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Expression of band 3 anion exchanger induces chloride current and taurine transport: structure–function analysis

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Most, but not all, cell types release intracellular organic solutes (e.g. taurine) in response to cell swelling to achieve cell volume regulation. Although this efflux is blocked by classical inhibitors of the electroneutral anion exchanger band 3 (AE1), it is thought to involve an anion channel. The role of band 3 in volume-dependent taurine transport was determined by expressing, in *Xenopus* oocytes, band 3 from erythrocytes which do (trout) or do not (mouse) release taurine when swollen. AE1 of both species elicited anion exchange activity, but only trout band 3 showed chloride channel activity and taurine transport. Chimeras constructed from trout and mouse band 3 allowed the identification of some protein domains critically associated with channel activity and taurine transport. The data provide evidence that swelling-induced taurine movements occur via an anion channel which is dependent on, or controlled by, band 3. They suggest the involvement of proteins of the band 3 (AE) family in cell volume regulation.

Keywords: anion exchanger/band 3/Cl channel/taurine transport/volume regulation

Introduction

Most cells possess mechanisms to regulate their volume during mitosis and osmotic challenge. Hypotonically swollen cells recover their volume (regulatory volume decrease, RVD) by releasing intracellular solutes (ions, organic compounds) and obligated water. Swelling-activated ion efflux has been studied extensively (Hoffman and Simonsen, 1989; Sarkadi and Parker, 1991). However, although the release of organic osmolytes may account for as much as half of the total RVD (Garcia-Romeu *et al.*, 1991) and has been described in a variety of cell types, including renal epithelial cells (see Jackson and Strange, 1993), relatively little is known about the mechanisms and regulation of the pathways involved. The primary organic osmolyte involved in RVD is taurine, which is present in mammalian cells at concentrations of up to 40 mM (Huxtable, 1992). In some cells, polyols such as sorbitol and myoinositol play an important role. Volume-activated amino acid and polyol efflux mechanisms share two characteristics (Strange and Jackson, 1995): (i) they are mediated by passive, Na^+-independent, low-affinity transport pathways, suggesting that a diffusion process may be involved; and (ii) they are similarly affected by various anion transport blockers, mainly inhibitors of band 3 proteins, suggesting that transport of these structurally unrelated solutes may be mediated by a single volume-activated pathway.

From studies performed on a number of different cell types, such as MDCK cells (Banderali and Roy, 1992), flounder erythrocytes (Kirk *et al.*, 1992), C6 glioma cells (Jackson and Strange, 1993), a human lung cell line (Kirk and Kirk, 1993) and Ehrlich cells (Lambert and Hoffman, 1993), it has been suggested that the transport of organic osmolytes occurs via a pathway unrelated to band 3 protein and having the characteristics of an anion channel. The block by band 3 inhibitors is considered as non-specific. Conversely, because of the nature of inhibitors, it has been proposed that volume-activated taurine efflux from skate erythrocyte might be via the anion exchanger, band 3 (Goldstein and Brill, 1991), which is an electroneutral transporter. We have suggested a third explanation, based on several observations obtained with trout erythrocytes (Motais *et al.*, 1991a,b): in response to swelling, various specific membrane transport systems (KCl co-transport and osmolyte channel) are activated, but this activation is under the control of band 3. This regulatory property of the anion exchanger is envisaged as resulting from the fact that band 3 protein is part of a network (cytoskeleton–band 3–membrane) which would be involved in the signal transduction for sensing and correcting volume perturbation. Any alteration in the network architecture, as is likely to be induced by inhibitors binding to band 3, could affect the RVD.

To evaluate the putative role of band 3 protein in the volume-dependent transport of taurine and to gain some insight into the nature of the pathway, we cloned the trout erythrocyte band 3 and expressed the protein in *Xenopus* oocytes. We also expressed the band 3 protein of mouse erythrocyte, a cell which, in contrast to the trout erythrocyte, does not release organic osmolytes in response to cell swelling. Both trout and mouse band 3 showed anion exchange activity, but only trout band 3 (TB3) elicited anion channel activity and taurine permeability. Chimeric constructs between trout and mouse band 3 allowed the definition of protein domains associated with channel activity and taurine transport.

Results

Cloning of two isoforms of TB3

DNA hybridization of a mouse erythrocyte band 3 cDNA probe with a trout cDNA library from circulating red cells led to the isolation of two isoforms of band 3. The first one, TB3a, is identical to the trout anion exchanger previously cloned by Hübner *et al.* (1992); the second

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b: Trout band 3 isoform TB3b
a: Trout band 3 isoform TB3a (Trout AE1 Hübner et al., 1992)

b: MENDLSFGEDVMSSEEESDSAFPSPTRP------GNYDLEQSRHEEDSNQAIQSIVIHTD  54
a: =============Y===========I==TPPGHS=========Q============V===  60

b: PEAYLNLNTNANTRGDAQAYVELNELIGSSWQETGRWVGYEENLNPATGKWGPSHVSYLT 114
a: ==========================M=N==============F==G============= 120

b: FKSLIQLRKIMSTGAIILDLQASSLSAVAEKVVDELRTKGEIRATDRDGLLRALLQRRSQ 174
a: ============================================A=============== 180

b: SEGAVAQPLGGDIEMQTFSVTKQRDTTDSVEASIVLSGVMDSLEKPAVAFVRLGDSVVIE 234
a: ============================================================ 240

b: GALEAPVPVRFVFVLVGPSQGGVDYHESGRAMAALMADWVFSLEAYLAQTNKELTNAIAD 294
a: ================================================P=========== 300

                    ↓
b: FMDCSIVIPPTEIQDKGMLQPIIDFQKKMLKDRLRPSDTRIIFGGGAKADEADEEPREDP 354
a: ====G==========E============================================ 360

b: LARTGIPFGGMIKDMKRRYRHYISDFTDALDPQVLAAVIFIYFAALSPAITFGGLLADKT 414
a: ============================================================ 420

b: EHMMGVSELMISTCVQGIIFAFIAAQPTLVIGFSGPLLVFEEAFFAFCKSQEIEYIVGRI 474
a: ============================================================ 480

a: WVGLWLVIIVVVIVAVEGSFLVKFISRFTQEIFSILISLIFIYETFSKLGKIFKAHPLIL 534
b: ==========================================================V= 540

                   ************************
b: NYEHLNDSLDNPFHPVVKERIEYQDDGNKTVHEVIHERAYPNTALLSMCLMFGCFFIAYF 594
a: ===================H===HE=================================== 600

b: LRQFKNGHFLPGPIRRMIGDFGVPIAIFFMIAVDITIEDAYTQKLVVPKGLMVSNPNARG 654
a: ============================================================ 660

b: WFINPLGEKKPFPAWMMGACCVPALLVFILIFLESQITTLIVSKPERKMVKGSGFHLDLL 714
a: ============================================================ 720

b: ILVTMGGIASLFGVPWLSAATVRSVTHANALTVMSKGPKPEIEKVLEQRISGMLVAAMVG 774
a: ============================================================ 780

b: VSILLEPILKMIPMTALFGIFLYMGITSLSGIQMWDRMLLLIVPRKYYPRDAYAQRVTTM 834
a: ===========================================================A 840

b: KMHLFTLIQMVCLGALWMVKMSAFSLALPFVLILTIPLRMAITGTLFTDKEMKCLDASDG 894
a: ============================================================ 900

b: KVKFEEEPGEDMYESPLP 912
a: ================== 918
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Fig. 1. Amino acid sequence alignment of the two isoforms of trout erythroid band 3. The = sign denotes an identical amino acid. Boxes represent the putative transmembrane-spanning domains. The black bar outlines the Z-loop structure between helices nos 5 and 6. Stars represent the deleted portion in mutant TB3z(–) and the arrow corresponds to the N-terminus of mutant TB3ΔN (see Figure 6).

isoform is termed TB3b. Amino acid translation of the two corresponding open reading frames gives two 99% homologous proteins with 17 substitutions scattered in the sequence and a six-residue gap in the N-terminal part of TB3b. Figure 1 shows the amino acid sequences of the two isoforms. To ensure that we did not miss any mRNA present in early developmental stages of red cells and absent in circulating erythrocytes, we also screened a cDNA library from head kidney (a major haematopoietic tissue in fish) of anaemic trout. Both TB3 isoforms were retrieved in this second cDNA library and no additional cDNA was found. The significance of the presence of two distinct but closely homologous band 3 isoforms in trout red cells is not known. By their molecular weight, the length of their N-terminal cytoplasmic domain and the sequence homology of their hydrophobic domain with the human or mouse counterparts (65%), TB3s are clearly members of the group of band 3 anion exchangers found

