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The Effects of Glycophorin A on the Expression of the Human Red Cell Anion Transporter (Band 3) in *Xenopus* Oocytes

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Abstract. The effects of human red cell glycophorin A (GPA) on the translocation to the plasma membrane and anion transport activity of the human erythrocyte anion transporter (band 3; AE1) have been examined using the *Xenopus* oocyte expression system. We show that band 3 accumulates steadily at the oocyte surface with time in the presence or absence of GPA, but this occurs more quickly when GPA is coexpressed. The amount of band 3 at the surface is determined by the concentrations of band 3 and GPA cRNA that are injected, with a higher proportion of total band 3 being translocated to the surface in the presence of GPA cRNA. The increased expression of DNDS-sensitive chloride transport is highly specific to GPA, and is not observed when the cRNA to the putative glycophorin E or a very high concentration of the cRNA to glycophorin C are coexpressed with band 3 in oocytes.

Key words: Band 3 — AE1 — Glycophorin A — Anion transporter — *Xenopus* oocytes

Introduction

The human erythrocyte anion transporter (band 3, AE1; *recently reviewed by* Jennings, 1989; Tanner, 1993) is a 911 amino acid integral membrane protein (Tanner, Martin & High, 1988) that is present at approximately 1.2×10^6 copies/red cell (Steck 1978). Band 3 is made up of two domains that are structurally and functionally distinct, and can be separated by mild proteolysis (Steck, Ramos & Strapazon, 1976): the NH_2-terminal 43 kD cytoplasmic domain acts as an anchorage site for the components of the red cell skeleton, and also binds several glycolytic enzymes and hemoglobin; the COOH-terminal 55 kD membrane domain is responsible for the anion exchange function of the protein.

Glycophorin A (GPA)[1] is the only membrane protein that is present in the red cell at similar abundance ($0.5–1.0 \times 10^6$ copies/cell) to band 3 (*reviewed by* Anstee, 1990). Several pieces of evidence have suggested a high affinity interaction between GPA and the membrane domain of band 3 at an early stage in their biosynthesis or processing (*reviewed by* Dahr, 1986; Tanner, 1993). In the mature erythrocyte membrane it would appear that the two proteins interact only weakly and a stable complex had not been identified. GPA and band 3 cRNAs have recently been coexpressed in *Xenopus* oocytes (Groves & Tanner, 1992) where the presence of GPA resulted in an increased level of band 3-mediated chloride transport. It was suggested that GPA facilitates the translocation of band 3 to the oocyte surface. The effect of GPA was particularly noticeable at low band 3 cRNA concentrations. Since even lower concentrations of band 3 mRNA occur in the developing erythroid cell (Koury, Bondurant & Rana, 1987), we have suggested (Groves & Tanner, 1992) that GPA could act as part of a switch mechanism for the insertion of band 3 into the plasma membrane at the appropriate time in the development of erythroid cells. However, healthy individuals have been described whose red cells lack GPA (Gahmberg et al., 1976; Tanner & Anstee, 1976; Tokunaga et al., 1979), and the presence of normal amounts of band 3 in these cells clearly shows

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[1] The abbreviations used are: DNDS, 4,4′-dinitrostilbene-2,2′-disulfonate; DADS, 4,4′-dinitro-2,2′-stilbene disulfonate; GPA, glycophorin A: GPB, glycophorin B; GPC, glycophorin C; GPE, glycophorin E; SDS–PAGE, SDS–polyacrylamide gel electrophoresis.

that GPA is not essential for the expression of band 3 in the erythrocyte.

Anion transport assays and treatment of intact oocytes with chymotrypsin have provided complementary, but independent means for determining the amount of band 3 expressed at the oocyte cell surface (Groves & Tanner, 1992). In this paper, we have used both these techniques to investigate further the rate of accumulation of functional band 3 in the plasma membrane of oocytes in the presence and absence of coexpressed GPA.

Materials and Methods

Preparation of cRNAs and Expression in Oocytes

The cDNA clones encoding human band 3 (pBSXG1.b3), the band 3 membrane domain (pBSXG1.b3mem), glycophorin A (pBSXG.GPA) and glycophorin C (pGEM-4.GPC) have been described previously (Groves & Tanner, 1992). The cDNA coding for the putative glycophorin E polypeptide (Kudo & Fukuda, 1990) was constructed in the vector pBSXG (Groves & Tanner, 1992) and provided by Dr. K. Ridgwell and C. Ratcliffe (University of Bristol, UK).

