Expression of ion transport-associated proteins in human efferent and epididymal ducts

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Abstract

Appropriate intraluminal microenvironment in the epididymis is essential for maturation of sperm. To clarify whether the anion transporters SLC26A2, SLC26A6, SLC26A7, and SLC26A8 might participate in generating this proper intraluminal milieu, we studied the localization of these proteins in the human efferent and the epididymal ducts by immunohistochemistry. In addition, immunohistochemistry of several SLC26-interacting proteins was performed: the Na⁺/H⁺ exchanger 3 (NHE3), the Cl⁻ channel cystic fibrosis transmembrane conductance regulator (CFTR), the proton pump V-ATPase, their regulator Na⁺/H⁺ exchanger regulating factor 1 (NHERF-1), and carbonic anhydrase II (CAII). Our results show that SLC26A6, CFTR, NHE3, and NHERF-1 are co-expressed on the apical side of the nonciliated cells, and SLC26A2 appears in the cilia of the ciliated cells in the human efferent ducts. In the epididymal ducts, SLC26A6, CFTR, NHERF-1, CAII, and V-ATPase (B and E subunits) were co-localized to the apical mitochondria rich cells, while SLC26A7 was expressed in a subgroup of basal cells. SLC26A8 was not found in the structures studied. This is the first study describing the localization of SLC26A2, A6 and A7, and NHERF-1 in the efferent and the epididymal ducts. Immunolocalization of human CFTR, NHE3, CAII, and V-ATPase in these structures differs partly from previous reports from rodents. Our findings suggest roles for these proteins in male fertility, either independently or through interaction and reciprocal regulation with co-localized proteins shown to affect fertility, when disrupted.

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Introduction

Optimal microenvironment in each segment of the male reproductive tract is essential for successful maturation and correct motility of sperm and, thereby, for male fertility. Testicular fluid carries immature spermatozoa to the efferent ducts (Wong *et al.* 2002), where majority (>95%) of the fluid is reabsorbed (Clulow *et al.* 1998, Wong *et al.* 2002). During passage through the epididymal ducts, the composition of the luminal fluid is further gradually changed by sequential absorption and secretion processes (Turner 2002, Wong *et al.* 2002). Water absorption in efferent ducts is allowed by interdependent transport of Cl⁻ and Na⁺, which may be mediated by an apical anion Cl⁻/HCO₃⁻ exchanger, in parallel with a Na⁺/H⁺ antiporter (Hansen *et al.* 2004).

Accurate control of intraluminal pH and HCO₃⁻ concentration is needed for inducing the sperm motility and, on the other hand, for keeping sperm quiescence during the storage in epididymis (Litvin *et al.* 2003, Gadella & Van Gestel 2004). In rats, luminal pH and HCO₃⁻ concentration decrease in the distal segments of the efferent ducts, and further diminish along the epididymal ducts (Levine & Marsh 1971, Levine & Kelly 1978, Newcombe *et al.* 2000), but only little is known about specific HCO₃⁻ transporters mediating these processes.

SLC26 (Solute carrier family no 26) anion exchangers are cell membrane proteins capable of transporting several anions (Cl⁻, HCO₃⁻, OH⁻, SO₄²⁻, and oxalate) with different specificities. Altogether, 11 human members of this gene family have been discovered, of which 4 (SLC26A2–A5) are known to cause distinct

hereditary diseases when mutated (Hastbacka *et al.* 1994, Hoglund *et al.* 1996, Everett *et al.* 1997, Lohi *et al.* 2000, 2002, Toure *et al.* 2001, Liu *et al.* 2003 and McKusick (ed) OMIM database which can be viewed online at www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=OMIM). Prior to this work, preliminary information about the expression of five SLC26 members in the human male reproductive tract was available: SLC26A1 (sulphate anion transporter 1; SAT-1), SLC26A3 (down regulated in adenoma; DRA, congenital chloride diarrhea (CLD)), SLC26A4 (pendrin), SLC26A7 and SLC26A8 (testis anion transporter 1; TAT1) in the testis, and SLC26A3 in the seminal vesicle, the efferent, and the epididymal ducts (Haila *et al.* 2000, Lacroix *et al.* 2001, Lohi *et al.* 2002, Regeer *et al.* 2003, Hihkala *et al.* 2006). However, excluding SLC26A3 (Hihkala *et al.* 2006), neither systematic review of the expression profiles of these proteins in the male reproductive system nor their specific expression in certain cell types of the epididymis has been published. Furthermore, subfertility due to low amount of sperm with aberrant mobility are common among male patients with CLD, known to be caused by mutations in A3 member of this SLC26 gene family (Hoglund *et al.* 2006). Therefore, we proposed that other members of the SLC26 family might play role in human fertility as well, provided that their expression profile would conform to this hypothesis.

