Complementation Studies with Co-expressed Fragments of the Human Red Cell Anion Transporter (Band 3; AE1)

THE ROLE OF SOME EXOFACIAL LOOPS IN ANION TRANSPORT*

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We constructed cDNA clones encoding fragments of band 3 in which the membrane domain was truncated from either the N or the C terminus within each of the first four exofacial loops. The truncations containing the C terminus of the protein were fused with the cleavable N-terminal signal sequence of glycophorin A to facilitate the correct orientation of the most N-terminal band 3 membrane span. Cleavage of the glycophorin A signal sequence was observed, except when the truncation was in the first exofacial loop where the signal peptidase cleavage site was probably too close to the membrane. The anion transport activity of co-expressed complementary pairs of truncations which together contained the entire band 3 membrane domain was examined. The pairs of fragments divided in the third and fourth exofacial loops yielded transport activity, but the pair separated within the second exofacial loop was not active. We conclude that the integrity of the second exofacial loop, but not the third and fourth exofacial loops, is necessary for transport activity. The unusually stable association between the fragments divided in the second exofacial loop suggests that interactions may occur between polar surfaces on amphiphilic portions of the third and fifth transmembrane spans.

The human erythrocyte anion transporter (band 3, AE1) is the most abundant integral membrane protein present in the red cell membrane (1–5). Band 3 has two domains with distinct structures and functions; the C-terminal transmembrane domain mediates anion transport, whereas the N-terminal cytoplasmic domain associates with ankyrin to anchor the erythrocyte skeleton to the membrane and also binds several structures and functions; the C-terminal transmembrane domain is necessary for transport activity. The unusually stable association between the fragments divided in the second exofacial loop suggests that interactions may occur between polar surfaces on amphiphilic portions of the third and fifth transmembrane spans.

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EXPERIMENTAL PROCEDURES

Construction of the Human Red Cell Band 3 Truncation Mutants—The constructs pBSXG1.b3 and pBSXG.GPA have been described previously (10). They contain the cDNAs encoding human red cell band 3 (AE1) and glycophorin A (GPA), respectively, flanked by the 5′- and 3′-noncoding regions of Xenopus β-globin. cDNA constructs encoding five pairs of complementary N- and C-terminally truncated band 3 mutants (Fig. 1) were prepared from pBSXG1.b3 and pBSXG.GPA by polymerase chain reaction (PCR) mutagenesis as follows.

The recombinants encoding fragments containing the N-terminal portion of band 3 (i.e. truncated at the C terminus) were constructed as described previously (9). Each antisense PCR primer annealed to the 3′-noncoding region of Xenopus b-globin. cDNA constructs encoding the fourth exofacial loops yielded transport activity, but the pair separated within the second exofacial loop was not active. We conclude that the integrity of the second exofacial loop, but not the third and fourth exofacial loops, is necessary for transport activity. The unusually stable association between the fragments divided in the second exofacial loop suggests that interactions may occur between polar surfaces on amphiphilic portions of the third and fifth transmembrane spans.
Complementation of Band 3 Fragments

Coding Glu-12 to Ala-19 in the sequence of pBSXG.GPA and incorporated an NheI restriction enzyme site (underlined) at the 3′-side of the GPA coding sequence. The sense primer in the PCR reaction annealed to a site upstream of the HindIII site in pBSXG.GPA. Both PCR products were digested with NheI and HindIII, ligated, and cloned into E. coli TG2 to yield pBSXG.ND1 (Fig. 2). The translation product of pBSXG.ND1 contained the signal peptidase cleavage site of the GPA signal sequence (between Ala-19 and Ser-20) followed by a glycine spacer before the start of the band 3-derived sequence. After signal peptidase cleavage, the resulting band 3 portion would be expected to have the N-terminal sequence Ser-Gly- followed by amino acid residues 453–911 of band 3.

pBSXG.ND1 was used to prepare the four mutants of band 3 that were truncated at the N terminus. A series of sense primers (each analogous to NtOut1-S) were used to incorporate an NheI site and a glycine codon upstream of the appropriate band 3 coding sequence. Following PCR in combination with the antisense primer Out-AS (described above), each PCR product was digested with NheI, recircularized, and cloned into E. coli TG2. After signal peptidase cleavage, the resulting band 3 fragments would be expected to have the N-terminal amino acid sequence Ser-Gly- followed by amino acid residues 482–911 of band 3 for the construct designated (4:14), residues 554–911 of band 3 for (6:14), residues 640–911 of band 3 for (8:14)a, or residues 647–911 of band 3 for (8:14)b, as shown in Figs. 1 and 2.