Protocols for synthesis of cRNA, oocyte isolation and cRNA injection, [^{35}S]-amino acid labeling and immunoprecipitation of band 3, chymotrypsin treatment of oocytes, SDS–PAGE, fluorography and the oocyte chloride influx assay were all as detailed previously (Groves & Tanner, 1992). Variations to the methods in individual experiments are detailed in the figure legends.

Results and Discussion

Low Levels of Glycophorin A Specifically Enhance Band 3-mediated Anion Transport in Oocytes

Xenopus oocytes were injected with the cRNA coding for human red cell band 3 either with or without coinjection of the cRNA for GPA. After 24 hr of incubation during which the band 3 was expressed in the plasma membrane of the oocyte, the DNDS-sensitive influx of $^{36}Cl^-$ over a 1 hr period was used as an estimate of the band 3-specific anion transport induced in oocytes. Using this method, we have shown previously that coexpression of low levels of the two cRNAs (less than 1.5 ng/oocyte of each) leads to higher levels of anion transport than expression of the same weight of band 3 cRNA alone (Groves & Tanner, 1992).

To investigate how little GPA cRNA is required for a significantly increased level of anion transport to be observed, oocytes were injected with a constant amount of band 3 cRNA (1.5 ng/oocyte) together with a range of low amounts of GPA cRNA. When chloride influx was measured after 24 hr, higher levels of transport were obtained with oocytes coexpressing GPA at any of the cRNA concentrations tested compared with oocytes expressing band 3 cRNA alone (Fig. 1*a*). In this experiment, the lowest concentration of GPA cRNA that was injected was 15 pg/oocyte (the transport increase observed in comparison with band 3 without GPA was significant at the 10% level in a *t*-test). This amount of GPA cRNA corresponds approximately to a 25-fold molar excess of band 3 cRNA. At GPA cRNA concentrations of 150 pg/oocyte and higher, the enhancement of DNDS-sensitive anion transport over that with band 3 alone was greatest (significant at the 1 or 0.1% levels). Injection of higher concentrations of GPA cRNA did not lead to further increases in anion transport activity. Coinjection of 1.5 ng band 3 cRNA and 150 pg GPA cRNA per oocyte corresponds approximately to proportions of 2.5/1 (by molarity of the cRNAs), which is of the same order as the relative abundance of the two proteins in the plasma membrane of red cells.

Glycophorin C (GPC) is a red cell sialoglycoprotein which is not related in sequence to GPA (Colin et al., 1986; High & Tanner, 1987) and is present in red cells with an abundance approximately 10% that of GPA (Dahr, 1986). In previous work (Groves & Tanner, 1992), we have shown that coexpression of GPC cRNA (at up to 1.5 ng/oocyte) with an equal weight of cRNA coding for either band 3 or the band 3 membrane domain (amino acid residues 360–911 of band 3) did not lead to enhancement of anion transport activity. Since the band 3 cDNA was cloned within the vector pBSXG1 (Groves & Tanner, 1992), whereas the GPC cDNA was cloned into a different vector (pGEM-4), the GPC cRNA may not have been translated as efficiently as the band 3 in oocytes. To eliminate this possibility, we have coexpressed higher concentrations of GPC cRNA with 1.5 ng/oocyte of band 3 cRNA in oocytes and measured the effects on DNDS-sensitive chloride influx (Fig. 1). When band 3 and GPC cRNAs were coinjected in proportions of 1/1, 1/5 and 1/20 (by weight), which correspond approximately to ratios of 1/4, 1/20 and 1/80 (by molarity of the cRNAs), the DNDS-sensitive chloride transport observed was not significantly different from that obtained with band 3 cRNA alone. However, in each case the anion transport activity was lower than that obtained when band 3 cRNA was coexpressed with an equal weight of GPA cRNA (significant at the 1 or 0.1% levels in *t*-tests). Hence, band 3-mediated anion transport is not enhanced by the coexpression of GPC cRNA at a concentration that is three orders of magnitude higher than that at which GPA cRNA does cause an enhancement.