As a primary step towards understanding the possible role of the SLC26 protein family in human efferent and epididymal ducts, we searched for the localization of SLC26A2 (diastrophic dysplasia sulfate transporter; DTDST), SLC26A6 (putative anion transporter-1; PAT-1, chloride-formate exchanger; CFEX), SLC26A7, and SLC26A8 in these structures by immunohistochemistry. Furthermore, we studied the human expression of several proteins shown to interact with SLC26 members on molecular level, and known to be involved in fluid and electrolyte absorption or pH regulation in efferent and/or epididymal ducts in animal studies. This set of proteins included: Na⁺/H⁺ exchanger 3 (NHE3, SLC9A3), cystic fibrosis transmembrane conductance regulator (CFTR), Na⁺/H⁺ exchanger regulating factor 1 (NHERF-1, SLC9A3R1, EBP50), carbonic anhydrase II (CAII), and V-ATPase (vacuolar-type H⁺ATPase). Since expression and function of some of these proteins varies greatly between different species (Chernova *et al.* 2005, Kujala *et al.* 2005), it is important to study them in human tissues as well.

Materials and Methods

Tissue samples

Formalin-fixed, paraffin-embedded human epididymis specimens were obtained from the archives of the Department of Pathology, HUSLAB, Helsinki University Central Hospital. We examined altogether 18 histologically normal adult (22–71 years old, mean value 37)

epididymides removed because of testicular tumors (ten seminomas, two teratocarcinomas, one mature teratoma, one embryonal carcinoma and seminoma, one cysta epidermalis, one cystadenoma of rete testis, one cicatrix, and one normal finding). Histological examination revealed complete spermatogenesis in all but two corresponding testis samples.

Antibodies

Antisera were raised in rabbits against the synthetic peptides TVRDSLNTGEYCKKEEN for SLC26A2 (anti-A2), MDLRRRDYHMERPLLNQEH for SLC26A6 (anti-A6), SHIHSNKNLSKLSHDSEV for SLC26A7 (anti-A7), and VEEVWLPNNSSRNSSPGLPD for SLC26A8 (anti-A8), corresponding to amino acids 689–706, 1–20, 639–656, and 680–699 respectively. Two rabbits were immunized with each peptide. Peptide synthesis and antibody production were purchased from Research Genetics (anti-A2) and Sigma Genosys Ltd (anti-A6, -A7, -A8). The anti-A2, -A6, and -A7 antibodies were purified from whole serum by affinity chromatography (Amersham Pharmacia Biotech). The specificity of these antibodies except for anti-A8 has been shown previously (Haila *et al.* 2001, Lohi *et al.* 2002, 2003, Kujala *et al.* 2005).

Commercial polyclonal rabbit antibodies used were: anti-human CAII (200-401-136; Rockland Immunochemicals, Gilbertsville, PA, USA), anti-rat NHE3 (NHE31-A; Alpha Diagnostics, San Antonio, TX, USA), anti-human vacuolar type H⁺-ATPase B1/2 (V-ATPaseB, sc-20943; Santa Cruz Biotechnology, Heidelberg, Germany), anti-human vacuolar type H⁺-ATPase E (V-ATPaseE, sc-20946; Santa Cruz Biotechnology), and anti-human NHERF-1 (EBP50, PA1-090; Affinity BioReagents, Golden, CO, USA). In addition, the mouse MAB anti-human CFTR (Ab-2 MM13-4; Neo Markers, Fremont, CA, USA) was used.