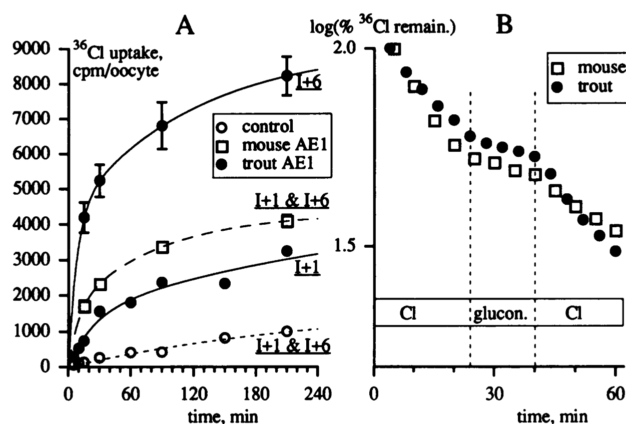

Fig. 2. (A) Kinetics of radioactive chloride uptake in water and in trout and mouse AE1 cRNA-injected oocytes (means ± SE, n = 8), measured 1 day (I+1) and 6 days (I+6) post-injection. (B) ^{36}Cl content of an oocyte expressing trout or mouse AE1 as a function of time when incubated in Cl-containing MBS, then in Cl-free medium (replacement of 85 over 87 mM Cl by gluconate) and finally in MBS.

in mammalian and avian erythrocytes, AE1s (Wood, 1992). However, the presence, between the putative transmembrane domains 5 and 6, of an extracellular loop carrying a glycosylation signal and which is twice the length of the corresponding segment in AE1 (47 versus 22 amino acids), should be noted. A similar enlarged and glycosylated loop between helices 5 and 6, known as the Z-loop (Demuth *et al.*, 1986), is a feature of the band 3-related proteins found in non-erythroid cells and called AE2s and AE3s. So far, there is no clue as to the physiological significance of the Z-loop which seems to play no essential role in the anion transport function (Karbach *et al.*, 1992). It is inserted near a very well conserved Lys residue, the site of covalent binding of 4,4′-Diisothiocyanatostilbene-2,2′-Disulfonic acid (DIDS). In this work we will concentrate on the role of TB3 Z-loop.

Expression of TB3 in Xenopus oocytes—Cl permeability

Figure 2A shows the kinetics of radioactive chloride uptake measured in oocytes injected with either water (control), TB3b (trout AE1) or mouse erythroid band 3 (mouse AE1; mAE1) cRNA, 1 and 6 days post-injection. There are several points to note. Firstly, the amount of chloride taken up by band 3-injected oocytes was much higher than in the control oocytes; chloride uptake was linear over 3 h in control oocytes but not in oocytes expressing band 3. This result is in agreement with similar experiments on human band 3 (Garcia and Lodish, 1989). Secondly, in oocytes expressing mAE1, the chloride permeability was the same when measured at day 1 or 6 post-injection, indicating that the expression level of mAE1 was maximal after 1 day and constant for 6 days. Conversely, the expression level of TB3b increased over the first 6 days, then became constant until at least day 8 (data not shown). The reasons for this difference in the time course of mAE1 and TB3 expression is unknown.

Since two isoforms of band 3 may co-exist in trout red cells, we measured the relative transport capacities of each and tested the possibility that a heteromultimer composed of TB3a and TB3b would be a more potent transporter. The two single isoforms gave a similar value: J_{in}Cl in nmol/min/oocyte (means ± SE, n = 8), TB3a: 0.266 ± 0.014; TB3b (same amount of cRNA): 0.227 ± 0.007. Co-injection of both (ratio 1/1) resulted in a very similar flux, TB3a + TB3b: 0.206 ± 0.010. Most subsequent experiments have been designed with TB3b.

Because band 3 mediates essentially one-for-one anion exchange, most of the efflux should come to a halt when the Cl in the medium is replaced by a non-penetrating or very slowly penetrating anion species. This was actually observed for both TB3 and mAE1 (Figure 2B). This result confirms previous data obtained by Passow *et al.* (1992) with erythroid mAE1, and Humphreys *et al.* (1994) with kidney mAE2.

Figure 3 shows the effects of several known band 3 inhibitors on chloride transport in injected oocytes. Both niflumic acid and flufenamic acid inhibited Cl uptake (Figure 3A). DIDS did not have any significant effect (Figure 3B) even at concentrations which fully inhibit Cl exchange in trout erythrocytes (Baroin *et al.*, 1984). However, DIDS did inhibit Cl uptake mediated by the chimeric construct TmT (Figure 3B), which is a protein identical to TB3b except that the extracellular Z-loop was replaced by its mAE1 counterpart m-loop (see below Figure 6). TmT was less sensitive to DIDS than was mAE1, the concentrations required for half-maximal inhibition, IC_{50}, being 0.44 μM and 0.02 μM respectively. 5-Nitro-2-(3-phenylpropylamino)-benzoate (NPPB) is a potent blocker of some types of epithelial Cl channels. However, it is also closely related to non-steroidal anti-inflammatory substances such as flufenamic acid and, if used at concentrations >10 μM, it has been shown to block band 3-mediated Cl exchange in human (Cabantchik and Greger, 1992) and in trout (unpublished data) red cells. Figure 3C shows that NPPB also inhibits mAE1 and TB3 expressed in oocytes: the pattern of inhibition of both proteins was very similar and the IC_{50} was ~100 μM. From these experiments, we concluded that TB3 is functionally expressed in oocytes. In subsequent experiments, the rate of ^{36}Cl uptake measured for the first 15 min was taken as an index of the level of expression of the protein.

Expression of TB3 also generates a channel activity

The membrane potential (E_m) of control oocytes was –56.8 mV ± 1.5 (SE) (n = 71). In TB3-expressing oocytes, E_m was shifted to –27.4 mV ± 2.3 (n = 19) and –17.1 mV ± 3.9 (n = 33) at day 1 and 6 post-injection respectively. Such a depolarization was not observed in oocytes expressing mAE1 (–55.6 mV ± 2.5, n = 14). Figure 4B shows the voltage–current relationship in TB3- and mAE1-expressing oocytes. Control oocytes (data not shown) and oocytes expressing mAE1 (open squares) had a similar and very small background current with a slope conductance of 0.70 μS ± 0.05, n = 67 (mean slope between –100 and –20 mV). Expression of TB3b in oocytes caused a dramatic increase in the membrane conductance, which rose progressively with time after injection. Thus, at day 6 post-injection, TB3 elicited a 100-fold increase in the slope conductance (80 μS ± 8, n = 41). The current did not display time-dependent inactivation even at membrane potentials above +60 mV (Figure 4A).

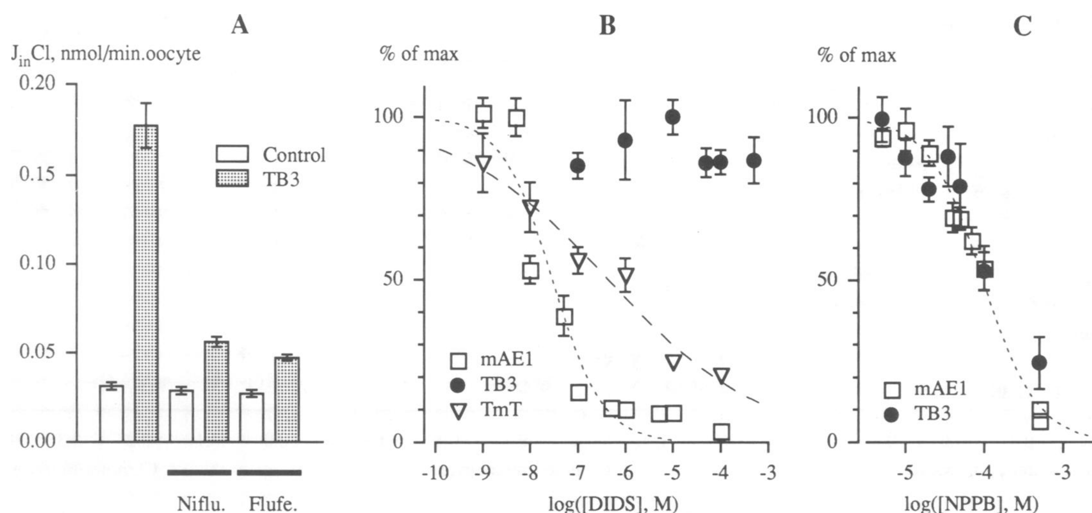

Fig. 3. Effect of band 3 inhibitors (means ± SE, $n = 8$). Effect of 10^{-4} M niflumic and flufenamic acids (**A**), DIDS (**B**) and NPPB (**C**) on J_{in}Cl of mouse and TB3-expressing oocytes. DIDS was also tested on chimera TmT.

