitations with BRIC155 and BRIC170 respectively.

Co-translations of the 1 + 13, 3 + 11, 5 + 9 and 7 + 7 span combinations were prepared (Figure 6a), solubilized in C₁₂E₈ buffer and immunoprecipitated. In each case, the N-terminal fragment was co-immunoprecipitated with its C-terminal partner using BRIC155 (Figure 6b) and the C-terminal fragment was co-immunoprecipitated with its N-terminal partner using BRIC170 (results not shown). Similar results were obtained using both antibodies in another experiment in which the microsomes were solubilized in IP buffer containing 1.5% (v/v) Triton. Control immunoprecipitations of each N-terminal fragment using BRIC155 and of each C-terminal fragment using BRIC170 contained little or no detectable polypeptides, even after prolonged autoradiography. An additional experiment was carried out to assess the relative strength of the interactions between the various pairs of complementary fragments. Pairs of cRNAs were combined at suitable concentrations to incorporate the fragments into microsomes at three different molar proportions. Quantification of the results of immunoprecipitations in Triton buffer using BRIC155 and BRIC170 is shown in Table 2. In each case, the amount of co-immunoprecipitated material was greatest in the samples that contained the co-immunoprecipitating fragment (i.e. not containing the antibody epitope) at the highest relative abundance. These results suggest that the association of pairs of fragments is a concentration-dependent process, in common with most other protein-protein interactions.

Figure 5(b) shows the result of co-translation of the 12 + 2 span combination. Microsomal membrane pellets containing a 2-fold excess of b3(1:12) (lane 5) were immunoprecipitated with BRIC155 (lane 6) and pellets containing a 5-fold excess of (13:14) (lane 7) were immunoprecipitated with BRIC170 (lane 8). Neither sample showed detectable co-immunoprecipitation, similarly to the result from oocytes (Figure 4).

Since co-expression of b3(1:12) + (13:14) in *Xenopus* oocytes generates functional band 3 [17], we investigated further the lack of a detectable association between these fragments. First, we

Figure 7 Fusion of microsomal membranes with PEG

(a) 200 ng of b3(1:8) cRNA and 80 ng of (9:14) cRNA (per 25 μ l mixture) were translated in the rabbit reticulocyte lysate cell-free system, either as two separate translations that were pooled after termination of the reaction (lanes 1–4) or as one co-translation (lanes 5–8). Each sample (100 μ l) was divided into four equal portions and microsomes were purified by Airfuge centrifugation. Pellets were then treated either with HKS buffer (lanes 1 and 5), three cycles of $-196^{\circ}\text{C}/37^{\circ}\text{C}$ freeze-thaw (lanes 2 and 6), PEG 4000 (Boehringer) (lanes 3 and 7) or PEG1500 (Sigma) (lanes 4 and 8), resuspended, pelleted through a sucrose cushion, solubilized in IP buffer containing 2% (w/v) Triton X-100 and immunoprecipitated with BRIC155, as detailed in the Materials and methods section. Each lane contains the immunoprecipitated material from a 12.5 μ l mixture on pre-cast 4–20% Laemmli gradient gels (Bio-Rad). The relative molar expression of b3(1:8)/(9:14) from separate translations was 0.36 and from the co-translation was 0.40. (b) 330 ng of b3mem cRNA (lanes 1, 3, 6 and 8), 110 ng of (8:14)_a cRNA (lanes 4, 5, 9 and 10) and 1350 ng of Glut1 cRNA (lanes 2–4 and 7–9) were translated in 25 μ l mixtures. Microsomes were purified from 10 μ l of translation mixture, treated with PEG 4000 and immunoprecipitated with BRIC155, as described in (a). Cell-free translations (0.75 μ l of mixture) (lanes 1–5) and immunoprecipitations (lanes 6–10) were analysed on 12% Laemmli gels. Immunoprecipitations of microsomal membranes that had been treated with HKS buffer (results not shown) were identical with those treated with PEG 4000 (lanes 6–10). The relative molar expression of Glut1/b3mem (lane 3) was approx. 15 and Glut 1/(8:14)_a (lane 4) was approx. 1.4. Owing to reduced expression of b3mem and (8:14)_a when co-translated with excess Glut1, fluorographs of these immunoprecipitations were exposed 20 times (lanes 7 and 8) or three times (lane 9) longer than those from the single translations (lanes 6 and 10). No co-immunoprecipitation of Glut1 was detected in any of the samples by scanning (molar association ratio $\ll 0.1$). (c) Translations containing (per 25 μ l of mixture) either: (i) 600 ng of b3(1:3) cRNA; (ii) 400 ng of (4:14) cRNA; or (iii) 800 ng of b3(1:3) cRNA plus 300 ng of (4:14) were treated as described for (a), except that each separately translated (lanes 1–2 and 5–6) and co-translated (lanes 3–4 and 7–8) sample was divided and treated either with HKS buffer (lanes 1, 3, 5 and 7) or with PEG 4000 (lanes 2, 4, 6 and 8), followed by immunoprecipitation with either BRIC155 (lanes 1–4) or BRIC170 (lanes 5–8). Each lane contains the immunoprecipitated material from 1.5 μ l of microsomes on 12% Laemmli gels. The relative molar expression of b3(1:3)/(4:14) from separate translations was 0.30 and from the co-translation it was 0.40. (d) Translations containing (per 25 μ l of mixture) either: (i) 550 ng of b3(1:5) cRNA; (ii) 400 ng of (6:14) cRNA; or (iii) 770 ng of b3(1:5) cRNA plus 300 ng of (6:14) cRNA were treated as described for (c). The relative molar expression of b3(1:5)/(6:14) from separate translations was 0.52 and from the co-translation was 0.55. Abbreviations: (0), none detectable; ST, separate translations; CT, co-translations.

