

Localisation of tight junction protein cingulin is temporally and spatially regulated during early mouse development

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SUMMARY

The molecular maturation of the tight junction in the mouse early embryo has been investigated by monitoring the distribution of cingulin, a $140 \times 10^3 M_r$ peripheral (cytoplasmic) membrane constituent of the junction, at different stages of development and in different experimental situations. Although tight junction formation does not begin until compaction at the 8-cell stage, cingulin is detectable in oocytes and all stages of cleavage, a factor consistent with our biochemical analysis of cingulin expression (Javed et al., 1992, *Development* 117, 1145-1151). Using synchronised egg and embryo stages and isolated cell clusters, we have identified three sites where cingulin is localised, the cytocortex, punctate cytoplasmic foci and tight junctions themselves. Cytocortical cingulin is present at the cumulus-oocyte contact site (both cell types), in unfertilised and fertilised eggs and in cleavage stages up to 16-cell morulae, particularly at microvillous domains on the embryo outer surface (eg. apical poles at compaction). Embryo manipulation experiments indicate that cortical cingulin is labile and dependent upon cell interactions and therefore is not merely an inheritance from the egg. Cingulin cytoplasmic foci are evident only

in outer cells (prospective trophectoderm) from the 32-cell stage, just prior to cavitation, and decline from approx. 8 hours after cavitation has initiated. The appearance of these foci is insensitive to cycloheximide treatment and they colocalise with apically derived endocytic vesicles visualised by FITC-dextran, indicating that the foci represent the degradation of cytocortical cingulin by endocytic turnover. Cingulin is detectable at the tight junction site between blastomeres usually from the 16-cell stage, although earlier assembly occurs in a minority (up to 20%) of specimens. Cingulin assembly at the tight junction is sensitive to cycloheximide and is identifiable approx. 10 hours after cell adhesion is initiated and ZO-1 protein assembles. Collectively, our results indicate that (i) cingulin from non-junctional sites does not contribute to tight junction assembly and (ii) the molecular maturation of the junction appears to occur progressively over at least two cell cycles.

Key words: mouse preimplantation embryos, mouse eggs, tight junctions, cingulin, ZO-1, epithelial biogenesis

INTRODUCTION

During early mammalian development, epithelia participate in morphogenetic processes that contribute to pattern formation and homeostatic control. Epithelia depend upon apicolateral tight junctions for polarised vectorial transport activity (reviewed in Cereijido, 1991). Tight junction formation in early embryos is therefore likely to be a crucial event not only for nascent epithelia to become functional but also for morphogenesis to proceed. During cleavage, the mouse embryo delaminates the trophectoderm epithelium, which functions initially to generate the blastocoele of the blastocyst (reviewed in Fleming and Johnson, 1988; Wiley et al., 1990; Fleming, 1992). We and others have

used this system to analyse the process of tight junction formation in vivo, which begins at compaction in the 8-cell embryo (see Introduction of Javed et al., 1992).

In the accompanying paper, we have investigated biochemically the expression of the tight junction peripheral membrane protein cingulin during early development (Javed et al., 1992). In contrast to our data on the tight junction protein ZO-1 (Fleming et al., 1989), cingulin is expressed maternally as well as embryonically. Here, we report on the localisation of cingulin during early development and in different experimental situations. In particular, we have investigated the significance of maternal and embryonic expression programmes, and the relationship between ZO-1 and cingulin during tight junction formation.

MATERIALS AND METHODS

Egg and embryo collection and culture

Procedures used for collection and culture of eggs, cleaving embryos and blastocysts (MF1 strain) following superovulation are as described previously (Fleming et al., 1991; Javed et al., 1992). Embryos were staged according to time post-hCG (human chorionic gonadotropin) injection (see Table 1), but blastocysts were also staged according to the time at which the blastocoele was first detectable following hourly inspection of stock cultures (Fleming et al., 1984). Trophoblast outgrowths were grown for 48 hours after initial attachment of blastocysts on sterile coverslips as described (Fleming et al., 1991).

Egg and embryo manipulations

Cumulus cells were removed from unfertilised eggs using hyaluronidase and removal of the zona pellucida from eggs and embryos was by acid Tyrode's incubation as described (Fleming et al., 1991; Javed et al., 1992). Embryos (4- and 8-cell stage) were disaggregated to single cells using a flame-polished micropipette following zona removal and culture in calcium-free M2+BSA for 15 minutes (Fleming et al., 1991). In some experiments, the isolated blastomeres (1/4 or 1/8 cells) from individual embryos were reaggregated back together by allowing them to touch and adhere during brief (1 minute) incubation in M2+BSA containing phytohaemagglutinin (PHA; Gibco; 1:20 dilution) before further culture in M16+BSA. These embryos at compaction were labelled with TRITC-conjugated concanavalin A (Sigma; 10 µg/ml in M2+BSA) to allow identification of the microvillous pole before fixation and immunolabelling. In other experiments, 1/4 and 1/8 cells were used to generate synchronised cell clusters. Several hundred single cells were cultured spaced apart and monitored hourly for evidence of division to 2/8 or 2/16 couplets, respectively. Newly divided couplets were collected and cultured in individual 10 µl M16+BSA drops for specific times before immunocytochemistry. In some cases, intact embryos and cell clusters were incubated for various times in M16+BSA containing cycloheximide (Sigma; 400 µM; this concentration reduces TCA-precipitable incorporation of [³⁵S]methionine to 6% normal level (Fleming et al., 1989) and inhibits cingulin synthesis (Javed et al., 1992). Blastocysts were also incubated in FITC-dextran

(Sigma; 9,400 *M_r*, 12.5 mg/ml in M16+BSA) for 4 hours to label the endocytic system before M2+BSA washing and fixation.

