Aquaporin AQP11 in the testis: molecular identity and association with the processing of residual cytoplasm of elongated spermatids

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Abstract

AQP11 is one of the latest aquaporin (AQP) family members found, which differs from the other AQPs by its intracellular localisation and unusual water pore nucleotides with unclear function. Despite the highest mRNA expression among organs having been reported in the testis, the testicular molecule has not been studied in detail. Immunohistochemistry of rat adult testis localised AQP11 to the elongated spermatids (ES) and no other cell types except residual bodies inside Sertoli cells. It was absent from early ES at least until stage 13, and after a first diffuse appearance in the caudal cytoplasm became concentrated in intracellular organelles by stage 17, was strongest in vesicles in the anterior cytoplasm at the final ES stages and appeared in residual bodies. Staining was detected on the distal quarter of the sperm tail only immediately before spermiation. A similar localisation was found in the mouse and developmental profiles for both the open reading frame mRNA and protein expression in 8–50 dpp testis pinpointed its first appearance coinciding with late stage ES. Sequencing of PCR products of testicular Aqp11 containing the open reading frames confirmed a full match with GenBank databases for rat, mouse and human. Western blotting revealed two or more molecular forms with the 26/27 kDa species dominating in the rat/mouse testis and the 33/34 kDa form selectively allocated to the spermatozoa. In view of intracellular vacuolation leading to polycystic kidney in Aqp11-null mice, a possible role of testicular AQP11 in the recycling of surplus cytoplasmic components of the ES and sustaining Sertoli cell capacity in the support of spermatogenesis was discussed.


Introduction

Aquaporins (AQPs) were first found to be channel proteins on plasma membranes allowing water transport driven by osmotic gradients. There are 13 members in the AQP family (AQP0–12), and the expression of one or more of these is ubiquitous in mammalian cells (see reviews: Jeyaseelan et al. (2006), Ishibashi et al. (2009)). These include the four aquaglyceroporins that also allow passage of glycerol (AQP3, 7, 9 and 10), the two superaquaporins (AQP11, 12) and the remaining seven are largely water-selective AQPs. Some can also conduct other molecules, such as hydrogen peroxide, urea and ammonia by AQP8 (Wu & Beitz 2007). The superaquaporins differ from the others not only in their unusual Asn-Pro-Ala (NPA) motifs, in that in the first of the two highly conserved hydrophobic NPA motif forming the water pore in the prototypical AQPs (Hub et al. 2009) contains a cysteine instead of an alanine, but also in their intracellular localisation (Gorelick et al. 2006, Ishibashi 2006).

It is well established that the testis expresses AQP7 and AQP8 both at the mRNA and protein levels. AQP7, which was first cloned from the testis (Ishibashi et al. 1997), is expressed initially in the round spermatids (Kageyama et al. 2001) and is also localised to the sperm tail in the rat (Ishibashi et al. 1997, Suzuki-Toyota et al. 1999, Kageyama et al. 2001), mouse (Skowronski et al. 2007) and human (Saito et al. 2004). The role of AQP7 in male reproductive physiology is unclear as knockout mice are fertile, producing normal functional spermatozoa (Sohara et al. 2007), whereas an association with sperm motility has been suggested in infertile men (Saito et al. 2004). Although there are many studies on AQP8 in the testis, reports on the cellular localisation are inconsistent, ranging from spermatogenic cells in general (Elkjaer et al. 2001, Kageyama et al. 2001, Yang et al. 2005), in elongated spermatids (ES), residual bodies and primary spermatocytes (Calamita et al. 2001) and exclusively in Sertoli cells (Badran & Hermo 2002). Our recent work has demonstrated localisation only in ES and provides evidence suggesting a role of AQP8 in...
sperm volume regulation (Yeung et al. 2009). AQP0, the AQP expressed specifically in the eye (Ishibashi et al. 2009), has also been found in the testis but only restricted to somatic cells including the Sertoli and Leydig cells (Hermo et al. 2004).

