Occludin TM4–: an isoform of the tight junction protein present in primates lacking the fourth transmembrane domain

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Summary

The tight junction protein occludin possesses four transmembrane domains, two extracellular loops, and cytoplasmic N- and C-termini. Reverse transcription-PCR analysis of human tissues, embryos and cells using primers spanning the fourth transmembrane domain (TM4) and adjacent C-terminal region revealed two products. The larger and predominant product corresponded in sequence to canonical occludin (TM4+), while the smaller product exhibited a 162 bp deletion encoding the entire TM4 and immediate C-terminal flanking region (TM4–). Examination of the genomic occludin sequence identified that the 162 bp sequence deleted in TM4– coincided precisely with occludin exon 4, strongly suggesting that TM4– is an alternative splice isoform generated by skipping of exon 4. Indeed, the reading frame of downstream exons is not affected by exclusion of exon 4. The presence of both TM4+ and TM4– occludin isoforms was also identified in monkey epithelial cells but TM4– was undetected in murine and canine tissue and cells, indicating a late evolutionary origin for this alternative splicing event. Conceptual translation of TM4– isoform predicts extracellular localisation of the C-terminus. Immunocytochemical processing of living human Caco-2 cells using a C-terminal occludin antibody revealed weak, discontinuous staining restricted to the periphery of subconfluent islands of cells, or islands generated by wounding confluent layers. In occludin immunoblots, a weak band at ~58 kDa, smaller than the predominant band at 65 kDa and corresponding to the predicted mass of TM4– isoform, is evident and upregulated in subconfluent cells. These data suggest that the TM4– isoform may be translated at low levels in specific conditions and may contribute to regulation of occludin function.

Key words: Occludin, Tight junction, Epithelium, Isoform, Alternative splicing, Embryo

Introduction

The tight junction (TJ) is a specialised multiprotein complex located at the apical contact point between cells in epithelial and endothelial tissues. This junction type acts to seal the intercellular space and thereby generate a permeability barrier required for transport processes (reviewed by Stevenson and Keon, 1998; Matter and Balda, 1999). In addition, by regulation of the TJ intercellular seal, the paracellular pathway may be open for selective transport of molecules and ions (Madara et al., 1992) and neutrophils (Huber et al., 2000). The TJ has also been identified as a contributor to epithelial cell polarity, acting as a fence to the movement of proteins and extracellular leaflet lipids between apical and basolateral membrane domains (van Meer and Simons, 1986).

TJs contain several interacting constituents which contribute to at least three types of transmembrane proteins, which contribute to the intercellular sealing process, and a series of cytoplasmic ‘plaque’ proteins with signalling and regulatory properties, which also mediate linkage to the cytoskeleton (Stevenson and Keon, 1998; Matter and Balda, 1999). The first transmembrane protein of the TJ to be discovered was occludin (Furuse et al., 1993), followed by claudins (Furuse et al., 1998a; Morita et al., 1999) and JAM (junction adhesion molecule (Martin-Padura et al., 1998)). Occludin (~65 kDa) has four membrane-spanning domains creating two extracellular loops, characteristically rich in glycine and tyrosine residues, and cytoplasmically located N- and C-termini (Furuse et al., 1993; Ando-Akatsuka et al., 1996). The longer C-terminal domain contains binding sites for plaque proteins including ZO-1, ZO-2, ZO-3 and cingulin (Furuse et al., 1994; Haskins et al., 1998; Fanning et al., 1998; Cordenonsi et al., 1999; Wittchen et al., 1999; Itoh et al., 1999) and is essential for occludin assembly at the TJ, mediated by ZO-1 association (Furuse et al., 1994; Chen et al., 1997; Matter and Balda, 1998; Mitic et al., 1999; Sheth et al., 1997; Sheth et al., 2000; Medina et al., 2000). Several studies have provided evidence that occludin contributes to both the structure and the sealing function of the TJ and to its role in maintaining epithelial membrane polarity (Furuse et al., 1996; Balda et al., 1996; McCarthy et al., 1996; Chen et al., 1997; Van Itallie and Anderson, 1997; Wong and Gumbiner, 1997; Bamforth et al., 1999; Lacaz-Vieira et al., 1999; Balda et al., 2000; Medina et al., 2000). However, since occludin null
embryonic stem cells and mice are capable of differentiating TJs (Saitou et al., 1998; Saitou et al., 2000), other TJ transmembrane proteins, notably claudins (Furuse et al., 1998b; Tsukita and Furuse, 1999; Sonoda et al., 1999; Furuse et al., 2002), may have a more direct role in regulating TJ activity.