Immunocytochemistry

Whole-mount eggs and embryos without zonae pellucidae, trophoblast outgrowths, manipulated embryos and synchronised cell clusters were fixed in 0.5% formaldehyde in phosphate-buffered saline (PBS) for 20 minutes and processed for immunolabelling using the procedure described previously (Fleming et al., 1989, 1991). Samples were incubated (1 hour) in polyclonal rabbit anti-cingulin antibody diluted 1:50 to 1:1000 (Citi et al., 1988) before washing and incubation (1 hour) in FITC- or TRITC-conjugated anti-rabbit Ig also containing Hoechst dye 33258 (50 µg/ml) to label nuclei and to confirm embryo stage especially at late cleavage. Various other fixation regimes (eg, chilled methanol, acetone or ethanol; 2% formaldehyde; 0.5% or 2% paraformaldehyde) did not appreciably influence staining patterns observed, nor did the inclusion of 1% BSA during primary antibody incubation. Cell clusters were also labelled with R26.4 rat monoclonal antibody to ZO-1 as described (Fleming et al., 1989). Ovary and oviducal ampullae containing cumulus masses from superovulated virgin MF1 mice (3-4 week old) at 8 and 18 hours post-hCG injection (or ovary from females either not injected or 48 hours post-PMS injection) were fixed in 1% formaldehyde in PBS and processed subsequently for cryosectioning or alcohol dehydrated and paraffin embedded. Sections (8 µm) were stained for cingulin as above. Specimens were viewed on a Leitz Diaplan microscope using appropriate filter systems; photographs were taken on Kodak T-max film.

RESULTS

Cingulin localisation in eggs and embryos

Unfertilised eggs and preimplantation embryos at precise stages were examined by immunofluorescence microscopy using anti-cingulin antibody (data summarised in Table 1). In unfertilised eggs, following removal of cumulus cells and the zona pellucida, cingulin staining was detectable associated with the cytoplasmic face of the oolemma (i.e. per-

Table 1. Cingulin localisation in embryos at different stages of development

| Developmental stage (hours post-hCG) | Embryo number | Cingulin staining pattern (% embryos) | | |
|---|------------------|---------------------------------------|-----------------|-------------------|
| | | cyt cortex* | tight junction† | cytoplasmic foci‡ |
| unfertilized eggs (14) | 48 | 100 | 0 | 0 |
| fertilized eggs (22) | 37 | 100 | 0 | 0 |
| 2-cell (48) | 29 | 69 | 0 | 0 |
| 4-cell (56) | 42 | 78 | 0 | 0 |
| precompact 8-cell (65) | 63 | 57 | 0 | 0 |
| compact 8-cell (68) | 55 | 85 | 0 | 0 |
| 16-cell morulae (80) | 49 | 67 | 75 | 0 |
| late morulae (92) | 78 | 0 | 100 | 70 |
| blastocysts (90-97)§ | | | | |
| 2 h post-cavitation (p-cav) | 69 | 0 | 100 | 100 |
| 8 h p-cav | 48 | 0 | 100 | 100 |
| 12 h p-cav | 51 | 0 | 100 | 65 |
| 20 h p-cav | 58 | 0 | 100 | 0 |
| trophoblast outgrowth (188) | 12 | 0 | 100 | 0 |

*Cytocortex: staining more intense at microvillous sites (see Figs 1A,B, 2B) and less so during early cleavage (Fig. 2A).

†Tight junction: staining faint up to early blastocyst stage (Fig. 3A), increasing in intensity thereafter (Fig. 3D,E).

‡Cytoplasmic foci: staining mostly perinuclear and punctate in appearance (Fig. 3A).

§Individual blastocysts staged from onset of cavitation (occurring between 90 and 97 hours post-hCG) and cultured for specified times thereafter (see Materials and methods).