The cDNA coding sequences of all 10 recombinants were verified using either a DuPont Genesis 2000 automated sequencer or manually by double-stranded dideoxy sequencing with Sequenase or cycle sequencing with Taq polymerase (both from U. S. Biochemical Corp.). DNA sequencing showed that all the recombinants had the correct sequences, except that fragments (8:14)a and (8:14)b each contained a mutation that was probably introduced in the PCR step (Arg-879 → Gly in (8:14)a; Phe-705 → Ile in (8:14)b; numbering based on the intact band 3 sequence) although neither of these appeared to affect the functional activity of the constructs (see “Results”).

Cell-free Translation and Expression in Xenopus Oocytes—The methods used for in vitro transcription of cRNA with T7 RNA polymerase, cell-free translation in the rabbit reticulocyte system with canine pancreatic microsomes, purification of microsomal membranes, isolation of N-glycosylation using the acceptor tripeptide N-benzylo-asn-Leu-Thr-N-methylamide (NLT), as well as the isolation of Xenopus oocytes, injection with cRNA, chloride transport assay, 35S-amino-acid labeling, immunoprecipitation and chymotrypsin treatment of oocytes have been described previously (9–11). Samples were separated by SDS-PAGE (12) using either linear or gradient gels (4–20% acrylamide, Bio-Rad Laboratories, Hemel Hempstead, UK) and subjected to autoradiography or fluorography as detailed in the figure legends. Fragments expressed in microsomes were treated with trypsin (5 μg/ml) as described previously (9).

Radiosequencing of Expressed N-terminally Truncated Fragments—Samples of three of the expressed fragments truncated at the N terminus (2:14), (4:14), and (6:14) were prepared by cell-free translation (50-μl reaction mixture) as above. Expressed protein was labeled with l-[35S]methionine at 0.9 Ci/mM. The fragment (4:14) was double-labeled with the addition of l-[4,5-3H]leucine at 0.5 Ci/mM. In this case l-[35S]methionine was added to enhance the visualization of the bands after autoradiography. After translation, the microsomes were purified by centrifugation and solubilized, and radiolabeled proteins were separated by SDS-PAGE and electroblotted onto Problott membrane (Applied Biosystems). After autoradiography to identify the positions of the radiolabeled proteins, the bands were excised from the Problott membrane and subjected to Edman degradation on an automatic protein sequencer (Applied Biosystems 477A).

RESULTS

Preparation of Recombinants—In previous studies we showed that two pairs of complementary band 3 fragments divided in cytoplasmically oriented loops were able to assemble into an anion transport-active structure (9). To investigate whether functional complementation was a general structural feature of the protein, a series of recombinants were constructed from pBSXG1.b3 (which encodes the intact band 3 polypeptide (10)) to allow co-expression of pairs of fragments divided in exofacial loops. Each pair of fragments taken together provides all 911 amino acid residues of human red cell band 3. Fig. 1 illustrates the five complementary pairs of fragments encoded in these constructs that were separated in the first, second, third, and fourth exofacial loops. The fourth exofacial loop of band 3 contains the N-glycosylation site, and two pairs of constructs were made with the division located at the N-terminal side (b3(1:7)a and (8:14)a) and at the C-terminal side (b3(1:7)b and 8:14)b of the N-glycan addition site (see Fig. 1).

It was unclear whether the sequence information present in the most N-terminal membrane span of the C-terminal partner of each pair would be sufficient to ensure this span became oriented in the membrane with its N terminus exofacial (see “Discussion”). To drive the N termini of these band 3 fragments to the luminal side of the ER membrane, we fused the cleavable N-terminal signal sequence of glycophorin A (GPA) to the N terminus of the band 3 fragments with a short linker (Fig. 2). After translocation into the ER lumen, we expected the GPA signal sequence to be cleaved by signal peptidase at Ala-19, leaving two amino acid residues of the linker (Ser-Gly) preceding the band 3 sequence.
Expression of Band 3 Fragments in a Cell-free Translation System—The band 3 fragments were expressed from cDNAs in the rabbit reticulocyte cell-free translation system. Fig. 3 shows the first 40 amino acids of nascent (2:14). The signal sequence of fragment (2:14) is shown, together with the signal sequence cleavage site (between Ala-19 and Ser-20) in the sequence. Residue numbers shown above the sequence are those of nascent (2:14). Residue numbers below the sequence are those of the band 3 coding sequence.

