Development 117, 1145-1151 (1993)
Printed in Great Britain © The Company of Biologists Limited 1993

Tight junction protein cingulin is expressed by maternal and embryonic genomes during early mouse development

Qamar Javed[1], Tom P. Fleming[1,*], Mark Hay[1] and Sandra Citi[2]

[1]Department of Biology, Biomedical Sciences Building, University of Southampton, Bassett Crescent East, Southampton SO9 3TU, UK
[2]Department of Cell Biology and Anatomy, Cornell University Medical College, 1300 York Avenue, New York, NY 10021, USA

*Author for correspondence

SUMMARY

The expression of the tight junction peripheral membrane protein, cingulin (140×10^3 M_r), was investigated in mouse eggs and staged preimplantation embryos by immunoblotting and immunoprecipitation. Polyclonal antibody to chicken brush border cingulin detected a single 140×10^3 M_r protein in immunoblots of unfertilised eggs and all preimplantation stages. Relative protein levels were high in eggs and early cleavage stages, declined during later cleavage and increased again in expanding blastocysts. Quantitative immunoprecipitation of metabolically labelled eggs and staged embryos also revealed a biphasic pattern for cingulin synthesis with relative net levels being high in unfertilised eggs, minimal during early cleavage, rising 2.3-fold specifically at the onset of compaction (8-cell stage, when tight junction formation begins), and increasing further at a linear rate during morula and blastocyst stages. Cingulin synthesis in eggs is not influenced by fertilisation (or aging, if unfertilised), but this level declines sharply after first cleavage. These results indicate that cingulin is expressed by both maternal and embryonic genomes. The turnover of maternal cingulin (unfertilised eggs) and embryonic cingulin at a stage before tight junction formation begins (4-cell stage) is higher ($t_{\frac{1}{2}}$~4 hours) than cingulin synthesised after tight junction formation (blastocysts; $t_{\frac{1}{2}}$~10 hours). This increase in cingulin stability is reversed in the absence of extracellular calcium. Cingulin synthesis is also tissue-specific in blastocysts, being up-regulated in trophectoderm and down-regulated in the inner cell mass. Taken together, the results suggest that (i) cingulin may have a role during oogenesis and (ii) cell-cell contact patterns regulate cingulin biosynthesis during early morphogenesis, contributing to lineage-specific epithelial maturation.

Key words: mouse preimplantation embryos, mouse eggs, tight junctions, cingulin, epithelial biogenesis, cell-cell adhesion, trophectoderm

INTRODUCTION

The junctional complex of polarised epithelia consists of an apicolateral tight junction (zonula occludens) beneath which are found the zonula adherens, desmosomes and gap junctions, each possessing distinct characteristics and molecular properties (reviewed in Staehelin, 1974; Stevenson and Paul, 1989; Garrod and Collins, 1992). Tight junction ultrastructure consists of circumferential membrane contact sites where the intercellular space is occluded and which, in freeze-fracture preparations, contain a network of branching ridges composed of linearly arranged intramembraneous particles (P-face) and complementary grooves (E-face) (Staehelin, 1974; Pinto da Silva and Kachar, 1982). The tight junction is thought to restrict paracellular passage of ions and macromolecules between mucosal and serosal compartments, and to contribute to the maintenance of epithelial membrane domain polarity (Claude and Goodenough, 1973; van Meer and Simons, 1986).

Although the molecular identity of integral membrane components of the tight junction are unknown, two peripheral tight junction proteins, ZO-1 and cingulin, have been identified at the cytoplasmic face of the junction in a variety of epithelia (eg, Stevenson et al., 1986; Citi et al., 1988, 1989; Schnabel et al., 1990; reviewed in Anderson and Stevenson, 1992). ZO-1 is a large, asymmetric, monomeric phosphoprotein (215-225$\times10^3$ M_r; Anderson et al., 1988), whereas cingulin (140×10^3 M_r) is an elongated, acidic, heat-stable dimeric phosphoprotein possessing an alpha-helical coiled-coil structure (Citi et al., 1988, 1989, 1990; Denisenko and Citi, 1991). These two immunologically unrelated tight junction proteins show overall similarities in their tissue distribution and subcellular localisation, although double-label immunogold analysis indicates ZO-1 is positioned closer to the membrane than cingulin (Stevenson et al., 1989).

The mouse preimplantation embryo provides a model system in which to study the expression and assembly of

tight junction components during development. The polarised epithelial features of the blastocyst trophectoderm are acquired progressively during cleavage and the tissue becomes functional from the late 32-cell stage when vectorial transport causes blastocoele formation (reviewed in Fleming and Johnson, 1988; Fleming, 1992). The assembly of tight junctions begins at compaction (8-cell stage), coinciding with the onset of cell polarity and the activation of cell-cell adhesion mediated by uvomorulin (E-cadherin). Freeze-fracture analysis indicates that the tight junction at compaction is focal rather than zonular, but subsequently extends laterally to become belt-like before the blastocyst is formed (Ducibella and Anderson, 1975; Ducibella et al., 1975; Magnuson et al., 1977; Pratt, 1985). We have previously reported on the expression and assembly of ZO-1 in the early embryo and identified mechanisms that appear to control its tissue-specificity (Fleming et al., 1989; Fleming and Hay, 1991). These studies indicated that the time of ZO-1 protein expression (4- to 8-cell stage) and membrane assembly at nascent tight junction sites (compaction) was closely related to the timing of the structural development of the tight junction, as seen in freeze-fracture.