Whereas the function of AQP11 is still unknown, its mRNA is expressed by many organs including mainly the testis, liver, brain, kidney, heart among others. Despite the finding that among these organs, testis has the highest expression detected by northern blotting (mouse: Morishita et al. 2005, rat: Gorelick et al. 2006), the cellular localisation or function of AQP11 in the testis is not known. In the report of AQP11 expression by the epithelial cells of the rat epididymis, positive staining of the luminal content was shown in the micrographs, but there was no description of expression by the spermatozoa in the text (Hermo et al. 2008). Although water permeability in the superaquaporins was in doubt in early expression studies, high water conductance has since been demonstrated in AQP11 when expressed in liposomes (Yakata et al. 2007) instead of plasma membranes (Gorelick et al. 2006). It is known that various AQPs are involved in transport of small molecules besides water, such as urea, chloride, heavy metal salts, ammonia, hydrogen peroxide and others (Wu & Beitz 2007), and thus may play unexpected cellular roles (Verkman 2005). The role of AQP11 in testicular function may turn out to be more than just conferring water permeability. Unfortunately, Aqp11 knockout mice do not survive beyond puberty, and the only phenotype described is confined to phenomena related to lethal renal failure (Morishita et al. 2005). The present study aimed to identify this little known AQP, which is highly expressed in the testis, at the mRNA and protein levels and to follow its cellular distribution during a normal spermatogenic cycle and pubertal development as a first step in the understanding of its role in testicular and sperm function.

**Results**

**Immunohistochemistry of AQP11 in testis and spermatozoa**

Throughout the testis in the adult rat, AQP11 was only expressed in the ES and the residual bodies dotting the stage I germinal epithelium after spermiation (Fig. 1A). In the spermatogenic cycle, positive staining was first discernable in a diffused pattern within the caudal cytoplasm of the elongating spermatids lining the lumen of the seminiferous tubule at around stage II and III when the ES development has already reached stage 16. Staining intensity increased and became concentrated in intracellular organelles in the caudal cytoplasm as the ES migrated towards the lumen (Fig. 1D and E). When the residual cytoplasm was located anterior to the head of the ES, the staining then accumulated into granules and vesicles (Fig. 1F). The elongating sperm tail was negative up to this stage of development. Just before spermiation, staining appeared on the distal part of the sperm tail (Fig. 1G). After the release of spermatozoa into the lumen, AQP11 was found in the residual bodies distributed within the Sertoli cells from the apex towards the base (Fig. 1H and stage I tubule in A). Both the commercial and the in-house antibodies demonstrated the same localisation of AQP11. In sections stained with the commercial antibody incubated with excess antigen peptide, there was only a slight hue of brown colour in the background but no particular cellular staining.

A similar pattern of AQP11 localisation was observed in the murine testis. Examination of the expression profile during pubertal development (Fig. 2A–F) revealed the absence of staining from the early germ cells in the testis of 8-, 14- and 22-day-old mice. In 30- and 37-day-old mice when ES could be found, staining of the ES, residual cytoplasm and residual bodies was observed (Fig. 2E–G) as described for the rats above. Staining of the membrane of the vesicles as well as the cytoplasm of the residual bodies was clearly visible in the mouse (Fig. 2G) and the rat (Fig. 1H).

As a confirmation of AQP11 expression on the distal sperm tail, washed spermatozoa obtained from the cauda epididymidis showed staining along the last quarter of the sperm flagellum (Fig. 2H and I).

**Detection of Aqp11 mRNA by RT-PCR**

In the rat testis, cDNA was detected as a single band product in RT-PCR containing the entire open reading frame (ORF; Fig. 3A) which was sequenced to show a match with the GenBank reference NM_173105.1 from nt228–1046 except at nt676 with an A-G alteration. This would imply a Lys-Glu conversion of the amino acid sequence at position 150 of the protein reference NP_775128.1. However, the presence of G at nt676 agreed with the published Aqp11 genomic sequences on chromosome 1 (AC_000069 and NC_005100), and the deduced amino acid sequence fully matched the Swissprot protein Q8CHM1.2 for rat AQP11.

