The testis anion transporter TAT1 (SLC26A8) physically and functionally interacts with the cystic fibrosis transmembrane conductance regulator channel: a potential role during sperm capacitation

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The Slc26 gene family encodes several conserved anion transporters implicated in human genetic disorders, including Pendred syndrome, diastrophic dysplasia and congenital chloride diarrhea. We previously characterized the TAT1 (testis anion transporter 1; SLC26A8) protein specifically expressed in male germ cells and mature sperm and showed that in the mouse, deletion of Tat1 caused male sterility due to a lack of sperm motility, impaired sperm capacitation and structural defects of the flagella. Ca2+1, Cl2 and HCO32 influxes trigger sperm capacitation events required for oocyte fertilization; these events include the intracellular rise of cyclic adenosine monophosphate (cAMP) and protein kinase A (PKA)-dependent protein phosphorylation. The cystic fibrosis transmembrane conductance regulator (CFTR) is expressed in mature sperm and has been shown to contribute to Cl− and HCO3− movements during capacitation. Furthermore, several members of the SLC26 family have been described to form complexes with CFTR, resulting in the reciprocal regulation of their activities. We show here that TAT1 and CFTR physically interact and that in Xenopus laevis oocytes and in CHO-K1 cells, TAT1 expression strongly stimulates CFTR activity. Consistent with this, we show that Tat1 inactivation in mouse sperm results in deregulation of the intracellular cAMP content, preventing the activation of PKA-dependent downstream phosphorylation cascades essential for sperm activation. These various results suggest that TAT1 and CFTR may form a molecular complex involved in the regulation of Cl− and HCO3− fluxes during sperm capacitation. In humans, mutations in CFTR and/or TAT1 may therefore be causes of asthenozoospermia and low fertilizing capacity of sperm.

INTRODUCTION

Sperm produced in the testis are non-motile and cannot fertilize the oocyte until they have undergone further maturation in the epididymis and in the female genital tract after ejaculation. The process of maturation in the epididymis renders the sperm motile, whereas that occurring during their transit in the female genital tract, known as capacitation, leads to the...
SLC26A8/TAT1 (testis anion transporter 1) was initially cloned in our laboratory (20) as a member of the SLC26 family expressed exclusively in adult human testis. TAT1 expression in the testis was shown to be restricted to male germ cells at the spermatoocyte and spermatid stages (20,21). The expression of TAT1 in COS-7 cells or *Xenopus laevis* oocytes facilitated the transport of SO\(_4^{2-}\), Cl\(^{-}\) and Ox\(_{2}^{2-}\) (20,21). However, the physiological substrates and functions of TAT1 in the germline remain to be demonstrated.

We previously investigated TAT1 function by generating mice with a targeted disruption of the gene (22). Tat1-null males were unable to sire offspring, although their mating behavior and reproductive organs were normal. Tat1-null sperm were entirely non-motile and displayed capacitation defects (lack of protein hyperphosphorylation and low frequency of acrosomal reaction). Tat1-null sperm also displayed severe structural defects, including hairpin-like bending of the flagella and structural defects of the annulus, a ring-shaped structure located at the junction of the midpiece and the principal piece of the flagellum. Consistent with this phenotype, Tat1 protein was found at the annulus of mature sperm, in both humans and mice (22,23).

The recent observation of CFTR expression in sperm and its involvement in sperm capacitation prompted us to investigate the possible physical and functional interaction between CFTR and the sperm-specific member of the SLC26 family, TAT1. We also analyzed in more detail the sAC/PKA signaling pathway in sperm of Tat1-null mice.

We report that TAT1 physically interacts with the CFTR channel and that TAT1 expression strongly stimulates the activity of the CFTR channel. Consistent with these findings, we show that Tat1 inactivation in mouse sperm results in the deregulation of the intracellular cAMP content and a failure to trigger the sAC/PKA pathway. Thus, it is likely that, in sperm, TAT1 and CFTR cooperate in the regulation of the Cl\(^{-}/HCO_3^2-\) fluxes required for motility and capacitation.

**RESULTS**

TAT1 and CFTR interact physically and functionally

We first investigated the physical interaction between TAT1 and the CFTR channel by co-immunoprecipitation experiments in COS-7 cells and CHO-K1 cells transiently transfected with plasmids encoding the TAT1 and CFTR full-length proteins. Unlike the control antibody directed against the transferrin receptor (TFR), the monoclonal M3A7 antibody directed against the CFTR channel specifically precipitated the TAT1 protein, indicating the existence of physical interactions between the two proteins (Fig. 1A). To test whether, as previously described for other SLC26 members, the sulfate transporter and anti-sigma antagonist (STAS) domain involved in the interaction with CFTR, we performed co-immunoprecipitation using a plasmid construct of the STAS domain (Myc-STAS construct). We observed that the M3A7 antibody specifically co-precipitates CFTR and the Myc-STAS fusion protein (Fig. 1B).