In this paper, we report on the pattern of expression and synthesis of the tight junction protein cingulin in mouse oocytes and early embryos using biochemical analyses. Complementary morphological studies are reported separately (Fleming et al., 1992). Our results indicate that cingulin, unlike ZO-1, is expressed by the maternal as well as the embryonic genome, and that cell interactions regulate the level of cingulin synthesis, stability and tissue-specificity.

MATERIALS AND METHODS

Egg and embryo collection, culture and manipulation

Three- to four-week old MF1 female mice (Olac-derived, Southampton University Animal House) were superovulated by intraperitoneal injections (48 hours apart) of pregnant mares serum (PMS, Folligon, Intervet) and human chorionic gonadotrophin (hCG, Chorulon, Intervet). Cumulus masses were collected from oviducts at 12 hours post-hCG and unfertilised eggs recovered following hyaluronidase treatment (100 mM in M2+BSA for 5-10 minutes). Embryos were obtained by mating female mice overnight with MF1 males, checking for copulation plugs and flushing oviducts at appropriate times post-hCG injection. Flushing times were 30 hours (early 2-cell), 46-48 hours (late 2-cell, 4-cell, 8-cell), or 65-70 hours post-hCG (16-cell, blastocysts) and embryos staged following washing in M2+BSA and culture for varying times in M16+BSA (up to blastocyst) or DMEM+FCS (blastocysts and ICMs) as described previously (Fleming et al., 1991). Fertilized eggs were obtained from cumulus masses at 18-20 hours post-hCG and checked for the presence of two pronuclei. Removal of the zona pellucida was by acid Tyrode's solution (Nicolson et al., 1975) and immunosurgery to isolate ICMs from blastocysts by the method described by Chisholm et al. (1985).

Western blotting

Eggs and staged embryos were washed three times in M2+PVP (see Fleming et al., 1991), boiled for 3 minutes in SDS sample buffer and stored at −70°C until required. The soft upper pellet from liver homogenate in 1 mM $NaHCO_3$, spun for 10 minutes at 2,000 ***g*** (Stevenson et al., 1986), was used as a positive control. Egg/embryo samples were separated on 7.5% SDS-polyacrylamide gels, electrophoretically transferred (Trans-Blot Semi-Dry Transfer Cell, BioRad Labs, UK) in 48 mM Tris, pH 9.2, 39 mM glycine, 1.3 mM SDS, 20% methanol at 20 V for 1.5 hours onto Hybond C-extra nitrocellulose (Amersham International, UK). Blots were treated with blocking buffer (BB; 1% dried milk powder, 0.02% Tween 20, 0.01% NaN_3 in phosphate-buffered saline (PBS)) for 3 hours before probing with rabbit polyclonal antibody to chicken brush border cingulin at 1:3000 dilution in BB for 16 hours at 4°C. The nitrocellulose was washed several times in BB, probed with ^{125}I-labelled goat anti-rabbit IgG (Dako Ltd, UK; approx. 3×10^5 cts/minute/ml) in BB for 1 hour at 4°C, and washed several times in BB for 1 hour before drying. Immunoblots were exposed to Fuji X-ray film for 3 days at −70°C before developing and fixing. Relative cingulin protein levels in egg/embryo samples were measured by scanning densitometry (Joyce Lobel densitometer) over a range shown to be linearly related to sample size defined by stage-specific embryo number. Embryo number rather than protein estimations were used for standardising blot (and immunoprecipitation) samples since protein levels are relatively consistent throughout preimplantation development (Brinster, 1967).

Metabolic labelling and immunoprecipitation

Eggs and staged embryos (200-1000 per sample) were labelled in 50 μl M16+BSA (for pre-blastocyst stages) or methionine-free Eagle's MEM+FCS (ICN Flow, UK; for blastocysts) containing 'Trans ^{35}S-label' (ICN Flow; specific activity >1000 Ci/mmol) for either 3 hours (1000 μCi ^{35}S/ml) or for 14 hours (100 μCi ^{35}S/ml), before washing four times in M2+PVP containing nonradioactive methionine (7.45 μg/ml) and cysteine (6.05 μg/ml; M2+PVP+MC). Immunoprecipitation was carried out as described previously (see Fleming et al., 1991); cingulin polyclonal antibody was used at saturating doses (1 or 10 μl per sample). Preliminary work showed that the SDS-buffer used for extraction of embryos (Fleming et al., 1991) solubilised at least 94% of total radiolabelled cingulin, indicating high efficiency. Immunoprecipitates were run on a 7.5% SDS-PAGE gel, which was soaked in Amplify (Amersham) for 15 minutes before drying and autoradiography at −70°C using Fuji RX-film. A pulse-chase metabolic labelling protocol was used for cingulin turnover analysis. Eggs and staged embryos (100-400 per sample) were labelled with 'Trans ^{35}S-label' (100 μCi ^{35}S/ml) for 3 hours in 50 μl M16+BSA, washed four times in M2+PVP+MC, and chased in M16+BSA+MC for various times before washing in M2+PVP and immunoprecipitation.

