Distict Regions of Human Glycophorin A Enhance Human Red Cell Anion Exchanger (Band 3; AE1) Transport Function and Surface Trafficking*

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Mark T. Young‡ and Michael J. A. Tanner
From the Department of Biochemistry, School of Medical Sciences, University of Bristol, University Walk, Bristol BS8 1TD, United Kingdom

Human red cell glycophorin A (GPA) enhances the expression of band 3 anion transport activity at the cell surface of Xenopus oocytes. This effect of GPA could occur in two ways, enhancement of band 3 anion transport function or enhancement of band 3 trafficking to the cell surface. We have examined the GPA effect using GPA mutants. We compared the sequences of GPA and its homolog glycophorin B (GPB; which does not facilitate band 3 cell-surface activity or trafficking) to identify candidate regions of GPA for study. We constructed several GPA or GPB mutants, including naturally occurring GPA/GPB hybrid molecules and insertion, deletion, and substitution mutants. We analyzed the effects of the mutant proteins on band 3-specific chloride transport and surface presentation using co-expression in Xenopus oocytes. We find that the C-terminal cytoplasmic tail of GPA enhances trafficking of band 3 to the cell surface, whereas the extracellular residues 68–70 increase the specific anion transport activity of band 3. In addition, examination of the oligomerization of GPA mutants showed that single amino acid substitutions N-terminal to the transmembrane domain greatly reduce SDS-stable GPA dimer formation, implying that regions outside the transmembrane domain of GPA are important for GPA dimer formation.

The red cell anion exchanger (band 3, AE1; for review, see Refs. 1–4) is a multifunctional polytopic membrane protein comprising two structurally and functionally distinct domains, a C-terminal transmembrane domain predicted to span the membrane up to 14 times and an N-terminal cytosolic domain. The transmembrane domain mediates chloride-bicarbonate exchange, whereas the cytosolic domain associates peripheral proteins with the membrane. Band 3 is present in the red cell membrane at ~1.2 x 10^6 copies/cell (5) and exists in both dimeric and tetrameric states (6).

Glycophorin A (GPA)1 is a bitopic integral membrane sialoglycoprotein that is present in the red cell in similar abundance to band 3 (for review, see Refs. 7 and 8), and there is substantial evidence for interactions between GPA and band 3 in the red cell membrane. Anti-GPA antibodies decrease the rotational mobility of band 3 in red cell membranes (9–13). Anti-Wr antibodies require the presence of both GPA and band 3 for their reactivity with red cells and co-immunoprecipitate both GPA and band 3 (10). GPA facilitates the movement of band 3 to the cell surface in Xenopus oocytes. Co-expression of crRNAs corresponding to wild-type band 3 and GPA induces an increased level of band 3-mediated chloride influx in the oocytes by facilitating the trafficking of band 3 to the oocyte surface and potentially also by enhancing the anion transport function of band 3 (14, 15). The enhancing effect of GPA on band 3 transport activity at the oocyte surface is independent of the oligomeric state of GPA; GPA mutants that are unable to form stable dimers act upon band 3 in the same fashion as wild-type GPA (16).

Other evidence also suggests an interaction between band 3 and GPA at an early stage in the biosynthesis and intracellular processing of the two proteins (1, 9, 17). The N-glycan chain of band 3 is increased in size in red cells that lack GPA (18–20) but is decreased in size in red cells that effectively have more GPA than normal (21). The sulfate transport activity of band 3 is diminished in GPA-deficient human red cells, although the amount of band 3 remains unchanged (20). The red cells of band 3 gene knock-out mice do not express either band 3 or GPA, although the erythropoietic precursors contain GPA mRNA, indicating that band 3 is also involved in the movement of GPA to the cell surface (22). In contrast to these interactions between GPA and band 3, there is no evidence that the closely related red cell glycophorin B (GPB) interacts directly with band 3. Co-expression of GPB with band 3 in Xenopus oocytes has no effect on band 3 anion transport activity (14).