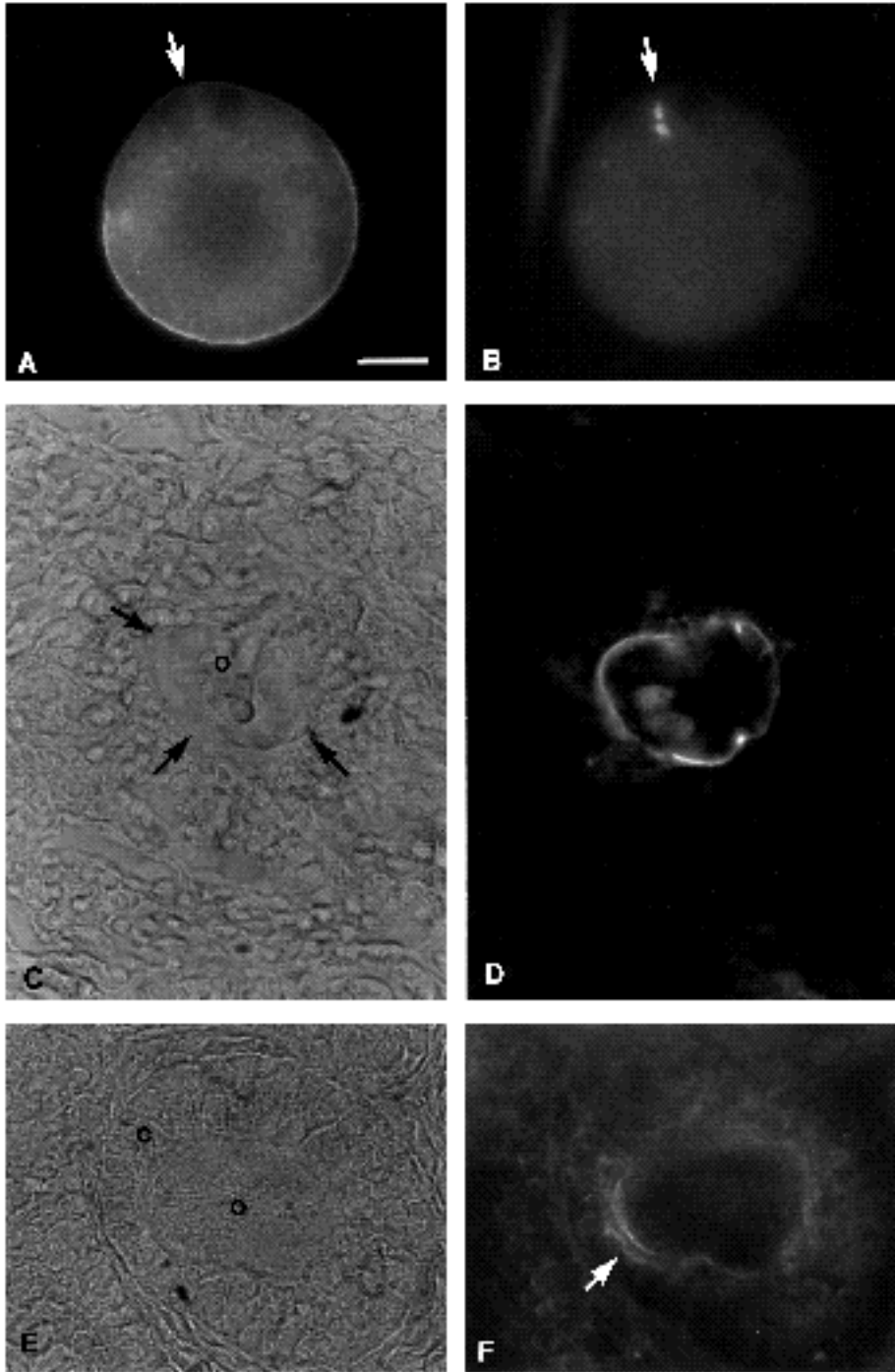


Fig. 1. Cingulin localisation in oocytes. (A) Unfertilised egg freed from cumulus cells showing cingulin reactivity associated with the oolemma except for region arrowed. (B) Same egg as A labelled with Hoechst dye showing that position of spindle chromosomes (arrowed) corresponds with region of low cingulin reactivity. (C-F) Paraffin sections of ovary fixed 8 hours post-hCG (C,E phase contrast, D,F cingulin labelling). (C,D) The outline of a young oocyte (o), sectioned tangentially, is arrowed (C) and corresponds to site of cingulin staining (D); the bulk of cumulus cells are negative. (E,F) Young follicle with oocyte (o) and surrounding cumulus cells (c); cingulin is localised to the oocyte cortex and in the contact region of adjacent cumulus cells (arrow in F). More diffuse staining on upper right side of oocyte is due to an unfavourable plane of section. Bar, 20 μ m.

meabilisation-dependent). This cytocortical reaction was heterogeneous in that the microvillous domain was uniformly labelled but the smooth membrane domain overlying the metaphase II spindle (identified by chromosome staining) was poorly stained or negative (Fig. 1A,B). Cryostat and paraffin sections of ovary tissue and cumulus masses were used to examine cingulin distribution in pre-ovulatory and postovulatory oocytes in relation to cumulus cells. At all stages of follicular maturation observed, cingulin was detectable at the oocyte surface but was absent from the bulk of cumulus cells except within the corona radiata where cells directly associated with the oocyte were

weakly stained at the oocyte contact site (Fig. 1C-F). Cingulin localisation in fertilised eggs (both experimentally and naturally depleted of cumulus cells) was comparable with unfertilised eggs except that cytocortical heterogeneity was not observed.

Early cleavage stages also displayed cingulin staining within the cytocortex. Staining was either uniform or occurred preferentially on the outer embryo surface and appeared as a fine membrane-associated reaction (Fig. 2A). In compacted 8-cell embryos, cytocortical cingulin was again heterogeneous, localising at the apical microvillous pole on the outer surface of each blastomere but, signifi-

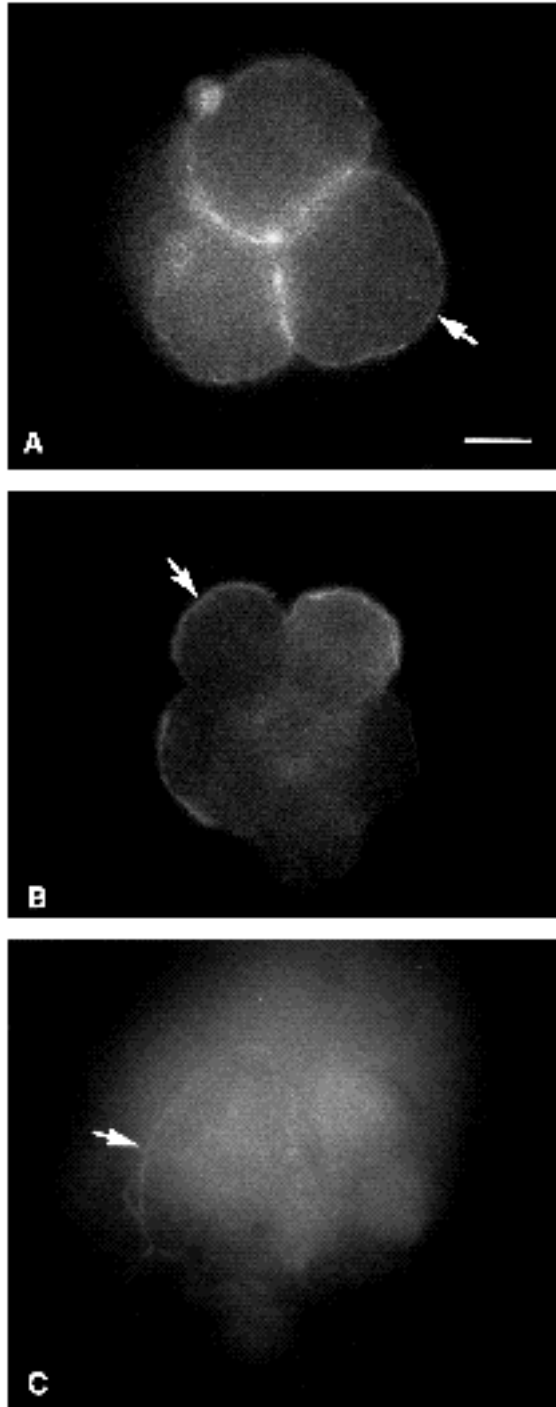


Fig. 2. Cingulin localisation during cleavage. (A) 4-cell embryo showing cytocortical staining for cingulin (arrow). (B) Compact 8-cell embryo in which cytocortical cingulin is localised in the outer membrane domain (arrow) corresponding to the apical microvillous pole. (C) 16-cell morula viewed tangentially in which faint cingulin staining is evident associated with the tight junction site between outer blastomeres (arrowed). Bar, 20 μ m.

cantly, was not detectable at the nascent apicolateral tight junction site (Fig. 2B). In 16-cell morulae, cytocortical staining of microvillous poles in outer blastomeres (trophectoderm lineage) was again evident but, in 75% moru-

lae, faint cingulin staining was also detectable at the forming tight junction site. In tangential section, staining at the tight junction site was either zonular or discontinuous around each blastomere (Fig. 2C).

