

Post-translational control of occludin membrane assembly in mouse trophoctoderm: a mechanism to regulate timing of tight junction biogenesis and blastocyst formation

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SUMMARY

The mouse blastocyst forms during the 32-cell stage with the emergence of the blastocoelic cavity. This developmental transition is dependent upon the differentiation and transport function of the trophoctoderm epithelium which forms the wall of the blastocyst and exhibits functional intercellular tight junctions (TJs) to maintain epithelial integrity during blastocoele expansion. To investigate mechanisms regulating the timing of blastocyst formation, we have examined the dynamics of expression of occludin, an integral membrane protein of the TJ. Confocal microscopy of intact embryos and synchronised cell clusters revealed that occludin first assembles at the apicolateral membrane contact site between nascent trophoctoderm cells usually during the early 32-cell stage, just prior to the time of blastocoele cavitation. This is a late event in the assembly of TJ-associated proteins within trophoctoderm which, from our previous data, spans from 8- to 32-cell stages. Occludin membrane assembly is dependent upon prior E-cadherin-mediated cell-cell adhesion and is sensitive to brefeldin A, an inhibitor of Golgi-to-membrane transport. Occludin is delivered to the TJ site in association with the

TJ plaque protein, ZO-1 α +, which we have shown previously is newly transcribed and translated during late cleavage. Immediately after assembly and before cavitation, occludin localised at the TJ site switches from a Triton X-100-soluble to -insoluble form indicative of actin cytoskeletal and/or membrane anchorage. Occludin mRNA and protein are detectable throughout cleavage by RT-PCR and immunoblotting, respectively, indicating that timing of membrane assembly is not controlled by expression alone. Rather, we have identified changes in the pattern of different occludin forms expressed during cleavage which, using phosphatase treatment of embryo lysates, include post-translational modifications. We propose that the phosphorylation of one form of occludin (band 2, 65-67 kDa) during late cleavage, which leads to its exclusive conversion from a Triton X-100-soluble to -insoluble pool, may regulate occludin association with ZO-1 α and membrane assembly, and thereby act to control completion of TJ biogenesis and the timing of blastocyst formation.

Key words: Epithelial differentiation, tight junction, occludin, trophoctoderm, mouse embryo, blastocyst.

INTRODUCTION

The first tissue to differentiate during mammalian development is the trophoctoderm epithelium, which forms the wall of the blastocyst and encloses the inner cell mass (ICM). Once formed, the trophoctoderm engages in vectorial transport driven by a basal membrane Na⁺/K⁺-ATPase, thereby generating the blastocoelic cavity and regulating exchange of ions, amino acids, sugars and other metabolites between the maternal uterine environment and the ICM (reviewed in Fleming et al., 1998).

Mouse trophoctoderm differentiation is characterised by the gradual acquisition of polarised epithelial features by blastomeres during cleavage. Differentiation begins at

compaction (8-cell embryo) when intercellular adhesion is initiated, mediated by activation of the E-cadherin complex (Hyafil et al., 1980; Vestweber et al., 1987; Ohsugi et al., 1996), and blastomeres become polarised in terms of surface morphology, membrane composition and cytoplasmic organisation (reviewed in Fleming et al., 1998). In E-cadherin null mutant embryos, trophoctoderm formation is effectively inhibited (Larue et al., 1994; Riethmacher et al., 1995). During 16- and early 32-cell stages, additional features of epithelial organisation are acquired by outer blastomeres, culminating in their ability to generate the blastocoelic cavity by the mid 32-cell stage (reviewed in Fleming et al., 1998).

One important structure regulating the transport capacity of epithelial cells is the zonula occludens or tight junction (TJ), a

belt-like site of close intercellular contact circumscribing the apicolateral border between cells. The TJ is responsible both for the adhesive permeability seal of epithelial sheets which controls paracellular solute diffusion and transepithelial resistance (barrier function) and for the maintenance of compositionally distinct apical and basolateral membrane domains (fence function) (reviewed in Citi and Cordenonsi, 1998; Mitic and Anderson, 1998; Matter and Balda, 1999). The TJ is a multiprotein complex that comprises integral membrane and peripheral cytoplasmic constituents. Occludin (~65 kDa), the first transmembrane TJ protein characterised, contains four membrane-spanning domains with two extracellular loops rich in tyrosine and glycine, a short N-terminal segment and a longer C-terminal domain involved in binding to TJ cytoplasmic proteins (Furuse et al., 1993; Ando-Akatsuka et al., 1996). Expression and mutagenesis studies have shown that occludin is directly involved both in the TJ adhesion process, mediated by the extracellular loops, and in the fence function, maintaining membrane polarity (Furuse et al., 1996; Balda et al., 1996; McCarthy et al., 1996; Van Itallie and Anderson, 1997; Wong and Gumbiner, 1997). The phosphorylation status of occludin has been implicated in its capacity to assemble at the TJ (Cordenonsi et al., 1997; Sakakibara et al., 1997; Wong, 1997). In addition, there is evidence from northern and western blotting that occludin may occur as different isoforms (Saitou et al., 1997; Hirase et al., 1997; Muresan et al., 1998). However, occludin-deficient embryonic stem cells remain capable of TJ formation (Saitou et al., 1998), indicating the presence of other transmembrane proteins with complementary roles to occludin. Indeed, recently, members of the claudin multigene family (~22 kDa), also with four membrane-spanning domains, have been identified at the TJ but are yet to be fully characterised (Furuse et al., 1998; Morita et al., 1999).

Several peripheral cytoplasmic proteins have been identified at the TJ including three members of the MAGUK family (Anderson, 1996), ZO-1 (Stevenson et al., 1986; Itoh et al., 1993; Willott et al., 1993), ZO-2 (Gumbiner et al., 1991; Jesaitis and Goodenough, 1994; Beatch et al., 1996; Itoh et al., 1999) and ZO-3 (Haskins et al., 1998), each of which binds to the C-terminal tail of occludin (Furuse et al., 1994; Haskins et al., 1998; Wittchen and Stevenson, 1998). ZO-1 occurs in two isoforms (ZO-1 α +, ZO-1 α -) derived from alternate splicing (Balda and Anderson, 1993). Other proteins reported to associate with or close to the TJ include cingulin (Citi et al., 1988, 1989), 7H6 antigen (Zhong et al., 1993), symplekin (Keon et al., 1996), the Ras target AF-6 (Yamamoto et al., 1997) and small GTPases rab13 (Zahraoui et al., 1994) and rab3b (Weber et al., 1994).

Previously, we have demonstrated that TJ formation during trophectoderm differentiation follows compaction and is characterised by sequential expression and membrane assembly of the constituents ZO-1 α - at the 8-cell stage (Fleming et al., 1989; Sheth et al., 1997), cingulin during the 16-cell stage (Fleming et al., 1993; Javed et al., 1993) and lastly ZO-1 α + isoform, during the late 16- and early 32-cell stages, immediately prior to blastocoele cavity formation (Sheth et al., 1997). We also demonstrated that ZO-1 α + initially co-localises with occludin intracellularly, the two proteins assembling at the membrane together (Sheth et al., 1997).