examined the effect of microsomal membrane solubilization at a lower concentration of detergent. cRNAs encoding b3(1:8) or b3(1:12) were translated in the cell-free system in the presence or absence of the cRNAs encoding their complementary partners. Microsomes were purified and solubilized in IP buffer containing either 2%, 0.5%, 0.2%, 0.1% (w/v) Triton X-100 or C₁₂E₈ and immunoprecipitated with BRIC155. In each case, samples were washed in 0.05% (w/v) detergent, which is at least 3-fold higher than the c.m.c. [28]. We found that the co-immunoprecipitation of either pair of fragments was not affected by the concentration of these detergents within this range (results not shown). These

results suggest that the interaction of b3(1:12)+(13:14) is sensitive to disruption by relatively low concentrations of detergent which do not denature the global structure of band 3.

Second, we compared the effect of a number of different detergents on the co-immunoprecipitation of b3(1:8)+(9:14) and b3(1:12)+(13:14) (results not shown). cRNAs were co-translated in the cell-free system and microsomal membranes were purified and solubilized with detergent at a concentration approximately 0.5% (w/v) greater than the c.m.c. [28]. Samples were immunoprecipitated with BRIC155 and washed in IP buffer containing detergent at 0.1% (w/v) in excess of the c.m.c. (9:14)

was immunoprecipitated with BRIC155 using any of the detergent solutions (listed below), except 0.5 % Tween 20. However, b3(1:8) was co-immunoprecipitated with (9:14) at a high level only in 0.5 % Triton, 0.5 % C₁₂E₈ or 0.5 % DDM. Little or no co-immunoprecipitation of b3(1:8) was obtained using 0.5 % dodecyl phosphocholine, 0.5 % lauroyl L- α -lysophosphatidyl choline, 0.5 % myristoyl L- α -lysophosphatidyl choline, 1.5 % octyl-D-glucopyranoside, 0.7 % nonyl-D-glucopyranoside or 0.5 % *N,N'*-dimethyl dodecylamine-*N*-oxide. Although fragment (13:14) was immunoprecipitated in the various detergent solutions similarly to (9:14), a high level of co-immunoprecipitation of b3(1:12) was not observed in any case. In the three optimal detergent systems (Triton, C₁₂E₈ and DDM), the level of co-immunoprecipitation of b3(1:12) with (13:14) was 10–20-fold lower than b3(1:8) and (9:14) in the same detergent.