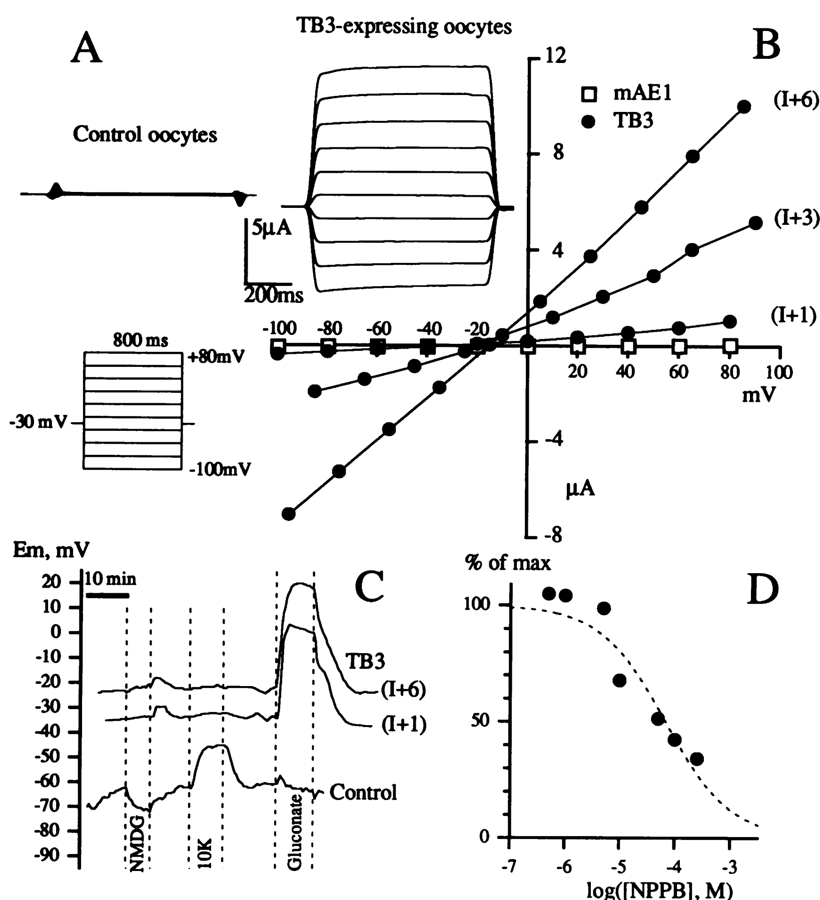

Fig. 4. Electrophysiological parameters. (**A**) Representative currents recorded from control and TB3-expressing oocytes. Oocytes were voltage clamped at –30 mV, and 800 ms potential pulses were applied from –100 to +80 mV in 20 mV increments. (**B**) Current–voltage relationship of mAE1- and TB3-expressing oocytes, measured at day 1 (I+1), 3 (I+3) and 6 (I+6) post-injection. These data are representative of 87 experiments. (**C**) Effect of changes in extracellular Na, K and Cl on membrane potential (E_m) of control and TB3-expressing oocytes measured 1 day (I+1) and 6 days (I+6) post-injection. Traces are representative of six experiments. Na substitution was obtained by replacement of 87 over 92 mM by *N*-methyl-D-glucamine. K was increased 10-fold from 1 to 10 mM, with simultaneous reduction of Na from 92 to 83 mM. Cl substitution was obtained by replacement of 78 over 87 mM by gluconate. (**D**) Effect of NPPB on the membrane conductance of TB3-expressing oocytes (mean slope of the current–voltage relationship between –100 and –20 mV).

Ion substitution experiments were carried out to determine the nature of this current. Figure 4C illustrates a representative experiment showing the effect of Na, K and Cl substitutions on the membrane potential of control and TB3-expressing oocytes. As previously reported (see review by Dascal, 1987), in control oocytes the membrane

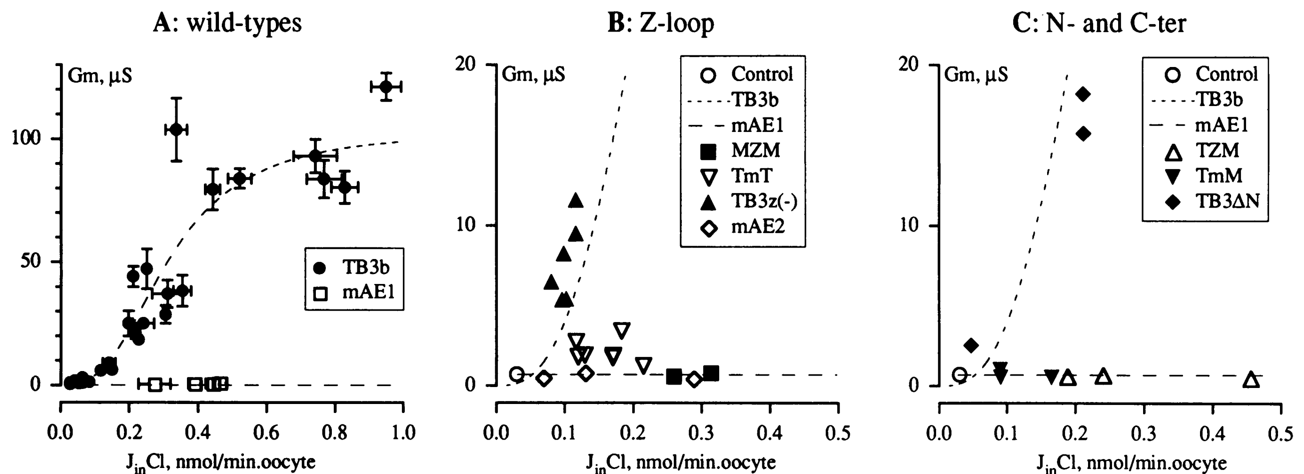

Fig. 5. Correlation between chloride permeability and membrane conductance. Means ± SE, n = 8 for J_{in}Cl and n = 6 for Gm. (**A**) Oocytes expressing TB3b and mAE1. (**B** and **C**) Oocytes expressing chimeras and deletion mutants (SE values omitted for the purpose of clarity).

potential is mainly imposed by the K current. In TB3-expressing oocytes, the E_m is no longer sensitive to changes in K and Na external concentrations, but is obviously determined primarily by the Cl current. The Cl conductance pathway was blocked by NPPB in a dose-dependent manner (Figure 4D) with an IC_{50} of 60 µM, and by niflumic acid (55% inhibition of I_{Cl} at a concentration of 10^{-4} M).

Figure 5 shows the relationship between TB3-induced chloride permeability and TB3-induced chloride conductance. To analyse this relationship over a wide range of chloride permeability, we took advantage of the fact that the expression level of the protein increases between day 1 and day 6 post-injection (Figure 1A), and that it is possible to inject various non-saturating concentrations of cRNA. Figure 5A shows that, in data pooled from several batches of eggs (17 toads), the TB3-induced ^{36}Cl uptake and Cl conductance were correlated, but with a non-linear relationship (closed circles). Conversely, mAE1 (open squares) did not generate Cl conductance whatever its level of expression (obtained by diluted cRNA injection).

Relationship between stucture of band 3 proteins and induction of a channel activity

When expressed in oocytes, both erythroid anion exchangers TB3 and mAE1 transported chloride, but only TB3 generated a significant chloride conductance. To try to define the structural domain(s) involved in the induction of the channel activity, we created chimeras from these two homologous members of the AE family. One marked difference between TB3 and mAE1 is the presence in the former of an enlarged extracellular loop (Z-loop) between transmembrane fragments 5 and 6. This segment is absent in all mammalian erythroid exchanger AE1s, but is a characteristic of the non-erythroid mammalian exchangers AE2 and AE3. Thus the Z-loop and its mAE1 counterpart, the so-called m-loop, were considered as pivotal elements, the subunits on both sides (i.e. N- and C-terminal parts) of TB3 and mAE1 being swapped to obtain six different cRNA constructs. Figure 6 is a schematic representation of these chimeric proteins; the letters T and M refer to subunits of trout and mouse origin respectively. With this terminology, the wild-type TB3 and mAE1 are designated as TZT and MmM respectively. With the exception of MZT and MmT, all the chimeric cRNA constructs (MZM, TZM, TmM, TmT) formed functional anion exchangers (as shown by Cl uptake) when expressed in *Xenopus* oocytes. This indicates that the N-terminal part of trout AE1 and the C-terminal part of mouse AE1 combined and folded correctly to transport chloride, whereas the reverse construct (mouse N-terminal + trout C-terminal) was not compatible with function or expression in the membrane. However, not all the chimeras which transported chloride exhibited ionic current.

Three essential messages emerge from comparing the relationship between chloride transport and Cl conductance in ooctyes injected with the different forms of band 3. Figure 5B focuses on the role of the Z-loop. The presence of the Z-loop was necessary to induce a channel activity: replacement of the Z-loop in TB3 by its mAE1 counterpart (TmT chimera, downward open triangles) did not impair chloride exchange but drastically reduced the chloride conductance to a value of 1.90 µS ± 0.31 (n = 30), very close to that measured in control oocytes (0.70 µS ± 0.05, n = 67). However the Z-loop, if necessary, was not sufficient to generate channel activity: after replacement in mAE1 of m-loop by Z-loop (MZM, closed squares), the electroneutral chloride transport capacity was preserved, but the chimera did not exhibit any chloride current (0.58 µS ± 0.04, n = 13). To assess further the role of the Z-loop, we created a TB3 mutant, TB3z(–), which was characterized by the deletion of 24 amino acids within the Z-loop (aa 551–574), thus reducing the length of the loop to that of its mAE1 counterpart. TB3z(–) behaved strictly as the wild-type TB3 (upward closed triangles), indicating that the amino acid composition of the Z-loop, and not simply its length, is crucial for the generation of channel activity. A similar conclusion can be drawn by expressing mAE2, a non-erythroid band 3 cloned from mouse kidney (Alper *et al.*, 1988; Humphreys *et al.*, 1994) which, like all other band 3-related proteins, possesses a Z-loop. Despite the presence of the Z-loop, mAE2 (open diamonds) behaved as mAE1, i.e. it transported chloride but did not induce any detectable Cl current.