Here, we examine the role of occludin in TJ maturation in the trophectoderm. We demonstrate that occludin first

assembles at the nascent TJ site as a late event, usually during the early 32-cell stage, and does so always in association with newly expressed ZO-1 α +. This assembly process is dependent upon E-cadherin adhesion and rapidly converts the assembled occludin from a detergent-soluble to -insoluble form. However, unlike any other TJ constituents studied in the embryo, occludin assembly does not appear to be regulated by the timing of its expression. Rather, we propose that post-translational modification of one form of occludin initiates its membrane assembly and anchorage to the cytoskeleton and membrane, thereby controlling completion of TJ biogenesis and the timing of blastocyst formation.

MATERIALS AND METHODS

Embryo collection, culture and manipulations

Late 2-cell embryos were collected from MF1 strain mated female mice (University of Southampton Biomedical Facility) following superovulation by intraperitoneal injection of 5 i.u. pregnant mares serum (PMS; Folligon, Intervet) followed 48 hours later by 5 i.u. human chorionic gonadotrophin (hCG; Chorulon, Intervet). Embryos were flushed from dissected oviducts using H6 medium (containing 4 mg/ml BSA) and cultured in T6 medium (containing 4 mg/ml BSA) in 5% CO₂ at 37°C until required, as described previously (Fleming et al., 1991; Sheth et al., 1997). Unfertilised eggs within cumulus masses were collected from superovulated unmated mice. Cumulus cells were removed from unfertilised eggs using hyaluronidase, the zona pellucida removed from eggs and embryos using acid Tyrode's solution, and ICMs were isolated from blastocysts by immunosurgery (see Fleming et al., 1991; Chisholm et al., 1985; Sheth et al., 1997 for details). To obtain synchronised embryo cell clusters, zona-free compact 8-cell embryos were cultured in Ca²⁺-free H6 medium for 15 minutes, disaggregated into single cells (1/8 cells) using a flame-polished micropipette, cultured in T6 medium and examined hourly for division to 2/16 cell couplets. Newly divided synchronised couplets were cultured for periods up to 24 hours during which division to 4/32 cells and formation of a blastocoele occurred (Johnson and Ziomek, 1983; Sheth et al., 1997). In other experiments, zona-free embryos at the late morula stage were incubated for varying periods in 5 µg/ml brefeldin A (BFA, Sigma, 10 mg/ml ethanol stock) in T6 plus BSA; control embryos were cultured with solvent alone (0.05% ethanol). In all experiments, embryos were staged as follows from the hCG injection time: 2-cell, 48 hours; 4-cell, 56 hours; early 8-cell, 68 hours; compact 8-cell, 70 hours; 16-cell morula, 78 hours; late morula, 90-96 hours; early blastocysts, 94-98 hours; late (expanded) blastocysts, 115 hours.

Antibodies

Rabbit polyclonal antibodies to chick and human occludin, mouse monoclonal antibody to human occludin (Fallon et al., 1995; Van Itallie and Anderson, 1997) and guinea-pig polyclonal antibody to mouse ZO-1 α + isoform (Sheth et al., 1997) were prepared as described previously. E-cadherin neutralising antibody, ECCD-1 (Shirayoshi et al., 1983), was used at 1:50 dilution of serum in T6 plus BSA.

Immunocytochemistry and confocal microscopy

Zona-free embryos or cell clusters were fixed in PBS containing 1% formaldehyde, attached onto coverslips coated with 1 mg/ml poly-L-lysine hydrobromide and processed for immunocytochemistry as described previously (Fleming et al., 1991). Staged embryos were stained with either rabbit anti-chick occludin (1:50), rabbit anti-human occludin or mouse anti-human occludin antibody (1:1000), prepared in and subsequently washed in PBS: 0.1% Tween 20, and

labelled with FITC-conjugated secondary antibody (Amersham) containing Hoechst dye 33258 (Sigma, 50 µg/ml) for labelling and counting nuclei. Occludin and ZO-1 α + double labelling was as described previously (Sheth et al., 1997). In detergent extraction experiments, embryos without zonae were attached to coverslips using 0.1 mg/ml Concanavalin A (Sigma) in H6 plus 6 mg/ml polyvinylpyrrolidone (PVP) and permeabilised with 0.5% Triton X-100 in H6 plus PVP at 37°C for 10 minutes before fixation with 1% formaldehyde and staining with mouse anti-human occludin antibody. Specimens were visualised using $\times 63$ oil-immersion objective on a Nikon inverted microscope linked to a Bio-Rad MRC-600 series confocal imaging system, equipped with a krypton-argon laser.

Reverse transcriptase-polymerase chain reaction (RT-PCR)

Single-staged embryos were lysed onto messenger affinity paper (mAP, Amersham) and processed for RT-PCR as described by Collins and Fleming (1995), with the exceptions that Vent exo⁻ DNA polymerase (Biolabs) and Dynawax (Dynal) were used. The 3'-antisense outer primer 2 for occludin C-terminal domain was used during the RT step and the entire first strand cDNA reaction mix was added to the first stage PCR. 4% (2 µl) of the first stage product was subsequently added to the second stage reaction and amplified using nested primers. The 5'-outer and inner sense primers were AGTCAACACCTCTGGTGC (primer 1), CTGACCTTGAGTGTG (primer 3), TGACCAAGTGACATCAGCC (primer 5) and CATGTCCGTGAGGCCTT (primer 7). The 3'-antisense outer and inner primers were CAGGCAGATGCACCTCTC (primer 2), GGCACTCTCTAAGG (primer 4), GGCGATGCACATACAAT (primer 6) and GAGCATAGACAGGATCCG (primer 8). Both first and second stage amplification was achieved using 25 cycles at 95°C (30 seconds), 55°C (40 seconds) and 72°C (40 seconds). cDNAs amplified from late blastocysts were either cloned into pBluescript (Stratagene) and sequenced in both directions using a Thermosequenase kit (Amersham) or directly sequenced using a BigDye Terminator kit (Applied Biosystems) and automated sequencing.

