Occludin is concentrated at tight junctions of mouse/rat but not human/guinea pig Sertoli cells in testes

SEIJI MOROI,1,2 MITINORI SAITOU,1 KAZUSHI FUJIMOTO,3 AKIRA SAKAKIBARA,1 MIKIO FURUSE,1 OSAMU YOSHIDA,2 AND SHOICHIRO TSUKITA1

1Departments of Cell Biology, Urology, and Anatomy, Faculty of Medicine, Kyoto University, Sakyo-ku, Kyoto 606, Japan
2Department of Anatomy, Faculty of Medicine, Kyoto University, Sakyo-ku, Kyoto 606, Japan
3Department of Cell Biology, Anatomy, Faculty of Medicine, Kyoto University, Sakyo-ku, Kyoto 606, Japan

The tight junction (TJ) is one mode of cell-to-cell adhesion in epithelial or endothelial cell sheets. It constitutes continuous, circumferential seals around cells, which serve as a physical barrier preventing solutes and water from passing freely through the paracellular spaces. TJs are also thought to play a role as a boundary between the apical and the basolateral plasma membrane domains to create and maintain cell polarity (for reviews, see Refs. 17, 18, 34). In thin-section electron microscopy, the TJ appears as a series of discrete sites of apparent fusion, involving the outer leaflet of the plasma membrane of adjacent cells (10). In freeze-fracture electron microscopy, the TJ appears as a set of continuous, anastomosing intramembranous strands or fibrils in the P face (outwardly facing cytoplasmic leaflet) with complementary grooves in the E face (inwardly facing extracytoplasmic leaflet) (35).

The testis has well-developed TJs between adjacent Sertoli cells in seminiferous epithelia (9). These junctions have been reported to constitute a major part of the so-called “blood-testis barrier” (BTB), which is thought to be involved in the maintenance of a special physiological milieu for spermatogenesis and in the protection of germ cells, especially postmeiotic cells, from the immune system. Although the morphological features of the BTB have been well described in several species (for review, see Ref. 31), information regarding the molecular architecture of the inter-Sertoli TJ is limited (3, 6, 23).

Recently, in addition to TJ-specific peripheral membrane proteins such as ZO-1, ZO-2, cingulin, 7H6 antigen, and symplekin (1, 7, 19, 23, 36, 39), a novel TJ-associated integral membrane protein, occludin, has been identified (2, 14). This molecule contains four transmembrane domains, a long COOH-terminal cytoplasmic domain, a short NH2-terminal domain, two extracellular loops, and an intracellular turn (14). Immunolabeled freeze-fracture replica analyses revealed that occludin is one of the constituents of the TJ strand itself (12, 20, 32). Occludin is possibly associated with the cytoskeleton through a direct interaction with ZO-1 (15).

In immunoblotting, occludin shows a characteristic multiple banding pattern (14, 32). We recently found that occludin is heavily phosphorylated at its serine or threonine residues and that phosphorylation shifts the occludin band upward, resulting in their multiple banding pattern (33). Phosphorylated occludin was resistant to NP-40 extraction and concentrated at the TJ proper. The functional importance of occludin in TJs has been revealed in several recent reports. When Madin-Darby canine kidney (MDCK) cells were transfected with occludin cDNA, they showed elevated transepithelial resistance and an increased number of TJ strands (25). Introduction of a COOH-terminal-truncated occludin into MDCK cells caused the increased paracellular flux and perturbation of the apico-basolateral intramembrane diffusion barrier (4). Furthermore, a synthetic peptide corresponding to the second extracellular loop of occludin disrupted the transepithelial permeability barrier (38).

In the present study, we demonstrated that occludin is present in TJ strands between adjacent Sertoli cells of adult mice but not those of humans or guinea pigs. Furthermore, we analyzed the developmental changes of the expression level and subcellular distribution of occludin in mouse testes.

MATERIALS AND METHODS

Antibodies. Rat anti-mouse occludin monoclonal antibodies (MAbs; MOC37 and MOC60) and rabbit anti-mouse occludin polyclonal antibodies (PAb; F4 and F5) were raised against the cytoplasmic domain of mouse occludin produced in Esherichia coli (32). MOC37 recognizes occludin in various mammalian species, whereas MOC60 is specific for mouse occludin.
occludin. Rabbit anti-rat ZO-1 PAb and mouse anti-rat ZO-1 MAb (T8–754) were raised and characterized previously (22).