In the adult murine testis, a major band of the expected size as well as two smaller and weaker bands was obtained (Fig. 3B). Sequencing of the major band revealed full match of the nucleotide bases from nt190–1239 according to the GenBank reference NM_175105.3, including the entire ORF. Alternative primers avoiding the CG-rich segment of the ORF produced only a single band (Fig. 3C). Aqp11 mRNA in the developing testis was first expressed at day 30 but only showed the two smaller bands, although the primer pair used spanned the entire ORF. By day 50, the expected complete ORF was evident while the smaller species were less prominent (Fig. 3G).
Sequencing revealed both smaller bands contained exons 2 and 3 of the ORF but omitted the parts of exon 1 which are rich in strings of CG nucleotide bases.

Identification of AQP11 in the human testis could only be achieved at the mRNA level since no specific antibody for the protein is available. RT-PCR of the human testis revealed AQP11 cDNA of the expected size whose sequence was verified from nt202–1211 according to the GenBank reference NM_173039.1, including the entire ORF. However, additional shorter PCR products with the omission of nt229–369 were persistently obtained using different primer pairs (Fig. 3D and E). This PCR product with 141 bp short was also obtained in RNA samples from ejaculated spermatozoa and kidney, although it was only a minor band compared to the full length product (Fig. 3E).

**Protein expression of AQP11 in the testis and spermatozoa identified by western blotting**

Using the commercial antibody and antigen peptide for adsorption, specific bands were found in the rat testis at 26 and 33 kDa and only the 33 kDa band in rat spermatozoa, all of which disappeared when the antibody used had been incubated with excess antigen peptide (Fig. 4, upper panel). For murine testis, three
specific strong bands were obtained at 43, 34 and 27 kDa using the commercial antibody (Fig. 4, middle panel). However, the 43 kDa band was non-specific in the spermatozoa, while the major band was at 34 kDa with two smaller bands at 22 and 24 kDa which also appeared in the testis, but only as minor bands. In-house antibody against mouse AQP11 (Fig. 4, bottom panel) also revealed bands at 43, 34 and 27 kDa proven specific by commercial antibody and peptide. Since the available antibodies did not have specific interaction with human testis in immunohistochemistry, they were not used for western blotting.

**Discussion**

AQP11 was not expressed by early germ cells and round spermatids. This was confirmed in the developmental profile of the protein expression demonstrated by immunohistochemistry in pre-pubertal mice, with AQP11 first detected in the testis at day 30 when ES first appeared. The present finding revealed that AQP11 was a marker for the process of spermatiation, being first detected in the caudal cytoplasm of the elongating spermatids. As spermatiation progressed, changes in the localisation and intensity of AQP11 staining paralleled the fate of the residual cytoplasm during the formation and elimination of the residual bodies. At the start of nuclear condensation and tail formation, the cytoplasm of the spermatid moves caudally and no AQP11 could be detected. Only when development of the spermatid reached around stage 16, AQP11 was first expressed. The stronger expression of the protein coincided with the contraction of the caudal cytoplasm, which gradually became invaginated by the Sertoli cell while it moved anterior to the sperm head. As this cytoplasm is secured by the Sertoli cell processes, the elongating spermatids migrate towards the lumen until they are eventually detached from the seminiferous epithelium, leaving behind their bulk cytoplasm which forms the residual bodies that are phagocytosed and gradually digested by the Sertoli cells (Kerr et al. 2006). The appearance of AQP11 in the distal sperm tail before spermatiation probably reflects a redistribution at the completion of flagella formation for a possible function discussed below.