Fig. 2. Map of pBSXG.ND1 and sequence of the linking region between the GPA leader and N terminus of band 3-derived portion in (2:14). A, map of plasmid pBSXG.ND1, which encodes the (2:14) band 3 construct. The preparation of the plasmid is described under “Experimental Procedures.” B, the DNA sequence in the region between the signal sequence of GPA and the band 3-containing portion of fragment (2:14) is shown, together with the NheI site (underlined) used to ligate the two PCR products (upper panel). The lower panel shows the first 40 amino acids of nascent (2:14). The signal sequence of GPA is boxed. The arrow indicates the signal sequence cleavage site (between Ala-19 and Ser-20) in the sequence. Residue numbers shown above the sequence are those of nascent (2:14). Residue numbers below the sequence are those of the band 3 coding sequence.

To establish whether the GPA signal sequence was cleaved from (2:14) and (4:14), the translation products were radioactively labeled at methionine or leucine residues. After purification the radiolabeled translation products were subjected to N-terminal amino acid sequencing. A parallel experiment was done with fragment (6:14) as a control, since the experiment in Fig. 3B demonstrated that the GPA signal sequence was cleaved from the N terminus of this fragment and that it is N-glycosylated. The results in Fig. 3B showed that (2:14) and (4:14) were N-glycosylated, but it was much more difficult to establish whether the GPA signal sequence was cleaved from these larger fragments because the cleaved and uncleaved polypeptides could not be resolved clearly from each other. The change in molecular weight observed for (6:14) in this experiment demonstrate that the signal sequence is cleaved from the N terminus of this fragment and that it is N-glycosylated. The results in Fig. 3B showed that (2:14) and (4:14) were N-glycosylated, but it was much more difficult to establish whether the GPA signal sequence was cleaved from these larger fragments because the cleaved and uncleaved polypeptides could not be resolved clearly from each other. The change in SDS-PAGE mobility resulting from the addition of the N-glycan chain (approximately 2.2 kDa) was much greater than that observed from the loss of the GPA signal sequence (approximately 1.8 kDa). This probably results from the well known anomalous SDS-PAGE mobility effects observed with integral membrane proteins. The GPA signal sequence probably behaves like an additional membrane span in the polypeptide.

Amino Acid Sequence of Uncleaved (2:14)

| 1 | 10 | 20 | 30 | 40 |
|---|----|----|----|----|
| MYGIKIFVLLS| EIVS| | | |

GPA signal sequence

signal peptidase cleavage site

Complementation of Band 3 Fragments

The series of band 3 fragments truncated at the N terminus (2:14), (4:14), (6:14), (8:14a), and (8:14b)) were also examined in the cell-free translation system (Fig. 3B). Translation was carried out (i) with microsomes, which should result in both N-glycosylation and cleavage of the GPA signal sequence; (ii) with microsomes and the NLT peptide, which should inhibit N-glycosylation but permit cleavage of the GPA signal sequence; and (iii) without either microsomes or the NLT peptide, so that neither N-glycosylation nor GPA signal sequence cleavage should occur. Since the cleavage of the GPA signal sequence from the N terminus of the band 3 fragments occurs only within the lumen of the microsomal membranes, this experiment should help establish whether the N terminus of the C-terminal band 3 fragments was correctly translocated to the luminal (exofacial) side of the membranes.

Fragment (8:14b) does not contain the band 3 N-glycan addition site and as expected did not show any difference in molecular weight when the NLT inhibitor peptide was present but did show the expected change in molecular weight resulting from cleavage of the GPA signal sequence (Fig. 3B). In contrast, (8:14a) was efficiently N-glycosylated, and the GPA signal sequence was cleaved by the microsomes. Since the N-glycan chain is at the extreme N terminus of the processed polypeptide derived from this construct, this confirms that the N terminus of the band 3 fragment produced is efficiently translocated to the luminal side of the microsomal membranes. The changes in apparent molecular weight observed for (6:14) in this experiment demonstrate that the signal sequence is cleaved from the N terminus of this fragment and that it is N-glycosylated. The results in Fig. 3B showed that (2:14) and (4:14) were N-glycosylated, but it was much more difficult to establish whether the GPA signal sequence was cleaved from these larger fragments because the cleaved and uncleaved polypeptides could not be resolved clearly from each other. The change in SDS-PAGE mobility resulting from the addition of the N-glycan chain (approximately 2.2 kDa) was much greater than that observed from the loss of the GPA signal sequence (approximately 1.8 kDa). This probably results from the well known anomalous SDS-PAGE mobility effects observed with integral membrane proteins. The GPA signal sequence probably behaves like an additional membrane span in the polypeptide.
includes some initial wash off of the intact labeled fragment from the membrane) indicates that >90% of the GPA signal sequence was cleaved.

