Red-cell glycophorin A–band 3 interactions associated with the movement of band 3 to the cell surface

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We have examined the mechanism by which glycoporphin A (GPA) facilitates the movement of the human red-cell anion exchanger (band 3, AE1) to the cell surface. GPA itself forms stable dimers in membranes and detergent solution. Four mutants of human GPA with impaired dimerization were prepared (L75I, I76A, G79L and G83L). All four GPA mutants enhanced band 3 translocation to the *Xenopus* oocyte plasma membrane in the same way as wild-type GPA, showing that the GPA monomer is sufficient to mediate this process. Cell-surface expression of the natural band 3 mutant G701D has an absolute requirement for GPA. GPA monomers also rescued the cell-surface expression of this mutant band 3. Taking into account other evidence, we infer that the site of GPA interaction with band 3 is located outside the GPA dimerization interface but within the GPA transmembrane

span. The results of examination of the cell-surface expression of GPA and band 3 in different K562 erythroleukaemia cell clones stably transfected with band 3 are consistent with the movement of GPA and band 3 to the cell surface together. We discuss the pathways by which band 3 moves to the cell surface in the presence and the absence of GPA, concluding that GPA has a role in enhancing the folding and maturation of band 3. We propose that GPA functions in erythroid cells to assist with the incorporation of large amounts of properly folded band 3 into the membrane within a limited time span during erythroid maturation.

Key words: anion exchanger, anion transport, dimerization, translocation.

INTRODUCTION

The red-cell anion transporter (band 3, AE1) (reviewed in [1–4]) is a multifunctional integral membrane protein that is present at approx. 1.2×10^6 copies per cell [5]. Band 3 is made up of two structurally and functionally distinct domains. The N-terminal 43 kDa cytoplasmic domain is involved in a number of functions unrelated to anion transport such as the binding to peripheral membrane proteins, including the red-cell cytoskeleton via ankyrin [6]. The C-terminal 52 kDa membrane domain (residues 360–911) is responsible for anion transport and remains functional either when translated from a cDNA clone encoding the isolated membrane domain [7,8] or after proteolytic removal of the cytoplasmic domain [9,10]. In the red-cell membrane, intact band 3 exists as dimers and tetramers [11], whereas the membrane domain alone can only form dimers. The membrane domain of band 3 is both necessary and sufficient for anion transport and self-association to a dimeric state.

Glycophorin A (GPA) is a bitopic integral membrane sialoglycoprotein that is present in the red cell at a similar abundance (10^6 copies/cell) to band 3 (reviewed in [12,13]) and there is 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 [14,15]. Anti-Wr^b antibodies require the presence of both GPA and band 3 for their reactivity with red cells [16–20] and co-immunoprecipitate both GPA and band 3 [18]. These particular antibodies seem to stabilize the interaction between band 3 and GPA because most anti-GPA or anti-(band 3) antibodies do not co-immunoprecipitate both proteins from red-cell membranes. These observations suggest that although band 3 and GPA interact in the red-cell membrane, the complex is not stable under the conditions and time scale of an immunoprecipitation experiment.

Evidence is accumulating that GPA has a role in facilitating the movement of band 3 to the cell surface. Co-expression of band 3 cRNA with GPA cRNA in *Xenopus* oocytes induces an increased level of band 3-mediated Cl[−] transport in the oocytes by facilitating the translocation of band 3 to the oocyte surface [8,21]. Enhancement of cell-surface expression of band 3 by GPA has also been demonstrated by co-transfection experiments in the yeast *Saccharomyces cerevisiae* [22].

Some cell-surface expression of human band 3 is observed in *Xenopus* oocytes in the absence of GPA when high concentrations of band 3 cRNA are employed. The cell-surface expression of band 3 has also been reported in transfected mammalian cell lines lacking GPA [23,24]. These observations show that band 3 can also move to the surface by a GPA-independent pathway, albeit less efficiently than when GPA is present. Band 3 is also expressed at the basolateral membrane of the α -intercalated cells in the distal tubules and collecting ducts of the kidney [25,26]. It moves to the cell surface by a GPA-independent route in the renal distal nephron, because there is no evidence for the expression of GPA in the kidney [27]. Recently the human band 3 mutation G701D has been associated with recessive familial distal renal tubular acidosis, a defect in the kidney acid secretion system that involves band 3 [27,28]. The mutant band 3 did not induce any band 3-specific anion transport in *Xenopus* oocytes when GPA was absent. However, co-expression of the mutant band 3 with GPA resulted in the rescue of anion transport activity in oocytes to levels similar to that achieved with the normal protein. Individuals with the disease had normal levels of red-cell band 3 and the condition probably arises from the lack of cell-surface expression of band 3 in the kidney.

