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Restricted Localization of Claudin-16 at the Tight Junction in the Thick Ascending Limb of Henle's Loop Together with Claudins 3, 4, and 10 in Bovine Nephrons

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ABSTRACT. Claudin-16 is one of the tight junction protein claudins and has been shown to contribute to reabsorption of divalent cations in the human kidney. In cattle, total deficiency of claudin-16 causes severe renal tubular dysplasia without aberrant metabolic changes of divalent cations, suggesting that bovine claudin-16 has some roles in renal tubule formation and paracellular transport that are somewhat different from those expected from the pathology of human disease. As the first step to clarify these roles, we examined the expression and distribution of claudin-16 and several other major claudin subtypes, claudins 1–4 and 10, in bovine renal tubular segments by immunofluorescence microscopy. Claudin-16 was exclusively distributed to the tight junction in the tubular segment positive for Tamm-Horsfall glycoprotein, the thick ascending limb (TAL) of Henle's loop, and was found colocalized with claudins 3, 4, and 10. This study also demonstrates that bovine kidneys possess segment-specific expression patterns for claudins 2–4 and 10 that are different from those reported for mice. Particularly, distribution of claudin-4 in the TAL and distal convoluted tubules was characteristic of bovine nephrons as were differences in the expression patterns of claudins 2 and 3. These findings demonstrate that the total lack of claudin-16 in the TAL segment is the sole cause of renal tubular dysplasia in cattle and suggest that the tight junctions in distinct tubular segments including the TAL have barrier functions in paracellular permeability that are different among animal species.

KEY WORDS: cattle, claudin-16, paracellular pathway, renal tubule, tight junction.

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Tight junctions (TJs) are located at the apicalmost region of lateral membranes of epithelial cells, and play a central role in sealing the intercellular space in epithelial cellular sheets. TJs create the primary barrier to the diffusion of solutes and water through the paracellular pathway and maintain cell polarity as a boundary between the apical and basolateral plasma membrane domains [7, 26, 29, 34]. On freeze-fracture electron microscopy, TJs are visualized as a continuous anastomosing network of intramembranous particle strands, *i.e.*, TJ strands, and complementary grooves [28]. The major components of TJ strands are the integral membrane proteins occludin and claudins, all of which have four transmembrane spans and two intervening extracellular loops [19]. Claudins consist of a family of more than 20 homologous subtypes, and they show tissue-specific and segment-specific distribution patterns in epithelia [19, 29] such as those of the gastrointestinal tract [23] and renal tubules [15].

Among these claudin proteins, claudin-16, formerly paracellin-1, has been shown to be responsible for inherited disease. Various mutations of the *CLDN16* gene were reported to be the cause for familial hypomagnesemia with hypercalciuria and nephrocalcinosis (FHHNC) in the human [27]. Simon *et al.* [27] first reported that claudin-16/paracellin-1 was exclusively expressed in the thick ascending limb (TAL) of Henle's loop where reabsorption of Mg^{2+} and Ca^{2+}, for 60 to 70% and 20% of total contents in the glomerular filtrate, respectively, occurred via the paracelluar pathway [16, 31]. Since FHHNC has been characterized by renal Mg^{2+} and Ca^{2+} wasting, claudin-16 is likely to form aqueous pores that function as the paracelluar pathway for Mg^{2+} and Ca^{2+} in claudin-based TJs [3, 27]. A recent report by Hou *et al.* [11] clearly demonstrated that claudin-16/paracellin-1 modulated the ion selectivity of the TJ by selectively increasing the permeability of Na^+ with no effect on Cl^- when transfected into LLC-PK1 epithelial cells, and suggested that a loss of claudin-16/paracellin-1 function in the TAL could lead to losing the lumen-positive potential as the driving force for the reabsorption of Mg^{2+}.

Claudin-16 is also responsible for renal tubular dysplasia with interstitial nephritis in Japanese black cattle [9, 20]. This disease is inherited in an autosomal recessive mode and characterized by renal failure, growth retardation, and early death due to renal dysfunction with a high incidence. Affected animals possess a 37-kb or 56-kb deletion in the *CLDN16* gene [9, 10], resulting in the total lack of claudin-16 protein. Kidneys from one-to two-month-old calves affected revealed the presence of immature renal tubules without lumina accompanied by moderate interstitial fibrosis, whereas the numbers and distribution of the glomeruli appeared to be nearly normal. As the animal grows, the progress of glomerular and tubular atrophy with interstitial fibrosis and lymphocytic infiltration becomes evident [24]. Tubular atrophy, immature glomeruli, and interstitial fibrosis are also observed in the kidneys from FHHNC patients

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although details have not been reported [35]. These histological findings suggest that the primary lesion of bovine renal dysplasia is an anomalous defect in differentiation of renal tubular epithelial cells and that claudin-16 therefore has some pivotal roles in differentiation and formation of renal tubule segment(s) in addition to the function in paracelluar transport. Interestingly, in contrast to FHHNC, decreases in serum Mg^{2+} and Ca^{2+} concentrations are not apparent in cattle with claudin-16 deficiency, while a reduction in renal reabsorption rate has been suggested for Mg^{2+} [21]. This suggests that there is a compensatory system(s) for metabolism of these divalent cations in the absence of claudin-16 or that bovine claudin-16 may have functions in paracellular transport of Mg^{2+} and Ca^{2+} distinct from those of the human counterpart despite their high homology in amino acid sequences [9, 20].

To explore the physiological and pathological significance of bovine claudin-16 in renal tubular formation and reabsorption of divalent cations, it is essential to determine the distribution of this claudin subtype in the bovine kidney. In addition, it is also important to determine the expression and distribution of other claudin species in the segment to which claudin-16 is localized, because heterogeneous claudins form individual TJ strands as heteropolymers and claudin molecules adhere to each other in both homotypic and heterotypic manners [4] at the cell-cell interface to generate consequential variations in the tightness of individual paired TJ strands [5, 30]. A distribution pattern of claudin-16 restricted to the TAL, consistent with the situation in the human kidney [27], was also demonstrated in mouse nephron segments where several other claudin isoforms, including claudin-3, -10, and -11, were also found in TJs together with claudin-16 [15]. However, no information about claudin-16 in the bovine kidney has been available till date.