With fragment (2:14), methionine would be expected at residue 5 of the cleaved form compared with position 1 of the uncleaved polypeptide (Fig. 4 B). The results of sequencing fragment (2:14) labeled with [35S]methionine are shown in Fig. 4B. Only about 40% of the radioactivity was released at cycle 5, with 60% of the radioactivity being released at cycle 1. This suggests that the GPA signal sequence is rather inefficiently cleaved from (2:14) with less than half of the fragment being processed by the signal peptidase. While the N terminus of correctly processed material is on the exofacial side of the membrane, we cannot be certain of the location of the N terminus of the uncleaved material, although it is likely that the N-terminal end of the band 3-containing portion forms part of an exofacial loop attached to the GPA signal sequence.

Signal peptidase-processed fragment (4:14) does not contain a methionine residue close to the N terminus but contains leucine at residue 5 (see Fig. 4C). The GPA signal sequence contains leucines at residues 9, 10, and 11. Fig. 4C shows the results of sequencing (4:14) labeled with [3H]leucine. A large peak of radioactivity was released at cycle 5, and there was a small peak released at cycle 9. The high background release of radioactivity in the later cycles of this sequencer run makes it difficult to quantify the release of radioactivity from the three leucines at residues 9, 10, and 11 of the GPA signal sequence, but the results suggest there was substantial, if not almost complete, cleavage of the GPA signal sequence from (4:14).

Taken together these results show that, with the exception of (2:14), the GPA signal sequences are cleaved in the microsomes from the fragments containing the C-terminal portions of band 3. This confirms that the N termini of these fragments are translocated to the luminal face of the microsomes. However, only a proportion of the GPA signal sequence is cleaved from (2:14).

**Proteolysis of Band 3 Fragments in Microsomes—**Trypsin proteolysis of microsomes containing the truncated constructs prepared by cell-free translation was used to assess whether they were integrated into the membrane in a manner similar to the intact protein using conditions described previously (9). Microsomes containing intact band 3 and the series of band 3 recombinant constructs truncated at the C terminus were treated in parallel with the protease (results not shown). As expected all the samples yielded the intact 43-kDa N-terminal cytoplasmic domain resulting from cleavage at Lys-360 (14), and fragments of 26, 24, and 21 kDa as well as traces of an 8.5-kDa fragment, all of which probably originate from further cleavage of the 43-kDa N-terminal cytoplasmic domain (15).

Intact band 3 also gave a 75-kDa fragment that results from cleavage within the N-terminal cytoplasmic domain (9) and the entire membrane domain comprising residues 361 to the C terminus. The b3(1) truncation did not show any additional fragment derived from the membrane-bound portion, since this portion (residues 361–432) does not contain any methionine residues and therefore does not carry any radiolabel. The constructs b3(1:3), b3(1:5), b3(1:7a), and b3(1:7b) showed a ladder of distinctive major proteolytic products of increasing size migrating at 8.5, 19, 25, and 30 kDa, which contain the portions from residue 361 to the C terminus of the constructs. The b3(1:7a) and b3(1:7b) truncations also showed a ladder of minor tryptic cleavage products at 12 and 16 kDa. As expected, the fragments derived from b3(1:7b) gave doublets from the presence of both N-glycosylated and nonglycosylated forms.

Microsomes containing the constructs truncated at the N terminus were also treated in parallel with intact band 3 (results not shown). All the samples yielded a 24-kDa band that represents the C-terminal fragment resulting from cleavage at Lys-743 (16). In the case of intact band 3 this fragment co-migrated with the 24-kDa fragment derived from the N-terminal cytoplasmic domain. A ladder of distinctive proteolytic fragments was again observed, migrating at approximately 30, 27, 24, 19, and 9 kDa for (2:14), (4:14), (6:14), (8:14)a, and (8:14)b, respectively. These represent the portions of the constructs from the N terminus to Lys-743. All the constructs except (8:14)b can be N-glycosylated and, as expected, (8:14)b gave a single band while the other fragments gave doublets resulting from the presence of both N-glycosylated and nonglycosylated forms.

