ABC1, an ATP Binding Cassette Transporter Required for Phagocytosis of Apoptotic Cells, Generates a Regulated Anion Flux after Expression in *Xenopus laevis* Oocytes*

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The ATP binding cassette transporter ABC1 is a 220-kDa glycoprotein expressed by macrophages and required for engulfment of cells undergoing programmed cell death. Since members of this family of proteins such as P-glycoprotein and cystic fibrosis transmembrane conductance regulator share the ability to transport ions, we have investigated the transport capability of ABC1 expressed in *Xenopus* oocytes using iodide efflux and voltage-clamp techniques. We report here that ABC1 generates an anion flux sensitive to glibenclamide, sulfobromophthalein, and blockers of anion transporters. The anion flux generated by ABC1 is upregulated by orthovanadate, cAMP, protein kinase A, and okadaic acid. In other ABC transporters, mutating the conserved lysine in the nucleotide binding folds was found to severely reduce or abolish hydrolysis of ATP, which in turn altered the activity of the transporter. In ABC1, replacement of the conserved lysine 1892 in the Walker A motif of the second nucleotide binding fold increased the basal ionic flux, did not alter the pharmacological inhibitory profile, but abolished the response to orthovanadate and cAMP agonists. Therefore, we conclude that ABC1 is a CAMP-dependent and sulfonlyurea-sensitive anion transporter.

ATP binding cassette (ABC) transporters are implicated in the vectorial movement of a wide variety of substrates across biological membranes (1, 2). Most of the mammalian ABC transporters identified so far have been associated with clinically relevant phenotypes (2). The human P-glycoprotein confers resistance to chemotherapeutic drugs on tumor cells (3). Members of the ABC family have also been implicated in the vectorial movement of a wide variety of substrates across biological membranes (1, 2). Most of the mammalian ABC transporters identified so far have been associated with clinically relevant phenotypes (2). The human P-glycoprotein confers resistance to chemotherapeutic drugs on tumor cells (3).

cystic fibrosis transmembrane conductance regulator (CFTR), a CAMP-dependent chloride channel (5, 6).

The basic structural unit of an ABC transporter consists of a pair of nucleotide binding folds (NBF) and two transmembrane domains, each composed as a rule of six transmembrane spanners (1, 2). Their activity as transporters is dependent on their interaction with ATP at the NBFs followed by its hydrolysis (see Fig. 1A) (7–16), and in some cases evidence has been provided for a further regulation via phosphorylation of serine/threonine residues in the region linking the two symmetric halves (6, 17–19). The NBF domains contain the highly conserved phosphate binding loop (20) that forms intimate contacts with the β- and γ-phosphates of bound ATP (21) and an additional diagnostic motif, the active transport signature, whose function is so far unknown.

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We have recently reported on the functional characterization of a novel ABC transporter, ABC1 (22, 23). This molecule, whose expression during embryonic development correlates with the occurrence of programmed cell death, is required by macrophages during engulfment of cells undergoing apoptosis (23, 24). From a structural standpoint, ABC1 possesses all of the typical features of ABC transporters, i.e. the two symmetric halves each equipped with six transmembrane spanners and an NBF. In addition, a long charged region, reminiscent of the regulatory domain of CFTR but unique in that it is equipped with an extra hydrophobic segment, links the two halves of the molecule (23). The purpose of this work was to analyze the ionic transport capability of ABC1 transporter using radiotracer efflux and electrophysiological techniques.

