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Primary structure of a novel 4-acetamido-4′-isothiocyanostilbene-2,2′-disulphonic acid (SITS)-binding membrane protein highly expressed in *Torpedo californica* electroplax

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Polyclonal rabbit antibodies were raised against 4-acetamido-4′-isothiocyanostilbene-2,2′-disulphonic acid (SITS), an inhibitor of a variety of anion transport proteins. These antibodies specifically recognize SITS-reacted erythrocyte band 3 in immunoprecipitations and Western blots. In Western blots of SITS-reacted membrane proteins derived from vesicles of the electric organ of *Torpedo californica* (known to express a SITS-sensitive Cl^- channel) the antibodies recognized two major species of ~ 93 kDa and ~ 105 kDa. The ~ 93 kDa protein was identified as the α-subunit of the Na,K-ATPase. The ~ 105 kDa protein (designated sp105) is a glycoprotein which binds to wheat-germ agglutinin and concanavalin A and is present as a disulphide-linked homodimer under non-reducing conditions. A partial amino acid sequence and a polyclonal antibody were used to clone the corresponding cDNA. sp105 is encoded in electroplax by two abundant mRNAs of ~ 6 and ~ 6.8 kb. A hybridizing mRNA of ~ 5 kb was over 200-fold and over 500-fold less abundant in brain and heart respectively. Sequence analysis of the cDNA predicted a novel protein of 697 amino acids containing eight potential *N*-linked glycosylation sites. Analysis of hydrophobicity indicated the presence of at least one, and possibly three, putative membrane-spanning domains. When expressed from the Sp6 message in *Xenopus laevis* oocytes, the protein was inserted into membranes, glycosylated and processed to form a dimer. However, no increase in ^{36}Cl uptake or in membrane conductance could be detected. We found no effect of hybrid depleting the specific message on expression of the *Torpedo* electroplax Cl^- channel in oocytes. Thus we conclude that this novel electroplax membrane protein is probably not a functional part of the chloride channel.

INTRODUCTION

The electric organ (electroplax) of the ray *Torpedo californica* is a good model system for neurobiology since it contains several ion-transport proteins and enzymes at exceptionally high concentrations. Thus, all four subunits of the nicotinic acetylcholine receptor (Noda *et al.*, 1982, 1983*a*,*b*; Ballivet *et al.*, 1982; Claudio *et al.*, 1983), an acetylcholinesterase (Schumacher *et al.*, 1986) and the two subunits of the Na,K-ATPase (Kawakami *et al.*, 1985; Noguchi *et al.*, 1986) have been purified and (the corresponding cDNAs) cloned from this source. Additionally, proteins of 43, 58 and 300 kDa copurifying with the acetylcholine receptor in the 'heavy vesicle' fraction have been identified and characterized (Frail *et al.*, 1987; Froehner *et al.*, 1987; Woodruff *et al.*, 1987). So far, however, the function of those proteins has not been definitely established.

Reconstitution of electroplax 'light vesicles' into planar lipid bilayers led to the observation of a voltage-gated Cl^- channel activity (White & Miller, 1979). Estimates based on the size of the vesicles and the frequency with which channel activity was observed suggested that the putative channel protein is an abundant membrane protein in the electroplax (White & Miller, 1980). Copurification of channel activity with the Na,K-ATPase in the 'light' membrane vesicle fraction suggested localization of the channel in the non-innervated membrane. Moreover, gating characteristics suggested a homodimeric form of the channel (Miller & White, 1984). Channel activity could be inhibited by low concentrations of the disulphonic stilbenes 4,4′-di-isothiocyanostilbene-2,2′-disulphonic acid (DIDS) and 4-acetamido-4′-isothiocyanostilbene-2,2′-disulphonic acid (SITS) (Miller & White, 1980). A similar channel is present in the related species *Narke japonica*, where the channel protein was estimated to constitute roughly 10% of the proteins in 'light vesicles' (Kanemasa *et al.*, 1987).

In addition to the chloride channel from *Torpedo* and possibly other chloride channels (Kondo *et al.*, 1987), SITS and DIDS are known to inhibit a variety of other anion transporters, including erythrocyte band 3 (Cl^-/HCO_3^- exchange) (Cabantchik & Rothstein, 1972,

Abbreviations used: DIDS, 4,4′-di-isothiocyanostilbene-2,2′-disulphonic acid; SITS, 4-acetamido-4′-isothiocyanostilbene-2,2′-disulphonic acid; PBS, phosphate-buffered saline; TBS, Tris-buffered saline (0.15 M-NaCl/20 mM-Tris/HCl, pH 7.5); HBS, Hank's balanced salt solution; BSA, bovine serum albumin; KLH, keyhole-limpet haemocyanin; PMSF, phenylmethanesulphonyl fluoride; TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone; TLCK, *N*-tosyl-L-lysine chloromethyl ketone; PAGE, polyacrylamide-gel electrophoresis; WGA, wheat-germ agglutinin; conA, concanavalin A; NP40, Nonidet P40; sp105, SITS-binding protein of ~ 105 kDa; GES-scale, Goldman, Engelman, Steitz scale.

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1974*a*,*b*; Lepke *et al.*, 1976), and (in other tissues) $Na^+HCO_3^-/Cl^-H^+$ exchange (Thomas 1976; Boron *et al.*, 1981) and $Na^+(HCO_3^-)$ symport (Boron & Boulpaep, 1983; Jentsch *et al.*, 1984). Due to their negative charge, SITS and DIDS are thought to interact electrostatically with sites next to the transport site of an anion transporter, and then to react covalently with nearby amino groups of lysine residues. Thus they can be used to tag and identify putative anion transporters. This approach has been used successfully for the red blood cell anion exchanger (Cabantchik & Rothstein, 1974*a*,*b*; Lepke *et al.*, 1976), and, based on [^{3}H]H_2DIDS binding, putative anion transporters have also been identified in other cells and tissues (Taguchi & Kasai, 1980; Ziegler *et al.*, 1984; Burckhardt *et al.*, 1985; Jessen *et al.*, 1986). In one case (Pimplikar & Reithmeier, 1988), the labelled protein (a 130 kDa membrane glycoprotein from canine kidney) could also be purified by affinity chromatography on a SITS-affigel column. Except for band 3, however, no proof that the labelled proteins are indeed functional anion transporters has been reported.

Here we describe the use of antibodies we have raised against SITS to identify two major SITS-binding proteins of the *Torpedo* electroplax light membrane vesicle fraction. One of these is very likely the α-subunit of the Na,K-ATPase, which is known to be inhibited by low concentrations ($\sim 3\ \mu$M) of H_2DIDS (Pedemonte & Kaplan, 1986). The other one is an integral membrane glycoprotein of 105 kDa apparent molecular mass (designated sp105) and is present as a disulphide-linked dimer under non-reducing conditions. We have purified this protein, cloned its cDNA and deduced its primary structure by sequence analysis.

EXPERIMENTAL

Materials

SITS was obtained from Fluka. *Torpedo* electroplax cDNA libraries in λgt10 and λgt11 were a generous gift from Dr. Toni Claudio, Yale University School of Medicine (Claudio, 1987), and the anti-(Na,K-ATPase) antibody (rabbit antiserum against purified rat brainstem axolemmal Na,K-ATPase, late bleed of antibody A2) was kindly provided by Dr. Kathleen Sweadner, Massachussetts General Hospital (Sweadner & Gilkeson, 1985).

Antibodies

SITS–keyhole-limpet haemocyanin (KLH) conjugate was prepared by reacting 60 mg of SITS with 10 mg of KLH (Calbiochem) in 6 ml of phosphate-buffered saline (PBS; 150 mM-NaCl / 1 mM-$MgCl_2$ / 10 mM-Na_2HPO_4 / 1.4 mM-NaH_2PO_4, pH 7.5) at 37 °C for 3 h. The resulting conjugate was extensively dialysed against PBS at 4 °C. Two rabbits were injected with 500 μg or 50 μg of KLH conjugate, respectively, suspended in complete Freund's adjuvant, and were boosted at biweekly intervals using incomplete adjuvant. Antibodies against DIDS were prepared following a similar protocol (A. M. Garcia & H. F. Lodish, unpublished work).

Polyclonal antibody against sp105 was prepared against protein purified by two-dimensional SDS/polyacrylamide-gel electrophoresis (PAGE). Membranes from the 'light vesicle' fraction (prepared as described below) containing about 11 mg of protein were dissolved without boiling in SDS/PAGE sample buffer containing 4.5% SDS and no reducing agent, and loaded on to a preparative (3 mm thick) 5% acrylamide gel (Laemmli, 1970). After the run, a longitudinal slice was stained with Coomassie Blue to identify the band at $\sim$ 200 kDa. The corresponding section of the unstained gel was cut out and equilibrated for 1 h in SDS sample buffer containing 5% mercaptoethanol and 6 mg of dithiothreitol/ml. This gel slice was then sealed with agarose on the stacking gel of a 7.5% acrylamide gel which was run with 0.1 mM-sodium thioglycollate in the upper running buffer (Hunkapiller *et al.*, 1983). After the run, part of the gel was stained with Coomassie Blue, while the rest was fixed in 25% isopropanol and 10% acetic acid for 1 h, and then 10% acetic acid for 1 h. A gel slice corresponding to the purified 105 kDa band was cut out, equilibrated in PBS (1 h), added to a 5-fold volume of PBS and crushed with a Polytron homogenizer (Kinematika AG, Switzerland). Rabbits were injected with the crushed gel suspended in complete Freund's adjuvant and boosted four times in incomplete adjuvant. The serum obtained after the fourth boost was taken for this study.