To confirm the above results, we performed co-immunoprecipitation experiments in testis protein extracts from the *Flag-Tat1*/++ transgenic mice and from the Tat1-null...
TAT1 and CFTR are likely to interact in vivo when compared with that of significant co-precipitation of the CFTR protein was obtained with whole-testis lysates from TFR null mice, indicating that it was related to CFTR activation (Fig. 2B). In control condition, oocytes expressing TAT1 alone were not affected by PKA stimulation or CFTRinh-172 treatment (Fig. 2A).

In control condition, oocytes expressing CFTR alone or co-expressing both TAT1 and CFTR exhibited $I-V$ curves that were indistinguishable (Fig. 2B). In contrast, in these oocytes, PKA stimulation resulted in a large increase in whole-cell conductance (Fig. 2B). This increase was sensitive to CFTRinh-172, indicating that it was related to CFTR activation (Fig. 2B).

Interestingly, in oocytes co-expressing TAT1 and CFTR, PKA-stimulated currents were significantly larger than those in oocytes expressing CFTR alone (Fig. 2B). Current amplitudes from these experiments, measured at $-100/+40$ mV, are shown in Figure 2C. Taken together, these findings strongly suggest that TAT1 expression had a stimulatory effect on CFTR-mediated currents in X. laevis oocytes.

We confirmed the stimulatory effect of TAT1 on the cAMP-dependent CFTR channel by performing iodide efflux experiments in CHO-K1 cells stably expressing CFTR and transiently transfected with TAT1 constructs. Iodide efflux was recorded for 10 min and CFTR was stimulated by addition of forskolin (Fsk) after 3 min. Curves were constructed by plotting the rate of $^{125}$I efflux against time (Fig. 3A and C); histograms, which are used for comparisons, indicate the maximal value to the time-dependent rates (Fig. 3B and D).

Using cells not transfected and cells transfected with the empty plasmid as controls, we found that TAT1 expression enhanced the iodide efflux mediated by the CFTR channel (Fig. 3A and B). We also tested SLC26A3 (DRA) construct and observed, as previously described (12), a stimulatory effect on CFTR activity (Fig. 3A and B). Interestingly, a truncated construct of TAT1 lacking the STAS domain (TAT1 STAS), although expressed at a similar level as wild-type TAT1 (Supplementary Material, Fig. S1A), did not stimulate CFTR activity in this way (Fig. 3B).

A set of control experiments was performed in CHO-K1 cells, which do not overexpress the human CFTR channel; as reported in previous studies on TAT1, TAT1 expression alone was unable to mediate any iodide efflux (20) and Fsk addition had no effect (Fig. 3C and D).

The total amount of CFTR and the amount presumably addressed to the plasma membrane (glycosylated and mature form), as assessed by western blotting, were not increased by the presence of TAT1 (Supplementary Material, Fig. S1). The total amount of CFTR was not either diminished by the expression of the STAS version of TAT1 (Supplementary Material, Fig. S1). Therefore, we conclude that TAT1 directly stimulates CFTR conductance and requires the STAS domain to do so.

**TAT1 and CFTR are co-localized in sperm**

Using an antibody directed against the carboxy-terminal region (amino acids 664–970) of the human TAT1 protein, X. laevis oocytes producing CFTR and TAT1 alone or in combination. Current–voltage relationships ($I-V$ curves) were established in control condition (superfusion of the oocytes with ND96) or during PKA stimulation [superfusion of the oocytes with the stimulating mixture Fsk/3-isobutyl-1-methyl-xanthine (IBMX)] in the presence or absence of the CFTR inhibitor (CFTRinh-172). We observed that $I-V$ curves from oocytes expressing TAT1 alone were not affected by PKA stimulation or CFTRinh-172 treatment (Fig. 2A).

We next tested for functional effects of TAT1 expression on CFTR activity by performing voltage-clamp experiments in CHO-K1 cells stably expressing CFTR and transiently transfected with TAT1 constructs. Iodide efflux was recorded for 10 min and CFTR was stimulated by addition of forskolin (Fsk) after 3 min. Curves were constructed by plotting the rate of $^{125}$I efflux against time (Fig. 3A and C); histograms, which are used for comparisons, indicate the maximal value to the time-dependent rates (Fig. 3B and D).

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**TAT1 and CFTR are co-localized in sperm**

Using an antibody directed against the carboxy-terminal region (amino acids 664–970) of the human TAT1 protein,
we previously showed that TAT1 protein co-localizes with members of the Septin family (Septin 1, 4, 6, 7 and 12) at the annulus of human and mouse sperm (22,24,25). We recently generated a new antibody directed against both amino- and carboxy-terminal peptides of the human TAT1 protein (amino acids 1–15 and 955–970), the specificity of which was confirmed based on (i) the correct immunodetection of the TAT1 protein on COS cells transiently transfected with a TAT1 expression plasmid and (ii) the similarity of the profiles obtained with both antibodies (data not shown). Using this antibody for immunofluorescence-based localization, we found that the TAT1 protein was present at both the annulus and the equatorial segment of the human sperm head (Fig. 4A). As expected from the divergence between mouse and human TAT1 protein sequences, this antibody did not react with the mouse Tat1 protein, precluding its use for the study of Tat1 in the mouse.

