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Tight junction assembly during mouse blastocyst formation is regulated by late expression of ZO-1 α^+ isoform

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SUMMARY

The mouse preimplantation embryo has been used to investigate the de novo synthesis of tight junctions during trophectoderm epithelial differentiation. We have shown previously that individual components of the tight junction assemble in a temporal sequence, with membrane assembly of the cytoplasmic plaque protein ZO-1 occurring 12 hours before that of cingulin. Subsequently, two alternatively spliced isoforms of ZO-1 (α^+ and α^-), differing in the presence or absence of an 80 residue α domain were reported. Here, the temporal and spatial expression of these ZO-1 isoforms has been investigated at different stages of preimplantation development. ZO-1α^- mRNA was present in oocytes and all preimplantation stages, whilst ZO-1α^+ transcripts were first detected in embryos at the morula stage, close to the time of blastocoele formation. mRNAs for both isoforms were detected in trophectoderm and ICM cells. Immunoprecipitation of ^{35}S-labelled embryos also showed synthesis of ZO-1α^- throughout cleavage, whereas synthesis of ZO-1α^+ was only apparent from the blastocyst stage. In addition, ^{33}P-labelling showed both isoforms to be phosphorylated at the early blastocyst stage. The pattern and timing of membrane assembly of the two isoforms was also distinct. ZO-1α^- was initially seen as punctate sites at the cell-cell contacts of compact 8-cell embryos. These sites then coalesced laterally along the membrane until they completely surrounded each cell with a zonular belt by the late morula stage. ZO-1α^+ however, was first seen as perinuclear foci in late morulae before assembling at the tight junction. Membrane assembly of ZO-1α^+ first occurred during the 32-cell stage and was zonular just prior to the early blastocyst stage. Immunostaining indicative of both isoforms was restricted to the trophectoderm lineage. Membrane assembly of ZO-1α^+ and blastocoele formation were sensitive to brefeldin A, an inhibitor of intracellular trafficking beyond the Golgi complex. In addition, the tight junction transmembrane protein occludin co-localised with ZO-1α^+ at the perinuclear sites in late morulae and at the newly assembled cell junctions. These results provide direct evidence from a native epithelium that ZO-1 isoforms perform distinct roles in tight junction assembly. Moreover, the late expression of ZO-1α^+ and its apparent intracellular interaction with occludin may act as a final rate-limiting step in the synthesis of the tight junction, thereby regulating the time of junction sealing and blastocoele formation in the early embryo.

Key words: tight junction, ZO-1, occludin, trophectoderm, mouse embryo, blastocyst, epithelial differentiation

INTRODUCTION

The first epithelium to form during mammalian development is the trophectoderm of the blastocyst. In the mouse, trophectoderm differentiation begins at the 8-cell stage following activation of E-cadherin cell-cell adhesion and polarisation of blastomeres during embryo compaction (Hyafil et al., 1980; Ziomek and Johnson, 1980). Over the next 24 hours (covering two cell cycles), epithelial differentiation continues in outer blastomeres and is completed during the 32-cell stage when the nascent trophectoderm first engages in transport activity, resulting in blastocoele cavity formation (Chisolm et al., 1985; reviewed in Fleming and Johnson, 1988; Fleming, 1992; Collins and Fleming, 1995a). This model system provides an opportunity to study in vitro the differentiation of epithelial characteristics and mechanisms regulating functional activity of the epithelium.

Here, we investigate the construction of the epithelial tight junction (zonula occludens; TJ) during trophectoderm differentiation. TJs form a continuous belt around the apicolateral border of adjacent cells and restrict paracellular passage of ions and small molecules (barrier function), thereby contributing to transepithelial electrical resistance and the maintenance of distinct fluid compartments (Claude, 1978). TJs also preserve

the asymmetry in protein and lipid composition of epithelial apical and basolateral membranes (fence function) (reviewed in Gumbiner, 1987; Citi, 1993; Anderson and Van Itallie, 1995). The TJ is a multimolecular complex comprising at least one integral membrane protein, occludin, which engages in intercellular adhesion (Furuse et al., 1993, 1994) and several associated peripheral membrane proteins. The latter form a cytoplasmic plaque which interacts with actin filaments (reviewed in Gumbiner, 1987; Citi, 1993; Anderson and Van Itallie, 1995).

The TJ peripheral membrane proteins include ZO-1 (M_r~220×10^3; Stevenson et al., 1986; Itoh et al., 1993; Willott et al., 1993), which binds to the cytoplasmic domain of occludin (Furuse et al., 1994), and ZO-2 (M_r~160×10^3), which co-precipitates with ZO-1 (Gumbiner et al., 1991; Jesaitis and Goodenough, 1994). ZO-1 and ZO-2 are both members of a diverse family of putative signal transduction proteins known as MAGuKs (membrane-associated guanylate kinase homologue proteins; reviewed in Anderson and Van Itallie, 1995; Anderson, 1996). Other MAGuKs include *Drosophila* discs-large protein (Dlg-A) found at septate junctions (Woods and Bryant, 1991), rat PSD-95 present at the postsynaptic surface of neurons (Cho et al., 1992), human p55 a component of the red blood cell cytoskeleton (Ruff et al., 1991), and *C. elegans* LIN-2A protein involved in vulval development (Hoskins et al., 1996). All family members share three common domains which may play a role in membrane signalling; a 90 amino acid repeat domain known as DHR (discs-large homology region) or PDZ (PSD-95, Dlg-A and ZO-1), a src homology region 3 (SH3) domain, and a region homologous to the enzyme guanylate kinase. In addition, ZO-1 and ZO-2 have a proline-rich carboxy terminal sequence and ZO-1 has alternatively spliced isoforms, the longer isoform having an extra 80 amino acid domain known as the α-domain (Willott et al., 1992, 1993; Anderson et al., 1995). Although the function of the α-domain is unclear, ZO-1α^+ isoform is found in conventional epithelial TJs while ZO-1α^- is present in endothelial junctions and highly specialised epithelial TJs characteristic of Sertoli cells and renal podocytes. This pattern of ZO-1 isoform expression may influence the plasticity of TJs (Balda and Anderson, 1993). Other proteins reported to occur at the TJ cytoplasmic plaque include cingulin (Citi et al., 1988, 1989), p130 (Balda et al., 1993), 7H6 antigen (Zhong et al., 1993), rab13 (Zahraoui et al., 1994) and rab3b (Weber et al., 1994).

Previously, we have shown that TJ formation during trophectoderm differentiation is a gradual process characterized by sequential expression and membrane assembly of ZO-1 (from 8-cell stage, approximately 1 hour after compaction; Fleming et al., 1989) and cingulin (from 16-cell stage, 10-12 hours later; Fleming et al., 1993a; Javed et al., 1993). However, the mechanism regulating completion of TJ assembly and sealing capacity during the 32-cell stage, when the blastocoele is formed, is unknown. Here, we report on the expression of the ZO-1 isoforms and demonstrate that the initiation of embryonic transcription, translation and membrane assembly of ZO-1α^- and α^+ variants at putative TJ sites is differentially regulated. ZO-1α^- assembly occurs as an early event in TJ formation, from the 8-cell stage onwards following compaction, whereas ZO-1α^+ assembly occurs as a very late step in TJ biogenesis, during the early 32-cell stage immediately prior to blastocoele fluid accumulation. Moreover, we have found that ZO-1α^+ co-localises with occludin within perinuclear sites before co-ordinated incorporation of both proteins occurs into putative TJs. These data provide new insight into the control of TJ formation, and the timing of TJ sealing and blastocoele formation in the early embryo.