In this paper, we define cingulin net synthesis as the relative amount accumulated after a defined metabolic labelling period and, therefore, this figure does not account for concurrent degradation, although turnover has also been assayed at specific stages. Quantitation in relative terms of cingulin net synthesis and turnover was analysed routinely by scintillation counting (Raytest 4700 counter) of replicated immunoprecipitates. This method was compared directly with scanning densitometry (over a range where sample and scan area are linearly related) and was found to be reliable and, importantly, more sensitive for small embryo samples. Thus, in preliminary time course experiments in which unfertilized eggs were metabolically labelled in a time course up to 9 hours, the level of newly synthesised cingulin measured by scintillation counting was within 8% of that determined by scanning densitometry and was directly related to sample size within 2% (data not shown).

RESULTS

Cingulin expression in mouse eggs and staged embryos

The relative levels of expression of cingulin in eggs and preimplantation embryos were studied by western blotting using polyclonal rabbit antiserum against purified chicken intestinal cingulin. In chicken intestine, cingulin antibody recognises two polypeptides of 108×10^3 and 140×10^3 M_r (Citi et al., 1988), the former thought to be a breakdown product (Stevenson et al., 1989). Two major bands, corresponding to these mobilities were detectable in mouse liver, together with a lower band (65×10^3 M_r) that also may be a breakdown product. However, in all egg and embryo stages, only a single protein band at 140×10^3 M_r was blotted (or immunoprecipitated, see later) with cingulin antibody (Fig. 1A). The radiolabelled bands on mouse embryo immunoblots were analysed by scanning densitometry (Fig. 1B). Unfertilised eggs (14 hours post-hCG) and early cleaving embryos (48-52 hours post-hCG) contained higher relative levels of cingulin compared with early and compacted 8-cell embryos (65-68 hours post-hCG). The cingulin level increased again in early blastocysts (96 hours post-hCG) and more so in later blastocysts (120 hours post-hCG) (Fig. 1A,B).

Cingulin synthesis in eggs and staged embryos

Cingulin synthesis was first examined in unfertilised eggs freed from cumulus cells before metabolic labelling and immunoprecipitation. A single radiolabelled protein was precipitated, migrating at 140×10^3 M_r (Fig. 2A). Cingulin synthesis in unfertilised eggs was inhibited by cycloheximide treatment (Fig. 2A). However, the level of synthesis in fertilised eggs (containing two pronuclei) and in aging unfertilised eggs was similar to that occurring in non-aged unfertilised eggs (Fig. 2C). Metabolic labelling and immunoprecipitation of cumulus cells freed from unfertilised eggs provided no evidence of cingulin synthesis (using equivalent or greater protein levels to eggs; Fig. 2B). Immunoblotting of freed cumulus cells also failed to detect cingulin protein in these cells (data not shown). Analysis of cingulin synthesis after first cleavage showed that a sharp decline occurred by the late 2-cell stage (Fig. 2C).

Immunoprecipitation of newly synthesised cingulin from synchronised populations of metabolically labelled cleavage-stage embryos revealed an increasing level of synthesis up to the blastocyst stage (Fig. 3A). Scintillation counting of triplicated immunoprecipitate samples from defined stages was used to quantify in relative terms changes in net cingulin synthesis during cleavage (Fig. 3B). This analysis indicated (i) a low and apparently unchanging net level of synthesis between late 2-cell and early (pre-compact) 8-cell stages, approx. 12% of that occurring in unfertilised eggs, (ii) a sharp rise in cingulin synthesis specifically at compaction in the 8-cell embryo (approx. 2.3-fold higher than pre-compact embryos) and (iii) a linear increase in net synthesis occurring throughout later cleavage and blastocyst stages (doubling time approx. 24 hours). Only during blastocyst expansion was the net level of synthesis evident in unfertilised eggs exceeded (Fig. 3B).

Turnover of newly synthesised cingulin

Unfertilised eggs (cumulus-free) and preimplantation embryos (4-cell and early blastocyst stages) were metabolically labelled for 3 hours and ‘chased’ in label-free medium for various times before immunoprecipitation and radiolabel counting (Fig. 4). Newly synthesised cingulin in unfertilised eggs and 4-cell embryos had a similar relatively