Occludin is the product of a single gene located on human chromosome band 5q13.1 (Saitou et al., 1997). However, several protein forms of occludin may be evident in epithelial cells, representing different states of phosphorylation or other post-translational modifications. These occludin forms have been implicated in the regulation of occludin assembly at the TJ (Cordenonsi et al., 1997; Sakakibara et al., 1997; Wong, 1997; Antonetti et al., 1999; Farshori and Kachar, 1999; Sheth et al., 2000). In addition to post-translational modifications, there is evidence that distinct occludin isoforms may be expressed as a result of alternative splicing. Thus, several occludin mRNA bands have been identified in northern blots of mouse cultured epithelial cells and tissues (Saitou et al., 1997). Occludin 1B variant has been discovered recently in canine cells, containing a 193 bp insertion corresponding to an alternatively spliced exon in the gene encoding a unique N-terminus sequence of 56 amino acids (Muresan et al., 2000).

In this paper, we provide evidence for an additional site for alternative splicing of occludin, located at the fourth transmembrane domain (TM4). From reverse transcription-PCR analysis of human, monkey, dog and mouse occludin mRNA and genomic analysis of the human occludin gene, together with western blotting and immunofluorescence data, we report an isoform of occludin detected only in primates in which the TM4 and immediate 3′-flanking sequence is deleted (TM4 minus isoform; TM4−). In the human, both isoforms are expressed ubiquitously and from the preimplantation stage of development when the trophectoderm epithelium differentiates. Our data also indicate that occludin TM4+ is expressed as a protein, albeit at low levels and in particular culture conditions associated with subconfluent cells. The potential for this isoform, in which the C-terminus becomes extracellular, as a regulator of occludin functional activity at the TJ is briefly discussed.

Materials and Methods

Tissues and cells

Human kidney and bronchial epithelium (kindly provided by J. E. Collins and P. M. Lackie, Southampton General Hospital, UK) and MF1 strain mouse kidney, liver and lung (Biomedical Facility, University of Southampton, UK) were used for RNA extraction. Caco-2 human colon epithelial cells, 16HBE human bronchial epithelial cells, HuVEC human vascular endothelial cells, Madin-Darby canine kidney (MDCK) epithelial cells, CMT64/61 mouse lung epithelial cells, HuVEC human vascular endothelial cells, Madin-Darby canine kidney epithelial cells, CMT64/61 mouse lung epithelial cells, HuVEC human vascular endothelial cells, Madin-Darby canine kidney epithelial cells were obtained from ECACC (Salisbury, UK). Caco-2 and BSC1 cells were cultured in EMEM (Sigma), 16HBE and HuVEC cells in MEM (Sigma), MDCK cells in DMEM (Gibco) and CMT64/61 cells in Waymouth medium (Gibco), all supplemented with 10% fetal calf serum, 1% nonessential amino acids, 100 IU/ml penicillin, 100 μg/ml streptomycin and 2 mM freshly added L-glutamine. Human preimplantation embryos were derived from superovulated, mated MF1 mice (Biomedical Facility, University of Southampton) and, after collection from oviducts, were cultured in T6 medium plus 4 mg/ml bovine serum albumin as described (Sheth et al., 2000).