In the present study, we examined expression of claudin-16 and several other major claudin subtypes, claudins 1–4 and 10, in specified tubular segments of normal bovine kidneys by immunofluorescence microscopy using antibodies specific to each claudin and segment marker proteins. Care was taken to ensure the specificities of the antibodies to claudins 16 and 1–4 by utilizing fusion proteins to confirm the disappearance of specific reactions when present. The results obtained demonstrate that claudin-16 has a distribution restricted to the tight junction in a specific tubular segment along with some other claudin subtypes.

MATERIALS AND METHODS

Tissue collection and preparation: Kidneys were obtained from healthy adult Japanese black cattle that were determined to be free of type-1 and type-2 *CLDN16* mutations by the methods described previously [9, 10] in our laboratory and cut into slices. Slices were frozen in liquid nitrogen with or without OCT compound (Sakura Finetechnical Co., Tokyo, Japan) for immunohistochemistry and RNA analyses, respectively.

Antibodies: Rabbit anti-bovine claudin-16 polyclonal antibody (pAb) was kindly provided by Drs. Akiko Takasuga and Yoshikazu Sugimoto (Shirakawa Institute of Animal Genetics, Fukushima, Japan). Rabbit pAbs to human claudin-1, -2, -3, -10, and -11 and mouse monoclonal antibody (mAb) to human claudin-4 and occludin were purchased from Zymed Laboratories (South San Francisco, CA, U.S.A.). Rabbit anti-aquaporin-1 (AQP1), anti-chloride channel-K (CLC-K), anti-Tamm-Horsfall glycoprotein (THP), and goat anti-AQP2 pAbs were purchased from Chemicon International (Temecula, CA, U.S.A.), Alomone Laboratories (Jerusalem, Israel), Biomedical Technologies (Stoughton, MA, U.S.A.), and Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.), respectively. Rabbit anti-rat thiazide-sensitive Na-Cl cotransporter (NCCT) pAb [14] was kindly provided by Dr. M. A. Knepper (Laboratory of Kidney and Electrolyte Metabolism, National Institute of Health, Bethesda, MD, U.S.A.). Rabbit anti-dog Na, K-ATPase pAb was described previously [12].

cDNA cloning and construction of plasmids: Total RNA was extracted from bovine liver, kidney, and small intestine using the SV total RNA isolation system (Promega, Madison, WI, U.S.A.). First strand cDNA was synthesized using 1 μg of total RNA in the SuperScript^{TM} first-strand synthesis system for RT-PCR (Invitrogen, Gaithersburg, MD, U.S.A.). cDNA fragments for bovine claudin-1, -2, -3, and -4 were amplified by RT-PCR using primers designed based on cDNA fragments of human or murine counterparts. The amplified cDNAs were cloned into pCRII-TOPO vector using a TOPO TA clonig kit (Invitrogen), and the nucleotide sequences were determined using a CEQTM 8800 DNA analysis system (Beckman Coulter, Fullertion, CA, U.S.A.). We searched the GenBank database for partial cDNA sequences, and found 13 independent bovine expressed sequence tag (EST) clones possessing nucleotide sequences identical to those obtained for bovine claudins 1–4. The EST clones were CB454425, CB455833, and BF040486 for claudin-1, AV604013, AV605969, BE480970, and BE667438 for claudin-2, CB419176, CB445175, and CB454984 for claudin-3, and BF073399, BI775226, and CK944271 for claudin-4. Since these overlapping clones appeared to contain the entire coding region of each claudin molecule, we amplified cDNAs for coding regions by PCR using primer pairs prepared based on the sequences of appropriate EST clones (Table 1). The amplified cDNAs were cloned into pCRII-TOPO vector and the sequences were determined. To generate recombinant proteins of the C-terminal cytoplasmic domains of bovine claudins 1–4 and 16 fused to glutathione S-transferase (GST), the corresponding nucleotide sequences of bovine claudins were amplified by PCR, cloned into pGEX-6P-1 vector (Amersham Biosciences, Piscataway, NJ, U.S.A.), and the recombinant proteins were isolated by affinity purification with glutathione-uniflow resin (Clontech, Palo Alto, CA, U.S.A.).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting: GST-fusion proteins of the cytoplasmic portions of bovine claudin species were subjected to SDS-PAGE. Separated proteins were stained

Table 1. Primers used to isolate cDNA clones for bovine claudins 1–4

Subtype	Sequence	EST clone
Claudin-1	F, 5'-TGCACCTGCTGCCCCTGAGCC-3'	CB454425
	R, 5'-ATTCCTTACGATAGAGGGGAG-3'	CB455833
Claudin-2	F, 5'-GAAATGAGGGATTAGAGGCCG-3'	AV604013
	R, 5'-TGGCTTCTGGAGGGGGGGTGG-3'	BE667438
Claudin-3	F, 5'-CGCCGCCCTTGTGTCCGTCCG-3'	CB419176
	R, 5'-GAGGAGGGCTAGCAGCTGGGG-3'	CB454984
Claudin-4	F, 5'-TGCGAAGTAGCCGTCCGCGGC-3'	BF073399
	R, 5'-CCATGGTCCACTGAGCACCCACTC-3'	BI775226

F and R indicate the forward and the reverse primers, respectively.
Nucleotide sequences of primer pairs to isolate cDNA clones for coding regions of bovine claudins 1–4 and the GenBank accession numbers of the EST clones containing the primer sequences are presented.

with Coomassie brilliant blue R-250 or transferred to a polyvinylidene difluoride membrane followed by immunoblotting for detection of claudin polypeptides using the ECL chemiluminescence detection system (Amersham Biosciences, Buckinghamshire, UK).

Immunofluorescence microscopy: Pairs of serial frozen sections of the kidney were prepared. One of the sections was incubated with the appropriate anti-claudin pAb and the other was reacted with a pAb to one of the segment markers. For the control experiment, the anti-claudin antibody was incubated with excess amounts of appropriate GST-fusion proteins containing the C-terminal cytoplasmic peptide prior to incubation with tissue sections. Sections were then washed three times with phosphate-buffered saline (PBS), followed by incubation for 30 min with secondary antibodies conjugated with AlexaFluor 568 or 488 (Molecular Probes, Inc., Eugene, OR, U.S.A.). After washing with PBS, sections were embedded in ProLong antifade reagent (Molecular Probes, Inc.) and examined under an ECLIPSE E800 microscope (Nikon, Tokyo, Japan) or a Zeiss LSM 5 PASCAL confocal laser-scanning microscope (Carl Zeiss Japan, Tokyo, Japan). In some experiments, tissue sections were double-stained with anti-claudin-4 mAb and the appropriate pAb (Fig. 4).