The experimental conditions for TAT1 and CFTR detection were different, so double labeling experiments were not feasible. However, in our investigations of the potential co-localization of TAT1 and CFTR in the flagella, we used the CF3 mouse monoclonal antibody against CFTR to determine the precise distribution of this protein in sperm preparations. We detected CFTR in both the equatorial segment of the head and the midpiece of mouse sperm. This finding reconciles the apparently conflicting reports of Xu et al. and Hernandez-Gonzalez et al., who reported CFTR expression in the head and midpiece, respectively (Fig. 4B). Interestingly, in about half of the sperm, we also observed a mass of CFTR protein close to the end of the midpiece (Fig. 4A). Double staining for Septin 4, a structural component of the annulus, revealed that CFTR did not precisely co-localize with the annulus but was rather located at the proximal border of the annulus (Fig. 4B).

**Tat1-null sperm display abnormal cAMP content**

Ca$^{2+}$, Cl$^-$ and HCO$_3^-$ are known to induce cAMP production, a key event in the initiation of capacitation, leading to downstream PKA-dependent protein phosphorylation cascades. Tat1-null mouse sperm displays a lack of motility (asthenozoospermia) and capacitation defects, as shown by the absence of protein hyperphosphorylation and a low acrosomal reaction capacity (22). We analyzed the cAMP content of wild-type and Tat1-null sperm in basal conditions (NO medium) and in capacitating conditions (CAP). The basal cAMP content was higher in Tat1-null sperm than in wild-type sperm and the increase in the cAMP concentration induced during sperm capacitation was smaller in Tat1$^{+/-}$ than in wild-type sperm (Fig. 5A). Altogether, the cAMP

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**Figure 2.** Current–voltage ($I$–$V$) relationships in *X. laevis* oocytes expressing human TAT1 and CFTR alone or both. Results from a representative two-electrode voltage-clamp experiment performed with *X. laevis* oocytes expressing TAT1 (n = 7) or CFTR alone (n = 7), or co-expressing TAT1 and CFTR (n = 11). Similar results were obtained in three independent experiments. (A) $I$–$V$ curves from oocytes expressing TAT1 alone in control conditions (superfused with ND96, black line) or in the presence of a PKA-stimulating mixture (1 μM Fsk and 100 μM isobutylmethylxanthine, Fsk + IBMX) supplemented (red line) or not (green line) with 10 μM of the CFTR inhibitor, CFTRinh-172. These treatments had no effect on whole-cell currents in TAT1-expressing oocytes, such that the three $I$–$V$ curves overlap. (B) $I$–$V$ curves from oocytes expressing CFTR alone (open circles) or co-expressing CFTR and TAT1 (filled circles), in control conditions (superfused with ND96, black line) or in the presence of the PKA-stimulating mixture supplemented (red line) or not (green line) with 10 μM of the CFTR inhibitor, CFTRinh-172. $I$–$V$ curves obtained from oocytes expressing CFTR alone or co-expressing CFTR and TAT1 are indistinguishable before PKA stimulation. Of note, PKA stimulation induced a larger increase in cell conductance in oocytes co-expressing CFTR and TAT1 than in oocytes expressing CFTR alone; this increase was sensitive to CFTRinh-172, indicating that it was related to CFTR activation. (C) Results from the same experiment presented as histograms of whole-cell currents measured at −100 and +40 mV values (Student’s $t$-test, *p < 0.05).
concentration upon capacitation was clearly lower in Tat1-null than in control sperm (Fig. 5B).

We next analyzed the expression and distribution of the soluble adenylate cyclase (sAC) in wild-type and Tat1-null sperm retrieved from the caudal region of the epididymis and capacitated in vitro. As previously described, sAC was found at the annulus in capacitated sperm (Fig. 6A). Furthermore, despite the bending of the flagella, sAC was correctly located at the annulus of Tat1-null sperm upon capacitation (Fig. 6B). This indicates that the motility and capacitation defects in Tat1-null sperm do not result from misproduction or mislocalization of the sAC but rather from its misactivation.

cAMP analogs restore hyperphosphorylation and flagellar beating in a PKA-dependent manner in Tat1-null sperm

We incubated wild-type and Tat1-null sperm in a medium not permissive to capacitation but supplemented with dibutyryl-cAMP and analyzed the tyrosine phosphorylation pattern associated with sperm capacitation, by western blot analysis. We observed partial restoration of the tyrosine phosphorylation pattern associated with sperm capacitation in Tat1-null sperm (Fig. 7A). Incubation with Sp-cAMP, another active permeant analog of cAMP, gave similar results; furthermore, control experiments involving co-incubation of Tat1-null sperm with both Sp-cAMP and Rp-cAMP, a specific competitive inhibitor of cAMP, prevented hyperphosphorylation rescue (data not shown). The re-establishment of hyperphosphorylation in Tat1-null sperm was also prevented by incubation with H89, a specific inhibitor of PKA activity, before the addition of dibutyryl-cAMP to the medium (Fig. 7B).