Other evidence also suggests an interaction between GPA and band 3 at an early stage in biosynthesis and intracellular processing of the two proteins (reviewed in [2,4,17]). Thus the N-

Abbreviations used: GPA, glycoporphin A; GPB, glycoporphin B; TM, transmembrane.

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glycan chain of band 3 is increased in size in red cells that lack GPA [29–31] but is decreased in size in red cells that effectively have more GPA than normal [32]. The anion transport activity of band 3 is diminished in GPA-deficient human red cells, although the amount of band 3 remains unchanged [31]. The red cells of band 3 gene knock-out mice do not express either band 3 or GPA, although the erythroid precursors contain GPA mRNA, indicating that band 3 is also involved in the movement of GPA to the cell surface [33].

GPA forms stable dimers by self-association of the transmembrane (TM) region. Dimerization of the GPA TM domain has been demonstrated in detergent micelles and in bacterial membranes [34–40]. Extensive mutagenesis studies [34–39] have shown that seven residues in the GPA TM span are critical for dimer formation: Leu⁷⁵, Ile⁷⁶, Gly⁷⁹, Val⁸⁰, Gly⁸³, Val⁸⁴ and Thr⁸⁷. A high-resolution structure of the GPA TM dimer obtained by NMR spectroscopy confirms this result [40].

In the present study we have examined the role of GPA dimers in the movement of band 3 to the cell surface in *Xenopus* oocytes. We find that dimerization-defective GPA mutants retain the ability to facilitate the translocation of normal and mutant band 3 to the surface of *Xenopus* oocytes, showing that monomeric GPA is sufficient to enhance the translocation of band 3 to the plasma membrane. These results indicate that the site of interaction of GPA with band 3 is different from the region involved in the formation of GPA dimers. Our results are also consistent with the view that GPA moves to the cell surface together with band 3 in transfected human erythroleukaemic cells. We discuss the involvement of GPA in the cell-surface movement of band 3 and suggest that the role of GPA in the red cell is to enhance the rate of folding and maturation of band 3 during band 3 biosynthesis in the maturing erythroid cell.

MATERIALS AND METHODS

Construction of GPA and band 3 mutants

The constructs pBSXG1.b3 and pBSXG.GPA have been described previously [8]. They contain the cDNA species encoding human red-cell band 3 and GPA respectively, flanked by the 5'- and 3'- non-coding regions of *Xenopus* β -globin. The GPA mutants L75I, I76A, G79L and G83L and the band 3 mutant B3G701D were prepared with the Seamless Cloning kit (Stratagene) [41,42]. The primers used for mutagenesis are shown in Table 1. The cDNA coding sequences of all five mutants were verified by either an automatic DNA sequencer or manually by double-stranded dideoxy sequencing with Sequenase (U.S. Biochemical Corp.).

Table 1 Primers used for mutagenesis

Primer	Type	Sequence
GPA L75I	Sense	5'-AGTTACTCTCAATCATTATTTTGGGGTGATG-3'
	Anti-sense	5'-AGTTACTCTTCAGATTGTTATCTCTGGTTCAGAG-3'
GPA I76A	Sense	5'-AGTTACTCTCAAGCTATTTTGGGGTGATGGCT-3'
	Anti-sense	5'-AGTTACTCTTCAGCTAGTGTATCTCTGGTTCAG-3'
GPA G79L	Sense	5'-AGTTACTCTCACTAGTGATGGCTGGTGTATT-3'
	Anti-sense	5'-AGTTACTCTTCATAGAAAAAATGAGTGTATCTC-3'
GPA G83L	Sense	5'-AGTTACTCTCACTAGTTATTGGAACGGATCCTC-3'
	Anti-sense	5'-AGTTACTCTTCATAGACCATCACCCAAAAAT-3'
B3 G701D	Sense	5'-AGTTACTCTCAGACTCCACCTGGACCTG-3'
	Anti-sense	5'-AGTTACTCTTCAGTCGGAGCCCTTGACCAT-3'

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, ³⁵S-amino acid labelling, immunoprecipitation and chymotrypsin treatment of oocytes have been described previously [8,21,43]. Samples were separated by SDS/PAGE [44,45] on linear gels and subjected to fluorography.