Individual nephron segments were identified by the use of antibodies to marker proteins specific to each segment based on previous studies: AQP1 for proximal tubules and thin descending limbs [2], CLC-K for thin ascending limbs [32, 36], THP for the TAL [8], NCCT for distal convoluted tubules [14], and AQP2 for collecting ducts [6].

RESULTS

Cloning of cDNAs encoding bovine claudins 1–4, specificities of antibodies and detection of claudin proteins: To prepare expression plasmids and GST-fused proteins for judging the specificities of commercially available antibodies to bovine claudins, we newly isolated cDNAs encoding major claudins 1–4.

The amino acid sequences of bovine claudins 1–4 deduced from their cDNAs are shown in Fig. 1A with that of bovine claudin-16, and are highly homologous to those of their human and murine counterparts. The molecular masses of claudins 1–4 and 16 are 22.8, 24.5, 23.3, 22.1, and 26.1 kDa, respectively. Multiple sequence alignments (Fig. 1A) also indicated that the amino acid sequences of the C-terminal cytoplasmic tail, the region used as the antigen for production of antibodies, were fairly diverse among different bovine claudin species.

Immunoblotting analysis of the GST-fused C-terminal tail regions of claudin-1~-4 and -16 (GST-claudin-16Ct, etc.) demonstrated that each antibody used in this study specifically recognized the corresponding claudin subtype (Fig. 1B). In immunofluorescence microscopy, claudin-16 protein was found as lines or dots near the apical surface of tubular epithelial cells, consistent with signals for occludin, a ubiquitous TJ protein, demonstrating that claudin-16 colocalized with occludin at the TJ (Fig. 1C). Fluorescent signals were specific to claudin-16, since the signals disappeared when sections were reacted with the anti-claudin-16 pAb in the presence of GST-claudin-16Ct but not in the presence of GST alone. Likewise, specificities in reactivity of antibodies to claudins 1–4 were also verified (data not shown).

Segment-specific expression of claudin-16 and claudins 1–4 and 10 in bovine nephrons: Expression of claudin-16 in different tubular segments was examined by immunofluorescence microscopy (Fig. 2). Because of the limited availability of antibodies to segment-specific marker proteins applicable to double staining of the tissue section in combination with the antibody to each claudin subtype, most of the present studies on distribution of claudins were performed on pairs of serial frozen sections, one of which contained the tubular segments corresponding to those in the other section. One of the sections was reacted with the appropriate anti-claudin antibody and the other one was stained for the marker protein.

In the cortex, claudin-16 was found at the cell-cell junctional areas of epithelial cells in some tubules. Tubular segments carrying positive signals of AQP1, NCCT, or AQP2, that is proximal tubules, distal convoluted tubules, and collecting ducts, respectively, had no specific signals corresponding to claudin-16 (Figs. 2A, 2E, and 2F). In contrast, tubules with positive signals for THP and claudin-16 on