Finally, we used videomicroscopy to analyze the behavior of Tat1-null sperm in the presence of dibutyryl-cAMP. Unlike untreated sperm and sperm treated with IBMX alone, sperm treated with dibutyryl-cAMP and IBMX displayed flagellar beating, although they did not fully recover their swimming capacity (Supplementary Material, Movies).

DISCUSSION

Sperm must undergo an activation process, called capacitation, during their transit in the female genital tract if they are to acquire their fertilization potential. This activation process involves multiple modifications to the sperm, including
plasma membrane phospholipid reorganization, an increase in intracellular cAMP concentration and protein phosphorylation, intracellular alkalinization and plasma membrane potential hyperpolarization.

Sperm capacitation is partly triggered by complex anion movements including Cl\(^-\) and HCO\(_3\)\(^-\) influxes. CFTR has recently been implicated in this process, so we investigated the role of TAT1, which, as a member of the SLC26 anion transporter family specifically expressed in the male germ line, appears as a candidate regulator of CFTR function in sperm. We found that (1) TAT1 and CFTR form a molecular complex and that upon PKA stimulation, TAT1 expression enhances CFTR-mediated currents in X. laevis oocytes and iodide transport in CHO-K1 cells; (2) CFTR was present in both the equatorial segment and the midpiece of the flagellum, where it was found close to the annulus; (3) TAT1 was also present in the equatorial segment of the sperm in addition to the annulus; (4) despite correct production and localization of sAC to the annulus, Tat1\(^{-/-}\) sperm displayed only a minor increase in cAMP concentration upon capacitation and were unable to trigger the sAC/PKA signaling pathway leading to protein phosphorylation. Consistent with these findings, we finally showed that membrane-permeant analogs of cAMP restored, in a PKA-dependent manner, protein hyperphosphorylation and flagellar beating in Tat1\(^{-/-}\) sperm.

Our results are consistent with the notion that like other SLC26 anion exchangers, TAT1 interacts with and stimulates the activity of CFTR. It is therefore possible that TAT1 and CFTR form a complex in vivo involved in the regulation of the Cl\(^-\) and HCO\(_3\)\(^-\) fluxes required for activation of the sAC/PKA pathway during sperm capacitation.
The mechanisms underlying the influx of Cl\(^-\) and HCO\(_3^-\) during sperm capacitation appear to be complex, and the molecular entities associated with these fluxes and their regulation remain unclear. The most likely physiological model involves a functional network of transporters consisting mostly of Cl\(^-\) transporters, Na\(^+\)/HCO\(_3^-\) cotransporters, Cl\(^-\)/HCO\(_3^-\) antiporters and K\(^+\) and Na\(^+\) channels (5,7). In this model, Cl\(^-\) influx mediated by CFTR leads to the inhibition of ENaC, which in turn leads to membrane hyperpolarization. Concomitantly, the inward influx of Cl\(^-\) mediated by Cl\(^-\) transporters is coupled to the action of Cl\(^-\)/HCO\(_3^-\) antiporters, generating the HCO\(_3^-\) influx required for activation of the sAC/PKA pathway.

Pioneering work by Muallem and coworkers demonstrated physical and functional interaction between CFTR and the anion exchanger SLC26A3 (12,19). Chen et al. (8) recently described the expression of SLC26A3 in the guinea pig sperm head and suggested a functional interaction, although they did not find precise co-localization of CFTR and SLC26A3 proteins in the sperm head (CFTR being found at the equatorial segment and SLC26A3 in the acrosome).

We show here that in addition to its localization at the annulus initially detected with the L2Cl4 antibody, TAT1 (SLC26A8) is also detected at the equatorial segment when using a novel antibody directed against both amino- and carboxy-peptides of the TAT1 protein. As both antibodies have been shown to be specific for TAT1, the accessibility of the epitopes could explain this difference. Taken together, these results indicate that TAT1 co-localizes with CFTR in sperm head and locates in close proximity to CFTR in the flagella. TAT1 has been reported to carry sulfate, chloride and oxalate anions (20,21) but no HCO\(_3^-\) transport activity has been detected (our unpublished data); the mechanism of TAT1-mediated ionic transport remains to be determined. We found that functional expression of TAT1 in X. laevis oocytes did not significantly affect whole-cell conductance. Therefore, as reported for other SLC26 members, TAT1 may act as an electroneutral anion exchanger. Importantly, we demonstrate in this study that like several other SLC26 members, TAT1 interacts physically with CFTR and stimulates its activity via the STAS domain. In addition to the primary role of the STAS domain for the interaction of SLC26 transporters with the R domain of CFTR, the contribution of a carboxy-terminal PDZ binding motif has been documented, in particular, for SLC26A3/CFTR interaction.
case of TAT1/SLC26A8, a class III PDZ motif (26) is also present at the carboxy terminus of human TAT1 protein and could be implicated. Altogether, the available data are consistent with SLC26A3 and TAT1 (SLC26A8), both contributing to anion flux regulation in sperm; this may involve their intrinsic anion transport activity and/or their capacity to stimulate the anion transport activity of CFTR.