Figure 5C gives information on the possible involvement of two other parts of the protein. First, in addition to the

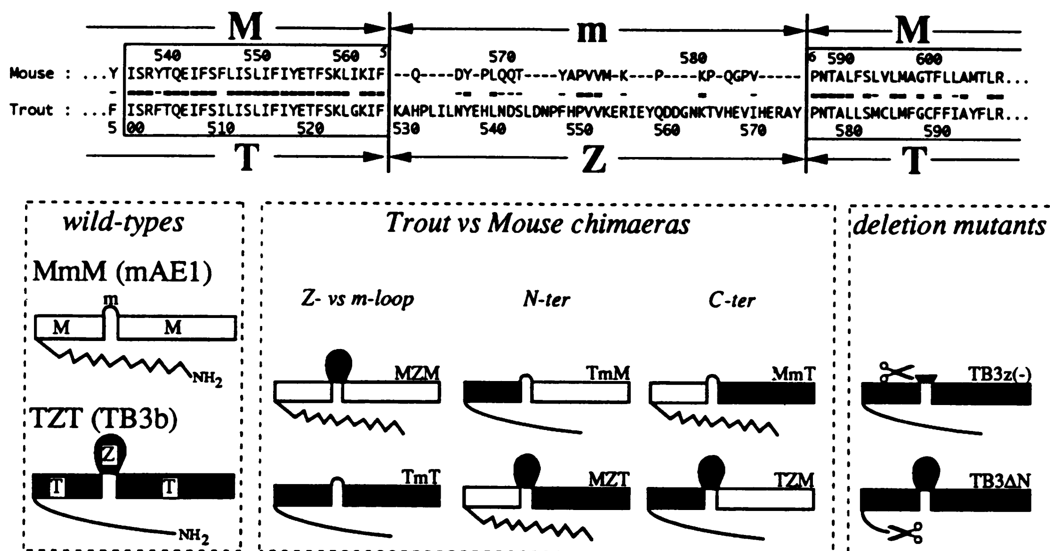

Fig. 6. Construction of chimeras and deletion mutants. Upper panel: amino acid alignment between mouse and TB3 proteins. Transmembrane domains 5 and 6 are boxed. Lower panel: wild-type proteins MmM (= mAE1), TZT (=TB3b). Trout versus mouse chimeras: MZM, TmT, TmM, MZT, MmT, TZM. Deletion mutants: TB3z(–) is devoid of amino acids 551–574, TB3ΔN is devoid of amino acids 1–311.

Z-loop domain, the C-terminal domain of TB3 is also of crucial importance in promoting channel activity: the chimera TZM (upward open triangles) did not exhibit any detectable ionic current, whereas Cl exchange activity was preserved. The same result was observed with TmM (downward closed triangles). Second, the hydrophilic N-terminus of AE1 is a domain which links the band 3 protein to the cytoskeleton. From experiments performed with mAE1, it is known that truncation of this domain does not abolish anion exchange activity (Lepke *et al.*, 1992). We designed a deletion mutant, TB3ΔN, devoid of its 311 N-terminal residues, and expressed it in the oocyte. As shown in Figure 5C (closed diamonds), the hydrophobic domain alone, as for mAE1, was sufficient to express Cl exchange but, in addition, TB3ΔN exhibited a Cl conductance with the ratio between Cl conductance and ^{36}Cl uptake fitting with the relationship established with TB3. Thus, the 311 N-terminal residues of the protein are not involved in the induction of TB3 channel activity. All the above experiments were performed using the isoform TB3b. The second TB3 isoform, TB3a, behaved exactly as TB3b (data not shown). This result was expected since the primary structure of the two proteins are 99% homologous and the differences are essentially located in the cytoplasmic N-terminus, the deletion of which had no apparent effect on either exchange or conductance activities.

Taurine transport is associated with a channel activity

The volume-dependent efflux of organic osmolytes, such as taurine, from a number of cell types has been suggested to occur via an anion channel sensitive to band 3 inhibitors. The kinetics of [^{14}C]taurine uptake measured in control and TB3b-expressing oocytes are illustrated in Figure 7A. These experiments show that control oocytes exhibited a small taurine permeability, but that taurine uptake was greatly increased in oocytes expressing TB3. [^{14}C]Taurine uptake was linear for many hours, and in subsequent experiments a 210 min incubation was used to quantify taurine permeability. Figure 7B shows that the taurine pathway observed in TB3-expressing oocytes was: (i) sensitive to NPPB (also to nifumic and flufenamic acids, not shown); (ii) independent of the presence of extracellular Na; and (iii) insensitive to hypotonicity. In control oocytes, taurine uptake was not significantly affected by NPPB, Na removal (data not shown) and hypotonicity (Figure 7B).

Figure 8 shows the relationship between TB3-induced conductance and TB3-induced taurine transport. As illustrated in Figure 8A, the taurine transport increased as a function of Cl conductance. To investigate this relationship further, we expressed in oocytes the chimeric constructs used previously and then measured both taurine transport and channel activity. As illustrated in Figure 8B, anionic exchangers (mAE1, mAE2) or chimeric constructs (MZM,TmM,TZM) which are devoid of channel activity (despite being well expressed in the membrane as indicated by their ^{36}Cl permeability) did not exhibit any taurine transport. Conversely, TB3b and TB3a, which generated a large channel activity, exhibited taurine transport. Replacement of the Z-loop by the mammalian m-loop (chimera TmT compared with the wild-type TZT) was sufficient to reduce channel activity drastically (1.90 μS) and to render taurine transport undetectable. Thus, there was a strong correlation between channel activity and taurine transport. The chimeras TB3z(–) and TB3ΔN, which exhibit a small but significant channel activity (8 and 16 μS respectively), also fitted with the conductance–taurine relationship established with TB3 (Figure 8A, open triangle, open diamond).

Oocytes possess a native volume-dependent taurine pathway

The question arises of whether the chloride current and taurine flux associated with TB3 expression in oocytes are mediated by the band 3 protein or result from the activation by band 3 of some pre-existing transport mechanism(s). A potential candidate for such an endogenous transporter in the oocyte has been reported by

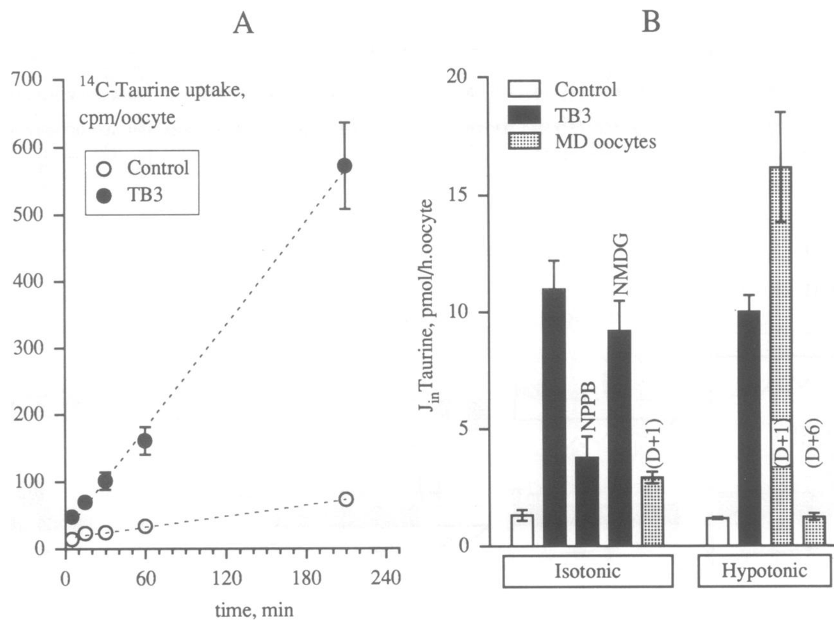

Fig. 7. Taurine permeability (means ± SE, $n = 8$). (**A**) Kinetics of radioactive taurine uptake in water and TB3 cRNA-injected oocytes. (**B**) Effect of 5×10^{-4} M NPPB, extracellular Na (substitution by NMDG as in Figure 4C) and hypotonicity (replacement of 42.5 mM NaCl by water) on J_{in} taurine of TB3-expressing oocytes. Effect of hypotonicity on J_{in} taurine of manually defolliculated (MD) oocytes, measured 1 (D+1) and 6 (D+6) days post-defolliculation.