Western blotting

Fresh rat testis was snap frozen in liquid nitrogen and stored in –18 °C. The frozen sample was homogenized, Laemmli buffer and β-mercaptoethanol were added and the sample was boiled in 100 °C for 5 min. The sample was run on 7.5% SDS-PAGE gel in several parallel wells. The separated proteins were transferred electrophoretically to Hybond-C extra membranes (Amersham Life Sciences). After blocking in 5% nonfat dry milk and 1% BSA in Tris-buffered Saline Tween-20 (TBST) for 60 min, the filters were incubated with A8 antiserum (1:50) in 2.5% nonfat dry milk and 0.5% BSA in TBST for 1 h. Horseradish peroxidase (HRP) conjugated swine anti-rabbit IgG (Dako, Glostrup, Denmark) was used as a secondary antibody. The corresponding rabbit preimmune serum (1:50) was used as a negative control. The protein bands were visualized by chemiluminescence (ECL Western Blotting Detection Reagents, Amersham Biosciences) detected on X-ray films.

Immunohistochemistry

Deparaffinized 5 µm human epididymis tissue sections were used in the experiments. The average number of distinct specimens stained with each antibody was nine, and all staining experiments were repeated several times. The peroxidase–antiperoxidase labeling kit (Vectastain Elite ABC Kit, Vector Laboratories, Burlingame, CA, USA) and the two-step peroxidase kit (EnVision+ System HRP, DAKO, Carpinteria, CA, USA) were used. The pretreatment for each of the antibodies was individual: boiling in microwave oven in 10 mM citrate buffer (pH 6.0) was used for anti-A2, anti-A6, anti-CAII, anti-CFTR, anti-NHE3, and anti-NHERF-1, but anti-A6, anti-A7, anti-VATPaseB, and anti-VATPaseE required no specific pretreatment. For anti-A8 antibody, several different pretreatments were examined, but none resulted in specific signal in the human epididymis.

The pretreatment was followed by blocking of endogenous peroxidase activity with H₂O₂, according to the kit manufacturer's directions. The dilutions of the antisera ranged in the ratio from 1:750 to 1:3000 and for the affinity purified antibodies from 0.1 to 20 µg/ml. Diaminobenzidine was used as the chromogenic substrate with Mayer's hematoxylin counterstaining. Preimmune sera, normal rabbit IgG or normal mouse IgG were used as the corresponding negative controls, with similar concentrations of IgG as for the purified antibodies.

Histological classification

Different cell types were determined by their morphology and location. Analyzing the different types of the efferent ducts was done by following the definitions by Yeung *et al.* briefly: 1) type I epithelium has an irregular profile with variable height of the epithelial cells, 2) type II epithelium is regular low cuboidal with very few vacuoles, 3) type III epithelium is distinguishable by its vacuolated epithelial cells forming tubules with a regular outline, 4) cuboidal type IV epithelium lines, with mosaic pattern, parts of short communal cavities, and 5) type V efferent duct epithelium consists of ciliated columnar cells. Only types I and III form longer segments, while type II is mainly found in short blind end tubules, type V in brief distal segments close to epididymal ducts, and type IV in short transition segments only. Each of the efferent duct tubule types has both ciliated and nonciliated cells. In addition, basal cells can be found in tubule types I–III (Yeung *et al.* 1991).

Distinct cell types of the human epididymal ducts were distinguished according to the descriptions of Palacios *et al.* and Regadera *et al.* briefly: three morphologically different cell types can be detected; principal cells, apical mitochondria rich cells (AMRC), and basal cells. The nucleus is located centrally in

principal cells and more apically in AMRC. AMRC are further divided into two histologically separable cell types: narrow cells, having a slender cytoplasm extending all the way from the basement membrane to the luminal edge, and apical cells, with a short cone-shaped morphology on the luminal side of the epithelium, with possibly no continuation to the basement membrane. The amount of AMRC decreases from caput to cauda (Palacios *et al.* 1991, Regadera *et al.* 1993). Different epididymal duct segments were categorized according to the classification of Holstein, briefly: types 2a and b are located in the caput and consist of tall columnar epithelium with regular outline and tall cilia. Both the cytoplasm and the cilia are taller in type 2b than 2a. Type 3 tubules belong to the corpus. In type 3a, the columnar epithelium becomes lower with shorter cilia. Small cysts formed by the epithelium appear in 3b tubules, and in 3c these cysts become larger, more frequent, and may include spermatozoa. The outline of the epithelium transforms into irregular in 3b–c. Tubules located in the cauda epididymis are classified as types 4a–c. In 4a tubules, the outline of the epithelium is irregular with no cysts, and the amount of cytoplasm diminishes when compared with the former segments. In 4b tubules, the lumen gets remarkably wider and the epithelium lower. The epididymal lumen is at its widest in type 4c tubules, where the epithelium is at its lowest (Holstein 1969).