These results show that the trypsin susceptibility of the constructs in microsomes is comparable with that of intact band 3. The limited protease susceptibility observed together with the results of the GPA signal peptide cleavage and N-glycosylation studies suggest that the constructs insert into membranes with the correct topology and with tightly folded structures similar to that adopted by the intact protein. However, a significant portion of the (2:14) construct retains the GPA signal sequence appended at the N terminus.

**Expression of Band 3-Mediated Anion Transport from Pairs of Fragments—**The cRNAs encoding band 3 fragments were injected into Xenopus oocytes either individually or co-injected as complementary pairs. Oocytes were co-injected with a high concentration of band 3 cRNA (15 ng/oocyte) or a molar equivalent of band 3 fragment cRNA, together with GPA cRNA (3.5 ng/oocyte). GPA is known to enhance the cell surface expression of intact band 3 (10, 11) and of complementary pairs of band 3 fragments (9) in Xenopus oocytes. After 48 h at 18 °C to allow expression, the uptake of 36Cl− into the cells was measured.
ured over a 1-h period. The band 3-specific chloride uptake was estimated from the difference in chloride uptake by the oocytes in the absence and presence of 4,4′-dinitro-2,2′-stilbene disulfonate (DNDS). The results of these experiments are shown in Fig. 5. None of the band 3 fragments induced significant DNDS-sensitive \( ^{36}\text{Cl}^- \) uptake when expressed individually with GPA. However, co-expression of equimolar amounts of the pairs of cRNAs encoding fragments b3(1:5) + (6:14), b3(1:7)a + (8:14)a, and b3(1:7)b + (8:14)b induced DNDS-sensitive \( ^{36}\text{Cl}^- \) uptake into the oocytes at levels similar to that obtained with intact band 3. In contrast, no significant DNDS-sensitive anion transport was obtained with either of the pairs of fragments b3(1) + (2:14) or b3(1:3) + (4:14). Similar results (not shown) were obtained in another experiment using the latter two pairs of complementary fragments but at lower cRNA concentrations (7.5 ng/oocyte).

To examine the rate at which stilbene disulfonate-sensitive chloride transport was expressed in the cells, oocytes were co-injected with relatively low concentrations (equimolar to 1.5 ng/oocyte of band 3) of the cRNAs encoding either intact band 3 or the three pairs of complementary fragments that had shown functional reconstitution in Fig. 5, together with 0.5 ng of GPA cRNA, and influx of chloride was measured over 15 min. The level of DNDS-sensitive chloride influx increased steadily over a period of 48-h expression of the cRNAs (data not shown). Stilbene-sensitive chloride influx of 3.6 nmol/oocyte over 15 min was obtained with intact band 3 after 48-h expression in this experiment. The stilbene-sensitive anion transport activities induced by the pairs of fragments b3(1:5) + (6:14) and b3(1:7)a + (8:14)a were comparable with that of intact band 3, suggesting that these fragments assemble with a functional activity similar to that of intact band 3. At these lower cRNA concentrations, after 48-h expression the pair of fragments b3(1:7)b + (8:14)b induced only 18% of the DNDS-sensitive chloride transport obtained with intact band 3.

The Cell Surface Expression of the Band 3 Truncations—It was important to establish whether the pairs of complementary constructs b3(1) + (2:14) and b3(1:3) + (4:14) were unable to induce DNDS-sensitive anion transport in oocytes because they could not assemble into an anion transport-active structure or because they were not translocated to the cell surface after biosynthesis. Band 3 in red cells is cleaved by chymotrypsin at a site in the third exofacial loop (Tyr-553) to yield a 60-kDa N-terminal fragment and a 35-kDa C-terminal fragment (17). The truncated band 3 constructs that contain the chymotrypsin cleavage site can be detected at the oocyte cell surface by the formation of either the 60-kDa chymotryptic product (from truncations containing the N terminus of the protein) or the 35-kDa chymotryptic product (from truncations containing the C terminus of the protein) after treating intact oocytes with chymotrypsin (9). Internally located truncated band 3 is not accessible to the protease and remains uncleaved. After expression of the cRNAs in the presence of \( ^{35}\text{S}-\text{amino-acids, chymo-} \)
Complementation of Band 3 Fragments