RT-PCR analysis

Total RNA was extracted from tissues using the RNeasy Midi kit (Qiagen) and from cells using Tri-Reagent (Sigma), according to manufacturer’s instructions. In addition, commercially available total RNA from human kidney, cervix and uterus (Ambion) was used. Poly A+ RNA was extracted from single human and mouse embryos using the Dynabeads mRNA DIRECT kit (Dynal A.S., Norway) as described previously (Eckert and Niemann, 1998; Holding et al., 2000), with modifications. Individual embryos were lysed in 150 μl lysis buffer for 10 minutes and transferred to tubes containing 20 μl Dynabeads previously washed in lysis buffer, and roller incubated for 15 minutes. Bead-mRNA complex was removed by magnet and washed twice in kit buffers A and B before cDNA synthesis either after mRNA elution (mouse embryos) in 20 μl RNase-free water at 65°C for 2 minutes or by solid-phase method (human embryos, see below). Total RNA (200-500 ng) and 80% of the PolyA+ mRNA from single mouse embryos was reverse transcribed in 20-50 μl RT reactions into cDNA using 0.5 mM of each dNTP, 5 μM random hexamers (Promega), 40 U RNAGuard (Pharmacia) and 200 U Superscript II reverse transcriptase (Gibco-BRL). To control for genomic DNA contamination of PolyA+ mRNA, 20% of the fraction eluted from the Dynabeads was treated similarly but in the absence of reverse transcriptase. Conditions for reverse transcription were 10 minutes at 25°C, 1 hour at 42°C and 5 minutes at 95°C. For solid-phase cDNA synthesis of single human embryos, a 50 μl RT reaction was used as above except containing 50 U RNAGuard and 250 U Superscript II. Supernatant was removed by magnet and bead-cDNA complexes washed in Tris-HCl.

cDNA derived from 50 ng total RNA or 95% mouse embryo cDNA was then amplified in 50 μl PCR reactions using 0.2 μM of each primer.

Table 1. Primer pairs used for RT-PCR analysis of occludin

<table>
<thead>
<tr>
<th>Primer</th>
<th>Position</th>
<th>Sequence (5'→3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>H1</td>
<td>966</td>
<td>TAG TGA GTG CTA TCC TGG GCA T</td>
</tr>
<tr>
<td>H2</td>
<td>1585</td>
<td>TGC AGG TGC TCT TTT TGA AGG T</td>
</tr>
<tr>
<td>H3</td>
<td>1020</td>
<td>TGG GAG TGA ACCCAA CTG CT</td>
</tr>
<tr>
<td>H4</td>
<td>1484</td>
<td>CTT CAG GAA CCG CGG TGG AT</td>
</tr>
<tr>
<td>H5</td>
<td>1260</td>
<td>ACA AGT CCA ATA TTT TGT GG</td>
</tr>
<tr>
<td>H6</td>
<td>1799</td>
<td>TAT TGA TCT CAT CAA GTT CT</td>
</tr>
<tr>
<td>H7</td>
<td>1281</td>
<td>ACA AGG AAC AAT AAT ATG AT</td>
</tr>
<tr>
<td>H8</td>
<td>1778</td>
<td>ATT GTA AGC TCT TGT ATT CC</td>
</tr>
<tr>
<td>M1</td>
<td>741</td>
<td>GAT CAT AGT CAG CGC TAT CCT</td>
</tr>
<tr>
<td>M2</td>
<td>1347</td>
<td>TGT CTC TAG GTT ACC ATT GCT</td>
</tr>
<tr>
<td>M3</td>
<td>914</td>
<td>TGT ATC ACT ACT GTG TGG T</td>
</tr>
<tr>
<td>M4</td>
<td>1319</td>
<td>CGA GGC TGG CTC AAG TCA</td>
</tr>
<tr>
<td>C1</td>
<td>565</td>
<td>CCA GAA CCA GAA GGT ACT ACT</td>
</tr>
<tr>
<td>C2</td>
<td>1278</td>
<td>TCG TGT AGT CTG CAT AGT</td>
</tr>
<tr>
<td>C3</td>
<td>766</td>
<td>TGT ACT ACT GTG TGG T</td>
</tr>
<tr>
<td>C4</td>
<td>1168</td>
<td>CGA GGC TCG CTC AAG TCA</td>
</tr>
</tbody>
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H1 to H8 are based on human occludin cDNA accession no. NM_002538.2; M1 to M4 are based on mouse occludin cDNA accession no. NM_008756.1; C1 to C4 are based on dog occludin cDNA accession no. U49221.1. Odd numbered primers are sense, even numbered primers are antisense.

*C3 is identical in sequence to M3.