Glycophorin B (GPB) is a sialoglycoprotein that is closely related to GPA (Blanchard et al., 1986; Siebert & Fukuda, 1987; Tate & Tanner, 1988) and is present in red cells with an abundance of about 15% that of GPA (Merry et al., 1986). In previous work (Groves &

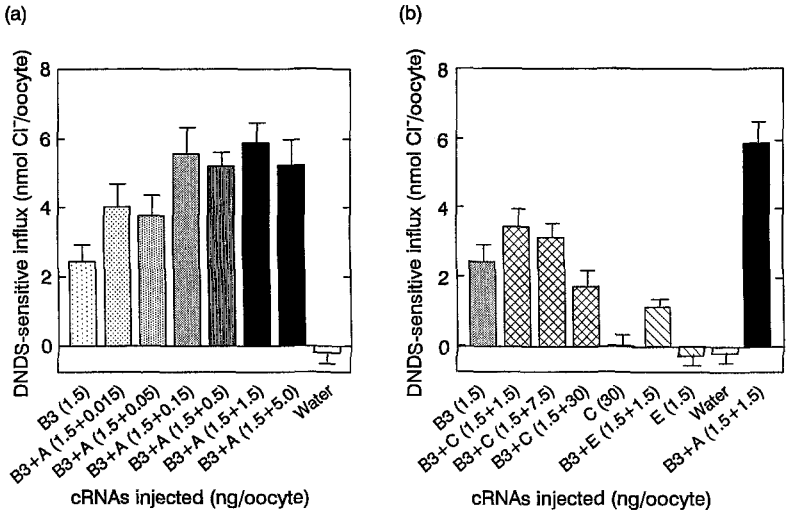


Fig. 1. Comparison of the effects of various glycophorins on the band 3-mediated chloride influx into oocytes. Oocytes were injected with 1.5 ng of the cRNA to band 3 (B3) with or without various amounts of the cRNAs to GPA (A), GPC (C) or the putative GPE (E). Chloride influx (60 min) was measured 24 hr after injection of cRNAs. Each value shows the mean DNDS-sensitive chloride influx estimated from the difference between the means of groups of 10–12 oocytes which were assayed with and without 400 μM DNDS treatment. The bar shows the standard error of the mean DNDS-sensitive chloride influx.

Tanner, 1992), we have shown that coexpression of GPB with band 3 does not enhance anion transport activity. The putative glycophorin E gene has recently been identified (Kudo & Fukuda, 1990) and shown to code for an amino acid sequence that is closely related to GPB, although its expression in red cells has not been unequivocally demonstrated to date. Coexpression of equal weights of band 3 and GPE cRNAs (1.5 ng/oocyte) did not enhance the level of band 3-specific chloride influx over that obtained by expression of band 3 alone (Fig. 1*b*). Taken together, the results shown in Fig. 1 provide further evidence that the interaction of band 3 with glycophorin A is specific to these two red cell membrane proteins.

GPA Increases the Rate of Accumulation of Functional Band 3 in the Plasma Membrane of Oocytes

To examine the effect of coexpression of GPA on the rate of accumulation of band 3 in the plasma membrane and the final level of anion transport attained, two types of chloride influx experiments were performed. In our first approach (Fig. 2), two time course experiments were carried out. Groups of oocytes were injected with band 3 cRNA at each of two different concentrations, with or without coinjection of an equal weight of GPA cRNA. The accumulation of band 3 in the plasma membrane was estimated from the band 3-mediated chloride transport activity at various times from one to four days after injection. In one of these experiments (Fig. 2*a*), an especially low band 3 cRNA concentration was injected (0.1 ng/oocyte). This resulted in a marked GPA-dependent enhancement of the DNDS-sensitive chloride influx after 24 hr expression time. However, the anion transport activity obtained with oocytes that had been injected with band 3 cRNA in the absence of GPA cRNA increased with the time of expression, whereas that observed with oocytes that were coexpressing band 3 and GPA remained equally high on each day. As a result, the difference between the levels of anion transport activity in the presence and absence of GPA became less marked with time. Although injection of this low concentration of band 3 cRNA resulted in a considerably greater level of band 3-specific chloride influx after four days expression without GPA than after one day without GPA, the amount of anion transport activity was still lower after four days without GPA than after one day in the presence of GPA.

To test whether the same level of DNDS-sensitive chloride transport activity was eventually attained by oocytes expressing band 3 cRNA with or without coexpression of GPA cRNA, a subsequent experiment was performed (Fig. 2*b*) in which the time courses for expression of anion transport activity were compared at two higher concentrations of cRNA. At the lower of these band 3 cRNA concentrations (0.5 ng/oocyte), the DNDS-sensitive chloride influx increased linearly from day 1 to day 2. After 2–3 days, the level of anion transport was similar to that obtained after 1 day in the presence of GPA and reached a plateau. Taken together, the results shown in Fig. 2 suggest that coexpression of GPA with band 3 increases the rate at which functional band 3 is translocated to the oocyte surface.