Results

SLC26A2

In all efferent ducts detected (types I–III), the cilia of the ciliated cells stained specifically for the anti-A2 antibody (Fig. 1J). In the epididymal ducts (types 2a–4a), all cell types remained negative for SLC26A2 (Fig. 2M and data not shown).

SLC26A6

The nonciliated cells of the types I and II efferent ducts gave strong positive signal for SLC26A6 on their apical edge (Fig. 1A and E), but the type III efferent ducts remained negative (Fig. 1A). In the epididymal ducts, a subgroup of AMRC was positive for SLC26A6 (Fig. 2A–C). Most of the narrow type AMRC and the early phase flat apical cells were negative, while the later phase apical cells extruding to the lumen stained apically (Fig. 2A–C). The epididymal tubules found in SLC26A6 stained samples were of types 2a–3c, and positive AMRC were found in all of these tubule types detected (Fig. 2A–C and data not shown). The positive staining in the epididymal ducts was seen clearest with no pretreatment of the slides, whereas the signal in the efferent ducts required 10 min microwaving in citrate buffer to become visible.

SLC26A7

All types of the efferent ducts were negative for anti-A7 antibody (Fig. 1K). However, SLC26A7 was detected in a subgroup of the basal cells in all the epididymal duct types found in the samples (duct types 2a–3c and 4b–c; Fig. 2K–L and data not shown). The strong positive staining followed the cell membrane of these basal cells (Fig. 2L). Rest of the epididymal cell types remained unstained (Fig. 2K–L).

SLC26A8

The specificity of the anti-A8 serum was tested by western blot made of adult rat testis, known to express this protein (Toure *et al.* 2001). The anti-A8 serum detected a strong specific band of approximately 100 kDa on the western blot made of adult rat testis (Fig. 3). This band corresponds well to the known 970 aa size of the SLC26A8 protein. In addition, a less intense 60 kDa band was observed (Fig. 3). This is probably a degradation product of the SLC26A8 protein. Negative control stained with the corresponding rabbit preimmune serum remained negative (Fig. 3). Despite several different pretreatments and concentrations of antiserum used, the anti-A8 sera did not give any specific signal in the immunohistochemical stainings of human efferent ducts (Fig. 1L) or epididymal ducts (types 2a–4c, Fig. 2O and data not shown).

CFTR

The CFTR antibody gave a strong apical signal in the nonciliated cells of the efferent ducts, while the ciliated cells remained unstained (Fig. 1F). The staining was strongest in the types I (Fig. 1B and F) and II epithelium of the efferent ducts, and only a portion of the type III tubules stained for this antibody (data not shown), while type V epithelium remained totally negative (Fig. 1B). In the epididymal ducts, a clear positive signal was seen on the apical border of the apical cells (Fig. 2D) of all duct types (2a–4a, 4c) detected (Fig. 2D and data not shown). The other cell types of the epididymal ducts remained negative (Fig. 2D).

NHE3

NHE3 gave a strong positive signal on the luminal edge of the nonciliated cells in the types I and II efferent ducts (Fig. 1C and G). In type III efferent ducts, faint staining could be seen in a few nonciliated cells, but the majority of ducts of this type were negative (Fig. 1D). All cell types in the epididymal ducts (types 2a–3c and 4c) were negative for NHE3 antibody with all antigen retrieval methods used (Fig. 2N and data not shown).

NHERF-1

NHERF-1 was found on the apical aspect of virtually all of the efferent duct cells, both ciliated and nonciliated, in all different epithelium types detected (Fig. 1H–I). The signal was generally slightly stronger in the nonciliated than the ciliated cells. In the epididymal ducts, NHERF-1 was found in the cytoplasm of the narrow and apical forms of AMRCs and in a few basal cells in duct types 2a–3c (Fig. 2E–F and data not shown). Only very few type 4a epididymal tubules were found in the samples studied, and they included no NHERF-1 signal (data not shown). However, considering the small amount of the detected 4a tubules and the low AMRC count in the distal epididymis, no reliable conclusion about the absence of NHERF-1 in the distal epididymis can be made based on these results.