Testes samples. Human testes were obtained from orchidectomies for patients with testicular tumors or trauma. Microscopically normal regions of these samples were used. Mice (ddY) and guinea pigs (Hartley) of various ages were purchased from Japan SLC (Shizuoka, Japan).

Immunofluorescence microscopy. Mouse, human, and guinea pig testes were frozen in liquid nitrogen. Sections –10 µm in thickness were cut in a cryostat, mounted on coverslips, and air dried. They were fixed with 95% ethanol for 30 min on ice and then with 100% acetone for 1 min at room temperature. Some frozen sections were fixed with 1% formaldehyde in PBS for 15 min and then soaked in 0.2% Triton X-100-PBS for 15 min at room temperature. Sections were washed three times with PBS, soaked in blocking solution (PBS containing 1% BSA) for 15 min, and then incubated with the first antibody for 1 h. As second antibodies, FITC-conjugated goat anti-rat IgG (Tago, Burlingame, CA), rhodamine-conjugated goat anti-mouse IgG, or rhodamine-conjugated goat anti-rabbit IgG (Chemicon International, Temecula, CA) was used. Sections were washed three times with PBS, mounted in PBS containing 1% p-phenylenediamine and 90% glycerol, and examined under a fluorescence microscope (Axiophot photomicroscope, Zeiss, Thornwood, NY).

SDS-digested freeze-fracture replica labeling technique. The SDS-digested freeze-fracture replica labeling technique was previously described in detail (12, 13). To remove collagenous tissues, decapsulated testes were first incubated and stirred in Hanks' balanced salt solution containing 0.5 mg/ml collagenase (types I and II, Sigma-Aldrich Japan, Tokyo, Japan) at 37°C for 10 min, and small fragments of seminiferous tubules were quickly frozen by contact with a copper block cooled with liquid helium (13). The frozen samples were fractured at –110°C and platinum-shadowed unidirectionally at an angle of 45° in Balzers Freeze Etching System (BAF...
The samples were immersed in sample lysis buffer containing 2.5% SDS, 10 mM Tris·HCl (pH 8.2), and 0.6 M sucrose for 12 h at room temperature, and replicas floating off the samples were washed with PBS. Under these conditions, integral membrane proteins were captured by replicas, and their cytoplasmic domain was accessible to antibodies. Replicas were incubated with anti-occludin MAb (MOC37) for 60 min and then washed several times with PBS. They were incubated with goat anti-rat IgG coupled to 10-nm gold (Amersham International, Buckinghamshire, UK). Replicas were washed with PBS, picked up on Formvar-coated grids, and examined in a Jeol 1200EX electron microscope at an accelerating voltage of 80 kV.

Immunoprecipitation. Occludin was recovered from testes by immunoprecipitation (IP) as previously described (33) with a slight modification. After careful removal of the epididymides, testes were minced and homogenized in 1 µl of ice-cold NP-40-IP buffer [25 mM HEPES-NaOH (pH 7.4), 150 mM NaCl, 4 mM EDTA, 25 mM NaF, 1% NP-40, 1 mM Na3VO4, 1 mM 4-amidinophenylmethanesulfonfonyl fluoride hydrochloride (APMSF), 10 µg/ml leupeptin, and 10 µg/ml aprotinin] using a Kontes homogenizer (Kontes, Vineland, NJ). The homogenate was gently rotated for 30 min at 4°C and centrifuged at 10,000 g for 30 min. After collection of the supernatant as the “NP-40-soluble fraction,” the pellet was resuspended by sonication in 100 µl of SDS-IP buffer [25 mM HEPES (pH 7.5), 4 mM EDTA, 25 mM NaF, 1% SDS, 1 mM Na3VO4, 10 µg/ml leupeptin, and 10 µg/ml aprotinin]. Then 900 µl of NP-40-IP buffer were added, followed by rotation for 30 min at 4°C. After centrifugation at 10,000 g for 30 min, the supernatant was collected as the “NP-40-insoluble fraction.” Occludin was not detected in the pellet by immunoblotting. The mixture of a half-volume of each fraction was designated “total fraction.” Anti-occludin antiserum (4 µl; mixture of F4 and F5) and a 15-µl bed volume of recombinant protein G-Sepharose 4B (Zymed Laboratories, South San Francisco, CA) were added to each fraction and rotated for 4 h at 4°C. Beads were washed five times with 1 ml of NP-40-IP buffer and boiled in SDS-PAGE sample buffer containing 1 mM Na3VO4 to elute the immunoprecipitates. Samples were separated by gel electrophoresis, followed by immunoblotting using anti-occludin MAb MOC60.

