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Anion transport in oocytes of *Xenopus laevis* induced by expression of mouse erythroid band 3 protein—encoding cRNA and of a cRNA derivative obtained by site-directed mutagenesis at the stilbene disulfonate binding site

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A vector was constructed containing a cDNA for mouse band 3 obtained from Demuth *et al.* (1986, *EMBO J.*, 5, 1205–1214), a synthetic linker (containing 5′-non-translated region, start codon and a coding region for the first 12 N-terminal amino acids), and RNA polymerase promoters suitable for *in vitro* transcription of cRNA. After injection of the cRNA into the cytoplasm of *Xenopus* oocytes and incubation for 16 h, expression of mouse band 3 was demonstrated by immunoprecipitation, immunohistochemical methods and influx or efflux measurements with $^{36}Cl^-$. Antisense cRNA inhibits the expression. Lysines 558 and 561 were replaced by asparagines using oligonucleotide-directed mutagenesis. Like the original band 3, the mutant shows stilbene disulfonate-inhibitable anion exchange. However, in contrast to the original band 3, inhibition by 4,4′-diisothiocyano dihydrostilbene-2,2′-disulfonate (H_2DIDS) is no longer irreversible. This indicates that thiourea bond formation between H_2DIDS and band 3 involves one of the two modified lysine residues. It also shows that the two lysine residues are not essential for the execution of the anion transport function of band 3. The results described suggest that the cDNA clone of Demuth *et al.* (1986) encodes a protein with properties that are representative for the properties of the bulk of the band 3 protein in the plasma membrane of the red cell of the mouse.

Key words: anion transport/cRNA expression/mouse erythroid band 3 protein/site-directed mutagenesis/*Xenopus* oocytes

Introduction

The band 3 protein (mol. wt 96 000) was first identified in red blood cells of humans and other vertebrate species where it constitutes the most abundant integral membrane protein (Fairbanks *et al.*, 1971; Steck, 1974, 1978). In the red blood cells, band 3 protein serves several different functions, such as the mediation of an electrically silent anion exchange (which includes the physiologically important exchange of Cl^- against HCO_3^-) and the fixation of the membrane skeleton to the lipid bilayer. Later work with immunological (Kay *et al.*, 1983; Drenckhahn *et al.*, 1985) and gentechnological (Alper *et al.*, 1987) methods revealed its ubiquitous occurance in many other cells and tissues. Up to the present time cDNAs of erythroid band 3 proteins of mouse (Kopito and Lodish, 1985; Demuth *et al.*, 1986), man (Tanner *et al.*, 1988) and chicken (Cox and Lazarides, 1988; Kim *et al.*, 1988), and non-erythroid band 3 proteins of liver, kidney and certain erythropoeitic precursors (Demuth *et al.*, 1986; Alper *et al.*, 1987, 1988) have been cloned and sequenced.

Although the anion transport function of band 3 has been studied in many laboratories (for reviews, see Jennings, 1985; Knauf, 1986; Passow, 1986), little is known about the role of specific amino acid residues in the translocation process. Studies of the exchange kinetics indicate that the mode of action of band 3 is different from that of ionic channels and involves substrate binding prior to substrate translocation. Work with site-specific inhibitors suggests that certain arginine, lysine and possibly histidine residues as well as some carboxyl groups of either aspartate or glutamate participate in the transport process. The function and localization of most of these groups in the amino acid sequence is still uncertain or completely unknown. In particular, nothing is known about the amino acids that directly participate in the formation of the substrate binding site. It has been speculated that arginine residues play a role (Zaki, 1981; Wieth and Bjerrum, 1982), but it cannot be excluded that uncharged regions of the peptide replace the hydration shell of the substrate anion, similar to valinomycin when it forms complexes with K^+.

The availability of cDNAs encoding band 3 opens up a new avenue to the study of the functional significance of individual amino acid residues by site-directed mutagenesis. The prerequisite for such studies is, however, the availability of a suitable expression system. Oocytes of *Xenopus laevis* have been used previously for the expression of many transport systems, notably ion channels (reviewed by Yellen, 1984). The activity of the channels can be measured with high sensitivity by electrophysiological methods, including patch-clamping. The transport mediated by band 3 does not contribute, however, to the electrical conductance and hence requires measurements of anion fluxes by means of radioactive tracers. In previous publications, we have demonstrated that microinjection into the oocytes of poly(A)$^+$ mRNA extracted from the spleens of anemic mice leads to the expression of band 3 protein with functional characteristics essentially similar to those observed in the red cell of the mouse (Morgan *et al.*, 1985; Grygorczyk *et al.*, 1987, 1989; Hanke-Baier *et al.*, 1988). In the present paper we report the successful expression in *Xenopus* oocytes of cRNA derived from mouse erythroid band 3 cDNA. We demonstrate that the expressed protein mediates chloride transport that is inhibitable by stilbene disulfonates which are known to be highly specific inhibitors of band 3-mediated anion exchange in the red cells of man and mouse. In addition, we apply site-directed mutagenesis to study the role of two lysine residues that are located near the cell's outer surface and are supposed to be candidates for the site of covalent binding to band 3 of the stilbene disulfonate H_2DIDS (4,4′-diisothiocyano dihydrostilbene-2, 2′-disulfonate).

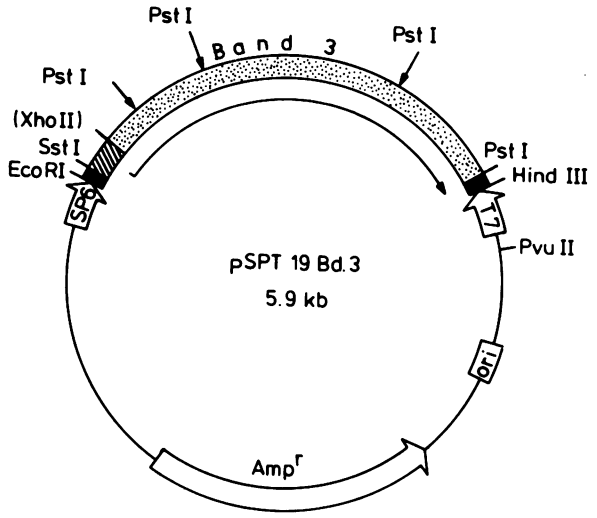

Fig. 1. Structure of pSPT19Bd.3 vector for *in vitro* synthesis of band 3 cRNA transcripts. Arrows indicate the approximate positions of relevant restriction enzyme cleavage sites. Relative positions and orientations of β-lactamase gene (*amp*, designated AMP^r) and band 3 cDNA insert are indicated. cDNA insert regions are represented as follows: hatched, synthetical oligonucleotide including 5′-untranslated region, start codon AUG and a translated region for the 12 N-terminal amino acids; stippled, coding region of band 3 (Demuth *et al.*, 1986); filled, polylinker sites. SP6 and T7 are promoters for SP6 and T7 RNA polymerases.