PEG-mediated fusion of canine pancreatic microsomes

A further series of experiments were conducted to investigate two aspects: first, to confirm that co-immunoprecipitation results from the association of fragments within individual microsomal membranes used in the cell-free translations (closed vesicles of diameter range 100–500 nm, [33]) and not as an artefact of detergent solubilization; and second, to investigate whether two independent populations of microsomes each containing a different fragment will fuse and whether the pairs of fragments will associate post-translationally. In an initial experiment (Figure 7a), equimolar quantities of b3(1:8) and (9:14) cRNAs were either separately translated in different tubes and then combined after termination of the translation (lanes 1–4) or co-translated in a single mixture (lanes 5–8). Each sample was divided, and microsomal membranes were purified and treated either with buffer only (lanes 1 and 5), buffer and three cycles of freeze-thaw (lanes 2 and 6), 50 % (v/v) PEG 4000 (lanes 3 and 7) or 50 % (v/v) PEG 1500 (lanes 4 and 8). Membranes were then washed by centrifugation, solubilized in Triton buffer, immunoprecipitated with BRIC155 and analysed by SDS/PAGE. Similarly to Figure 5, co-immunoprecipitation of b3(1:8)+(9:14) was observed in each of the co-translated samples (Figure 7a, lanes 5–8). In contrast, the separately translated samples that had been mixed after completion of the cell-free reaction did not show co-immunoprecipitation of b3(1:8), except after treatment with PEG 4000 (lane 3) or, much less effectively, with PEG 1500 (lane 4). To confirm that treatment with PEG 4000 did not induce any non-specific co-immunoprecipitation, control experiments were performed (Figure 7b). The membrane domain of band 3 (b3mem) or fragment (8:14)_a cRNAs were co-translated in the presence or absence of an excess of Glut1 cRNA (lanes 1–5), microsomal membranes were purified, treated either with PEG 4000 or buffer only, and then co-immunoprecipitated with BRIC155 as above. Glut1 was not co-immunoprecipitated after treatment with PEG (lanes 6–10) or with buffer (identical results, not shown).

Subsequently, the PEG 4000 membrane-fusion technique was performed with the four exofacially divided pairs of fragments, using both BRIC155 and BRIC170. Results for the 3+11 and 5+9 span combinations are shown (Figures 7c and 7d) and other combinations were similar (results not shown). Scanning densitometry showed that treatment with PEG 4000 of samples that had been translated separately and then mixed resulted in at least an 8-fold increased level of co-immunoprecipitation (lanes 1–2 and 5–6); in the absence of PEG, the amount of co-immunoprecipitation in these samples (lanes 1 and 5) was not above background level. Taken together, our results indicate that band 3 fragments can associate within individual microsomes and that

co-immunoprecipitation does not result from associations that form after solubilization of membranes with detergent. In some samples, subsequent treatment of the co-translated samples with PEG 4000 further increased the molar association ratio. This suggests that each microsome may contain unequal numbers of the two fragments or perhaps only a few individual fragment molecules. This microsomal membrane fusion technique may therefore provide a useful means of increasing the association of fragments within these relatively small membrane vesicles.

DISCUSSION

Rationale for co-immunoprecipitation studies

In previous studies, we have used two criteria to investigate the assembly of pairs of co-expressed complementary fragments. First, the ability to regenerate the functional (i.e. transport active) protein, which provides compelling evidence that the fragments can insert independently into the endoplasmic reticulum membrane, associate with each other and adopt the native structure. We have shown by co-expression in *Xenopus* oocytes that cRNAs encoding pairs of complementary fragments of band 3 with discontinuities in the fourth or sixth putative intracellular loops [17] and in the third or fourth extracellular loops [16] can generate stilbene disulphonate-sensitive anion transport. However, the absence of functional activity may result from the position of the breakage (e.g. in a mechanistically important loop) rather than a failure to assemble correctly or achieve stable expression. Pairs of complementary fragments divided in the first or second extracellular loops were not functionally active, despite using a construct containing the cleavable, leader sequence of GPA at the N-terminus of each C-terminal fragment [16].