Preparation of *Torpedo* electroplax membrane vesicles

Membrane vesicles from the electric organ of *Torpedo californica* (obtained alive from Pacific Biomarine, Venice, CA, U.S.A.) were prepared as described (White & Miller, 1981). The final vesicle suspension in 10% sucrose/10 mM-Mops, pH 7.4, was layered on a step gradient of 39% and 35% (w/w) sucrose in 10 mM-Mops, pH 7.4. After overnight centrifugation at 24000 rev./min ($r_{av.}$ = 118.2 mm) in a SW28 rotor (Beckman) the material at the 35%/10% interface ('light vesicles') and the 35%/39% interface ('heavy vesicles') was collected, diluted with 2 vol. of water, centrifuged in a SW28 rotor (2 h, 24000 rev./min), resuspended in 0.4 M-sucrose/5 mM-Mops/KOH, pH 7.4, and stored in liquid N_2.

SITS labelling of red blood cells

Mouse red blood cells were washed twice in PBS and reacted as a $\sim$ 10% cell suspension in Hank's balanced salt solution (HBS)/20 mM-Hepes, pH 7.4, with 100 μM-SITS (Calbiochem) for 1 h at 37 °C. The cells were then washed twice in HBS/20 mM-Tris, pH 7.6, and twice in HBS/20 mM-Tris/HCl, pH 7.6, containing 0.5% bovine serum albumin (BSA) [to remove and quench unreacted SITS (Cabantchik & Rothstein, 1972)], and once with HBS at room temperature. For proteolytic treatment, labelled intact cells were treated as a $\sim$ 10% cell suspension in HBS with 1 mg of chymotrypsin/ml (A4, Boehringer Mannheim) for 1 h at 37 °C. The cells were then lysed in ice-cold lysis buffer (5 mM-sodim phosphate, pH 7.9/1 mM-EDTA/1 mM-phenylmethanesulphonic acid (PMSF)/0.2 mM-TPCK/0.2 mM-TLCK). The membranes were recovered by centrifugation and taken up in sample buffer for subsequent analysis by SDS/PAGE (Laemmli, 1970) and Western blotting.

Labelling of *Torpedo* membrane vesicles with disulphonic stilbenes

Torpedo membrane 'light vesicles' were labelled at $\sim$ 100–300 μg of protein/ml in PBS/1 mM-PMSF with varying amounts of SITS (5–50 μM) or DIDS (1–10 μM) for 5–60 min at either room temperature or 37 °C. The membranes were then cooled on ice and recovered by centrifugation [15 min at 4 °C and 75000 rev./min ($r_{av.}$ = 31.8 mm) in a TLA100.2 rotor in a TL100 centri-

fuge (Beckman)]. During this time, some additional reaction might occur. The membrane pellet was resuspended in PBS + 20 mM Tris/HCl, pH 7.5, and sequentially washed in PBS + 20 mM-Tris/HCl, pH 7.5; PBS-20 mM-Tris/HCl, pH 7.5, +0.5% BSA; and PBS alone, with centrifugation steps as above. The resulting pellet from the last wash was suspended either in sample buffer for SDS/PAGE (Laemmli, 1970) or in the appropriate buffer for lectin affinity chromatography.

Lectin affinity chromatography

Washed membranes labelled with SITS as described above were dissolved (at 200 μg of protein/ml) for 15 min on ice in Tris-buffered saline (TBS) containing 1% Nonidet P40 (NP40), 0.25% sodium deoxycholate, 1 mM-PMSF and 0.2 mM each of TPCK and TLCK. For affinity chromatography on concanavalin A (conA) the buffer contained additionally $CaCl_2$ (1 mM) and $MnCl_2$ (1 mM). Insoluble material was removed by a 40 min centrifugation at 100000 *g*. The soluble material (4 ml) was incubated batchwise with 1 ml of WGA–agarose or conA–agarose (EY Labs, San Mateo, CA, U.S.A.) for 4 h at 4 °C. The agarose was then extensively washed with TBS containing 1% NP40 (and divalent cations for conA as above). The bound material was eluted with identical solutions containing additionally 0.5 M-*N*-acetylglucosamine for WGA or 0.2 M-α-methylmannoside for conA.

For WGA-chromatography with SDS-denatured protein, 400 μl of membrane suspension ($\sim$ 2 mg of protein) were combined with 400 μl of 10% SDS and boiled for 5 min. Buffer containing 0.15 M-NaCl, 20 mM-Tris/HCl, pH 7.5, 1% NP40 and 0.25% sodium deoxycholate and protease inhibitors as above was added to 8 ml (final SDS concentration 0.625%), and 100 mg of BSA was added to further reduce the free SDS concentration. Affinity chromatography on WGA was performed as above. The eluate and flow-through fractions were analysed by SDS/PAGE according to Laemmli (1970) with subsequent silver staining or Western blottting.

Western blots

Proteins from polyacrylamide gels were electrophoretically transferred to nitrocellulose membranes (BA85, Schleicher & Schuell). The membranes were washed (3 × 10 min, in TBS plus 0.1% Tween-20), blocked (2 h, in TBS containing 5% BSA), incubated with antiserum (1:200 dilution) overnight at 4 °C (in TBS containing 0.2% NP40 and 5% BSA), washed (3 × 10 min, TBS with 0.1% NP40), blocked as above for 1 h, incubated with ^{125}I-Protein A (Amersham) (2 kBq/ml in TBS containing 5% BSA and 0.1% NP40), and finally washed as above and used for autoradiography.

Phosphorylated intermediate of the Na,K-ATPase

Preparation and analysis of the phosphorylated intermediate of Na,K-ATPase was carried out as described elsewhere (Sweadner, 1979).

RNA preparation, hybrid depletion and Northern blots

RNA was isolated (either from fresh tissue or from tissue shock-frozen in liquid N_2 and stored at −80 °C) by homogenization in 5 M-guanidinium isothiocyanate (Fluka) and centrifugation through a CsCl cushion (Chirgwin *et al.*, 1979). Poly(A)$^+$ RNA was enriched by chromatography on oligo(dT) cellulose (Type 3, Collaborative Research, Lexington, MA, U.S.A.) and subsequent ethanol precipitation. RNA concentration was quantified by measuring absorbance at 260 nm.

Hybrid depletion was performed by a modification of the solution hybridization/CsCl gradient separation of Lübbert *et al.* (1987). A 20 μg portion of total electroplax RNA was hybridized at 65 °C for 15 min with 150 μg of single-stranded M13 (non-linearized) containing the corresponding insert in either sense or antisense orientation. RNA was separated from DNA and DNA/RNA hybrids by a CsCl-gradient centrifugation (Lübbert *et al.*, 1987). The RNA was precipitated with ethanol, dissolved in 30 μl of RNAse-free water and used for Northern blots or oocyte injections.

Northern blots were done by separating RNA on denaturing formaldehyde gels (1.5% agarose) and transferred by blotting to Nylon membranes (Biotrans, ICN, Costa Mesa, CA, U.S.A.). The RNA was fixed by u.v. irradiation and baking at 80 °C for 2 h. Hybridization with probes labelled by oligonucleotide-primed ^{32}P incorporation was carried out at 42 °C in 50% formamide, 5 × SSC (1 × SSC = 150 mM-NaCl/15 mM-sodium citrate, pH 7.0), 5 × Denhardt's (1 × Denhardt's = 0.02% each of Ficoll, BSA and polyvinylpyrrolidone) and 0.1% SDS overnight. The washes were performed at 50 °C in 0.1 × SSC/0.1% SDS (2 h), and the filter was processed for autoradiography.