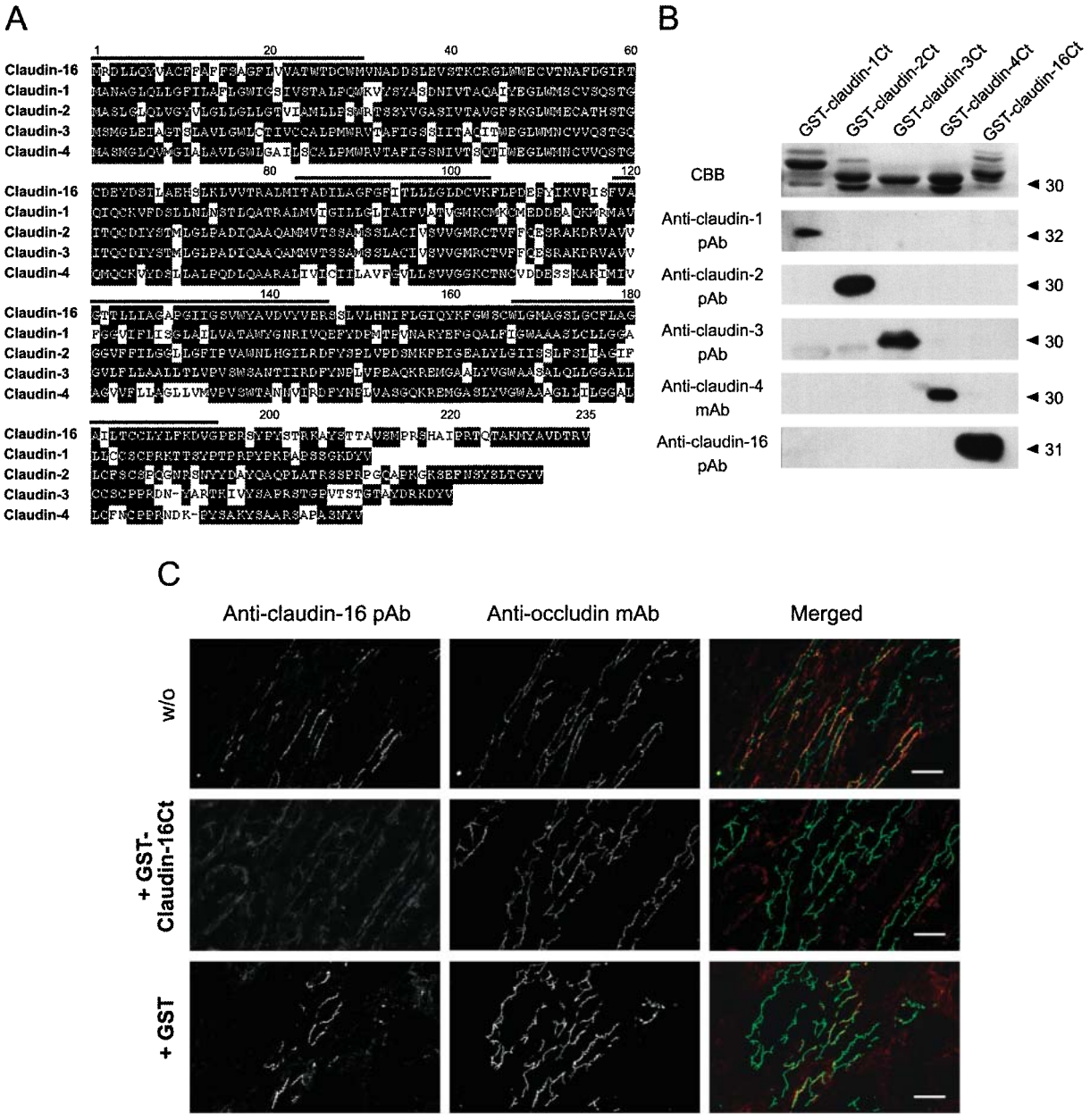

Fig. 1. Amino acid sequences and detection of bovine claudins. A, amino acid sequences of bovine claudin-16 and claudins 1–4 deduced from their cDNA sequences are presented. Bars indicate putative membrane-spanning regions depicted based on previous reports (19). Amino acid residues of each claudin subtype that are conserved among cattle, humans, and mice are highlighted with black backgrounds. The amino acid sequences of C-terminal cytoplasmic domains are fairly diverse. The nucleotide sequences of cDNA for bovine claudins 1–4 determined in the present study have been deposited in the GenBank with accession numbers AB178476, AB115779, AB115781, and AB185928 for claudins 1–4, respectively. B, immunoblotting analysis of GST-fused C-terminal peptides of claudin-16, and claudins 1–4 (GST-claudin-16Ct, etc.). GST-fused recombinant proteins were separated by SDS-PAGE followed by staining with the Coomassie brilliant blue (CBB) or immunoblotting with appropriate antibodies. Apparent molecular masses of each protein are indicated in kDa. C, immunofluorescence detection of bovine claudin-16 in kidney cryosections with anti-claudin-16 pAb. Frozen sections were reacted with the anti-claudin-16 pAb (red) and anti-occludin mAb (green) in the absence (*w/o*) and presence of GST-claudin-16Ct (*+GST-claudin-16Ct*) or GST alone (*+GST*). Merged images represent colocalization of claudin-16 and occludin (*w/o* and *+GST*). Bars = 20 μm.

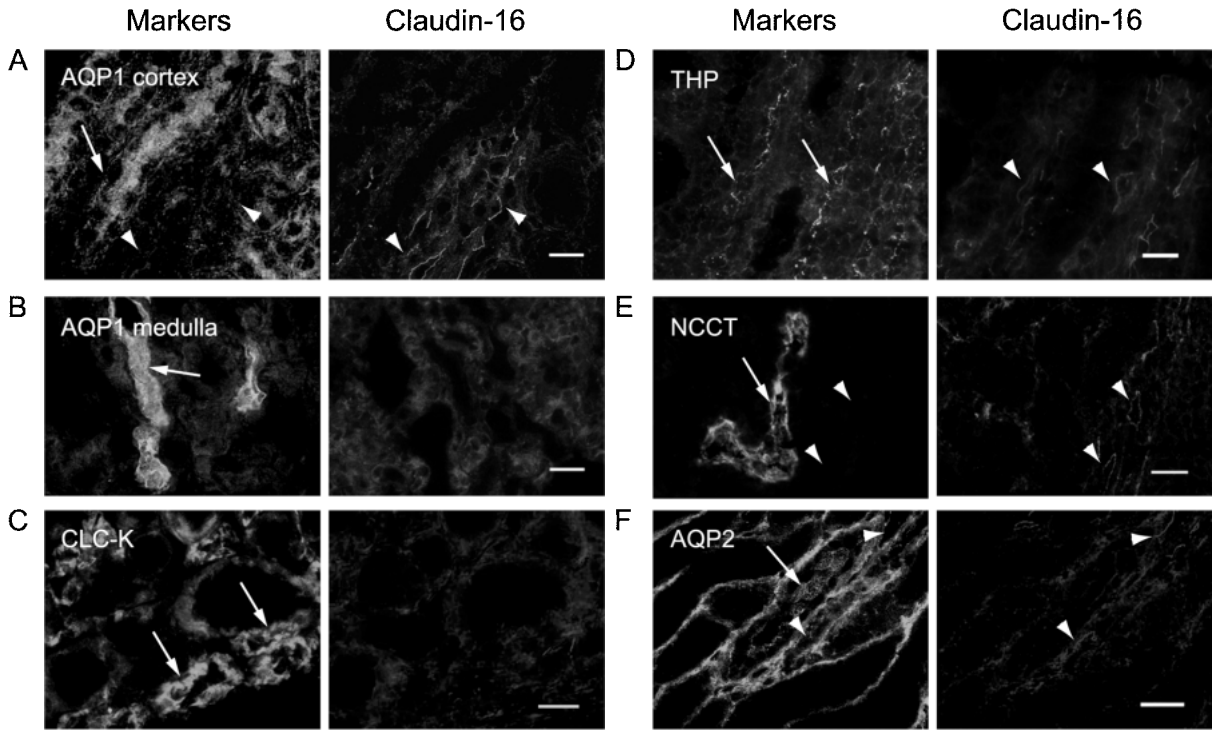

Fig. 2. Segment-specific expression pattern of claudin-16 in bovine nephrons. One of each pair of serial frozen sections was reacted with the pAb for segment markers, AQP1 (*A* and *B*), CLC-K (*C*), THP (*D*), NCCT (*E*), and AQP2 (*F*), and the other was visualized with the anti-claudin-16 pAb (*Claudin-16*). Although the anti-AQP2 pAb used in this study gave immunospecific signals when reacted with murine renal tissue sections, it showed unexpected staining of connective tissues of basement membranes in frozen sections from bovine kidneys presumably due to some non-specific reaction. Specific staining by this AQP2 pAb is observed at the apical surface of some tubules with relatively large lumen, the collecting duct (*F*, also see Figs. 3G and 3K). Arrows indicate tubules identified with segment markers and arrowheads indicate signals of claudin-16 and corresponding regions in the section for marker proteins. Scale bars = 20 μm.

serial sections were coincident with each other, indicating that claudin-16 was expressed in the TAL region (Fig. 2D). In the medulla, some tubules exhibited distribution of claudin-16 at the apical regions of epithelial cells. These tubules were positive for THP, but were negative for AQP1 (Fig. 2B), CLC-K (Fig. 2C), and AQP2 (data not shown), indicating that claudin-16 was present in the TAL segment but not in thin descending and ascending limbs of Henle's loop and collecting ducts.