**MATERIALS AND METHODS**

*Plasmid Construction—*The ABC1 full-length cDNA was constructed from the 13C, A10F, and 8a4 overlapping plaque clones (22) using the prokaryotic cloning vector pBluescript KS (Stratagene). The NsoMI-EcoRI fragment from the 13C clone, which extends in the 5′ region of ABC1 (EMBL accession number X75926, nucleotides 1–2567), was juxtaposed to the EcoRI-HindIII fragment from A10F (nucleotides 2568–5665) and the HindIII-XhoI fragment of 8a4 (nucleotides 5666–6916). The final construct, pABC1KS, was excised by NotI-Apal in the vector polylinker and then cloned into the pSP64TN poly(A) vector (pABC1TN modified from pSP64T poly(A) (a generous gift of A. Ceriotti, Milano, Italy) (25) by the insertion of an oligonucleotide linker (GATCTGCGG-CCACTCGAGTTAACGCGGCCGCA) conferring ampicillin resistance to the ampicillin selectable marker in pSP64TN poly(A) (underlined). The final construct contained an additional unique site. The CFTR cDNA construct pACF23 was provided by J. R. Riordan, Scottsdale, AZ. (26).

In Vitro Mutagenesis Procedure—*The Lys1892 in the second ATP binding cassette of ABC1 was mutated to methionine by polymerase chain reaction amplification of the wild-type cDNA with a mutated 5′ primer spanning nucleotides 5854–5874 and a vector-specific 3′ primer down-stream from the multiple cloning sites linker of pSP64TN poly(A). The 5′ sense primer whose sequence is GTTTACGAGACTGGAATGTCGCA includes the AAG (lysine) to ATG (methionine) single mutation (in bold) and the wild-type HpaI restriction site (underlined). The final con-

Oocytes*

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struct, K1892M, was obtained after insertion into pABC1TN of the amplification product digested by HpaI-ApaI and verified by sequencing.

In Vitro Transcription—10 µg of linearized DNA template was transcribed in vitro by SP6 RNA polymerase following standard protocols (27) for 1 h at 37 °C. After removal of DNA template by treatment with DNase I, the capped RNA was precipitated and resuspended in diethyl pyrocarbonate-water. The final concentration was adjusted to 200 ng/µl for ABC1 and K1892M cRNA and to 100 ng/µl for CFTR cRNA after analysis on formaldehyde-agarose gel.

Oocyte Expression Studies—Stage V Xenopus laevis oocytes were defolliculated by collagenase and microinjected with 50 nl of water or aqueous solutions of cRNA encoding ABC1, K1892M, or CFTR. Oocytes were incubated in modified Barth’s saline medium (88 mM NaCl, 1 mM KCl, 0.41 mM CaCl2, 0.33 mM Ca(NO3)2, 0.82 mM MgSO4, 2.4 mM NaHCO3, and 10 mM HEPES, pH 7.4) containing 5 µM penicillin/streptomycin (Life Technologies, Inc.). The protein expression was monitored systematically for every batch of injected oocytes. After overnight metabolic labeling the oocytes were lysed in 0.1M NaCl, 0.1M Tris, pH 8.0, 10 mM EDTA, 1% Triton X-100, and 1 mM phenylmethylsulfonyl fluoride. The proteins of interest were immunoprecipitated according to the standard protocols (28) and eluted from protein A- or protein G-Sepharose in 20 µl of sample buffer (supplemented with 8 µl urea) for 2 min at 50 °C. ABC1 and K1892M were immunoprecipitated using Ab16, a rabbit polyclonal antiserum recognizing the first NBF domain of ABC1 (dilution 1:250) (23), and CFTR with 1 µg of monoclonal mouse anti-human regulatory domain-specific antibody (Genzyme, Cambridge, MA) (28).