In the second approach (Fig. 3), oocytes were injected with both very low and high concentrations of band 3 cRNA, with or without coinjection of an equal amount of GPA cRNA. This experiment was performed with oocytes that were isolated and injected with cRNA at the same time as those used for the experiment described in Fig. 2*a*. As observed previously (Groves & Tanner, 1992), anion transport was enhanced greatly by coexpression of GPA at low levels of band 3 cRNA, but

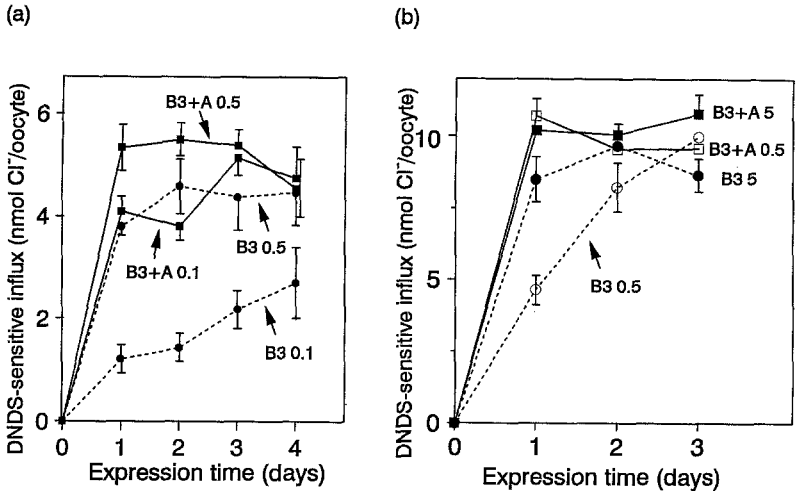


Fig. 2. Time courses for expression of band 3-mediated chloride influx into oocytes. Oocytes were injected with various amounts of band 3 cRNA (B3), with or without coinjection of an equal weight of GPA cRNA (A). Band 3-specific chloride influx (60 min) was measured at various times after injection of cRNAs using groups of 12–16 oocytes (*a*) or 10–14 oocytes (*b*) which were assayed with or without 400 μM DNDS treatment, as described in Fig. 1. The bar represents the standard error of the mean DNDS-sensitive chloride influx in each case. The data in *a* were obtained from the same set of experiments as those in Fig. 3.

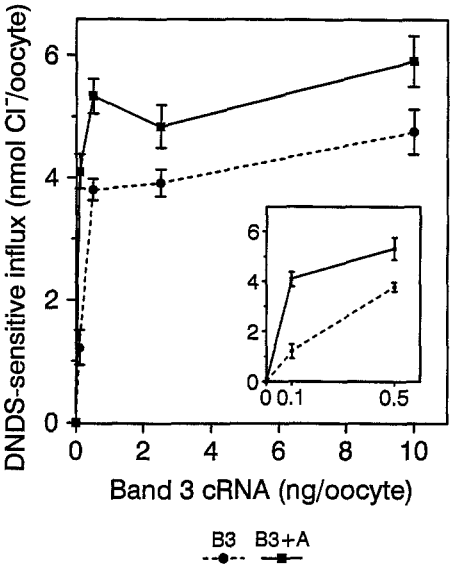


Fig. 3. Effect of glycophorin A on DNDS-sensitive chloride influx into oocytes. Oocytes were injected with various quantities of band 3 cRNA (B3), with or without coinjection of an equal weight of GPA cRNA (A). Band 3-specific chloride influx (60 min) was measured 24 hr after injection of cRNAs using groups of 11–17 oocytes which were assayed with or without 400 μM DNDS treatment, as described in Fig. 1. The bar represents the standard error of the mean DNDS-sensitive chloride influx in each case.

was less marked at high band 3 cRNA concentrations. However, at all the cRNA concentrations tested, anion transport was at least 20% higher than that with the same weight of band 3 cRNA alone (significant at the 5, 2, and 1% levels for 10, 2.5, and 0.5 ng of each cRNA, respectively). In a previous experiment, a similar increase in the presence of GPA was also observed (significant at the 1% level with 15 ng of each cRNA; Fig. 2*a* of Groves & Tanner, 1992). It is possible that coexpression of GPA with band 3 may enable the radiolabeled chloride to access a larger average volume in the oocyte after a 24 hr expression time than when band 3 is expressed alone. In this case, the GPA-dependent effect could be mediated by entry of chloride into an additional intracellular compartment or by an increase in overall cell volume available to the chloride in the presence of GPA.