Chymotrypsin cleavage of fragments (2:14) and (4:14) yields the C-terminal 35-kDa fragment of band 3 that can be immunoprecipitated by BRIC 155 (Fig. 6A). When the (2:14) polypeptide was expressed alone (lane 1), a low level of cleavage was obtained (9%) similar to the background level when chymotrypsin was not used (6%). The co-injection of GPA (lane 2) or the complementary partner b3(1:5) or both GPA and b3(1:3) (lanes 3 and 4) caused only a small increase in the proportion of (2:14) that was cleaved by chymotrypsin. These results suggest that there was little or no surface expression of (2:14) when expressed alone or with GPA, and surface expression of (2:14) was not enhanced by co-expression with its complementary partner b3(1:5) or both b3(1:3) and GPA. When the (4:14) polypeptide was expressed alone (lane 5), it was cleaved by chymotrypsin to a greater extent than (2:14), indicating a significant level of cell surface expression. Cleavage was enhanced by co-expression of either GPA (lane 6) or the complementary fragment b3(1:3) (lane 7), and an even higher degree of cleavage of (4:14) was obtained when co-expressed with both GPA and the complementary fragment b3(1:3) (lane 8). The cell surface expression of (4:14) was enhanced by co-expression either of GPA or its complementary partner b3(1:3) and most markedly when both of these are also co-expressed. Interestingly, Fig. 6A shows that expression of b3(1:3), which does not contain the BRIC 155 antibody epitope, with its C-terminal partner leads to co-precipitation of b3(1:3) in BRIC 155 immunoprecipitations of (4:14) (lanes 7 and 8). Quantitation from the scanned gels (after correcting for the number of labeled methionine residues in each fragment) showed that b3(1:3) was present at a 0.9 molar ratio compared with (4:14). This demonstrates that the association between b3(1:3) and (4:14) is sufficiently stable to resist dissociation even after solubilization of the oocytes in the presence of SDS.

Chymotrypsin cleavage of b3(1:7)a and b3(1:7)b yields the N-terminal 60-kDa product that can be immunoprecipitated with BRIC 170 (Fig. 6B). Both these constructs were expressed at the cell surface at a relatively high levels even when expressed alone (lanes 1 and 5). The cell surface expression of b3(1:7)a was slightly enhanced by the presence of GPA (lane 2)

or of its complementary partner (8:14)a (lane 3), whereas co-expression of both GPA and (8:14)a increased the cell surface expression of b3(1:7)a substantially (lane 4). The cell surface expression of b3(1:7)b was not increased by co-expression of with GPA alone (lane 6), whereas co-expression with its complementary partner (8:14)b significantly increased the cell surface expression of this construct (lane 7). Co-injection of GPA in addition to (8:14)b did not appear to increase the relatively high level of cell surface expression of b3(1:7)b any further.

DISCUSSION

In this study we investigated the functional complementation of five pairs of truncated fragments separated at different points in exofacial loops of the membrane domain of band 3 as shown in Fig. 1. These regions were chosen as exofacial on the basis of current models for the topology of the band 3 polypeptide chain in the membrane (1, 2).

The constructs containing the C-terminal portion of the protein needed to be inserted into the membrane with their N
Complementation of Band 3 Fragments

Some of the N-terminally truncated constructs described in this paper, especially (2:14) and (4:14), do not have a marked charge bias across the most N-terminal span. We therefore appended the cleavable N-terminal signal sequence of GPA, with the linker sequence Ser-Gly-, at the N-terminal end of the constructs to assist the translocation of the N termini of the band 3-containing portion of the constructs to the luminal side of the ER membrane. We expected that the GPA signal sequence would then be cleaved by signal peptidase at its junction with the N terminus of the band 3 portion, creating an N-terminal sequence of Ser-Gly- followed by the appropriate band 3 fragment polypeptide sequence. The studies using SDS-PAGE and radiosequencing of the labeled constructs expressed in the cell-free system showed that all the C-terminal fragments, except for (2:14), were cleaved efficiently by signal peptidase. This confirmed that the N termini of the band 3-containing portions were translocated to the exofacial side of the membrane. Radiosequencing showed that rather less than half of the (2:14) construct was cleaved by signal peptidase. Although we interposed the Ser-Gly linker sequence between the signal peptidase cleavage site and the N terminus of the band 3-containing sequence to provide greater flexibility to this region of polypeptide chain, the signal peptidase cleavage site in the (2:14) construct is rather close to the point at which the band 3 polypeptide chain is predicted to enter the membrane (at Met-435). It seems likely that the short exofacial loop formed between the GPA leader sequence and the band 3 sequence in this construct is cleaved rather inefficiently by signal peptidase. This may be because the signal peptidase cleavage site is either too close to the membrane or the loop is insufficiently flexible for the active site of the enzyme to gain good access. The exofacial loop that would be formed between the GPA leader sequence and the band 3 sequence is much larger in the other four constructs, all of which are cleaved efficiently by signal peptidase.