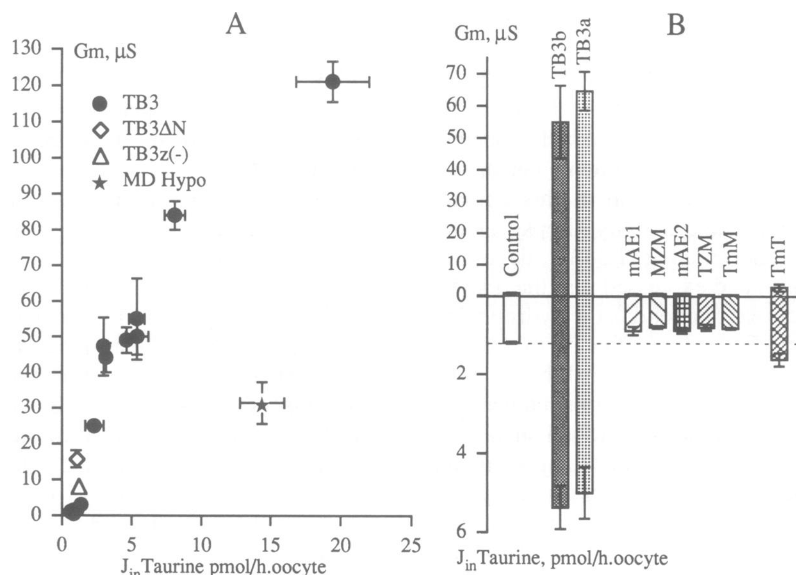

Fig. 8. Correlation between taurine permeability and membrane conductance. Means ± SE, $n = 8$ for J_{in} taurine and $n = 6$ for Gm. (**A**) Oocytes expressing TB3, TB3ΔN, TB3z(–) and hypotonic manually defolliculated oocytes (MD Hypo, $n = 23$ for J_{in} taurine and $n = 17$ for Gm). (**B**) Control oocytes and oocytes expressing TB3a, TB3b and low-conductance anion exchangers.

Ackerman *et al.* (1994) in manually defolliculated (MD) oocytes. Swelling of MD oocytes elicited a Cl current, called $I_{Clswell}$, which has similar characteristics to swelling-induced chloride currents observed in epithelial cells. However, such a Cl current cannot be triggered by hypotonic swelling in oocytes defolliculated by collagenase treatment (Ackerman *et al.*, 1994), the procedure we used in all the above experiments. Nevertheless, $I_{Clswell}$ could be a possible candidate for activation by TB3 in oocytes.

We also tried to detect the presence of an endogenous volume-dependent taurine pathway, and measured Cl current and taurine permeability in MD oocytes before and after hypotonic swelling. We observed that, in response to hypotonic swelling, a chloride channel with the characteristics described by Ackerman *et al.* for $I_{Clswell}$ was activated (data not shown). However, hypotonic swelling of MD oocytes also resulted in a strong activation of a taurine transport pathway (Figure 7B). By contrast, swelling of oocytes defolliculated by collagenase treatment did not induce any taurine transport (Figure 7B). We also confirmed the observation of Ackerman *et al.* that MD oocytes become less capable of evoking $I_{Clswell}$ in response to hypotonicity over time: $I_{Clswell}$ can no longer be activated 6 days after defolliculation (data not shown). The hypotonic-induced taurine transport also decreased over time, becoming inactivable 6 days after manual defolliculation (Figure 7B).

These data suggest that the endogenous volume-activated anion channel of MD oocytes mediates taurine transport. This is consistent with the possibility that TB3 acts as an activator of this endogenous channel. However, the characteristics of the TB3-induced and the endogenous channels are quite distinct. Not only did conductance and taurine transport induced by TB3 increase over time, whereas the endogenous channel showed the opposite time course, but the capacities of the two channels to carry taurine (ratio taurine flux/channel conductance) are very different: the taurine transport capacity of the endogenous channel (star in Figure 8A) is much higher than that of the TB3-induced channel.

Discussion

There is increasing evidence that volume regulatory taurine release occurs via an anion channel. On the other hand, volume-activated taurine transport is blocked by a number of inhibitors of the AE1, a protein which is normally electrically silent. The question therefore arises of whether these effects of AE1 inhibitors are unspecific, or whether AE1 is involved in taurine transport, directly as a carrier or indirectly as a regulator of a specific volume-dependent osmolyte channel. In this study, we have addressed this question by cloning the band 3 protein from the trout erythrocyte, a cell showing high levels of volume-activated taurine transport, and then expressing it in *Xenopus* oocytes. Two major conclusions can be drawn from the results.

First of all, it appears that some members of the AE1 family can elicit Cl channel activity when expressed in *Xenopus* oocytes. Expression of mAE1 induced a Cl exchange but did not elicit Cl current, as expected for an electrically silent exchanger. By contrast, expression of either TB3b or TB3a, the two isoforms of the trout erythrocyte anion exchanger, was associated with the induction of a chloride-conducting pathway, as evidenced by its reversal potential approximating E_{Cl} in oocytes and depending on extracellular chloride. Removal of the N-terminal, cytoplasmic domain of the protein did not alter this property, indicating that the signal for induction of the chloride conductance is located in the membrane-spanning domain. That difference between mAE1 and TB3 in the ability to express chloride conductance is striking, given the conservation in the primary sequence (65% identity between the membrane-spanning domains). One significant difference between the two proteins is the extracellular loop between the fifth and the sixth transmembrane helices, which is not only different but also twice as long in TB3 (Z-loop) as in mAE1. Chimeric constructs between TB3 and mAE1 clearly indicated that expression of the channel activity is fully dependent on the nature, but not the length, of the Z-loop. Thus, a small change in the sequence of AE1 is able to affect this function drastically. However, some other element(s) of the protein, located in the C-terminal membrane-spanning domain, is also required to induce the channel activity. The fact that modifying either the Z-loop or the C-terminus affects the ability of trout AE1 to act as a channel (or a channel regulator) but preserves its ability to act as an anion exchanger strongly supports the conclusion that the channel activity or the channel regulatory activity of trout AE1 is not artefactual but is related to the structure or conformation of the protein.

The second major observation was that expression of a TB3-induced channel activity in oocytes was accompanied by appearance of a Na-independent taurine transport. The behaviour of both wild-type exchangers and chimeric constructs showed a very tight correlation between both activities. Moreover, the same inhibitors, in a similar range of concentrations, affected both channel activity and taurine permeability. These results strongly support the view that taurine movements induced by cell swelling would occur via an anion channel, as previously suggested by others, but that this anion channel is dependent on, or controlled by, band 3.

A volume-dependent taurine pathway has been described previously in several fish red cells (Fugelli and Thoroed, 1986; Goldstein and Brill, 1991; Kirk *et al.*, 1992). In trout erythrocyte, this taurine transport is quiescent in normal conditions and is activated in response to swelling (Garcia-Romeu *et al.*, 1991). It has been demonstrated that this activation by swelling is dependent on intracellular ionic strength: lowering the ionic strength while swelling the cells favours the taurine transport activation (Motais *et al.*, 1991b). Moreover, this swelling-induced taurine pathway is sensitive to DIDS and other inhibitors of anionic exchangers (Motais *et al.*, 1991a). A striking difference between expression of taurine transport in trout erythrocytes and in oocytes expressing TB3 is the effect of DIDS, which blocks swelling-induced taurine movements in trout red cells but has no effect on taurine movements in oocytes expressing TB3. However, it must be pointed out that DIDS has no effect on Cl permeability of TB3-expressing oocytes, whereas other inhibitors of anion exchangers, which affect Cl permeability, also block taurine transport in TB3-expressing oocytes. Another key difference is the manner in which taurine transport is elicited: in erythrocytes, it is activated only in response to an osmotic challenge, whereas in oocytes the TB3-associated Cl current and taurine transport were elicited in isotonic medium and remained insensitive to osmotic variations. The reason for these differences is unclear. Regarding the sensitivity to osmotic challenge, at least two possibilities must be considered. First, because it is expressed in a different environment (membrane lipid composition, ionic strength, cytoskeletal anchorage), TB3 could spontaneously adopt a conformation which, in red cells, is induced only after swelling. In this respect, the fact that when expressed in oocytes TB3 is no longer inhibited by DIDS would indicate indeed that the conformation of the protein in oocytes differs from that in red cells. Furthermore, the recovery of DIDS inhibition following replacement of the Z-loop by the m-loop suggests that this putative conformational change is dependent on the nature of the loop. An alternative possibility is suggested by a similar situation recently reported by Clapham and collaborators. They showed that expression of a 235 amino acid protein from MDCK (pI_{Cln}) induced a chloride current in oocytes maintained in an isotonic bath (Paulmich *et al.*, 1992) but that the same chloride current could be induced in control oocytes by hypotonicity (Ackerman *et al.*, 1994). Subsequently, Krapivinsky *et al.* (1994) showed that a protein homologous to pI_{Cln} is present in the oocyte and this endogenous pI_{Cln} regulates