Alkaline phosphatase treatment. Alkaline phosphatase (AP) treatment was performed as previously described (33). After IP, beads were washed three times with 1 ml of NP-40-IP buffer and then three times with 1 ml of AP buffer [50 mM Tris·HCl (pH 8.2), 50 mM NaCl, 1 mM MgCl2, 1 mM dithiothreitol, and 1 mM APMSF]. They were then resuspended in 200 µl of AP buffer containing 20 U of calf intestine AP (Takara Shuzo, Ohtsu, Japan). To some aliquots, phosphatase inhibitors (25 mM NaF, 100 mM β-glycerophosphate, 4 mM EDTA, and 1 mM Na2VO4) were also added. After a 1-h incubation at 30°C, beads were washed and processed for SDS-PAGE as described above.

Immunoblotting. Samples were resolved by one-dimensional SDS-PAGE according to the method of Laemmli (24) and were electrophoretically transferred onto nitrocellulose membranes (Protran, 0.45 mm pore size; Schleicher and Schuell, Dassel, Germany). They were then incubated with the first antibody. For antibody detection, a blotting detection kit (Amersham) was used.

RESULTS

Occludin in adult mouse testis. When frozen sections of adult mouse testes were immunofluorescently stained with anti-occludin MAb, intense signals were detected.
in a linear fashion from the most basal region of lateral membranes of adjacent Sertoli cells, where Tj's were reported to be well developed (Fig. 1, A and B). Endothelial cells of microvessels were also intensely stained. The localization of occludin in Sertoli cells was then analyzed at the electron microscopic level by the SDS-digested freeze-fracture replica labeling technique (Fig. 1C). As reported previously in other tissues, anti-occludin MAb MOC37 specifically labeled the TJ strand itself in Sertoli cells.

Next, occludin expression in adult mouse testis was examined by immunoblotting with anti-occludin PAb (Fig. 2). When the same amounts of total protein from mouse kidney and testis were resolved by SDS-PAGE followed by immunoblotting, a band of ~65 kDa was detected in both tissues, and the level of expression of occludin in the testis was lower than that in the kidney (Fig. 2A). Occludin was reported to be resolved as multiple bands in SDS-PAGE, and slowly moving bands were concentrated in the NP-40-insoluble fraction in cultured epithelial cells (33). Adult mouse testes were homogenized in the presence of 1% NP-40, and both the NP-40-soluble and -insoluble fractions were immunoprecipitated with anti-occludin PAb followed by immunoblotting with anti-occludin MAb MOC60 (Fig. 2B). Under these conditions, the multiple banding pattern of occludin was clearly identified in mouse adult testis, and slowly moving bands resistant to the NP-40 extraction were also seen in the testis. AP analyses showed that phosphorylation shifted the occludin bands upward and made occludin resistant to NP-40 extraction also in the testis (Fig. 2C).

Developmental changes of occludin in mouse testis. We next examined the developmental changes of occludin distribution in mouse testis. Frozen sections of testis from 1-, 2-, 3-, and 7-wk-old mice were stained with anti-mouse occludin MAb MOC37. In the testis of 1-wk-old mice, occludin was distributed discontinuously from apical to basal regions of cell-cell borders of adjacent Sertoli cells (Fig. 3A). With the development of testis, i.e., spermatogenesis, occludin was gradually restricted to the basal part of the lateral membranes of the epithelia, where Tj's between Sertoli cells were reported to be located in the adult testis (Fig. 3, B–D). When these samples were doubly stained with anti-ZO-1 PAb and MOC37, occludin was mostly colocalized with ZO-1, but some ZO-1-positive and occludin-negative structures were detected in the interstitium (Fig. 4).