The expression and distribution of claudins 1–4 and 10 were also analyzed. As demonstrated in Fig. 3A, claudin-1 was concentrated at the apical regions of epithelial cells of Bowman's capsule. Claudin-2 was localized at both the apical and basolateral regions of epithelial cells of AQP1-positive tubules in the cortex (Fig. 3B) and medulla (Fig. 3C), that is, the proximal tubule and thin descending limb, respectively. Claudin-3 was detected in epithelial cells of Bowman's capsule (Fig. 3D) as observed for claudin-1. Claudin-3 was also detected at the TJ region in the distal nephron involving the TAL, distal convoluted tubules, and collecting ducts, which were positive for THP, NCCT, and AQP2, respectively (Figs. 3E-3G). Moreover, claudin-4 was found in distal nephrons including CLC-K-positive tubules in the inner medulla (thin ascending limb), the TAL, distal convoluted tubules, and collecting ducts with both apical and basolateral distributions in all of these segments (Figs. 3H-3K). All the signals for claudins 1–4 disappeared or were markedly reduced to the background level in the serial sections reacted with each antibody in the presence of the appropriate GST fusion proteins described above, indicating that the signals obtained were specific to each claudin subtype.

In addition, claudin-10 was visualized at the apical side of epithelial cells of the tubules positive for AQP1 in the medulla (Fig. 3L) but not in the cortex (data not shown). Claudin-10 was also detected in the cells of THP-positive tubules with apicalmost distribution (Fig. 3M). These findings indicated the presence of claudin-10 in the thin descending limb and the TAL of Henle's loop, although we found no evidence to show specificity in reactivity of the anti-claudin-10 with bovine counterpart. We could not detect immunofluorescent signals for claudin-11, a claudin subtype that has been shown to localize in the TAL of mouse nephrons [15], when the sections were reacted with an anti-claudin-11 antibody that exhibited weak but significant signals in mouse tissues (data not shown). These data

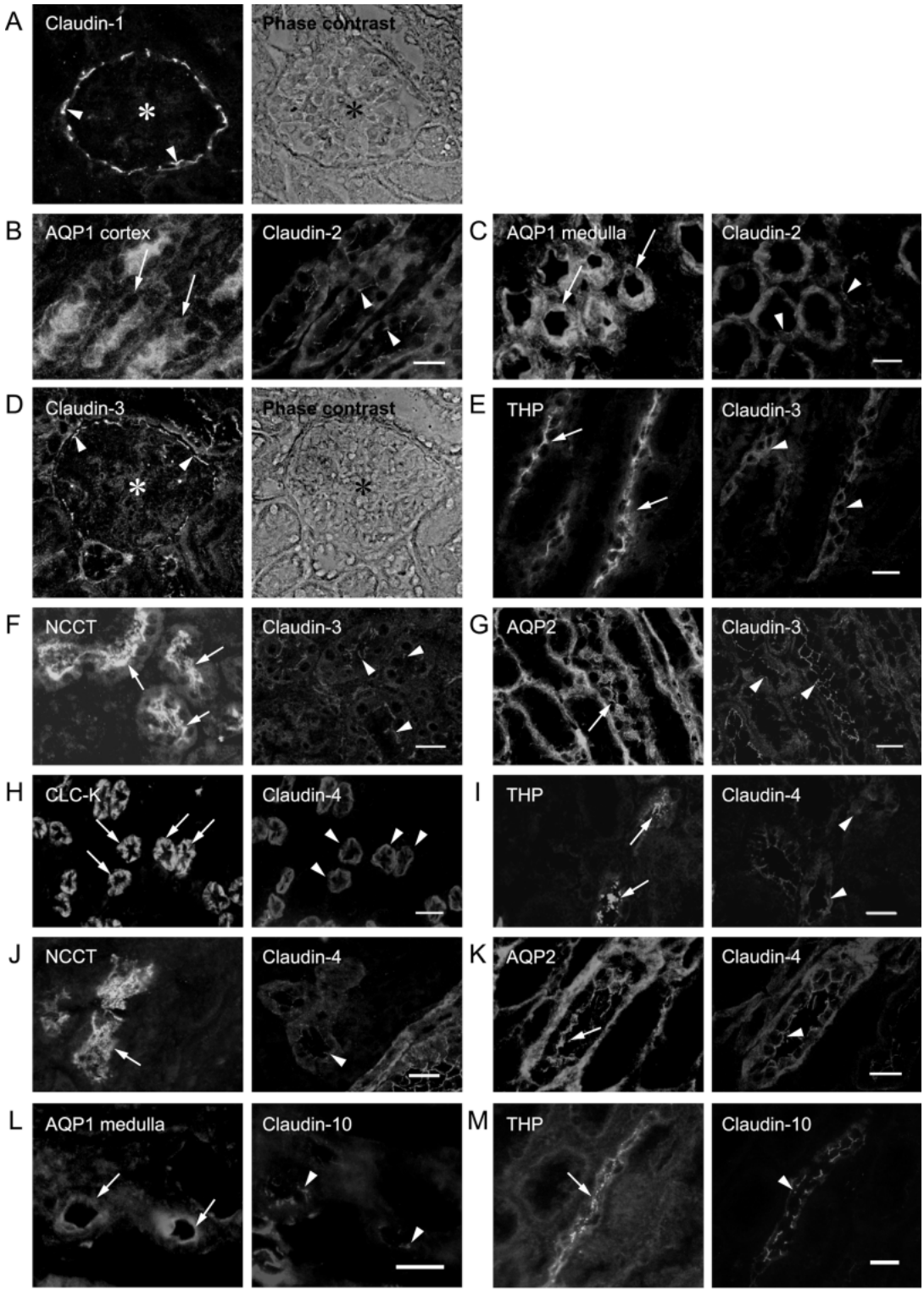

Fig. 3. Segment-specific expression of claudins 1–4 and 10 in bovine nephrons. Claudins 1–4, 10 and segment markers were detected by immunofluorescence microscopy in serial frozen sections. To reveal the presence of claudin-1 and claudin-3 in Bowman's capsules, corresponding phase-contrast images are shown (*A* and *D*). Asterisks indicate glomeruli. Arrows and arrowheads indicate signals for marker proteins and claudins, respectively. Bars = 20 μm.