Electrophoresis and blotting

Mouse lung 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. Mouse eggs and embryos were briefly washed in H6 plus PVP (Sigma) before solubilisation in boiling SDS-sample buffer for 5 minutes. Samples were run on 4-12% polyacrylamide gradient gels (Novex) and blotted onto Hybond-C nitrocellulose (Amersham) at 300 mA overnight in 25 mM Tris-HCl:150 mM glycine buffer, pH 8.3 containing 0.1% SDS and 20% methanol. Blots were blocked in PBS: 0.3% Tween 20: 10% non-fat milk, before incubating with rabbit anti-human occludin antibody diluted 1:1000 in blocking buffer for 1 hour. Antibody binding was detected with HRP-labelled secondary antibody (Sigma) diluted in blocking buffer. Blots were developed using ECL chemiluminescence (Amersham). Blots were scanned (Nikon Scantouch 210) and analysed by densitometry using NIH Image. For phosphorylation studies, eggs and blastocysts were solubilised in 10 mM Hepes, 150 mM NaCl, 0.1% Triton X-100 and 1% NP40, pH 7.4 for 30 minutes at 4°C, centrifuged (10,000 g, 10 minutes, 4°C) and treated with either (a) 0.1 unit/µl sweet potato acid phosphatase (Sigma) in 0.2 M sodium acetate, 10 mM MgCl₂, pH 5.5 in the presence or absence of 20 µM phenyl arsine oxide inhibitor (PAO; Sigma), or (b) 0.1 unit/µl calf alkaline phosphatase (Boehringer-Mannheim) for 1 hour at 37°C in 50 mM Tris-HCl, 0.1 mM EDTA, pH 8.5, before occludin immunoblotting. Detergent-soluble and-insoluble embryo and lung fractions were generated by solubilisation in 0.5% Triton X-100 in 10 mM Hepes, 150 mM NaCl buffer, pH 7.4, centrifugation (10,000 g, 10 minutes, 4°C) and removal of the soluble fraction, washing the pellet in the above Triton X-100 containing buffer, and solubilising in 1% SDS, 10 mM Hepes, 150 mM NaCl buffer, pH 7.4.

RESULTS

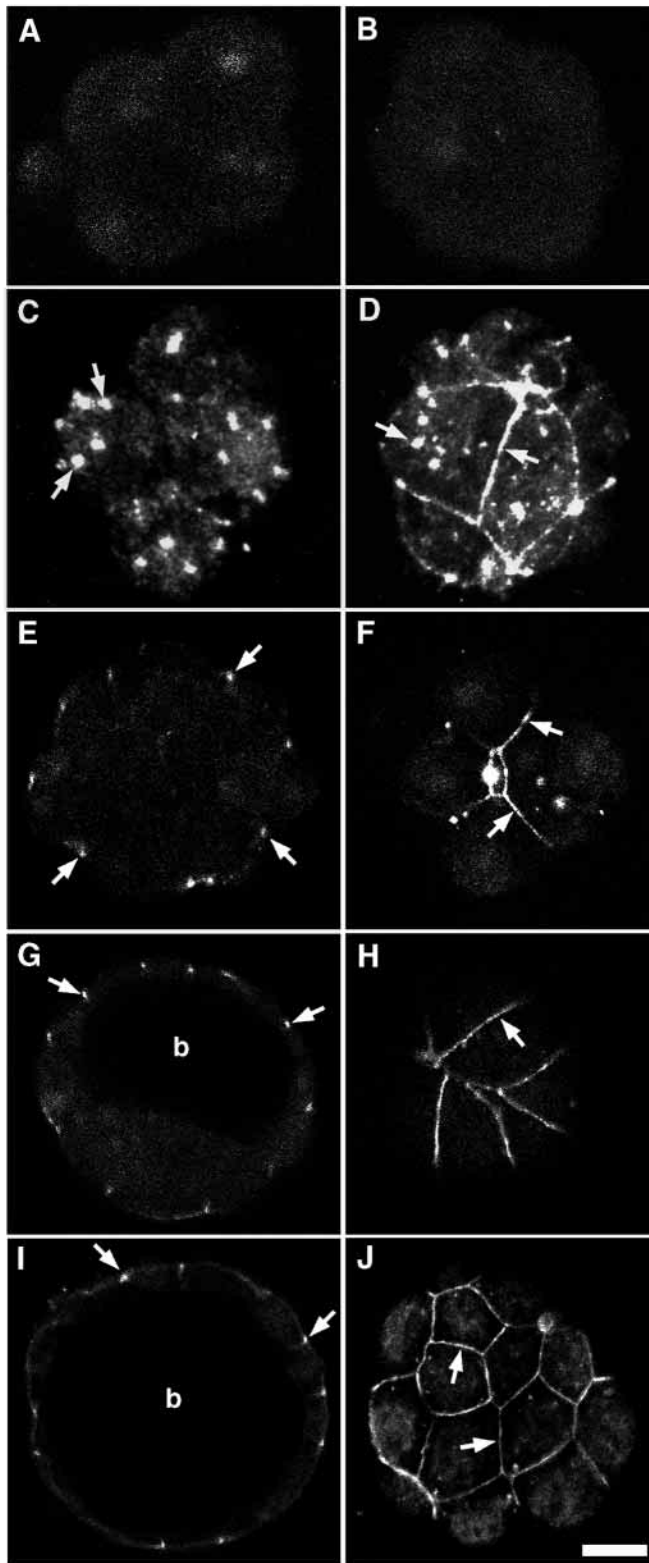
Membrane assembly of occludin

Embryos at precise stages of cleavage were examined by confocal microscopy to determine the timing of occludin membrane assembly. Results using three different antibodies to occludin (see Methods) were essentially the same except where indicated below. Early cleavage and both pre-compact (Fig. 1A) and compact (Fig. 1B) 8-cell stage embryos showed weak diffuse cytoplasmic staining with all of the occludin antibodies. The first distinct staining of occludin occurred in 16-cell embryos using the anti-chick occludin antibody where punctate, perinuclear cytoplasmic foci were evident in outer blastomeres (Fig. 1C, arrows). In late morulae, the pattern of occludin staining changed, with reactivity now also present at the apicolateral TJ site between cells (Fig. 1D, arrows). Comparison between late morulae indicated that as cell-cell contact staining was initiated, cytoplasmic staining gradually decreased. The anti-human occludin antibodies did not stain the intracellular pool of occludin (possibly reflecting an altered protein conformation in the cytoplasm), however, zonular staining of occludin at apicolateral cell contact sites was evident in late morulae (Fig. 1E,F). Examination of early blastocysts, including those with very small blastocoels, revealed occludin staining at TJs in all specimens and minimal or no staining of cytoplasm. In mid-plane optical sections, spots of reactivity were evident at the TJ sites between all trophoblast cells in both polar (adjacent to ICM) and mural (adjacent to blastocoel) regions (Fig. 1G). In tangential optical sections of early blastocysts, occludin was distributed as a continuous belt around each trophoblast cell (Fig. 1H). A similar pattern was evident in late blastocysts (Fig. 1I,J) although staining was consistently weaker than in early blastocysts. In all blastocysts, occludin was not detectable within or between ICM cells (Fig. 1G). Using Hoechst dye to label nuclei in morulae, occludin was first evident at the TJ site in embryos with more than 16 cells (Table 1), indicating that occludin assembly first occurred during either the 16- or 32-cell stages.