The expression studies using cell-free translation and proteolysis assays using trypsin suggest that the individual fragment constructs insert into membranes with the correct topology and form tightly folded structures with a limited protease susceptibility similar to that of the intact protein. Co-expression in Xenopus oocytes using high concentrations of cRNAs demonstrated that the complementary pairs of fragments b3(1:5) + (6:14), b3(1:7)a + (8:14)a, and b3(1:7)b + (8:14)b induced stillbene disulfonate-sensitive chloride uptake into the oocytes at levels similar to that obtained with intact band 3. However, the individual fragment constructs were unable to induce DNDS-sensitive chloride uptake into oocytes. Although two of the constructs, (8:14)a and (8:14)b, each had single amino acid difference from the wild type protein (see “Experimental Procedures”), these polypeptides were able to interact with their complementary partners to yield band 3-specific anion transport in the oocytes (Fig. 5). Expression studies using low cRNA concentrations showed the anion transport activity of b3(1:7)a + (8:14)a was similar to that of intact band 3, but the anion transport activity of the pair b3(1:7)b + (8:14)b was lower than intact band 3. Although we cannot exclude the possible influence of the stated amino acid change in (8:14)b on the level of anion transport, the rather lower efficiency of glycosylation of b3(1:7)b could also be a cause of the reduced anion transport observed with this pair of constructs at low cRNA concentrations. Only the glycosylated form of b3(1:7)b was observed in the oocyte experiment in Fig. 6B (the unglycosylated form of b3(1:7)b has the same mobility on SDS-PAGE as b3(1:7)a, see Fig. 3), suggesting that the unglycosylated form of b3(1:7)b may be rapidly turned over by the oocytes. This would reduce the concentration of b3(1:7)b that is able to interact with (8:14)b and give reduced DNDS-sensitive anion transport in the oocytes. This effect is consistent with our oocyte transport data in which the reduction in DNDS-sensitive chloride influx mediated by b3(1:7)b + (8:14)b was observed only at low cRNA concentrations with chloride influx measured over 15 min and not at high cRNA concentrations with chloride influx measured over 1 h (as in Fig. 5). Under the latter conditions, the chloride influx was around its maximal value of about 10 nmol of Cl⁻/oocyte (10, 11), and this saturation of the oocyte influx assay obscures quantitative differences between the transport-active samples (discussed in Ref. 11).

Band 3-specific anion transport was not observed when the complementary pairs of fragments b3(1) + (2:14) or b3(1:3) + (4:14) were co-expressed in oocytes. Although the cell-free translation studies demonstrated that the GPA signal sequence was cleaved from only about 40% of the (2:14) construct, we might have expected co-expression of b3(1) + (2:14) to have given a small but detectable level of DNDS-sensitive anion transport from the expression of the correctly processed (2:14) with b3(1) at the oocyte cell surface. However, the chymotrypsin-cleavage studies on 35S-amino-acid-labeled oocytes demonstrated that little (2:14) was expressed at the oocyte cell surface even when its complementary partner b3(1) and GPA were also co-expressed. This was in contrast with the other truncated fragments investigated in the experiments shown in Fig. 6. We cannot conclude whether (2:14) assembles with its complementary partner into an anion transport-active structure, because of the absence of cell surface expression of (2:14) in these oocyte experiments.