RESULTS

Dimerization of intact GPA polypeptides carrying mutations in the TM domain

We have examined the effects of mutations that disrupt GPA dimerization. Figure 1 (upper panel) indicates the location of the dimer interface on the TM helix of GPA [34–39]. Four GPA

Figure 1 Dimerization of GPA and GPA mutants

Upper panel: helical net diagram of the GPA TM span. The dimer interface is enclosed in the box marked 'dimer'. Filled circles indicate residues mutated for this study. Lower panel: cell-free translation of GPA and GPA mutants in the presence of microsomes. The microsomes were purified and dissolved in SDS sample buffer containing 10% (v/v) 2-mercaptoethanol and subjected to PAGE [12% (w/v) gel] [45]. Lane a, normal GPA; lane b, GPA mutant L75I; lane c, GPA mutant I76A; lane d, co-translated mutants L75I and I76A; lane e, co-translated normal GPA and L75I; lane f, GPA mutant G79L; lane g, GPA mutant G83L. The position of the glycosylated GPA monomer (glyc. mon.), unglycosylated GPA monomer (monomer) and partly resolved mixture of glycosylated and unglycosylated dimers (dimer) are indicated. The percentage of dimer (% dimer) was estimated by using the sum of glycosylated and unglycosylated monomers to represent the total monomer, and the mixture of glycosylated and unglycosylated dimers to represent the amount of dimer.

Figure 2 Anion transport activity of band 3 with GPA and GPA mutants

³⁶Cl⁻ influx over a 1 h period was measured with groups of 10–15 oocytes in Barth's saline or in Barth's saline containing 400 μ M 4,4'-dinitrostilbene-2,2'-disulphonate (DNDS), as described previously [8]. The bars represent the DNDS-sensitive Cl⁻ influx per oocyte over 1 h; the error bars show the S.E.M. of the influx measurements. (A) *Xenopus* oocytes were injected with 0.4 ng of band 3 (B3) cRNA per oocyte, with or without 0.04 ng of GPA cRNA per oocyte or 0.04 ng per oocyte of the mutant GPA indicated in the label. (B) Oocytes were injected with 0.2 ng B3 cRNA per oocyte and various amounts of GPA (A) or GPA mutant G83L (B3) cRNA. The concentration of GPA or GPA G83L cRNA used (ng of cRNA per oocyte) is indicated in parentheses in the label to each bar.

mutations (L75I, I76A, G79L and G83L) were prepared for study. The effects of mutations at these residues on GPA dimerization have been examined previously, by using fragments of GPA containing the TM domain fused with other proteins. One group of studies assessed dimerization in SDS [34,37], whereas the other studies measured dimerization in the *Escherichia coli* inner membrane [35,38,39]. GPA TM mutant G83L did not dimerize in either SDS or membranes; I76A and G79L did not dimerize in SDS but partly dimerized in membranes; and L75I partly dimerized in SDS and in membranes.

We confirmed the effects of these mutations on dimer formation of the complete GPA polypeptide by translating cRNA species corresponding to wild-type GPA and the four GPA mutants in the rabbit reticulocyte lysate system with microsomes. The [³⁵S]methionine-labelled GPA in the purified microsomes was separated by SDS/PAGE to resolve the monomer and dimer forms (Figure 1, lower panel). The proportion of dimer present in each sample was estimated by scanning the fluorographs of the gels (Figure 1, lower panel). GPA is not quantitatively N-glycosylated in the cell free-system and some unglycosylated GPA monomer is also evident in Figure 1 (lower panel). A substantial fraction (31%) of normal GPA was present as a dimer under these conditions but little or no dimer was observed with any of the GPA mutants. These results agree with previous results on the dimerization in SDS of GPA fusion proteins containing the same mutations in the TM span [34,37]. They demonstrate that disruption of dimerization also occurs with these GPA mutants in the context of the complete GPA polypeptide chain.

Mixtures of normal GPA and GPA L75I yielded substantially more than half the dimer obtained from normal GPA alone [78% relative to normal GPA (Figure 1, lower panel)]. This shows that normal GPA and GPA L75I heterodimers form, but at a lower efficiency than normal GPA homodimer formation. When mixed together, L75I and I76A yielded more dimer than when each mutant was examined separately (Figure 1, lower panel), showing that even though the two mutant proteins cannot individually form homodimers, they have the ability to

form heterodimers. These results confirm previous reports of the formation of mixed dimers between the TM domains of normal GPA and a different GPA L75V mutant (L75V), and heterodimer formation between GPA L75V and GPA I76A [34].