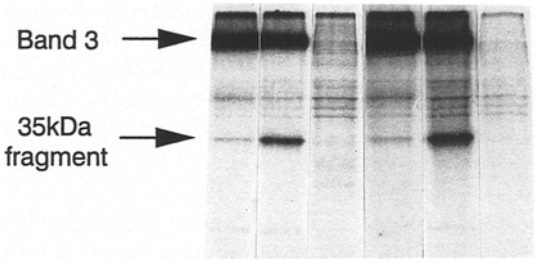
GPA Facilitates the Cell Surface Expression of Band 3 at a Wide Range of Band 3 cRNA Concentrations

Chymotrypsin cleavage has been used previously to probe for the band 3 expressed at the surface of oocytes (Groves & Tanner, 1992). Band 3 in red cells is cleaved by chymotrypsin at its extracellular surface to yield an N-terminal 60 kD and a C-terminal 35 kD fragment (Steck et al., 1976). We used this assay to examine the expression of cell surface band 3 at high concentrations of band 3 cRNA (5 ng/oocyte) as well as lower concentrations. Groups of oocytes were injected with band 3 cRNA at both 1.5 ng/oocyte and 5 ng/oocyte, either with or without coinjection of an equal weight of GPA cRNA. After pulse-labeling with [^{35}S]-amino acids and a 48 hr chase in the presence of nonradioactive amino acids, intact oocytes were treated with chymotrypsin. Subsequently, the cells were homogenized and the radiolabeled band 3 was immunoprecipitated using the monoclonal antibody BRIC 155 (which reacts with the COOH-terminus of band 3). The immunoprecipitates were separated by SDS–PAGE (Fig. 4) to distinguish the uncleaved band 3 (100–105 kD) from the C-terminal (35 kD) fragment which is derived from band 3 cleaved at the cell surface by chymotrypsin. Fluorographs were scanned by absorbance densitometry and the percentage of band 3 that had been cleaved by chymotrypsin was estimated. The results of treatment with chymotrypsin for 3 hr are shown (Fig. 4).

Parallel sets of oocytes that had been treated with chymotrypsin for 1 hr were also immunoprecipitated, and the results were essentially identical (*data not shown*) which showed that chymotrypsin digestion was complete in this experiment.

When band 3 was coexpressed with GPA, a higher proportion of pulse-chase labeled band 3 was cleaved by chymotrypsin than when band 3 was expressed alone (Fig. 4). This GPA-dependent increase in cleavage was marked at both the cRNA concentrations examined. The results (Fig. 4) indicate that coexpression of GPA caused a higher proportion of the band 3 that was synthesized during the 3 hr pulse-labeling to be translocated to the plasma membrane after 48 hr at the higher band 3 cRNA concentration (5 ng/oocyte), as well as at lower levels (Groves & Tanner, 1992). We did not examine chymotrypsin cleavage of oocytes injected with cRNA at a concentration of less than 1.5 ng/oocyte as the amount of labeled band 3 obtained in the immunoprecipitates is too small to derive accurate estimates of the proportion of band 3 that is in the cleaved 35 kD band.

ng each cRNA	1.5			5.0		
Band 3 cRNA	+	+	-	+	+	-
GPA cRNA	-	+	+	-	+	+



Cleaved band 3 (%)	2	21	-	2	29	-

Fig. 4. Effect of band 3 cRNA concentration on the cleavage of pulse-labeled band 3 by chymotrypsin. Groups of 10 oocytes were injected with either band 3 cRNA (B3), GPA cRNA (A) or both cRNAs (B3 + A) at 1.5 or 5 ng each cRNA/oocyte. Cells were pulse-labeled with [^{35}S]-labeled amino acids and chased with unlabeled amino acids for 48 hr. Oocytes were then treated with chymotrypsin for 3 hr and immunoprecipitated with BRIC 155 (reactive with the COOH terminus of band 3) as detailed in Groves and Tanner (1992). Each lane represents immunoprecipitated protein from the equivalent of five oocytes, separated on 11% SDS–PAGE gels. The faint bands which are also visible in the GPA-injected oocyte samples are due to low level nonspecific precipitation of other proteins. The position of the COOH-terminal 35 kD fragment of cleaved band 3 is indicated. The proportion of band 3 cleaved by chymotrypsin was determined by scanning densitometry of the SDS–PAGE fluorographs. Expressed protein was estimated from the areas under the peaks. The 35 kD band 3 fragment was assumed to contain 60% of the radioactivity incorporated into intact band 3, based on methionine and cysteine content. Values are percentage of total band 3 (i.e., intact + fragment) which was fragment. The absorbance readings for intact band 3 increase linearly with time in this density range (Groves & Tanner, 1992).