*C4 is identical in sequence to M4.
(H1/H2 and H3/H4) amplify two products, the upper corresponding to the published cDNA sequence while, in the lower band, the TM4 and immediate 3'-flanking sequence are deleted. B, embryo blastocyst stage; BE, bronchial epithelium; C, cervix; Ca, Caco-2 cells; HBE, 16HBE cells; HuV, HuVEC cells; K, kidney; M, marker lane; Mo, embryo morula stage; U, uterus.

sequence-specific primer for tissue/cell-line RNA and 1 μM for mouse embryos (Table 1), 2 mM MgCl₂, 0.2 mM dNTPs and 2.5 U native Taq Polymerase (Gibco-BRL). PCR conditions were as follows: 95°C for 5 minutes followed by 72°C for 1 minute. After addition of the enzyme at 72°C (hot start), cDNAs were amplified for 40-45 cycles at 95°C for 30 seconds; 58°C for 60 seconds and 72°C for 90 seconds. A two-stage PCR reaction was also employed, using nested primers and generally 2 μl (4%) of the first-stage product. For solid-phase PCR (human embryos), 50 μl PCR reactions comprised 0.2 μM of each primer in Dynabead PCR buffer (×1), 1 mM dNTPs and 2.5 U Taq Polymerase and were heated at 94°C for 3 minutes before two cycles at 94°C for 30 seconds, 58°C for 1 minute, 72°C for 1 minute, and finally 94°C for 2 minutes before placing on ice to prevent renaturation of strands. Beads were removed using the magnet and sufficient supernatant placed in a new tube for a further 40 cycles of amplification.

For detection of human occludin mRNA, four pairs of primers were designed, two pairs flanking the fourth transmembrane domain (TM4) including the beginning of the C-terminal domain, and two pairs exclusively within the C-terminus (Table 1). Human primers were also used successfully to amplify monkey occludin. Mouse and dog primers included two pairs flanking the TM4 domain and adjacent region of C-terminus, in each case (Table 1). Primers for RNA polymerase A were used as a positive control in embryo samples (Eckert and Niemann, 1998). PCR products were directly sequenced using a BigDye Terminator kit (Applied Biosystems) and automated sequencing.

Antibodies and western blotting
Rabbit polyclonal antibody to the C-terminus of human occludin (Van Itallie and Anderson, 1997) was kindly provided by J. M. Anderson (Yale University, CT) and was used in immunoblotting of cells and tissues. Cells were washed in PBS and solubilised in boiling SDS-sample buffer for 5 minutes. Tissue extract for electrophoresis was generated using frozen tissue powder boiled for 5 minutes in PBS:1% SDS and centrifuged at 10,000 g for 3 minutes. Samples were run on 4-12% polyacrylamide gradient gels (Invitrogen) and blotted onto Hybond-C nitrocellulose (Amersham) at 300 mA overnight before immunoblotting and ECL chemiluminescence (Amersham) as described previously (Sheth et al., 2000). Densitometric analysis of immunoblots was performed using AlphaImager (Alpha Innotech Corporation).

Immunocytochemistry
Caco-2 cells were cultured on coverslips at 3×10⁵/cm² and 6×10⁵/cm² for up to 4 days to induce confluent and sub-confluent monolayers. Confluent layers were also wounded with a fine sterile forceps tip to create cell islands, washed in EMEM and cultured further for 30 minutes, 1 hour and 2 hours. Confluent, sub-confluent and wounded layers were processed for localisation of occludin using a rabbit polyclonal antibody recognising the C-terminal domain of human occludin. Rabbit polyclonal antibody to mouse ZO-1 (Sheth et al., 1997) and ZO-2 (Zymed) were also used for immunostaining. Cells were processed either living (using EMEM for antibody and washing
steps at 4°C, fixing finally in paraformaldehyde) or after 10 minutes fixation in 1% Triton X-100 in PBS, permeabilised in 0.25% Triton X-100 in PBS, washed three times in PBS:1.8 mM CaCl₂ and treated with occludin antibody (1:1000 in PBS:1.8 mM CaCl₂:0.1% NaN₃, 1 hour) followed by washing three times in PBS and labelling with Alexa 488-conjugated secondary antibody (1:500 in PBS:1.8 mM CaCl₂; Cambridge Bioscience). Specimens were viewed on an MRC-600 series confocal imaging system (BioRad, UK) or a Leitz Fluovert epifluorescence microscope using 63 objectives and appropriate filters.

