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Mediation of inorganic anion transport by the hydrophobic domain of mouse erythroid band 3 protein expressed in oocytes of *Xenopus laevis*

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A cDNA clone of the mouse erythroid band 3 protein encoding the 556 amino acid residues of the hydrophobic domain from Thr-374 to the C-terminal Val-929 is shown by immunoprecipitation to be expressed in *Xenopus* oocytes. Measurements of $^{36}Cl^-$ efflux indicate that the translation product mediates Cl^- transport, which is inhibitable reversibly by DNDS or H_2DIDS, specific inhibitors of band 3-mediated transport. The apparent K_I values are 3.6 μM and 0.094 μM, respectively, and hence similar to those found in the wild type band 3-mediated anion transport. The rapid reversible inhibition by H_2DIDS slowly changes to irreversible inhibition. The rate of change increases with increasing pH, again similar as to the wild-type band 3. It is concluded that the hydrophobic domain of band 3 is capable of executing anion transport essentially similar to the full-length band 3, although minor differences with respect to transport and inhibition kinetics cannot be ruled out.

Introduction

The erythroid band 3 protein (molecular mass approx. 100 kDa) consists of two domains of about equal size: the C-terminal, hydrophobic transmembrane domain which resides in the lipid bilayer and the N-terminal, hydrophilic domain, which protrudes into the cytosol [7,13,18]. The hydrophilic domain includes binding sites for hemoglobin and certain glycolytic enzymes, and for ankyrin, a protein which links spectrin to the band 3 protein. It also contains a powerful antigenic determinant (Ref. 12, reviewed in Refs. 9,15,17). The hydrophobic domain accomplishes anion transport. It has been shown that the hydrophilic domain can be removed by tryptic cleavage with little loss of anion transport activity [5,11]. The anion transport mediated by the truncated transporter could still be inhibited by stilbene disulfonates or the disulfonate APMB with the same typical sidedness as in the unaltered transporter [11]. Nevertheless, certain interactions between the two domains have also been described. For example, H_2DIDS binding to the outward-facing surface of band 3 increases the strength of binding of both hemoglobin [16] and ankyrin to their respective binding sites in the inward – pointing hydrophilic domain [8].

Recently, it was shown that after microinjection of band 3-encoding cRNA, functional erythroid band 3 of mouse and man can be expressed in oocytes of *Xenopus laevis* [1,3]. Here we report the expression and functional characterization of a mouse band 3 cDNA clone truncated at a restriction site which, in the translated protein, is homologous to the trypsin cleavage site in the human band 3.

Successful attempts to express truncated non-erythroid band 3 proteins from various sources in COS 7 cells have been made previously [20,21]. However, similar attempts with erythroid band 3 (AE1) have been published only as an abstract without data [4].

Materials and Methods

The cRNA used for microinjection into the oocytes was derived from the modified cDNA clone pSPT19.Bd3 previously described by Bartel et al. [1]. This clone encodes the full length of mouse band 3, flanked at the 5′ end by the first 30 untranslated nucleotides proximal to the start codon, and at the 3′

Abbreviations: APMB, 2-(4′-aminophenyl)-6-methylbenzenethiazol-3′,7-disulfonic acid; DNDS, 4,4′-dinitrostilbene-2,2′-disulfonate; H_2DIDS, 4,4′-diisothiocyanodihydrostilbene-2,2′-disulfonate; BSA, bovine serum albumin.

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end by 204 untranslated nucleotides. Using appropriate conventional techniques, the nucleotides encoding Arg-5 up to Thr-374 were replaced by a synthetic nucleotide sequence encoding the amino acid sequence EVERERDVPPPAP. This sequence is an antigenic determinant of the band 3-like protein of human K 562 cells, where it is located between Glu-104 and Pro-116 in the sequence published by Demuth et al. [2]. This determinant was introduced into our construct since it is difficult to raise good antibodies against the hydrophobic domain of the erythroid band 3 protein and since Dr. Phil Low of Purdue University was kind enough to provide his antibody A 33, which was raised against the sequence listed above.

The construct described above was prepared as follows: the clone pSPT19.Bd3 was treated with the restriction enzyms *Sst* I and *Xba* I. The restriction site of *Sst* I is located in the polylinker upstream of the 5′ end of the band 3-encoding DNA sequence. The restriction site of *Xba* I resides between the nucleotides encoding Thr-374 and Leu-375. Thus, this treatment leads to a release of the nucleotide sequence encoding band 3 up to Thr-374. The removed part of the DNA sequence was replaced by a synthetic oligonucleotide comprising the nucleotides of the non-translated part of the sequence, the nucleotides encoding the first five amino acid residues of band 3 (up to Arg-5) and the nucleotides encoding the amino acid residues that constitute the antigenic determinant described above. The oligonucleotides ended with sequences that matched the restriction sites for *Sst* I and *Xba* I. Thus, the band 3 amino acid sequence is interrupted at Arg-5 and begins again at Thr-374, while the gap between the two amino acid residues 5 and 374 is filled with the antigenic determinant, as described above.