GPA forms SDS-stable dimers by self-association of the transmembrane (TM) region (residues 73–95). Dimerization has been demonstrated both in detergent micelles and in bacterial membranes (23–30). Extensive mutagenesis studies (23–28, 30) show that seven residues in the GPA TM span are critical for dimer formation, and a high resolution NMR structure of the GPA TM dimer confirms this result (29). Although the NMR structure of the GPA dimer showed the transmembrane region clearly, the structure of residues 62–71 (N-terminal to the transmembrane domain) was poorly defined.

In the present study we have examined the role of different regions of the GPA molecule in its interaction with band 3 by performing an extensive mutational analysis of the GPA molecule. Detailed examination of the sequences of GPA and GPB, a molecule with a close sequence relationship to GPA that does not enhance band 3-mediated chloride transport (14), allowed identification of target regions for mutagenesis. We examined the effects of the GPA mutations on both band 3 function in

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1 The abbreviations used are: GPA, human erythrocyte glycophorin A; GPB, human erythrocyte glycophorin B; TM, transmembrane span.
**Xenopus oocytes and GPA dimerization.** We find that the cytosolic C-terminal tail of GPA is responsible for enhancing trafficking of band 3 to the cell surface, whereas the extracellular residues 68–70 of GPA (N-terminal to the transmembrane domain) have a role in increasing the specific activity of band 3 for anion transport. Our data demonstrate that the presence of GPA results in the enhancement of band 3 anion transport. In addition, we find that single amino acid substitutions N-terminal to the transmembrane domain of GPA greatly reduce SDS-stable GPA dimer formation, implying that regions outside the transmembrane domain of GPA are important for GPA dimer formation.

**EXPERIMENTAL PROCEDURES**

**Construction of GPA Mutants**—The constructs pBSXG.1.B3, pBSXG.GPA, and pBSXG.GPB have been described previously (14). They contain the cDNAs encoding human intact red cell band 3, GPA, and GPB, respectively, flanked by the 5′ and 3′-non-coding regions of *Xenopus* β-globin. The three GPA/GPB hybrid molecules MIV (GPA-(1–58)-GBP (27–72)), Sta (GBP-(1–26)-GPA-(59–131)), and Dantu (GPA-(1–97)-GBP) were also used. GPA-(1–97) has been described previously (CtDelA (14)). GPA-(1–101) was constructed by PCR amplification of the GPA-coding region with a 3′ primer-containing sequence annealing to the region of GPA from Arg-97 to Lys-101 including a stop codon and a BobEI site. The PCR product was restricted with BstXI and BobEI and ligated into BstXI- and BobEI-digested pBSXG vector. The GPA mutants H66Q, H67Q, F68C, S69C, E70C, P71C, E72C, and R97M were prepared using the Seamless Cloning kit (Stratagene) (31, 32). Two C-terminal deletion mutants of GPA were also used. GPA-(1–97) has been described previously (ClDelA (14)). GPA-(1–101) was constructed by PCR amplification of the GPA-coding region with a 3′ primer-containing sequence annealing to the region of GPA from Arg-97 to Lys-101 including a stop codon and a BobEI site. The PCR product was restricted with BstXI and BobEI and ligated into BstXI- and BobEI-digested pBSXG vector. The GPA mutants H66Q, H67Q, F68C, S69C, E70C, P71C, P72C, R73M were prepared using the Seamless Cloning kit (Stratagene) (31, 32). The Seamless Cloning kit was also used to generate the deletion constructs GPA\_Phe-68–Glu-70, GPA\_Ala-65–Glu-70, GPA\_Arg-61–Glu-70, and GPA\_Pro-41–Val-43.