Confirmation that a non-functional fragment complex is capable of translocation to its expected final cellular destination (the plasma membrane) provides a second line of evidence for the correct membrane insertion and folding as a pseudo-native structure. Proteolytic digestion of intact oocytes [7] was used to demonstrate that this occurred with truncated fragments of band 3 [16,17] and with naturally occurring non-functional band 3 variants [34,35]. Since this method relies upon specific proteolysis of the cell surface band 3 (but not the protected intracellular band 3) at a characterized location in the third extracellular loop, the approach is restricted in applicability to polypeptides that contain this portion of the molecule. Whereas both functional complementation and cell-surface expression assays provide important means to study the assembly of polytopic membrane protein fragments, we have sought a more direct and widely applicable approach that would enable us to investigate the interactions of any combinations of fragments in internal membranes, particularly those that fail to meet the above two criteria.

Expression and stability of band 3 fragments

In the cell-free system, each of the C-terminal fragments was translated more efficiently than its complementary N-terminal partner. The extent to which the C-terminal fragment polypeptide was over-represented was broadly similar whether the fragments were translated individually or as complementary pairs. Since the cell-free system contains no mechanism for selective proteolysis of certain proteins, either differences between the nucleotide sequence of N- and C-terminal fragments or the translation of the cytoplasmic domain and/or first transmembrane span may contribute to this effect.

In contrast with the cell-free system, co-injection of oocytes with equimolar quantities of the cRNAs encoding the 1+13, 3+11, 5+9, 7+7 or 8+6 combinations resulted in expression of

the N-terminal fragment at a 2–6-fold excess over the C-terminal fragment (Table 1). Many of these fragments were expressed at a broadly similar level (less than 2-fold differences), whether the complementary partners were present or absent. However, b3(1:3) was present at a 4–6-fold lower level in the absence of (4:14) (Figure 3b) and, most strikingly, (9:14) could not be detected in the absence of b3(1:8) (Figure 2). When expressed alone, (9:14) was present at a level that was at least 50 times lower than b3(1:8) expressed alone. It seems likely that (9:14) is stabilized against proteolysis by association with co-expressed b3(1:8). This phenomenon has been reported with truncated C-terminal fragments of *Lac* permease from *Escherichia coli*, where the final six spans [21,36] or final ten spans [37] were not expressed stably *in vivo* unless some or all of the complementary N-terminal spans were co-expressed. Although we cannot be certain whether (9:14) is competent to insert into the oocyte endoplasmic reticulum, this fragment was inserted into microsomal membranes in the absence of b3(1:8) in the cell-free system. It seems likely that (9:14) is rapidly targeted for degradation as an incorrectly folded protein and that the association with b3(1:8) occludes or modifies the structure of the portion of the molecule that acts as the trigger for proteolysis. A recent paper by Popov et al. [13] has re-evaluated the transmembrane topology of band 3 using glycosylation scanning mutagenesis in the cell-free system. Their work provides evidence that Lys-743 (which is partially accessible from the cytosolic side of the membrane in the mature protein and has been assigned to the fifth cytoplasmic loop in the 14-span model of band 3) is luminal at certain stages of biosynthesis. They propose that the luminal exposure of this loop may be a feature of an intermediate in the folding pathway of band 3. It is possible that the interaction of certain parts of band 3 contained in b3(1:8) may be required for fragment (9:14) to undertake this transition to the mature folded form and that this is required for stable expression in oocytes. Since fragment (8:14)_a is expressed relatively stably in oocytes in the presence or absence of b3(1:7)_a, the proximity of span 8 and its preceding luminal loop during biosynthesis may be sufficient for maturation of this part of the band 3 molecule to proceed. Some degradation of (9:14) may still take place in oocytes co-expressing both fragments, since assembly of the fragments within internal membranes takes a finite time. The lower level of anion transport that we observed previously with this pair of fragments [17] may reflect this partial degradation of nascent (9:14) in oocytes, despite co-expression of b3(1:8).