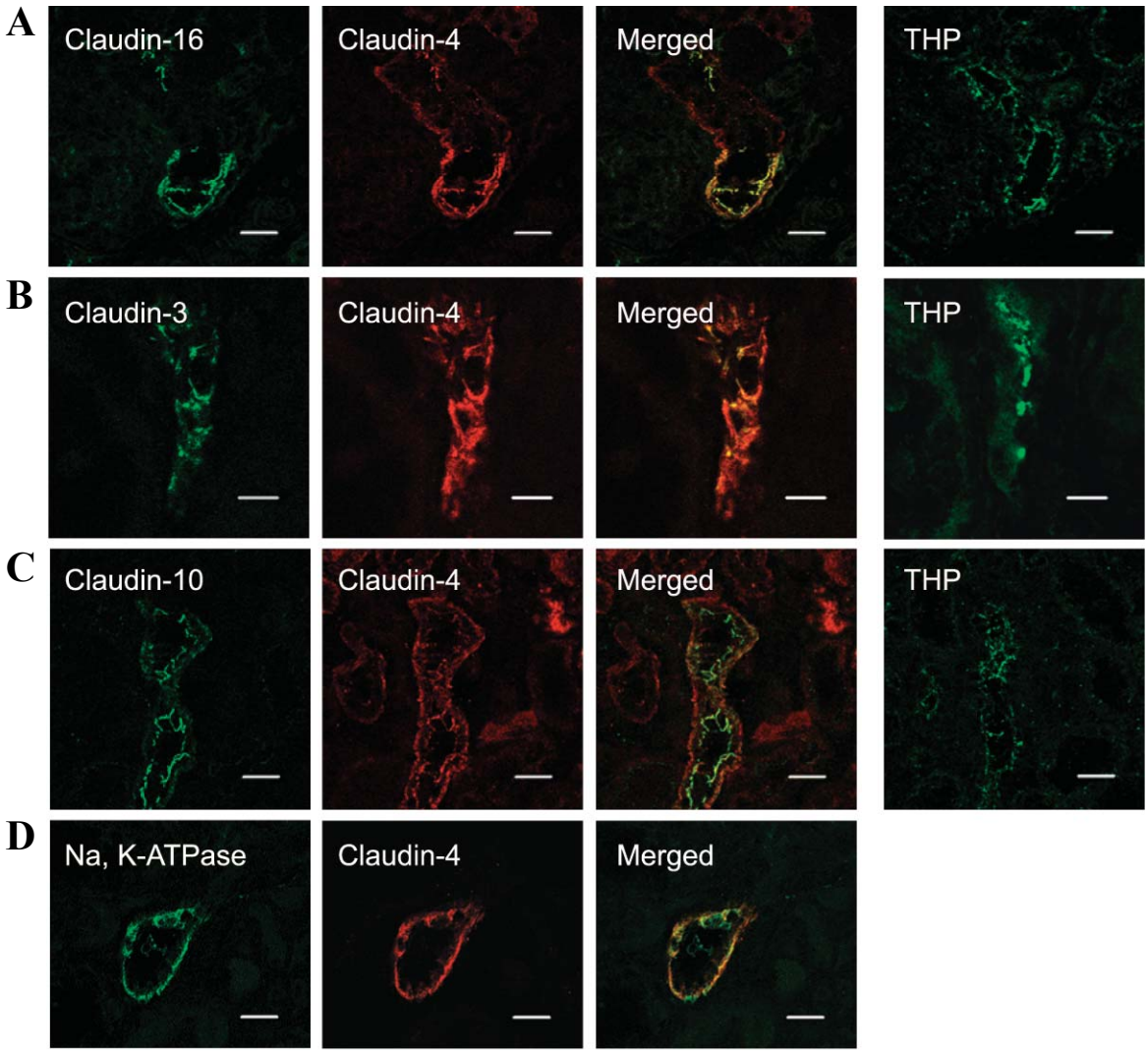

Fig. 4. Localization of claudin-16, -3, -4, and -10 in TAL segments. One of the serial frozen sections was reacted with the anti-THP pAb to detect the TAL segment (*A*-*C*, right panels). The other section was double-stained with the anti-claudin-4 mAb and the anti-claudin-16 pAb (*A*), anti-claudin-3 pAb (*B*), or anti-claudin-10 pAb (*C*). Merged images show that claudin-16 colocalizes with claudin-4 at TJ region of the TAL segment (*A*). Colocalization of claudin-4 with claudin-3 and -10 at TJ region of the TAL are also demonstrated (*B* and *C*). The frozen section was also reacted with anti-claudin-4 mAb and anti-Na,K-ATPase pAb to show colocalization of claudin-4 with Na,K-ATPase at basolateral membranes (*D*). Bars = 20 μm.

are summarized and compared with the previously reported segment-specific distributions of corresponding claudins in mouse nephrons [15] in Fig. 5.

Claudins expressed in TAL of Henle's loop: The data reported above indicated that bovine claudins 3, 4, and 10 were present at the TJ area in epithelia of the TAL segment where claudin-16 was expressed, although claudin-4 had both apical and basolateral localization (Figs. 3H-3K). This is inconsistent with the previous observation that TJ in the TAL region are comprised of claudins 3, 10, 11, and 16 but not claudin-4 in mouse nephrons [15]. To confirm our observation for bovine tissues, colocalization of claudin-4 with other subtypes was analyzed by double staining on the same frozen section. Immunofluorescence detection of claudin-16 and claudin-4, shown in Fig. 4A, confirmed that claudin-4 colocalized with claudin-16 at the apical regions of epithelial cells in the tubules positive for THP, the TAL segment. Likewise, immunofluorescent signals of claudin-4 were partly merged with those of claudin-3 or -10 at the apical regions of the cells in the TAL (Figs. 4B and 4C). Disagreement of the signals between claudin-4 and other claudins appeared to reflect dispersed distribution of claudin-4 at the basolateral domain that was demonstrated by colocalization of Na,K-ATPase, a typical enzyme present in the basolateral membranes, with claudin-4 (Fig. 4D).