Synchronised cell clusters (see Methods) were double labelled for occludin and ZO-1 α + to determine more precisely the timing of occludin membrane assembly. The analysis revealed that, in all cases, occludin was only detectable at membrane TJ sites in cell clusters when precisely co-localised with ZO-1 α + (Fig. 2). After 12 hours culture, 2/16 couplets had not divided and over 90% were negative for both proteins (Figs 2A-C, 3). However, by 21 hours culture, nearly 70% couplets had divided to 4/32 clusters of which over 40% had gone on to cavitate (Fig. 3). At both 21 and 24 hour culture periods, the majority of 4/32 clusters without a cavity and all

Table 1. Relationship between cell number and occludin junctional staining in late morulae and early blastocysts

Cell number range	<i>n</i>	% junctional staining
Late morulae		
16-20	12	33
21-25	10	60
26-30	16	100
31-35	5	100
Early blastocysts		
32-45	9	100



those with a cavity were positive for both proteins (Figs 2D-F, 3). A smaller proportion of 2/16 couplets at these later time points that had failed to divide were also positive for both proteins. These data indicate that occludin membrane assembly first occurs in a complex with ZO-1 α usually during the early 32-cell stage, just prior to blastocoele cavity formation.

Fig. 1. Localisation of occludin during cleavage in whole-mount embryos using immunofluorescence and confocal microscopy. (A) Precompact 8-cell and (B) compact 8-cell embryos in optical midplane section showing weak diffuse labelling with rabbit anti-human occludin antibody. (C,D) Using anti-chick occludin antibody, occludin is evident in outer cells of 16-cell morula (shown in tangential optical section) at perinuclear sites (C, arrows) while in late morulae (tangential section), cell contact staining in addition to perinuclear staining is present (D, arrows). (E-J) The rabbit anti-human occludin antibody did not stain the perinuclear pool of occludin in late morulae but the continuous zonular staining of occludin at cell-cell contacts was evident (arrows; E, mid-plane, F, tangential plane). (G) Mid-plane and (H) tangential sections of early blastocyst showing continuous zonular staining of occludin (arrows) around each trophectoderm cell but absent from the ICM. (I,J) A similar localisation is evident in late blastocysts. B, blastocoele. Bar, 20 μ m.

Properties of occludin membrane assembly in late morulae

Culture of embryos in the presence of E-cadherin neutralising antibody (ECCD-1, Shirayoshi et al., 1983) from compact 8-cell stage (70 hours post-hCG) to late morula stage (92 hours post-hCG) was used to investigate the role of E-cadherin adhesion in occludin membrane assembly. Control late morulae showed typical zonular staining of occludin ($n=27$; Fig. 4A). However, ECCD-1 treated embryos were partially or wholly decompact and were either negative (28%, $n=29$) or only weakly stained for occludin, with just 1-2 cell contact sites per embryo positive (72% Fig. 4B). If ECCD-1 culture was extended for a further 4 hours at which time most (64%, $n=25$) control embryos had cavitated, embryos tended to overcome the block to adhesion as has been shown previously (Johnson et al., 1986; Collins et al., 1995) and showed staining for occludin at contact sites between several blastomeres (75%, $n=28$; data not shown). These results indicate a requirement for E-cadherin mediated cell adhesion for normal membrane assembly of occludin in late morulae.

Late morulae at 92 and 96 hours post-hCG were compared for the detergent solubility of occludin junctional staining. At 92 hours post-hCG, most control embryos (83%, $n=18$) were stained in a zonular pattern at apicolateral contact sites (Fig. 4C) but after Triton X-100 extraction prior to fixation, only 38% ($n=37$) were weakly positive at one or two contact sites per embryo (Fig. 4D). At 96 hour post-hCG, all control ($n=18$) and detergent-extracted ($n=18$) late morulae were stained positively for occludin in a zonular pattern around each outer cell (Fig. 4E). Equivalent detergent-insoluble staining was evident in early blastocysts at 96 hours post-hCG (data not shown). These results indicate that, in late morulae, membrane assembly of occludin is shortly followed by its conversion from a Triton X-100-soluble to -insoluble form before blastocoele cavity formation occurs.

The close temporal relationship between occludin TJ assembly and blastocoele formation is indicative of a functional requirement. To test this proposal, late morulae were cultured for 4 hours in the presence of brefeldin A (BFA, 5 μ g/ml), a drug shown to inhibit specifically transport between Golgi and the cell membrane (Klausner et al., 1992). This treatment, which we have shown previously inhibits blastocoele formation (Sheth et al., 1997), reduced the proportion of late morulae showing membrane assembly of occludin by 38% ($n=22$) of the control level (Fig. 4F).

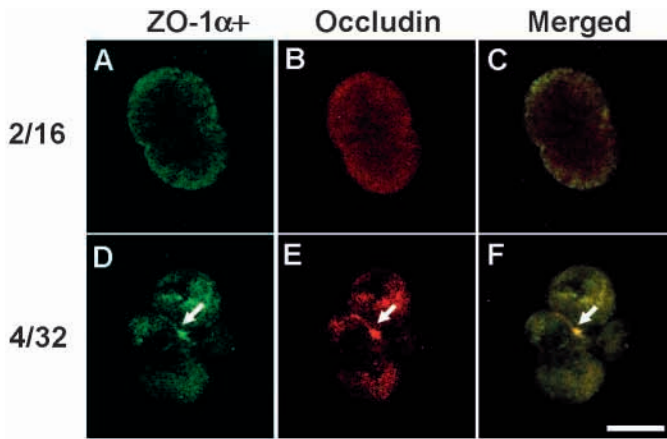


Fig. 2. Synchronised cell clusters double labelled for occludin (red) and ZO-1 α + (green). (A-C) 2/16 couplet 12 hours post-division, negative for both proteins. (D-F) Non-cavitated 4/32 cluster, 21 hours post-division to 2/16, showing co-localisation of occludin and ZO-1 α + at cell contact site (arrows). Bar, 10 μ m.

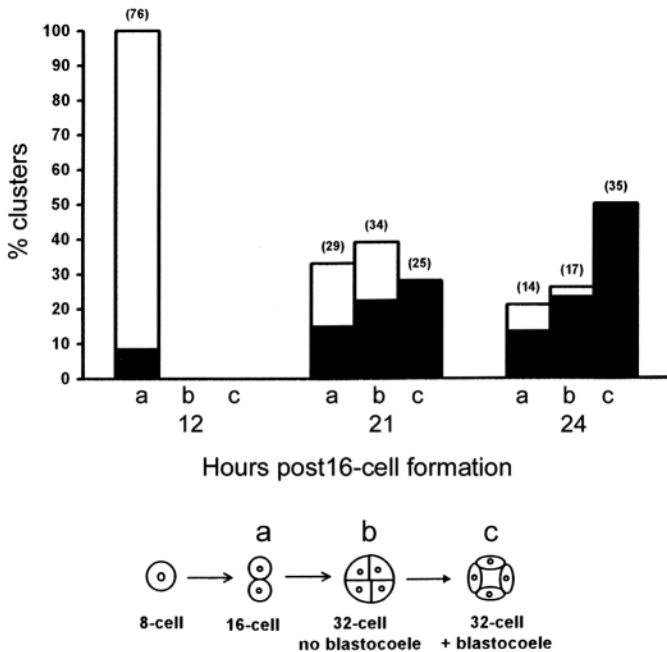


Fig. 3. Relationship between onset of occludin and ZO-1 α + membrane assembly and cell cycle using synchronised cell clusters derived from 2/16 couplets cultured for 12, 21 or 24 hours. Assembly of both proteins occurs at the same time, in most cases after division to 32-cell stage and prior to cavitation. \square Indicates negative and \blacksquare junctional staining for both proteins. Numbers in parenthesis indicate sample size.