Consistent with a potentially important role for TAT1 in sperm capacitation and as reported for Cftr/+− sperm by Xu et al. (6), we show that the increase in the intracellular cAMP concentration in Tat1-null sperm is significantly impaired. However, sAC is correctly localized at the annulus of Tat1-null sperm, suggesting that the lack of motility and the capacitation defects result from their inability to trigger the PKA signaling pathway.

Surprisingly, in basal conditions, Tat1-null sperm had higher cAMP contents than wild-type sperm. We have no information about the basal levels of cAMP in Cftr/+− sperm, but Nolan et al. (27) reported that basal levels of cAMP were high in Ca2+-PKA-null sperm. Furthermore, Hess et al. (28) observed that sAC-null sperm had abnormal hyperphosphorylation patterns in conditions that were not permissive for capacitation. These various observations strongly suggest the existence of negative feedback mechanisms regulating cAMP production, involving either stimulation of phosphodiesterase activity or inhibition of sAC activity.

The location of sAC and TAT1 at the annulus, in close proximity to CFTR, suggests the existence of a spatially confined Cl−/HCO3−-dependent pathway resulting in local cAMP production close to the annulus. The reason for such low local cAMP production is unclear and, indeed, direct (PKA) and indirect (sAC) are found along the length of the flagella. Nevertheless, these findings are consistent with the hypothesis of a functional interaction between TAT1 and CFTR in vivo, with both proteins being required for sAC/PKA activation.

We also show that TAT1 and CFTR strictly co-localize at the equatorial segment of the sperm head, suggesting that interaction between both proteins could be involved in capacitation events required for the acrosomal reaction. Consistent with this, we have previously described a significant reduction of the acrosomal reaction in Tat1-null sperm (22); furthermore, inhibition of CFTR in human and mouse sperm was shown to impair the acrosomal reaction (6,7).

These results are collectively potentially valuable for improving our understanding of the genetic etiological factors responsible for human infertility. CFTR mutations cause CF, a genetic disease affecting the homeostasis of Cl− and HCO3− in epithelial cells in the airways, intestine and genital tract (29). In addition to displaying respiratory and digestive symptoms, both men and women with CF display impaired fertility (30); >95% of male CF patients are infertile due to a congenital bilateral absence of vas deferens (CBVAD) (31) and many women with CF are subfertile due to a viscous mucus in the cervix that may create a barrier to sperm passage, and/or to defective bicarbonate secretion in the genital tract, preventing successful sperm capacitation (32,33).

Several studies have reported a significantly higher frequency of heterozygous CFTR mutations in infertile male populations without CBVAD and with infertility of unknown origin (34,35). This observation raises the possibility of CFTR being involved in other (non-CBVAD) sperm disorders. By regulating the ion exchanges required for sperm capacitation, CFTR and TAT1 are clear candidate genes for infertility, and mutations in these genes may be involved in human cases of asthenozoospermia and/or an inability of sperm to fertilize oocytes.

In conclusion, we describe here a new model of the physical and functional interactions between CFTR and members of the SLC26 family. This is of particular interest to sperm physiology and pathophysiology and may also be useful to improve our understanding of the molecular basis of CFTR function and dysfunction.

MATERIALS AND METHODS

All experiments with human and animal samples were conducted in accordance with the ethical guidelines and were approved by the Ethics Evaluation Committee (CQI) of the Institut National de la Santé et de la Recherche Médicale, INSERM (Authorization No. 01-013).

Co-immunoprecipitation and immunoblotting

COS-7 cells cultured in 10 cm plates were transiently transfected, according to the standard FuGENE (Roche) protocol, with 5 µg of the following plasmids in a 1:1 ratio: pRK5-TAT1 and green fluorescent protein (GFP)-tagged CFTR or empty vector. CHO-K1 cells cultured in 10 cm plates were transiently transfected, according to the standard JetPEI (Polyplus Transfection) protocol, with 10 µg of the following plasmids in a 1:1 ratio: pRK5-Myc-STAS and GFP-tagged CFTR or empty vector.

After 24 h, cells were washed twice in cold phosphate-buffered saline (PBS) and lysed in 500 µl of lysis buffer (50 mM Tris–HCl, pH 8, 150 mM NaCl, 10 mM MgCl2, 0.5% NP40, 1 × Complete protease inhibitor cocktail (Roche)) for 30 min at 4°C. Cellular residues were eliminated by centrifugation at 13 000 g, 4°C, for 20 min. We removed 50 µl of supernatant, which 30 µg of proteins was used to check transfection efficiency. Then, 2 µg of anti-CFTR antibody (M3A7 mouse monoclonal antibody) or 2 µg of anti-TFR was added to the remaining 450 µl of supernatant and the mixture incubated at 4°C overnight.