**In Vitro Transcription, Cell-free Translation, and Expression in *Xenopus Oocytes***—Transcription of cRNA in vitro was performed with the mMessage mMachine kit (Ambion). The methods used for cell-free translation in the rabbit reticulocyte system with canine pancreatic microsomes, purification of microsomes, isolation of *Xenopus* oocytes, injection with cRNA, Cl− transport assay, and chymotrypsin treatment of oocytes have been described previously (14, 15, 33, 34). Where possible chloride transport studies were carried out in parallel on the same batch of oocytes as those used for the cell surface expression assays using chymotrypsin, but different amounts of the cRNAs were injected for the transport and chymotrypsin assays as indicated in the legends to Figs. 2 and 3. For the oocyte chymotrypsin assay, samples were separated by SDS-PAGE (35) and subjected to Western blotting using the ECL method as described in the figure legends. The chymotrypsin assays were done in parallel on matched groups of oocytes from the same batch (injected with band 3 cRNA alone, band 3 plus normal GPA cRNAs, band 3 plus GPA mutant cRNAs and uninjected oocytes). The quantitative data shown in Table I were obtained by scanning films exposed so that the band density was within the linear range of the film.

**RESULTS**

**Analysis of GPA and GPB Sequences for Regions Involved in Band 3 Interactions**—Fig. 1A shows a “bubble diagram” representation of the human GPA molecule in the membrane, with the C terminus at the cytosolic face. Fig. 1B shows a sequence

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**FIG. 1. Sequences of GPA, GPB, and mutant constructs.** Panel A, bubble diagram representation of GPA in the membrane. The C terminus is cytosolic. A diamond indicates the N-glycosylation site. Panel B, sequence alignment of GPA and GPB. The mature proteins are shown (without their identical 19-amino acid leader sequences). Amino acid identities are indicated with *asterisks*. The transmembrane domain of GPA is shown underlined. In Panel C, sequences of constructs used. Hyphens indicate constructs. The sequences are aligned around the GPA transmembrane span (underlined). Mutations are shown in bold. FGLC, F78L/G79C.
alignment of GPA and GPB, its close homolog. Because GPB does not enhance band 3-mediated anion transport in oocytes (14), this alignment was used to examine regions of sequence difference that could be important for the action of GPA on band 3. Analysis of the two sequences indicates four main differences. First, GPA has a 32-amino acid insertion within its extracellular N terminus (amino acids 27–58). Second, there is considerable sequence difference in the extracellular region N-terminal to the transmembrane domain (Arg-61–Glu-72 in GPA; Thr-29–Val-43 in GPB). GPB has a 3-amino acid insertion (Pro-41–Val-43) N-terminal to the transmembrane domain. Third, there are three sequence differences within the transmembrane spans of GPA and GPB (F78L, G79C, and V84I from GPA to GPB). Fourth, GPA has an additional 30-amino acid C terminus (amino acids 102–131).

Many naturally occurring hybrids of GPA and GPB in human red cells have been reported (36), and some of these molecules were also used in analysis of regions of interest on the GPA molecule. MiV (GPA-(1–58)-GPB-(27–72)), Sta (GPB-(1–26)-GPA-(59–131)), and Dantu (GPB-(1–39)-GPA-(72–131)) hybrids were constructed. Several other GPA mutants were also constructed (Fig. 1C) for this study. A double substitution mutant where two residues within the GPA transmembrane span were changed to their corresponding residues in GPA (GPA F78L/G79C) was prepared. Six amino acids from the portion of GPB N-terminal to its transmembrane domain (Val-38–Val-43) were inserted into the same position in GPA (BintoA). The 3-amino acid region Pro-41–Val-43 was deleted from GPB (GPBΔPro-41–Val-43). Two GPA C-terminal deletions (GPA-(1–97) and GPA-(1–101)), a truncation series where 3, 6, and 10 amino acids were deleted were constructed from the region of GPA N terminus to the transmembrane span (GPAΔPhe-68–Glu-70, GPAΔAla-65–Glu-70, and GPAΔArg-61–Glu-70) and several single amino acid substitutions of GPA were constructed.