In blastocysts, cingulin staining at tight junction sites in the trophectoderm gradually increased in intensity as blastocoele expansion progressed; however, cytocortical cingulin staining was no longer evident. Instead, punctate cytoplasmic foci of cingulin reactivity were present that tended to surround trophectoderm nuclei and were not detectable within the inner cell mass (Fig. 3A-C). Since aspects of tight and desmosome junction formation have previously been implicated with the onset of cavitation in the early embryo (Magnuson et al., 1978; Fleming et al., 1991), we investigated the time of appearance of cingulin cytoplasmic foci in embryos prior to, and at different times after, the onset of blastocoele formation (Table 1). These experiments indicated that cingulin cytoplasmic sites (i) first appeared in late morulae before the accumulation of blastocoele fluid and (ii) gradually declined from after 8 hours post the onset of cavitation, coincident with the gradual increase in cingulin staining associated with the tight junction. Thus, blastocysts at 12 hours post-cavitation contained a mixture of trophectoderm cells, either with or without cytoplasmic foci (Fig. 3D), while in well-expanded blastocysts at 20 hours post-cavitation, foci were entirely absent (Fig. 3E). In trophoblast outgrowths, cingulin was localised exclusively at junctional contact sites between cells (Fig. 3F).

Significance of cingulin cytocortical sites

The stability of cytocortical cingulin in relation to altered cell contacts was investigated. Late 4-cell (62 hours post-hCG) and early 8-cell embryos (67 hours post-hCG) were disaggregated to single cells and then reaggregated (using PHA) so that the original orientation and position of cells would have been randomised. A similar treatment has been shown previously to alter the position at which the microvillous pole develops (Johnson and Ziomek, 1981). Reaggregated embryos were cultured for 3-13 hours and those that had compacted were labelled with TRITC-concanavalin A to define the position of microvillous poles and then examined for cingulin distribution using FITC-conjugated secondary antibody (Table 2). Cytocortical cingulin in nearly all reaggregated embryos cultured for only a short period (3 hours) was fragmented in appearance, occurring as small isolated tracts of reactivity at both cell contact and contact-free membrane sites (Fig. 4C). However, in most embryos cultured for 13 hours cytocortical cingulin colocalised with microvillous poles on the outer embryo surface (Fig. 4A,B). Both staining patterns in approx. equal proportions were evident in embryos cultured for an intermediate period (8 hours; Table 2). These results suggest that cytocortical cingulin sites are labile and can reposition following changes in cell contact patterns.

Significance of cingulin cytoplasmic foci

A mixed population of late morulae and early blastocysts (88 hours post-hCG; approx. 32-cell stage) were cultured in the presence of cycloheximide (400 μ M) for 7 hours

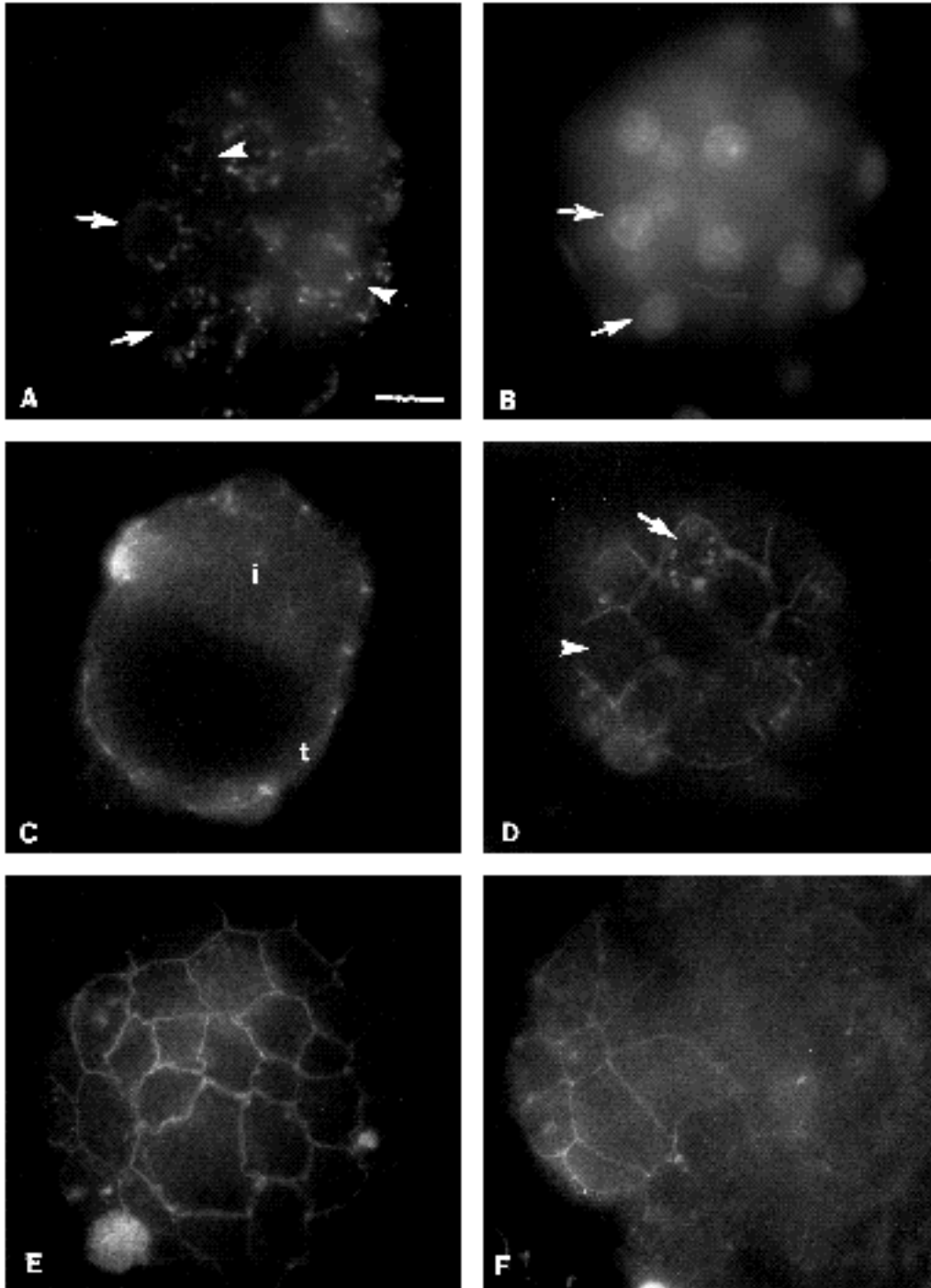


Fig. 3. Cingulin localisation in blastocysts. (A) Blastocyst at 2 hours post-cavitation, viewed tangentially, showing weak tight junction staining between trophectoderm cells (arrowheads) and more prominent cytoplasmic foci of cingulin reactivity (arrows). (B) Same as A, stained with Hoechst dye; cingulin cytoplasmic foci surround trophectoderm nuclei, identified here (arrows). (C) Mid-sectional view of blastocyst at 8 hours post-cavitation; cingulin staining is associated with trophectoderm (t) but not inner cell mass (i). (D) Tangential view of 12 hour post-cavitation blastocyst; cingulin at the tight junction between trophectoderm cells is now more prominent and cells with (arrow) or without (arrowhead) cytoplasmic foci are evident. (E) Late blastocyst (20 hour post-cavitation) viewed tangentially in which cingulin is restricted to trophectoderm tight junction sites. (F) Trophoblast outgrowth showing cingulin localisation at junction sites between trophoblast cells. Bar, 20 μ m.