**Results**

RT-PCR analysis of occludin in human tissues and cells

RNA from human tissues (kidney, cervix, uterus, bronchial epithelium), preimplantation embryos and cell lines (Caco-2, 16HBE and HuVEC cells) were analysed by RT-PCR for occludin expression using the different primer sets shown in Table 1. Primer pairs H5/H6 and H7/H8 located within the C-terminus consistently produced a single product at 539 bp and 497 bp, respectively, which, upon sequencing, corresponded to the human occludin published sequence (accession no. NM_002538.2; Fig. 1). However, primer pairs H1/H2 and H3/H4, which flank the TM4 domain and immediate proximal region of the C-terminus, produced two predominant products at 620 bp and 458 bp for H1/H2, and 465 bp and 303 bp for H3/H4, the larger product being the major band in each case (Fig. 1). This pattern was observed when these primers were used either in single-stage PCR or in a two-stage reaction. In human embryos, two products were detected using the primer sets spanning TM4 from as early as embryonic compaction (8- to 16-cell stage morula) up to and including expanded blastocysts (Fig. 1). Of 25 embryos positive for occludin expression in either one- or two-stage PCR analysis, 12 showed both isoforms, with the TM4⁺ variant being the predominant band. In ten embryos, only the TM4⁺ variant was detectable and in three, only the TM4⁻ variant.

The sequence of the larger RT-PCR product from human tissues, embryos and cells using the primer sets spanning the
TM4 region corresponded exactly to the published coding sequence. However, in the sequence for the smaller product, a 162 bp region from 1166 to 1327 bp of the published occludin cDNA sequence was deleted. The deleted region encodes the entire TM4 segment and adjacent C-terminal sequence (Fig. 2A,B). We designate these two forms of occludin as TM4 plus (+) and TM4 minus (−) variants.

Human occludin genomic sequence analysis
In order to determine whether the TM4− variant occludin cDNA was due to differential splicing, we initially compared the published human occludin cDNA sequence to the working draft of the human genome using the Blast program at the NCBI website. This revealed the presence of nine exons in the human occludin mRNA (not shown). The positioning of these exons is confirmed by the current annotation of the pertinent genomic contig (NT_006497.6) as viewed on the NCBI website. Remarkably, the sequence alignment showed that human occludin exon 4 of 162 nucleotides precisely coincided with the 162 bp region deleted in the smaller TM4− RT-PCR product observed with primers H1/H2 and H3/H4 (Fig. 3A). This observation strongly supported the view that the TM4− variant is a genuine mRNA isoform due to alternative splicing. In addition, the data indicated skipping of exon 4 as the mechanism to generate the TM4− isoform (Fig. 3B). While the TM4− mRNA isoform is predicted to encode a shorter protein product, the deletion of the 162 bp of coding sequence contained in exon 4 does preserve the reading frame in the downstream exon 5 (Fig. 3A). The predicted peptide sequence of the TM4−-encoded protein would therefore be identical to canonical occludin, except for the deletion of the 54 amino acids encoded by exon 4.

Species variation in occludin TM4 isoforms
We next investigated whether the presence of the TM4− isoform could be demonstrated in species other than human. RNA extracted from the African Green Monkey (Cercopithecus aethiops) kidney epithelial cell line BSC1 was employed in single and nested RT-PCR using human occludin primer pairs H1/H2 and H3/H4. Two products were ampliﬁed, the larger being predominant, and which corresponded in size to the human TM4+ and TM4− cDNAs, respectively (Fig. 4). Both of the Cercopithecus putative TM4+ and TM4− products were sequenced. Alignment to each other and to the corresponding human sequences conﬁrmed that size and position of the 162 bp deletion are precisely conserved between Cercopithecus and Homo (Fig. 2B). We concluded that the TM4− isoform in monkey cells was homologous to the human and likely generated by an exon skipping mechanism similar to the one in human tissues. There was divergence between the monkey and human nucleotide sequences, ruling out human contamination as a source of the monkey cDNAs and predicting three amino acid substitutions in the 54 amino acid sequence encoded by occludin exon 4 (Fig. 2B).