Effect of GPA and GPB Mutants on Band 3-specific Anion Transport and Band 3 Surface Presentation in Xenopus Oocytes—In vitro transcribed mRNA was co-injected into Xenopus oocytes with mRNA for human erythrocyte band 3 and either wild-type GPA, wild-type GPB, GPA mutant, or GPB mutant mRNA, and the band 3-specific anion transport was estimated from the 4,4′-dinitrostilbene-2,2′-disulfonate (DNDS)-sensitive chloride uptake induced in the oocytes (Fig. 2). Chloride transport of all the mutants was measured in individual oocytes using a total of ~15 oocytes per experimental sample and in duplicate sets of experiments using different batches of oocytes. In each experiment control samples comprising oocytes injected with band 3 mRNA alone and oocytes co-injected with both band 3 and wild-type GPA mRNAs were analyzed to allow normalization of data sets. The graphs in Fig. 2 show the percentage enhancement of band 3-mediated chloride transport induced in the oocytes by co-injection of band 3 and the GPA mutant mRNAs (over basal band 3 chloride transport activity induced in the oocytes by band 3 mRNA alone) with transport due to band 3 mRNA alone set at 0% and transport due to band 3 mRNA co-injected with wild-type GPA mRNA set at 100%. Data from the transport assays are shown in Table I. Because GPA is known to increase the amount of band 3 at the oocyte surface, we also measured the proportion of band 3 protein that moved to the oocyte surface using a protease accessibility assay (33). Band 3 has a single extracellular site susceptible to extracellular chymotrypsin cleavage, which is located between transmembrane spans 5 and 6. Intact oocytes expressing band 3 and glycophorin mutants were treated with chymotrypsin, and the 35-kDa C-terminal band 3 fragment generated by extracellular cleavage of band 3 at the oocyte surface was separated from the uncleaved band 3 located within the cells using SDS-PAGE. Previous studies of the time course of chymotrypsin cleavage show that extracellular band 3 cleavage is complete under the conditions used in these experiments whether GPA is present or absent (14). Immunoblotting with a monoclonal antibody was used to detect and estimate the relative proportions of the 35-kDa fragment (derived from surface band 3) and intact band 3 (internal band 3) in the oocytes (Fig. 3). Films exposed so that the band density was in the linear range of the film were scanned, and the quantitative data are shown in Table I (the images shown in Fig. 3 are from
Band 3-Glycophorin A Interactions

Values for chloride transport are expressed as percentage enhancement of the basal level of band 3 chloride influx (% enhancement of chloride transport), with transport due to band 3 alone set at 0% and transport due to band 3 co-expressed with wild-type GPA set at 100%. Values for surface presentation of band 3 co-expressed with GPA mutants (% enhancement of surface band 3) are expressed as percentage enhancement of the basal amount of band 3 at the oocyte surface, with surface presentation of band 3 alone (9 ± 8% from 10 experiments) set at 0%. Surface presentation of band 3 co-expressed with wild-type GPA (38 ± 8% from 12 experiments) set at 100%. Average values with S.D. are shown, and the number of samples averaged is shown in parentheses. For samples with only two repeated results both numbers are shown.

### Table I

| Constructs | % enhancement of chloride transport | % enhancement of surface band 3 |
|------------|-------------------------------------|---------------------------------|
| Band 3     | 0 ± 8.6                             | 0                               |
| Band 3 + GPA | 100 ± 12.0                         | 100                             |
| Band 3 + GPB | 7 ± 2.2                            | 9*                              |
| Band 3 + MIV | −22 ± 11.0                         | −2 ± 9.2 (3)                    |
| Band 3 + Sta | 71 ± 8.4                           | 60, 75                          |
| Band 3 + Dantu | 150 ± 11.7                        | 225, 238                        |
| Band 3 + GPΔPro41–Val43 | 4 ± 0.5                      | 13*                            |
| Band 3 + GPA(1–97) | 25 ± 5.0                      | −11, −33                        |
| Band 3 + GPA(1–101) | 51 ± 12.9                  | 5 ± 9 (3)                       |
| Band 3 + GPA R97M | 133 ± 12.1                | 23, 37                          |
| Band 3 + BintoA | −3 ± 0.1                        | 117*                           |
| Band 3 + GPAΔphe68–Glut70 | 90 ± 6.0                 | 210, 158, 158                  |
| Band 3 + GPAΔAlu65–Glut70 | 18 ± 5.1                  | 100*                           |
| Band 3 + GPAΔArg61–Glut70 | 19 ± 9.1                   | 183, 140                       |
| Band 3 + F78LG79C | 80 ± 8.7                  | 143*                           |
| Band 3 + GPA H66Q | 83 ± 7.7                      | 158, 105                       |
| Band 3 + GPA H67Q | 95 ± 13.6                   | 158, 105                       |
| Band 3 + GPA F68C | 61 ± 4.1                      | 84*                            |
| Band 3 + GPA S69C | 76 ± 9.0                      | 59*                            |
| Band 3 + GPA E70C | 96 ± 10.9                    | 140*                           |
| Band 3 + GPA P71C | 104 ± 9.1                    | 83 ± 23 (4)                     |
| Band 3 + GPA E72C | 130 ± 8.0                     | 78*                            |