before fixation and cingulin staining. These embryos ($n=32$) displayed cingulin cytoplasmic foci similar to controls but the protein was undetectable or very weakly reactive at tight junction sites suggesting that cytoplasmic foci were not newly synthesised cingulin. We next preincubated late morulae in medium containing FITC-dextran (12.5 mg/ml), a marker for fluid-phase endocytosis, for 4 hours before fixation and cingulin staining (using TRITC-conjugated secondary antibody). These embryos displayed numerous dextran-containing endocytic vesicles throughout the trophectoderm; cingulin cytoplasmic foci consistently co-

localised with these endocytic vesicles (Fig. 5A,B). These results suggest that cingulin cytoplasmic foci are derived from apical endocytosed membrane.

Cingulin expression in synchronised cell clusters

Intact embryos, especially in later cleavage, contain asynchronous blastomeres, either at different stages of the same cell cycle or in different cycles (eg. Chisholm et al., 1985). Cell clusters, synchronised from specific cell divisions, were therefore used to define more precisely the time of cingulin assembly at the maturing tight junction in relation

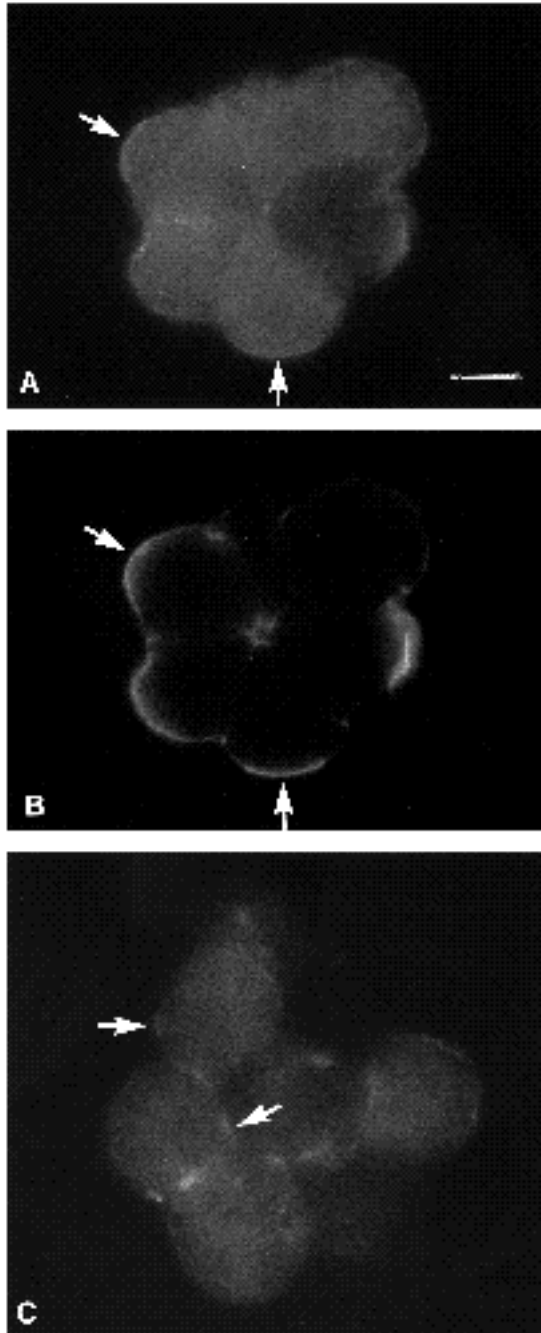


Fig. 4. Cytocortical cingulin in compact 8-cell embryos following disaggregation and reaggregation of blastomeres either at late 4-cell stage and cultured for 13 hours (A,B) or at early 8-cell stage and cultured for 3 hours (C). (A,B) Cingulin is localised to outer embryo surface (arrows in A), corresponding to microvillous poles identified by concanavalin A staining (arrows in B). (C) Cingulin occurs as small fragmented sites at both contact and contact-free membranes (arrows). Bar, 20 μ m.

to cell cycle and other relevant events (cell adhesion, ZO-1 assembly, blastocoele formation). Late 4-cell embryos (58 hours post-hCG) were disaggregated to single blastomeres (1/4 cells) and allowed to divide in culture to 2/8 couplets. These were synchronised from division of 1/4 cells and cultured for times up to 35 hours during which compaction, further division and cavitation took place as in intact embryos. Clusters were then examined for cingulin (or ZO-1) distribution, cell number (identified by nuclear staining) and state of fluid accumulation. In short-term experiments (2-10 hours post 2/8 formation), nearly all couplets engaged in cell-cell adhesion and began to assemble ZO-1 at the nascent tight junction site (periphery of the disc-shaped contact region) as has been shown previously (Fleming et al., 1989), but only a small minority showed evidence of cingulin tight junction staining (Figs 6A, 7A).

In longer-term experiments, the proportion of cell clusters showing cingulin associated with the tight junction site increased, but only after division to the 16-cell stage (Fig. 8). Initially, most clusters showed a punctate junctional staining pattern for cingulin (early/mid 16-cell stage; Fig. 7B) before a linear (ie, zonular) pattern was established (mid/late 16-cell stage; Fig. 7C). Cingulin junctional staining was restricted to contact sites between outer cells in clusters while any internal cells were negative (Fig. 7D-F). Nearly all clusters contained linear cingulin junctional staining before division occurred to the 32-cell stage (Fig. 8). However, most clusters contained 32-cell stage blastomeres before cytoplasmic foci of cingulin were detectable (Figs 7G,H, 8). These foci occurred prior to the time that clusters began to accumulate blastocoele fluid (Fig. 8), confirming the data from intact embryos.