It was recently reported that the phosphorylation level of occludin correlated well with the development of Tj's in epithelial cells (33). We then examined the developmental changes of the phosphorylation level of occludin in mouse testis. As shown in Fig. 5A, even in...
1-wk-old mice, highly phosphorylated occludin was clearly detected in the NP-40-insoluble fraction. As development of the testis proceeded, an amount of both NP-40-soluble and -insoluble occludin increased. Judged from the banding patterns of NP-40-insoluble occludin, the relative content of the most slowly moving band (i.e., most heavily phosphorylated occludin) gradually increased until 4 wk and then decreased with development (Fig. 5B).

Species differences of occludin in testis. Unexpectedly, when guinea pig testes were immunofluorescently stained with anti-mouse occludin MAb MOC37 (A and D) and anti-ZO-1 PAb (B and E), C and F: phase-contrast images. In both cases, occludin was mostly colocalized with ZO-1 in seminiferous tubules, but ZO-1 was also detected in occludin-negative sites in interstitium. Asterisks represent center of each tubule. Bar, 25 \( \mu \)m.

![Image](http://ajpcell.physiology.org/)

**Fig. 4.** Colocalization of occludin and ZO-1 in mouse testis. Frozen sections of testis from 1 (A–C) and 6-wk-old (D–F) mice were doubly stained with anti-mouse occludin MAb MOC37 (A and D) and anti-ZO-1 PAb (B and E). C and F: phase-contrast images. In both cases, occludin was mostly colocalized with ZO-1 in seminiferous tubules, but ZO-1 was also detected in occludin-negative sites in interstitium. Asterisks represent center of each tubule. Bar, 25 \( \mu \)m.
Furthermore, we examined the expression level of occludin in guinea pig testis by immunoblotting with anti-occludin PAb F4 (Fig. 8). When the same amounts of total protein from mouse or guinea pig kidney and testis were resolved by SDS-PAGE followed by immunoblotting, in mouse kidney and testis and guinea pig kidney, an intense occludin band of ~65 kDa was detected. By contrast, in guinea pig testis, only a weak occludin band that appeared to be derived from endothelial cells was detected. Among the several species examined, occludin was detected in the seminiferous epithelia of mouse and rat testes but not detected in those of guinea pig or human testes.

**DISCUSSION**

When some substances, such as dyes, are administered via blood vessels, they do not so readily reach the interior of the seminiferous tubules (31), suggesting the existence of a unique barrier system in the testes, which is now called the BTB. The BTB is believed to play a crucial role in maintaining a favorable milieu for spermatogenesis. Many types of tracer studies have demonstrated that well-developed TJs of Sertoli cells primarily constitute the BTB (5, 8, 16, 30, 31, 37), although other structures, such as myoid cells around the seminiferous tubules (9, 11) or some microvessels in the interstitium, were also reported to be involved in the BTB to some extent.

In the present study, we demonstrated that occludin is present in the TJ strands of murine Sertoli cells, and we followed the developmental changes in its subcellular distribution and expression level. Occludin in the early prepubertal period was located diffusely on the lateral membranes of Sertoli cells and later gradually became concentrated in their basal part. These findings were consistent with previous observations of TJs in the developing testes of mice as well as other species by freeze-fracture electron microscopy (26–29). In the early prepubertal period, short and blind-ended TJ
Fig. 6. Lack of occludin staining in guinea pig Sertoli cells. Frozen sections of testes from adult guinea pig (A–F) were doubly stained with anti-ZO-1 MAb T8–754 (B and E) and anti-mouse occludin MAb MOC37 (A) or anti-mouse occludin PAb F4 (D). C and F: phase-contrast images. As controls, frozen sections of guinea pig kidney were singly stained with anti-mouse occludin MAb MOC37 (G) or anti-mouse occludin PAb F4 (H). In testes, occludin was not detected from ZO-1-positive seminiferous epithelia (arrows) and was detected only in ZO-1-positive structures in interstitium (arrowheads). In kidney, TJ's were clearly stained with MAb MOC37 (G) as well as PAb F4 (H). G, glomerulus. Bar, 25 µm.
strands were reported to be scattered around the lateral membranes of Sertoli cells, and the continuous network of TJ strands was gradually developed in their basal part. Interestingly, even in testes with poorly developed TJs, the heavily phosphorylated and NP-40-insoluble occludin was significantly detected, although in small amounts. With the development of the testis, the de novo synthesis of occludin in Sertoli cells may be upregulated, resulting in a relative increase of non- or less-phosphorylated occludin in both NP-40-soluble and -insoluble fractions. Of course, to correctly interpret the data on the phosphorylation of occludin in testis, we must carefully evaluate the contribution to the immunoblots of occludin coming from the endothelium of vessels.