The Proportion of Band 3 Translocated to the Cell Surface Increases with the Band 3 cRNA Concentration

It is clear from autoradiographs of the immunoprecipitations (Fig. 4) that the amount of radiolabeled band 3 synthesized by pulse-chase labeled oocytes that have been injected with 5 ng band 3 cRNA is greater than the amount made by cells injected with 1.5 ng band 3 cRNA (with or without coinjection of GPA cRNA). Although we did not confirm that these immunoprecipitations were quantitative, we have shown previously that coexpression of GPA and band 3 causes an apparent reduction in net band 3 synthesis due to competition with GPA cRNA in the synthesis of band 3 at cRNA concentrations of greater than approximately 5 ng/oocyte (Groves & Tanner, 1992). Taken together, these results suggest that the amount of band 3 expressed in the plasma membrane of oocytes increases with the concentration of band 3 cRNA injected when less than 5 ng band 3 cRNA is injected per oocyte. When GPA is coexpressed, this concentration-dependent effect is enhanced at all cRNA concentrations examined.

In the above experiments, we studied the translocation of band 3 to the plasma membrane by injecting oocytes with cRNA, pulse-labeling with [^{35}S]-amino acids and then subjecting them to various chase periods (Fig. 4). The advantages of this technique are that the translocation of a particular group of band 3 molecules can be followed and a higher proportion of the labeled band 3 is cleaved by chymotrypsin. The disadvantages of this approach are that a smaller amount of band 3 becomes radiolabeled than in a steady [^{35}S]-amino acid labeling and hence the assay is less sensitive to low band 3 cRNA concentrations. In addition, care is required in relating the observations to anion transport data, since the latter is derived from the band 3 synthesized over the entire period from injection to assay.

To study the translocation of band 3 to the plasma membrane in the presence and absence of GPA at a wide range of band 3 cRNA concentrations, oocytes were injected with cRNAs at several concentrations in the range 0.5–5 ng/oocyte. To ensure sufficient radiolabeled amino acids were incorporated into the band 3 for detection of the 60 kD chymotrypsin fragment, cells were incubated with [^{35}S]-labeled amino acids for a continuous 24 or 48 hr period. Intact oocytes were treated with chymotrypsin and then immunoprecipitated with BRIC 170 (directed against the NH_2-terminal domain of

band 3). Cell surface band 3 was estimated from the proportion of total band 3 that was present as the 60 kD fragment. At all concentrations of band 3 cRNA, a higher proportion of total band 3 was cleaved when GPA was coexpressed (Fig. 5). The level of cell surface expression in the presence of GPA was up to about twice that with band 3 alone. At 48 hr after injection, the proportions of total band 3 that are present at the oocyte surface are greater than the corresponding values after 24 hr of expression. The synthesis and translocation of a measurable proportion of band 3 to the plasma membrane of oocytes has been shown to take at least 10 hr at 18°C, even in the presence of GPA (Groves & Tanner, 1992) and implies that band 3 molecules accumulate continuously in the plasma membrane over the first 48 hr after injection. The results of Fig. 5 confirm the data from pulse-chase labeled oocytes (48 hr chase) in Fig. 4. Taken together, these results suggest that GPA facilitates the translocation of band 3 to the plasma membrane even at high cRNA concentrations (>3 ng/oocyte) where these differences are not apparent from the chloride influx assay (Fig. 2). In both the presence and absence of GPA, the increased proportion of band 3 expressed at the cell surface after 48 hr compared with 24 hr (Fig. 5) correlates with the levels of anion transport observed at these two expression times with lower cRNA concentrations (Fig. 3). The anion transport results in Fig. 2*a* suggest that this process of accumulation of band 3 at the surface may continue in oocytes for up to four days, at least at low cRNA concentrations.

In both the presence and absence of GPA cRNA, the amount of band 3 expressed at the oocyte surface increased with the concentration of band 3 cRNA (Figs. 4 and 5). This results from both the synthesis of a greater amount of total band 3 and the translocation of a slightly higher proportion of total band 3 to the surface at higher band 3 cRNA concentrations. Our results suggest that translocation may be a concentration-dependent effect. This may indicate that dimerization, for example, is involved in making the band 3 competent for translocation. Any interaction or modification which stabilizes the dimer (such as association with GPA) could therefore encourage a higher proportion of the band 3 to adopt a translocation-competent form.