We added 40 µl of Protein G agarose beads (previously washed three times in cold 1× PBS) to each sample, which was then incubated for 2 h at 4°C, with shaking. The beads were then washed twice in washing buffer (50 mM Tris–HCl, pH 8, 150 mM NaCl, 10 mM MgCl2, 0.1% NP40, 1 × Complete protease inhibitor cocktail (Roche)), and proteins were eluted in 30 µl of 2× sample buffer. Samples were incubated for 5 min at 95°C to denature proteins and centrifuged for 5 min at 13 000 g and 4°C. The supernatant was subjected to sodium dedecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), and the resulting bands were transferred to nitrocellulose for immunoblotting (mouse monoclonal M3A7 antibody, rabbit polyclonal L2CL4-TAT1 antibody, mouse anti-TFR).
The Flag-Tat1+/+ transgenic mice were obtained by targeted insertion of a cDNA construct comprising of Tat1 mouse partial open reading frame fused to the Flag epitope, into the Tat1 locus. Tat1-null mice were previously described (22). Testis from Flag-Tat1+/+ transgenic mice and Tat1-null mice were dissected and proteins were extracted in lysis buffer: 50 mM Tris–HCl, pH 8, 150 mM NaCl, 10 mM MgCl2, 0.5% NP40, 1× protease inhibitor cocktail (following a ratio of 10 μl of lysis buffer per milligram of testis). Immunoprecipitation was performed with 1 mg of proteins incubated with 8.4 μg of mouse monoclonal M2 antibody (Sigma-Aldrich, Lyon, France). In this case, 50 μl of supernatant was removed, of which 50 μg of proteins was used to check the transfection efficiency.

Heterologous expression of TAT1 and CFTR in X. laevis oocytes

Human cDNA sequences coding for TAT1 and CFTR were inserted into pGH19 (kindly provided by Dr W.F. Boron, Case Western University, Cleveland, OH, USA) and pT7TS plasmids, respectively. The pGH19-TAT1 and pT7TS-CFTR constructs were linearized with appropriate restriction enzymes (NcoI and Smal, respectively; New England BioLabs), and cRNAs were transcribed in vitro using mMESSAGE MACHiNE® (Ambion).

Defolliculation of oocytes (obtained from the partial ovariectomy of female X. laevis) was achieved by gentle shaking (2 h, at room temperature) in calcium-free ND96 (composition of ND96 in mmol/l: NaCl, 96; KCl, 2; MgCl2, 1; CaCl2, 1 buffered to pH 7.5 with 5 HEPES/NaOH) supplemented with 2 U/ml type 1A collagenase (Sigma-Aldrich); stage V–VI oocytes were injected (Inj ect + Matic microinjector, Geneva, Switzerland) with 1 ng of cRNA coding for CFTR and/or 20 ng of cRNA coding for TAT1 dissolved in RNAase-free water (50 nl) or with water alone (control oocytes). The oocytes were incubated at 18°C in ND96 supplemented with penicillin (100 U/ml)/streptomycin (100 μg/ml) for 3–4 days and then used for experiments. Heterologous protein expression was studied by western blot analysis of total and membrane proteins, as described elsewhere (36).

Electrophysiological measurements

Two-electrode voltage clamp experiments were performed with oocytes expressing either TAT1 or CFTR or both. The oocytes were placed in a microchamber and punctured with two low-resistance (0.5–3 MΩ), 3 k KCl-filled microelectrodes. The microelectrodes and a virtual ground amplifier (Axoclamp 2B, Union City, CA, USA); the electrical circuit was closed with two bath electrodes (an agar-3 M KCl bridge electrode and an Ag–AgCl pellet) to minimize serial resistance-induced errors (37). The oocytes were superfused with ND96 or with test solution: a stimulating mixture (1 μM Fsk and 100 μM isobutylmethylxanthine IBMX) was added to ND96 to stimulate PKA; 10 μM of CFTRinh-172 (Calbiochem) was added to ND96 to inhibit CFTR (9).

Current–voltage (I–V) relationships were obtained by applying voltage steps of ±20 mV (range –100 to +50 mV, 5 s duration) from the resting membrane potential value using the Clampex9-generated protocol (Axon Instruments). Results were analyzed using P-Clamp9 software program (Axon Instruments).

Iodide efflux assays

CHO-K1 cells stably expressing human CFTR or CHO-K1 cells were transiently transfected with the following plasmid constructs: pRK5, pRK5-TAT1 wt, pRK5-TAT1 ΔSTAS and pCMV-SLC26A3 (DRA) wt for 48 h. The CFTR Cl– channel activity was assayed by measuring the rate of iodide (125I) efflux from the cells as described previously (38). Time-dependent rates of 125I efflux were calculated from the following: ln(125I1/125I2)/(t1 − t2), where 125I1 is the intracellular 125I at time t, and t1 and t2 are the successive time points. Curves were constructed by plotting rates of 125I against time. All comparisons were based on maximal values of the time-dependent rates (k = peak rates, min⁻¹), excluding the points used to establish the baseline (kpeak−kbasal min⁻¹).