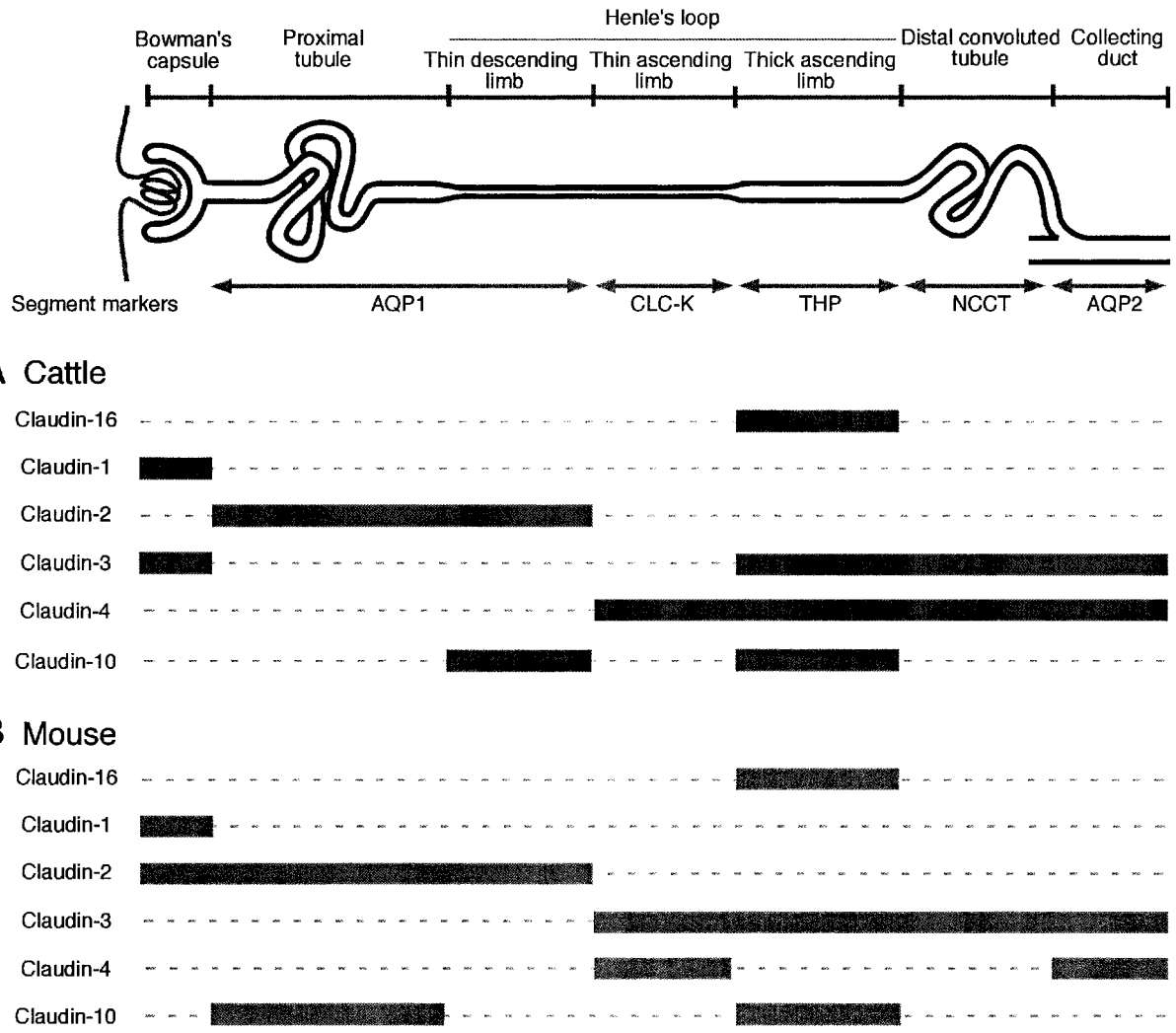

Fig. 5. Summary of the segment-specific expression of claudins 1–4, 10, and 16 in the bovine nephron. Tubular segments, marker proteins for each segment, and distribution of claudins 1–4, 10, and 16 (indicated by closed boxes) determined in the present study for the bovine nephron are summarized and compared with those for the mouse nephron [15].

DISCUSSION

The present study demonstrated that the expression and distribution of bovine claudin-16 were exclusively limited to the renal tubular segment TAL (Fig. 2) as has been reported for the human [27] and mouse [15]. A previous study on the murine kidney [15] showed that claudin-3, -10, and -11 were present at TJ in epithelial cells of the TAL segments in addition to claudin-16. We could not detect claudin-11, as described above, but instead found the distribution of claudin-4 at the TJ in this segment of the bovine kidney, in contrast to its absence in the TAL of the mouse nephron. It is unlikely that the anti-claudin-11 antibody used in the present study reacted with mouse claudin-11 but not with the bovine one, since bovine and mouse claudin-11 share an identical C-terminal amino acid sequence (GenBank accession numbers BC105204 and NM_008770, respectively) to which the antibody was raised. Rather, the absence of the signals for claudin-11 in bovine tissues may be explained by the extremely low levels of expression of this claudin subtype in adult kidneys compared with fetal tissues that was demonstrated by RT-PCR (data not shown) as has been shown in our previous study for mouse kidneys (Ohta, H., Adachi, H., and Inaba, M., accepted for publication in J. Vet. Med. Sci.). Actually, weak but significant immunofluorescent signals for claudin-11 were observed at the apical side of some tubules in developing kidneys from a bovine fetus at embryonic days 100 (data not shown). Thus, we conclude that claudin-16, -3, -4, and -10 are the major components of TJ strands of TAL regions in adult bovine kidneys (Fig. 5).