We investigated whether the TM4− occludin isoform was present in two other mammalian orders, rodents and carnivores. Based on the published murine occludin cDNA sequence (accession no. NM_008756.1), a primer pair spanning the 162 bp TM4 was synthesized (M1/M2, Table 1). When these primers were used in RT-PCR experiments on mouse tissue (kidney, lung and liver), embryos and cell line (CMT64/61 cells) RNA, a band corresponding in size to the canonical TM4+ occludin mRNA was observed (and conﬁrmed by sequencing), but no smaller product corresponding to the hypothetical mouse occludin TM4− isoform was detectable (Fig. 4). The same result was found using M3/M4 primers either alone or in nested PCR after M1/M2. Likewise, we designed suitable primers (C1/C2 and C3/C4, Table 1) based on the published canine occludin cDNA sequence (accession no. U49221.1) and used them on MDCK cell line RNA. Again, while the product expected from the canonical TM4+ mRNA was readily ampliﬁed as a strong band (and conﬁrmed by sequencing), the smaller product predicted for the hypothetical TM4− isoform could not be detected (Fig. 4).

Immunocytochemistry
Preliminary experiments were conducted on human cells to investigate whether the occludin TM4− isoform was expressed as protein. Conceptual translation of the TM4− isoform indicates extracellular localisation of the C-terminus. Confluent and subconfluent monolayers of ﬁxed and permeabilised Caco-2 cells showed strong staining for occludin by confoocal microscopy at apicolateral contact sites using the C-terminal antibody (Fig. 5A,B). A similar junctional staining pattern was evident for cytoplasmic plaque TJ proteins, ZO-1 (Fig. 5C,D) and ZO-2. Junctional staining using these antibodies was not evident in living confluent monolayers but in subconfluent islands, the occludin antibody resulted in

![Composite image of RT-PCR analysis of occludin TM4− isoform expression in different species using primer sets shown in Table 1. Both TM4+ and TM4− isoforms are detectable in African Green Monkey (AGM) BSC1 epithelial cells but only the canonical TM4+ isoform is detectable in mouse tissues, cells and embryos, and in canine-derived MDCK cells. B, embryo blastocyst stage; BS, AGM BSC1 cells; CM, CMT64/61 cells; K, kidney; L, liver; L, marker lane; MK, MDCK cells; Mo, embryo morula stage.](image-url)
intermittent, weak staining around the periphery of the island in 13/17 (76%) islands examined (Fig. 5E,F). This pattern of staining was not observed using either the ZO-1 (Fig. 5G,H) or ZO-2 antibodies (0/15), or in the negative control of the secondary antibody alone (Fig. 5I,J), employed in all staining reactions. In addition, when confluent monolayers were wounded to generate islands of cells and subsequently washed and cultured for up to 2 hours, peripheral staining of occludin was observed in living cultures, appearing to increase in intensity between 30 minutes and 1 hour of culture (Fig. 5K,L).

At high magnification, discontinuous spots of occludin staining were present in outermost and adjacent enclosed cells of the islands, at both contact-free and cell-cell contact surfaces (Fig. 5K,L). In the absence of a specific antibody for occludin TM4–, these data, showing weak extracellular exposure of occludin C-terminus in living cells, indicate the potential for protein expression of this isoform in specific conditions.

Western blotting
Human, monkey, mouse and canine epithelial cells and mouse lung were probed with occludin antibody in immunoblots. Several bands of occludin were identified around 58-72 kDa, as shown previously (e.g. Sakakibara et al., 1997; Wong, 1997; Sheth et al., 2000), and represent potential post-translational states (Fig. 6). The lowest band of the complex migrated at ~58 kDa, some 7 kDa below the predominant band at 65 kDa, corresponding to the reduction in protein mass anticipated for the TM4– isoform. A similar pattern of occludin bands was evident across species although the position of individual bands varied slightly. We investigated the level of expression of the lowest band in Caco-2 cells from confluent and subconfluent culture using equal protein loading and densitometry. While the expression of the predominant 65 kDa band was equivalent in both confluent and subconfluent cells, the 58 kDa band was significantly upregulated in subconfluent