Cloning and sequencing of the cDNA for sp105

To obtain a partial amino acid sequence, sp105 was purified by two-dimensional gel electrophoresis as described above, and cleaved by limited proteolysis with V8 protease (Boehringer Mannheim) in the stacking gel of a 5–18% gradient polyacrylamide SDS gel (Cleveland *et al.*, 1977) (0.5 μg of protease per lane). The fragments were electrophoretically transferred to polyvinylidene difluoride membrane (Immobilon; Millipore, Bedford, MA, U.S.A.), stained with Coomassie Blue, cut out and the peptides bound to the membrane were sequenced using an Applied Biosystems model 470A sequenator as described (Matsudaira, 1987). Based on these peptide sequences, four oligonucleotide probes were designed. Two long antisense probes were based on *Torpedo* codon usage using the rules outlined by Lathe (1985): a 35 mer TTACCAGGCA GATAAATATC ATAATGCACT TTACC (oligonucleotide 1, amino acid GKVHYDIYLPG) and a 41 mer TGATGCTGAG CCTGACCCAC ATAAGGGGAA CCCACACCCAC (oligonucleotide 2, amino acid VGVGSPYVGQAQHH) and two oligonucleotide mixtures allowing for all possible codons: GCCAT(C,T)TT(C,T)TT(T,A,C,G)GC (oligonucleotide 3) encoding for part of an *N*-terminal sequence, AKKM, and TT(T,G,A)AT(G,A)TA(G,A)TC(G,A)TC (oligonucleotide 4) for amino acids DDYI (brackets mean that mixtures of the indicated bases were used at these positions). These oligonucleotides were synthesized on an Applied Biosystems 380A DNA synthesizer, gel-purified, end-labelled from [^{32}P-γ]ATP (> 260 TBq/mol; New England Nuclear, Boston, MA, U.S.A.) using T4 polynucleotide kinase, and used to screen a *Torpedo* electroplax cDNA library in λgt10 (obtained from Dr. Claudio, Yale University) (Claudio, 1987) using the method of Wood *et al.* (1985). In parallel, a cDNA library in λgt11 (Claudio, 1987) was screened using the anti-sp105 antibody and alkaline-phosphatase-

coupled secondary antibodies (Promega) as a detection system. Out of 5×10^5 plaques of the λgt10 library screened, none was positive with two different oligonucleotides. One clone (F44) hybrdizing with oligonucleotide 2 also hybridized with oligonucleotide mixture 4 in a Southern blot of purified phage. This clone also hybridized at high stringency with the insert of a clone isolated by immunoscreening from the λgt11 cDNA library. Fragments from this clone (F44) were labelled (by oligonucleotide-primed polymerization with Klenow fragment) and used to screen the library under high stringency conditions (hybridize in 50% formamide/ 5 × SSPE (1 × SSPE = 0.15 M-NaCl/10 mM-NaH_2PO_4/ 1 mM-EDTA, pH 7.4)/5 × Denhardt's/0.1% SDS at 42 °C; wash in 0.1 × SSC/0.1% SDS at 50 °C) to obtain the remaining clones shown in Fig. 5(*a*). The inserts from the λ phages were subcloned into the plasmids pGem3 and pGem-5Zf (Promega, Madison, WI, U.S.A.) using standard methods. The DNA was sequenced by the dideoxy chain terminator method (Sanger *et al.*, 1977) with ^{35}S-dATP using double-stranded sequencing of restriction fragments subcloned into pGem3 and pGem-5Zf using Klenow enzyme and reverse transcriptase, where necessary.

Expression in oocytes

Proteins were expressed in oocytes essentially as described (Colman, 1984). Ovaries from *Xenopus laevis* frogs (obtained from Nasco or Xenopus I) were removed by surgery, washed in ND96 (96 mM-NaCl/2 mM-KCl/1.8 mM-$CaCl_2$/1 mM-$MgCl_2$/5 mM-Hepes, pH 7.6), treated with collagenase [20 mg of collagenase/ml (type IV, Sigma) for 30 min at room temperature, or collagenase type Ib (2 mg/ml, Sigma) for 2 h], extensively washed in ND96 and manually dissected. The oocytes were injected on the same day with 50 nl of RNA dissolved in water, and incubated at room temperature in ND96 containing 100 units of penicillin/ml and 100 μg of streptomycin/ml. Functional expression of chloride channels was assayed on each of the next two days using a high voltage two-electrode voltage clamp (Dagan model 8500, Minneapolis, MN, U.S.A.) by stepping the holding potential from −50 mV in 10 mV steps for 5 s each up to +10 mV and down to −110 mV. The clamp current was recorded by a chart recorder.

RESULTS

Anti-SITS antibodies

Antibodies against DIDS can be used to identify plasma membrane proteins covalently labelled by DIDS (A. M. Garcia & H. F. Lodish, unpublished work). In this work, we generated antibodies against SITS as an alternative tool to identify and purify SITS-binding proteins, among them putative anion transporters. Although in several systems (including the electroplax chloride channel) DIDS has a higher affinity for anion transporters than does SITS, we chose to produce antibodies against SITS since, in contrast to DIDS which has two reactive isothiocyano groups, this inhibitor cannot cross-link proteins, and might be more exposed as a hapten.

These antibodies were first tested using SITS-labelled mouse erythrocytes. Previous studies showed that DIDS and H_2DIDS react specifically with band 3 (molecular mass ~ 95 kDa), and that after chymotrypsin treatment most of the label is found in the 65 kDa fragment (Cabantchik & Rothstein, 1974 *a*,*b*). In a Western blot of erythrocyte membranes labelled with 100 μM-SITS (Fig. 1), the antibody specifically recognized a 95–98 kDa protein, which shifted to ~ 65 kDa upon chymotrypsin treatment of the intact cell. Red blood cell membranes unreacted with SITS were not recognized (results not shown). These results suggest that our anti-SITS antibody is a useful tool to identify and eventually purify SITS-binding proteins in other systems as well.

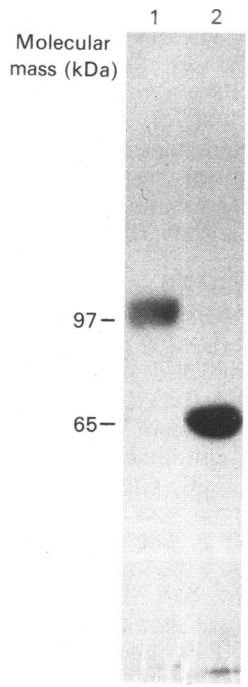


Fig. 1. Immunoblot of SITS-reacted erythrocyte membranes probed with anti-SITS antibody

Lane 1, intact membranes; lane 2, membranes from cells digested with 1 mg of chymotrypsin A4/ml. Reaction conditions are given in the Experimental section, and membrane proteins were analysed by SDS/PAGE (7.5% acrylamide) and subsequent immunoblotting with anti-SITS serum.

Labelling of *Torpedo* electroplax vesicles with SITS

Membranes from the electric organ of *Torpedo californica* are known to contain high concentrations of a Cl^- channel which is highly sensitive to disulphonic stilbenes (White & Miller, 1979). In addition, they may contain other SITS-sensitive anion transporters such as a Cl^-/HCO_3^--exchanger or $Na^+HCO_3^-/H^+Cl^-$-exchanger, which serve as important mechanisms for pH regulation in a variety of tissues (Roos & Boron, 1981). Thus, we were interested in whether SITS-labelling of the 'light vesicle' fraction, which is enriched in the Cl^- channel activity, could detect putative anion-transport proteins.

The result of a typical experiment is shown in Fig. 2. Incubation of membranes with 10 μM-SITS resulted in the predominant labelling of two proteins with approximate molecular masses of ~ 93 kDa and ~ 105 kDa (Fig. 2, lane 2). Detection of SITS-reacted proteins by Western blotting is specific, since no species are detected in membranes not reacted with SITS (lane 3 versus lane 4). In Western blots of some vesicle prepar-

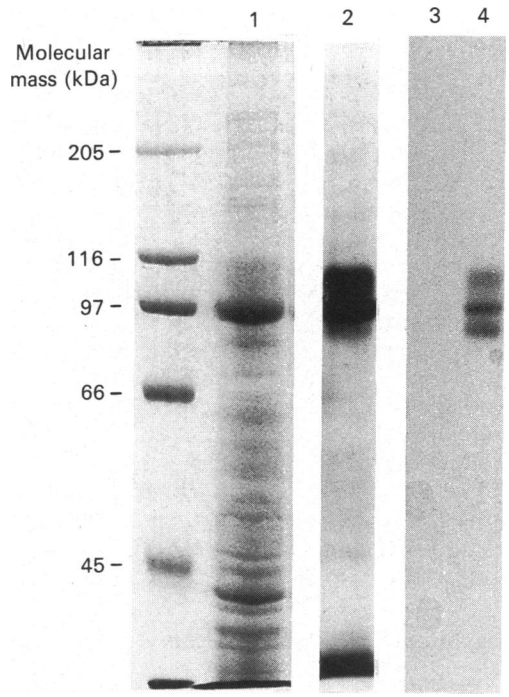


Fig. 2. Gel electrophoresis of SITS-labelled membranes from the *Torpedo* electroplax 'light vesicle' fraction

Lane 1, Coomassie Blue stain of total membrane proteins; lane 2, Western blot of membranes reacted with 10 μM-SITS for 1 h at 37 °C and probed with the anti-SITS antibody. Lanes 3 and 4, Western blot of a different batch of *Torpedo* light vesicles either reacted with 5 μM-SITS (lane 4) or not (control, lane 3). Proteins were separated by reducing SDS/PAGE (7.5% acrylamide).