V-ATPase

All types of the efferent duct epithelium lacked V-ATPase signal (Fig. 1N). Instead, it was clearly detected both in the narrow and the apical forms the AMRC of the epididymal ducts (Fig. 2I–J) in all duct types (2a–4c; Fig. 2I–J and data not shown). Both the antibody against B and the one against E subunit of V-ATPase gave similar results (data not shown).

CAII

All human efferent duct types were negative for the anti-CAII antibody (Fig. 1M). In the types 2a–3c and 4c, epididymal ducts found in the CAII-stained samples (Fig. 2G–H and data not shown), the apical and narrow cells showed cytoplasmic signal for the CAII (Fig. 2G–H).

Figure 1 Immunohistochemical stainings of human efferent ducts. (A) Anion transporter SLC26A6 (A6) is expressed in the type I but not in the vacuolated type III efferent ducts. (B) Cl^- channel CFTR is located to the type I but not in the type V efferent ducts. (C) Sodium/proton exchanger 3 (NHE3) is clearly positive in type I efferent ducts (arrow), while (D) in type III vacuolated efferent ducts NHE3 staining is very faint (arrow). With higher magnification, it is evident that (E) SLC26A6, (F) CFTR, and (G) NHE3 are all located to the apical face of the nonciliated cells (arrows) and the ciliated cells remain negative (arrowheads) for all these three antibodies. (H) NHE3 regulatory factor 1 (NHERF) is expressed both in types I and III efferent ducts. (I) Higher magnification from type II efferent duct reveals that all of the nonciliated cells (arrows) and the majority of the ciliated cells (arrowheads) are positive for NHERF-1 antibody. (J) Anion transporter SLC26A2 (A2) is detected specifically in the cilia of the ciliated cells (arrowheads). The nonciliated cells are negative for this protein (arrows). Stainings for anion transporters (K) SLC26A7 and (L) SLC26A8, as well as (M) CAII and (N) vacuolar-type H^+ ATPase subunit B (VATPase) remained negative for both ciliated (arrowheads) and nonciliated (arrows) cells. Negative controls stained with (O) normal rabbit serum (NRS), (P) normal rabbit serum's IgG fraction (NRIgG) and (Q) normal mouse serum's IgG fraction (NMIgG) remained negative in all experiments. Scale bars: A and H, 100 μm ; B–D and N, 50 μm ; E–G, I–M and O–Q, 20 μm .

Figure 3 Western blot made of normal rat testis and stained with anti-human SLC26A8 antiserum (A8) or the corresponding preimmune serum (Pre). The anti-A8 serum detected a strong specific band of approximately 100 kDa, which corresponds well to the known 970 aa size of the SLC26A8 protein. In addition, a less intense circa 60 kDa band is seen. This is probably a degradation product of the SLC26A8 protein. Negative control (Pre) remained totally negative.

Other epididymal cell types remained negative for this antibody (Fig. 2G–H).

Discussion

Many complex physiological processes take place in the male efferent and epididymal ducts, but little is still known about the particular roles of the various cell types and proteins expressed by them. In this study, we immunolocalized for the first time the anion exchanger proteins SLC26A2, A6, and A7 and the regulatory protein NHERF-1 in the efferent and epididymal ducts, and further characterized the expression of CFTR, NHE3, and V-ATPase in these structures in human, and compared their localization with that of CAII.