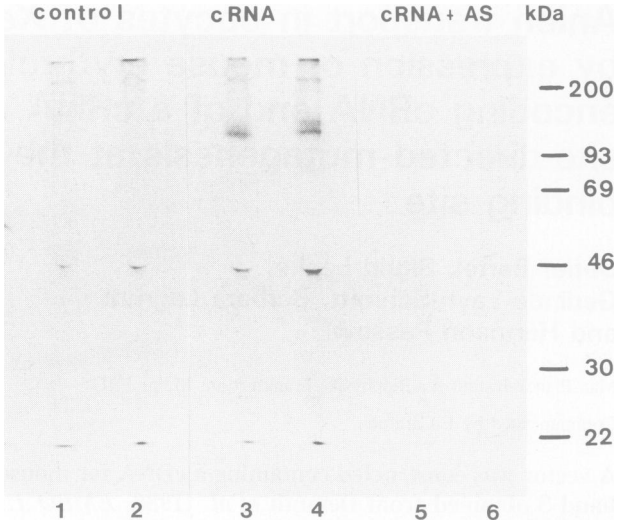

Fig. 2. Autoradiogram of an SDS–polyacrylamide gel (10%) used to fractionate band 3 immunoprecipitated from microinjected oocytes. **Lanes 1** and **2**, non-injected oocytes (controls); **lanes 3** and **4**, band 3 cRNA-injected oocytes; **lanes 5** and **6**, oocytes injected with sense–antisense cRNA hybrids. Two different concentrations of antibodies were used: 15 ng for lanes 1, 3 and 5, 25 ng for lanes 2, 4 and 6.

Preliminary reports about our work have been presented elsewhere (Bartel *et al.*, 1989; Passow *et al.*, 1989).

Results

Source of cRNA

Plasmid pMEB3.18 was kindly supplied by Dr Peter Curtis of the Wistar Institute in Philadelphia and was used for the *in vitro* synthesis of cRNA. This clone encoded the amino acid sequence of mouse band 3, commencing at Leu 11 (using the designations of Kopito and Lodish, 1985) up to the C-terminal Val 929 plus 212 bp of the 3′-untranslated region: altogether 2970 bp. The cDNA obtained was cloned into the *Pst*I site of pBR322. Since the base sequence encoding band 3 also contains three *Pst*I sites (Figure 1), it was necessary to subclone overlapping fragments obtained after hydrolysis with suitable restriction enzymes. Subsequently, a synthetic oligonucleotide was ligated to the 5′ end of the cDNA to supplement the remaining amino acid residues up to the N terminus plus 30 additional bases upstream of the start codon. In addition, the frame shift mutation in pMEB3.18 of Gly 385 was amended by inserting a desoxythymidine at position 1125 of the cDNA sequence (in the notation of Demuth *et al.*, 1986). The completed cDNA sequence was cloned into pSPT19, a vector which has a SP6 promoter at the 5′ end of the cDNA insert and a T7 promoter at the 3′ end (Figure 1). This enabled us to transcribe *in vitro* full-length sense and antisense cRNA using the corresponding polymerases. In a number of experiments, we used an antisense cRNA derived from cDNA extending from position −30 of the 5′-untranslated region to nucleotide 344 of the band 3-coding sequence. The sense cRNA was capped with a monomethyl cap but not polyadenylated (see Materials and methods).

Expression of the cRNA in a cell-free translation system

Using a reticulocyte lysate as a cell-free translation system, a [^{35}S]methionine-labeled band appeared at the location expected for mouse band 3 protein. The appearance of this band could be suppressed when the lysate contained either full-length antisense cRNA or the smaller fragment of antisense cRNA derived from the 5′ region of the cDNA described above (not shown).

The absence of band 3-encoding mRNA in the Xenopus oocytes

Oocytes of *Xenopus* exhibit endogenous Cl^- transport (Richter *et al.*, 1984), which is however, only slightly, if at all, inhibitable by band 3-specific stilbene disulfonates (Morgan *et al.*, 1985). Polyclonal antibodies against mouse band 3 produce no detectable immunoprecipitation of the solubilized oocyte proteins. Hybridization with two different cDNA fragments provided no evidence for the occurrence of the endogenous mRNA in the cytosol of the oocyte that encodes for band 3 or band 3-like peptides. Hence the injection of mouse cRNA is most unlikely to trigger the expression of a *Xenopus*-specific band 3.

The biosynthesis of mouse band 3 protein in the Xenopus oocyte

The biosynthesis of the mouse band 3 protein in the foreign host cell was demonstrated by means of polyclonal antibodies against band 3. Figure 2 shows that 24 h after microinjection of cRNA (~30 ng/oocyte) and incubation at 18–20°C, immunoprecipitates can be observed that are located on SDS–PAGE at the mol. wt expected for mouse band 3. The occurrence of the precipitate is suppressed when the cRNA is microinjected together with antisense cRNA, regardless of whether one uses full-length antisense cRNA or only the small (374 bases) fragment derived from the 5′ region of

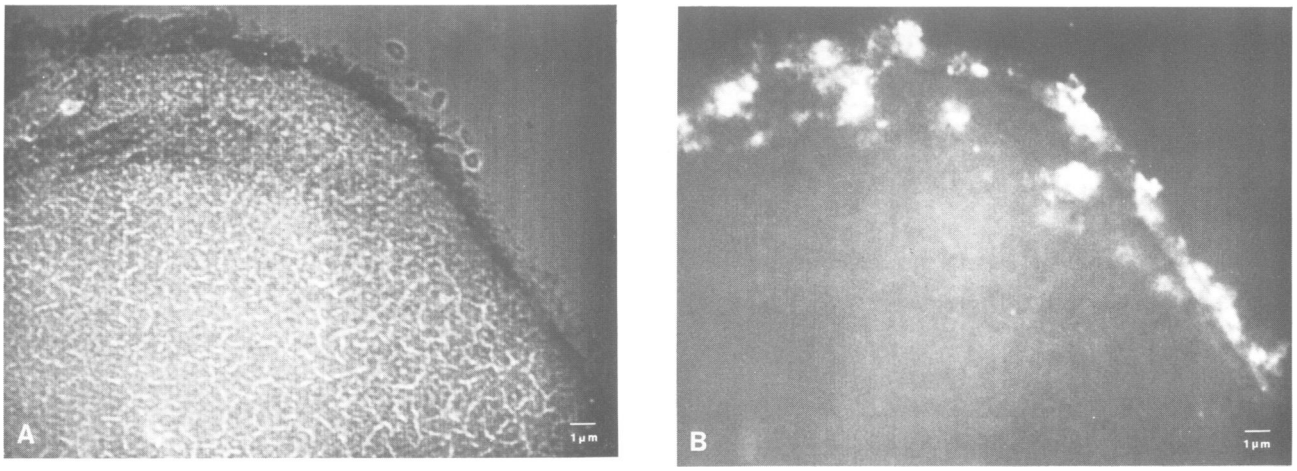

Fig. 3. Phase-contrast (**A**) and immunofluorescence microscopy (**B**) of cryosections from band 3 cRNA-injected oocytes. Expression of mouse band 3 in the oocyte membrane was detected by an antibody to mouse band 3 raised in rabbits and FITC labeled goat-anti-rabbit IgG.

the cDNA (see above). Similar results were obtained by observing the immunofluorescence of cryoslices from oocytes. The controls without microinjected cRNA show no detectable fluorescence (not shown) while the fluorescence of the slices obtained from cRNA-microinjected oocytes is quite pronounced (Figure 3).