MATERIALS AND METHODS

Embryo collection, culture and manipulations

Embryos were collected from mated 3- to 5-week-old females of the MF1 mouse strain (University of Southampton Animal House) following superovulation by intraperitoneal injection of 5 i.u. pregnant mares serum (PMS) followed 48 hours later by 5 i.u. human chorionic gonadotrophin (hCG) as described previously (Fleming et al., 1991). Embryos were flushed at the late 2-cell stage from dissected oviducts using H6 medium containing 4 mg/ml bovine serum albumin (BSA) and cultured in T6 plus BSA until required. Unfertilised eggs within cumulus masses were collected from superovulated unmated mice. Cumulus cell removal from unfertilised eggs using hyaluronidase, zona pellucida removal from eggs and embryos using acid Tyrode's solution, and isolation of inner cell masses (ICMs) from blastocysts by immunosurgery were achieved as described previously (Chisholm et al., 1985; Fleming et al., 1991). 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, 92 hours; blastocysts, 94-98 hours; late (expanded) blastocysts, 115 hours.

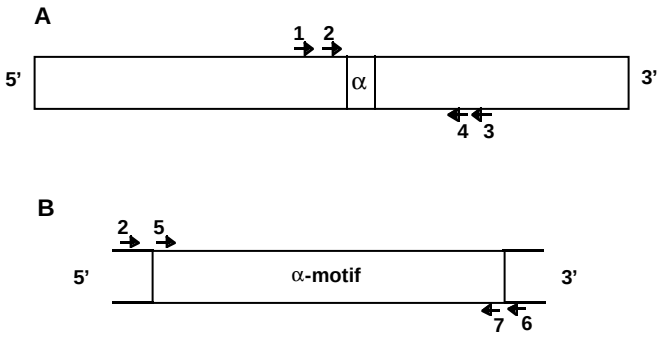
To obtain synchronised embryo cell clusters, zona-free compact 8-cell embryos were cultured in calcium-free H6 plus BSA for 15 minutes, disaggregated into single cells (1/8 cells) using a flame-polished micropipette, cultured in T6 plus BSA and examined hourly for division to 2/16 cell couplets. Synchronised 2/16 couplets were cultured individually up to the 4/32 cell stage and formation of a miniature blastocoele. Previous studies have established that couplets prepared in this manner maintain their developmental programme as would occur in the intact embryo (Fleming et al., 1989, 1993a). 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).

Reverse transcriptase-polymerase chain reaction (RT-PCR)

A two-stage RT-PCR method was employed essentially as described by Collins and Fleming (1995b) with the exceptions that Vent exo$^-$ DNA polymerase (Biolabs) and Dynawax (Dynal) were used, the 3′-antisense primer used during the RT step was ZO-1 specific and all the first strand cDNA reaction mix was added to the first stage amplification. Primers used for PCR and their positions on the full-length mouse ZO-1 sequence are shown in Fig. 1. Primers 1, 3 and 2, 4 were used to amplify both isoforms whilst primers 2, 6 and 5, 7 were used to amplify the α-motif. 25 cycles of amplification were carried out at 95°C (30 seconds), 55°C (40 seconds) and 72°C (40 seconds) for both first- and second-stage reactions. DNA amplified from blastocysts was cloned into pBluescript (Stratagene) and sequenced in both directions using [^{35}S]dATP and the Sequenase II kit (Stratagene).

Whole-mount fluorescent in situ hybridisation (FISH)

Sequence common to both mouse ZO-1 isoforms (bp3179-3646) and sequence coding the α-motif (bp2767-3003) were cloned separately into pGEM3Z at the *Eco*RI site in both directions. These constructs were then transcribed using T7 RNA polymerase and a digoxigenin (DIG) RNA labelling kit, according to the manufacturer's instructions (Boehringer Mannheim) to produce sense and antisense probes. Zona-



C Primer sequences

1 GCTATTCATAGAATAGACTC
2 CCTGGACTTAAGCCAGC
3 TGCAGCACCATGGAGAG
4 GCTCGTAATGGCCTGTTA
5 GCAGAAGCCTCATCT
6 CCTTCCTGTACACCTTTGC
7 TGCTGGGTCTGCATG

Fig. 1. Representation of mouse ZO-1 sequence, showing the position of the α-domain and nested primers used for cDNA amplification of (A) both isoforms and (B) α-motif. Arrows mark the position and direction of primers used for cDNA synthesis. (C) cDNA sequences of primers in a 5′→3′ direction.

free embryos were fixed in 3.7% formaldehyde in PBS for 20 minutes, dehydrated sequentially in 25%, 50%, 75% and 100% methanol made up in PBS:0.1%Tween20 (PBS/T) and rehydrated in the reverse order. After proteinase K treatment (10 μg/ml in PBS/T, 30 minutes), the embryos were refixed with 3.7% formaldehyde plus 0.2% glutaraldehyde in PBS/T for 10 minutes, washed in PBS/T, prehybridised for 2 hours at 70°C and then 2 hours at 64°C in hybridisation buffer (50% formamide, 5× SSC, 1× Denhardt's, 1% SDS, 160 μg/ml total yeast RNA, 100 mg/ml heparin and 2% Boehringer Mannheim blocking buffer), before hybridisation overnight at 64°C in the presence of 0.5-1 μg/ml DIG-labelled RNA probe. The embryos were then washed for 20 minutes each in (a) hybridisation buffer, (b) solution 1 (50% formamide, 5× SSC, 1%SDS), (c) solution 2 (50% formamide, 2× SSC, 1%SDS), (d) solution 3 (50% formamide, 2× SSC) and (e) Tris-buffered saline:0.1% Tween 20 (TBS/T; 25 mM Tris-HCl pH 7.6, 140 mM NaCl and 2.7 mM KCl). Embryos were heated for 20 minutes at 70°C to inactivate endogenous alkaline phosphatases before staining with alkaline phophatase-labelled anti-DIG antibody (Boehringer Mannheim) overnight at 4°C. These samples were finally washed with TBS/T containing 2 mM levamisole and stained with HNPP, a fluorescent substrate for alkaline phosphatase, according to manufacturer's conditions (Boehringer Mannheim). Embryos were examined by confocal microscopy.

GST-fusion protein and antibody preparations

Two glutathione-S-transferase (GST) fusion proteins were generated according to manufacturer's instructions (Pharmacia). The first contained the rat sequence rZ1 (Willott et al., 1992) common to both ZO-1 isoforms (equivalent to base pair 2610-3624 on the mouse sequence; Itoh et al., 1993). This construct was made before the availability of a mouse sequence and subsequent alignment of rZ1 showed 85% identity at the amino-acid level with mouse ZO-1. The second fusion protein was derived from an RT-PCR product of mouse blastocysts and contained sequence exclusive to the α-motif (base pair 2767-3003). Both sequences were cloned into the *Eco*RI site of pGEX:1λT (Pharmacia) and electroporated into XL-1 blue (Stratagene). Production of the fusion proteins was induced with 0.1 mM IPTG for 4 hours at 37°C before purification. The ZO-1 portion of the fusion protein was cleaved off with thrombin (Sigma) and the dialysed proteins were injected in adjuvant, using standard procedures to generate polyclonal antisera in rabbits. The α-motif construct was also injected into guinea-pigs, to raise antibodies for use in double-labelling experiments. In addition, a rabbit polyclonal antibody was raised to a synthetic peptide (ASQQVYRKEPC) corresponding to the mouse ZO-1 splice junction and present only in ZO-1α^- isoform (Itoh et al., 1993). The COOH-terminal cysteine was added to the peptide in order to couple it to maleimide-activated Keyhole Limpet Haemocyanin (Pierce). Rabbit antibody used in our experiments to the carboxy-terminal of chick occludin has been characterised previously (Fallon et al., 1995). All antibodies were affinity purified on their respective fusion protein, or peptide, coupled to cyanogen bromide-activated sepharose (Pharmacia).