At molecular level, the studies of regulatory domains of distinct transporters have revealed complex interaction networks linking specific transporters spatially together, enabling, e.g. their reciprocal regulation. As an example, binding of the sulfate transporter and anti-sigma factor antagonist (STAS) domain of the SLC26 family members SLC26A3 and A6 with the R domain of the Cl^- channel CFTR, promotes activation of both proteins, which is

further facilitated by their PDZ ligands (Ko *et al.* 2004). The regulator NHERF-1 is an apical PDZ protein shown to interact with SLC26A6, CFTR, the apical Na^+/H^+ exchanger NHE3, and B1 subunit of proton transporting V-ATPase (V-ATPaseB1; Weinman *et al.* 1995, Wang *et al.* 1998, Breton *et al.* 2000, Lohi *et al.* 2003), thus NHERF-1 may act as a shared spatial link for these transporters. Although only SLC26A6 of the SLC26 family members has been studied for NHERF-1 interactions so far, also SLC26A2, A3, A7, and A9 have consensus PDZ-binding sequences at their carboxyl terminus, offering a possibility to interact with PDZ networks (Lohi *et al.* 2002, 2003). Moreover, the H^+ and HCO_3^- formation catalyst CAII was recently shown to form a transport metabolon with SLC26A6, affecting the HCO_3^- transport activity of SLC26A6 (Alvarez *et al.* 2005).

This study shows that, in human efferent ducts, SLC26A6, CFTR, NHE3, and NHERF-1 are expressed on the apical edge of the nonciliated cells (Fig. 4), which are responsible for the major fluid reabsorption (Hess 2002). In addition, the anion transporter SLC26A3 has recently been located in these cells in human (Hihnal *et al.* 2006), enabling the proposed model of paired function of SLC26A3 and A6, activated by CFTR (Ko *et al.* 2002). Our results further coincide with the previous functional data from rat indicating that major absorption of Cl^- , Na^+ , and water in the efferent ducts is mediated by an apical $\text{Cl}^-/\text{HCO}_3^-$ exchanger, acting in parallel with a Na^+/H^+ antiporter (Hansen *et al.* 2004). Thus, we propose a model that in human efferent ducts, SLC26A3 and A6 work in conjunction with CFTR to secrete HCO_3^- and absorb Cl^- , and this action is further facilitated by the Na^+ absorption of the Na^+/H^+ exchanger NHE3. All of these transporters may be regulated and spatially linked by NHERF-1, also localized in the nonciliated cells. Interestingly, estrogen regulates SLC26A3, NHE3, CFTR, and NHERF-1 (Rochwerger & Buchwald 1993, Ediger *et al.* 1999, Lee *et al.* 2001, Zhou *et al.* 2001, Ediger *et al.* 2002), further suggesting that these proteins may form a functional unit. Whether SLC26A6 is also regulated by estrogen, remains to be clarified.

Figure 2 Immunohistochemical stainings of human epididymal ducts. (A–C) Anion transporter SLC26A6 (A6) is positive in the out bulging forms of apical cells (arrows), while the flat apical cells (arrowheads) are negative. The more the cell is bulging into the lumen, the more positive the staining tends to be. The positive signal is concentrated on the apical face of the cells. (D) Cl^- channel CFTR gives a strong apical signal in the apical cells (arrow). (E) A low magnification picture showing an overview of both efferent ducts type I (I) and epididymal ducts (Ep) stained with NHE3 regulatory factor 1 (NHERF). (F) NHERF gives strong pancytoplasmic staining in both apical (arrowheads in (E) and narrow (arrows in (F) types of AMRC. In addition, few basal cells are stained (arrowheads in (F)). (G–H) CAII was detected in the cytoplasm of both (G) apical and (H) narrow forms of AMRC. (I–J) Vacuolar-type H^+ ATPase subunit B (VATPase) is positive in all AMRC as well, both in apical (arrowheads) and narrow (arrow) cell types. The staining is mainly pancytoplasmic, but in some cells apical concentration could be seen (arrow). (K and L) Anion transporter SLC26A7 (A7) is located to a subgroup of the basal cells (arrowheads). Some of the basal cells are negative for this protein (arrows). (M) Anion transporter SLC26A2 (A2) is not detected in any epididymal cell types. (N) NHE3 is negative in all cells of human epididymal ducts, including the apical cells (arrowhead). (O) Antibody against anion transporter SLC26A8 (A8) leaves all cells of human epididymal ducts negative. (P–R) Negative controls stained with (P) normal rabbit serum (NRS), (Q) normal rabbit serum's IgG fraction (NRIgG), and (R) normal mouse serum's IgG fraction (NMIgG) remained negative in all experiments. The epididymal tubule types shown here (classified according to Holstein, 1969): A, C and Q, type 2a; E, M–N, P and R, 2b; B, D, F–H, K–L and O, 3a; I–J, 3b. Scale bars: A, I–K and N, 50 μm ; B–D, F–H, L–M and O–R, 20 μm ; E, 100 μm .