Human sperm smear preparation

Semen samples were obtained from normal fertile volunteers by masturbation, after a period of 2–5 days of abstinence; samples were then incubated at 37°C for 30 min, for liquefaction. Sperm viability, morphology and motility were checked by eosin staining, Giemsa staining and CASA analysis, respectively.

We spread 20 μl of liquefied sperm onto Superfrost Plus slides (Menzel Glasbearbeitungswerk, GmbH & Co. KG, Braunschweig, Germany). The sperm was allowed to dry briefly and fixed by incubation for 10 min in cold methanol/acetone (3:1). Slides were stored at −80°C until immunodetection.

Mouse sperm smear preparation

The caudal regions of the mouse epididymis were dissected in 1 ml of preheated (37°C) NO medium (120 mM NaCl, 2 mM KCl, 1.2 mM MgSO4, 0.36 mM Na2HPO4, 5.56 mM glucose, 1 mM sodium pyruvate, 18.5 mM sucrose, 50 mM HEPES, pH 7.3) and incubated at 37°C for 15 min. Paraformaldehyde was then added to a final concentration of 3.6% and the sperm was fixed by incubation for 30 min at room temperature. We spread 20 μl of fixed sperm onto Superfrost Plus slides (Menzel Glasbearbeitungswerk, GmbH & Co. KG) and allowed it to dry briefly. Slides were stored at −80°C until immunodetection.

Immunodetection in sperm preparations

The fixed slides were treated with 0.2% Triton in PBS for permeabilization and were then blocked by incubation in 1% bovine serum albumin (BSA; for human sperm analysis) or 10% normal goat serum (for mouse sperm analysis) in PBS for 1 h. Slides were then incubated with primary antibodies for 2 h at room temperature (TAT1 and sAC antibodies) or
at 37°C (CFTR and Septin 4 antibodies). They were washed three times in PBS, for 5 min each, and incubated with Alexa Fluor® 568 goat anti-rabbit antibody (Invitrogen, Life Technologies SAS, Villebon sur Yvette, France), Alexa Fluor® 594 goat anti-mouse antibody (Invitrogen) or fluorescein isothiocyanate (FITC) goat anti-rabbit (Dako, Trappes, France) antibody for 1 h at room temperature. Slides were then washed three times in PBS, for 5 min each, and mounted in Vectashield medium (Vector Laboratories, Burlingame, CA, USA) supplemented with 0.5 mg/ml 4’,6-diamidino-2-phenylindole (DAPI).

Slides were analyzed with a Zeiss Axiophot epifluorescence microscope. Digital images were acquired with a cooled charge-coupled device camera (Hamamatsu Co., Japan), under identical instrument settings, with MetaMorph® software (Molecular Devices, Inc., USA). Several Z acquisitions were performed for TAT1 detection at both the annulus and the equatorial segment; Z projections were then performed with Image J 1.44c® software (National Institute of Health, USA).

Measurement of cyclic AMP content
We collected Tat1+/+ and Tat1−/− sperm by dissecting the caudal region of the epididymis in 1 ml of a medium not supporting capacitation (NO medium). After a short incubation at 37°C, the sperm were collected and counted. We incubated 0.5–1.5 × 10⁶ sperm for 10 min in a medium not supporting (NO) or supporting (CAP) capacitation, supplemented with 100 μM IBMX, in a final volume of 400 μl.

After incubation, the sperm were centrifuged at 13 000g for 10 min at room temperature and resuspended at a concentration of 1 × 10⁸ sperm/100 μl in 0.1 M HCl, 1% Triton X-100. The sperm suspension was centrifuged at 13 000g for 10 min at room temperature and 100 μl of the resulting supernatant was assayed for cAMP concentration according to the kit manufacturer’s protocol (Direct Cyclic AMP Enzyme Immunoassay Kit Assay Designs).

Hyperphosphorylation assay
We collected Tat1+/+ and Tat1−/− sperm by dissecting the caudal region of the epididymis in 1 ml of NO medium, followed by a short period of incubation at 37°C. Sperm was counted and diluted to 2 × 10⁶ sperm in 400 μl of the appropriate medium: medium not supporting capacitation (NO), capacitation medium (CAP) or NO medium supplemented with 1 mM db-cAMP and 100 μM IBMX. The sperm were incubated in the media for 90 min at 37°C, under an atmosphere containing 5% CO₂.

For H89 inhibition assays, 2 × 10⁶ spermatozoa were incubated in 200 μl of NO medium. H89 [diluted in dimethyl sulfoxide (DMSO)] was added to a final concentration of 200 μM and sperm were incubated for 10 min at 37°C, under an atmosphere containing 5% CO₂. The medium was then supplemented with 200 μl of capacitation medium (CAP) or a medium not supporting capacitation with 1 mM db-cAMP and 100 μM IBMX and for the resulting sperm suspension was incubated for 50 min at 37°C under an atmosphere containing 5% CO₂.