Expression of occludin mRNA

To determine whether the timing of membrane assembly of occludin was regulated by its pattern of expression, a sensitive two-stage RT-PCR method was used. The position of nested primers (1, 3 and 2, 4) at the COOH-terminal of full-length mouse occludin is shown in Fig. 5A. A 550 base pair product was amplified using mRNA derived from cumulus cells, single unfertilised eggs and single preimplantation embryos at all

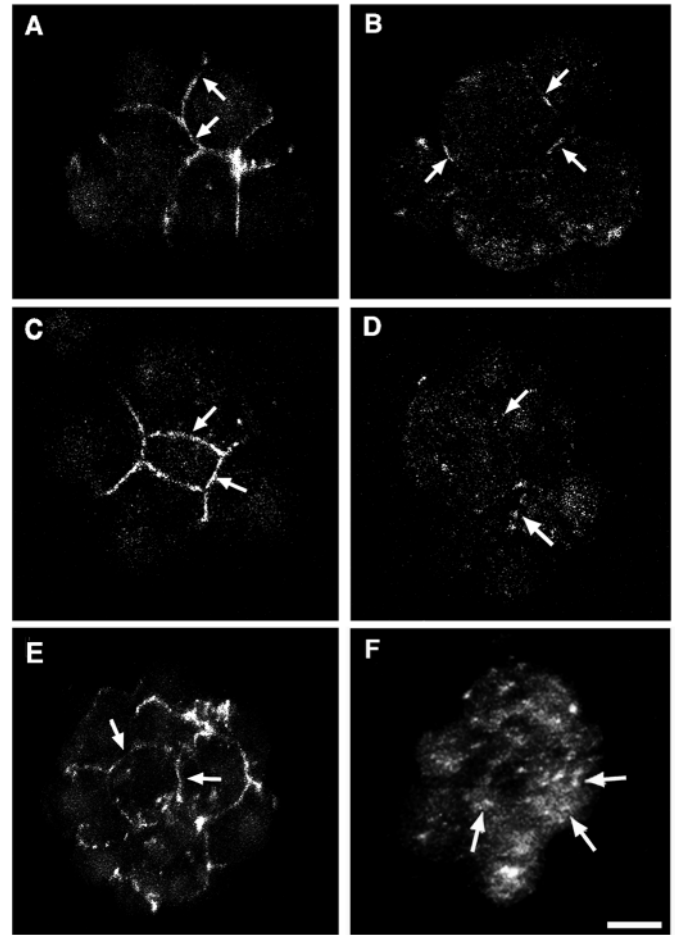


Fig. 4. Properties of occludin membrane assembly. (A,B) Control and experimental late morulae in tangential optical section, cultured in the presence of ECCD-1 (E-cadherin neutralising antibody) for 22 hours from compaction. Control morula has extensive zonular staining of occludin at contact sites (A, arrows) while in the ECCD-1 treated morula, cell contacts are usually negative except for occasional sites (B, arrows). (C-E) Non-extracted control (C) and Triton X-100-extracted late morula (D) in tangential section at 92 hours post-hCG for occludin. Control morula has typical zonular staining at contact sites (C, arrow) while extracted embryo is mainly negative except for rare, isolated sites (D, arrow). (E) Later staged morula (96 hour post-hCG) after Triton X-100 extraction showing more extensive occludin staining at cell contact sites (arrows). (F) Late morula cultured in the presence of brefeldin A (5 μ g/ml, 4 hours) results in retention of occludin at intracellular sites (arrows) and inhibition of membrane assembly. Bar, 20 μ m.

stages up to and including the late blastocyst (Fig. 5B). The intensity of the amplified band in 4-cell embryos was consistently weaker than at other stages. Cloning and sequencing of blastocyst-derived cDNA confirmed 99.8% identity with the published mouse occludin sequence (Ando-Akatsuka et al., 1996). Occludin transcripts were also readily detected in ICMs isolated from early blastocysts by immunosurgery (Fig. 5C). These results indicate that the pattern of occludin mRNA expression cannot account for the timing and tissue specificity of occludin protein membrane assembly.

We have also investigated the possibility that occludin is expressed as different isoforms (Saitou et al., 1997; Hirase et

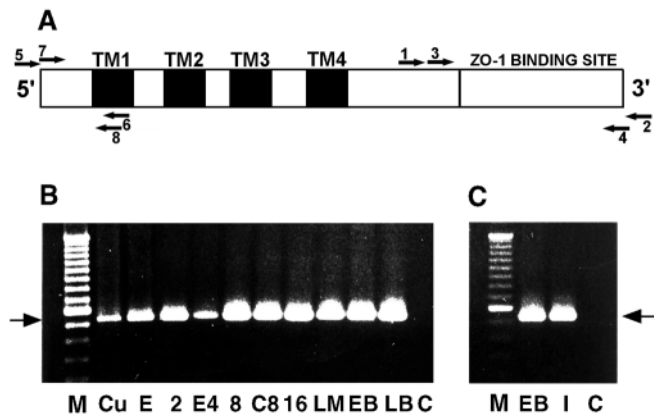


Fig. 5. Detection of occludin mRNA in embryos. (A) Occludin structure showing transmembrane domains (TM1-4) and position (arrows) of outer (1, 2) and inner (3, 4) primers used at the C-terminal cytoplasmic tail with data shown in B. Positions of other primer sets used are also shown (see text). (B) 550 bp RT-PCR product (arrows) amplified using primers 1-4, from single eggs and embryos at all stages examined. Lanes from left, M, markers; Cu, cumulus cells; E, unfertilised egg; 2, 2-cell embryo; E4, early 4-cell; 8, precompact 8-cell; C8, compact 8-cell; 16, 16-cell embryo; LM, late morula; EB, early blastocyst; LB, late blastocyst; C, control with no template. (C) RT-PCR product in blastocysts. M, markers; EB, single early blastocyst; I, three ICMs processed immediately following immunosurgery from early blastocysts; C, control no template.

al., 1997; Muresan et al., 1998). Primers were designed to span three different regions of the mouse coding sequence (Ando-Akatsuka et al., 1996). Primers 5 and 7 (Fig. 5A) together with primers 2 and 4 were used to amplify the complete coding sequence in single unfertilised eggs and blastocysts. Primers 5 and 6 together with 7 and 8 were used to amplify the first 213 base pairs of the coding sequence where, in the canine, a second occludin isoform, Occ-2, has been identified (Muresan et al., 1998). In addition, primers at the beginning of the 5' untranslated region were also employed in RT-PCR with primers 6 and 8. In each case, only a single cDNA product corresponding to the published mouse sequence (Ando-Akatsuka et al., 1996) was generated (data not shown). These data indicate but do not confirm that a single mRNA is expressed within preimplantation embryos.