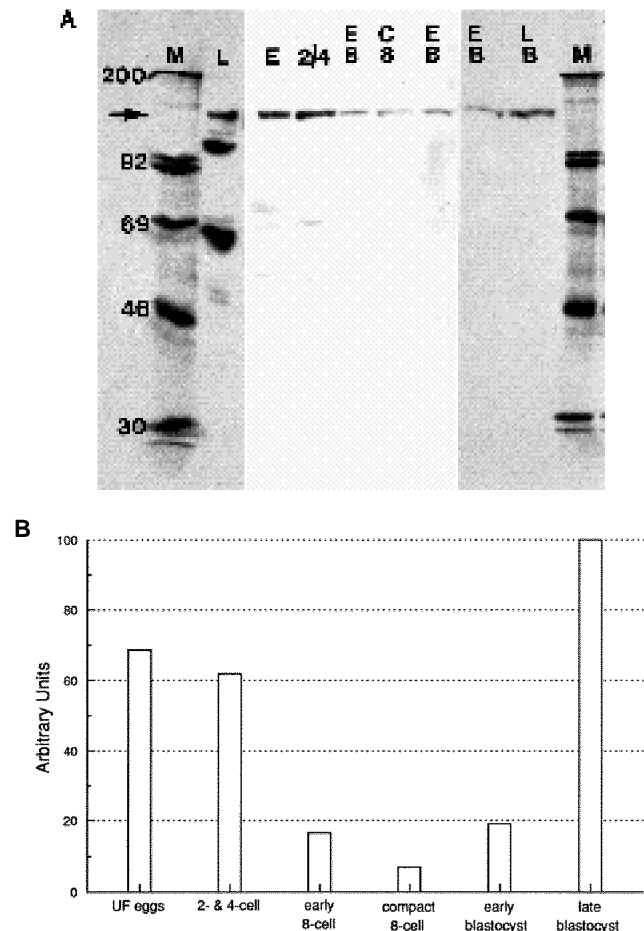

Fig. 1. (A) Western blot analysis of relative cingulin levels in unfertilised mouse eggs (E; 14 hours post-hCG), mixed late 2-cell and early 4-cell embryos (2/4; 48-52 hours post-hCG), precompact 8-cell embryos (E8; 65 hours post-hCG), compact 8-cell embryos (C8; 68 hours post-hCG), early blastocysts (EB; 96 hours post-hCG) and late blastocysts (LB; 120 hours post-hCG). 1,000 eggs/embryos were loaded per lane of 7.5% SDS-polyacrylamide gels, transferred to nitocellulose and incubated in polyclonal cingulin antibody; iodinated anti-rabbit IgG was used for detection (see Materials and methods). At each stage, a single prominant band at approx. 140×10^3 M_r is detectable (arrow), while in liver homogenate (L) additional bands at approx. 108×10^3 and 65×10^3 M_r are evident. M, ^{14}C-labelled molecular weight standards as indicated in $\times10^3$ M_r. (B) Relative levels of cingulin present in eggs and embryo stages following scanning densitometry of representative autoradiograms. The blotting experiment was repeated twice. For each, densiometric data from UF eggs up to early blastocyst stages were from a single gel and blot; the result for the late blastocyst stage was from a separate gel but an additional early blastocyst sample was used for standardisation.

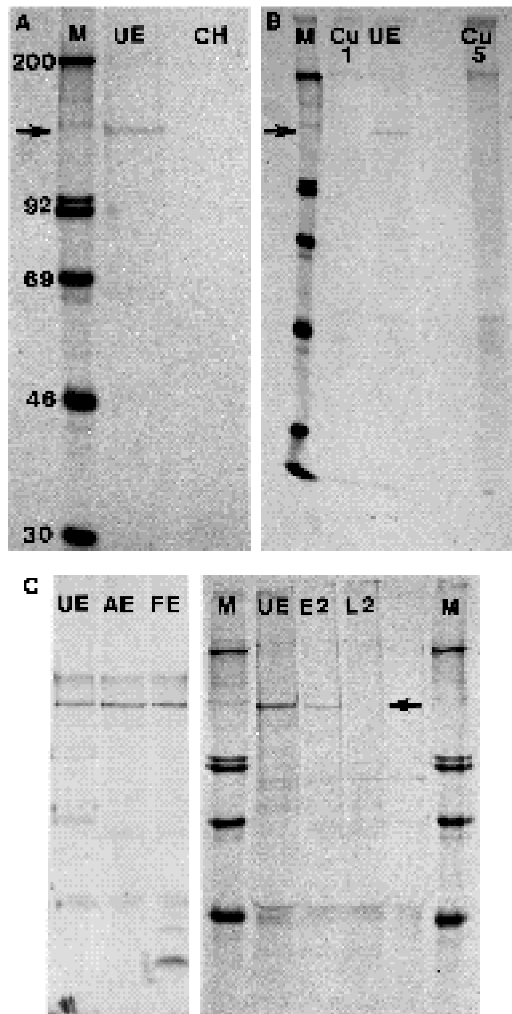

Fig. 2. (A) Immunoprecipitation of newly synthesised cingulin from mouse unfertilised eggs (UE; 14 hours post-hCG; *n*=900), following ^{35}S-metabolic labelling for 3 hours (see Materials and methods for details). A single band at approx. 140×10^3 M_r has been precipitated (arrow). Metabolic labelling in the presence of cycloheximide (CH; 400 μM) inhibits cingulin synthesis. M, molecular weight markers as indicated ($\times10^3$ M_r). (B) Cingulin immunoprecipitation from unfertilised eggs (UE) and cumulus cells either at equivalent total protein concentration to the UE lane (Cu1) or five-fold higher (Cu5). Arrow indicates position of 140×10^3 M_r band only identifiable in UE sample. M, molecular weight markers. (C) On left, cingulin immunoprecipitation from unfertilised eggs (UE; 14 hours post-hCG), aging unfertilised eggs (AE; 24 hours post-hCG) and fertilised eggs (FE; 24 hours post-hCG); all samples contain 900 eggs, following metabolic labelling for 3 hours. In each, a 140×10^3 M_r band is evident (arrow on right). On right, cingulin immunoprecipitation from unfertilised eggs (UE), early 2-cell embryos (E2; 34 hours post-hCG) and late 2-cell embryos (L2; 46 hours post-hCG), 1000 eggs or embryos per lane; the cingulin band at 140×10^3 M_r declines in intensity during the 2-cell stage. M, molecular weight markers.

high rate of turnover ($t\frac{1}{2}$~4 hours) compared with early blastocysts ($t\frac{1}{2}$~10 hours). To investigate whether cell-cell adhesion influenced this change in stability, the net level of synthesis and turnover of newly synthesised cingulin was compared between early blastocysts cultured either in the presence or absence of extracellular calcium (Fig. 4). In these experiments, calcium depletion (i) reversed adhesion between blastomeres and reduced net cingulin synthesis by 25% (mean of three experiments) and (ii) increased cingulin turnover rate to approx. that detected in eggs and 4-cell embryos ($t\frac{1}{2}$~4 hours).