Electrophoresis and blotting

Soluble mouse lung extract for electrophoresis was generated using frozen tissue powder boiled for 5 minutes in PBS:1% SDS followed by centrifugation at 10,000 ***g*** for 3 minutes. Mouse blastocysts were solubilised by boiling in SDS-sample buffer for 5 minutes. 60 μg lung protein and 1000 blastocysts were loaded per lane on a large 7.5% polyacrylamide gel (Bio-Rad). Proteins were blotted onto Hybond-C nitrocellulose (Amersham) at 300 mA overnight in 25 mM Tris: 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 0.1 μg/ml primary antibody or control preimmune serum, diluted in the blocking buffer. The antibody binding was then detected with HRP-labelled goat anti-rabbit secondary antibody (Sigma), diluted 1:10,000 in blocking buffer. All incubations were carried out for 60 minutes at room temperature. Blots were developed using an ECL chemiluminescence kit (Amersham).

Metabolic labelling and immunoprecipitation

Staged embryos were radiolabelled in Trans [^{35}S]-label (ICN Flow; 1000 μCi/ml in T6 plus BSA for 4 hours) and immunoprecipitated as described (Fleming et al., 1991). For each immunoprecipitation, lysates from 500 embryos were precleared using 40 μl of preimmune serum and 75 μl of protein A-sepharose. 40 μl of specific polyclonal antiserum was then added overnight at 4°C, followed by 75 μl of protein A-sepharose for 2 hours. 16-cell embryos and early blastocysts were also labelled with 1 mCi/ml [^{33}P]orthophosphate (ICN Flow) in phosphate-free T6 plus BSA, for 4 hours. Embryos were washed in phosphate-free H6 plus 6 mg/ml PVP (Sigma) and immunoprecipitated as above. Samples were electrophoresed on a 7.5% polyacrylamide gel which was then fixed, incubated in Amplify (Amersham, 30 minutes), dried and exposed to X-ray film (Kodak) for 2-4 weeks at –70°C.

Immunocytochemistry and confocal microscopy

Zona-free embryos were fixed in either 1% formaldehyde in PBS or ice-cold methanol, then attached to coverslips within processing chambers pretreated with 1 mg/ml poly-L-lysine hydrobromide or 0.1 mg/ml Concanavalin A in PBS, respectively, and processed for immunocytochemistry as described previously (Fleming et al., 1991). Staged embryos were stained with 20 μg/ml affinity-purified ZO-1α^+ or ZO-1α^- rabbit antibodies, washed in PBS/T and labelled with 1:100 dilution of FITC-conjugated secondary antibody (Amersham). Controls using preimmune serum were included at each stage of cleavage. Guinea-pig ZO-1α^+ antibody and rabbit occludin antibody were used in double-labelling experiments followed by FITC and Texas Red-conjugated secondary antibodies (Amersham). Specimens were visualised using a plan apo ×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. Hoechst dye 33258 (Sigma, 50 μg/ml) added to the secondary antibody solution was used for staining and counting of nuclei.

RESULTS

ZO-1 mRNA detection

Using a sensitive RT-PCR method, transcripts for both ZO-1 isoforms were detectable in preimplantation embryos (Fig. 2A). ZO-1α^- was present in follicular cumulus cells, unfertilised eggs and in all cleavage stages including blastocysts. In contrast, ZO-1α^+ was found in cumulus cells but was undetectable in eggs and in early/mid cleavage stages, but occurred again at the morula and blastocyst stages. This pattern of ZO-1 expression was detectable using single eggs/embryos as templates at the appropriate stage and with nested primers either side of the α domain, which resulted in one or two bands corresponding in size to the ZO-1 isoforms (Fig. 2A). Since amplification of a shorter cDNA may occur faster and at the expense of a longer cDNA, we also designed primers within the α domain (see Fig. 1) for separate detection of ZO-1α^+. In this case, no transcripts were detected at or before the 8-cell stage, whilst 44% of 16-cell morula, 89% of late morulae and 100% blastocysts (*n*=9 each, Fig. 2B) were positive for ZO-1α^+. RT-PCR employing both primer sets as previously was also used to investigate tissue specificity of ZO-1 isoforms. Both isoforms were readily detected in single early blastocysts and groups of three ICMs (approximately the same cell number as one blastocyst) examined immediately after immunosurgery (Fig. 2C), indicating that transcription of ZO-1 is not tissue specific.

Fluorescent in situ hybridisation (FISH) on whole-mount embryos at different stages of cleavage was also used for localisation of ZO-1 isoform expression. Antisense RNA probe, recognising both isoforms, hybridised to 2- and 4-cell embryos weakly but above background as assessed by comparison with control sense RNA (Fig. 3A-D). Hybridisation to 8-cell embryos was more pronounced than in early cleavage stages (Fig. 3E,F) and the signal was stronger again at the early blastocyst stage (Fig. 3G,H). Staining in both trophectoderm and ICM cells was evident; however, in late blastocysts mural trophectoderm labelling was consistently weaker than polar trophectoderm (Fig. 3I,J). Hybridisation of the antisense α-motif probe was not above background in 4- and 8-cell embryos (compare Fig. 4A-D). However, at the blastocyst stage, both

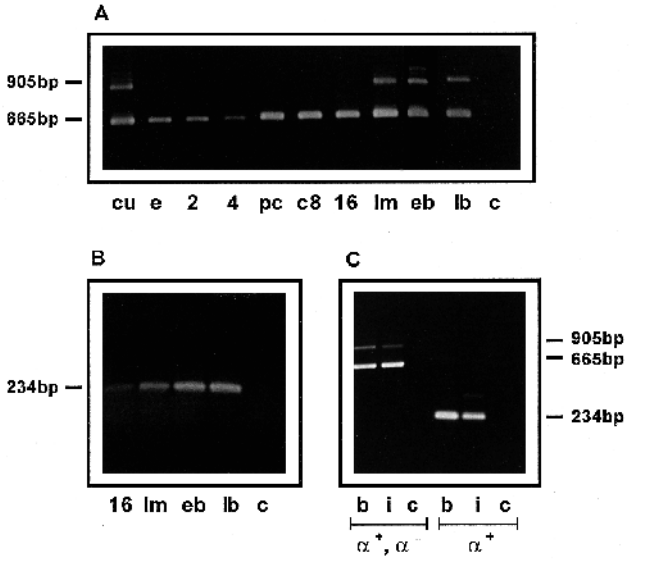


Fig. 2. RT-PCR of ZO-1 from single eggs and preimplantation embryos at different stages of cleavage using primers designed to detect both ZO-1α^+ and ZO-1α^- (A) or only ZO-1α^+ (B). Lanes are (cu) cumulus cells, (e) unfertilised egg, (2) 2-cell embryo, (4) 4-cell embryo, (pc) pre-compact 8-cell, (c8) compact 8-cell, (16) 16-cell morula, (lm) late morula, (eb) early blastocyst, (lb) late blastocyst and (c) negative control. cDNA product sizes were ZO-1α^+, 905bp; ZO-1α^-, 665bp and α-motif, 234bp. (C) Detection of both isoforms (α^+, α^-) and α-motif (α^+) from one blastocyst (b) and three inner cell masses (i).