* Only one value was obtained for these constructs.

Co-expression of GPA increased the amount of band 3 protein at the cell surface (38% compared with 9% for band 3 alone; Fig. 3A and Table I legend) as has been described previously (14, 15). Co-expression of band 3 with GPB had little effect on either the band 3-specific anion transport or the surface presentation of band 3 protein, as has been previously observed.
Band 3-Glycophorin A Interactions

The GPA/GPB hybrid MiV showed two bands corresponding to non-glycosylated and di-glycosylated dimer. GPB is known to form homodimers and GPB-GPA heterodimers in red cells (9). However, in previous experiments, the GPA/GPB hybrid MiV showed two bands corresponding to non-glycosylated and N-glycosylated monomer (Fig. 4A, seventh lane). This indicates that GPB expressed in cell-free translation could not form SDS-stable dimers under the conditions of the experiment. A single monomer band was observed for GPB because the molecule does not contain an N-glycosylation site. The GPA/GPB hybrid MiV showed two bands corresponding to non-glycosylated and N-glycosylated monomer (Fig. 4A, second lane) but no dimer bands. MiV did not form an SDS-stable dimer in cell-free translation because it

Glycosylated monomer, and the upper band, representing the more abundant N-glycosylated monomer. Three SDS-stable dimer bands were visible, representing non-glycosylated dimer, mono-glycosylated dimer, and di-glycosylated dimer. The top band (di-glycosylated dimer) predominated. GPB is known to form homodimers and GPB-GPA heterodimers in red cells (9). However, only one band corresponding to the GPB monomer was observed in our cell-free translation experiments, (Fig. 4B, seventh lane). This indicates that GPB expressed in cell-free translation could not form SDS-stable dimers under the conditions of the experiment. A single monomer band was observed for GPB because the molecule does not contain an N-glycosylation site. The GPA/GPB hybrid MiV showed two bands corresponding to non-glycosylated and N-glycosylated monomer (Fig. 4A, second lane) but no dimer bands. MiV did not form an SDS-stable dimer in cell-free translation because it

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contains the GPB transmembrane span. The two GPB/GPA hybrids Sta and Dantu showed two bands corresponding to monomer and dimer (Fig. 4A, third and fourth lanes). These constructs lack the extracellular N-glycosylation add-on site since they possess the GPB N terminus but contain the GPA transmembrane span and, thus, can form SDS-stable dimers. The double substitution mutant F78L/G79C showed partial SDS-stable dimer formation (Fig. 4A, fifth lane). The GPA insertion mutant BintoA (the two GPA C-terminal deletion mutants GPA-(1–97) and GPA-(1–101), and the three substitution mutants GPA H66Q, GPA H67Q, and GPA R97M all showed wild-type levels of dimer formation (Fig. 4, A and B). SDS-stable dimer formation was markedly reduced after 6 or 10 amino acids were deleted from the region of GPA N-terminal to the transmembrane span (GPAAla-65–Glu-70 and GPAΔArg-61–Glu-70; Fig. 4B, fifth and sixth lanes). This result was of interest as it clearly shows that regions outside the GPA transmembrane span may affect SDS-stable GPA dimer formation. Dimer formation may have been affected either by loss of important interacting residues or, for example, by the charged cluster of three glutamic acid sequences (Glu-55–Glu-57) in the N-terminal region of each GPA monomer being brought too close together in the dimer. Deletion of the three-amino acid insertion in GPB (GPBDPro-41–Val-43; Fig 4C, eighth lane) did not give rise to any detectable SDS-stable dimer formation. The GPA single substitution mutants F68C, S69C, P71C, and E72C all exhibited substantially reduced SDS-stable dimer formation compared with wild-type GPA. It appears that single substitution of these residues for cysteine may affect SDS-stable dimer formation, implying that this region of the GPA molecule N-terminal to the transmembrane span is involved in dimer formation. In contrast GPA E70C formed a similar proportion of SDS-stable dimers as wild-type GPA (Fig. 4C). Because single cysteine mutants potentially could be capable of forming a disulfide bridge in a dimer, the samples were also separated by SDS-PAGE under non-reducing conditions, but no increase in dimer formation was observed (results not shown).