Transport Assays—Groups of five oocytes were incubated for 30 min in a 6.5-mm diameter porous bottom dish (Transwell®, Costar) in 1 ml of ND96 buffer (96 mM NaCl, 2 mM KCl, 1.8 mM CaCl2, 1 mM MgCl2, and 5 mM HEPES titrated with NaOH to pH 7.4) containing 1 µCi KI (1 µCi of trace [3H]-labeled KI, DuPont) at room temperature (adapted from Ref. 30). After the loading period, the oocytes were sequentially moved at 1-min intervals through a series of wells containing 1 ml of buffer. 3H Iodide released from the oocytes in each well was measured by radioactivity counting (Compu Gamma, LKB). The first two wells were used to establish a stable baseline in ND96 buffer (time 0). After eight 1-min washes, the oocytes were solubilized in 1 M NaOH and the residual iodide content was measured. The total amount of 3H Iodide (900–1200 cpm/oocyte) at time 0 was calculated as the sum of radioactivity recovered in each 1-min sample plus the residual iodide content. Efflux curves were constructed by plotting the percentage of total radioactivity released in the medium versus time. The percentage of total at 8 min (T8) was used for further analysis. The appropriate drug or modified ND96 buffers (Cl−-free ND96 medium, all external Cl− was replaced by gluconate; Na+−free ND96 medium, 96 mM NaCl was replaced by 96 mM LiCl) were applied after the stable baseline in ND96 buffer was obtained. Oocyte ion conductance was measured by conventional two-electrode voltage clamp (26) and bathed in ND96 medium. Data are presented as the mean ± S.D. of n observations. Statistical significance was assessed at the 95% confidence level with Student’s t test.

In Vitro Phosphorylation—Ab16 immunoprecipitates from samples of 10 unlabeled oocytes were equilibrated in 50 mM Tris, pH 7.5, 10 mM MgCl2, and 100 mg/ml bovine serum albumin and then allowed to react in the presence of 50 ng of catalytic subunit of protein kinase A (Sigma) and 10 µCi of [γ-32P]ATP for 1 h at 30 °C. The reaction was stopped by the addition of sample buffer. The patterns of phosphorylation in oocytes injected either with ABC1 or water were analyzed by SDS-polyacrylamide gel electrophoresis on 7.5% polyacrylamide gels and autoradiography.

Pharmacological Agents—N-(2-(b-Bromocinnamylamino)ethyl)-5-isoquinolinesulfonamide (H89) and okadaic acid were from RBI (Natick, MA), and A27187, forskolin, and cAMP were from Boehringer Mannheim. The drugs were dissolved in dimethyl sulfoxide stock solutions and used at a final dimethyl sulfoxide concentration of 0.1%. All other reagents were from Sigma.

RESULTS

Expression of ABC1 in Xenopus Oocytes—After injection of cRNA, the synthesis of ABC1 at the predicted size (220 kDa) was monitored after immunoprecipitation by Ab16 (Fig. 1B, lane 2). The expression of K1892M, an ABC1 mutant in which the LyS1892 of the Walker A motif in the second nucleotide binding fold (NBF2) has been replaced by a methionine, was monitored in the same way (Fig. 1B, lane 3). No variations with respect to the wild-type protein were detected either in the temporal onset of protein synthesis or in the ratio of produced protein to injected cRNA. The expression of CFTR in oocytes was monitored by immunopurification with a monoclonal mouse anti-human regulatory domain-specific antibody (Genzyme, Cambridge, MA) (28).

ABC1 Generates an Anion Efflux in Xenopus Oocytes—Since the activity of CFTR and P-glycoprotein is associated with chloride transport (6, 18, 19, 26, 31), we wished to determine the properties of ABC1 expressed in Xenopus oocytes using the radiotracer iodide efflux technique. First, we analyzed the activity of two well characterized Cl− channels in Xenopus oocytes. The endogenous Ca2+- dependent Cl− channel can be activated by promoting Ca2+- influx using A23187 (29, 32) as shown in Fig. 1C. Indeed, the presence of 10 µM A23187 in the buffer increased (∼3-fold) the iodide efflux in water-injected oocytes after 8 min, T8 = 68 ± 9%, n = 4) compared with basal oocytes (T8 = 21 ± 5%, p < 0.01, n = 4). After expression in Xenopus oocytes, CFTR generates a cAMP-dependent chloride current (26, 29). In our experiments the iodide efflux in CFTR-expressing oocytes was increased (p < 0.01) upon addition of cAMP agonists (Fig. 1D, CFTR basal T8 = 31 ± 5%, n =
was also sensitive to bumetanide ($T_b = 42 \pm 6\%$, 200 mM, $n = 3$, $p < 0.05$) and furosemide ($T_b = 33 \pm 7\%$, 200 mM, $n = 3$, $p < 0.01$) (Fig. 2B)). The prostaglandin transporter (33) inhibitor sulfobromophthalain (BSP) (500 mM, $n = 8$) inhibited ~80% of the ABC1 activity ($T_b = 24 \pm 6\%$, $p < 0.01$ (Fig. 2, A and B)). The sulfonamide compound glibenclamide (100 mM), which is an inhibitor of the activity of KATP (34) and CFTR (35) channels, almost completely blocked the activity of ABC1 ($T_b = 21 \pm 7\%$, $n = 6$, $p < 0.01$ (Fig. 2, A and B)) and CFTR (CAMP +