Effect of GPA mutants on band 3-specific anion transport induced in *Xenopus* oocytes

Xenopus oocytes were co-injected with cRNA species coding for human red-cell band 3 and either normal GPA or each of the four GPA mutants. The stilbene disulfonate-sensitive ³⁶Cl⁻ uptake induced in the oocytes was measured to estimate the band 3-specific anion transport induced in the oocytes (Figure 2). Most previous studies on the enhancement of band 3-specific anion transport by GPA in oocytes have employed equal weights of band 3 cRNA and GPA cRNA [8,43]. However, the enhancing effect of GPA on band 3-dependent anion transport is decreased significantly only when the ratio of GPA cRNA to band 3 cRNA is less than 1:10 (w/w) [21]. This does not imply a catalytic effect of GPA on the induced band 3-dependent anion transport. Not only is band 3 cRNA 4.2 times larger than GPA cRNA, but also more molecules of the small GPA protein are translated in the oocytes in the same period than are those of the much larger band 3. Expression of a 1:10 (w/w) ratio of GPA cRNA to band 3 cRNA probably results in similar molar quantities of the two proteins being produced in oocytes; these conditions were employed for our initial experiments. Measurement of the band 3-specific Cl⁻ transport induced in the oocytes (Figure 2A) showed that all the GPA mutants with impaired dimerization were able to enhance band 3-specific anion transport activity induced in the oocytes to the level obtained with normal GPA. The same result was obtained in four separate experiments employing a range of different ratios of GPA cRNA to band 3 cRNA.

It was possible that, even at the low GPA cRNA concentrations used in these experiments, a sufficiently high concentration of GPA protein was synthesized by the oocytes to drive the dimerization of the GPA mutants. We therefore compared

Figure 3 Effects of normal and mutant GPA on amount of oocyte cell-surface band 3

Xenopus oocytes were injected with 0.4 ng of band 3 (B3) cRNA per oocyte with or without 0.04 ng of normal or mutant GPA cRNA per oocyte. The oocytes were pulse-labelled with ^{35}S -labelled amino acids for 24 h at 18 °C, then chased with unlabelled amino acids for 24 h. Groups of oocytes were treated with chymotrypsin for 1 h at 4 °C and then immunoprecipitated with antibody BRIC 155 (against the C-terminus of band 3), as described previously [8]. Each lane represents the immunoprecipitated protein from five oocytes, separated by SDS/PAGE [10% (w/v) gel] [44]. The percentage of band 3 that was cleaved by chymotrypsin (to yield the 35 kDa C-terminal fragment) was determined as described previously [8]. Results are corrected for the presence of 13 methionine and 2 cysteine residues in the 35 kDa fragment and 24 methionine and 5 cysteine residues in intact band 3. Lane a, control oocytes injected with water; lane b, band 3 alone; lane c, band 3 plus normal GPA; lane d, band 3 plus GPA L75I; lane e, band 3 plus GPA I76A; lane f, band 3 plus GPA G79L; lane g, band 3 plus normal GPA; lane h, band 3 plus GPA G83L. The samples from lanes a–f and lanes g and h were derived from two different experiments. B3 and 35 kDa indicate the positions of intact band 3 and the 35 kDa C-terminal chymotryptic fragment of band 3 respectively.

the effects of a wide range of mutant and normal GPA cRNA concentrations, using a constant concentration of band 3 cRNA (Figure 2B). The GPA G83L mutant was employed in this experiment because mutations at this residue disrupt dimerization in membranes as well as in SDS [34,35,38,39]. A range of GPA cRNA concentrations of 0.2–0.0008 ng per oocyte was tested by using 0.2 ng of band 3 cRNA per oocyte (range in ratios of GPA cRNA to band 3 cRNA from 1:1 to 1:250). Normal GPA cRNA used at the lowest concentration induced very little stilbene disulphonate-sensitive Cl^- transport additional to that obtained when GPA cRNA was omitted (Figure 2B). GPA G83L gave the same enhancement of band 3-specific Cl^- transport as normal GPA over the entire range of GPA concentrations employed. These results show that the GPA monomer is sufficient to enhance band 3-specific anion transport in oocytes.