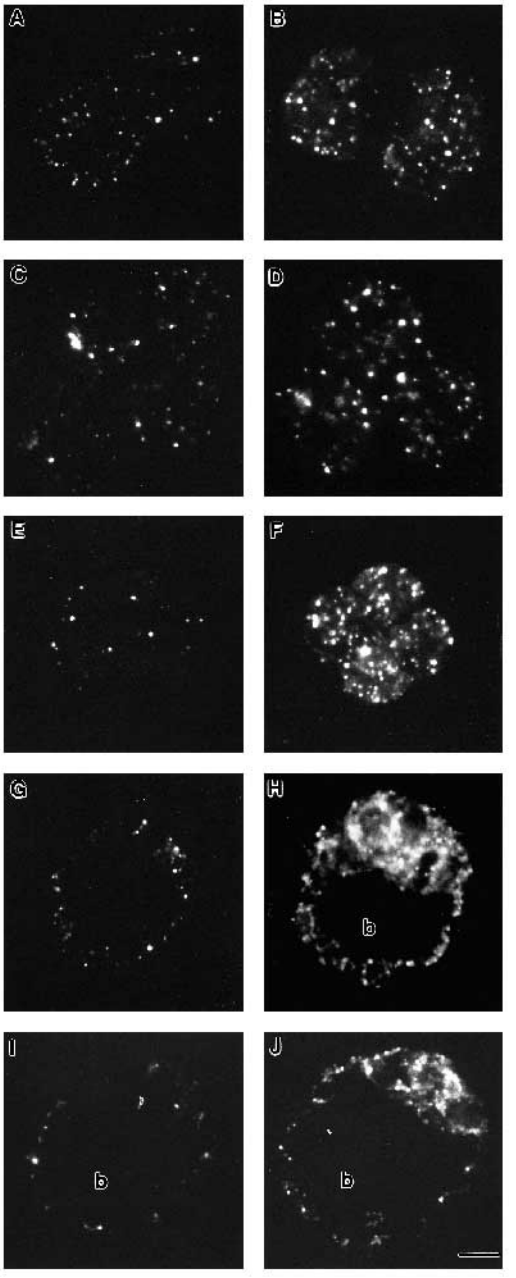


Fig. 3. In situ hybridisation of control sense (A,C,E,G,I) and antisense (B,D,F,H,J) probes to both ZO-1 isoforms. Confocal images of mid-plane optical sections of 2-cell (B) and 4-cell embryos (D) show weak positive reaction compared with controls (A,C) whereas 8-cell (F), early blastocyst (H) and late blastocyst (J) stages show stronger staining compared with controls (E,G,I). b, blastocoele. Bar, 20 μm.

trophectoderm and ICM tissues were positive for ZO-1α$^+$ mRNA (compare Fig. 4E and F). The FISH and RT-PCR data therefore both indicate that mRNA expression of ZO-1 isoforms is differentially regulated temporally but not spatially in the early embryo.

ZO-1 isoform protein expression and phosphorylation

Rabbit polyclonal antibodies raised against (a) rZ1 sequence found in both isoforms (here designated 'pan' antibody), (b) the α-motif fusion protein (ZO-1α$^+$ antibody) and (c) the splice-site peptide (ZO-1α$^-$ antibody), were tested for specificity on mouse lung extracts using western blotting (Fig. 5A). Pan antibody recognised both ZO-1 isoforms (lane 1), whereas ZO-1α$^+$ antibody recognised only the slower migrating isoform (lane 2) and the ZO-1α$^-$ antibody reacted preferentially with the shorter isoform (lane 3). Metabolic labelling and immunoprecipitation of late blastocysts with pan and ZO-1α$^+$ antibodies confirmed their specificity as above (Fig. 5B). No reactions were seen with preimmune sera used at equivalent dilutions.

Metabolic labelling and immunoprecipitation of staged embryos with the pan antibody revealed synthesis of ZO-1α$^-$ occurring throughout preimplantation development (Fig. 6A). Synthesis in eggs and during early cleavage (until the 4-cell

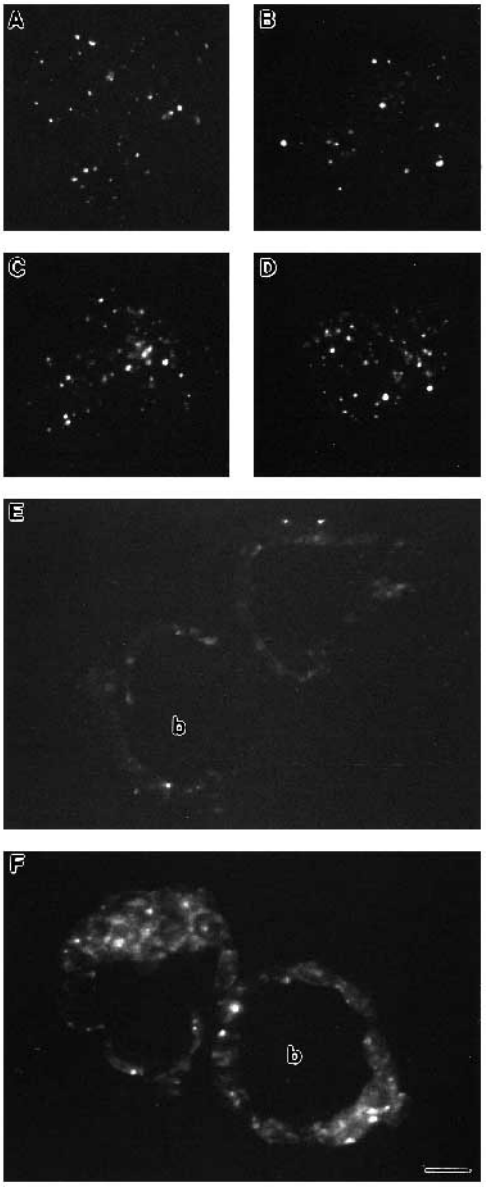


Fig. 4. In situ hybridisation of control sense (A,C,E) and anti-sense (B,D,F) probes to α-motif. Confocal images of mid-plane optical sections of 4-cell (A,B) and 8-cell (C,D) embryos are negative whereas early blastocysts (F) show positive staining for α-motif mRNA compared to the control (E). b, blastocoele. Bar, 20 μm.

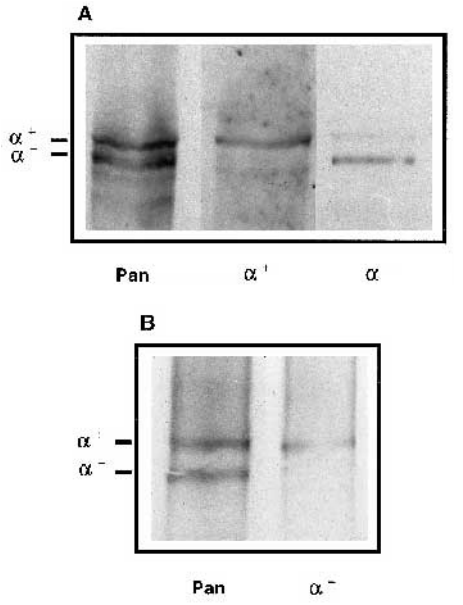


Fig. 5. Specificity of ZO-1 polyclonal antibodies using (A) western blotting with mouse lung extracts and (B) immunoprecipitation of late blastocysts. 'Pan' ZO-1 antibody recognises both α$^+$ and α$^-$ isoforms, α$^+$ antibody only recognises the upper ZO-1α$^+$ band and α$^-$ antibody recognises the lower ZO-1α$^-$ band.

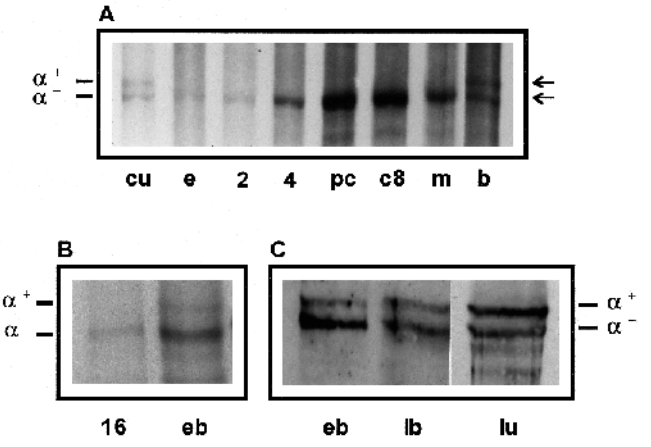


Fig. 6. Expression of ZO-1 in embryos detected using the 'pan' antibody. Immunoprecipitation of (A) [^{35}S]methionine/cysteine and (B) [^{33}P]orthophosphate-labelled embryos. (C) Western blot. Lanes are (cu) cumulus cells; (e) unfertilised eggs; (2) 2-cell embryos; (4) 4-cell embryos; (pc) pre-compact 8-cells; (c8) compact 8-cells; (m); late morulae; (b) blastocysts; (16) 16-cell morulae; (eb) early blastocysts; (lb) late blastocysts; (lu) mouse lung extract.

stage) was low compared with later cleavage and blastocyst stages. Synthesis of ZO-1α$^+$ (upper band Fig. 6A) was undetectable in eggs and cleavage stages and was first evident at the blastocyst stage. Interestingly, both isoforms were synthesised in cumulus cells (Fig. 6A).