Cingulin assembly at the tight junction was sensitive to cycloheximide treatment. For example, newly formed 2/8 couplets cultured in medium containing cycloheximide for 6-10 hours ($n=47$ or more per treatment) were all negative

Table 2. Distribution of cytocortical cingulin in compact 8-cell embryos following zona removal, disaggregation and reaggregation of blastomeres at 4-cell or early 8-cell stages and culture for specified times

| Stage reaggregated (hours post-hCG) | Culture time (hours) | <i>n</i> | % compacted | Cingulin cytocortical staining pattern (%)* | | |
|--|-------------------------|----------|-----------------|---|------------|----------|
| | | | | apical | fragmented | negative |
| 4-cell (62) | 13 | 47 | 87 | 80 | 5 | 15 |
| precompact 8-cell (67) | 8 | 56 | 77 | 42 | 46 | 12 |
| precompact 8-cell (67) | 3 | 63 | 52 [†] | 0 | 97 | 3 |
| <i>controls</i> [‡] | | | | | | |
| 4-cell (62) | 13 | 29 | 96 | 93 | 0 | 7 |
| 8-cell (67) | 3 | 24 | 67 [†] | 87 | 0 | 13 |

*Only compacted embryos analysed. Apical, cingulin only at microvillous poles (Fig. 4A,B); fragmented, cingulin at several membrane sites, randomly distributed (Fig. 4C).

[†]95% (experimental) and 92% (control) embryos had compacted by 75 hours post-hCG.

[‡]Zona removed but otherwise not manipulated.

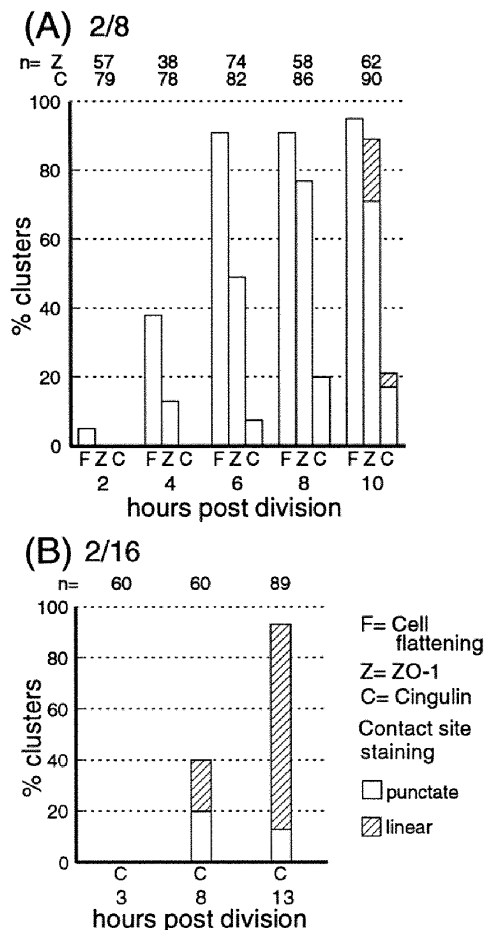


Fig. 6. (A) Incidence of cell flattening (adhesion), ZO-1 and cingulin assembly at the tight junction site between 2/8 couplets at different times post-division of 1/4 cells. (B) Incidence of cingulin assembly at junction site between 2/16 blastomeres at different times post-division of 1/8 cells. (Data from three experiments).

for cingulin at the tight junction although weak cytotortical staining was retained. Moreover, when clusters at 15 hours post 2/8 formation (corresponding to early 16-cell stage, see Fig. 8) were cultured in cycloheximide for a further 5 or 10 hours, cingulin staining at the tight junction site was evident in 77% ($n=65$) and 75% ($n=80$) clusters, respectively. Thus, the increase in the proportion of clus-

Fig. 5. Nascent blastocyst following incubation in FITC-dextran for 4 hours to label endocytic vesicles before fixation and cingulin staining. (A) Cingulin is detectable at tight junctions and in cytoplasmic foci (large arrow); one cell without foci is indicated by small arrow. (B) FITC-dextran-labelled endocytic vesicles (large arrow) colocalise with cingulin cytoplasmic foci; the same cell lacking foci also lacks endocytosed dextran. Bar, 20 μ m.

ters displaying junctional cingulin evident in controls at 20 and 25 hours post 2/8 formation (94% and 98% respectively, Fig. 8) was inhibited by cycloheximide.

Cingulin assembly at the tight junction was also investigated in cell clusters derived from single 8-cell blastomeres (1/8 cells) that were synchronised from division to 2/16 couplets (Fig. 6B). Here, cingulin junctional staining was not evident until 8 hours post-division in only 40% couplets. This represents a delay of several hours compared with data from clusters derived from 1/4 cells (Fig. 8).

DISCUSSION

This study has shown that the distribution pattern of cingulin during early development is complex and, in contrast to ZO-1 (Fleming et al., 1989; Fleming and Hay, 1991), the protein is not confined to maturing tight junctions and associated regions of cell-cell contact. We have identified three distinct sites of cingulin reactivity: the cytocortex, cytoplasmic foci, and tight junctions themselves. The use of synchronised embryo and blastomere populations has revealed that these sites are reactive at different times. In combination with the accompanying biochemical analysis of cingulin expression (Javed et al., 1992), we have attempted to characterise these sites and to interpret their significance in the molecular events underlying tight junction assembly.

The cytocortex is the earliest detectable location of cingulin, being present throughout oogenesis and early embryogenesis up to the morula stage, and is evident particularly at microvillous membrane as found on eggs and on the outer embryo surface. The expression of cingulin during oogenesis has been confirmed by immunoblotting and immunoprecipitation (Javed et al., 1992), despite the absence of tight junctions at this time. Although the function of cortical cingulin in oocytes has not been established, a role in cellular interactions appears likely since cingulin is also detectable in adjacent cumulus cells at their contact site with the oocyte, but not in remaining cumulus cells. The cumulus-oocyte interaction is essential for oocyte growth and the regulation of meiotic maturation (Eppig, 1991), yet little is known of its molecular organisation. The cell adhesion glycoprotein, uvomorulin is present here (Vestweber et al., 1987) and, like cingulin, is synthesised