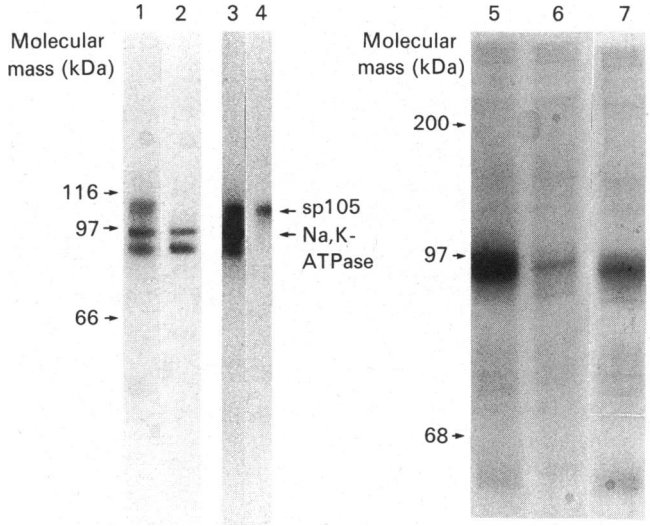


Fig. 3. Distinctions between SITS-binding proteins

Lanes 1 and 2, Western blot of SITS-reacted *Torpedo* membranes (light vesicle fraction). Lane 1 was probed with anti-SITS antibody, lane 2 with anti-(Na,K-ATPase) antibody. Lanes 3 and 4, Western blot (probed with anti-SITS antibody) of material specifically eluted from a WGA-column with *N*-acetylglucosamine. In these experiments, *Torpedo* membranes were labelled with 10 μM-SITS, solubilized either in 1% NP40 and 0.25% deoxycholate (lane 3) or initially in 5% SDS (lane 4) and passed over a WGA column and eluted with *N*-acetylglucosamine as specified in the Experimental section. Lanes 5–7, detection of phosphorylated intermediate of the Na,K-ATPase. *Torpedo* electroplax membranes (light vesicle fraction) were phosphorylated with [γ-^{32}P]ATP under control conditions (lane 5), or in the presence of 0.1 M-potassium (lane 6) or 1 mM-ouabain (lane 7).

ations (e.g. Fig. 2, lane 4), a third band at ~ 85 kDa could be detected, which turned out to be a proteolytic degradation product of the ~ 93 kDa polypeptide. This is supported by: (i) the variable occurrence of this band, which was more often seen in preparations stored a longer time; (ii) the fact that it reacts with the antibody directed against the α-subunit of the Na,K-ATPase (Fig. 3, lane 2); and (iii) identical characteristics of binding to WGA as the α-subunit (Fig. 3, lanes 3 and 4). Besides these major bands, some minor labelling of other proteins can also be detected (Fig. 2, lane 2). While the labelled protein of ~ 93 kDa comigrates with (and might be identical to) the major protein of this membrane fraction, it is clear that SITS is not incorporated into all membrane proteins in proportion to their abundance.

The same polypeptides were detected when the membranes were labelled with only 5 μM-SITS (Fig. 2, lane 4), and anti-DIDS antibodies also detected the same proteins after labelling of membranes with 1 μM-DIDS (results not shown).

Distinction between SITS-binding proteins

Pedemonte & Kaplan (1986) showed that Na,K-ATPase function can be irreversibly inhibited by low concentrations of H_2DIDS ($K_i \sim 3$ μM). This suggested that one of the labelled bands could be the α-subunit of the Na,K-ATPase, especially since this enzyme is the single most abundant protein complex in the non-innervated membrane from *Torpedo* electroplax.

The experiment of Fig. 3 shows that this is the case. Lane 1 is a Western blot of SITS-reacted membranes probed with the anti-SITS antibody. In lane 2, an identical preparation was probed with an antiserum directed against rat brainstem Na,K-ATPase which shows a broad species cross-reactivity (Sweadner & Gilkeson, 1985). This antibody recognized two bands at ~ 93 and ~ 85 kDa, suggesting that the corresponding bands recognized by the anti-SITS antibody represent the α-subunit of the Na,K-ATPase and its degradation product.

Additional evidence for this identity was obtained by lectin affinity chromatography. The β-subunit but not the α-subunit of the Na,K-ATPase is known to bind to WGA, while neither binds to conA (Omori *et al.*, 1983). When SITS-labelled *Torpedo* membranes were solubilized with 1% NP40 and 0.25% deoxycholate in PBS, all three SITS-reactive species bound specifically to WGA (lane 3). However, the Na,K-ATPase α_2/β_2 complex might still be intact at the conditions of solubilization used, and the α-subunit might therefore bind to WGA via the carbohydrate part of the β-subunit. Therefore we dissociated the subunits by boiling the labelled membranes in 5% SDS and performed WGA affinity chromatography as above (after reducing the SDS concentration by dilution). Analysis of the WGA-bound fraction (Fig. 3, lane 4) showed only the presence of the

~ 105 kDa SITS-binding protein, strongly suggesting that the SITS-reactive ~ 93 kDa protein is indeed the α-subunit of the Na,K-ATPase. This is also in line with the observation (results not shown) that only the ~ 105 kDa protein (and not the ~ 93 kDa protein) bound to conA. Additionally, a band at ~ 93 kDa could be specifically phosphorylated from [γ-^{32}]ATP in a potassium- and ouabain-sensitive manner (Fig. 3, lanes 5–7), being characteristic of the Na,K-ATPase. Thus, we have obtained strong evidence that the SITS-binding protein of ~ 93 kDa is identical to the α-subunit of the Na,K-ATPase.

Dimeric structure of the 105 kDa SITS-binding protein

Taguchi & Kasai (1980), working with *Narke japonica* [which expresses a similar chloride channel to *Torpedo* (Kanemasa *et al.*, 1987)], have identified in that electric fish a [^{3}H]H$_2$DIDS-binding protein which existed as a disulphide-linked dimer of ~ 180 kDa and shifted to ~ 90 kDa upon reduction. Furthermore, biophysical investigations have been interpreted as evidence that the *Torpedo* chloride channel exists as a homodimer (Miller & White, 1984) [however, see Labarca *et al.* (1985)]. We therefore performed two-dimensional non-reducing/reducing SDS/PAGE to determine whether the 105 kDa SITS-binding protein is part of a larger complex linked by disulphide bonds. When analysed by Coomassie Blue staining (Fig. 4*b*), two major off-diagonal proteins (at ~ 105 and ~ 66 kDa) are evident. The ~ 66 kDa protein may be the δ-subunit of the acetylcholine receptor, which in *Torpedo* exists as a disulphide-linked dimer (Suarez-Isla & Hucho, 1977; McCrea *et al.*, 1987) and which is an innervated-face contaminant of the non-innervated face membrane vesicle fraction we have used. Examination of an equivalent two-dimensional gel of SITS-reacted membranes processed for immunoblotting with the anti-SITS antibody (Fig. 4*a*) revealed that the ~ 105 kDa SITS-binding protein is present as a disulphide-linked dimer and is identical to the major disulphide-linked protein in the 'light vesicle' fraction. As expected, the ~ 93 kDa SITS-binding protein (the α-subunit of the Na,K-ATPase) is not linked by disulphide bonds to other peptides or to itself. The observation that the 105 kDa protein is a disulphide-bonded homodimer suggests two-dimensional non-reducing/reducing PAGE as a simple purification procedure for sp105.

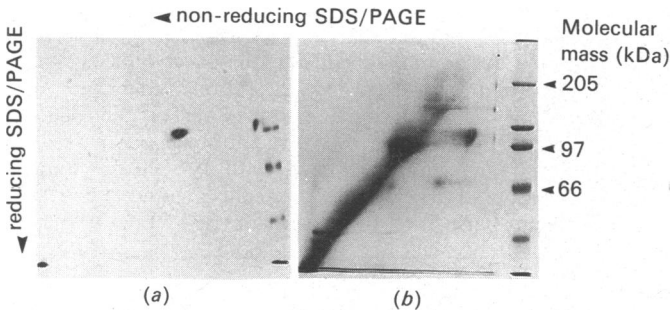


Fig. 4. Two-dimensional non-reducing/reducing gel electrophoresis of SITS-reacted *Torpedo* electroplax membranes

(*a*) Western blot using anti-SITS antibody, (*b*) total membrane proteins as detected by Coomassie Blue staining. 7.5% Acrylamide gels were used for both dimensions. Markers were run at the right end of the two-dimensional gels. In the Western blot (*a*), molecular mass standards reacted with SITS at high concentrations (2 mM, 2 h at 37 °C) were used [rabbit phosphorylase *b* (approx. 97 kDa), bovine albumin (approx. 66 kDa) and egg albumin (approx. 45 kDa)].