Immunoprecipitation of [^{33}P]orthophosphate-labelled embryos indicated that ZO-1α$^-$ was phosphorylated at the 16-cell stage (the earliest stage assayed) while both ZO-1α$^-$ and ZO-1α$^+$ were phosphorylated at the early blastocyst stage (Fig. 6B). Western blotting with pan ZO-1 antibody indicated that, at the early blastocyst stage, ZO-1α$^-$ was the predominant isoform (Fig. 6C, lane 1) whilst at the late blastocyst stage (lane 2) both isoforms were present in approximately equal amounts. The stronger labelling of ZO-1α$^-$ in ^{33}P-labelled early blastocysts clearly reflects the relative quantity of the two isoforms present.

ZO-1 isoform localisation and membrane assembly during tight junction formation

Embryos at specific cleavage stages were immunolabelled with the ZO-1α$^-$ antibody and examined by confocal microscopy (Fig. 7). Staining was first detectable after compaction in 8-cell embryos (compare Fig. 7A and B). At this stage, the protein was seen as a series of discrete spots at the cell-cell contacts between blastomeres, preferentially along the apicolateral margin.This pattern changed to a continuous belt-like assembly around each outer blastomere by the late morula stage (Fig. 7C,D). Similarly, early and late blastocysts displayed ZO-1α$^-$ as a zonular belt around the apicolateral contact site of each trophectoderm cell but was absent from the ICM (Fig. 7E-H). An identical profile of staining was obtained with a guinea-pig antibody raised against the human ZO-1α$^-$ peptide (Balda and Anderson, 1993, data not shown).

Embryos labelled with ZO-1α$^+$ antibody were negative prior to the late morula stage (Fig. 8A). ZO-1α$^+$, unlike the shorter isoform, was first detected at perinuclear sites in the outer (trophectoderm lineage) cells of late morulae (Fig. 8B). In other late morulae of equal age, ZO-1α$^+$ was also present as a weak reaction at the apicolateral cell contact sites of some or all outer cells (Fig. 8C). All late morulae without a cavity possessed perinuclear ZO-1α$^+$ while 46% (n=44) also displayed cell contact staining. In nascent blastocysts (even with a very small cavity, Fig. 8D,E), ZO-1α$^+$ was detected at the apicolateral cell contact sites in both polar and mural trophectoderm; in tangential view (Fig. 8F), ZO-1α$^+$ staining was continuous around the cell borders. A similar zonular staining pattern was observed in late expanded blastocysts (Fig. 8G,H). ZO-1α$^+$ staining was not detected in the ICM throughout blastocyst expansion.

ZO-1α$^+$ membrane assembly and coordination with blastocoele formation

A functional TJ is essential for the formation of the blastocoele cavity. The cavity begins to form when the embryo has approximately 32 cells (Chisholm et al., 1985). To determine the exact cell cycle when ZO-1α$^+$ first assembles at the TJ, late morulae were double labelled for nuclei (Hoechst dye) and ZO-1α$^+$. Assembly of ZO-1α$^+$ at TJs first occurred in embryos with greater than 16 cells (ie, a mixture of 16- and 32-cell stage blastomeres; Fig. 9A). To counteract the effect of asynchrony in intact embryos, cell clusters derived from a single 8-cell blastomere were synchronised from the time of division to 2/16 couplets and used to assess more precisely ZO-1α$^+$ membrane assembly in relation to cell

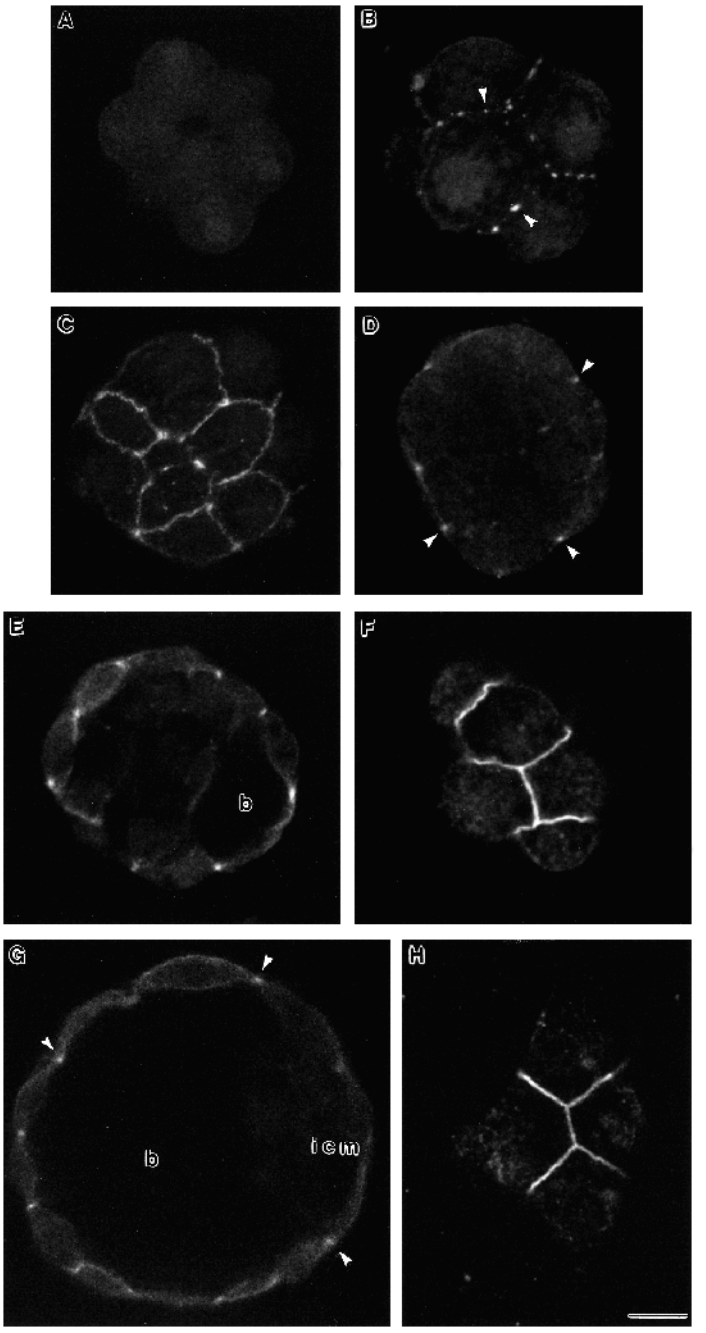


Fig. 7. Timing of expression and localisation of ZO-1α$^-$ protein during cleavage. Optical mid-plane section taken through a pre-compact 8-cell embryo (A) shows no labelling of ZO-1α$^-$. Z-series projection through a compact 8-cell embryo (B) shows ZO-1α$^-$ initially assembles at punctate sites along the cell contacts (arrowheads). Z-series (C) and mid-plane section(D) through a late morula indicate that assembly at the cell contacts is complete by this stage and zonular. Mid-plane (E) and tangential (F) sections of an early blastocyst show ZO-1α$^-$ in a zonular pattern around each trophectoderm cell but absent from the ICM. A similar localisation is evident in late blastocysts (G, H). b, blastocoele. Bar, 20 μm.

cycle and cavitation. These data revealed that ZO-1α+ always assembled at the TJ after division of blastomeres to the 32-cell stage but before blastocoele fluid accumulation occurred (Figs 9B, 10A-E).