Mouse band 3-mediated chloride transport across the plasma membrane of the Xenopus oocyte

In a first set of experiments, the cRNA-injected (~30 ng/oocyte) oocytes were first incubated for 16 h to allow for band 3 expression. They were then resuspended in Barth's medium containing $^{36}Cl^-$. After another 3 h of incubation at 20°C, the oocytes were collected, washed free of external radioactivity and placed individually in counting vials containing liquid scintillator. After microinjection of cRNA, $^{36}Cl^-$ uptake was several-fold higher than in uninjected control oocytes (Figure 4). This extra uptake was inhibited when the external medium contained the band 3-specific inhibitor 4,4′-dinitrostilbene-2,2′-disulfonate (DNDS) and was absent when the mRNA was microinjected together with antisense cRNA (Figure 5A). The antisense cRNA was effective, regardless of whether the full-length antisense cRNA or the fragment complementary to the 5′ terminal end of the sense cDNA was used. These findings confirm that the cRNA derived from the cDNA clone causes the expression of functional mouse band 3 in the oocyte.

The successful functional expression of band 3 was further substantiated by continuous recordings of efflux from single oocytes, using a previously described experimental arrangement (Grygorczyk *et al.*, 1987, 1989). Such measurements are much less sensitive than influx measurements since a small percentage decrease of radioactivity in the cells from a high initial value is more difficult to detect than the uptake of $^{36}Cl^-$ by a cell that was initially devoid of radioactivity. However, the influx measurements suffer from the disadvantage that the behavior of cRNA-injected oocytes has to be compared with that of others that had received no cRNA. Since the variability of band 3 expression in individual oocytes is fairly large, large numbers of oocytes have to be microinjected and subjected to flux measurements in order to obtain statistically

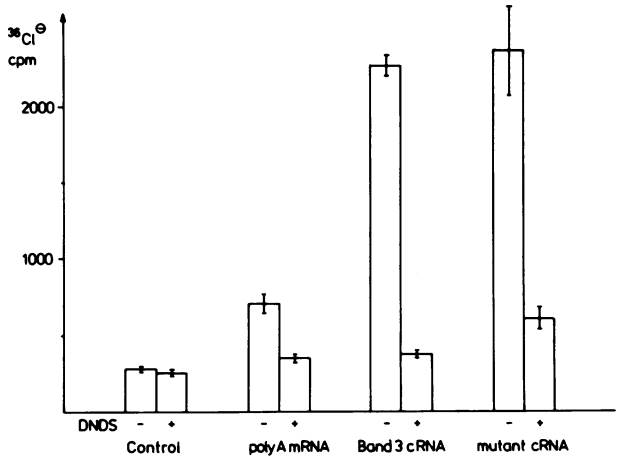

Fig. 4. $^{36}Cl^-$ uptake by oocytes microinjected with poly(A)$^+$ mouse spleen RNA, band 3 cRNA, mutated cRNA, measured in the presence (+) or absence (−) of DNDS. Control: non-injected oocytes. Error bars indicate standard errors of the mean.

significant differences. Measuring $^{36}Cl^-$ efflux does not encounter this problem, since each oocyte can be used as its own control by observing the effect of reversibly acting inhibitor, like the band 3-specific stilbene disulfonate DNDS (4,4′-dinitrostilbene-2,2′-disulfonate). The residual flux persisting after maximal inhibition yields a measure of the band 3-independent 'leakiness' for $^{36}Cl^-$. Evidently, efflux measurements require a higher expression of band 3 than influx measurements. The high expression can be achieved by microinjection of sufficiently large amounts of synthetic cRNA (~20−30 ng/oocyte), prior to the beginning of the 16 h incubation period at 18−20°C.

The protocol of the efflux measurements is illustrated in Figure 6. At the end of the incubation period required for band 3 expression, an oocyte received an injection of $^{36}Cl^-$. It was then placed into a perfusion chamber the bottom of which is formed by the mica window of a Geiger−Müller tube. The chamber was perfused at room temperature with Barth's solution containing either 0, 2 or 500 µM of the reversibly binding inhibitor DNDS. These media carried away the radioactivity that leaked out of the

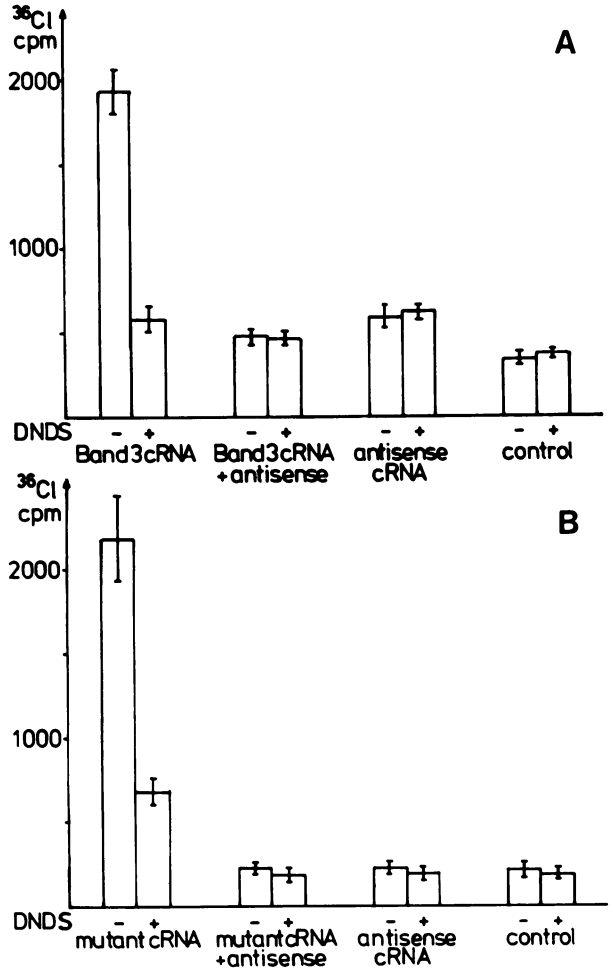

Fig. 5. Effect of full-length antisense cRNA on the development of DNDS-sensitive (i.e. band 3-mediated) $^{36}Cl^-$ transport in sense cRNA-injected oocytes. Same experimental arrangement as in Figure 4.