Primary structure of sp105 deduced from its cDNA sequence

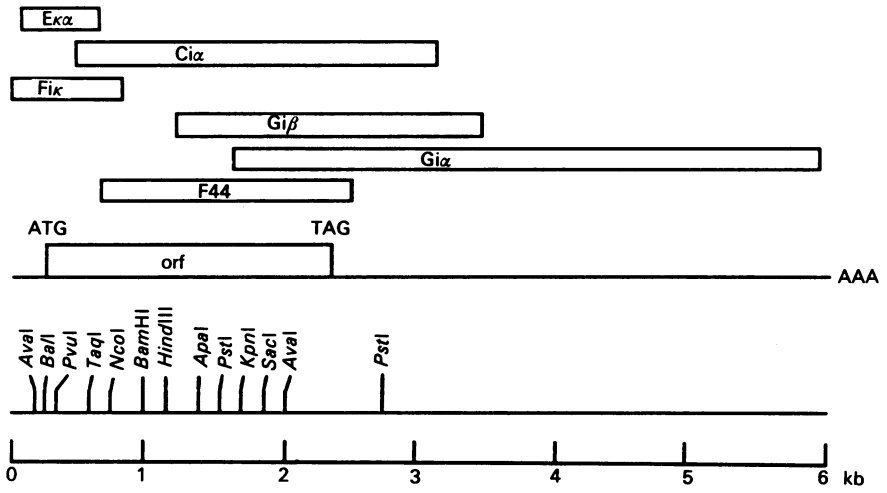
To characterize sp105 further, we cloned the corresponding cDNA. The dual cloning strategy employed polyclonal antibodies against sp105 to screen an expression library in λgt11. We also obtained partial peptide sequence to design oligonucleotides (as described in the Experimental section) for screening a cDNA library in λgt10.

sp105 was purified by two-dimensional non-reducing/reducing SDS/PAGE and used to obtain polyclonal antibodies in rabbits. Protein sequencing of intact, similarly purified sp105 yielded a sequence of ten amino acids. Internal peptide sequences of 5–25 amino acids were obtained from fragments generated by proteolytic digestion with V8-protease in the gel (results not shown). These were used to design oligonucleotide probes (specified in the Experimental section) to screen a cDNA library in λgt10. A clone was isolated, its identity confirmed by DNA sequencing, and used to obtain other overlapping clones (Fig. 5*a*). The overall length of the overlapping cDNA is about 6 kb, as judged by DNA sequencing and gel electrophoresis. This corresponds closely with the lower band observed in Northern blots from electroplax (see below). Sequence analysis (Fig. 5*b*) predicts an open reading frame of 2090 bases, corresponding to a 697 amino acid protein with a predicted

Fig. 5 (*a*) Clones coding for sp105, and (*b*) primary structure of sp105

(*a*) The initial clone, F44, was identified by hybridization to oligonucleotides 2 and 4, and by cross-hybridization to a clone (not further analysed) obtained by antibody screening of a λgt11 library. Rescreening of the λgt10 library with F44 gave clones Ciα, Giα and Giβ. A second round of screening with the 5′ *Bam*HI fragment of F44 gave clone Fiκ, and a third round using the 5′ *Ava*I fragment of Fiκ as a probe led to the identification of Eκα. Extensive restriction mapping was consistent with identity of overlapping sequences. A subset of restriction sites (not necessarily unique) is shown in the second line from the bottom. Clones F44 and Fiκ were fully sequenced on both strands, while the other clones were sequenced only at both ends. (*b*) Primary structure of sp105 as deduced by the sequence of its cDNA. Except for the last line (3′ end of the clone preceding the poly(A)$^+$ tract), both strands were sequenced. As estimated by gel electrophoresis, the length of the unsequenced part of the 3′ untranslated region (gap between bases 2345 and x1) is about 3.5 kb. Stretches confirmed by protein sequencing are highlighted by underlining with ===, the proposed membrane spanning domain by *****, less hydrophobic putative transmembrane regions by (* * * *) and the polyadenylation signal by -----. Gaps in the confirmed protein sequence at positions 450 and 453 are due to the inability to detect cysteine in the protein sequencing procedure. Potential *N*-linked glycosylation sites are marked by CHO.

(a)



(b)

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-233  GAGTAGGTTACCCGCCAGCGCAAATTCTCAGGCTTACCCGTGAATCCTCCGCACTGCCCAGCGTTTGGTTTACTTAATTCAAGCCCTGCTCGGCGCGCTTGGTCCGGCTCCAGT  -120

-119 CCGGGTCAAGTGCCGCGGCAGTTCAGCGGCAGTTCGCAGCGGGGAGCACGGGGCTCGGGGTAGGAGCGGGCGAGTGCAGCTCCCGCGGCGCCCGTGCGCCGAGCCCAGCGCCGGGACGAG     0
    1                 10                  20                  30                  40
  1 ATGGCAAGACGGGCCAAGAAAATGGCCAGTAATTCCGGCGACTCCAGCCCAGAGCCCGGTATCAAAGAGATAAACGAGACGTGGAAGGGGGCGATCGCCTGCCTGGGGGTCGCCTTACTT   120
    MetAlaArgArgAlaLysLysMetAlaSerAsnSerGlyAspSerSerProGluProGlyIleLysGluIleAsnGluThrTrpLysGlyAlaIleAlaCysLeuGlyValAlaLeuLeu
       ==============================          (CHO)          ******************************
                      50                  60                  70                  80
 121 TTTCTGATGACCATCGGCGTCCTTTACTGGCAGGTGGTGGAGAAGCCGGACACCAACTGGGTTCTGAGGGGGCAGGTCAGCGGGCTGGTGTGGGACAAGAAAAGCCTCTCCTTCTCATTC   240
    PheLeuMetThrIleGlyValLeuTyrTrpGlnValValGluLysProAspThrAsnTrpValLeuArgGlyGlnValSerGlyLeuValTrpAspLysLysSerLeuSerPheSerPhe
    ****************************
                      90                  100                 110                 120
 241 AAGTCCCTCAGCCAGGATAAGACTTTCGCTCGCATCGACGCCCGTCACTTGCCGGTCCGAGGAGGCGCCCTGCTCAACAACCACTGCTGGTTCAACACAACCCGTTTCTGCCACACATGG   360
    LysSerLeuSerGlnAspLysThrPheAlaArgIleAspAlaArgHisLeuProValArgGlyGlyAlaLeuLeuAsnAsnHisCysTrpPheAsnThrThrArgPheCysHisThrTrp
                                                                        CHO
                      130                 140                 150                 160
 361 GACGGCGTGGCGAATCTCCAGGTGACGCTGGATACACCGAACCAGAGTGTGGCGGAGTGTTACCGGGTGGAGTGGACACCCCTTAACTGCCAAGTGGCGCTTCAGGACTGCTTTTCCATG   480
    AspGlyValAlaAsnLeuGlnValThrLeuAspThrProAsnGlnSerValAlaGluCysTyrArgValGluTrpThrProLeuAsnCysGlnValAlaLeuGlnAspCysPheSerMet
                          CHO
                      170                 180                 190                 200
 481 GTGAACACCTCCTGGTATGGAGGAGGAAGTATGCGCTTACAGTACTGGCCCATAAACAATGCCAACATTAACTCACAGCCGTTCGTTATCAGTGACCTACAGGACACACCCACGGGCTAT   600
    ValAsnThrSerTrpTyrGlyGlyGlySerMetArgLeuGlnTyrTrpProIleAsnAsnAlaAsnIleAsnSerGlnProPheValIleSerAspLeuGlnAspThrProThrGlyTyr
       CHO
                      210                 220                 230                 240
 601 GGATCTGTATTAGAGAGATACTTCCTTGGATCAACAGGTGTGGCTGTTCGTGTTCATCAGGAAGTCCCATTACATGTTGGAATTGAGAGCAGAAAGCAGTTGTGCTTGGGGATCCCACCC   720
    GlySerValLeuGluArgTyrPheLeuGlySerThrGlyValAlaValArgValHisGlnGluValProLeuHisValGlyIleGluSerArgLysGlnLeuCysLeuGlyIleProPro

                      250                 260                 270                 280
 721 AATGCTGAGATGCAACCCCTGAGGTACACAATATGTGTGAGTGACAGTCTGAGATCAGCCCACCAACAATTCGGCACTGTGATCCCTGTGGTTCAGCCTGACCAGCCTGACACCAGCATC   840
    AsnAlaGluMetGlnProLeuArgTyrThrIleCysValSerAspSerLeuArgSerAlaHisGlnGlnPheGlyThrValIleProValValGlnProAspGlnProAspThrSerIle

                      290                 300                 310                 320
 841 CTGAGACTGCCACATTGGAGGTCGCAAAGGGTTGCTGACATTGCATTGAAGCTGGAGCACAATCTGAAGACTTTTACCAGGAAACTGAAGCTTCACAGGCTGGGAGAGGGAATAATGGAT   960
    LeuArgLeuProHisTrpArgSerGlnArgValAlaAspIleAlaLeuLysLeuGluHisAsnLeuLysThrPheThrArgLysLeuLysLeuHisArgLeuGlyGluGlyIleMetAsp