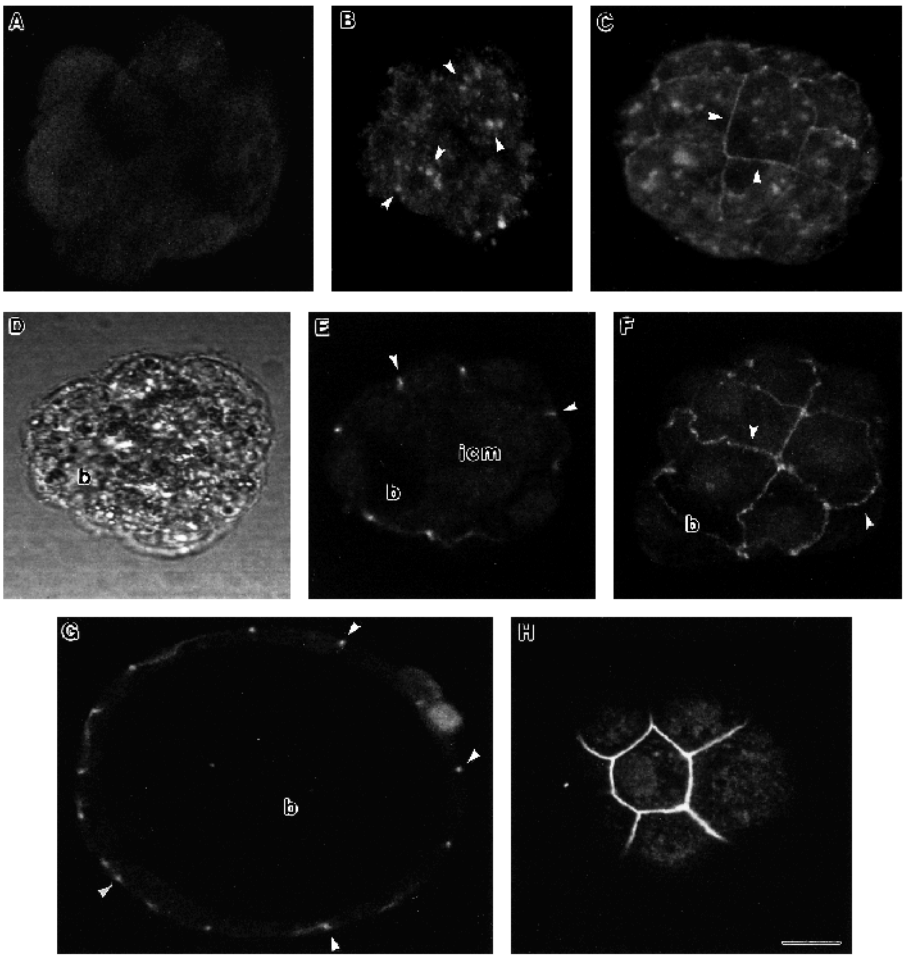


Fig. 8. Timing of expression and localisation of ZO-1α+ protein during cleavage. Optical section through a representative embryo shows no staining for ZO-1α+ in 16-cell morula (A). Staining is first evident at perinuclear sites (arrowheads) in late morulae (B); some late morulae also showed staining at cell contacts (C, arrowheads). (D) Brightfield image of an early blastocyst showing a small blastocoele cavity (b) and (E) mid-plane section of the same blastocyst stained for ZO-1α+ present at cell contacts (arrowheads). Z-series through early blastocyst shows this staining is zonular (F). A similar ZO-1α+ staining pattern is present in late blastocysts (G mid-plane, H tangential). Bar, 20 μm.

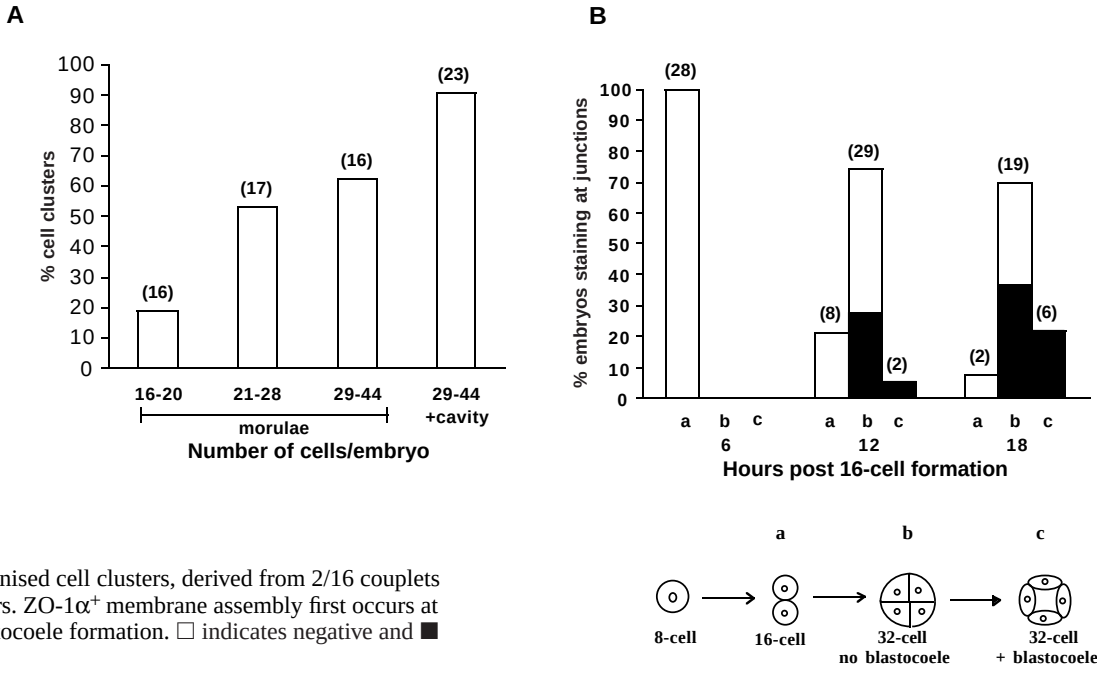


Fig. 9. (A) Relationship between embryo cell number (Hoechst dye) and membrane assembly of ZO-1α+. ZO-1α+ staining was first evident in embryos with more than 16 cells. Numbers in paranthesis indicate sample size.
(B) Relationship between cell cycle and ZO-1α+ junctional staining in synchronised cell clusters, derived from 2/16 couplets cultured for 6, 12 and 18 hours. ZO-1α+ membrane assembly first occurs at the 32-cell stage prior to blastocoele formation. □ indicates negative and ■ positive staining for ZO-1α+.

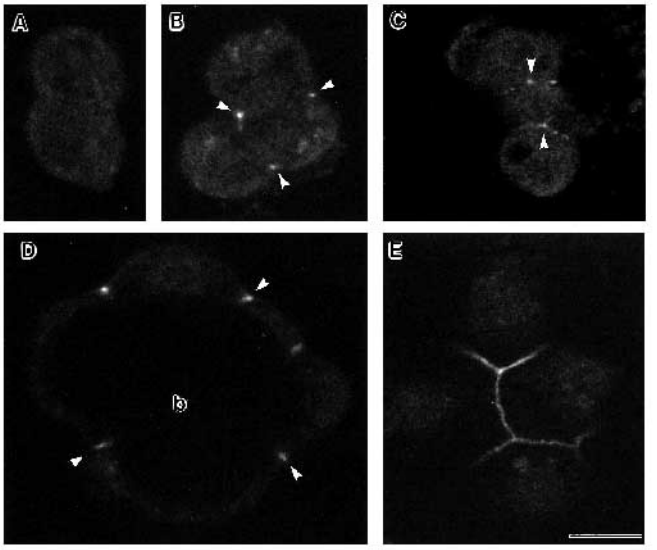


Fig. 10. ZO-1α^+ staining in cell clusters derived from synchronised 2/16 cell couplets. Optical sections show no expression of ZO-1α^+ in 2/16 couplet (A), punctate staining (arrowheads) at apicolateral cell contacts in mid-plane section of 4/32 cell cluster without a blastocoele (B), tangential section of the same 4/32 cluster shows discontinuous staining (arrowheads) at cell contacts (C). 8/64 cell cluster with a blastocoele (b) and ZO-1α^+ localised at cell contacts (arrowheads) in mid-plane (D) and in a zonular pattern in tangential (E) sections. Bar, 10 μm.