Tissue-specificity of cingulin synthesis

Since tight junction formation is restricted to the trophectoderm lineage of the blastocyst, we next compared cingulin net synthesis between trophectoderm and inner cell mass (ICM) tissues in both early (96 hours post-hCG) and expanded (120 hours post-hCG) blastocysts (Fig. 5). These experiments indicated that the level of cingulin synthesis in early blastocysts is over three-fold higher in the trophectoderm than in the ICM, while in late blastocysts, this difference increases to fifteen-fold.

DISCUSSION

In this paper, we report on biochemical studies concerned with the expression of the tight junction peripheral membrane protein cingulin in the early stages of mouse development. The timing and characteristics of cingulin expression in the embryo, where tight junction formation takes place de novo during cleavage, is relevant to the processes controlling the assembly of different junction components. One important finding from our work is that cingulin expression occurs in two phases that appear to involve sequential maternal and embryonic gene expression programmes. Cingulin is detectable at relatively high levels by both immunoblotting and by immunoprecipitation in unfertilised eggs. This maternal expression programme is maintained after fertilisation and declines during the 2-cell stage, coincident with the global degradation of inherited maternal RNA and the transition to embryonic gene activity (Flach et al., 1982; Bensaude et al., 1983). The embryonic programme of cingulin expression initiates during early cleavage at a relatively low level of net synthesis which then increases significantly at compaction and at later cleavage stages.

The maternal expression of cingulin was unexpected since tight junctions have not been identified in unfertilised eggs. Moreover, expression of another tight junction peripheral membrane protein, ZO-1, was not detectable (either by immunoblotting or by immunocytochemistry) in unfertilised eggs (Fleming et al., 1989) although uvomorulin, the cell adhesion molecule involved in compaction, is synthesised at this stage (Sefton et al., 1992). Our data suggest, therefore, that cingulin may occur at sites where tight junctions are not definable by electron microscopy (see Citi et al., 1989), as has recently been shown for ZO-1 (Howarth et al., 1991). Although cingulin expression was detectable in eggs, it was not evident within cumulus cells, either by immunoprecipitation or immunoblotting. This result is consistent with immunolocalisation of maternal cingulin in the oocyte cortex and corona radiata layer but not in bulk cumulus tissue (Fleming et al., 1992).

To understand the mechanisms of tight junction

B

Fig. 3. (A) Immunoprecipitation of cingulin from unfertilised eggs (UE), late 2-cell/early 4-cell embryos (2/4), compact 8-cell embryos (C8) and early blastocysts (EB). See Fig. 1A for staging times. 600 eggs/embryos per lane. Arrow indicates position of 140×10^3 M_r band of precipitated cingulin which is evident in eggs, but declines in intensity during early cleavage before increasing again in later cleavage. Arrowheads indicate position of molecular weight markers (from top, 200, 92, 69, 46, 30×10^3 M_r). (B) Relative net levels of cingulin synthesis during early development assayed by immunoprecipitation (3 hours labelling time) and scintillation counting (see Materials and methods). Each point represents the mean (±s.e.) of three samples of 200 eggs/embryos at specified stages/time post-hCG as indicated: UF, unfertilised eggs; E8,C8 early and compact 8-cell embryos; EB,LB early and late blastocysts.

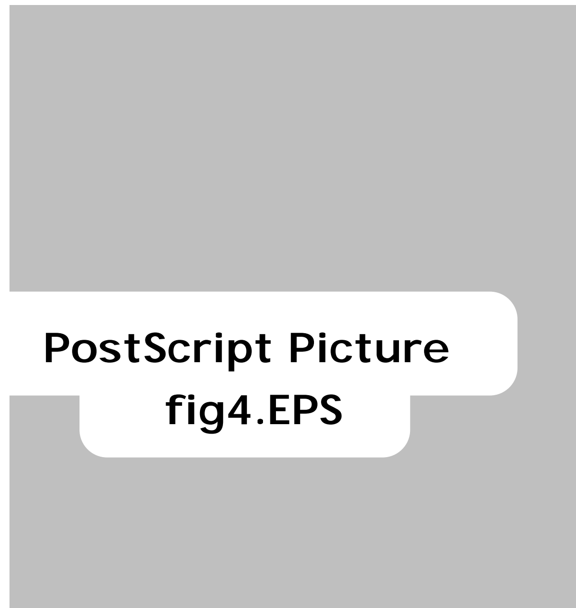

Fig. 4. Turnover of newly synthesised cingulin in unfertilised eggs, 4-cell embryos and early blastocysts. 400 eggs or embryos at each stage were metabolically labelled for 3 hours and ‘chased’ in the presence of excess unlabelled methionine and cysteine for the indicated times before immunoprecipitation and scintillation counting (see Materials and methods). Blastocysts were also cultured in calcium-free medium for 6 hours prior to metabolic labelling and throughout the ‘chase’ period. Mean of three experiments (±s.e.); note that newly synthesised cingulin increases in stability by the blastocyst stage ($t_{\frac{1}{2}}$~10 hours) but reverts to an unstable pattern ($t_{\frac{1}{2}}$~4 hours), typical of eggs and 4-cell embryos, in the absence of extracellular calcium.