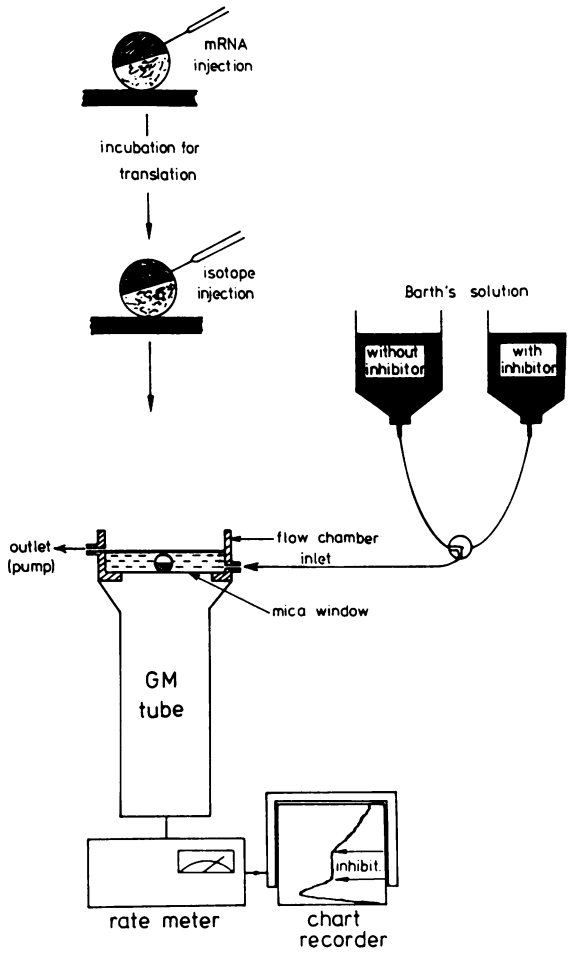

Fig. 6. Experimental set up for measuring $^{36}Cl^-$ efflux in a single *Xenopus* oocyte. After microinjection of cRNA and incubation for 16 h, an oocyte is microinjected with $^{36}Cl^-$ and placed on a Geiger–Müller (GM) tube. The counting rate is continuously determined by a ratemeter and recorded by a chart recorder during perfusion with $^{36}Cl^-$-free solutions (adapted from Grygorczyk *et al.*, 1989).

oocyte. A ratemeter attached to the GM tube permitted the continuous recording of the $^{36}Cl^-$ content of the oocyte as a function of time.

Figure 7(A) shows that there is little if any $^{36}Cl^-$ efflux from control oocytes that had not received cRNA. Similarly (Figure 7B), in oocytes that had received enough cRNA to produce sufficient mouse band 3 for detection by the immunological methods described above, little efflux is seen as long as the perfusing Barth's solution contains the inhibitor DNDS at a concentration of 500 μM (1). When we turn, however, to perfusion with DNDS-free Barth's solution, rapid $^{36}Cl^-$ efflux is observed (2). The rate of efflux is significantly reduced when the perfusion is continued with Barth's solution containing DNDS at a concentration of 2.0 μM, which is close to the concentration known to produce about half maximal inhibition in mouse red cells (3) (Hanke-Baier *et al.*, 1988). When the DNDS concentration is again augmented to 500 μM, the rate of $^{36}Cl^-$ efflux is reduced to about the same low level found during the first observation period at this concentration of DNDS (4). Another perfusion period with DNDS-free Barth's solution confirms that the changes observed in the course of the experiment were entirely reversible (5).

The time course of band 3-mediated Cl^- efflux follows a single exponential which, for short observation times, can be approximated by a straight line (Grygorczyk *et al.*, 1987, 1989). From the slope of the straight line normalized with respect to the radioactivity at the mid-point of the line [which is approximately equal to $(t_1 - t_2)^{-1} \ln(y_{t1}/y_{t2})$], one obtains rate constants with the dimension min^{-1} (see legend to Figure 7). Using these constants it is possible to make a rough estimate of the number of band 3 molecules inserted into the plasma membrane, provided one introduces the following assumptions:

(i) The turnover number of each band 3 molecule in the oocyte membrane is the same as in the mouse red blood cell membrane, namely 490 s^{-1} at 0°C (Raida *et al.*, 1989).
(ii) The Arrhenius activation energy for band 3-mediated transport is similar to that observed in the red cells of mouse and other species over the temperature range 0–20°C (~30 kcal/mol).
(iii) The Cl^- concentration inside the oocytes is equal to the Cl^- concentration in Barth's medium (90 mM).
(iv) The volume of one oocyte is ~1 μl, and 70% of this volume is accessible to Cl^-.

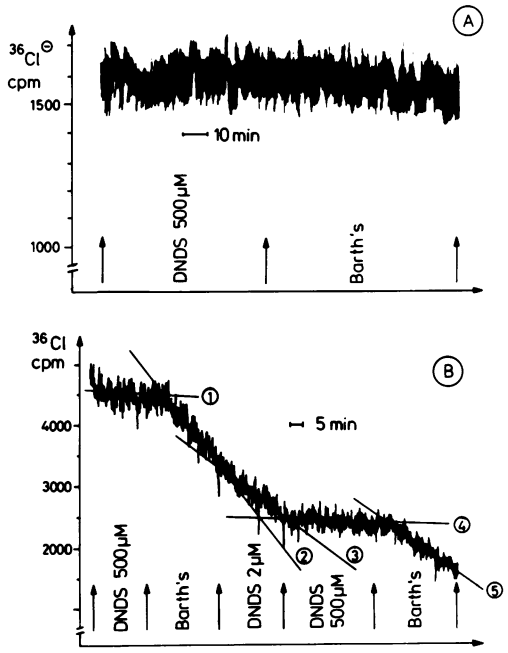

Fig. 7. $^{36}Cl^-$ efflux from a single *Xenopus* oocyte determined by the method represented in Figure 6. (**A**) $^{36}Cl^-$-efflux from a control oocyte which had not been injected with cRNA; (**B**) efflux from a band 3 cRNA-injected oocyte which had been incubated overnight before flux measurement. The rate constants for $^{36}Cl^-$ efflux (relative units) DNDS, 500 μM: 0.0023; Barth's (DNDS, 0 μM): 0.082; DNDS, 2 μM: 0.063; DNDS, 500 μM: 0.00294; Barth's DNDS, 0 μM): 0.094.

The result of such estimate is ~20–40 band 3 molecules/μm^2. This should be compared with ~7000 band 3 molecules/μm^2 in the red cell membrane.

Cl^- transport mediated by a band 3-derived mutant

For the identification of the anion transport function of erythroid band 3, the use of non-penetrating, covalently binding stilbene disulfonate derivatives was essential. These derivatives include H_2DIDS, which has the same structure as DNDS except that the two nitro groups are substituted by two isothiocyanate groups and the double bond is hydrogenated. One of these isothiocyanate groups combines readily with a lysine residue. It has been suggested that this residue is either Lys 558 or Lys 561, both of which are located in a region of the amino acid sequence which shows a high degree of sequence homology between mouse and man (Tanner *et al.*, 1988). In the chicken red cell, only the lysine residue homologous to Lys 558 is present (Cox and Lazarides, 1988; Kim *et al.*, 1988); this has been considered to point to Lys 558 as the site of reaction with H_2DIDS (Tanner *et al.*, 1988).