GPA mutants retain the ability to enhance the translocation of band 3 to the oocyte cell surface

GPA has been shown to enhance band 3-specific anion transport by increasing the proportion of band 3 present at the oocyte cell surface. However, a contribution to the enhancing effect might arise from an interaction between GPA and band 3 at the cell surface, which increases the specific anion transport activity of band 3 [8,21]. We confirmed that the mutant GPA molecules acted by increasing band 3 movement to the cell surface by using a protease-based assay to measure the cell-surface band 3 in the oocytes. The membrane domain of band 3 contains an extracellular site that is susceptible to cleavage with chymotrypsin. Measurement of the amount of band 3 cleavage in chymotrypsin-treated intact oocytes can be used to estimate the proportion of band 3 at the cell surface [8]. Oocytes were

Figure 4 Anion transport activity of band 3 and band 3 G701D with normal GPA and GPA mutants

Oocytes were injected with 0.2 ng of band 3 (B3) or band 3 G701D (B3 701) cRNA per oocyte, with or without 0.002 ng of normal GPA or mutant GPA cRNA per oocyte. $^{36}\text{Cl}^-$ influx was measured over a 1 h period with groups of 10–15 oocytes in Barth's saline or in Barth's saline containing 400 μM 4,4'-dinitrostilbene-2,2'-disulphonate (DNDS), as described previously [8]. The bars represent DNDS-sensitive Cl^- influx per oocyte over 1 h; the error bars show the S.E.M. of the influx measurements.

metabolically labelled with ^{35}S -labelled amino acids, treated with chymotrypsin, and the C-terminal 35 kDa chymotrypsin fragment of band 3 and intact band 3 protein were immunoprecipitated with a monoclonal antibody reactive with the C-terminal portion of band 3. The radiolabelled proteins in the immunoprecipitate were separated by SDS/PAGE and the proportion of band 3 cleaved was estimated from the amounts of radioactivity in the intact band 3 and 35 kDa fragment in scans of the fluorographs (Figure 3). The results show clearly that each of the dimerization-impaired GPA mutants substantially increases the proportion of cleaved band 3 present at the surface of the chymotrypsin-treated oocytes. The GPA mutants all retain the ability to increase the translocation of band 3 to the oocyte cell surface, indicating that the GPA monomer is able to perform this process.

Is the GPA dimer required for movement of mutant band 3 to the cell surface?

The band 3 mutation G701D (associated with renal tubular acidosis) renders the protein unable to reach the cell surface when expressed alone in oocytes. However, GPA completely restores the movement of the mutant protein to the cell surface [27,28]. The dimerization of GPA might have a role in the rescue of the anion transport activity of band 3 mutants that are defective in translocation to the cell surface.

We examined the possible role of GPA dimers in this process by studying the effects of the dimerization-impaired GPA mutants on the anion transport activity induced in oocytes by band 3 G701D (Figure 4). The experiment was performed under conditions in which the enhancement of normal band 3 anion transport activity is sensitive to the concentration of normal GPA (ratio of GPA cRNA to band 3 cRNA of 1:100). In this experiment, normal band 3 alone induced substantial amounts of stilbene disulphonate-sensitive anion transport in the oocytes; this was further enhanced by the small amount of normal GPA employed in the experiment (Figure 4). Band 3 G701D gave no stilbene disulphonate-sensitive anion transport in the absence of GPA but the presence of normal GPA resulted in the partial

Figure 5 Surface expression of band 3 and GPA in K562 cell clones

Three DNA constructs were used: the previously described pBpB3 construct [47] containing band 3 cDNA in the pBabe puro vector [57]; empty pBabe neo vector [57] and the construct pBnGPA, containing GPA cDNA in pBabe neo. The pBnGPA construct was made by inserting a 469 bp *Bgl*/II fragment of the construct pBSXG.GPA [8] into the compatible *Bam*HI restriction site in pBabe neo. K562 cells were first transfected with the pBpB3 construct; a clone expressing functional band 3 at the cell surface (K562/B3 clone 1) was isolated by flow cytometry, as described previously [47]. Cells of this K562/B3 clone were subsequently transfected either with empty pBabe neo vector (yielding 16 stable K562/B3 + pBn subclones resistant to puromycin and G418), or with the pBnGPA construct (yielding 10 stable K562/B3 + GPA subclones). The cell-surface expression levels of band 3, GPA and Wr^b antigen of each of the K562/B3 + pBn and K562/B3 + GPA clones were determined by flow-cytometric analysis, with methods described previously [47]. Three samples were analysed for each clone. For the first sample, cells were incubated with the murine monoclonal anti-(band 3), BRIC 6 [58], as primary antibody; for the second sample, cells were incubated with the murine monoclonal anti-GPA, R18 [59]; for the third sample, cells were incubated with the murine monoclonal anti-Wr^b, BRIC 14 [60]. For all samples, FITC-labelled F(ab')₂ fragments of goat anti-mouse IgG were used as the secondary antibody. The mean fluorescence intensity (FL1) was used as the measure of antibody binding. (A) FL1 values obtained with anti-GPA plotted against those obtained with anti-(band 3) for each of the 26 K562/B3 + pBn and K562/B3 + GPA clones (●). The line of best fit as determined by linear regression analysis ($r^2 = 0.923$) is shown. The position of the original K562/B3 clone 1 clone is shown by +. (B) FL1 values obtained with anti-Wr^b plotted against those obtained with anti-(band 3) for each of the 26 K562/B3 + pBn and K562/B3 + GPA clones (●). The line of best fit as determined by linear regression ($r^2 = 0.976$) is shown. The position of the original K562/B3 clone 1 clone is shown by +.