Claudin-16/paracellin-1 was first identified as the prod-

uct of the gene (*CLDN16/PCLN1*) responsible for FHHNC in humans [27] followed by the observation that renal tubular dysplasia in cattle was linked to deleterious mutations of the *CLDN16* gene [9, 20]. Although the significance of claudin-16 function in Mg^{2+} and Ca^{2+} metabolism remains uncertain in cattle [21], the affected animals [24] and FHHNC patients [35] share several histopathological findings, including tubular atrophy, immature tubules, and interstitial fibrosis. These renal lesions are observed in affected animals with claudin-16 deficiency at 1 to 2 months of age, and appear to progressively lead to decreases in the number of glomeruli [24]. These observations suggest that the barrier function in the paracelluar pathway, function in cell-cell adhesion, or both of claudin-16 in the TAL segment are important in the differentiation, formation, and/or maintenance of renal tubules. However, it is not likely that the absence of claudin-16 disrupts or eliminates TJ formation, since the TAL segment in bovine kidneys contains claudin-3, -4, and -10 in addition to claudin-16 as demonstrated above, and each of these claudins can form TJ-like structures to adhere to each other between adjacent cells when transfected into fibroblast cell line L cells [4], although this does not rule out the possibility that claudin-16 strengthens the cell-cell adhesion. Hence, the major causes for renal lesions in bovine claudin-16 deficiency likely involve dysfunction of the TJ at TAL segments in regulating the paracellular permeability, disabling the maintenance of tubular architecture.

A recent study by Hou *et al.* [11] has shown that human claudin-16/paracellin-1 modulates the ion selectivity of the TJ to profoundly increase the permeability of Na^+ without significant effect on Cl^- in transfected LLC-PK1 cells, and suggested that claudin-16/paracellin-1 controls the level of transepithelial voltage that is the primary driving force for Mg^{2+} and Ca^{2+} reabsorption in TAL epithelia [25]. They also demonstrated that the amino acid sequence in the first extracellular loop, consisting of Thr^{26}-Leu^{81}, was critical for the function of claudin-16/paracellin-1 in the Na^+-selective permeability [11]. Since the corresponding region of bovine claudin-16 possesses an amino acid sequence identical to that in the human homologue, with the only exception being that the 72nd residue is Ser in cattle (Fig. 1A) but Pro in the human, it is not unreasonable to expect that bovine caludin-16 has a similar ion selectivity to generate transepithelial potential for passive paracellular flow of divalent cations. Thus, the lack of claudin-16 in the TAL segments in the bovine kidney would lead to the loss of the lumen-positive potential and subsequent reduction in reabsorption of Mg^{2+} and Ca^{2+}, as has been suggested for FHHNC [11, 27]. In the present study, we found coexistence of claudin-3, -4, and -10 with claudin-16 in the TAL of the bovine nephron. In contrast to claudin-16, claudin-4 has been shown to selectively decrease the permeability of Na^+ [33], although the ion selectivities of claudin-3 and -10 are as yet undefined. Therefore, the presence of claudin-4, in addition to the lack of claudin-16, in TAL segments of affected animals could greatly enhance the transcellular concentration gradient of NaCl from peritubular space to the lumen compared to that formed under normal conditions because of its permeability barrier to Na^+. This may cause some metabolic changes in TAL epithelial cells and consequent cellular death, leading to renal tubular dysplasia in cattle. Moreover, even under physiological conditions, the presence of claudin-4 seems to affect the function of claudin-16 in generating the lumen-positive potential in the TAL segments of bovine nephrons, suggesting that reabsorption of divalent cations in TAL regions is less fundamental in cattle. This may partly explain why the affected animals do not have aberrant phenotypes in Mg^{2+} and Ca^{2+} metabolisms [21]. However, the physiological role and the regulatory mechanism for functional interaction of claudin-16 and claudin-4 in cattle remain to be clarified.

The present study also showed that the bovine kidney had a segment-specific expression pattern for claudins that was basically the same as that observed for mouse kidneys but with some discrepancies (Fig. 5). First, as described above, this study showed that bovine claudin-4 was found throughout the distal nephron, while it was present in the thin ascending limb of Henle's loop and the collecting duct in the mouse kidney [15]. In these segments, bovine claudin-4 showed distribution both at the TJ and the basolateral membranes. Many investigators have reported basolateral distribution of some claudins, including claudin-4 [1, 17, 22, 23], although the precise roles of claudins in the plasma membrane compartment other than TJ remain unknown; they may simply represent a storage pool capable of being recruited to the TJ when required, or they may have some novel functions in intercellular adhesion and/or adhesion between cells and the extracellular matrix.

Distributions of claudin-2 and -3 showed differences between cattle and mice. Claudin-2 was absent but claudin-3 was present in Bowman's capsule in the bovine kidney (Fig. 3), whereas the former, but not the latter, was found there in mice [15]. Since Bowman's capsule contains claudin-1 in both bovine and mouse kidneys (Fig. 5), and claudin-3 strands but not claudin-2 strands can interact with claudin-1 strands [4], there may be some differences between cattle and mice in the permeability of the TJ in Bowman's capsule although no substantial information about the paracellular pathway in this part is available. Furthermore, bovine claudin-3 was observed in the TAL, distal convoluted tubules, and collecting ducts, but exhibited no signal in the thin ascending limb, although it was found in all of these segments in the mouse [15]. The anti-CLC-K antibody used in this study detects both CLC-K1 and CLC-K2, and recognizes whole distal nephron segments, including the thin ascending limb [32, 36]. Thus, our conclusion that the thin ascending limb lacks claudin-3, is based on the absence of immunofluorescence signals for claudin-3 in the tubules stained positively with the anti-CLC-K antibody in the inner medulla. These differences in segment-specific distribution of claudins 1–4 may give us relevant information about physiological functions of renal tubules characteristic to different animal species, as previous studies

demonstrated that some claudins, including claudin-1 [13, 18] and -4 [33] had low-conductance characteristics, whereas claudin-2 likely constituted aqueous pores with high conductance [5].

In conclusion, the present study shows that the bovine kidney has a segment-specific distribution pattern for claudin-16 and claudins 1 through 4 and 10, suggesting distinct characteristics of the TJ concerned. The expression of claudin-16 is exclusively restricted to the TJ in the TAL region where claudins 3, 4, and 10 colocalize, indicating that the aberrant lesions found in renal dysplasia in Japanese black cattle are subsequent to some undefined events in this restricted area, the TAL, presumably due to the total lack of claudin-16 function in paracellular transport.

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