**DISCUSSION**

GPA enhances both the anion transport function and the movement of band 3 to the cell surface. We have carried out an extensive mutational study of GPA to understand which regions of the molecule are involved in the above functions by co-expression of GPA mutants with band 3 in *Xenopus* oocytes and analysis of the effects of the mutants on band 3 anion transport and surface presentation. GPA also forms an SDS-stable dimer, and we have expressed GPA mutants using cell-free translation into canine pancreatic microsomes to establish that they insert efficiently into the endoplasmic reticulum with the correct orientation and examine their ability to form SDS-stable dimers.

**Different Regions of the GPA Molecule Have Different Effects on Band 3 Anion Transport Functional Activity and Band 3 Protein Movement to the Cell Surface**—There are four main regions of sequence difference between GPA and GPB (see “Results”). Because GPB appears to have no significant effect on either band 3 anion transport function or surface expression, some or all of the four main regions of sequence difference in GPA must be involved in the effects of GPA on band 3.

**The Cytoplasmic C Terminal of GPA Is Involved in the Enhancement of Band 3 Protein Movement to the Cell Surface**—Deletion of the cytoplasmic C terminus of GPA led to the loss of ability to increase band 3 movement to the cell surface, but these mutants retained the ability to enhance band 3-specific chloride transport. The single point mutation GPA R97M (located on the cytoplasmic side of the TM domain) also exhibited an impaired ability to increase the surface presentation of band 3 but efficiently enhanced band 3 anion transport activity. These results imply that the cytoplasmic C terminus of GPA, including the region close to the TM domain, is responsible for enhanced trafficking of band 3 protein to the cell surface. The GPA-mediated enhancement of band 3 surface presentation could be achieved either by direct interaction of the GPA C terminus with band 3 or by interaction of the GPA C terminus with other proteins that may facilitate band 3 trafficking. Because the C-terminal GPA deletions retain the ability to enhance band 3 anion transport functional activity, it is clear this activity of GPA resides in the remaining part of the GPA molecule.

**Extracellular GPA Residues 61–70 Are Involved in the Enhancement of Band 3 Anion Transport Activity**—The region of GPA encompassed by the extracellular amino acid residues Arg-61–Glu-70 is thought to form a shared antigen epitope with residue 658 on band 3 (the Wrβ antigen; (13)). Insertion or deletion of residues within this region severely reduced the ability of the GPA mutants to enhance the anion transport function of band 3 without affecting their ability to increase the movement of band 3 to the cell surface. In fact, deletion of Phe-68–Glu-70 reduced enhancement of band 3-specific chloride transport by 80%, whereas the insertion of the 5 residues of GPB in this region (BintoA, Fig. 1) showed normal GPA enhancement of band 3 movement to the cell surface without a similar large enhancement of band 3 anion transport activity above the basal level. Although the transport and cell surface expression assays are not directly comparable, these results suggest that these GPA mutations inhibit the transport activity of band 3 relative to the situation where GPA is absent.