Fig. 5. Immunocytochemical analysis of occludin and ZO-1 in Caco-2 cells. (A,B) Brightfield and occludin labelling of subconfluent island after fixation showing typical TJ pattern of staining at cell borders. (C,D) Brightfield and ZO-1 staining of subconfluent island after fixation showing typical TJ pattern. (E-J) Brightfield and protein labelling of subconfluent islands immunolabelled prior to fixation. (E,F) Occludin labelling; note weak staining of the island periphery. (G,H) ZO-1 labelling showing absence of peripheral staining. (I,J) Negative control (secondary antibody only) showing absence of peripheral staining. (K,L) Confluent monolayer wounded to produce island and cultured for 30 minutes (K) or 1 hour (L) before labelling for occludin prior to fixation, showing weak staining around cells close to the wound edge (arrows). Bars, 20 μm.
culture (Fig. 6). Collectively, these data indicate that occludin TM4+ in human and monkey cells may migrate as a component of the 58 kDa band and be expressed in subconfluent cells.

Discussion

In this paper, we identify a new splice isoform of the TJ membrane protein, occludin, in which a 162 bp sequence encoding the fourth TM domain and adjacent C-terminal region is deleted. Our RT-PCR analysis used primers spanning this region of occludin to identify two cDNA products, corresponding in sequence to the TM4+ and TM4– isoforms. The presence of the TM4– isoform was identified in all human epithelial-based tissues and cells examined, including preimplantation stage embryos, indicating a ubiquitous expression profile. The TM4– isoform was also apparent in HuVEC endothelial cells, indicating its presence in endothelial as well as epithelial lineages. Our study of human genomic sequence supports the model that the TM4– isoform is generated by exon skipping in which the entire exon 4 (162 bases) is deleted with the reading frame preserved in the downstream exon 5.

Recent analysis of the human genome indicates that a large proportion (around 42%) of genes are alternatively spliced, in particular for cell surface receptors (Modrek et al., 2001). The vast majority of detected splice variants appeared to produce highly specific, biologically meaningful control of function forms (Modrek et al., 2001). Modulation of intercellular adhesion by expression of alternate splice variants of adhesion molecules is well documented, including, in particular, the deletion of TM domains. Mouse neural cell adhesion molecule (N-CAM) of the immunoglobulin superfamily, is expressed as at least three splice variants, one of which lacks the TM domain, altering localisation and adhesive function (Santoni et al., 1987; Owens et al., 1987; Powell et al., 1991). Other examples of adhesion molecules in which TM domain deletion by alternative splicing has been identified include integrin aIIb subunit (Trika et al., 1998), the leukocyte selectin adhesion molecules GMP-140 and P-selectin (Johnston et al., 1990; Ushiyama et al., 1993), kit ligand (Flanagan et al., 1991), vascular cell adhesion molecule (Terry et al., 1993; Cybulsky et al., 1993; Pirozzi et al., 1994), receptor tyrosine phosphatase β (Barnea et al., 1994; Maurel et al., 1995) placental sialoadhesion molecule CD33L (Takei et al., 1997), PECAM-1 (Goldberger et al., 1994) and ICAM-1 (Wakatsuki et al., 1995). However, our data is the first report of a TM deletion within a TJ membrane protein. The importance of adhesion systems in intercellular signalling further raises the possibility of alternative splice forms acting as functional regulators.

Comparative analysis of human, monkey, mouse and canine occludin cDNA illustrated that the TM4– isoform was detectable in the primate species examined but not elsewhere. A plausible explanation is that such a splicing mechanism was present in the common ancestor of Homo and Cercopithecus, which is thought to have lived 23.3 million years ago (Kumar and Hedges, 1998). Since Homo and Cercopithecus belong to different superfamilies within the infraorder Catarrhini (Fleagle, 1999), it appears that the occurrence of occludin TM4 differential splicing may be conserved among the Catarrhini, which comprise humans, great apes, gibbons, and Old World monkeys.