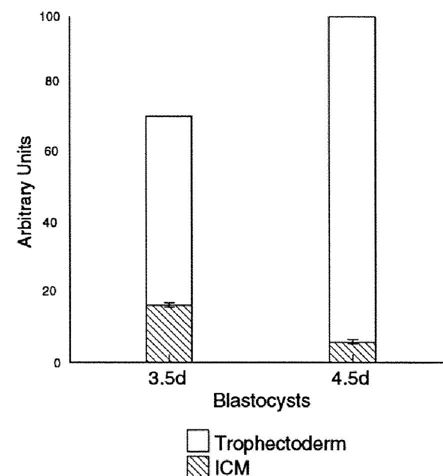

Fig. 5. Tissue-specific cingulin synthesis in blastocysts. Blastocysts (200 per sample) at 96 hours post-hCG (3.5 days of development) and 120 hours post-hCG (4.5 days) were metabolically labelled for 3 hours before washing and immunosurgery to isolate ICMs, which were then used for immunoprecipitation and scintillation counting. Trophectoderm data were obtained by subtraction of ICM values from whole blastocyst samples. Mean of three experiments (±s.e.); note that cingulin synthesis declines in the ICM lineage but increases in the trophectoderm lineage during blastocyst expansion.

assembly during cleavage, we must consider whether maternal cingulin may act as a store of protein that is available at the time of junction formation. The relatively long half-life of many proteins expressed in mouse eggs and early embryos is consistent with this possibility (eg. Merz et al.,

1981; Barron et al., 1989). We also interpret the relatively high levels of cingulin detected in blots of 2- and 4-cell embryos when synthesis is minimal as a consequence mainly of maternal inheritance. A similar expression pattern has recently been revealed for uvomorulin (Sefton et al., 1992). However, our turnover data for maternal cingulin ($t_{\frac{1}{2}}$~4 hours) indicate that the protein is too short-lived for involvement in tight junction assembly some 24-48 hours later in development. This view is further supported by the fact that cingulin appears to associate with the tight junction mainly during the 16-cell stage rather than at compaction in the 8-cell embryo when ZO-1 assembles at nascent junction sites (Fleming et al., 1992). Since our metabolic labelling of oocytes in pulse-chase experiments utilised cumulus-free oocytes, it is possible that newly synthesised cingulin may have a longer half-life if cumulus cells were present, possibly providing (indirectly) a binding site to promote cingulin stability. However, if this was so, the detachment of cumulus cells from fertilised eggs that occurs in vivo some 36 hours prior to compaction would in turn induce an increase in cingulin turnover.

The net level of cingulin synthesis from the embryonic genome is enhanced significantly when 8-cell embryos engage in compaction (Fig. 3B). Previously, we have estimated that the time at which ZO-1 expression in the embryo is initiated is at the late 4-cell or early 8-cell stage, some 5-8 hours earlier than compaction (Fleming et al., 1989). Thus, a temporal sequence appears to exist in which ZO-1 precedes cingulin both in expression and in junction assembly times (Fleming et al., 1992). The most likely explanation for the increase in cingulin synthesis level specifically at compaction is that one or more aspects of cell-cell adhesion are stimulatory. This increase in cingulin synthesis is presumably required for assembly of cingulin at the tight junction.

The requirement for cell-cell adhesion for tight junction assembly and subsequent stability has been demonstrated in various morphological and biophysical studies (eg, Curran et al., 1961; Galli et al., 1976; Gumbiner and Simons, 1987; Gumbiner et al., 1988). In assembly, cell-cell adhesion can provide conditions for molecular components to associate with the junctional complex; this has been shown for ZO-1 in epithelial cell lines (Siliciano and Goodenough, 1988; Anderson et al., 1989) and in mouse blastomeres (Fleming et al., 1989). It has also been shown that upon provision of cell-cell adhesion in Caco-2 cells, ZO-1 protein levels gradually increase over an extended culture period, which may result from either increased synthesis and/or increased stability (Anderson et al., 1989). Similarly, the rise in net cingulin synthesis at compaction may reflect either a real increase in synthesis rate and/or enhanced stability. Our results favour a rise in the synthesis rate as the most likely explanation, for two reasons. First, the increase in cingulin stability in blastocysts ($t_{\frac{1}{2}}$ ~10 hours, Fig. 4), if initiated at compaction, would not account for more than a minor proportion of the 2.3-fold rise in the net synthesis data compared with precompact 8-cell embryos. Second, since cingulin assembly at the developing tight junction in most embryos is evident some 5-10 hours later in the next cell cycle (Fleming et al., 1992), a case for junction assembly promoting stability at compaction cannot be made. However, it should be noted that cell adhesion stimulation of synthesis appears effective only to **initiate** the rise in expression (compaction) rather than to **maintain** it, since inhibition of cell adhesion in blastocysts (by extracellular calcium depletion) reduces net synthesis by 25%, a level that **can** be explained by the observed reduction in stability. Thus, cell-cell adhesion in the embryo (or events associated with it, such as ZO-1 assembly) appears to influence the onset of expression as well as the stability of junctional components, a concept that has been established for other proteins in various differentiation systems (eg, Ben Ze'ev, 1986; Bendori et al., 1987).