All other procedures were as described previously [1]. In particular, the flux measurements were performed in single *Xenopus* oocytes [6]. After expression of wild type or truncated band 3, 75 nl of $^{36}Cl^-$ (0.11 mCi/ml) were microinjected. The oocyte was placed into a perfusion chamber the bottom of which was formed by the mica window of a Geiger Müller tube. The radioactivity escaping from the oocyte was continuously washed away and the remaining radioactivity was recorded as a function of time. Rate constants of efflux were determined after digitalization and fitting a single exponential to the data by a nonlinear curve fitting procedure [6].

Results and Discussion

2 days after microinjection of cRNA encoding the truncated construct described above, the biosynthesis of the expected peptide can be demonstrated by immunoprecipitation (Fig. 1). The insertion of the construct into the plasma membrane can be inferred from the finding that it accomplishes Cl^- transport (Fig. 2). On a semilog scale a plot of the residual $^{36}Cl^-$ in the microinjected oocyte against time yields a straight line relationship, as expected for a transport process under conditions where the penetration through the plasma

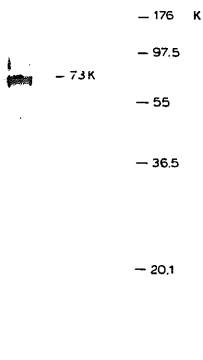

Fig. 1. Immunoprecipitation of truncated band 3 from mouse red blood cells expressed in *Xenopus* oocytes. 10 oocytes were microinjected with 50 ng/cell of the cRNA derived from the cDNA construct described under Materials and Methods. This construct encodes the 556 C-terminal amino acid residues of mouse AE1 and 13 amino acid residues of an antigenic determinant from human erythroleucemic AE2. The oocytes were incubated in Barth's medium at 18°C for 2 days and subsequently used for immunoprecipitation by the method described previously in extenso [6]. The antibody against the antigenic determinant was a gift of Dr. Ph. Low. The calculated molecular mass of the construct was 64.8 kDa. In a cell free translation system containing a reticulocyte lysate (see Ref. 1) we observed 66.8 kDa unglycosylated on SDS-PAGE (not shown). After expression in the oocytes we find two rather distinct bands at 67.5–71.0 kDa and 75.0–79.5 kDa. After SDS-PAGE, full length band 3 from mouse red blood cells yields a molecular mass of 108 kDa, as measured at the center of the rather diffuse band [22]. The difference between this value and the value calculated from the amino acid composition (~ 102 kDa) represents an approximate measure for the carbohydrate content of mouse band 3. We would expect to find the glycosylated form of the truncated band 3 at about 73 kDa, i.e., somewhere between the two bands shown in lane 2. The appearance of two distinct bands indicates the existence of two distinct populations of band 3 molecules below and above the calculated average of 73 kDa with different carbohydrate contents. Such populations have been described previously [24,23]. Lane 1: control oocytes, not cRNA injected. Lane 2: oocytes with microinjected cRNA. The molecular weight markers were not radioactively labeled. Their locations are indicated to the right of the autoradiograms.

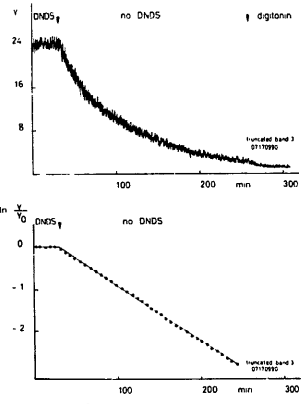

Fig. 2. Mediation of $^{36}Cl^-$ efflux by the truncated band 3 protein. 2 days after microinjection of a cRNA encoding the truncated band 3 protein, $^{36}Cl^-$ was microinjected into the same oocyte. The upper panel shows the original record of the $^{36}Cl^-$ content of the oocyte as a function of time. During the initial perfusion with Barth's solution in the presence of 500 μM-DNDS, little if any $^{36}Cl^-$ is released, indicating the absence of a leak. After changing to Barth's medium without DNDS, Cl^- efflux is initiated. After averaging and digitalizing, a replot of the data on a semilog scale yields the straight line shown in the lower panel. The addition of digitonin (5 μM) renders the plasma membrane leaky and shows that most of the Cl^- inside the oocyte is readily diffusable. Barth's medium contained 88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 0.82 mM $MgSO_4$, 0.33 mM $Ca(NO_3)_2$, 0.41 mM $CaCl_2$, 5.0 mM Hepes (pH 7.6), 20°C.