                      330                 340                 350                 360
 961 CTCGGTGAACAGTCTACGCTGCTACTGTCCAATGAGGTAGACGAGACGCTTCAGTCACAGAACAGATACCGTGGCCTACGTCAGCTGAGGTTGTCCATCACCCTCTCTCCTTTCACGAGC  1080
    LeuGlyGluGlnSerThrLeuLeuLeuSerAsnGluValAspGluThrLeuGlnSerGlnAsnArgTyrArgGlyLeuArgGlnLeuArgLeuSerIleThrLeuSerProPheThrSer

                      370                 380                 390                 400
1081 ATTGATTCCCATCATTTCCAAACCACTCTACAAGAAGGGCGAGAGAATTTATGGCTTGGTCTTCCATCAGCAGCAAATGGATCTCAAGGGCCCCTCTTGATGAAATGGAAGGGGAAGTTT  1200
    IleAspSerHisHisPheGlnThrThrLeuGlnGluGlyArgGluAsnLeuTrpLeuGlyLeuProSerAlaAlaAsnGlySerGlnGlyProLeuLeuMetLysTrpLysGlyLysPhe
                                                                  CHO
                      410                 420                 430                 440
1201 GCAGTGAAGTTGAACATCAGCAATGAGGAGGCTCAGGACTGGTTCATCGAGCAGGTCCACAGCCTGCAGAGGAGGCTGGAGGTCGACTATGTTAACCTGGAGGTTGGAGTGGGAAGCCCC  1320
    AlaValLysLeuAsnIleSerAsnGluGluAlaGlnAspTrpPheIleGluGlnValHisSerLeuGlnArgArgLeuGluValAspTyrValAsnLeuGluValGlyValGlySerPro
                CHO                                                                 ==================
                      450                 460                 470                 480
1321 TATGTGGGGCAAGCACAGCATCATTCCTGCCGATTGTGTGGAGATGACTACATCAATCAGTTTGCACTGCTGGCTGAGAAGTTGGGAAATGGAACAACTGTATCAGCTGCTACCAGGACT  1440
    TyrValGlyGlnAlaGlnHisHisSerCysArgLeuCysGlyAspAspTyrIleAsnGlnPheAlaLeuLeuAlaGluLysLeuGlyAsnGlyThrThrValSerAlaAlaThrArgThr
    ============================   ======   ==================          CHO
                      490                 500                 510                 520
1441 GCTCACTTGCCAGTGTTTGTTCGAATGGTACCTCGACAGTCTGACTGGAGTCATGCAGGATTAAAGGGCCTCATTCCATCTATTCTTCACTACAGCTTGCTAGGATACAGCTTCTTCATT  1560
    AlaHisLeuProValPheValArgMetValProArgGlnSerAspTrpSerHisAlaGlyLeuLysGlyLeuIleProSerIleLeuHisTyrSerLeuLeuGlyTyrSerPhePheIle
                                                          * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * *
                      530                 540                 550                 560
1561 CCAGATGTTATAGGAGGTTCTCTGACAGATGGGTTTCTGGCAGATGAGGAGCTCTTTGTCCGGTGGATGCAGATTGCGACTTTTCTACCTGTGATGTCTTTCAGTACTCCGCCTTGGGTC  1680
    ProAspValIleGlyGlySerLeuThrAspGlyPheLeuAlaAspGluGluLeuPheValArgTrpMetGlnIleAlaThrPheLeuProValMetSerPheSerThrProProTrpVal
    * *                                                 * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * *
                      570                 580                 590                 600
1681 TTTGGGGAGACCTGGATTGTGAACGTGACCCGGTCATGTATACACAGACACCAAACATTTGTTGTGCCACTGCTGATGAAATACGCCGCTGAGTGGACATCCCTCGGGCACCCTGTGTTT  1800
    PheGlyGluThrTrpIleValAsnValThrArgSerCysIleHisArgHisGlnThrPheValValProLeuLeuMetLysTyrAlaAlaGluTrpThrSerLeuGlyHisProValPhe
     * * *                CHO
                      610                 620                 630                 640
1801 CGGCCGCTGTGGTGGGTCAGCCCAAGTGACCCAAATACCTTTACCATCAATGACGAGTTTCTGATTGGTGATGAGGTACTGGTGGCGCCGGTGACAGAGTCAGGCAAAGTCCACAGGGAC  1920
    ArgProLeuTrpTrpValSerProSerAspProAsnThrPheThrIleAsnAspGluPheLeuIleGlyAspGluValLeuValAlaProValThrGluSerGlyLysValHisArgAsp
                                                                        ====================
                      650                 660                 670                 680
1921 ATCTACCTCCCAGGAAACAGCTTCCAGTGGAAGGACATGAACACAGCGCAGGTGTTTGAGGGTGGGACCCTGCTGCGCGAGTACCCCGTGGCCTTGACTGAAGTGGCAGTCTTCATCAGG  2040
    IleTyrLeuProGlyAsnSerPheGlnTrpLysAspMetAsnThrAlaGlnValPheGluGlyGlyThrLeuLeuArgGluTyrProValAlaLeuThrGluValAlaValPheIleArg
    ============================
                      690
2041 CAGAAATCCAAGTATACGCCAGCAATTGCAGAGATCCAACAAGCAACAACCTAGGCCTGAGGGACCAAAACTCTTCACCATTCTCAAACACTCAACCGGACTTTTGTTACTGACCATAGC  2160
    GlnLysSerLysTyrThrProAlaIleAlaGluIleGlnGlnAlaThrThrstop

2161 TTGGCTGCCTGCACTGTCTTAAGTGCCAGCAGGTGCCGTTCATGGAAAGGATAAGTAAACAGCACAGTCCATGATCACCAGCTCCCCAGGGCAGCTTTGCAAAAACCAGCTTTCCCTCCA  2280

2281 CCTGCTGCCGGCCTGAACCATCCACAACCATGCCTCGACGATGCCCGTGAAGCTGAACTGTTTCG  2345


 x1 TTGAGAACCTTGCCTGGACAGAATCCAGTGTATTGGACTGTGTCTTATTGCACAACTCCCAGGTTGCATACACATATTTGTACTTTTGCAAACAATAAATAGATGGTGAGAAAAAAAAAA  x120
                                                                        ------
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molecular mass of 78 kDa. The initiator methionine is preceded by a 5′ untranslated region of 233 bases. The stop codon is followed by an untranslated 3′ region of about 3.4 kb (as judged by gel electrophoresis). The clone ends with a poly(A)$^+$ tract preceded by a polyadenylation signal. The initiator methionine is encoded by the first ATG after stop codons in all three reading frames and displays the consensus sequence for initiation of translation by eukaryotic ribosomes (Kozak, 1986). The initiator methionine is immediately followed by the *N*-terminal sequence as determined by peptide sequencing of sp105. Thus, only the first methionine is removed post-translationally, and there is no cleavable signal peptide for membrane translocation. Alanine as the first amino acid of the mature protein is compatible with the known sequence specificity of *N*-terminal methionine aminopeptidase (Tsunasawa *et al.*, 1985; Flinta *et al.*, 1986).

The sequence predicts eight potential *N*-linked glycosylation sites, suggesting that heavy glycosylation may account for the difference between the apparent molecular mass observed by SDS/PAGE (~ 105 kDa) and that predicted by the cDNA-sequence (78 kDa). This is compatible with the results from lectin affinity chromatography reported above and with translation *in vitro* (results not shown). The sequence of every peptide fragment occurred in the derived protein sequence, thus unambiguously establishing that these clones encode the 105 kDa SITS-binding protein.

Hydrophobicity analysis was performed using the GES-scale (Engelman *et al.*, 1986) with a window size of 19 amino acids, which is thought to yield the most reliable predictions for potential membrane spanning helices (von Heijne, 1987). The hydrophobicity plot (Fig. 6) predicts a highly hydrophobic stretch of ~ 20 amino acids next to the *N*-terminus, which clearly satisfies the usual criteria for a transmembrane region. The plot reveals two other hydrophobic stretches of ~ 19 amino acids length with centres at positions ~ 513 and ~ 552, which, however, are somewhat less hydrophobic and hence less convincing candidates for membrane spanning domains. [When analysed by the algorithm of Kyte & Doolittle (1982), these stretches get scores of ~ 1.1, which, in contrast with the values obtained by the GES analysis, is considered to be somewhat below the limit assumed for a typical transmembrane domain. However, both predictions should be viewed with caution considering our current limited understanding of the primary structural determinants of transmembrane segments.]