In contrast, the chymotrypsin cleavage experiments showed that the (4:14) truncation was translocated to the oocyte cell surface when expressed alone, and the movement to the cell surface was increased further by co-injection either of GPA or of its complementary partner b3(1:3). Co-expression of (4:14) with both GPA and b3(1:3) enhanced the cell surface expression of (4:14) still further. B3(1:3) was co-immunoprecipitated with (4:14) in almost stoichiometric proportions, even though the samples had been treated with SDS. Co-immunoprecipitation under these conditions was not found for the other pairs of complementary fragments studied in the experiments shown in Fig. 6. These results indicate that the fragments b3(1:3) and (4:14) form a stable association with each other and suggest that the site(s) of association between these fragments has properties that are different from those that determine the association of the other complementary pairs of fragments. The resistance to dissociation by SDS also suggests that these sites are located in regions within the membrane interior. We examined the amino acid sequence of band 3 for unusual features within membrane-spanning segments that could participate in intermolecular interactions between b3(1:3) and (4:14) but are likely to be involved in intramolecular interactions in the other pairs of complementary fragments studied. The region around the pair of adjacent glutamic acid residues at amino acids 472 and 473 in putative membrane span 3 of b3(1:3) and the region around Glu-555, Lys-539, and Lys-542 in putative membrane span 5 located in (4:14) are likely candidates. Both regions are in strongly conserved portions of potentially amphiphilic α-helices and are expected to be located toward the exofacial edge of the lipid bilayer (19, 20). The two regions could interact by forming charge pairs and hydrogen bonds between the polar surfaces of the amphiphilic portions of the two α-helices. It should be possible to test this hypothesis by studying the ef-
effects of mutagenesis of residues in these regions on the associations between the fragments. Since b3(1:3) and (4:14) clearly associate with each other, the lack of anion transport activity of the b3(1:3) + (4:14) complex suggests that the integrity of the exofacial loop between putative transmembrane helices 3 and 4 of band 3 is required for the operation of the anion-transport mechanism. This may indicate a role for residues in this loop in the anion transport process, including Glu-485 and Arg-490, both of which are conserved throughout all the members of the AE gene family (19). The ability of the b3(1:5) + (6:14) and both b3(1:7) + (8:14) complementary pairs of constructs to yield DNDS-sensitive anion transport when expressed in Xenopus oocytes demonstrates that the integrity of the loops between membrane spans 5 and 6, and also 7 and 8, is not required for transport. The former result is not surprising since it is known that chymotrypsin cleavage of band 3 in intact red cells, which occurs at the site that separates b3(1:5) and (6:14), does not affect red cell anion transport (21). We have previously demonstrated that the integrity of the loops between membrane spans 8 and 9 and 12 and 13 are not required for anion transport (9).

Kalo (22) has suggested that Tyr-486 is located at the intracellular surface of band 3, contrary to the conventional models for band 3 topography (1, 2) which place this residue at the exofacial surface. The truncations used to form the b3(1:3) and (4:14) constructs are in the loop containing Tyr-486. Our data show that the N terminus of processed (4:14) is clearly exofacial when its synthesis is directed by the GPA signal sequence. The protease resistance of (4:14) and its ability to associate with b3(1:3) suggest it is not misfolded as might be expected if the N terminus of native (4:14) was intracellular. The proposal by Kalo (22) was based on the isolation of band 3 pentapeptide YIVGR radioiodinated when lactoperoxidase was applied to the cytoplasmic surface of erythrocyte membranes. However, the peptide was rather weakly labeled, and the band 3 preparation was crudely purified by gel filtration. It is possible that the radioiodinated peptide may have originated from a contaminating protein containing this sequence that was also susceptible to cytoplasmic labeling. Scanning of the Swissprot data base showed that at least four non-band 3 proteins contain the exact band 3 pentapeptide sequence, and there are a large number of proteins that contain closely related pentapeptide sequences that might behave similarly to the band 3-derived pentapeptide on chromatography.

The chymotrypsin cleavage experiments (Fig. 6) allow us to assess the movement to the cell surface of some of the fragment constructs without their complementary partners and the influence of GPA on this movement. All the fragments studied moved rather inefficiently to the oocyte cell surface when expressed alone. Although GPA enhanced the movement of the (4:14) construct to the cell surface, it had less effect on the movement of the two b3(1:7) constructs. These observations support our conclusion that the facilitating effect of GPA on band 3 movement to the surface involves the portion of band 3 containing transmembrane segments 9–12 (9). Expression of the constructs together with their complementary partners also enhanced their movement to the cell surface, but maximal cell surface expression was obtained when the constructs were expressed with both their complementary partners and GPA. This is consistent with our earlier results (9, 10) and suggests that both GPA and features in the entire band 3 molecule are required for the efficient translocation of band 3 to the cell surface.

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Complementation Studies with Co-expressed Fragments of the Human Red Cell Anion Transporter (Band 3; AE1): THE ROLE OF SOME EXOFACIAL LOOPS IN ANION TRANSPORT

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