Expression and modification of occludin protein

Western blotting techniques were used to determine whether regulation of occludin membrane assembly was controlled by the pattern of protein expression and/or modification. Equivalent numbers of unfertilised eggs and embryos at different stages of development, together with an equivalent protein level of mouse lung, were probed in immunoblots using the rabbit anti-human occludin antibody (Fig. 6A,B). Four major bands of occludin were detectable (bands 1-4 at ~72-75, 65-67, 62 and 58 kDa respectively) which varied in intensity and breadth at different developmental stages. In eggs, a broad band 1 was the predominant form which gradually declined in intensity during cleavage, particularly in late blastocysts where it migrated as a narrow band at 72 kDa, the low end of its kDa range. Band 4 also declined in intensity during development, similar to band 1. In contrast, band 2 increased in intensity during cleavage from

a relatively minor band in eggs to become the predominant band in blastocysts; it also became a broader band at the blastocyst stage (Fig. 6A,B). Band 3 was undetectable before the blastocyst stage and remained a minor form in blastocysts. In mouse lung, bands 2 and 3 were the predominant forms although bands 1 and 4, plus other minor bands, were also detectable (Fig. 6A,B). These bands were not detectable in the fibroblast cell line 3T3 or in negative controls (data not shown). Thus, during cleavage, the relative levels of occludin forms changed with stage of development and, by the late blastocyst stage, acquired a pattern similar to that present within lung.

To determine whether the above banding pattern represented different occludin phosphorylation states, lysates were treated with acid or alkaline phosphatase (ACP, ALP) for 1 hour before immunoblotting (Fig. 6C,D). Generally, ACP resulted in weaker antibody reactivity in occludin blots. Both ACP and ALP treatment slightly reduced the apparent molecular mass of the predominant band 1 in eggs from 72-75 to 71-72 kDa which was inhibited by ACP inhibitor (PAO; Fig. 6C). Remaining occludin bands in eggs were not detectable due to the reduced sample loading and weaker antibody reactivity after phosphatase treatment. However, the narrower and smaller-sized band 1 in late blastocysts was unaffected by ACP or ALP. The major broad band 2 (65-67 kDa) in late blastocysts was reduced in breadth by ACP, migrating at the low end of its range at 65 kDa, but was unaffected by ALP (Fig. 6D). Bands 3 and 4 in blastocysts were unaffected by ACP but ALP treatment caused band 3 to disappear whilst band 4 remained unchanged (Fig. 6D). Collectively, these experiments indicate (a) band 1 is phosphorylated in eggs but is relatively unphosphorylated in late blastocysts; (b) in blastocysts, band 2 is phosphorylated while band 3 may be a phosphorylated form of band 4.

The different forms of occludin in embryos and lung were also investigated to determine their presence or absence within Triton X-100-soluble and -insoluble protein fractions (Fig. 6E,F). At the compact 8-cell stage, nearly all the expressed occludin (bands 1, 2 and 4 at this stage) was present within the detergent-soluble fraction with only a trace pool of band 1 and 2 (3% and 2% of total respectively, Fig. 6F) detectable within the insoluble fraction. In contrast, in blastocysts, whilst all four expressed occludin bands were present within the detergent-soluble fraction, a prominent pool of band 2 (representing 59% of total, Fig. 6F) was detectable within the insoluble fraction. In lung, the bulk of occludin protein is present within the insoluble fraction, particularly bands 2 and 3. In both blastocysts and lung, the insoluble pool of band 2 migrated at a slightly higher position than the soluble pool (Fig. 6E). These results suggest that modifications within occludin band 2 may be important for membrane assembly and cytoskeletal and membrane stabilisation of occludin within the developing TJ complex of the trophectoderm lineage.

DISCUSSION

In this paper, we have examined in detail the expression of occludin in the early embryo to interpret mechanisms controlling the timing and biogenesis of the tight junction (TJ) during trophectoderm differentiation. Our confocal microscopy analysis on staged embryos indicated that occludin first assembled at apicolateral membrane contact sites between