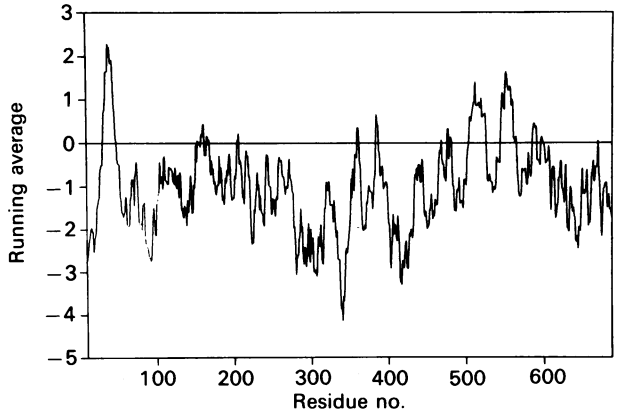

Fig. 6. Predicted hydrophobicity of sp105

Hydrophobicity was analysed using the GES-hydrophobicity scale (Engelman *et al.*, 1986) with a window of 19 amino acids.

Searching of three protein databases (Genbank release # 55, EMBL release # 14, and Institut Pasteur protein databases release # 2) using the algorithm of Lipman & Pearson (1985) did not yield proteins with a significant degree of sequence identity with sp105.

Tissue distribution of sp105

RNA was extracted from various tissues of *Torpedo californica* and hybridized in a Northern blot to a radioactively labelled cDNA coding for sp105 (Fig. 7). In electroplax, the probe hybridized to two messages of approx. 6 and 6.8 kb under high stringency conditions (for better resolution, see Fig. 8, lane 1). Both bands were recognized irrespective of whether a probe encompassing predominantly coding sequence (clone F44), 5′ untranslated region (5′ *Bal*I fragment) or 3′ untranslated region (3′ *Pst*I fragment) was used. With electroplax, the signal was very strong even with total RNA. In contrast, when using poly(A)$^+$-enriched RNA from other tissues, only a comparatively faint signal could be observed for *Torpedo* brain and a still fainter band for heart. The size of these mRNAs appears to be smaller (~ 5 kb) than in

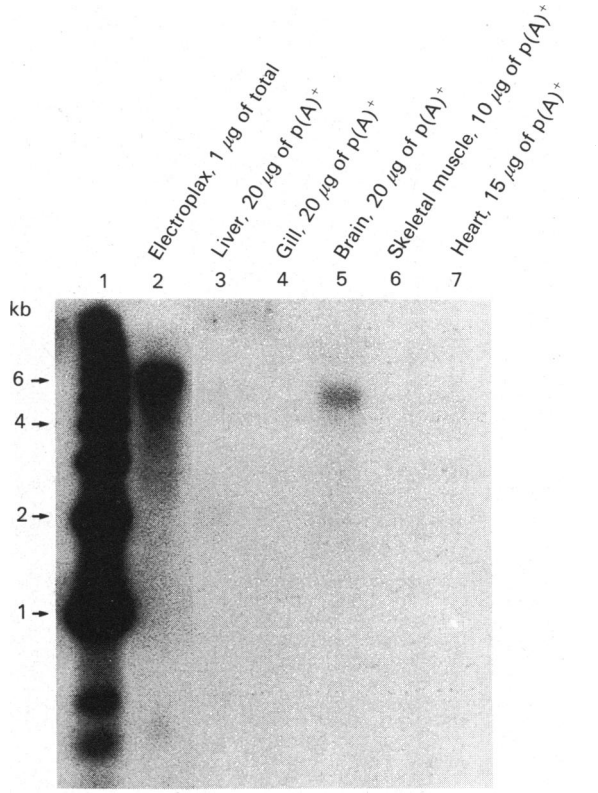

Fig. 7. Tissue distribution of RNA coding for sp105

RNA was extracted from several *Torpedo* tissues, electrophoresed under denaturing conditions, and analysed using high stringency conditions with a probe derived from sp105 cDNA (bp −233 to 2345). Note that only ~ 1 μg of total RNA was used for electroplax, while RNA from other sources was enriched for polyadenylated species. p(A)$^+$, poly(A)$^+$.

electroplax. Within the sensitivity of the assay, no hybridizing mRNA could be detected in liver, gill or skeletal muscle (in the last case, however, only ~ 10 μg of poly(A)$^+$ RNA was loaded). Assuming that a single selection step on oligo(dT) cellulose leads to a > 10-fold enrichment of mRNA, electroplax tissue contains at least 200-fold more sp105 RNA than brain and over 500-fold more than heart.

Expression in *Xenopus* oocytes

Immunoprecipitation of membranes from oocytes injected with Sp6 RNA encoding sp105 generates two major bands of about 98 and 92 kDa apparent molecular mass, which migrate as a ~ 200 kb band in non-reducing gels (results not shown). The 92 kDa but not the 98 kDa species was completely endoglycosidase-H-sensitive (results not shown). Thus, the difference in molecular mass might be explained by differences in glycosylation.

Since SITS- or DIDS-binding proteins are potential candidates for anion transporters, we investigated whether there was an increase in anion transport in oocytes injected with Sp6 RNA coding for sp105. We injected oocytes with total poly(A)$^+$ RNA from electroplax and measured ^{36}Cl uptake and membrane conductance. As reported elsewhere (Sumikawa *et al.*, 1984), oocytes injected with electroplax poly(A)$^+$ RNA, but not mock-injected controls, had membrane potentials close to the Cl^- equilibrium potential in oocytes (−30 to −40 mV compared to −50 to −60 mV of controls) (results not shown). Membrane conductance in oocytes injected with poly(A)$^+$ RNA was increased by a factor of up to 10, and displayed the typical voltage-dependence of the *Torpedo* chloride channel (Sumikawa *et al.*, 1984). In oocytes injected with Sp6 RNA, no increase in $^{36}Cl^-$ fluxes or membrane conductance could be observed (results not shown), despite the fact that large amounts of the protein were being made. This strongly suggests that sp105 by itself is unable to transport chloride.

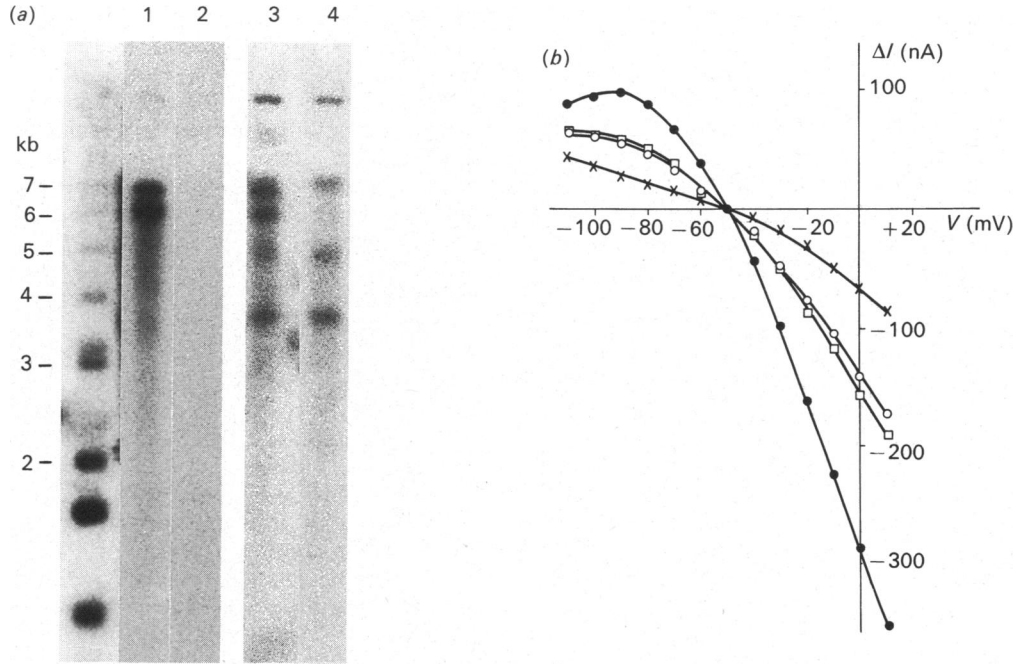

Fig. 8. Hybrid depletion of sp105 message and functional analysis after expression in *Xenopus* oocytes

(*a*) Northern blot of control and hybrid-depleted *Torpedo* electroplax RNA. A fragment of the sp105 cDNA (bp −233 to 2345) was subcloned into M13 in both orientations to give single-stranded DNA in both antisense (for hybrid-depletion) and sense (for control = mock-depletion) orientation. Hybrid depletion was performed using a modification of the method of Lübbert *et al.* (1987) as given in the Experimental section. RNA-containing fractions of the CsCl gradient were analysed by Northern blotting using the 3′ *Pst*I fragment of the sp105 clone as a probe. Lane 2, hybrid-depleted RNA; lane 1, mock-depleted RNA. The band at ~ 10 kb represents contamination by M13 (most of which is found on top of the gradient) in the RNA fraction. To control for integrity and overall abundance of RNA, the nylon membrane was subsequently stripped and hybridized to an anonymous probe recognizing RNAs at ~ 7, ~ 5 and ~ 4.5 kb. This demonstrated that hybrid depletion was specific, but that the control fraction (lane 3) contained about twice as much RNA as the hybrid-depleted fraction (lane 4). (*b*) Current–voltage relationships of *Xenopus* oocytes injected with hybrid-depleted and mock-depleted *Torpedo* electroplax RNA. Measurements using a two-electrode voltage clamp were performed one day after injection. The cells were initially clamped to −50 mV, and the voltage then symmetrically clamped for 5 s intervals in 10 mV steps to de- and hyperpolarizing values. The resulting current was measured, and normalized to the current needed to clamp the oocyte membrane at −50 mV. Each point represents the average of measurements of four different oocytes. X, mock-injected oocytes (injected with water); ●, injected with mock-depleted RNA; □, injected with mock-depleted RNA diluted 1:1 with water; ○, injected with hybrid-depleted RNA (corresponding to lane 2, Fig. 1*a*). Injection with mock-depleted RNA gave a ~ 3-fold increase in membrane current versus control with a current–voltage relationship typical of the *Torpedo* chloride channel (Sumikawa *et al.*, 1984). Since oocytes injected with 1:2 diluted mock-depleted RNA gave about the same amount of current as hybrid-depleted RNA (which contains approximately the same amount of total RNA), no effect of sp105 depletion on Cl^- channel expression could be observed.