Brefeldin A sensitivity of ZO-1α^+ membrane assembly and co-localisation of ZO-1α^+ with occludin.

The perinuclear staining pattern of ZO-1α^+ in late morulae prior to membrane assembly is suggestive of a Golgi location but ZO-1 is not a transmembrane or secreted protein. We next investigated the effect of brefeldin A (BFA), an inhibitor of protein trafficking beyond the Golgi complex (Klausner et al., 1992) on ZO-1α^+ TJ assembly and blastocoele formation. Culture of late morulae in the presence of BFA inhibited blastocoele formation almost entirely (1% cavitated during 6 hours culture, *n*=80) compared with controls (100% cavitated, *n*=80). Late morulae cultured in BFA for 4 hours showed inhibition of ZO-1α^+ assembly at TJ sites (7% positive, *n*=23) compared with controls (74% positive, *n*=26). In support of an effect on trafficking, BFA treated embryos showed ZO-1α^+ staining in large perinuclear foci within blastomeres (Fig. 11A,B).

The effect of BFA on ZO-1α^+ membrane assembly could be due to an inhibition of co-transport with a transmembrane protein. We therefore examined the co-localisation of ZO-1α^+ with the TJ transmembrane protein occludin, in late morulae and early blastocysts by dual labelling confocal microscopy. ZO-1α^+ and occludin were found precisely co-localised in perinuclear spots and in regions of cell contact between blastomeres (Fig. 12), thus supporting this possibility.

DISCUSSION

In this study, we provide evidence that TJ formation during trophectoderm differentiation is a multistep cellular process

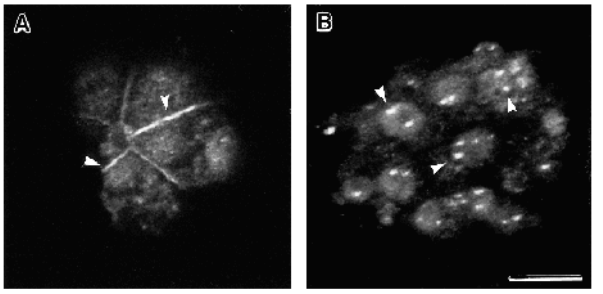


Fig. 11. ZO-1α^+ staining of late morula in the absence of brefeldin A treatment showing junctional staining (A, arrowheads), and in the presence of BFA (5 μg/ml, 4 hours) ZO-1α^+ is retained within large perinuclear foci (B; arrowheads). Bar, 20 μm.

involving sequential expression of protein constituents. Previously, we reported that initial assembly of ZO-1 protein at blastomere contact sites was detected 1-2 hours following compaction at the 8-cell stage, but did not distinguish between isoforms (Fleming et al., 1989). Approximately 10 hours later, during the 16-cell stage, cingulin assembles at the TJ contact sites (Fleming et al., 1993a) and co-localises with ZO-1 (Fleming et al., 1993b). A further cell cycle (approximately 12 hours) elapses before completion of TJ assembly occurs and vectorial transport results in the formation of a blastocoel cavity. During this time, fluid phase markers such as unconjugated HRP are able to permeate between outer cells of the embryo (Fleming and Pickering, 1985) whilst, in blastocysts, the TJ is finally sealed (Fleming and Goodall, 1986). In this paper, we have now identified what is likely to be this final and rate-limiting step in TJ formation, involving expression of the ZO-1α^+ isoform, its intracellular localisation with occludin and the co-ordinated transport of both proteins to the TJ site.

Using molecular techniques (RT-PCR, FISH) in conjunction with biochemical and confocal microscope analyses, we have found that expression and membrane assembly of ZO-1α^- and α^+ isoforms are differentially regulated. ZO-1α^- transcripts were detected in unfertilised eggs and all cleavage stages while ZO-1α^+ transcripts were detected much later, from the 16-cell stage onwards. Although the PCR method employed here is not quantitative, the amount of ZO-1α^- cDNA amplified from single embryos consistently declined during the 4-cell stage and then increased again during the pre-compact 8-cell stage. These changes probably reflect the true levels of ZO-1 mRNA present since embryos show no growth during cleavage and have a consistent protein content (Sellens et al., 1988). In addition, degradation of maternal RNA during early cleavage is well documented in the mouse embryo (Giebelhaus et al., 1983; Paynton et al., 1988; Rappolee et al., 1988; Bachvarova et al., 1989), including that representing other epithelial junction genes (Collins et al., 1995). The simplest interpretation of this pattern, therefore, is that ZO-1α^- transcripts detected until the 2-cell stage are expressed from the maternal genome; since transition from the maternal to the embryonic genome in the mouse has been shown to occur at the 2-cell stage (Flach et al., 1982; Telford et al., 1990), our results indicate that zygotic transcription of ZO-1α^- probably begins during the 4-cell stage and is maintained into the late blastocyst stage. ZO-1α^- protein synthesis, presumably from the maternal genome, was detected at relatively low levels in unfer-

tilised eggs and 2-cell embryos but the protein was not evident at the cell surface (Fleming et al., 1989). Interestingly, mRNA and protein for both isoforms were present within cumulus cells. Thus, ZO-1 may contribute to the oocyte-cumulus cell interaction during oogenesis as do other adhesion and junctional proteins (Anderson and Albertini, 1976; Eppig, 1991; Fleming et al., 1993a).

Our results also show that both ZO-1 isoforms were phosphorylated in late cleavage. Although, the exact nature of the relationship between ZO-1 phosphorylation and TJ formation is not as yet known, it is clear that aspects of ZO-1 function are regulated by tyrosine phosphorylation. The variant form of TJs found in the kidney can be chemically induced to form a typical TJ. Using this system, Kurihara et al. (1995) have suggested that transient tyrosine phosphorylation of ZO-1 is an early event in TJ formation. Transient phosphorylation of both isoforms has also been reported when the human epidermal carcinoma cell-line, A431, is treated with epidermal growth factor (Van Itallie et al., 1995). Furthermore, increased tyrosine phosphorylation in MDCK cells has been correlated with decreased cell adhesiveness (Takeda et al., 1995) and decreased transepithelial resistance (Staddon et al., 1995) and, in both cases, ZO-1 was tyrosine phosphorylated. Before a role for ZO-1 phosphorylation can be established in the mouse embryo, further investigation into the timing of ZO-1 phosphorylation with respect to a funtional TJ is required.

The first occurrence of ZO-1α^- at blastomere cell contact sites was in the compact 8-cell embryo, consistent with our previous study using an isoform non-specific antibody (Fleming et al., 1989). ZO-1α^- first appeared as dots at apicolateral and lateral membrane domains but then extended into a continuous zonular belt around each blastomere by the late morula stage. This early TJ assembly event is dependent upon E-cadherin cell adhesion for normal localisation, and is sensitive to microfilament disruption (Fleming et al., 1989). Using the Ca^{2+} switch assay in MDCK cells, Rajasekaran et al. (1996) have shown that a transient complex exists between ZO-1, E-cadherin and the catenins both intracellularly and at the adherens junction during the early stages of TJ formation. This association is lost prior to complete polarisation of the cell layer and segregation of ZO-1 to the TJ. ZO-1α^- expression in the embryo is distinct from that of ZO-1α^+, not only in timing, but also in the initial perinuclear localisation pattern found only with ZO-1α^+ and its intracellular colocalisation with occludin. Occludin protein expression pattern in the embryo is similar to that reported here for ZO-1α^+ in that it is not detectable at membrane contact sites at mid-cleavage when ZO-1α^- first assembles (B. Sheth, M. Hay, B. Moran, J. Anderson and T. Fleming, unpublished data). It appears, therefore, that in the embryo the first wave of ZO-1 assembly is independent of occludin binding and may be facilitated by E-cadherin/catenin complexes. Thus, our data for a native epithelium are comparable to those described above for MDCK cells during TJ formation (Rajasekaran et al., 1996). E-cadherin and the catenins are present at the cell-contact sites in 8-cell embryos (Ohsugi et al., 1996) and we are currently investigating this possible similarity in assembly.