Although the occurrence of species-specific differences in alternative splicing of genes amongst mammals is uncommon (reviewed by Lu et al., 1999), a growing number of human- or primate-specific splicing events mediated by exon skipping and with functional implications have been reported. Thus, a splice variant of hormone-sensitive lipase generated by skipping of exon 4 has been detected in human but not rat, mouse, dog or rabbit tissues (Laurell et al., 1997). Similarly, deletion of exon 4 resulting in a truncated splice variant of 5-aminolevulinate synthase mRNA is evident in human differentiating erythroid cells but not in dog or mouse (Conboy et al., 1992). The estrogen receptor-α also exhibits exon-deleted variants in the human but not mouse (Lu et al., 1999). Other examples of human-specific alternative splicing by exon skipping include the EGF1 domain of cartilage aggrecan (Fulop et al., 1996) and glucocorticoid receptor-β (Otto et al., 1997). It would appear, therefore, that exon skipping has remained a viable mechanism for modifying protein function during mammalian evolution.

Alternative splicing by exon skipping has also been identified in TJ-associated proteins, notably in the MAGUK proteins ZO-1 (Balda and Anderson, 1993) and ZO-2 (Chlenski et al., 2000). Moreover, the species-specific nature of exon skipping to generate the TM4– isoform of occludin is not unique for this gene. Thus, the 1B variant of canine occludin that results in a unique N-terminus is generated by insertion of exon 2B, not expressed in canonical occludin.
(Muresan et al., 2000). This exon is not present in the human genome database and is undetectable in mouse following extensive sequence analysis of cDNAs generated by RT-PCR (B.S., unpublished), indicating a canine-specific modification. Given the breadth of modulation that occurs at the TJ to regulate transcellular electrical resistance, paracellular transport and signalling activity (Stevenson and Keon, 1998; Matter and Balda, 1999), it is not surprising that alternative splicing may be used to expand the repertoire of protein function. This may be true in particular for occludin as a single copy gene while variability in claudin TJ transmembrane protein activity is known to be derived from gene multiplicity.

We have made a preliminary investigation of the potential for human occludin TM4– isoform to be expressed as a protein, which would be indicative of a possible functional role. In the absence of a specific antibody recognising occludin TM4–, we used a C-terminal antibody on living cells since, if TM4– is expressed as a protein, the C-terminus switches from cytoplasmic to extracellular domains. This might be an interesting biological phenomenon since the occludin C-terminus is recognised as a site of interaction with several TJ cytoplasmic plaque proteins including ZO-1, ZO-2, ZO-3 and cingulin (Furuse et al., 1994; Haskins et al., 1998; Fanning et al., 1998; Cordenonsi et al., 1999; Wittchen et al., 1999; Itoh et al., 1999). Thus, adhesivity of occludin TM4– in the absence of plaque protein interaction might act to negatively regulate TJ integrity.

We found no convincing evidence of occludin immunostaining in living confluent Caco-2 cell cultures using the C-terminal antibody. However, living islands of cells in subconfluent or wounded cultures showed weak, intermittent membrane staining in the periphery corresponding to the outermost cells, a staining pattern not reproduced in controls using other TJ antibodies with the same secondary antibody. The extent of peripheral staining in wounded islands also appeared to increase with time. These data indicate that TM4– occludin may be expressed at low levels in certain cellular conditions such as subconfluency, although it is possible that reduced antibody accessibility may also contribute to the failure to detect expression in living confluent cells. Further support for expression of TM4– at the protein level was provided by immunoblotting analysis. Here, a weak positive band for occludin at the size appropriate for the TM4– splice variant (58 kDa) was present in both primate and nonprimate species. Significantly, the level of expression of the 58 kDa band was upregulated in subconfluent versus confluent culture of human epithelial cells.

Epithelial cells are known to become more motile and proliferative in response to wounding, which may involve changing their adhesive state (Martin, 1997). For example, in a culture model similar to the one employed here, peripheral cells in subconfluent islands after wounding have been shown to alter their state of desmosome adhesion (Wallis et al., 2000). We speculate that induction of increased motility and proliferative potential in epithelial cells will also include mechanisms to downregulate TJ integrity. Our data indicate that such a response may in part be mediated by expression of occludin TM4– to modulate intercellular adhesion. In conclusion, we have identified a new mRNA splice variant of the TJ membrane protein, occludin, within human and monkey epithelial cells in which the fourth TM domain is deleted, resulting in misalignment of the C-terminus with respect to the membrane. Preliminary evidence suggests weak expression of this isoform also occurs at the protein level in conditions of cellular subconfluency, which may regulate TJ intercellular adhesion.

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References


Occludin TM4 isoform