Figure 4 A diagram of the possible interactions of SLC26A6 (A6), CFTR, NHE3, and NHERF-1 in a nonciliated cell of the efferent ducts of the human epididymis.

In contrast to the other proteins studied, expression of the SLC26A2 protein was found strictly in the cilia of the ciliated cells in the efferent ducts. As an epithelial $\text{Cl}^-/\text{SO}_4^{2-}$ exchanger (Satoh *et al.* 1998), its putative role in the male reproductive tract is less clear. However, diastrophic dysplasia male patients with mutations in *SLC26A2* have not been reported to be subfertile, thus the function of SLC26A2 in male fertility may be less crucial than SLC26A3's, which causes male subfertility when mutated (Hoglund *et al.* 2006).

In the epididymal ducts, we found co-expression of SLC26A6, CFTR, NHERF-1, CAII, and B and E subunits of V-ATPase in the AMRC (Fig. 5), while SLC26A7 was localized in a subgroup of basal cells. Strict control of luminal HCO_3^- concentration by epididymal epithelium is essential to avoid premature sperm motility, membrane instability and thus impaired fertility (Litvin *et al.* 2003, Gadella & Van Gestel 2004). AMRC have been suggested to have a critical part in regulating the intraluminal pH in human epididymal ducts (Palacios *et al.* 1991), which would coincide with localization of HCO_3^- transporting SLC26A6, proton transporting V-ATPase, and CAII that catalyzes formation of these ions and affects the activity of HCO_3^- transport by SLC26A6 (Alvarez *et al.* 2005) in this cell type.

Expression of the SLC26A7 protein, a transporter for Cl^- , SO_4^{2-} , and oxalate (Lohi *et al.* 2002), was found in a subgroup of basal cells of the human epididymal ducts. Physiological function of the basal cells is unclear; roles in regulation of anion secretion of nearby principal cells

Figure 5 A diagram of SLC26A6 (A6), CFTR, CAII, NHERF-1, and V-ATPase in an apical form of AMRC of human epididymal ducts and their possible interactions.

and local immune defense have been proposed (Yeung *et al.* 1994, Cheung *et al.* 2005), and interestingly, SLC26A7 is also found in the nonspecific defense cells, macrophages (Kujala unpublished observations). However, the detailed function of SLC26A7 in these processes is yet to be clarified.

The localization of some of the proteins studied here in human differs partly from the earlier studies in rodents. Unexpectedly, none of the proteins were found in the principal cells of human epididymal ducts. For instance, CFTR was located specifically on the apical border of the apical form of AMRC in human, while earlier findings showed immunolocalization in the principal and clear cells of the epididymis in rats (Ruz *et al.* 2004). However, in efferent ducts, CFTR was found in the apical surface of the nonciliated cells in human, similarly as previously shown for rat (Ruz *et al.* 2004). In rat, NHE3 is found on the apical pole of the nonciliated cells of the efferent ducts and principal cells of the epididymal ducts (Pushkin *et al.* 2000, Bagnis *et al.* 2001, Kaunisto *et al.* 2001, Leung *et al.* 2001, Kaunisto & Rajaniemi 2002), while we found it only in the former in human. Rat V-ATPase is expressed widely in the male reproductive tract, e.g., in the efferent ducts and epididymis, (Herak-Kramberger *et al.* 2001) but the human ortholog was found in AMRC of epididymal ducts only. Based on functional data derived from rodents, NHEs may be more important in the proximal parts of epididymis, whereas V-ATPase in more distal structures (Breton *et al.* 1996, Bagnis *et al.* 2001, Herak-Kramberger *et al.* 2001, Yeung *et al.* 2004), which would be in harmony with our immunohistochemical data.

Discrepancies in the transporter expression profiles between different species make functional differences possible on tissue level. Unfortunately, relevant

functional studies in human reproductive tract are, and will be, challenging. However, our immunohistochemical results together with previous functional data suggest that the proteins studied in this work may form interaction units in the human epididymis, and possibly affect human male fertility, if mutated.

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