Functional Expression of ABC1 in X. laevis oocytes. A, activation of ABC1 by cAMP (500 mM) + IBMX (1 mM) (ABC1 + cAMP) in oocytes bathed in ND96 medium. B, effects of forskolin (10 mM), cAMP (500 mM) alone or associated with IBMX (1 mM), H89 (20 mM), okadaic acid (10 mM), and orthovanadate (1 mM) alone or with glibenclamide (100 mM). Bars show means and S.D. of iodide efflux after 8 min for 3–6 separate experiments. C, incorporation of $[^{35}P]$phosphate into ABC1 after immunoprecipitation with Ab16 in the absence (lane 1) or presence (lane 2) of protein kinase A.

FIG. 3. Regulation of ABC1 activity by orthovanadate and cAMP in X. laevis oocytes. A, activation of ABC1 by cAMPCAMP was then investigated whether an iodide efflux from water-injected oocytes (glucagon, 100 mM) determined that our technique offers a convenient way to evaluate the activity of both endogenous and expressed chloride channels in oocytes, we then investigated whether an iodide efflux is detectable in ABC1-injected oocytes. Fig. 1, E and F, shows that the expression of ABC1 in oocytes is associated with an increase ($p < 0.01$) of the iodide efflux amplitude ($T_b = 49 \pm 7\%$, $n = 12$) greater than that seen in water-injected oocytes ($T_b = 19 \pm 7.5\%$, $n = 14$) in Ca$^{2+}$-free ND96 medium. Similar results were obtained in ND96 medium containing 2 mM Ca$^{2+}$ (ABC1, $T_b = 50 \pm 3\%$, $n = 7$; water, $T_b = 20 \pm 6\%$, $n = 9$, $p < 0.01$). The amplitude of the iodide efflux appeared to be directly dependent on the amount of injected ABC1 mRNA because 10-fold less mRNA led to a reduced efflux ($T_b = 36 \pm 7\%$, $n = 6$).

The equimolar substitution of extracellular chloride with the nonpermeant anion gluconate did not affect the amplitude of the A23187-activated efflux from water-injected oocytes (glucagon + A23187, $T_b = 67.4 \pm 7\%$, $n = 2$; ND96 + A23187, $T_b = 68 \pm 9\%$, $n = 4$ (not shown)). In contrast, a similar procedure decreased the magnitude of the efflux from ABC1-injected oocytes (glucagon, $T_b = 34.2 \pm 6\%$, $n = 8$ (Fig. 1F)), whereas substitution of extracellular sodium with lithium had no effect ($T_b = 49.8 \pm 5\%$, $n = 5$ (Fig. 1F)).