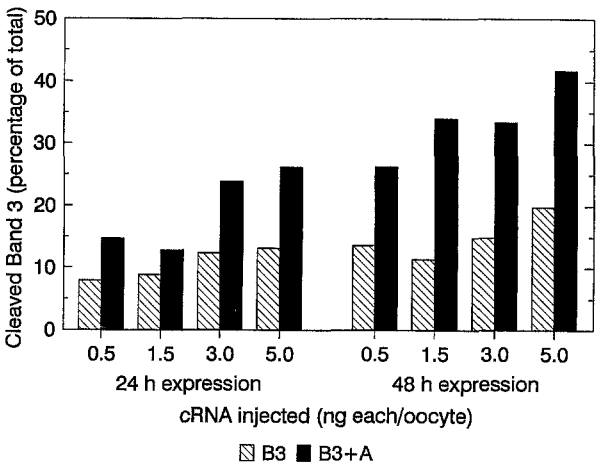


Fig. 5. Effect of band 3 cRNA concentration and time for expression on the appearance of band 3 at the oocyte surface. Groups of 10 oocytes were injected with either band 3 cRNA (B3), GPA cRNA (A) or both cRNAs (B3 + A) at four different cRNA concentrations. Cells were steady-labeled with [^{35}S]-labeled amino acids for either 24 or 48 hr, treated with chymotrypsin for 1 hr and then homogenized and immunoprecipitated with BRIC 170 (reactive with the NH_2-terminus of band 3), and immunoprecipitated protein from the equivalent of five oocytes was separated on 10% SDS–PAGE gels, as detailed in Groves and Tanner (1992). The proportion of band 3 cleaved by chymotrypsin was determined by scanning densitometry of the SDS–PAGE fluorographs as described in Fig. 4.

Limitations to the Chloride Influx Assay

Two types of approach have been used previously to study the expression of band 3-mediated anion transport activity in oocytes. In both techniques, oocytes are microinjected with exogenous band 3 cRNA (or mRNA) and the cells are incubated for at least 24 hr to allow the protein to be expressed. In the first technique, oocytes expressing band 3 are injected with a solution of $Na^{36}Cl$ and the rate of efflux of the radiolabeled chloride is measured (Bartel et al., 1989; Grygorczyk et al., 1989). Individual oocytes are placed immediately into a continuously flowing wash chamber which is positioned adjacent to a Geiger-Müller detection tube. Since the inhibitor content of the washing solution may be changed repeatedly during a single efflux run, this technique is particularly amenable to the study of the kinetics of anion transport in individual oocytes. However, the approach becomes very labor intensive when a large number of different expression conditions need to be studied simultaneously.

The second approach is to measure the influx of radiolabeled chloride into labeled cells (Garcia & Lodish, 1989). In this technique, groups of 10–20 oocytes are bathed in radiolabeled chloride for a prescribed period, washed in nontransporting buffer and counted individually in scintillation vials. Large numbers of oocytes may be studied simultaneously and the method is sensitive to the expression of very small amounts of band 3 cRNA, which are closer to the concentration of mRNA in the developing erythroid cell (Koury et al., 1987).

In this paper we provide further evidence, using the *Xenopus* oocyte expression system, that GPA specifically facilitates the translocation of band 3 to the plasma membrane. Although the effect of GPA on DNDS-sensitive chloride influx is more noticeable at low band

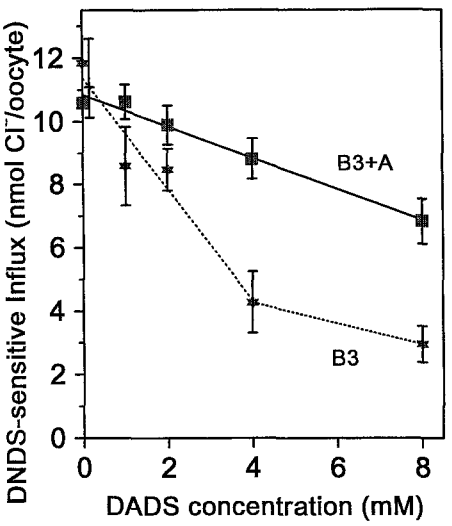


Fig. 6. Effect of DADS concentration on band 3-mediated chloride influx into oocytes. Oocytes were injected with band 3 cRNA (B3) at 5 ng/oocyte, with or without coinjection of GPA cRNA (A) at 1.5 ng/oocyte. Band 3-specific chloride influx (60 min) was measured 24 hr after injection of cRNAs using groups of 10–13 oocytes which were assayed with or without 400 μM DNDS treatment, as described in Fig. 1. The bar represents the standard error of the mean DNDS-sensitive chloride influx in each case.