The residual bodies are rich in granules and vesicles. They contain remnants of the Golgi complex, endoplasmic reticulum (ER), mitochondria and lipid inclusion as well as remnants of the chromatoid body and the microtubular structure, the manchette (Kerr et al. 2006). Among the intracellular organelles in somatic cells, the ER has been identified as the location of AQP11 (Morishita et al. 2005). Since cells of the proximal renal tubule of AQP11-null mice show vacuolation leading to the formation of polycystic kidney, a role in the efflux of water or non-metabolisable substances across membranes of organelles has been
proposed, although the identities of the transported molecules proved elusive (Nozaki et al. 2008). The expression of AQP11 by the intracellular organelles and vesicular membrane of the residual bodies found at present strengthens such a suggested role. In the testis, spermiogenesis and spermiation produce a large redundant cytoplasmic volume and components which confer a heavy burden on the Sertoli cell to maintain homeostasis of the germinal epithelium. AQP11 may be important in facilitating the elimination of these surplus intracellular organelles and their contents without causing damage to the Sertoli cells after their phagocytosis and degradation. This would allow not only recycling of surplus proteins and organelles of spermatid origin, but also homeostasis of the Sertoli cells in the support of spermatogenesis, as suggested for the clearance of apoptotic spermatogenic cells, the failure of which may result in decreased sperm production (Maeda et al. 2002). Malfunction of the clearance mechanism may even cause a breakdown of self-tolerance leading to autoimmune orchitis (Pelletier et al. 2009). On the other hand, it has been demonstrated most recently that apoptotic spermatogenic cells and the residual bodies can be used to produce ATP by the Sertoli cells after phagocytosis (Xiong et al. 2009).

Unfortunately, a speculative role of AQP11 from this aspect cannot be investigated in knockout mice because of their pre-mature death due to kidney failure. In this respect, a conditional knockout model would be useful to study the involvement of AQP11 in residual body processing and germinal epithelial homeostasis.

As localisation of AQP11 in somatic cells has been confined to intracellular organelles, it is interesting that AQP11 was found to be expressed on the distal sperm tail where cytoplasm is extremely sparse, and plasma membrane is the major cell component. Whether AQP11 is really in the plasma membrane and what role it plays are still to be answered. The appearance of this channel protein at the final stages of tail formation in the testis may imply a function in the elimination of the excess components of outer dense fibres and axonemal microtubules at completion of the assembly of the flagellum, as well as excess cytoplasmic water in the newly formed distal part of the tail.

Characterisation of the distribution of AQP11 by immunohistochemistry was followed up by analysis of its molecular identity at the mRNA and protein levels. In the testis of the rat, mouse and human, cDNA with nucleotide sequences including the complete ORFs identical to those published in GenBank were identified.
by RT-PCR. Whereas only a single PCR product was obtained from rat testicular cDNA, three cDNA species were detected in the mouse testis using a primer pair spanning the entire ORF, with a difference of ca. 500 bp between the largest and smallest variants. In this respect, northern blotting revealed a wide band covering such a range in size in both rat and mouse testis, whereas liver and kidney yielded narrow bands at the lower size range (mouse: Morishita et al. 2005; rat: Gorelick et al. 2006). However, since the omissions by the shorter PCR products involved only the parts of exon 1 that contains strings of CG segments, it is likely a result of PCR artefacts. Nevertheless, the different preponderance of these variants depending on pubertal age may reflect differences in the stability of the cDNA structure in testicular development. On the other hand, the postnatal absence of Aqp11 gene expression until day 30 was consistent with the finding of protein localisation in late spermatids only.

The RNA sequence of human AQP11 is 91–92% similar and 82–83% identical to that of rat and mouse (Gorelick et al. 2006). In our analysis of human testicular mRNA, the complete ORF was identified. The additional PCR products with the omission of nt229–369 were most likely an artefact of PCR. The omitted region was within exon 1 and contained the start site, hence unlikely a result of alternative splicing. The CCGCT immediately upstream from this omission is identical in sequence to the 5’ ending nucleotides of the omitted sequence. This stretch of nucleotides is made up of 76% CG, which would render this segment very difficult to amplify by PCR. A loop-like configuration between the two identical CCGCT segments would facilitate such an omission in the amplification reaction.