$I_{Clswell}$ in control oocytes when osmotically challenged. The authors concluded that pI_{Cln} is a regulator of the endogenous chloride current ($I_{Clswell}$) rather than a channel itself, and they raised the question why, in isotonic conditions, pI_{Cln} from MDCK is able to activate $I_{Clswell}$ whereas the endogenous pI_{Cln} from the oocyte is inoperative. In addition, there is increasing evidence that some transport proteins, containing multiple transmembrane helices (as does AE1), can influence channel activity when expressed in heterologous systems. This includes several members of the ABC transporter family, like CFTR protein. One of these is the human multidrug resistance P-glycoprotein, an ATP-dependent active transporter which excretes cytotoxic drugs out of cells (Horio *et al.*, 1988). It has been reported that expression of P-glycoprotein in NIH3T3 fibroblasts generates a volume-activated chloride channel (Valverde *et al.*, 1992) and evidence was first provided that P-glycoprotein acts as a bifunctional protein with both transport and channel activities (Gill *et al.*, 1992). However, recent data strongly support the view that P-glycoprotein is, in fact, acting as a channel regulator rather than as a channel itself (Hardy *et al.*, 1995). The exact nature of the association between the cell volume-regulated chloride channel and expression of P-glycoprotein is still a subject of debate (Higgins, 1995).

In this respect, our data can be interpreted in the following way: TB3, when expressed in oocytes, acts as an activator of an endogenous chloride current. The volume-dependent chloride current $I_{Clswell}$ is obviously a potential candidate even in isotonic conditions (Paulmich *et al.*, 1992). By contrast, mAE1 would not be able to act in such a way. This possibility is supported by our observation that induction of $I_{Clswell}$ is accompanied by the appearance of taurine transport (Figure 7). It is noteworthy that if TB3 acts as a channel regulator, the regulation could not involve a signal transmission through the cytoskeleton since deletion of the N-cytoplasmic domain containing the ankyrin site did not alter this property. The following experimental data must be considered regarding the hypothesis that TB3 acts as a regulator of $I_{Clswell}$. (i) Oocytes defolliculated by collagenase treatment failed to elicit $I_{Clswell}$ and taurine transport in response to an osmotic challenge. In contrast, induction of channel activity and taurine transport after TB3 expression was always obtained after collagenase treatment. (ii) The endogenous volume-dependent chloride current and taurine transport cannot be activated by osmotic challenge 6 days after manual defolliculation of oocytes. By contrast, TB3-induced currents were maximally expressed 6 days after defolliculation by collagenase and cRNA injection. (iii) A number of the characteristics of TB3-induced current are quite distinct from those of $I_{Clswell}$: these include absence of time-dependent inactivation at membrane potential above +30 mV and insensitivity to nucleotides like cAMP. (iv) The taurine transport capacities of TB3-induced and endogenous channels are very different (Figure 8A). Despite these differences which argue against TB3 acting as a regulator of $I_{Clswell}$, we cannot exclude the possibilities that TB3 modifies the characteristics of $I_{Clswell}$, or that TB3 regulates another endogenous channel.

The alternative interpretation of our results is that TB3 is a bifunctional protein with both exchange and channel functions. The ability of some AE1 to express chloride conductance may appear unexpected, but old data indicate that it could be a general but very discrete property of all AE1: a very tiny anion net flux ($1/10^4$ of the exchange flux) has been systematically observed in red cells. This flux is susceptible to inhibition by DIDS and is hence believed to be band 3-mediated (Knauf *et al.*, 1977; Schwartz *et al.*, 1992). It has been suggested that band 3 would undergo, as rare events, a conformational change which converts the protein from an exchanger to a channel (Schwartz *et al.*, 1992). Small differences in the protein primary sequences could increase the ability of some AE1 to express conductance, with the transition from exchanger to channel being modulated by parameters such as cell swelling. However, there is little or no sequence homology between the AE family and the families of anion channel proteins cloned so far (Jentsch *et al.*, 1990). The channel function of TB3 appears to depend, in part, on the Z-loop which is adjacent to the funnel giving access to the exchange transport site. This loop (which is much longer than its counterpart in mouse band 3) could confer some flexibility to the protein when cells swell, allowing enlargement of the transport domain which would then act as a channel and not as an exchanger. However, the fact that reduction of Z-loop length [TB3 z(–)] did not impair channel activity suggests that the activating process must be more subtle. The channel function could also result from the capacity of TB3 to form channel-like structures when several monomers aggregate, and it has been observed that hypotonic-induced volume expansion of shark erythrocytes leads to formation of band 3 tetramers (Musch *et al.*, 1994). In this context, it is interesting to note that in oocytes the channel activity increased as a function of the chloride permeability, but in a sigmoidal fashion.

The question of whether TB3 acts as a channel or a channel regulator is still open. Nevertheless, the finding that some subtypes of AE1 could be involved, either as a channel or as a regulator of channels, in the volume-activated transport of organic solutes, has important implications. Firstly, most epithelial cells which regulate their volume are known to contain band 3-related proteins, characterized by the presence of a Z-loop (Alper, 1992). Even though, in a preliminary experiment, we did not succeed in inducing channel activity by expressing mAE2 in oocytes, the possibility remains that band 3-related proteins may be involved in epithelial cell volume regulation. Secondly, there are a number of intriguing similarities between the volume-activated channel of fish erythrocytes and the pathway responsible for the enhanced permeability of malaria-infected human erythrocytes (Kirk *et al.*, 1992). The question is still open as to whether this malaria-induced pathway occurs by *de novo* synthesis and membrane targeting or by modification of native red cell membrane constituents. Since our data suggest that small changes in AE1 structure can promote such a channel, it is tempting to speculate that the malaria-induced pathway results from an alteration of the human erythrocyte band 3 protein by the parasite.

Materials and methods

Isolation of trout red cell band 3 cDNA

Blood samples were withdrawn from the caudal vessel of anaesthetized 250 g rainbow trout, pooled and subsequently washed 10 times in fish

saline, taking care to discard white cells, as described in Motais *et al.* (1991b). Anaemic fish were obtained by phenylhydrazine infusion (Smith *et al.*, 1971) and sacrificed after 6 weeks for cephalic kidney removal. Total RNA was extracted from circulating red cells and cephalic kidney according to Chomczynski and Sacchi (1987), mRNA was then purified by affinity chromatography on oligo(dT)-cellulose and reverse transcribed using oligo(dT) priming and a Riboclone® cDNA synthesis kit from Promega. The cDNA inserts were size-selected above 1 kb by agarose gel electrophoresis and electroeluted from the gel slice. A red cell cDNA λgt10 library and a cephalic kidney cDNA λ-ZAP library were then constructed using λgt10 and λ-ZAP kits respectively from Promega, according to the manufacturer's instruction. The mAE1 cDNA (gift from Professor H.Passow, Max Planck Institut of Frankfürt) was labelled by random priming using [α-^{32}P]dCTP and a DNA labelling kit from Boehringer. It was used as a probe to screen 6×10^5 plaques from the trout red cell cDNA library and 4×10^5 plaques from the cephalic kidney cDNA library according to Church and Gilbert (1984). Seven and four positive clones were analysed from the circulating red cell and the cephalic kidney libraries respectively. After three rounds of plaque purification, the cDNA inserts were subcloned into pBluescript II (Stratagene) and analysed by restriction mapping. DNA sequencing of both strands of restriction fragments was performed according to the dideoxynucleotide chain termination method (Sanger *et al.*, 1977), and using the T7 DNA sequencing kit from Pharmacia.

In vitro cRNA transcription

The TB3b cDNA was digested by *Mse*I, blunted and subcloned into pSP64poly(A) (Promega), previously digested by *Sma*I, downstream of the SP6 RNA polymerase promoter. After linearization by *Eco*RI, 2 μg of DNA was transcribed for 1 h at 40°C, using SP6 RNA polymerase, rRNasin (Promega) and the following rNTP mixture [rATP, rCTP, rUTP: 500 μM; rGTP: 50 μM; m^{7}G(5′)ppp(5′)G: 250 μM; from Boehringer]. DNA template was then removed by RNase-free DNase I (Boehringer) treatment for 15 min at 37°C, and the capped cRNAs were recovered with two phenol–chloroform–isoamyl alcohol (25:24:1) extractions followed by two ammonium acetate–ethanol (0.7 M/2.5 vol.) precipitations. The cRNA pellet was resuspended in diethylpyrocarbonate-treated H_2O and an aliquot was analysed by agarose–formaldehyde gel electrophoresis. Known amounts of bacterial rRNA (Boehringer) were used as standards for concentration determination. cRNA was stored frozen in aliquots at –80°C. The mAE1 cDNA was inserted into the plasmid pSPT19 (Pharmacia), downstream of the SP6 RNA polymerase promoter. The mouse kidney related band 3 mAE2 cDNA was inserted downstream of the T7 RNA polymerase promoter of pBluescript II (Stratagene). *In vitro* transcription of these was carried out as described above after plasmid linearization by *Hin*dIII and *Eco*RI respectively. Other band 3 cDNA constructs in this study were inserted into the pSP64poly(A) or pSPT19 plasmids and their cRNA transcriptions were performed accordingly.