In our present attempt to establish the procedures for the study of site-directed mutations we chose to change the AAG codons for Lys 558 and 561 into AAC codons for asparagine residues. These mutations should show whether these lysine residues are essential for the transport process and if one or both of them are in fact involved in the covalent binding of H_2DIDS.

In the cell-free translation system the mutated band 3 cRNA directed the synthesis of a full-length translation product as observed after cell-free translation of the non-mutated cRNA. We expressed the mutated cRNA in the oocytes as described above for the non-mutated cRNA. Measurements of $^{36}Cl^-$ influx showed a considerable increase above the control level observed in oocytes which had received no cRNA. This increase was completely prevented when the sense cRNA was injected together with antisense cRNA (see Figure 5B). This suggests that the increment of the flux was due to the expression of the band 3 derivative.

The flux increment could be inhibited considerably by 500 μM DNDS, indicating that the mutations left the capacity for anion transport essentially intact and did not lead to the disappearance of the susceptibility to inhibition by the reversibly binding stilbene disulfonate.

A comparison of the effects of H_2DIDS on the non-mutated and the mutated band 3 was made in the experiments represented in Table I. Oocytes that had been incubated overnight after microinjection of ~30 ng cRNA coding for band 3 ('wild-type') or for the mutated band 3 ('mutant') were incubated at 20°C in Barth's medium, pH 7.4, containing $^{36}Cl^-$ for 3 h. They were then collected, washed free of external radioactivity and counted individually after dissolution in liquid scintillator. Table I shows that both the 'wild type' and 'mutant' are able to promote DNDS-inhibitable $^{36}Cl^-$ uptake. When this uptake is measured after previous exposure of the oocytes to H_2DIDS at pH 8.75 for 1 h and subsequent washings with albumin-containing Barth's medium, one observes an irreversible inhibition in the wild-type but no inhibition in the mutant. This shows that a replacement of Lys 558 and Lys 561 by Asn residues at the corresponding locations leads to the loss of the capacity of irreversible inhibition by H_2DIDS of band 3-mediated Cl^- transport.

The observations made by measuring $^{36}Cl^-$ influx were confirmed by measuring $^{36}Cl^-$ efflux from single oocytes. In a set of control experiments we first demonstrated that the Cl^- efflux induced by the cRNA derived from unaltered cDNA and measured at pH 7.6 can be inhibited by H_2DIDS and that this inhibition is reversible when the exposure to the H_2DIDS takes place at pH 6.8 (Figure 8A) but becomes irreversible when the exposure takes place at pH 8.5 (1 h, 20°C, Figure 8C). Analogous experiments with oocytes in which the mutated band 3 was expressed showed a rapid Cl^- efflux when no inhibitor was present, confirming that the mutation does not destroy the capacity of executing Cl^- transport (Figure 8B). The inhibition by H_2DIDS can still be seen; however, the effect remains reversible even when the cells had been exposed to H_2DIDS at pH 8.5, under the same condition under which the Cl^- efflux from the control is irreversibly blocked (Figure 8D). These results confirmed that either Lys 558 or Lys 561 are involved in covalent binding of H_2DIDS to band 3 and show clearly that the two lysine residues are not essential for the transport of the anions in the absence of H_2DIDS or DNDS.

Discussion

The main result of the present work consists of the observation that after minor amendments performed on the basis of the cDNA-deduced band 3 sequence published by Kopito and Lodish (1985), the cDNA clone isolated by Demuth *et al.* (1986) can be used to generate a cRNA that can be expressed in a functional state in the plasma

Table I. Effect of mutation of Lys 558 and 561 on inhibition by H_2DIDS of mouse band 3-mediated Cl^- influx into *Xenopus* ooxytes

cRNA	No pretreatment with H_2DIDS				Pretreatment with H_2DIDS			
	No DNDS	*n*	500 μM DNDS	*n*	No DNDS	*n*	500 μM DNDS	*n*
Wild-type	2243 ± 260	10	171 ± 30	14	362 ± 75	10	171 ± 27	14
Mutant	1869 ± 286	12	415 ± 84	13	2370 ± 300	9	406 ± 69	14
No cRNA injected	226 ± 33	15	162 ± 24	17	–	–	–	

The figures in the table represent radioactivity uptake per oocyte, in c.p.m. and the corresponding standard deviations of the means. *n* = number of individual oocytes counted. Time period of uptake is 3 h. Pretreatment of the oocytes prior to the influx measurements with 20 μM H_2DIDS, pH 8.75, 20°C, for 1 h. $^{36}Cl^-$ uptake was measured after removal of non-covalently bound H_2DIDS by washes in albumin-containing (0.25%) Barth's medium at 20°C. Mutant: oocytes microinjected with a cRNA derived from a band 3 encoding cDNA in which the codons coding for Lys 558 and Lys 561 had been mutated into codons coding for Asn 558 and Asn 561.