Whereas the calculated size of AQP11 is 30 kDa for both mouse and rat, western blotting in earlier studies revealed signal band of 26 kDa in the mouse (Morishita et al. 2005) and two bands (25 and 32 kDa) in the rat with different preponderance in different organs (Gorelick et al. 2006). Specific bands of similar sizes (26/27 and 33/34 kDa) were also found in the rodent testis in the present study, the smaller being predominant. On the contrary, the 33/34 kDa form being the only (rat) or major (mouse) band in epididymal spermatozoa suggests selective allocation of this form to the spermatozoa at spermiation.

Despite the recognition of high expression of Aqp11 in the testis compared with the other organs, there has been no detail study of testicular AQP11 so far. The present work sequenced the testicular mRNA of this AQP in rodents and men, confirmed the protein identities with western blotting and documented the dynamic cellular localisation during late stages of spermiogenesis and the retention of some form of the molecule on the sperm tail. The role of AQP11 in the testis and spermatozoa remains to be investigated.

Materials and Methods

Animals and tissues

Adult male Sprague–Dawley rats and developing male CD mice from 8 days old to adults were obtained from the Animal Centre of the University of Münster. Human ejaculates were produced in the Centre of Reproductive Medicine and Andrology Clinic by masturbation after at least 2 days of abstinence by healthy donors who had given written consent for use of their semen in research, which was approved by the Ethics Committee of the University Medical Faculty and the Chamber of Physicians of Westfalen-Lippe. RNA samples from human testes and kidneys were purchased from BioCat GmbH (Heidelberg, Germany). All chemicals used were purchased from Sigma–Aldrich unless stated otherwise.
Table 1 Forward (F) and reverse (R) oligonucleotide primers flanking the open reading frame of aquaporin 11 used for RT-PCR and intron-spanning primers (F1 and R1) used to confirm sequences in case there were variants of unexpected size.

<table>
<thead>
<tr>
<th></th>
<th>Mouse Aqp11 (NM_175105.3)</th>
<th>Mouse β-actin (NM_007393.3)</th>
<th>Rat Aqp11 (NM_173105.1)</th>
<th>Human AQP11 (NM_173039.1)</th>
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<tbody>
<tr>
<td>F</td>
<td>TCGGCTCTGGTTCCAACA</td>
<td>AGAGGGAAATCGTGCGTGAC</td>
<td>GACGCTCCTTTTCCCCCTGAC</td>
<td>CGGGGAGGACTTCTTAAAGTG</td>
</tr>
<tr>
<td>R</td>
<td>GCCGGACTCATGCAGTCTCCT</td>
<td>GACGCTCCTTTTCCCCCTGAC</td>
<td>GCCGTAGCTGTCACGTACTT</td>
<td>CCCGGTGTGTTCCCATATGAGG</td>
</tr>
<tr>
<td>F1</td>
<td>AGCCTCACATCTGCACAGC</td>
<td>AGACGGAAAATCGTGCGTGAC</td>
<td>GACGCTCCTTTTCCCCCTGAC</td>
<td>CCCGGTGTGTTCCCATATGAGG</td>
</tr>
<tr>
<td>R1</td>
<td>GTTCGAGTCTTTGGGAGTGG</td>
<td>GCCGGACTCATGCAGTCTCCT</td>
<td>GCCGTAGCTGTCACGTACTT</td>
<td>ACCACGCTGAGCCAAATGCCC</td>
</tr>
</tbody>
</table>