The delay in expression of ZO-1α^+ isoform until the morula stage was demonstrated in our studies at the level of transcription, translation and assembly of the protein at cell contact sites. The RT-PCR analysis revealed that this late step in TJ assembly is probably regulated by de novo transcription. ZO-1α^+ protein was first detected in embryos with cell numbers greater than 16. However, because of inherent cycling asynchrony within embryos, it was unclear from this data in which cycle ZO-1α^+ was first expressed. Using synchronised cell clusters, we found that ZO-1α^+ protein was first assembled at the junctions during the 32-cell stage, immediately prior to the formation of a nascent blastocoele. In fact, our data imply that assembly of ZO-1α^+ at the junctions is a prerequisite for blastocoele formation.

Both ZO-1α^+ and occludin were first evident in late morulae,

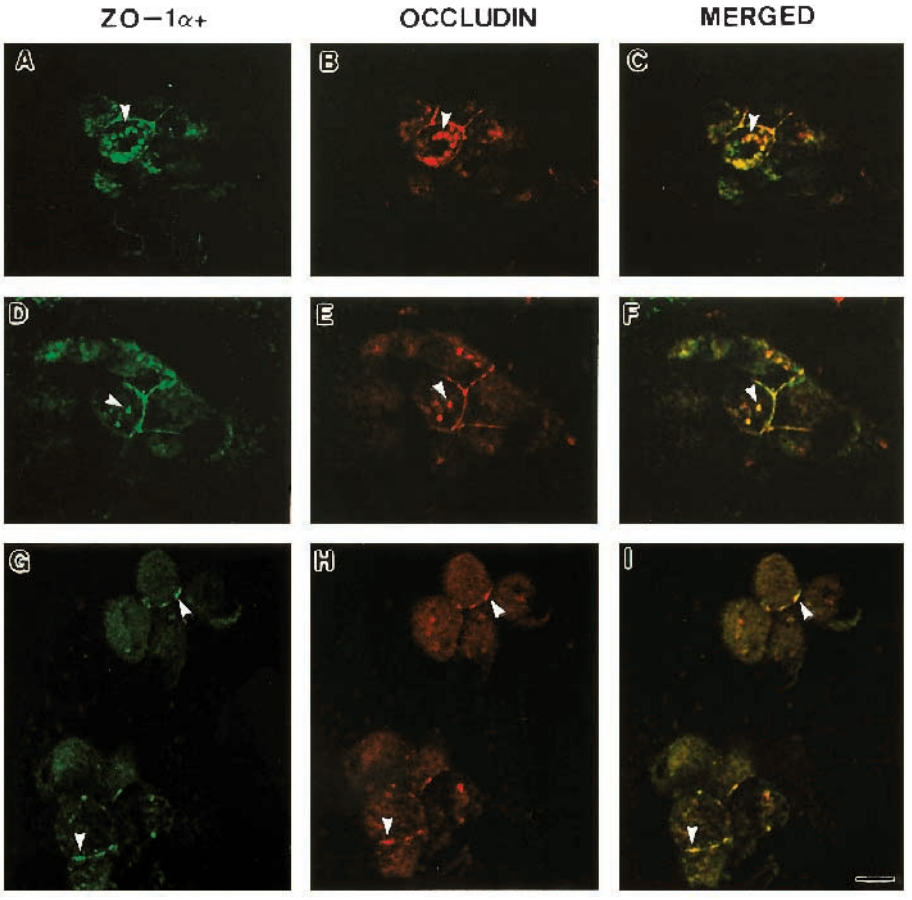


Fig. 12. Double-labelling of late morulae and early blastocysts with ZO-1α^+ (A,D,G), occludin (B,E,H) and merged images (C,F,I). The proteins are co-localised within morula outer blastomeres at peri-nuclear sites (A-C, arrowheads) and in blastocysts at intracellular sites (D-F, arrowheads) and at cell contact sites seen in tangential section (G-I, arrowheads). Bar, 20 μm.

co-localised at punctate perinuclear sites and junctions. BFA inhibited both cavitation and the movement of ZO-1α$^+$ from these perinuclear sites to the junction. Chick occludin transfected into MDCK cells has also been reported to co-localise intracellularly with canine ZO-1, in subconfluent cultures (McCarthy et al., 1996). These observations suggest that ZO-1 and occludin are processed together and associated with the same intracellular vesicles during de novo synthesis of TJs. Whether these proteins exist as a preformed complex in the Golgi is not yet known, but if these proteins are linked intracellularly, it may be that one or both proteins require the other for translocation to the membrane. Balda et al. (1996) found that a carboxy-terminal truncated occludin, unable to bind ZO-1, was targeted to the membrane normally in transfected MDCK cells. However, this occludin mutant exhibited discontinuous staining at the junctions whilst ZO-1 formed a continuous junctional ring. Although these findings suggest that interaction of the two proteins is not required for targeting to the membrane, the possibility that ZO-1 is transported to the junctions by endogenous occludin in these cells cannot be excluded. We are investigating whether occludin is required for the correct targeting of ZO-1α$^+$ in late morulae, in our present studies.

The RT-PCR and FISH analysis demonstrated that mRNAs for the two ZO-1 isoforms were present in both trophectoderm and ICM cells of the blastocyst, while immunolabelling revealed ZO-1 protein only within the trophectoderm lineage. The ICM lineage is derived from two rounds of differentiative division when some of the polarised 8- and 16-cell-stage blastomeres on the embryo surface allocate one daughter cell to the embryo interior (Johnson and Ziomek, 1981; Fleming, 1987). Consequently, ZO-1 mRNA within ICM cells may be derived either by inheritence from the parent cell undergoing differentiation into trophectoderm, or by de novo transcription. Previously, we have shown that suppression of ZO-1 membrane assembly within ICM cells is regulated by the absence of a contact-free cell surface (Fleming and Hay, 1991) which presumably acts to reduce levels of translation, as shown for the TJ protein, cingulin (Javed et al., 1993). In contrast to ZO-1, desmosomal desmocollin (DSC2) mRNA was found preferentially within trophectoderm cells of the blastocyst (Collins et al., 1995). Down-regulation of epithelial junction formation in the ICM is therefore controlled by both transcriptional and translational mechanisms.

In this paper, we have shown that both ZO-1 isoforms are incorporated into the TJs of preimplantation embryos and that the interaction between ZO-1α$^+$ and occludin (at the Golgi/ER level) is likely to be the final rate-limiting step in the formation of the TJ. Although the precise function of the two ZO-1 isoforms is not yet clear, our data indicate that they may differ in their ability to associate in vivo with known binding partners such as occludin and β-catenin during epithelial differentiation. Hence ZO-1α$^-$ may be involved in maintaining membrane polarity (established at compaction), whilst the late expression of ZO-1α$^+$ at the junctions may play an important role in sealing the TJ and thereby contribute to the establishment of a blastocoele cavity. Kidder and McLachlin (1985) showed that α-amanitin, an inhibitor of transcription, was only capable of inhibiting cavitation if added before the late morula stage. Since ZO-1α$^+$ and the β1-subunit of Na,K-ATPase (Watson et al., 1990) are two mRNAs expressed unusually late in cleavage, inhibition of their transcription would undoubtedly result in the failure of the embryo to cavitate, indicating that co-ordination between distinct features of differentiation regulates the timing of epithelial cell function.

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