To investigate the possibility that sp105 is an indispensable component of a Cl^- channel, we depleted the message encoding sp105 from total electroplax RNA using the method of Lübbert *et al.* (1987). The degree of depletion was checked by Northern blots using a DNA probe for sp105 (Fig. 8*a*). sp105 mRNA concentration was decreased by a factor of > 20 (lane 2) versus mock-depletion (lane 1). Injection of equivalent amounts of RNA (as judged by hybridization of the Northern blot with a control clone, lanes 3 and 4) led to a similar conductance increase in the oocytes irrespective of whether or not the mRNA for sp105 had been depleted (Fig. 8*b*). This suggested that sp105 is not a limiting functional component of the *Torpedo* chloride channel.

DISCUSSION

We have developed antibodies against SITS to identify and purify SITS-binding proteins commonly considered to be candidates for anion-transport proteins. Applied to SITS-reacted membrane vesicles from *Torpedo* electroplax, these antibodies recognized two major SITS-binding proteins. One of these is very likely the α-subunit of the Na,K-ATPase, while the other one (sp105) was shown to be a novel, heavily glycosylated dimeric integral membrane protein. Sequence analysis of its cloned cDNA indicated the presence of one to three putative membrane spanning domains. While functional assays were unable to demonstrate any role of sp105 in anion transport, its abundance and highly specific expression in electroplax, and to a lesser degree in brain and heart, suggests an important function for this protein, which will have to be elucidated in future studies.

The disulphonic stilbenes SITS and DIDS are inhibitors of a variety of anion transport processes and will often react covalently with their target proteins after binding. Inhibition constants have been determined to be between 1 μM and 100 μM, depending on the transporter. However, these compounds will bind also to a variety of other proteins unrelated to anion transport. Thus, recent physiological studies have shown that SITS can also modify K^+ conductance in the squid axon (Inoue, 1986) and can irreversibly inhibit Na,K-ATPase (Pedemonte & Kaplan, 1986), a finding supported by the present study (Fig. 3). Nevertheless, in the absence of a more specific high-affinity ligand for anion-transport proteins, labelling of membrane proteins by $[^3H]H_2DIDS$ [and more recently, affinity chromatography on a SITS-affigel column (Pimplikar & Reithmeier, 1986, 1988)] is a common approach to identify putative anion transporters (Cabantchik & Rothstein, 1974*a*; Taguchi & Kasai, 1980; Ziegler *et al.*, 1984; Burckhardt *et al.*, 1985; Jessen *et al.*, 1986).

In this work, we have developed a novel approach by raising polyclonal antibodies against SITS, enabling us to detect SITS-binding proteins by sensitive techniques such as Western blotting and immunoprecipitation. Furthermore, this allows purification of SITS-binding proteins by immunoaffinity chromatography on an anti-SITS column. In comparison to the SITS–affigel column procedure (Pimplikar & Reithmeier, 1986, 1988), this has the potential advantage that reaction with SITS can be performed with intact cells (binding only to extracellular sites) under conditions identical with those used in functional studies. However, the recognition of SITS by the antibodies could depend quantitatively on the molecular environment of the bound molecule. This problem is minimized by using polyclonal antibodies against SITS bound to a complex molecule (such as KLH in the present study) and by detecting the proteins after denaturation (e.g. Western blots). Indeed, control experiments (Fig. 1) gave identical results to those obtained with $[^3H]H_2DIDS$ labelling (Cabantchik & Rothstein, 1974*a*).

The identification of band 3 as the erythrocyte Cl^-/HCO_3^- exchange protein is so far the only published report in which DIDS has been successfully used to identify an anion transporter (Cabantchik & Rothstein, 1974*a*,*b*). Of course, the abundance of band 3 in red blood cells and its relatively high affinity for disulphonic stilbenes make it an especially simple system to study. In other cases, the labelled proteins have been claimed to be anion transporters or good candidates for these proteins (Taguchi & Kasai, 1980; Ziegler *et al.*, 1984; Burckhardt *et al.*, 1985; Jessen *et al.*, 1986; Pimplikar & Reithmeier, 1988), but no further functional studies have been published to prove or disprove these claims. Recently Pimplikar & Reithmeier (1988) have identified by SITS-affinity chromatography and $[^3H]H_2DIDS$ binding a highly glycosylated 130 kDa protein from dog kidney brush borders, which they consider to be an excellent candidate for a renal anion exchanger, without, however, proving this point. Interestingly, our studies with SITS-labelled BSC-1 cells and other cell lines also indicated major SITS- and WGA-binding proteins in the 120–140 kDa molecular mass range (T. J. Jentsch & H. F. Lodish, unpublished work). Also, the open reading frame of a recently cloned protein homologous to murine erythroid band 3 and transcribed in many tissues predicts a protein of roughly that size (Alper *et al.*, 1988). However, the latter protein is most likely to mediate some form of Cl^-/HCO_3^--exchange, and the presence of this activity in cortical renal brush border membranes used by Pimplikar & Reithmeier (1988) is controversial (Schild *et al.*, 1988).

Though *Torpedo* electroplax membranes are more complex than erythrocyte membranes, it is a favourable system in which to use DIDS or SITS for identifying putative anion transporters, since it presumably contains high concentrations of a disulphonic stilbene sensitive Cl^- channel (White & Miller, 1979). Estimates for the related species *Narke japonica*, which expresses a similar channel, showed that the channel protein could constitute as much as 10% of the protein of the comparable membrane (Kanemasa *et al.*, 1987). The same group also studied $[^3H]H_2DIDS$ incorporation into that membrane and identified as the major DIDS-binding protein a glycoprotein of ~ 180 kDa which shifted to ~ 90 kDa upon reduction (Taguchi & Kasai, 1980). It was resistant to alkaline extraction and thus was probably an integral membrane protein. The authors named it 'anionin' to suggest that it is the electroplax chloride channel. The similar molecular mass and the dimeric structure strongly suggest that 'anionin' is the *Narke* homologue of the sp105 we have cloned and analysed in the present study. The minor difference in molecular mass might be due to species differences. However, it is unclear why Taguchi & Kasai (1980) did not observe labelling of the Na,K-ATPase as we did. Possible explanations are again species differences (which seems unlikely) or different labelling conditions, which were not specified in their paper. Since the present study demonstrates that the two major SITS-

binding proteins identified by our antibodies are (very likely) the α-subunit of the Na,K-ATPase and sp105, which is not a limiting subunit of the chloride channel, it appears that SITS- or DIDS-binding as a criterion for identifying anion transporters is a risky approach even in *a priori* favorable systems like the electroplax.

The fact that sp105 (and probably its *Narke* homologue) is a very abundant protein highly specific for the electroplax suggests that it has an important function in this specialized organ. This protein is enriched in 'light' as opposed to 'heavy' vesicles, suggesting a localization to the non-innervated face of the electrocyte (results not shown). Unfortunately, our polyclonal antibodies recognize only the SDS-denatured protein, so that we were unable to use immunofluorescence to determine its cellular localization. Both the hydropathy plot and the resistance to alkali extraction for the likely *Narke* homologue are strong evidence that sp105 is an integral membrane protein. In contrast with ion channels cloned so far, sp105 contains only one to possibly three transmembrane domains. Thus, also on the basis of its structure, it seems unlikely that sp105 by itself is an ion channel. sp105 might cross the membrane three times, or, which we consider somewhat more likely, it is anchored in the membrane by its *N*-terminal hydrophobic stretch. Since the protein is heavily glycosylated, we believe that the *N*-terminus is cytoplasmic and that most of the protein, including seven potential glycosylation sites, is located extracellularly. The potential glycosylation site at position 25 would then not be used. A potential phosphorylation site (position 10), preceded by a cluster of positive charges (two arginines and two lysines) (Kemp *et al.*, 1975; Pearson *et al.*, 1985) is also found in this putative cytoplasmic stretch. Future studies will be required to define the function of this protein that is highly specific for neuromuscular tissue.

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