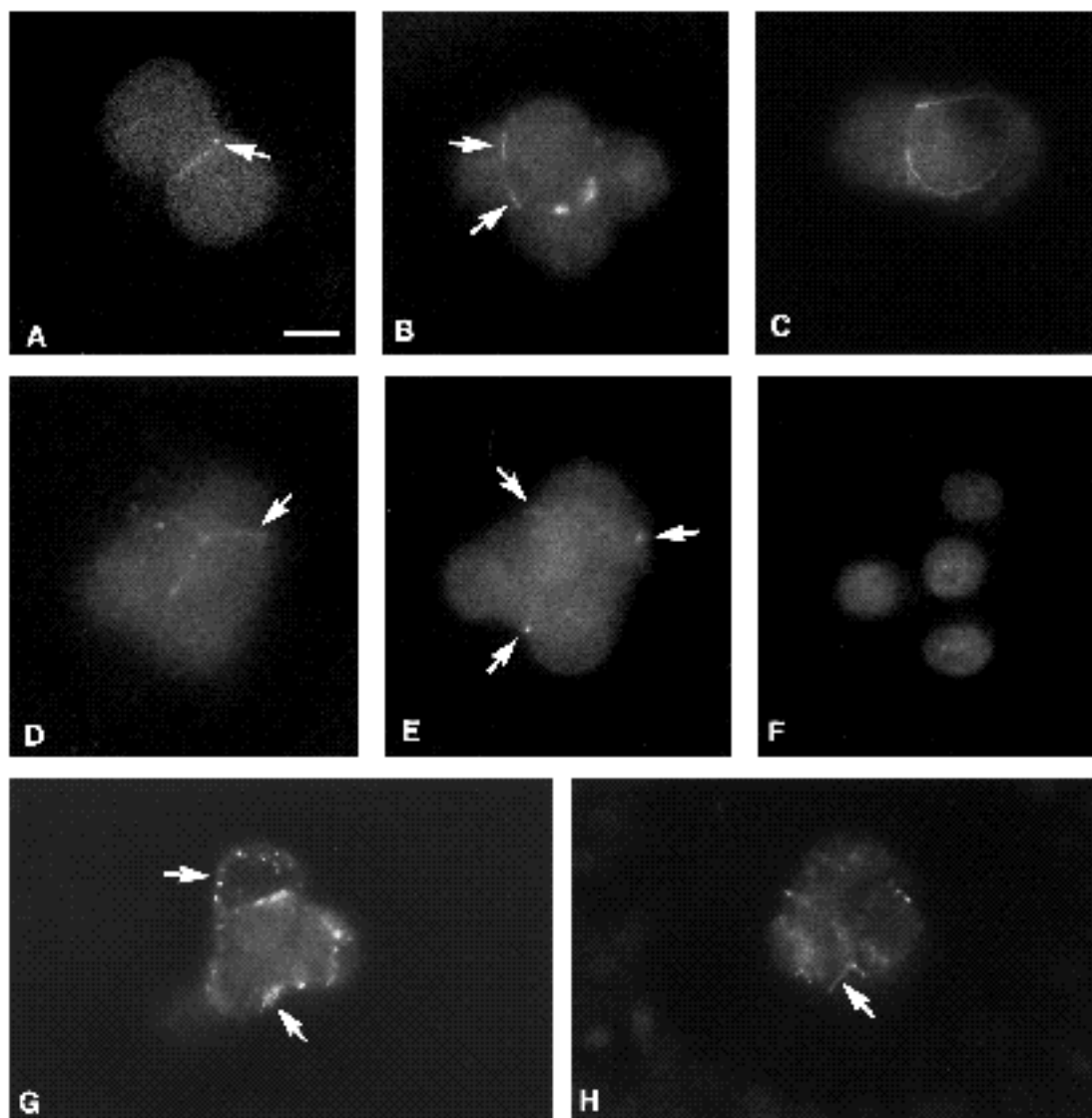


Fig. 7. Cingulin in synchronised cell clusters. (A) 2/8 couplet 8 hours post-division viewed tangentially showing punctate cingulin (arrow) at tight junction site (periphery of contact region). (B) 4/16 cluster (15 hours post-division of 1/4 cell) viewed tangentially showing punctate cingulin (arrows) between blastomeres. (C) 4/16 cluster (20 hours post-division of 1/4 cell) viewed tangentially showing linear cingulin staining around one blastomere. (D-F) 4/16 cluster (20 hours post-division of 1/4 cell) viewed tangentially (D), in mid optical section (E), and stained with Hoechst dye (F). The cluster contains three outer blastomeres and one internal cell. Cingulin is localised between outer cells only (arrows in D,E). (G,H) 8/32 clusters (30 hours post-division of 1/4 cell). (G) Mid-sectional plane showing cingulin cytoplasmic foci (arrows) restricted to outer cells. (H) Tangential plane showing cingulin in cytoplasmic foci and at tight junction site between blastomeres (arrow). Bar, 20 μ m.

in the oocyte (Sefton et al., 1992). Gap and desmosome-like junctions have also been identified at the cumulus-oocyte contact site (Anderson and Albertini, 1976; Gilula et al., 1978).

Since membrane-associated cingulin persists in the absence of cumulus cell attachment, the protein may also participate in the structural organisation of the cytocortex during early development (see also below). The cytocortex of follicular oocytes, unfertilised eggs and cleavage-stage blastomeres contains a number of cytoskeletal proteins such as actin, myosin, vinculin and spectrin (reviewed in Lehtonen et al., 1988). However, in unfertilised eggs, vinculin is

uniformly distributed in the cortex (Lehtonen and Reima, 1986), while actin and spectrin are localised preferentially at the smooth membrane domain overlying the spindle (Maro et al., 1984; Schatten et al., 1986), in contrast to cingulin, which is depleted in this area. The distribution of cingulin in the cortex more closely resembles that of myosin, which also associates preferentially with the outer embryo surface (Sobel, 1983). The shape and physicochemical properties of cingulin resemble that of the myosin rod (Citi et al., 1989) and it is possible that cingulin and myosin may share binding sites in the cytocortex of eggs and embryos.