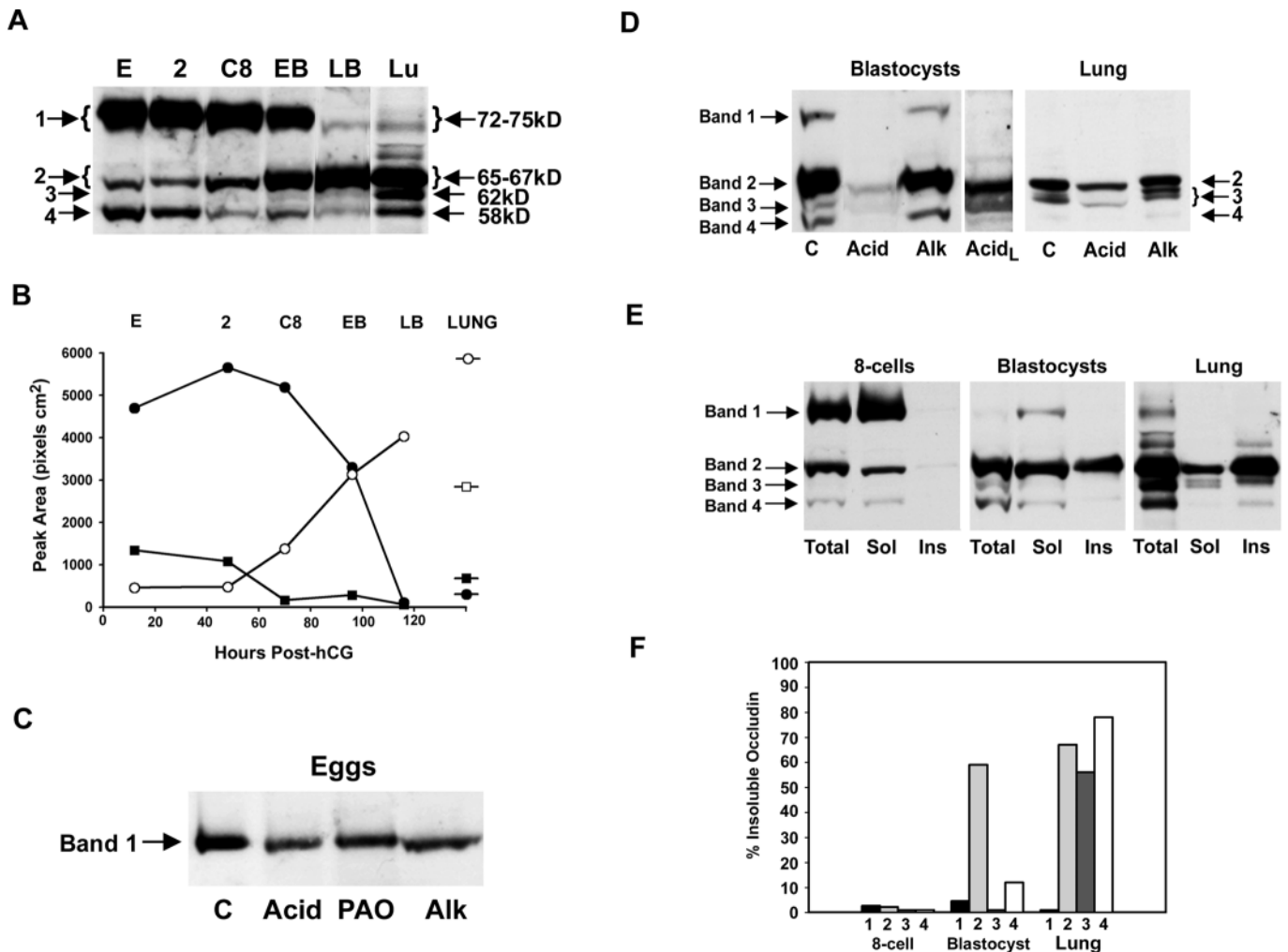


Fig. 6. Biochemical analysis of occludin expression in embryos. (A) Immunoblot of eggs, embryos at specific stages and lung tissue using rabbit anti-human occludin antibody. The same number of eggs and embryos ($n=600$) were added to each lane and an equivalent amount of total protein (12 μ g) added to lung lane. Arrows indicate position of the four main occludin bands 1-4. Lanes, E, unfertilised eggs; 2, 2-cell embryos; C8, compact 8-cell embryos; EB, early blastocysts; LB, late blastocysts; Lu, mouse lung extract. (B) Densitometry scan of above occludin blot showing changing pattern of expression of different bands post-hCG (note: band 3 in blastocysts at 96 and 115 hours post-hCG is below level of densitometric detection). Symbols represent ●, Band 1; ○, band 2; □, band 3; ■, band 4. (C) Lysates of eggs (200 per lane) immunoblotted for occludin following no treatment (lane C), acid or alkaline phosphatase treatment (Acid, Alk) or inhibitor (PAO). Band 1 is reduced in size following both phosphatase treatments. (D) Lysates of late blastocysts (500 per lane) and lung tissue (10 μ g protein per lane) immunoblotted for occludin following acid or alkaline phosphatase treatment. Blastocyst bands 1 and 4 are unaffected by either phosphatase (lane Acid_L, longer exposure to show bands 3 and 4 while band 1 is not detectable and requires further exposure), acid phosphatase causes band 2 to migrate at the low end of its range, and alkaline phosphatase causes band 3 to disappear. Similar changes occur in lung except that band 3 is stable to alkaline phosphatase treatment. (E) Triton X-100-soluble (Sol) and -insoluble (Ins) fractions of 8-cell embryos, blastocysts and lung immunoblotted for occludin (350 embryos or 7 μ g protein per total sample). (F) Densitometry scan of above fractions to show proportion of occludin bands 1-4 occurring within the insoluble pool. Note that whilst nearly all occludin is detergent soluble at the 8-cell stage, band 2 specifically enters the insoluble pool in blastocysts. Biochemical experiments were carried out at least three times with representative results shown here.

cells in late morulae. Prior to this stage, occludin was detected as weak diffuse cytoplasmic staining with the exception of the 16-cell stage when punctate perinuclear sites were evident. Using nuclear-labelled intact embryos and synchronised cell clusters derived from 2/16 couplets, we have found that occludin membrane assembly usually coincided with the early 32-cell stage, just prior to blastocoel cavitation. However, in some of our late-dividing cell clusters, occludin assembly occurred late in the 16-cell stage, indicating that cytokinesis itself is unlikely to contribute to the timing mechanism.

The timing of occludin membrane assembly indicates this to be a late event in trophoblast differentiation, later than the activation of E-cadherin-mediated adhesion and establishment of cell polarisation during compaction at the 8-cell stage (Hyafil et al., 1980; Johnson et al., 1986; reviewed in Fleming et al., 1998). It is also a late event in the maturation of the TJ itself, following the assembly of ZO-1 α isoform (8-cell stage; Fleming et al., 1989; Sheth et al., 1997) and cingulin (early 16-cell stage; Fleming et al., 1993; Javed et al., 1993). We found that occludin assembly always occurred in association with the

ZO-1 α + isoform. This isoform is transcribed and translated de novo during late cleavage (Sheth et al., 1997) indicating that the timing of ZO-1 α + expression may in turn regulate delivery of occludin to the TJ membrane site. This result is consistent with the properties of occludin targeting identified in cell culture studies. Thus, truncated chick occludin minus the ZO-1 binding site, in contrast to full-length occludin, failed to assemble at the TJ in transfected epithelial cells (Furuse et al., 1994). Moreover, full-length human occludin transfected into occludin-null fibroblasts will only assemble at cell-cell contact sites if ZO-1 is already concentrated at these sites (Van Itallie and Anderson, 1997). Although these in vitro studies do not discriminate between ZO-1 isoforms in occludin binding it is well established that in conventional epithelia it is the ZO-1 α + isoform that is predominantly expressed and located at the TJ (Balda and Anderson, 1993). It has been shown recently that an N-terminal ZO-1 construct could bind occludin independent of the α motif (Fanning et al., 1998). The fundamental role we propose for ZO-1 α + in occludin membrane assembly in the early embryo may therefore be an interaction regulated indirectly, for example by conformational changes in either partner controlling accessibility of binding sites.

Membrane assembly of occludin was shown to be dependent upon prior E-cadherin adhesion and was inhibited if adhesion from compaction was blocked using a neutralising E-cadherin antibody. The earlier assembly of TJ-associated proteins in the embryo, ZO-1 α - (Fleming et al., 1989) and cingulin (Javed et al., 1993), were shown to require E-cadherin adhesion for their normal timing, localisation and/or stabilisation from turnover. Thus, embryo compaction appears to act as a permissive state for all steps in TJ construction during trophectoderm differentiation. It is also from compaction that, in freeze-fracture ultrastructural analysis, nascent intramembranous fibrils have been detected in blastomeres (Ducibella and Anderson, 1975; Pratt, 1985). Although this precedes the timing of membrane assembly of occludin, at present, assembly of other TJ membrane proteins (claudin and JAM) have not been reported in the early embryo. Membrane assembly of occludin in late morulae was also inhibited by brefeldin A treatment, a drug that specifically inhibits vesicular transport between the Golgi and the cell membrane (Klausner et al., 1992). Brefeldin A-treated embryos exhibited a block at the stage when occludin is localised within perinuclear foci, indicating that these sites represent Golgi complexes which are known to have this localisation within blastomeres in morulae (Fleming and Pickering, 1985).