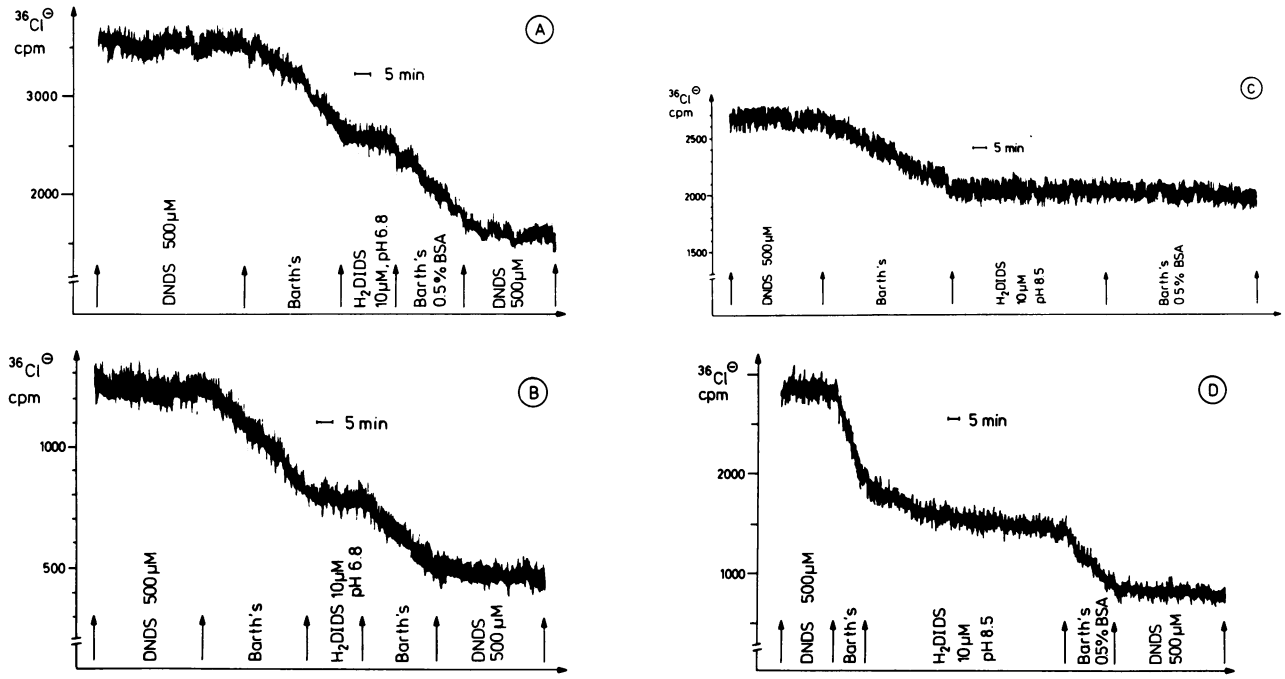

Fig. 8. Efflux measurements in oocytes microinjected with band 3-encoding cRNA (**A** and **C**) or band 3 mutLys encoding cRNA (**B** and **D**) and the inhibition of $^{36}Cl^-$ flux by H_2DIDS. The efflux is inhibited by H_2DIDS at pH 6.8 in normal and mutated band 3. (C) shows the irreversibility of inhibition of $^{36}Cl^-$ efflux from a band 3 cRNA-microinjected oocyte after treatment with H_2DIDS at pH 8.5 prior to the efflux measurement in the absence of H_2DIDS. The inhibitor cannot be washed away when perfusion with H_2DIDS-free Barth's medium containing 0.5% BSA is resumed. (D) shows the same measurement as (C) but with a band 3 mutLys cRNA-microinjected oocyte. H_2DIDS binding to the mutated protein remains reversible, even at pH 8.5. The efflux returns to nearly the original value after washing with Barth's medium containing 0.5% BSA. The recovery after inhibition by H_2DIDS shown in (A), (B) and (D) can be expressed quantitatively by calculating rate constants from the efflux curves prior and after perfusion with H_2DIDS. Using the expression $(t_1 - t_2)^{-1} \ln(y_{t1}/y_{t2})$ (where y_{t1} and y_{t2} represent the radioactivity in the oocyte at times t_1, t_2 respectively; Grygorczyk *et al.*, 1987, 1989), we find in (A) 0.014 and 0.016 min^{-1}, in (B) 0.013 and 0.014 min^{-1}, in (D) 0.018 and 0.014 min^{-1}. The first and second in each pair of figures refers to the rate constant of efflux before and after H_2DIDS respectively. The rate constant during perfusion with H_2DIDS in (D) was 0.0018 min^{-1}, representing 90% inhibition. The rate constant in (C) was 0.0062 min^{-1}. The differences of the rate constants of the different oocytes are within the range of scatter shown in Table I. The fluxes in the presence of DNDS or H_2DIDS could not be determined accurately. In all cases the inhibition was >95%.

membrane of *Xenopus* oocytes. After substitution of two specific lysine residues on the band 3 protein by asparagine residues, the execution of the transport function and the reversible inibition by stilbene disulfonate derivatives is still possible while irreversible inhibition by the isothiocyanate derivative H_2DIDS can no longer be achieved. To put these results in proper perspective with respect to present discussions about band 3-mediated anion transport, it seems appropriate to make two comments, one concerning the possible heterogeneity of band 3 and another concerning the significance of the modified lysine residues for the mechanism of band 3-mediated anion transport.

(i) On SDS–PAGE, band 3 appears rather diffuse and extends over a mol. wt range of ~10 kd. This may raise speculations about its heterogeneity. At first glance such heterogeneity would seem to be simply related to the well-known differences of the carbohydrate moieties in the band 3 population. This is supported by the observation that treatment with an appropriate glycosidase leads not only to the expected reduction in mol. wt but also to a considerable narrowing of the band width (Mueller *et al.*, 1979). Moreover, peptides obtained after tryptic or chymotryptic digestion of human (Markowitz and Marchesi, 1981) or mouse (Raida *et al.*, 1989) band 3 show uniform N-terminal

Table II. Effect of mutation of Lys 558 only on inhibition of Cl^- exchange by H_2DIDS

After expression of:	Inhibition of $^{36}Cl^-$ efflux		
	1st perfusion period (with H_2DIDS)	2nd perfusion period (no H_2DIDS)	n
non-mutated band 3	93% ± 6.5%	82% ± 7%	5
mutated band 3	92% ± 5%	12% ± 13%	7

sequences, which lends no support for the assumption of heterogeneity of the amino acid sequence. However, more recently, using isoelectric focusing (Ideguchi *et al.*, 1982) or phase-separation techniques (Swanson *et al.*, 1988), band 3 subfractions were obtained. Possibly these subfractions represent different stable conformeric states, but differences of the primary structure cannot be excluded. It is also still unclear whether or not the band 3 derivatives occurring in precursors of the red cells will still be expressed in the mature cell together with larger quantitives of erythroid band 3. It is clear, however, that our demonstration of the functional expression of the erythroid band 3 clone of Demuth *et al.* (1986) and the result of the mutagenesis strongly suggest that this clone is representative for the behavior of the bulk of the anion transport protein in the membrane of the red cell. This conclusion is in agreement with the well-known findings about the relationship between H_2DIDS binding and inhibition of anion exchange. The earliest measurements suggested that only ~350 000 out of the 1 million band 3 molecules per cell possess the capacity of combining covalently with H_2DIDS (Cabantchik and Rothstein, 1974). Later work has shown, however, that the number of H_2DIDS molecules bound at complete inhibition of anion transport amounts to $1-1\ 2 \times 10^6$ molecules per cell (Passow *et al.*, 1975; Zaki *et al.*, 1975; Lepke *et al.*, 1976; Ship *et al.*, 1977) and thus is indistinguishable from the number of band 3 molecules determined by an independent technique (Steck, 1974).

(ii) It is evident from our experiments that the replacement of both Lys 558 and Lys 561 by asparagine residues does not abolish the ability of band 3 to execute its transport function. It remains open, however, whether or not a quantitative change of the transport rate occurs. We are still unable to determine quantitatively the number of band 3 molecules expressed in the plasma membrane of the oocyte by an independent method, and hence no turnover numbers can be calculated. Nevertheless, it is useful to know that our observation is in qualitative accord with previous observations from this and other laboratories. We have shown that the lysine residue involved in covalent H_2DIDS binding is also capable of reacting with 1-fluoro 2,4-dinitrophenol (DNFB; Rudloff *et al.*, 1983). The reactivity of this residue (originally called Lys *a*) is abnormally high such that under suitable conditions no more than this and one additional residue amongst the 36 lysine residues in the hydrophobic domain of band 3 become labeled with [^{14}C]DNFB. The rate of labeling increases with increasing Cl^- concentration in the medium. This was considered to indicate that the lysine residue *a* (i.e. either Lys 558 or Lys 561) is not involved in Cl^- binding (Passow *et al.*, 1980). This conclusion was confirmed by $^{35}Cl^-$ NMR measurements (Falke *et al.*, 1984). It is in keeping with the result of the present mutagenesis experiments which definitely show that neither the presence of Lys 558 nor of Lys 561 represents an essential requirement for substrate binding. Our results also indicate that one of the sites of covalent attachment of H_2DIDS is one or the other of these lysine residues which is in accord with previous suspicions. Further mutations are needed to resolve which of the two neighboring residues is involved.