Does cytocortical cingulin contribute to tight junction

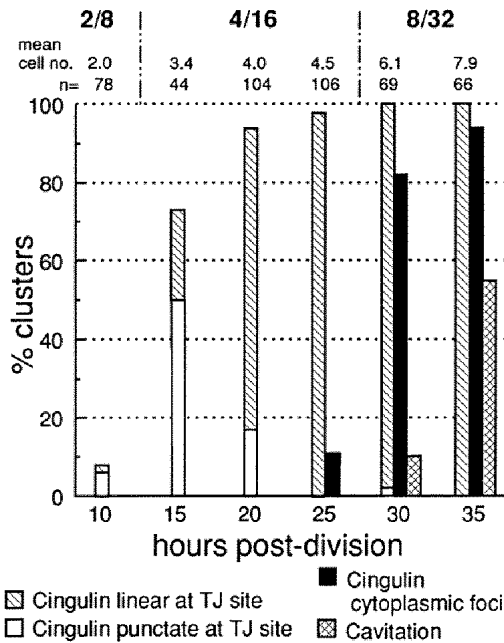


Fig. 8. Incidence of cingulin tight junction and cytoplasmic staining in relation to cell cycle and cavitation in cell clusters synchronised from division of 1/4 cells. Mean cell number at each time point identified by Hoechst staining. Data from three experiments.

formation? It is possible that cortical cingulin during cleavage represents a stable pool of protein inherited from the oocyte surface. Our experiments in which 4-cell and early 8-cell embryos were disaggregated and reagggregated such that new cell-cell contact positions were formed demonstrated that cortical cingulin sites reestablished on the outer embryo surface at compaction. Thus, cortical cingulin during cleavage is labile and dependent upon cellular interactions. Moreover, the half-life of cingulin synthesised in unfertilised eggs and in 4-cell embryos, when only the cortical site is present, is short (approx. 4 hours) (Javed et al., 1992). Also, the assembly of cingulin at the tight junction in embryos is sensitive to cycloheximide treatment, suggesting that the junction-associated protein does not derive from preexisting cortical cingulin. Collectively, these results indicate that cortical cingulin (i) cannot represent a protein store for junctional assembly and (ii) is likely to derive firstly from the maternal cingulin expression programme and later from the embryonic expression programme (see Javed et al., 1992). The precise role of cortical cingulin during cleavage is presently obscure. Possibly, cortical cingulin may function exclusively in the cumulus-oocyte interaction but the cortical binding site may persist up until the time the early embryo is capable of radically modifying cytocortex composition by endocytic turnover; the endocytic system does not mature fully until the morula stage (Fleming and Pickering, 1985). Alternatively, cingulin may indeed be required for cyto-cortical maturation during cleavage prior to cavitation. Interestingly, a similar cortical localisation of cingulin is evident in single, freshly plated MDCK cells (S. Citi, unpublished observation), suggesting that this site is not

embryo-specific and is available in the absence of a tight junction binding site.

A second site for cingulin in the early embryo is cytoplasmic foci surrounding nuclei in the trophectoderm lineage. This site is transient, evident from the 32-cell stage just prior to cavitation and declines some 12 hours after cavitation has initiated. At this time of development, cingulin is also detectable at maturing tight junctions. However, our experiments involving cycloheximide and dextran endocytic labelling indicated that cytoplasmic foci were not newly synthesised cingulin en route to the junctions but rather were associated with endocytic vesicles derived from the apical surface. These results suggest that they represent the degradation of cyto-cortical cingulin and its binding site.

The third site for cingulin in the embryo, the tight junction itself, was investigated with particular attention to its time of appearance. Whole embryo studies indicated that cingulin first associated with the junction between outer blastomeres during the 16-cell stage. However, studies with synchronised cell clusters suggested that, in a small minority of the population, junctional assembly occurred earlier, during the 8-cell stage following compaction. The time of junctional assembly is therefore unlikely to be linked directly with the cell cycle, as has been found for other epithelial maturation events in the embryo (eg. cytokeratin filament assembly; Chisholm and Houliston, 1987).

Direct comparison between the time of ZO-1 and cingulin assembly at the tight junction indicated that cingulin associates after ZO-1 and that ZO-1 assembles once compaction (cell-cell adhesion) has occurred (see also Fleming et al., 1989). Although, on a population basis, junctional assembly for both proteins is accumulative with time, it can be estimated from our current data that, on average, cingulin assembles some 10 hours after ZO-1 (for 50% population to be labelled). This period is characterised by a significant increase in the level of cingulin synthesis, from compaction onwards (Javed et al., 1992). Moreover, in 2/16 couplets, cingulin junctional assembly was further delayed compared with 4/16 clusters. For example, only 40% 2/16 couplets showed junctional staining at 8 hours post-division (Fig. 6B) while over 90% 4/16 clusters were positive at an equivalent time point (20 hours post 2/8 formation, Fig. 8). The most likely reason for this delay is that cell-cell contact in 2/16 couplets is initiated only from the time of division of isolated 1/8 cells while, in larger clusters (and intact embryos), cell adhesion persists relatively undisturbed from compaction. Thus, the 2/16 couplet is a useful assay for comparing the time course of assembly of different proteins because here both cell-cell adhesion and the cell cycle are coordinately synchronised. Previously, we have shown that all 2/16 couplets exhibit ZO-1 labelling immediately after division of 1/8 cells but that some 3 hours are required for cell-cell adhesion to take place before ZO-1 localises at the junction site (Fleming and Hay, 1991). Here, we show that a further 5-10 hours are required before cingulin is evident at the junctional domain in 2/16 couplets, data consistent with the results from intact embryos and larger cell clusters.

Immunogold double-labelling studies on chick and rat epithelia indicate that the localisation of cingulin at the tight junction is more cytoplasmic than that of ZO-1 (Stevenson

et al., 1989). Our results suggest, therefore, that tight junction formation in the mouse embryo occurs progressively with components closer to the membrane assembling before those located more internally. However, conclusive evidence of sequential assembly of ZO-1 and cingulin in embryos awaits double-labelling immunofluorescence analysis. Moreover, recent studies on MDCK cells indicate an exact co-localisation of ZO-1 and cingulin under several assembly/disassembly and modulated conditions (Denisenko and Citi, 1992). The apparent contrast between early embryos and MDCK cells in tight junction assembly patterns may reflect a difference in gene expression states between a developmental system and a mature epithelial cell line.

From our current data, it appears that the mechanism of construction of the tight junction in embryos is distinct from that of desmosomes. Desmosomes form from cavitation in the trophectoderm and, from that time, appear 'complete' in terms of their ultrastructure, composition and association with cytokeratin filaments, with assembly apparently regulated by expression of desmosomal glycoproteins (Fleming et al., 1991). This apparent distinction in the mechanism of tight and desmosome junction biogenesis *in vivo* may reflect the difference in their gross morphology. In contrast to the smaller, punctate desmosome, the more extensive zonular configuration of the tight junction may be incompatible with rapid construction. It may be for this reason that tight junction formation in the embryo begins well in advance of desmosome formation, although they both become competent to function at a similar time, when the blastocoele begins to accumulate.

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