Another issue to be discussed here is the relatively high level of occludin expression in testis endothelial cells. Although the microvessels of the testis are not considered to be directly involved in the BTB (11, 30), their permeability was reported to be lower than that of blood vessels in nontestis and nonneuronal tissues (21). Moreover, they express some of the marker molecules associated with barrier properties detected in brain microvessels (21). Therefore our results are consistent with the recent report by Hirase et al. (20) that the level of occludin expression correlated well with the tightness of endothelial sheets.

We conclude that occludin is an important structural and functional component in well-developed TJ strands of the mouse testis, as previously shown in other tissues. To our surprise, however, occludin was immunofluorescently detected in mouse and rat testis, whereas it was not detectable in human or guinea pig testis. Of course, in these occludin-negative testes, ZO-1 was concentrated in inter-Sertoli junctions, where TJ strands were reported to be well developed. The possibility was excluded that our anti-occludin MAbs or PAbs did not recognize human or guinea pig occludin, since these antibodies clearly stained TJs in kidney epithelial cells of these species. Furthermore, immunoblot analyses also suggested the absence of occludin in Sertoli cells of guinea pig testis. It is generally accepted that the intensity of immunofluorescent staining with anti-occludin antibodies is correlated well with the number of TJ strands detected by freeze-fracture electron microscopy (14, 32), but there were two exceptions. First, in MDCK cells transfected with COOH-terminal-truncated occludin, TJ strands were observed to continuously surround each epithelial cell, but occludin was not detected from ZO-1-positive seminiferous epithelia (arrows). In kidney, TJ strands were clearly stained with MAb MOC37 (D). Asterisk represents center of each tubule. Bar, 25 µm.

![Fig. 7. Lack of occludin staining in human Sertoli cells. Frozen sections of testes from adult humans (A–C) were doubly stained with anti-mouse occludin MAb MOC37 (A) and anti-ZO-1 MAb T8–754 (B). C: phase-contrast images. As controls, frozen sections of human kidney were singly stained with anti-mouse occludin MAb MOC37 (D). In tests, occludin was not detected from ZO-1-positive seminiferous epithelia (arrows). In kidney, TJ strands were clearly stained with MAb MOC37 (D). Asterisk represents center of each tubule. Bar, 25 µm.](http://ajpcell.physiology.org/)

![Fig. 8. Immunoblots of whole lysate of adult mouse or guinea pig kidney (K) and testis (T) with anti-occludin PAb F4 (30 µg proteins in each lane). Compared with expression level of occludin in mouse kidney and testis and guinea pig kidney, that in guinea pig testis was much lower. Note that molecular mass of mouse occludin is slightly larger than guinea pig occludin (see Ref. 32).](http://ajpcell.physiology.org/)
Concentrated at cell-cell borders in a discontinuous dotted pattern (4). Second, although occludin was highly concentrated at cell-cell borders of brain endothelial cells, where TJ’s were reported to be well developed, it was hardly detected in nonbrain endothelial cells, where TJ strands were observed (20). The identification of TJ strands lacking occludin in human or guinea pig testis in the present study represents clearer exceptions. We were led to speculate that, in guinea pigs or humans, there are some testis-specific isoforms of occludin. Inasmuch as all MAbs and PABs used here recognized the COOH-terminal cytoplasmic tail of occludin, it is possible that some alternatively spliced form of occludin specifically occurs in guinea pig or human testis. Alternatively, it is also possible that there are some other TJ-associated integral membrane proteins which differ from occludin. These putative novel TJ-associated integral membrane proteins must fulfill the following requirements: 1) they constitute TJ strands without occludin; 2) they associate with ZO-1 directly or indirectly; and 3) they may interact with occludin, as seen in TJ strands of mouse or rat testis. Studies are currently under way in our laboratory to evaluate these speculations.

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