Addendum

In the time between the submission of the paper and its acceptance for publication a mutant was made in which only the codon pertaining to Lys 558 was mutated to encode an asparagine residue by the oligonucleotide-directed mutagenesis described under Materials and methods. Lys 561 was left unaltered. This single mutation was sufficient to produce the same effect as the mutation of both Lys 558 and Lys 561 (Bartel *et al.*, 1989).

The effect of the mutation on inhibition of Cl^- exchange by H_2DIDS was measured by following $^{36}Cl^-$ release from single oocytes by the method illustrated in Figure 6 and calculating rate constants by means of the semi-log relationship indicated in the legend to Figure 8. The records of the time course of $^{36}Cl^-$ efflux were essentially similar to those shown in Figure 8c and d. It was possible to calculate from the rate constants the inhibition observed during a first perfusion period of 1 h at pH 8.75 with H_2DIDS in the Barth's solution and during a subsequent perfusion period with Barth's medium containing bovine serum albumine and no H_2DIDS (pH 7.4). The results obtained are shown in Table II.

These results indicate that in accordance with inferences from comparison of the band 3 sequence from several different species (Tanner *et al.*, 1988) Lys 558 is responsible for covalent H_2DIDS binding and irreversible inhibition of band 3 - mediated Cl^- exchange. We attribute the slight persisting inhibition after washout of H_2DIDS to a reaction of H_2DIDS with another as yet unidentified lysine residue which, according to Jennings and Passow (1979) is located in close juxtaposition to Lys 558 and involved in the formation of a cross-link via the two isothionate groups of H_2DIDS.

Materials and methods

Restriction endonucleases, SP6 and T7 RNA polymerase, T7 DNA polymerase, T4 DNA ligase, *Escherichia coli* DNA polymerase, T4 polynucleotidkinase, DNase I, human placental ribonuclease inhibitor and gp 32 protein were obtained from Gibco BRL, Pharmacia and New England Biolabs. Calf intestinal alkaline phosphatase and Klenow DNA polymerase were from Boehringer Mannheim. [α-^{32}P]ATP, [α-^{35}S]dATP, [^{35}S]methionine and $^{36}Cl^-$ were from Amersham. Synthetic oligonucleotides were synthesized on an Applied Biosystems 380A synthesizer using phosphoamidite chemistry and were purified on 7 M urea − 12% polyacrylamide gel followed by an octadecyl-C_{12} reversed-phase column chromatography (available from Baker Chemicals, Weiterstadt).

Bacterial strains and vectors

Plasmid pMEB3.18, including 2969 bp mouse band 3 cDNA was kindly supplied by Dr P.Curtis (Wistar Institute of Anatomy and Biology, Philadelphia). *Escherichia coli* strains BMH71-18, BMH71-18 mutS and MK30-3, phages M13mp18am and M13mp18rev were kindly supplied by

Professor J.Engels (Universität Frankfurt am Main). *Escherichia coli* strains JM109 and BMH71-18 were transformed according to the procedure of Mandel and Higa (1970).

Construction of plasmid DNAs

A 2631 bp *Pst*I fragment (nucleotides 339–2970) from mouse band 3 cDNA was isolated by limited hydrolysis of pMEB3.18 (Demuth *et al.*, 1986) and cloned into the *Pst*I site of pSPT19 (Pharmacia), a vector that contains the SP6 and T7 RNA polymerase promoters. A second fragment of band 3 cDNA (nucleotides 6–1190) was generated from pMEB3.18 by subcloning three overlapping fragments obtained by restrictions with *Nar*I, *Bal*I, *Eco*RV and *Xho*II. From these two fragments nucleotides 6–1169 and 1170–2970 were combined and inserted into the *Bam*HI/*Pst*I site of pSPT19. A synthetic oligonucleotide designed according to the sequence of mouse band 3, published by Kopito and Lodish (1985), containing 36 bp downstream from the start-codon AUG (encoding the first 12 N-terminal amino acids) and 30 bp upstream (5 '-non-translating region) was added. The missing base (frameshift mutation in pMEB3.18) at position 1125 (notation of Demuth *et al.*, 1986) was inserted by replacement by a chemically synthesized *Apa*I/*Stu*I fragment which included an additional T at the correct position. Large-scale plasmid preparations were performed according to the procedure of Hillen *et al.* (1981). For transcription, pSPT19Bd.3 and the mutant derivative pSPT19Bd.3mutLys were linearized with *Hin*dIII or *Eco*RI restriction endonucleases.

In vitro transcription

Transcription was carried out as described by Melton *et al.* (1984) and Drummond *et al.*, (1985). The typical reaction volume was 25 μl, containing 40 mM Tris–HCl, pH 7.9, 6 mM $MgCl_2$, 2 mM spermidine–HCl, 100 mM dithiotreitol, 1 U/μl human placental ribonuclease inhibitor, 500 μM rATP, rCTP and rUTP, 50 μM rGTP, 300 μM $m^7G(5')ppp(5')G$ (monomethyl cap, Pharmacia), 40 μg/ml linearized DNA templates and 600 U/ml SP6 or T7 RNA polymerase. Sense cRNA was made from *Hin*dIII (see Figure 1), antisense cRNA was transcribed from *Eco*RI digested pSTP19Bd.3 (but without cap-molecule and in the presence of 500 μM GTP). Template DNA was removed by subsequent DNase digestion (DNase pretreated with proteinase K according to Tullis and Rubin, 1980) for 10 min at 37°C. cRNAs were purified by phenol–chloroform extraction, precipitated twice with 0.7 M ammonium acetate, pH 5.8, and 2.5 vol ethanol, and resuspended in 15 μl diethylpyrocarbonate-pretreated water.

RNA isolation and Northern blots

Total cellular RNA was prepared from spleens of anemic mice (Sabban *et al.*, 1981) and from *Xenopus* oocytes using guanidine isothiocyanate (modified according to Glisin *et al.*, 1974). Poly(A)$^+$ RNA was enriched by oligo(dT)–cellulose column chromatography (Aviv and Leder, 1972). Denatured RNA was separated by denaturing agarose gel electrophoresis (McMaster and Carmichael, 1977). After transferring RNA to nitrocellulose in 20 × SSPE overnight, pre-hybridization and hybridization were performed at 42°C in 5 × SSPE, pH 7.0, 50% formamide, 1 × Denhardt's solution, 0.1% SDS, 1% glycine and 250 μg/ml denaturated herring sperm DNA. The hybridization probe was ^{32}P radiolabeled by nick-translation according to Weinstock *et al.*, (1978). Hybridization was carried out overnight. Hybridized filters were washed twice in 0.2 × SSPE, 0.1% SDS at 42°C. The filters were blotted dry and exposed overnight to Kodak XRP5 X-ray film at −70°C using two intensifier screens. For hybridization either a 1409 bp *Pst*I fragment or a 622 bp *Bgl*II fragment of band 3 cDNA were employed, using different amounts (3 or 15 μg) of mouse spleen or oocyte poly(A)$^+$ or A$^-$ mRNA. Both fragments formed hybrids with A$^-$ and poly(A)$^+$ mRNA from mouse spleen but did hybridize to mRNA$^-$, but not to mRNA$^+$ of the oocyte. This latter finding would suggest that in the oocyte no band 3-like transcripts are stored.