After incubation, the sperm were washed once with 1 mM Na₃VO₄–5 mM PPi in PBS and proteins were extracted by adding Laemmli buffer (4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.004% bromophenol blue, 0.125 mM Tris–HCl). These protein extracts were subjected to SDS–PAGE (1 × 10⁶ cells/lane) and transferred to a nitrocellulose membrane for immunoblotting with antibodies against phosphorylated tyrosine (4G10 mouse monoclonal antibody; Upstate).

Plasmid constructions
The pRK5-Myc-STAS construct comprises the entire STAS domain corresponding to amino acids 543–795 of the TAT1 protein. pRK5-Myc-STAS construct was generated by PCR using the primer pair STASBamFw: 5'-GCAGAGGGATCGATTATCGGGAGATCATC and STASHindRv: 5'-TATATAAAGCTTCTAGGCAAACAGCACGGCGTC, followed by BanHI and HindIII digestion and subcloning into the pRK5-Myc plasmid.

The STAS-truncated TAT1 construct was generated by engineering NheI sites flanking the STAS domain of TAT1 (between 1626 and 1627 bp and between 2385 and 2386 bp of the cDNA sequence). The STAS region (1627–2385 bp, ...DYREII...DAVLFA... ) was next removed by restriction digest with NheI and subjected to agarose gel purification. The resulting fragment was then religated and resulted in the STAS-truncated form, pRK5-TAT1 ΔSTAS.

pCMV-SLC26A3 (pCMVhDRA) construct is a gift from Shmuel Mualem (University of Texas, Dallas, TX, USA) and PT77s-CFTR is a gift from Cherif-Zahar (INSERM U845, Paris, France).

Media
Medium not supporting capacitation (NO): 120 mM NaCl, 2 mM KCl, 1.2 mM MgSO₄, 0.36 mM NaH₂PO₄, 5.56 mM glucose, 1 mM sodium pyruvate, 18.5 mM succrose, 50 mM HEPES, pH 7.3. Capacitation medium (CAP): 120 mM NaCl, 2 mM KCl, 1.2 mM MgSO₄, 0.36 mM NaH₂PO₄, 5.56 mM glucose, 1 mM sodium pyruvate, 18.5 mM succrose, 50 mM HEPES, 1.8 mM CaCl₂, 25 mM NaHCO₃, 3% BSA, pH 7.3. COS-7 cell medium (Dulbecco’s modified Eagle medium) and CHO-K1 cell medium (minimum essential medium) were purchased from Gibco.

Chemicals and antibodies
db-cAMP, IBMX, H89, Na₃VO₄ and PPI were purchased from Sigma-Aldrich. Sp-cAMP and Rp-cAMP were purchased from BioLog (Bremen, Germany). FuGENE transfection reagent and Complete Protease Inhibitor Cocktail were obtained from Roche Applied Science (Meylan, France). JetPEI transfection reagent was purchased from Polyplus Transfection.

Fsk was purchased from LC Laboratories (PKC Pharmaceuticals, USA). The CFTR inhibitor CFTRinh-172 (3-[(3-trifluoromethyl)-phenyl]-5-(4-carboxyphenyl)methylene]-2-thioxo-4-thiazolidinone) was from VWR International (Fontenay-sous-Bois, France).

The anti-TAT1 antibody (L2CL4) directed against the carboxy-terminal region of human TAT1 (amino acids 664–970) has been described elsewhere (20). The novel antibody
directed against amino acids 1–15 and amino acids 955–970 of human TAT1 was raised in rabbit and purified against both peptides (Eurogentec, Angers, France). Anti-phosphotyrosine clone 4G10 and anti-CFTR clone M3A7 were obtained from Millipore SAS (Molsheim, France); anti-CFTR clone CF3 was obtained from Abcam (Paris, France). Anti-Myc clone 9E10 was purchased from Roche Applied Science and anti-Flag clone M2 from Sigma-Aldrich. The anti-SepTin 4 antibody H-120 was generated against the 50 kDa splice variant of human sAC and was previously described (39). Horseradish peroxidase (HRP)-conjugated anti-mouse immunoglobulin and HRP-conjugated anti-rabbit immunoglobulin were purchased from Dako. Alexa 568 anti-rabbit and Alexa 594 anti-rabbit antibodies were purchased from Invitrogen. FITC anti-rabbit antibody was purchased from Dako.

Statistics

Results are expressed as mean ± SEM of n observations. Sets of data were compared with a Student’s t-test. Differences were considered statistically significant when P < 0.05. All statistical tests were performed using GraphPad Prism version 4.0 for Windows (Graphpad Software) and Origin version 5.0. ns, not significant; *P < 0.05, **P < 0.01 and ***P < 0.001.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG online.

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Conflict of Interest statement. The authors have no conflict of interest to declare.

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