Newly assembled occludin in late morulae was shown to rapidly convert from a Triton X-100-soluble to -insoluble form prior to blastocoel formation. The most likely explanation is that membrane and cytoskeletal association of the TJ complex is coordinated with occludin assembly and is required before the TJ can function in providing a permeability seal. Actin associated with the TJ has been implicated in controlling epithelial paracellular permeability (reviewed in Mitic and Anderson, 1998). Possibly, the association between occludin and ZO-1 α + may permit cytoskeletal anchorage of the TJ since the proline-rich C-terminal half of ZO-1 where the alpha domain resides has been shown to cosediment with F-actin (Fanning et al., 1998).

We investigated the pattern of expression of occludin during cleavage to determine whether this regulated its timing of

membrane assembly in the late morula. Using RT-PCR, we have demonstrated the presence of occludin mRNA in unfertilised eggs, cumulus cells and all preimplantation embryo stages, confirmed by cloning and sequencing of the product. In view of our protein data showing different forms of occludin, and recent data from northern and western analysis for the presence of different occludin isoforms (Saitou et al., 1997; Hirase et al., 1997; Muresan et al., 1998), we have investigated this possibility by RT-PCR. Despite using a series of primer combinations, only a single product was detected and shown by sequencing to be identical to the published mouse sequence (Ando-Akatsuka, 1996). Although the simplest interpretation is that a single mRNA transcript is expressed during cleavage, this cannot be confirmed until more detailed studies using northern and cDNA library analysis have been carried out. Although the PCR method employed here is not quantitative, the amount of occludin cDNA amplified from single embryos consistently declined during the 4-cell stage and then increased again during the precompact 8-cell stage. This decrease in the amount of mRNA has also been shown to occur in the case of other transcripts (Collins et al., 1995; Houghton et al., 1996; Sheth et al., 1997) and is likely to reflect the degradation of maternal RNAs in early cleavage stages (Paynton et al., 1988; Bachvarova et al., 1989).

Occludin transcripts were also detected within ICMs isolated by immunosurgery from early blastocysts, indicating that transcription of this gene is not tissue-specific within early embryos, despite the fact that, according to our confocal data, the protein is not assembled in these internal cells. Similarly, transcripts for both isoforms of ZO-1 are present within trophectoderm and ICM cells of blastocysts although these proteins only assemble at trophectoderm TJ sites (Sheth et al., 1997). We have previously shown that ZO-1 (Fleming and Hay, 1991) and cingulin (Fleming et al., 1993; Javed et al., 1993) membrane assembly within ICM cells can be activated by creating a contact-free cell surface, such as occurs following immunosurgical isolation and culture of ICMs. In the case of cingulin, the absence of a contact-free cell surface on ICM cells in situ has been shown to reduce the level of translation (Javed et al., 1993). Presumably similar mechanisms of downregulation also account for the lack of detection here of occludin protein within ICM cells.

The immunoblotting analysis demonstrated the presence of occludin protein at all stages of development examined. However, different forms of occludin were detectable and their relative abundance changed with developmental stage. Similarly, multiple bands of occludin have been detected in confluent MDCK cells (Sakakibara et al., 1997; Wong, 1997), mouse cell lines (Saitou et al., 1997) and during *Xenopus* embryo development (Cordenonsi et al., 1997), which may represent different isoforms (Saitou et al., 1997; Hirase et al., 1997; Muresan et al., 1998). In view of our RT-PCR data, we consider the different forms of occludin detected in the embryo to be products of a single mRNA, but this has yet to be confirmed. Mouse occludin consists of a series of putative phosphorylation and two glycosylation sites which, collectively, may permit post-translational processing resulting in the presence of different forms. The most important protein changes were the decline in intensity of bands 1 (72-75 kDa) and 4 (58 kDa) and the increase in intensity and breadth of band 2 (65-67 kDa) during cleavage and blastocyst formation,

and the emergence of band 3 (62 kDa) at the blastocyst stage itself. Moreover, phosphatase treatments further indicated that occludin post-translational modifications contributed to the developmentally regulated banding pattern. This changing profile is likely to be significant in the trophoblast epithelial differentiation programme since it gradually acquired a similarity to those features evident in mature lung tissue.

Do one or more of these changes to occludin forms explain the protein's capacity in late morulae to incorporate into the nascent TJ and contribute to the establishment of a permeability seal? Potential mechanisms identified from our phosphatase results would include the apparent dephosphorylation of band 1, phosphorylation of band 2 or the emergence of band 3, all of which occur at the blastocyst stage. Alternatively, the relative proportions of different isoforms or the threshold level(s) of one or more forms may be important in this respect. One significant result from the confocal microscopy study was the rapid switch after membrane assembly of occludin from a Triton X-100-soluble to -insoluble pool (see also above). Immunoblotting examination of detergent-soluble and -insoluble fractions at two embryo stages, either before (compact 8-cell stage) or after (late blastocyst stage) occludin membrane assembly and TJ sealing, revealed that occludin band 2 exclusively underwent a major switch in its state of Triton X-100 solubility with most of this form entering the detergent-insoluble pool. Moreover, in mature lung tissue, band 2 is the most abundant of the occludin forms and again is mainly associated with the insoluble pool. These results indicate that occludin band 2 is the form that engages in membrane assembly and incorporation into the trophoblast TJ complex. According to our data, this form is detectable throughout cleavage but becomes broader at the blastocyst stage, a modification that is reversed by acid phosphatase treatment indicating it is due to phosphorylation. We therefore propose that the apparent post-translational modification of band 2 may be significant both in its capacity to associate intracellularly with ZO-1 α + and to assemble at the TJ. Moreover, the insoluble pool of band 2 in blastocysts and lung migrates at a slightly higher position than that of the soluble pool, suggesting a correlation between phosphorylated and insoluble states of the protein. Such a correlation has been identified in MDCK cells between phosphorylation of occludin and its detergent insolubility, coinciding with occludin's ability to incorporate into the TJ (Wong, 1997; Sakakibara et al., 1997).

In conclusion, we have shown that the integral membrane protein, occludin, incorporates into the TJ complex usually during the early 32-cell stage of trophoblast differentiation. This event is a late step in TJ maturation and is rapidly followed by blastocoel cavity formation, which is dependent upon the integrity of the TJ intercellular seal. The timing of occludin membrane incorporation, unlike other TJ-associated proteins examined so far in the embryo model, appears not to be regulated by its pattern of transcription or translation but rather by post-translational modifications. In particular, we identify the apparent phosphorylation of one form (65-67 kDa, band 2) as a potential regulator of occludin membrane assembly which may act to permit its association with the newly expressed TJ plaque protein, ZO-1 α +, leading to the occludin-rich TJ complex becoming anchored to the actin cytoskeleton and membrane. Thus, we propose that occludin

modification, together with de novo transcription of ZO-1 α + (Sheth et al., 1997), acts as a developmental mechanism to regulate the sealing of the TJ and, thereby, the timing of blastocyst formation.

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