Translation and protein analysis

cRNAs were translated in reticulocyte lysate, prepared from blood of anemic white New Zealand rabbits as described by Jackson and Hunt (1983). [^{35}S]Methionine-labeled proteins were separated in an SDS–polyacrylamide gradient gel (7.5–18%) (Laemmli, 1980). Fluorography was performed after treatment with Amplify™ (Amersham) using Kodak XRP5.

RNA hybrid formation

Sense–antisense cRNA duplexes were formed by mixing a 10- to 12-fold mass excess of antisense cRNA with sense cRNA and incubation at 37°C for 1 h. The hybrid formation was examined using cell-free translation system and immunoprecipitation of microinjected *Xenopus* oocytes.

Microinjection and measurements of $^{36}Cl^-$ flux in Xenopus oocytes

After filtering the cRNAs through polycarbonate filters (pore size 0.2 μm), a volume of 50 nl containing ~30 ng cRNA was injected into prepared oocytes (Colman, 1984) as described previously (Morgan *et al.*, 1985; Grygorczyk *et al.*, 1987). The oocytes were washed and transferred to Barth's medium containing $^{36}Cl^-$ (0.113 mCi/ml) with or without 500 μM DNDS. After incubation for 3 h, oocytes were washed and placed individually into vials for liquid scintillation counting. For studying the H_2DIDS binding activity, the oocytes were first incubated for 1 h in 20 μM H_2DIDS/Barth's, pH 8.75 or pH 6.8, then washed three times in Barth's pH 7.6, containing 0.5% BSA and three times in Barth's without BSA. The influx was done as described above. For efflux measurement, one oocyte was microinjected with 50–75 nl of $^{36}Cl^-$ (~0.113 mCi/ml) and placed on the window of a Geiger–Müller tube (see Figure 6) as described previously (Grygorczyk *et al.*, 1987).

Immunoprecipitation

Microinjected oocytes were incubated for 24 h at 19°C in 10 μl/oocyte Barth's medium containing 0.02 g/l penicillin, 0.025 g/l streptomycin and 3 μCi/oocyte of [^{35}S]methionine. After washing three times the oocytes were solubilized in 10 μl/oocyte 50 mM Tris–HCl, pH 7.2, 50 mM NaCl, 4% SDS and 10 U/ml Trasylol® (Anderson and Blobel, 1979). After incubation for 1 min at room temperature, debris and lipids were removed by two centrifugations at 10 000 *g*. The sample was heated at 100°C for 4 min, cooled on ice and diluted with 1 vol H_2O and 4 vol of 2.5% Triton X-100, 190 mM NaCl, 6 mM EDTA, 60 mM Tris–HCl, pH 7.5, and 10 U/ml Trasylol, followed by an overnight incubation at 4°C with protein A–Sepharose purified rabbit IgG (15 or 25 μg) directed to mouse band 3. The immunocomplex was bound to protein A–Sepharose at 4°C for 4 h. The Sepharose pellets were washed four times in 0.1% Triton X-100, 0.02% SDS, 150 mM NaCl, 50 mM Tris–HCl, pH 7.5, 5 mM EDTA and 10 U/ml Trasylol. The final washing was performed without detergent. After heating at 100°C for 4 min in SDS sample buffer, the supernatant was applied to an SDS–polyacrylamide gel (10%) (Laemmli, 1980). Fluorography was performed as described above.

Indirect immunofluorescence test

Cryosections of microinjected oocytes of ~8 μm were fixed on gelatine-coated slides with acetone, and incubated at room temperature with a drop of the protein A–Sepharose purified IgG fraction (80 μg/ml PBS) of rabbit antiserum raised against mouse band 3 in a humid chamber for 45 min. The slides were rinsed for 10 min in PBS, dried and incubated with 1:100 diluted FITC-labeled goat anti-rabbit IgG (H+L) (Dianova, Hamburg). The slides were washed for 10 min in PBS, counterstained with 0.01 mg/ml Evan's blue, dissolved in PBS (Sigma, Deisenhofen) for 60 s, rinsed in distilled water and dried. The preparations were observed by a Zeiss phase-contrast epifluorescence microscope.

Oligonucleotide-directed construction of mutants

The *Ppu*MI fragment (nucleotides 1430–1855; Demuth *et al.*, 1986) of band 3 cDNA was inserted into the *Xba*I site of the M13mp18am phage. The gapped-duplex DNA was constructed by *in vitro* DNA–DNA hybridization of single-stranded virion DNA of this recombinant phage with *Xba*I-cleaved, double-stranded DNA of phage M13mp18rev (Kramer and Fritz, 1987). After annealing the synthetic 3' phosphorylated mutagenic primer DB4 to the gapped-duplex DNA, the remaining gaps and nicks were filled in simultaneously *in vitro* with DNA polymerase and DNA ligase in a reaction mixture containing 40 mM KCl, 260 mM NAD, 330 mM dATP, dCTP, dGTP and dTTP, 50 mM Tris–HCl, pH 8.0, 60 mM ammonium acetate, 5 mM $MgCl_2$, 5 mM dithiotreitol, 5 U/μl *E.coli* DNA ligase, 1 U/μl T4 DNA polymerase, 250 pM/ml mutagenic primer and ~100 pM/ml gapped duplex DNA. The reaction mixture was incubated for 15 min at 25°C, gp32 protein was added to a final concentration of 2 mg/ml, incubated again for 90 min at 25°C. The generated DNA was first transfected (method adapted from Cohen *et al.*, 1972) in *E.coli* BMH71-18 mutS (deficient in mismatch repair) for production of mixed phage progeny, and thereafter in *E.coli* MK30-3 for enriching the synthetic marker together with the minus strand progeny. Several recombinant clones were sequenced by the dideoxy chain-termination method of Sanger *et al.* (1977) for screening the presence of the desired mutation and DNA sequence verification. The mutagenic primer was optimized to avoid cross-hybridizations to M13mp18 sequence (Yanisch-Perron *et al.*, 1985) by using the UWGCG group of programs (Devereux *et al.*, 1984) and EMBL/GenBank libraries. Mutagenic primer DB4: 5'-CTGGAAAATGTTGATCAGGTTGGAGAAAGT-3'.

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