Immunocytochemistry of AQP11 in the rodent testis and spermatozoa

Rat spermatozoa were obtained by dissection and isolation of tubule lobules of the cauda epididymis and the release of luminal contents into PBS of osmolality 340 mmol/kg. Cells were washed three times by centrifugation at 600 g for 5 min, resuspended and smeared on polylysine-coated slides. Air-dried smears were fixed in Bouin’s solution for 30 min, washed and processed for antigen retrieval together with testicular sections. Bouin-fixed testicular tissue sections were deparaffinised and heated in 10 mM citrate buffer containing 0.05% Tween 20 at 70 °C for 25 min for antigen retrieval. After blocking with 25% goat serum and 0.1% BSA in PBS for 30 min, sections were incubated overnight at 4 °C with the primary antibody (Aqp115-A, 1:100; Alpha Diagnostic International, San Antonio, TX, USA) or the antibodies generated by Dr Jeppe Praetorius (Gorelick et al. 2006; rat #2981 1:25 or 12.5; mouse #3986 1:20 or 10). Binding was visualised by the biotinylated secondary antibody (Sigma B7389, 1:100 at room temperature for 15 min) followed by streptavidin–HRP conjugate (Sigma S5512, 1:500 at room temperature for 15 min). To check for specificity of positive staining, tissue sections were also stained with the commercial antibody that had been absorbed with 33 times excess antigen peptide purchased from Alpha-Diagnostics. Films of blots were analysed using the 1D Gel Analysis Software, LabImage 1D (Kapelan Bio-Imaging, Leipzig, Germany).

Analysis of AQP11 protein by western blotting

Animals were killed by CO2 asphyxiation and testes were isolated and snap-frozen in liquid nitrogen. Murine spermatozoa were obtained by cannulation of the vas deferens followed by retrograde luminal perfusion of the cauda epididymis using PBS at the osmolality of murine epididymal fluid (430 mmol/kg). Rat spermatozoa were obtained by dissection and isolation of tubule lobules of the cauda epididymis and release of luminal contents into PBS of osmolality 340 mmol/kg. Collected rodent spermatozoa were washed three times by centrifugation at 600 g for 5 min at 4 °C, counted and stored as pellets of known cell number at −80 °C until analysis. Protein was extracted by vortexing and incubation in 3% SDS in Tris buffer at pH 6.7 for 10 min at room temperature. Testicular tissue pulverisation, protein extraction, protein concentration estimation and separation on 4–12% Bis–Tris precast gels and western blotting were as previously described (Klein et al. 2006), except that each lane was loaded with protein from 10×10⁶ rat spermatozoa, 12×10⁶ murine spermatozoa or 40–80 μg testicular protein after heating at 40 °C for 10 min in the sample buffer. Primary antibody for AQP11 (Alpha-Diagnostics) was used at 1:500 and the secondary antibody (goat anti-rabbit, Sigma A9169) at 1:12 000. To check for specificity of the ECL Plus signals, parallel gel electrophoresis and blotted membranes were processed with primary antibody adsorbed with 33 times excess antigen peptide purchased from Alpha-Diagnostics. Films of blots were analysed using the 1D Gel Analysis Software, LabImage 1D (Kapelan Bio-Imaging, Leipzig, Germany).

Analysis of rodent Aqp11 and human AQP11 expression by RT-PCR using sperm and testicular RNA

Extraction of RNA from rodent testicular tissues was as previously described (Yeung et al. 2009). For human spermatozoa, elimination of any leukocytes by anti-CD45-coated Dynabeads after Percoll washing, the subsequent purification of RNA, quality control and check for any contaminating leukocyte RNA were performed as previously described (Yeung & Cooper 2008). In RT-PCR analysis of these RNAs and commercially purchased human testis and kidney RNAs (BioCat GmbH, Heidelberg, Germany), each sample was processed in parallel with and without the RT as negative control for any possible DNA contamination. Primers pairs (Table 1) were designed to generating one or overlapping sequence segments spanning the entire length of the ORF. PCR products which were generated using FailSafe buffers (Biozym Diagnostik GmbH, Hessisch Oldendorf, Germany) were separated in 2% agarose gel and blotted membranes were processed with primary antibody and blotted membranes were processed with primary antibody adsorbed with 33 times excess antigen peptide purchased from Alpha-Diagnostics. Films of blots were analysed using the 1D Gel Analysis Software, LabImage 1D (Kapelan Bio-Imaging, Leipzig, Germany).

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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