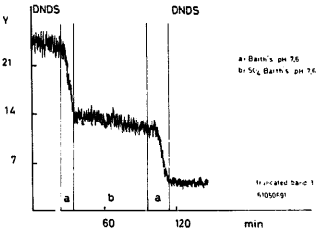

Fig. 3. Effect of replacement of Cl^- in Barth's medium by SO_4^{2-}. After translation of the cRNA encoding the truncated band 3 and microinjection of $^{36}Cl^-$, the flow chamber containing the oocyte is perfused with Barth's medium containing 500 μM DNDS. Efflux is inhibited; there is no leak. After replacement of the inhibitor-containing medium by inhibitor-free medium, $^{36}Cl^-$ in the oocyte decreases. Averaging, digitalizing and fitting a single exponential to the data yields the rate constants ($^{a}k_{Cl}$) for $^{36}Cl^-$ efflux of 0.33 min^{-1} for the first perfusion period with Cl^- Barth's solution and 0.31 min^{-1} for the second period. After changing to Barth's medium in which the NaCl (90 mM) had been isosmotically replaced by Na_2SO_4 (60 mM), the rate of decrease of intracellular $^{36}Cl^-$ is reduced. The rate constant for efflux indicated in the figure amounts to about 5% of the rate constant observed in the presence of Cl^-. When perfusion is changed back to Barth's solution $^{36}Cl^-$ efflux is resumed. The absence of a leak follows from the observation that after perfusion with 500 μM DNDS, inhibition reappears. Ordinate: radioactivity in the oocyte. Abscissa: time in min.

membrane rather than the diffusion inside the oocyte is rate limiting. The slope of the line yields the rate constant for efflux.

In the red blood cell, band 3-mediated Cl^- transport represents essentially a 1 : 1 exchange with little if any net Cl^- flux. Fig. 3 shows that this is also true when the $^{36}Cl^-$ efflux is mediated by the truncated band 3: when external Cl^- is isosmotically replaced by SO_4^{2-}, which is known to exchange about four orders of magnitude more slowly than Cl^-, then $^{36}Cl^-$ efflux is largely inhibited. Subsequent return to the original, Cl^--containing medium induces the resumption of the Cl^- efflux, indicating the reversibility of the inhibition. A systematic study of the effects of gradual Cl^-/SO_4^{2-} substitution will be presented elsewhere in a different context [19].

TABLE I

Reversible plus irreversible inhibition = inhibition measured during perfusion with 10 μM H_2DIDS. Irreversible inhibition = inhibition that persisted after perfusion with 10 μM H_2DIDS for 1 h during subsequent perfusion with H_2DIDS-free Barth's medium, containing 0.2% BSA. The data for irreversible inhibition in the wild type were calculated from the data of Kietz et al. [10].

pH	Wild type		Mutant			
	H_2DIDS total inhibition [a] (%)	H_2DIDS irreversible inhibition (%)	H_2DIDS total inhibition [a] (%)	H_2DIDS irreversible inhibition (%)	±S.E.	*n*
9.0	-	97.3	96.0	77.2	3.0	12
8.5	98.5	93.9	93.4	71.1	3.7	10
8.0	98.5	80.3	94.6	74.7	4.2	11
7.5	98.5	50.2	96.5	70.9	3.6	13
6.5	98.5	7.9	96.4	7.5	4.7	21

[a] Reversible plus irreversible inhibition.

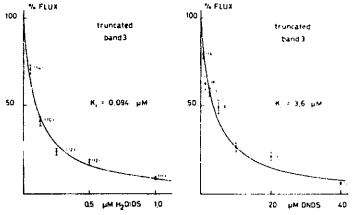

Fig. 4. $^{36}Cl^-$ efflux mediated by the truncated mouse band 3 protein as a function of DNDS or H_2DIDS concentration in the medium. Ordinate: $^{36}Cl^-$ efflux as a percent of efflux in the absence of inhibitor. Abscissa: inhibitor concentration. Inhibition by DNDS was measured at pH 7.4, inhibition by H_2DIDS at pH 6.5. Note that according to Fig. 2 in Ref. 10, at pH 6.5 there is virtually no irreversible H_2DIDS binding during the time required for the measurements. The drawn curves represent nonlinear least square fits to the data of the equation $({}^{e}k_{Cl}/{}^{e}k_{Cl(DNDS-0)})\cdot 100 = 100\cdot K_I/(K_I + [I])$, where K_I represents the inhibitor concentration [I] at half maximal inhibition. ${}^{e}k_{Cl}$ and ${}^{e}k_{Cl(DNDS-0)}$ indicate, respectively, the rate constants of efflux in the presence and absence of DNDS at the concentration [I].

Fig. 4 illustrates the relationship between reversible inhibition of Cl^- transport and stilbene disulfonate concentration in the medium. The concentration at half maximal inhibition is 3.6 μM for DNDS and 0.094 μM for H_2DIDS. This is similar to the results obtained with the wild type band 3 in red cells or after expression in *Xenopus* oocytes. In Table I data are compiled which show that the rapid reversible inhibition by H_2DIDS slowly becomes irreversible. The pH dependence of the transition from reversible to irreversible inhibition in the construct is rather similar to that in the wild type band 3 [10]. There is a tendency for the construct to allow a somewhat faster covalent reaction with H_2DIDS than the wild type. In addition, the maximal inhibition that can be achieved seems to be higher in the wild type than in the mutant. The significance of these differences is, however, doubtful.

On the basis of the experiments described we conclude that the hydrophobic domain of erythroid band 3 can be successfully inserted into the plasma membrane of *Xenopus* oocytes where it is capable of executing Cl^- transport similar to the wild type band 3. Small quantitative differences cannot be excluded.

Acknowledgements

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