3 cRNA concentrations (Fig. 3; Fig. 2*a* of Groves & Tanner, 1992), chymotrypsin treatment of intact cells has been used to show that greater amounts of band 3 protein are expressed at the surface in the presence of GPA at all the concentrations of band 3 cRNA we have studied (Figs. 4 and 5).

When low concentrations of band 3 cRNA are injected without the coinjection of GPA cRNA, then the amounts of band 3 in the plasma membrane are sufficiently low (even after four days expression time; Fig. 2*a*) that the assay of $^{36}Cl^-$ influx over a 60 min time period is required for sufficient radioactivity to be transported into the oocytes for accurate measurement. However, at high band 3 cRNA concentrations and in the presence of GPA cRNA, it appears that the amount of functional band 3 that is expressed at the surface of oocytes is sufficiently large (after only a 24 hr expression period) that the endogenous-labeled chloride in the oocyte and the radiolabeled chloride in the surrounding Barths saline may reach a near-equilibrium over a 60 min influx period (Figs. 2 and 3). Such "saturation" of the assay would cause the anion transport measurements not to reflect quantitatively the amounts of band 3 in the plasma membrane at these higher band 3 cRNA concentrations. In these circumstances, it is therefore likely that the apparent rates of anion transport are underestimates.

To investigate whether the amount of band 3 expressed at the cell surface (assessed by chymotrypsin assay) correlates with the amount of functional band 3 (anion transport assay) at high band 3 concentrations (>1.5 ng/oocyte), two modifications to the anion transport assay are possible. The first option is to measure chloride transport using a shorter influx time. An influx period of between 5 and 15 min may be appropriate. When oocytes were coinjected with band 3 cRNA (4.5 ng/oocyte) and GPA cRNA (1.5 ng/oocyte) and anion transport was measured after one, two or three days expression time using a 10 min influx, the DNDS-sensitive chloride influx was determined to be 4.57 ± 0.42 nmol Cl^-/oocyte (day 1), 7.31 ± 0.41 nmol Cl^-/oocyte (day 2) and 8.21 ± 0.28 nmol Cl^-/oocyte (day 3). These results demonstrate that the chloride transport assay does not show saturation 24 hr after coinjection of a high level of band 3 cRNA with GPA cRNA, and confirm that functional band 3 accumulates steadily in the plasma membrane with time under these conditions. Short influx times (especially 5 min or less) tend to lead to greater variability between individual oocytes. To ensure the significance of the results of a 10 min influx, we use at least 13–15 replicate oocytes for each test.

Secondly, it is possible to partially inhibit the anion transport activity of the band 3 using a relatively low affinity noncovalent inhibitor of band 3, such as 4,4,′-diamino-2,2′-stilbene disulfonate (DADS). After injection of oocytes with a high concentration of band 3 cRNA (5 ng/oocyte) in either the presence or absence of GPA cRNA (1.5 ng/oocyte), we measured the DNDS-sensitive chloride influx in the presence of various concentrations (0–8 mM) of DADS, which has a K_i of approximately 1.3 mM (Barzilay, Ship & Cabantchik, 1979). Oocytes coexpressing band 3 and GPA showed higher levels of anion transport in the presence of DADS than those expressing band 3 alone (Fig. 6). This difference was most clearly seen in the presence of 4 mM DADS, where the chloride influx in the presence of both band 3 and GPA was about twice as great as that with band 3 alone. Although we cannot exclude the possibility that this result may be influenced by differences in K_i between the band 3/GPA complex and band 3 alone in the plasma membrane, partial inhibition of anion transport (perhaps in combination with shorter influx times) may provide a useful tool to enable differences in band 3-specific anion transport to be detected at high concentrations of cRNA.

Conclusions

In this paper we have shown that GPA facilitates the expression of band 3 at the plasma membrane of oocytes more specifically, and at a wider range of band 3 cRNA concentrations than was previously indicated (Groves & Tanner, 1992). We have demonstrated that band 3 accumulates steadily in the plasma membrane of oocytes with time in both the presence and absence of

coexpressed GPA, but that the rate of accumulation is slower when GPA is not present.

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