Our immunoprecipitation data indicate that trophectoderm and ICM lineages in blastocysts exhibit distinct cingulin synthesis patterns with synthesis being steadily down-regulated in the ICM. Again, cell-cell interactions are likely mediators of this diversification. We have shown previously that ZO-1 can be inherited by ICM cells (or their precursors) when polarised 8- or 16-cell blastomeres undergo differentiative division (Fleming and Hay, 1991). In such circumstances, inherited ZO-1 is initially distributed randomly on ICM cell surfaces in the embryo interior, before disappearing. Various manipulations on ICMs isolated from early blastocysts have indicated that ZO-1 down-regulation in the ICM is reversible, and is regulated at the translational level by the loss of outward-facing, contact-free membrane surfaces (Fleming and Hay, 1991). The ICM cingulin synthesis data is therefore consistent with this model for how cell-cell contact patterns may control the tissue-specific level of expression of tight junction molecules during early development.

In summary, our results indicate that during early development, the expression of the tight junction protein cingulin is temporally regulated, involving both maternal and embryonic transcripts. This pattern distinguishes cingulin expression from that of ZO-1 and suggests a role specifically for cingulin during oogenesis. Cingulin synthesis is up-regulated at compaction, apparently mediated by events associated with cell-cell adhesion. Cellular interactions also appear to influence cingulin expression during later cleavage, to control its tissue-specificity and stabilisation at tight junction sites, events essential for blastocyst morphogenesis.

We are grateful for the financial support provided by The Wellcome Trust for reseach in Tom Fleming's laboratory. Sandra Citi is in receipt of a Cornell Scholar Award and an A. T. Mellon Teacher-Scientist Award. Thanks also to Charlotte McFadden for technical assistance.

REFERENCES

Anderson, J. M. and Stevenson, B. R. (1992). The molecular structure of the tight junction. In *Tight Junctions* (ed. M. Cereijido), pp. 77-90, Florida: CRC Press.

Anderson, J. M., Stevenson, B. R., Jesaitis, L. A., Goodenough, D. A. and Mooseker, M. S. (1988). Characterization of ZO-1, a protein component of the tight junction from mouse liver and Madin-Darby canine kidney cells. *J. Cell Biol.* **106**, 1141-1149.

Anderson, J. M., Van Itallie, D. M., Peterson, M. D., Stevenson, B. R., Carew, E. A. and Mooseker, M. S. (1989). ZO-1 mRNA and protein expression during tight junction assembly in Caco-2 cells. *J. Cell Biol.* **109**, 1047-1056.

Barron, D. J., Valdimarsson, G., Paul, D. L. and Kidder, G. M. (1989). Connexin32, a gap junction protein, is a persistent oogenetic product through preimplantation development of the mouse. *Dev. Genet.* **10**, 318-323.

Bendori, R., Salomon, D. and Geiger, B. (1987). Contact-dependent regulation of vinculin expression in cultured fibroblasts: a study with vinculin-specific cDNA probes. *EMBO J.* **6**, 2897-2905.

Bensaude, O., Babinet, C., Morange, M. and Jacob, F. (1983). Heat shock proteins, first major products of zygotic gene activity in the mouse embryo. *Nature* **305**, 331-333.

Ben Ze'ev, A. (1986). The relationship between cytoplasmic organization, gene expression and morphogenesis. *Trends Biochem. Sci.* **11**, 478-481.

Brinster, R. L. (1967). Protein content of the mouse embryo during the first five days of development. *J. Reprod. Fertil.* **13**, 413-420.

Chisholm, J. C., Johnson, M. H., Warren, P. D., Fleming, T. P. and Pickering, S. J. (1985). Developmental variability within and between mouse expanding blastocysts and their ICMs. *J. Embryol. Exp. Morph.* **86**, 311-336.

Citi, S., Kendrick-Jones, J. and Shore, D. (1990). Cloning of cingulin cDNA: evidence for a tight junction protein with a coiled-coil structure. *J. Cell Biol.* **111**, 409a.

Citi, S., Sabanay, H., Jakes, R., Geiger, B. and Kendrick-Jones, J. (1988). Cingulin, a new peripheral component of tight junctions. *Nature* **333**, 272-276.

Citi, S., Sabanay, H., Kendrick-Jones, J. and Geiger, B. (1989). Cingulin: characterization and localization. *J. Cell Sci.* **93**, 107-122.

Claude, P. and Goodenough, D. A. (1973). Fracture faces of zonulae occludentes from "tight" and "leaky" epithelia. *J. Cell Biol.* **58**, 390-400.

Curran, P. F., Zadunaisky, J. and Gill, J. R. (1961). The effect of ethylene-diaminetetraacetate on ion permeability of the isolated frog skin. *Biochim. Biophys. Acta* **52**, 392-395.

Denisenko, N. and Citi, S. (1991). Cingulin phosphorylation and localization in confluent MDCK cells treated with phorbol esters and protein kinase inhibitors. *J. Cell Biol.* **115**, 480a.

Ducibella, T., Albertini, D. F., Anderson, E. and Biggers, J. D. (1975). The preimplantation mammalian embryo: characterization of intercellular junctions and their appearance during development. *Dev. Biol.* **45**, 231-250.