recovery of some band 3-specific anion transport (to approx. 40% of that obtained with the same amount of normal band 3 with normal GPA). It has previously been reported that normal GPA rescues the band 3-specific anion transport activity of band 3 G701D expressed in oocytes to wild-type band 3 levels. However, equal weights of GPA cRNA and band 3 cRNA were employed in these experiments [27], which would result in a large excess of GPA being present. Figure 4 shows clearly that under conditions in which the enhancement of normal band 3-specific anion transport activity is sensitive to the concentration of GPA, normal GPA is unable to effect a complete rescue of the anion transport activity of band 3 G701D to the level obtained with normal band 3. When the same experiment was performed with band 3 G701D and the dimerization-impaired GPA mutants, all the GPA mutants enhanced the band 3-specific anion transport obtained with band 3 G701D to the same level as that obtained with normal GPA. These experiments show that dimerization of GPA is not required for the functional rescue of the mutant band 3 molecule.

Does GPA move to the cell surface with band 3?

Although GPA and band 3 are found together at the red-cell surface, there have been no studies of whether the two proteins move to the cell surface in concert. The human K562 erythroleukaemic cell line expresses GPA but does not express band 3 [46,47]. We have previously described the cell-surface expression of band 3 in transfected K562 cells [47]. Although we noted a doubling in cell-surface expression of GPA in some cloned lines of K562 cells transfected with band 3, increases of this magnitude were not always observed when other cell clones were examined. Individual K562 cells exhibit variable levels of endogenous GPA expression [48]. We attributed our results to differences in the

level of endogenous GPA expression in the individual transfected K562 cells from which each of the band 3-containing cloned cell lines had been derived, together with variability in the level of expression of the transfected band 3 in each of these clones.

We examined this question further by taking a cloned cell line derived from K562 cells transfected with band 3 (K562/B3 clone 1 [47]) and co-transfecting these cells with either GPA or empty vector. Cloned cell lines of these doubly transfected cells were isolated and the expression of cell-surface band 3 and GPA in each clone was examined by FACS analysis. Surprisingly, the K562/B3 clone 1 cells co-transfected with empty vector showed variable levels of band 3 expression, with some showing no band 3 expression at all. It is likely that these were derived from a small fraction of K562/B3 clone 1 cells that had lost band 3 expression before co-transfection with the vector. These band 3-negative cells would be selectively amplified during the transfection and cloning procedure because the doubling time of K562 cells expressing band 3 is twice that of K562 cells that do not express band 3 [47]. As would be expected, a similar degree of variability in band 3 expression was also observed in the K562/B3 clone 1 cells co-transfected with GPA.

Figure 5 shows the results of a FACS analysis of all the cloned cell lines obtained after co-transfection of K562/B3 clone 1 cells with GPA, and of K562/B3 clone 1 cells with empty vector. The results clearly show a linear correlation between the cell-surface band 3 and the cell-surface GPA in the cells (Figure 5A). The clones that expressed smaller amounts of band 3 than the original K562/B3 clone 1 cells showed a corresponding decrease in the amount of cell-surface expression of GPA. Anti-Wr^b antibodies require the presence of both band 3 and GPA for reactivity [16–20]. Figure 5(B) shows a similar correlation between the amount of cell-surface band 3 and the amount of Wr^b antigen at the surface of the cell. These results are consistent with

the idea that band 3 and GPA moved together to the cell surface in these transfected cells.