Examination of the sequence in this region of the GPA/GPB hybrids Sta and Dantu (which both behave in the same way as wild-type GPA on band 3) shows that in Sta this sequence is FSE, and in Dantu this sequence is FTV, with residue Phe-68 in common. The mutant GPA F68C is somewhat impaired in its ability to enhance band 3 anion transport (61% enhancement). These observations suggest that residue Phe-68 of GPA may be important in the interaction between GPA and band 3, which results in enhanced band 3 anion transport activity. We conclude that the portion of GPA responsible for mediating the enhancement of band 3 anion transport activity (independently of effects on band 3 movement to the surface) is within the Phe-68–Glu-70 extracellular region N-terminal to the GPA transmembrane span. This is located in the region of the Wrβ antigen, which is implicated by other data (10–13) to be involved in interactions between GPA and band 3.

Our observations that the presence of GPA results in the enhancement of the anion transport functional activity of band 3 confirm earlier results that showed that human red cells that lack GPA (and GPB) contain the normal number of band 3 molecules but have only 60% normal anion (sulfate) transport activity (20). The lower transport activity resulted from an increase in the apparent $K_{\text{s}}$ of the band 3 for sulfate. The band 3 in these cells has an abnormally long N-glycan chain, suggesting that there is an intracellular effect of GPA on band 3 during the biosynthetic pathway of the two proteins (20). GPA may facilitate the maturation of newly synthesized band 3 into a fully transport-active structure either directly or by recruiting other components that aid this process (16, 20). It is not clear at present whether GPA also has a direct effect in increasing the transport activity of mature band 3 already present at red cell surface. The region around Phe-68–Glu-70 of GPA clearly has a major role in these effects on the transport activity of band 3.

**The N Terminal and Transmembrane Domain of GPA**—The GPA/GPB hybrid MiV (GPA-(1–58)-GPB-(27–72)) behaved in
the same fashion as GPB, indicating that the region of GPA from amino acids 1–58 is not involved in the band 3-GPA interaction. The two GPB/GPA hybrids Sta (GPB-(1–26)-GPA-(59–131)) and Danu (GPB-(1–39)-GPA-(72–131)) both enhanced band 3 presentation at the cell surface. There is a high degree of homology between amino acids 26 and 39 of GPB and 59 and 72 of GPA (Fig. 1B) and therefore portions of this region of GPA may still be important for the band 3-GPA interaction. Double substitution in the GPA transmembrane span (F78G79→LC) had no effect on the ability of GPA to interact with band 3, and we conclude from this that the membrane-spanning region of GPA is unlikely to be involved in the GPA-band 3 interaction.

### The Site of Interaction between Band 3 and GPA on the Band 3 Molecule

The above results show that the extracellular portion of the GPA molecule next to the plasma membrane is involved in enhancing band 3 anion transport function independently of effects on band 3 trafficking to the cell surface. Because this region is within the Wt<sup>a</sup> region of the protein, the site of interaction between band 3 and GPA on the band 3 molecule is likely to be close to the region of band 3 involved in the Wt<sup>e</sup> epitope, band 3 residue Glu-658 (15). According to recent topology models of band 3, Glu-658 is positioned at the extracellular end of transmembrane span 8 (4). It has also been observed that GPA enhances the surface movement of C-terminal fragments of band 3 but not the N-terminal fragments of band 3 (37). Further evidence suggests that the minimum fragment of band 3 on which GPA may act to enhance band 3 surface presentation is the portion encompassing putative TM 9–12 (residues Met-696–Tyr-824; (37)). The band 3 mutant G701D is totally dependent upon GPA for its cell-surface expression in oocytes (38, 39). Band 3 Gly-701 is located at the cytoplasmic face of TM 9. It is conceivable that the band 3 mutation G701D leads to local misfolding of band 3 around TM 9, which GPA may refold by a chaperone-like effect. However, it is also possible that band 3 G701D is more generally misfolded and that GPA is able to rectify this problem in some other fashion.