Oocyte injection

Oocytes were removed from ice-anaesthetized *Xenopus* and maintained at 18°C in modified Barth's medium [MBS composition in mM: NaCl: 85; KCl: 1; $NaHCO_3$: 2.4; $MgSO_4$: 0.82; $Ca(NO_3)_2$: 0.33; $CaCl_2$: 0.41; HEPES: 10; NaOH: 4.5; pH 7.4; Po: 215 mOsm; supplemented with penicillin: 10 U/ml and streptomycin: 10 μg/ml]. After washing with MBS, defolliculation was obtained by 16 h incubation at 18°C in MBS containing 1.3 mg/ml collagenase (SERVA) followed by 30 min incubation in Ca-free MBS [$Ca(NO_3)_2$ and $CaCl_2$ replaced by water]. Stage V–VI oocytes were then injected with 50 nl of 70 ng/μl cRNA (3.5 ng/oocyte) and maintained at 18°C. Expression was tested 1 day post-injection and followed for 6 days. Dose–response analysis with TB3b cRNA showed saturation of the expression signal from 1 ng/oocyte.

Radioactive flux measurements

For chloride influx measurements, eight oocytes were incubated at 18°C in 100 μl MBS containing ^{36}Cl (Amersham) with a specific activity of 350 c.p.m./nmol chloride. The oocytes were washed twice in ice-cold MBS and transferred individually into counting vials. Excess extracellular fluid was quickly removed and 20 μl of 20% SDS was added before vortexing. Radioactive chloride uptake in each oocyte was determined after scintillation counting with an external standard procedure to check for quenching. The incubation medium was counted in duplicate on 5 μl aliquots, using the same protocol to determine the specific activity in each experiment. Chloride uptake was calculated as the mean of the eight values and expressed as nmol/min/oocyte. For chloride efflux measurements, the oocytes were loaded by overnight incubation in ^{36}Cl as described above. Individual oocytes were then placed in counting vials and 100 μl fresh MBS was added at 5 min intervals and removed for counting. Remaining radioactivity inside the oocyte was determined at the end of the procedure. The results are expressed as the logarithm of percentage of the total c.p.m. content in the oocyte as a function of time. Taurine influx measurements were carried out as for chloride influx in MBS supplemented with 0.5 mM taurine (Merck) and containing [^{14}C]taurine (Dupont NEN Products, Boston, MA) with a constant specific activity of 40×10^3 c.p.m./nmol taurine.

Electrophysiology

Electrophysiological parameters were measured at room temperature using the two-electrode voltage clamp technique with a TEV 200 amplifier (Dagan, Minneapolis, MN) monitored by computer through Digidata 1200 A/D converter/PClamp software (Axon Instruments Inc., Foster City, CA). Current and potential electrodes filled with 3 M KCl had a resistance of 1.8–2.3 MOhm. Following resting potential (E_m) determination, oocytes were clamped at a holding potential of –50 mV (control oocytes) or –30 mV (high Cl-conductive TB3-expressing oocytes) and 800 ms clamping potentials from –100 to +80 mV with 20 mV increments were applied and the current's values recorded. I_m was plotted versus V_m and the mean slope of the *I–V* curve between –100 mV and –20 mV was taken as an index of the membrane conductance and expressed as μS. Substitution experiments were carried out by recording the membrane potential (E_m) with one electrode inside the oocyte whilst a second one was kept outside to record and subtract possible junction potential. A KCl agar–agar bridge was used to minimize junction potentials which were found to be +8 mV for Cl decade substitution by gluconate. Junction potentials could be neglected when K concentration was increased 10-fold and when Na was substituted by *N*-methyl-D-glucamine (see figure legends for media compositions).

Chimeric and deletion mutant constructions

To construct the different chimeras between mouse (mAE1) and trout (TB3b) band 3, the following cassettes were designed: the mAE1 cDNA was separated into three restriction fragments, M5′: *Eco*RI (5′ end)–*Nco*I, M: *Nco*I–*Xma*I and M3′: *Xma*I–*Hin*dIII (3′ end), and TB3b cDNA into T5′: *Eco*RI (5′ end)–*Kpn*I, T: *Kpn*I–*Xho*I and T3′: *Xho*I–*Hin*dIII (3′ end). Thus the different points where the genes had to be fused to construct the chimeras are located within fragments M (mouse) and T (trout). Using PCR with Vent® DNA polymerase (New England Biolabs), synthetic oligonucleotides (Eurogentec, Belgium) as primers and mAE1 and TB3b cDNAs as templates, gene fusions between subfragments of M and T were produced as described in Higuchi (1990); see TZM example below. PCR products were verified by nucleotide sequencing. Reconstitution of the complete cDNAs was finally achieved by ligation with the suitable original M5′, M3′ (mAE1), T5′ or T3′ (TB3b) restriction fragments. The same strategy was used to construct the deletion mutant TB3z(–), which lacks residues 551–574, with the two 'outermost' primers sense (5′-G ACA GGC ATT CCC TTC GGC GGG-3′) and anti-sense (5′-G CAG GTC CAG ATG GAA ACC AGA GCC C-3′) and the two 'deletion-flanking' primers anti-sense (5′-GT GTT GGG.CAC CGG GTG GAA AGG-3′) and sense (5′-AC CCG GTG.CCC AAC ACT GCC CTG-3′), as described in Higuchi (1990). TB3ΔN was obtained simply by digestion of pSP64poly(A)-TB3b by *Pst*I, which cuts the plasmid upstream and within the TB3b cDNA insert at position 891/start codon, followed by recircularization. See Figure 6 for a schematic description of chimeric and deletion mutant band 3 proteins. The complete sequence of all the DNA clones and constructs was confirmed by nucleotide sequencing.

PCR strategy for chimera construction.

Construction of chimera TZM, which corresponds to the TB3 N-terminal half up to the Z-loop (amino acids 1–574) plus mouse band 3 C-terminal half (amino acids 586–929), is given as an example of the PCR protocol. Fragment TZ was produced from the sense primer 'tu' (5′-G ACA GGC ATT CCC TTC GGC GGG-3′) and the anti-sense primer 'tzmL' (5′-GCC TAC CCC AAC AC.A GCC CTC-3′) with TB3b cDNA as template, and fragment M was produced from the sense primer 'tzmR' (5′-GCC TAC.CCC AAC ACA GCC CTC-3′) and the anti-sense primer 'md' (5′-G CTG TAT AGA CTT TTC CCC ACG TGG-3′) with mAE1 cDNA as template. Excess primers and DNA template were removed by purification of PCR products on agarose gel electophoresis and β-agarase treatment of the gel slice (New England Biolobs). Mixed together, these two primary products, TZ and M, hybridize in the fusion point region where they overlap, and 3′ elongation of this heteroduplex produces the desired TZM gene fusion region. A secondary PCR was

performed on this mixture with primers 'tu' and 'md' to amplify the TZM gene fusion region. This secondary product was digested with *Kpn*I and *Xma*I and ligated between T5′: *Eco*RI (5′ end)–*Kpn*I and M3′: *Xma*I–*Hin*dIII (3′ end) to reconstitute the integral cDNA encoding for TZM.

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References

Ackerman,M.J., Wickman,K.D. and Clapham,D.E. (1994) Hypotonicity activates a native chloride current in *Xenopus* oocytes. *J. Gen. Physiol.*, **103**, 153–179.

Alper,S.L. (1992) Molecular and cell biological aspects of the AE anion exchanger gene family. In Bamberg,E. and Passow,H. (eds), *The Band 3 Proteins: Anion Transporters, Binding Proteins and Senescent Antigens. Progress in Cell Research.* Elsevier, Amsterdam, Vol. 2, pp. 9–15.

Alper,S.L., Kopito,R.R., Libresco,S.M. and Lodish,H.F. (1988) Cloning and characterization of a murine band 3-related cDNA from kidney and from a lymphoid cell line. *J. Biol. Chem.*, **263**, 17092–17099.

Banderali,U. and Roy,G. (1992) Anion channels for amino acids in MDCK cells. *Am. J. Physiol.*, **263**, C1200–C1207.

Baroin,A., Garcia-Romeu,F., Lamarre,T. and Motais,R. (1984) A transient sodium–hydrogen exchange system induced by catecholamines in erythrocytes of rainbow trout, *Salmo gairdneri*. *J. Physiol.*, **356**, 21–31.

Cabantchik,Z.I. and Greger,R. (1992) Chemical probes for anion transporters of mammalian cell membranes. *Am. J. Physiol.*, **262**, C803–C827.

Chomczynski,P. and Sacchi,N. (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate–phenol–chloroform extraction. *Anal. Biochem.*, **162**, 156–159.