Pharmacological Specificity—Compared with the basal activity of ABC1 (Fig. 2B), noted as DMSO, $T_b = 51 \pm 4\%$, $n = 5$), the Cl$^-$ channel blockers DIDS (500 mM, $n = 6$ (Fig. 2A)), diphenylamine-2-carboxylic acid (500 mM, $n = 3$), and flufenamidic acid (500 mM, $n = 4$) blocked 50–80% ($p < 0.01$) of the iodide efflux generated by the expression of ABC1 (Fig. 2B) ($T_b = 22.4 \pm 4\%$, 29 \pm 1\% and 33 \pm 8\% respectively). ABC1 activity
ABC1 Is Not a Chloride Channel—We then examined the electrophysiological properties of oocytes expressing ABC1 compared with water- and CFTR-injected oocytes (Fig. 5). The membrane potential ($E_m$) was not affected by the expression of the different transporters (ABC1, $E_m = -42 \pm 5$ mV, $n = 13$; CFTR, $E_m = -45 \pm 5$ mV, $n = 5$; water, $E_m = -43 \pm 6$ mV, $n = 10$). The holding current was similar in water (−13 ± 3.4 nA, $n = 7$) and ABC1 (−14 ± 4 nA, $n = 12$) oocytes voltage clamped at −60 mV and bathed in ND96 medium. The Ca2+–activated Cl− channel activity induced by A23187 (10 μM, −126 ± 48 nA at −60 mV, $p < 0.01$, $n = 5$) and inhibited by DIDS (500 μM, −30 ± 8 nA at −60 mV, $n = 3$) was not affected by the presence of ABC1 (A23187, −114 ± 50 nA at −60 mV, $n = 6$; A23187 + DIDS, −18 ± 4 nA at −60 mV, $n = 3$ (Fig. 5A)). When cAMP (500 μM), IBMX (1 mM), or forskolin (10 μM) was added to the perfusion medium, no modification of the holding current was observed in the presence of ABC1 (−15 ± 4 nA, $n = 14$) or in its absence (−14.5 ± 6 nA, $n = 20$). In contrast, and as already reported (26, 29), CFTR generated a cAMP-dependent current ($n = 10$ (Fig. 5B)) inhibited by glibenclamide (100 μM, $n = 4$ (not shown)). Like wild-type ABC1, K1892M also failed to generate a current either under resting conditions or in the presence of cAMP agonists (data not shown).

**DISCUSSION**

We report here on the characterization, pharmacological profile, and regulation of ABC1 as a cAMP-dependent and glibenclamide-sensitive anion transporter. Among the ABC proteins involved in anion transport, CFTR is a chloride channel (5, 6, 26, 36) and the P-glycoprotein regulates an endogenous chloride channel (2, 18, 19, 31). In this paper, we provide evidence that ABC1 shares with CFTR (5, 6, 26, 36) the ability to transport anions and to be up-regulated by cAMP, IBMX, okadaic acid, and vanadate. However, as opposed to CFTR, ABC1 behaves as an electroneutral anion exchanger since no significant chloride current was detected from oocytes expressing ABC1 and the ABC1-generated anion efflux is dependent on extracellular chloride concentration. In fact, the substitution of extracellular chloride by gluconate reduces the efflux from ABC1-injected oocytes as would be expected from a parallel chloride current. Therefore, ABC1 appears to be up-regulated by cAMP-dependent and glibenclamide-sensitive anion transporter function, and regulation of ABC1 as a cAMP-dependent and glibenclamide-sensitive anion transporter.
The corresponding mutation in ABC1 (i.e. K1892M) produces a transporter with an increased basal ionic flux and a conserved pharmacological inhibitory profile (i.e. inhibition by glibenclamide, DIDS, and BSP). In contrast, the K1892M mutant is unaffected by cAMP, and a reduced efflux is observed after vanadate treatment. A reasonable interpretation of these results is that vanadate in the wild-type ABC1 primarily impairs the function of NBF2, as is the case in the K1892M mutant. The reversed effect of vanadate treatment on the mutant might result from other actions of this compound either at NBF1 and/or as a phosphatase inhibitor. Similar effects of vanadate on CFTR and its mutants have been reported and led to a model of the transport cycle in which hydrolysis at NBF2 plays an inhibitory role (14–16, 36).

The identification of ABC1 as a cAMP-dependent anionic transporter does not provide any information on the nature of the transported substrate, nor does it shed light on the role played by ABC1 during engulfment of apoptotic corpses. Nonetheless, the finding of an ABC1-dependent anionic flux with a clear-cut pharmacological profile provides an extremely useful experimental tool. We can in fact readily investigate how modulations of ABC1 transporter activity in phagocytic cells, like mouse peritoneal macrophages, affect their responses to specific physiopathological challenges.

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