DISCUSSION

GPA dimerization and band 3 movement to the cell surface

Our results suggest that the effects of GPA on band 3 biosynthesis and movement to the cell surface are mediated by the GPA monomer and not by the GPA dimer. Our previous suggestion [8] that the dimers formed by GPA might enhance the oligomerization of band 3, and so facilitate the movement of band 3 to the cell surface, is clearly incorrect. This proposal was based on the observation that enhancement of the cell-surface movement of band 3 in oocytes by GPA is more marked at low concentrations of band 3 cDNA than at high concentrations of band 3 cDNA [8]. The present results also show that the dimerization of GPA is not required for rescue of the functional competence of mutant band 3 molecules. The biological significance of the stable dimers formed by GPA remains unclear.

Region of GPA involved in the movement of band 3 to the cell surface

Previous studies indicated that the C-terminal cytoplasmic domain of GPA is not part of the region involved in the biosynthetic interaction with band 3 [8]; however, the GPA TM domain was suggested to be responsible for this association [20]. In addition, red cells that carry a hybrid glycophorin in addition to normal levels of GPA (described as St^a and Dantu) (reviewed in [12,13]) contain band 3 with a lower apparent molecular mass on SDS/PAGE [12,32]. This is probably because of a decrease in the number of *N*-acetyl-lactosamine units in the glycan chain resulting from a more rapid transit of band 3 through the Golgi system to the cell surface (see below). The C-terminal portion of the St^a hybrid glycophorin contains residues 57–131 of GPA, whereas the C-terminus of the Dantu glycophorin contains residues 71–131 of GPA. In these red cells the hybrid glycophorins effectively provide additional GPA that can interact with band 3 and increase its rate of movement to the cell surface. This suggests that residues 71–131 of GPA contained in the Dantu glycophorin carry the site of interaction with band 3. Because residue 71 is at the extracellular boundary of the glycophorin TM span, this provides confirmatory evidence that the site of interaction of GPA with band 3 that is involved in band 3 movement to the cell surface is located within the GPA TM domain.

The results in the present paper suggest that the interacting site in the GPA TM domain is located outside the GPA dimerization interface, because the mutation of four different sites within the GPA dimer interface did not alter the ability of GPA to facilitate the cell-surface expression of band 3. The recent report that, although human and mouse GPA are unable to form heterodimers, human and mouse GPA compete for band 3 during their biosynthesis in erythroid cells [49] adds support to this view.

Although glycophorin B (GPB) has very close sequence similarity to GPA [12,13], GPB does not enhance band 3 movement to the cell surface [8]. GPB forms homodimers and also forms heterodimers with GPA, both of which are stable under the conditions of SDS/PAGE [50,51], suggesting that the dimerization interface in GPB is similar to that in GPA. Two differences between the TM domains of GPA and GPB might account for the inability of the latter to enhance band 3 movement to the cell surface. First, the amino acids corresponding to Thr⁷⁴ and Phe⁷⁸ of GPA, which lie outside the dimerization interface, are altered in GPB. Secondly, the TM span of GPB is probably

longer than that of GPA (because eight potentially membrane-embedded residues extend the GPB TM region at the N-terminal end) and this could alter its interaction with band 3.

Band 3 and GPA associations during their movement to the cell surface

The major route for band 3 movement to the cell surface in normal red cells most probably involves GPA. Because the GPA monomer is sufficient to enhance the cell-surface movement of band 3, we suggest that GPA monomers bind band 3 in an intracellular compartment. Band 3 undergoes several post-translational modifications (fatty acylation, phosphorylation and *N*-glycan processing), and GPA is also extensively modified post-translationally by *N*-glycan processing and *O*-glycosylation. One or more of these modifications might influence the association of band 3 with GPA monomers. The association of GPA monomers with band 3 could modify the structure of band 3 or occlude exposed retention signals so as to allow the rapid passage of the complex of both proteins to the cell surface. During the late stages of movement to the cell surface, further post-translational modification of one or both proteins, or associations of band 3 with ankyrin or other peripheral proteins, could weaken the interaction between band 3 and GPA monomer. This would cause GPA monomers to dissociate from band 3 and reform GPA homodimers. The weaker association of band 3 with GPA dimers would account for the inability to isolate complexes of the two proteins by immunoprecipitation from red-cell membranes.

The biosynthesis of chicken band 3 (AE1) in nucleated embryonic erythroid cells has recently been studied [52]. After first moving to the plasma membrane, the band 3 was internalized and recycled to the Golgi, where it became associated with cytoskeletal ankyrin. Although chicken red cells contain glycophorin-like glycoproteins rich in sialic acid [53], it is not known whether any of these are related to GPA and have a role in the cell-surface movement of band 3 in these nucleated cells.