Church,G.M. and Gilbert,W. (1984) Genomic sequencing. *Proc. Natl Acad. Sci. USA*, **81**, 1991–1995.

Dascal,N. (1987) The use of *Xenopus* oocytes for the study of ion channels. *CRC Crit. Rev. Biochem.*, **22**, 317–387.

Demuth,D.R., Showe,L.C., Ballantine,M., Palumbo,A., Fraser,P.J., Cioe,L., Rovera,G. and Curtis,P.J. (1986) Cloning and structural characterization of a human non-erythroid band 3-like protein. *EMBO J.*, **5**, 1205–1214.

Fugelli,K. and Thoroed,S.M. (1986) Taurine transport associated with cell volume regulation in flounder erythrocytes under anisosmotic conditions. *J. Physiol.*, **374**, 245–261.

Garcia,A.M. and Lodish,H.F. (1989) Lysine 539 of human band 3 is not essential for ion transport or inhibition by stilbene disulfonates. *J. Biol. Chem.*, **264**, 19607–19613.

Garcia-Romeu,F., Cossins,A.R. and Motais,R. (1991) Cell volume regulation by trout erythrocytes: characteristics of the transport systems activated by hypotonic swelling. *J. Physiol.*, **440**, 547–567.

Gill,D.R., Hyde,D.R., Higgins,C.F., Valverde,M.A., Mintenig,G.M. and Sepulveda,F.V. (1992) Separation of drug transport and chloride channel functions of the human multidrug resistance P-glycoprotein. *Cell*, **71**, 23–32.

Goldstein,L. and Brill,S.R. (1991) Volume-activated taurine efflux from skate erythrocytes: possible band 3 involvement. *Am. J. Physiol.*, **260**, R1014–R1020.

Hardy,S.P., Goodfellow,H.R., Valverde,M.A., Gill,D.R., Sepulveda,F.V. and Higgins,C.F. (1995) Protein kinase C-mediated phosphorylation of the human multidrug resistance P-glycoprotein regulates cell volume-activated chloride channels. *EMBO J.*, **14**, 68–75.

Higgins,C.F. (1995) Volume-activated chloride currents associated with the multidrug resistance P-glycoprotein. *J. Physiol.*, **482.P**, 31S–36S.

Higuchi,R. (1990) Recombinant PCR. In Innis,M.A., Gelfand,D.H., Sninsky,J.J. and White,T.J. (eds), *PCR Protocols. A Guide to Methods and Applications*. Academic Press, Inc., San Diego, pp. 177–183.

Hoffman,E. and Simonsen,L.O. (1989) Membrane mechanisms in volume and pH regulation in vertebrate cells. *Physiol. Rev.*, **69**, 315–382.

Horio,M., Gottesman,M.M. and Pastan,I. (1988) ATP-dependent transport of vinblastine in vesicles from human multidrug-resistant cells. *Proc. Natl Acad. Sci. USA*, **85**, 3580–3584.

Hübner,S., Michel,F., Rudloff,V. and Appelhans,H. (1992) Amino acid sequence of band-3 protein from rainbow trout erythrocytes derived from cDNA. *Biochem. J.*, **285**, 17–23.

Humphreys,B.D., Jiang,L., Chernova,M.N. and Alper,S.L. (1994) Functional characterization and regulation by pH of murine AE2 anion exchanger expressed in *Xenopus* oocytes. *Am. J. Physiol.*, **367**, C1295–C1307.

Huxtable,R.J. (1992) Physiological actions of taurine. *Physiol. Rev.*, **72**, 101–163.

Jackson,P.S. and Strange,K. (1993) Volume-sensitive anion channels mediate swelling-activated inositol and taurine efflux. *Am. J. Physiol.*, **265**, C1489–C1500.

Jentsch,T.J., Steinmeyer,K. and Schwarz,G. (1990) Primary structure of *Torpedo marmorata* chloride channel isolated by expression cloning in *Xenopus* oocytes. *Nature*, **348**, 510–514.

Karbach,D., Hanke-Baier,P., Wendel,J., Svetina,S., Müller,W., Hübner,S., Münchow,T., Appelhans,H. and Passow,H. (1992) Anion transport in the red blood cell of the mouse and in erythroleukaemic K562 cells. In Bamberg,E. and Passow,H. (eds), *The Band 3 Proteins: Anion Transporters, Binding Proteins and Senescent Antigens. Progress in Cell Research.* Elsevier, Amsterdam, Vol. 2, pp. 295–305.

Kirk,K. and Kirk,J. (1993) Volume regulatory taurine release from human lung cancer cell line: evidence for amino-acid transport via a volume-activated chloride channel. *FEBS Lett.*, **336**, 153–158.

Kirk,K., Ellory,J.C. and Young,J.D. (1992) Transport of organic substrates via a volume-activated channel. *J. Biol. Chem.*, **267**, 23475–23478.

Knauf,P.A., Furhmann,G.F., Rothstein,S. and Rothstein,A. (1977) The relationship between anion exchange and net anion flow across the human red blood cell membrane. *J. Gen. Physiol.*, **69**, 363–386.

Krapivinsky,G.B., Ackerman,M.J., Gordon,E.A., Krapivinsky,L.D. and Clapham,D.E. (1994) Molecular characterization of a swelling-induced chloride conductance regulatory protein, pI_{Cln}. *Cell*, **76**, 439–448.

Lambert,I.H. and Hoffman,E.K. (1993) Regulation of taurine transport in Ehrlich ascites tumor cells. *J. Membr. Biol.*, **131**, 67–79.

Lepke,S., Becker,A. and Passow,H. (1992) Mediation of inorganic anion transport by the hydrophobic domain of mouse erythroid band 3 protein expressed in oocytes of *Xenopus laevis*. *Biochim. Biophys. Acta*, **1106**, 13–16.

Motais,R., Fiévet,B., Borgese,F. and Garcia-Romeu,F. (1991a) Some functional properties of band 3 protein in nucleated red cells. In Bamberg,E. and Passow,H. (eds), *The Band 3 Proteins: Anion Transporters, Binding Proteins and Senescent Antigens. Progress in Cell Research.* Elsevier, Amsterdam.

Motais,R., Guizouarn,H. and Garcia-Romeu,F. (1991b) Red cell volume regulation: the pivotal role of ionic strength in controlling swelling-dependent transport systems. *Biochim. Biophys. Acta*, **1075**, 169–180.

Musch,M.W., Davis,E.M. and Goldstein,L. (1994) Oligomeric forms of skate erythrocyte band 3—effect of volume expansion. *J. Biol. Chem.*, **269**, 19683–19686.

Passow,H., Lepke,S. and Wood,P.G. (1992) Exploration of the mechanism of mouse erythroid band 3-mediated anion exchange by site-directed mutagenesis. In Bamberg,E. and Passow,H. (eds), *The Band 3 Proteins: Anion Transporters, Binding Proteins and Senescent Antigens. Progress in Cell Research.* Elsevier, Amsterdam, Vol. 2, pp. 253–262.

Paulmichl,M., Li,Y., Wickman,K.D., Ackerman,M.J., Peralta,E. and Clapham,D.E. (1992) New mammalian chloride channel identified by expression cloning. *Nature*, **356**, 238–241.

Sanger,F., Nicklen,S. and Coulson,A.R. (1977) DNA sequencing with chain-terminating inhibitors. *Proc. Natl Acad. Sci. USA*, **74**, 5463–5467.

Sarkadi,B. and Parker,J.C. (1991) Activation of ion transport pathways by changes in cell volume. *Biochim. Biophys. Acta*, **1071**, 407–427.

Schwartz,W., Gu,Q. and Passow,H. (1992) Potential dependence of mouse band 3-mediated anion exchange in *Xenopus* oocytes. In Bamberg,E. and Passow,H. (eds), *The Band 3 Proteins: Anion Transporters, Binding Proteins and Senescent Antigens. Progress in Cell Research.* Elsevier, Amsterdam, Vol. 2, pp. 161–168.

Smith,C.E., McLain,L.R. and Zaugg,W.S. (1971) Phenylhydrazine-induced anemia in chinook salmon. *Toxicol. Appl. Pharmacol.*, **20**, 73–81.

Strange,K. and Jackson,P.S. (1995) Swelling-activated organic osmolyte efflux: a new role for anion channels. *Kidney Int.*, in press.

Valverde,M.A., Diaz,M., Sepulveda,F.V., Gill,D.R., Hyde,S.C. and Higgins,C.F. (1992) Volume-regulated chloride channels associated

with the human multidrug-resistance P-glycoprotein. *Nature*, **355**, 830–833.
Wood,P.G. (1992) The anion exchange proteins: homology and secondary structure. In Bamberg,E. and Passow,H. (eds), *The Band 3 Proteins: Anion Transporters, Binding Proteins and Senescent Antigens. Progress in Cell Research.* Elsevier, Amsterdam, Vol. 2, pp. 325–352.

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Note added in proof

The EMBL accession number for the second isoform of trout erythroid band 3 TB3b reported in this paper is Z50848.