### Regions of the GPA Molecule N-terminal to the Transmembrane Span Influence GPA Dimer Formation—Cell-free translation of the GPA mutants showed that deletion of the extra-cellular six-amino acid region Ala-65–Glu-70 led to a large reduction in SDS-stable GPA dimer formation. This reduction could be explained in terms of the deletion bringing the N-terminal domains of the two GPA monomers too close together, causing steric hindrance and dissociation of the dimer. The fact that significant reduction in SDS-stable dimer formation was also observed for the four GPA substitution mutants F68C, S69C, P71C, and E72C suggests that for these mutants at least steric hindrance was not the reason for reduced dimer formation. A previous study using the GPA transmembrane domain fused at its N terminus to staphylococcal nuclease investigated the effect of truncating residues N-terminal to the transmembrane domain on SDS-stable dimer formation (30). The study employed the portion of GPA including residues Glu-60–Ile-99 in the fusion protein with staphylococcal nuclease. SDS-stable dimer formation was significantly reduced upon truncation of the N-terminal 9 residues (Glu-60–Phe-68) or 11 residues (Glu-60–Glu-70). The addition of two alanine residues N-terminal to the nine-amino acid truncation did not significantly restore dimer formation, but the addition of four alanine residues N-terminal to the 11-amino acid truncation did. The authors concluded that the region His-67–Glu-70 was acting simply as a flexible linker between the GPA transmembrane span and the staphylococcal nuclease fusion (30). The effect of residues Pro-71 and Glu-72 upon GPA dimer formation has not been tested previously. Because our data indicate that both deletions and single substitutions within the region of Ala-65–Glu-72 of GPA affect SDS-stable dimer formation of the full-length protein, we infer that this region of the GPA molecule is also important for GPA dimer formation. The GPA transmembrane span is capable of forming dimers without the presence of the adjacent extracellular region of the protein, but in the full-length protein it appears that the amino acid sequence of this region is critical for dimer formation. This result has implications for in vitro studies of GPA dimer formation.

It is also interesting that although the SDS-stable dimer formation of the double substitution mutant F78L/G79C was impaired, it was not completely abolished. Mutants of GPA where Gly-79 alone is changed to Leu, Val, or Thr exhibit essentially no dimer formation (16, 23). It is possible that G79C is a more stable substitution or that F78G79→LC partially compensates for the deleterious G79 mutation. The GPA transmembrane span, which exhibits no SDS-stable dimer formation in our assay but has been shown to form dimers when extracted from red cell membranes (9), has two further amino acid differences compared with the F78L/G79C mutant (Thr-741 and Val-841). This suggests that GPA dimer formation is significantly weaker than GPA dimer formation when expressed in endoplasmic reticulum microsomes because of the dual effect of the FG→LC double mutation and the two further sequence differences in GPA. Our results also provide further confirmation of other data indicating that the oligomeric state of GPA has no bearing on its ability to interact with band 3 (16), as the GPA mutants S69C, P71C, and E72C all exhibit markedly reduced SDS-stable dimer formation but still enhance the expression of band 3-specific chloride transport in oocytes in a similar manner to normal GPA.

In summary, we have used site-directed mutagenesis to analyze the role of GPA in band 3 anion transport activity and cell surface expression, and our results lead to two main conclusions; first, that the role of the cytoplasmic C terminus of GPA is to enhance the cell surface movement of band 3 either by direct interaction with band 3 itself or by interaction with other proteins; second, that the extracellular region including residues 68–70 of GPA close to the TM span is at least partially responsible for enhancement of band 3 anion transport function activity, and this result leads to the conclusion that the Wt<sup>e</sup> antigen is a structurally and functionally important area of the GPA-band 3 interaction. Our results provide further evidence that GPA increases band 3 anion transport functional activity, consistent with previous observations that band 3-specific anion transport was reduced in glycoporphin-deficient cells (20). These data also indicate that when expressed in oocytes, GPA acts upon band 3 in the same fashion as when physiologically expressed in red cells to both increase the anion transport activity of band 3 and band 3 trafficking to the cell surface. Finally, using cell-free translation experiments we provide evidence that the extracellular portion of GPA adjacent to the TM span (encompassing residues 61–72) is involved in dimer formation of intact GPA in addition to the transmembrane region.

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