GPA movement to the cell surface

Although the results in Figure 5 suggest that GPA moves with band 3 to the cell surface when the latter is present, GPA can move to the cell surface in the absence of band 3. Human K562 cells express cell-surface GPA but do not express band 3 [46,47]. In addition, GPA appears at the cell surface before band 3 during the maturation of human erythroid progenitor cells in culture [54]. However, the red cells of band 3-null mice do not express cell-surface GPA, suggesting that band 3 is required for the cell-surface expression of GPA in these cells [32]. These different observations might reflect differences in the expression of GPA in man and mouse. Nevertheless, it is clear that in both species band 3 and GPA act on each other to enhance the cell-surface expression of both proteins.

Band 3 biosynthesis in the absence of GPA

Band 3 moves to the membrane in the absence of GPA, albeit more slowly than when GPA is present. This situation occurs in the α -intercalated cells of the distal nephron of human kidney. Rare individuals are known who do not express GPA as a result of deletions in the GPA gene [described as En(a-) and M^k/M^k] (reviewed in [17]). Although the red cells of these individuals contain normal amounts of band 3, evidence suggests that band 3 moves more slowly to the cell surface in this case and has an altered structure [31]. The average length of the *N*-glycan chain of band 3 in GPA-null human red cells is greater than in normal red cells, because of an increase in the average number of

repeating *N*-acetyl-lactosamine units in the oligosaccharide [29,30]. This reflects a longer residence time of band 3 in the trans-Golgi network, where the glycosyl transferases involved in the addition of the *N*-acetyl-lactosamine units are localized [31]. The slower movement of band 3 to the surface in these cells probably results from a recycling of band 3 within the Golgi stack or a decreased export of band 3 from the Golgi system to the plasma membrane [31].

The anion transport activity of band 3 is also decreased in the human red cells that lack GPA. We have argued that this arises because biosynthesis in the absence of GPA yields band 3 with an altered structure (and therefore a changed functional activity) and not because GPA at the cell surface normally enhances the specific transport activity of band 3 [31]. Additional support for this view comes from the ability of GPA to partly rescue the cell-surface anion transport activity of mutant band 3 molecules such as band 3 G701D (Figure 4). Band 3 G701D is unstable in oocytes in the absence of GPA, suggesting that the mutant protein is improperly folded and turned over by the protein quality-control systems in the cell [28]. This indicates clearly that the structure of the band 3 G701D protein is altered by the presence of GPA, in a manner that allows it to pass through the cellular quality-control systems and also allows movement of the mutant band 3 to the cell surface. Similar effects are observed with band 3 mutants lacking the N-glycan chain [55] and co-expressed complementary fragments of band 3 [43], which exhibit much decreased anion transport activity in *Xenopus* oocytes in the absence of GPA. In the presence of GPA the anion transport activity of these constructs is restored to almost normal levels.

Role of GPA in erythroid cells is to enhance the folding and maturation of band 3 in red-cell precursors

We conclude that GPA acts by modifying the folding pathway of band 3 so that it attains the fully active, native conformation in the red cell. This effect of GPA might result directly from the interaction of GPA with band 3, or GPA might recruit other proteins that aid protein folding and maturation to the intracellular complex of band 3 with GPA. One consequence of this is that the properly folded band 3 protein is able to move rapidly to the red-cell surface, probably in concert with GPA. In red cells lacking GPA, band 3 is imperfectly folded and moves only slowly to the plasma membrane, where it exhibits impaired anion transport.

This role of GPA is likely to be especially important in erythropoiesis, in which the very large amount of band 3 that must be inserted into the surface membrane of the developing erythroid cell within a limited time might overwhelm the capacity of the normal protein folding and maturation system. In erythroid cells the presence of GPA, which is expressed in the cells before band 3 [54], probably results in an increased capacity for folding band 3. In kidney cells the abundance of band 3 and rates of band 3 synthesis are likely to be much lower than those in erythroid cells. The normal protein folding system in renal cells is probably sufficient to allow normal band 3 to be folded to its fully active native structure. Although adequate for normal band 3, the system in these cells might be inadequate for folding band 3 mutants such as band 3 G701D. The enhanced folding capability resulting from the presence of GPA is probably essential for the folding of these mutant band 3 molecules.

Erythroid cells are the only tissue known to express GPA. Although GPA was one of the first integral membrane proteins to be completely characterized [56], its functional role in the red cell has been unclear. It is likely that GPA helps to fulfil the

specific need of rapidly developing red-cell progenitors to incorporate large amounts of properly folded band 3 into the plasma membrane in a limited time during their maturation.

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