Ducibella, T. and Anderson, E. (1975). Cell shape and membrane changes in the 8-cell mouse embryo: prerequisites for morphogenesis of the blastocyst. *Dev. Biol.* **47**, 45-58.

Flach, G., Johnson, M. H., Braude, P. R., Taylor, R. and Bolton, V. N. (1982). The transition from maternal to embryonic control in the 2-cell mouse embryo. *EMBO J.* **1**, 681-686.

Fleming, T. P. (1992). Trophectoderm biogenesis in the preimplantation mouse embryo. In *Epithelial Organization and Development* (ed. T.P. Fleming), pp. 111-136, London: Chapman and Hall.

Fleming, T. P., Garrod, D. R. and Elsmore, A. J. (1991). Desmosome biogenesis in the mouse preimplantation embryo. *Development* **112**, 527-539.

Fleming, T. P. and Hay, M. J. (1991). Tissue-specific control of expression of the tight junction polypeptide ZO-1 in the mouse early embryo. *Development* **113**, 295-304.

Fleming, T. P., Hay, M. J., Javed, Q. and Citi, S. (1992). Localization of tight junction protein cingulin is temporally and spatially regulated during early mouse development. *Development* **117**, 1135-1144.

Fleming, T. P. and Johnson, M. H. (1988). From egg to epithelium. *Ann. Rev. Cell Biol.* **4**, 459-485.

Fleming, T. P., McConnell, J., Johnson, M. H. and Stevenson, B. R. (1989). Development of tight junctions *de novo* in the mouse early embryo: control of assembly of the tight junction-specific protein, ZO-1. *J. Cell Biol.* **108**, 1407-1418.

Galli, P., Brenna, A., DeCamilli, P. and Meldolesi, J. (1976). Extracellular calcium and the organization of tight junctions in pancreatic acinar cells. *Exp. Cell Res.* **99**, 178-183.

Garrod, D. R. and Collins, J. E. (1992). Intercellular junctions and cell adhesion in epithelial cells. In *Epithelial Organization and Development* (ed. T. P. Fleming), pp. 1-52, London: Chapman and Hall.

Gumbiner, B. and Simons, K. (1987). The role of uvomorulin in the formation of epithelial occluding junctions. *Ciba Found. Symp.* **125**, 168-186.

Gumbiner, B., Stevenson, B. and Grimaldi, A. (1988). The role of the cell adhesion molecule uvomorulin in the formation and maintenance of the epithelial junctional complex. *J. Cell Biol.* **107**, 1575-1587.

Howarth, A. G., Hughs, M. R. and Stevenson, B. R. (1991). Detection of the tight junction-associated protein ZO-1 in astrocytes and other nonepithelial cell types. *Am. J. Physiol.* **262**, C461-C469.

Magnuson, T., Demsey, A. and Stackpole, C. W. (1977). Characterization of intercellular junctions in the preimplantation mouse embryo by freeze-fracture and thin-section electron microscopy. *Dev. Biol.* **61**, 252-261.

Merz, E. A., Brinster, R. L., Brunner, S. and Chen, H. Y. (1981). Protein degradation during preimplantation development of the mouse. *J. Reprod. Fertil.* **61**, 415-418.

Nicolson, G. L., Yanigimachi, R. and Yanigimachi, H. (1975). Ultrastructural localisation of lectin binding sites on the zonae pellucidae and plasma membranes of mammalian eggs. *J. Cell Biol.* **66**, 263-274.

Pinto da Silva, P. and Kachar, B. (1982). On tight junction structure. *Cell* **28**, 441-450.

Pratt, H. P. M. (1985). Membrane organization in the preimplantation mouse embryo. *J. Embryol. Exp. Morph.* **90**, 101-121.

Schnabel, E., Anderson, J. M. and Farquhar, M. G. (1990). The tight junction protein ZO-1 is concentrated along slit diaphragms of the glomerulus epithelium. *J. Cell Biol.* **111**, 1255-1263.

Sefton, M., Johnson, M. H. and Clayton, L. (1992). Synthesis and phosphorylation of uvomorulin during mouse early development. *Development* **115**, 313-318.

Siliciano, J. D. and Goodenough, D. A. (1988). Localization of the tight junction protein, ZO-1 is modulated by extracellular calcium and cell-cell contact in Madin-Darby canine kidney cells. *J. Cell Biol.* **107**, 2389-2399.

Staehelin, L. A. (1974). Structure and function of intercellular junctions. *Int. Rev. Cytol.* **39**, 191-283.

Stevenson, B. R., Heintzelman, M. B., Anderson, J. M., Citi, S. and Mooseker, M. S. (1989). ZO-1 and cingulin: tight junction proteins with distinct identities and localizations. *Am. J. Physiol.* **257**, C621-C628.

Stevenson, B. R., Siliciano, J. D., Mooseker, M. S. and Goodenough, D. A. (1986). Identification of ZO-1: a high molecular weight polypeptide associated with the tight junction (zonula occludens) in a variety of epithelia. *J. Cell Biol.* **103**, 755-766.

Stevenson, B. R. and Paul, D. L. (1989). The molecular constituents of intercellular junctions. *Curr. Opin. Cell Biol.* **1**, 884-891.

van Meer, G. and Simons, K. (1986). The function of tight junctions in maintaining differences in lipid composition between the apical and the basolateral cell surface domains of MDCK cells. *EMBO J.* **5**, 1455-1464.

(Accepted 24 November 1992)