The interaction between pairs of band 3 fragments

Since each pair of N- and C-terminal fragments was expressed at a broadly similar ratio in oocytes, we used the association ratios to gain an indication of the relative strength of interaction between the pairs of fragments. Using the BRIC155 immunoprecipitation data (Table 1), the strength of interaction between fragments may be ordered (weakest < strongest) approximately:

$$\begin{aligned} & \text{b3(1:12) + (13:14)} \ll \text{b3(1) + (2:14)} = \text{b3(1:7)}_{\text{a}} + (8:14)_{\text{a}} \\ & < \text{b3(1:5) + (6:14)} = \text{b3(1:8) + (9:14)} < \text{b3(1:3) + (4:14)} \end{aligned}$$

It is clear that b3(1) associates with (2:14) within the membrane, and hence that the integrity of the relatively short first exofacial loop is not essential for this process. Since the complex formed by these fragments was not translocated to the oocyte plasma membrane [16] the question of whether this pair of fragments is capable of generating band 3-specific anion transport remains unresolved.

In both oocytes and the cell-free system, the interaction between pairs of fragments was generally disrupted by SDS,

since co-immunoprecipitation was not observed after solubilization in SDS/Triton. The only exception to this rule was b3(1:3) + (4:14), which showed significant co-immunoprecipitation (relative molar association = 0.9) in SDS/Triton using BRIC155 and the strongest of the associations in Triton. In previous work [16], we have reported that this pair of fragments is resistant to dissociation by SDS and have speculated that this could be due to the specific interaction of two amphipathic α -helices (spans 3 and 5) that flank the second exofacial loop.

The fragments b3(1:5) and (6:14) are divided in the third exofacial loop, which contains the site (Tyr-553) at which band 3 in red cells is cleaved by chymotrypsin to yield two fragments [38]. The observation that b3(1:5) + (6:14) generated by *de novo* biosynthesis are able to associate is consistent with early studies that showed that chymotryptic fragments of band 3 prepared from red cell ghosts remained associated in non-denaturing detergent solutions [39] and that chymotrypsin digestion of red cells has no effect on band 3-mediated anion transport [40].

Surprisingly, co-immunoprecipitation of b3(1:12) and (13:14) was not detected in either oocytes or the cell-free system using Triton X-100 or C₁₂E₈. Since the co-expression of these two fragments generates a high level of band 3-mediated anion transport [17], a proportion of the two fragment populations must interact in oocyte membranes. Fragment (13:14) is of particular interest because it contains two rather short hydrophobic segments, yet in the intact protein it is accessible from both sides of the membrane. In addition, Lys-851 (span 13) and Lys-539 (span 5) comprise the extracellular cross-linking sites of 4,4'-di-isothiocyanato-2,2'-dihydrostilbene disulphonate (H₂DIDS), indicating that these two amino acid residues are in proximity (15–20 Å) within the folded structure of band 3 [41]. Span 14 contains the site of the human red cell band 3 HT variant (Pro-868 → Leu) that is associated with a structural change that causes an increased rate of anion transport and altered binding of H₂DIDS [42]. It is therefore likely that (13:14) is closely packed with the remainder of the molecule rather than located in an isolated part of the structure. We have suggested previously [17] that these short transmembrane spans could adopt an extended structure only within the assembled molecule, and an example of this phenomenon has now been reported in a membrane protein crystal structure [43]. It is possible that the final two transmembrane spans of band 3 could adopt two alternative structures with respect to the rest of the molecule. If the higher-affinity interaction between (13:14) and b3(1:12) requires the presence of e.g. non-bilayer phospholipids [44] for 'tight' binding, and these are extracted under all the detergent solubilization conditions we have examined, then the discontinuous sixth cytoplasmic loop may provide insufficient constraint for co-immunoprecipitation to be observed. The structure of the final two transmembrane spans remain one of the intriguing features of the band 3 protein.

In conclusion, this paper supports the 'two-stage model' [25], by demonstrating that pairs of band 3 fragments that have independently completed the processes of translation, insertion and folding have sufficient structure to diffuse within the lipid bilayer, interact and assemble. The PEG fusion experiments show that the pairs of fragments undergo post-translational assembly within the membrane. The results we present here and previously [16,17] concur with the findings of a recent extensive fragmentation study of bovine opsin [24]. It would appear that many pairs of complementary fragments can assemble within the membrane bilayer without undertaking major structural rearrangements. However, divisions are permitted only at certain locations in loops of limited mechanistic importance if functional activity is to be observed.

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