# Development of Tight Junctions De Novo in the Mouse Early Embryo: Control of Assembly of the Tight Junction–specific Protein, ZO-1

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*Abstract.* Tight junction development during trophectoderm biogenesis in the mouse preimplantation embryo has been examined using monoclonal antibodies recognizing the tight junction–specific peripheral membrane protein, ZO-1. In immunoblots, mouse embryo ZO-1 had a molecular mass (225 kD) equivalent to that in mouse liver, was barely detectable in four-cell embryos although later stages exhibited increasing levels. ZO-1 was first detected immunocytochemically at the compacting eight-cell stage, coincident with or just after the expression of basolateral cell adhesion and apical microvillous polarity. Initially, ZO-1 was present as a series of spots along the boundary between free and apposed cell surfaces in intact embryos or cell couplets, but subsequently staining became more linear with blastocyst trophectoderm cells being bordered by a continuous ZO-1 belt. Inhibition of cell adhesion at the 8-cell stage delayed ZO-1 appearance and randomized its surface distribution in a reversible manner. Microfilament disruption, but not microtubule depolymerization, produced major disturbances in ZO-1 distribution. ZO-1 assembly de novo appeared to be independent of proximate DNA and RNA synthesis but was inhibited substantially in the absence of protein synthesis during the eight-cell stage, a treatment that did not prevent intercellular adhesion and polarization. ZO-1 surface assembly, but not adhesion and polarization, was also perturbed when single eight-cells were combined with single four-cells. The results suggest that tight junction development in mouse embryos is a secondary event in epithelial biogenesis, being dependent upon cell adhesion and cytoskeletal activity for normal expression, and can be disrupted without disturbing the generation of a stably polarized phenotype.

EPITHELIA form boundaries between distinct biological environments and regulate exchange between them. Their activity relies upon an underlying polarity in the organization of individual epithelial cells involving both cytocortical and cytoplasmic features. Thus, the apical and basolateral surfaces of these cells, facing mucosal and serosal environments, respectively, can be distinguished by their membrane protein and lipid composition (40), and the contents and architecture of their membrane skeletons (33, 34). The basolateral surface is also characterized by cell:cell and cell:matrix adhesion systems (46) and the presence of specialized cell junctions (see below). In addition, cytoplasmic components (cytoskeleton, organelles) are also polarized along the apicobasal cell axis (2, 39).

The tight junction (*zonula occludens*) is an important component of epithelial polarity and is situated at the distal extremity of the basolateral contact surface (for recent reviews see 16, 43), where it overlies other junctional types (*zonula adherens*, desmosomes, gap junctions). In thin section, it consists of a series of punctate membrane "kisses" where the intercellular space is occluded (8), while in freeze-fracture replicas, these sites correspond to a network of intramembraneous fibrils and complementary grooves that circum-

scribe the apicolateral cell border (e.g., reference 42). The tight junction appears to perform two fundamental roles in epithelial function. First, it restricts the paracellular passage of ions and small molecules between mucosal and serosal compartments, thereby contributing in large part to the transepithelial electrical resistance (5). Second, the tight junction appears to act as a barrier to the lateral diffusion of integral proteins and exoplasmic (outer leaflet) lipids between the two membrane domains of the epithelium, thereby helping to preserve the asymmetry in membrane composition that is essential for polarized function (e.g., references 48, 52).

The molecular organization of the tight junction is poorly understood. It remains controversial whether the fibrillate intramembraneous elements are predominantly lipidic in nature (37) or represent a linear aggregation of integral protein units (42), although recent evidence strongly favors the latter model (44, 47). However, two peripheral membrane proteins localized specifically at the cytoplasmic face of the tight junction in a variety of epithelia have been discovered recently (1, 4, 45). One of these, ZO-1, is an asymmetric, high molecular weight (215–225 kD), monomeric phosphoprotein (1, 45). The peripheral position of ZO-1 implies that it may contribute to the linkage between putative intramem-
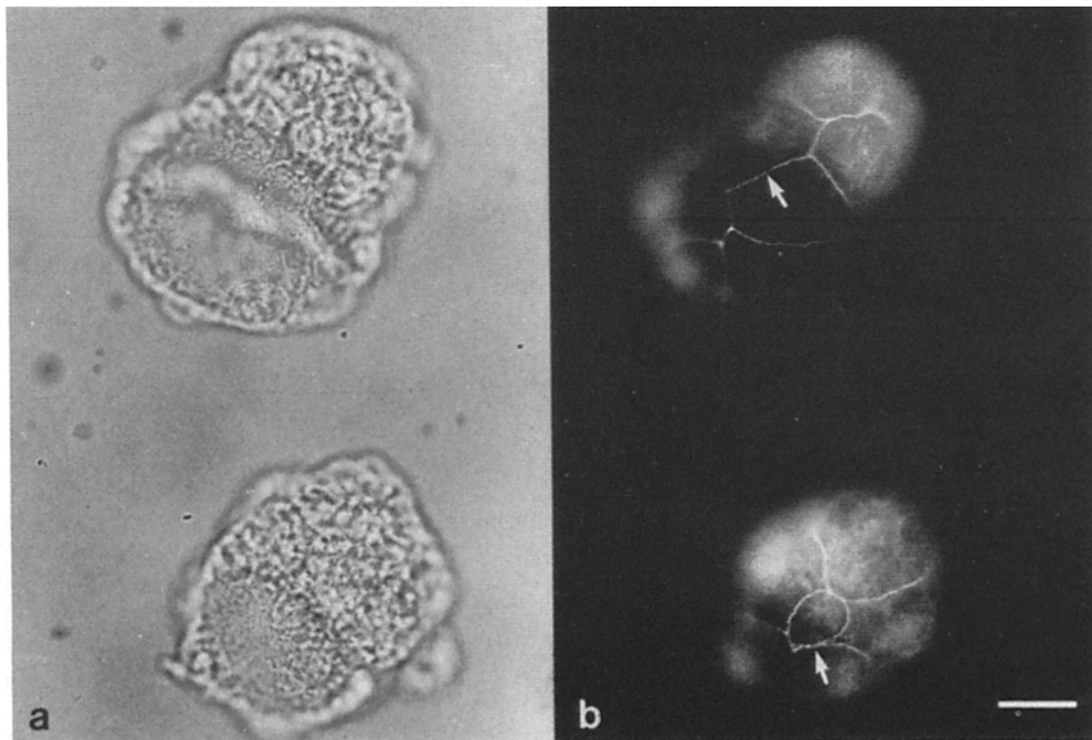
*Figure 1.* Immunofluorescent localization of ZO-1 in blastocysts. (*a*) Brightfield. (*b*) Immunofluorescence. The apicolateral cell borders in the trophectoderm are demarcated by continuous beltlike ZO-1 labeling (*arrows*). Bar, 20 $\mu$m.

braneous fibril particles and cytoskeletal elements that in detergent extracted specimens have been detected at the intracellular face of the tight junction (28).

The identification of tight junction subcomponents makes possible an examination of junction assembly processes at the molecular level. In the present paper, we report on the development of ZO-1 expression in cells of the mouse preimplantation embryo undergoing morphogenesis into blastocysts. The formation of the outer trophectoderm epithelium of the blastocyst, responsible for the vectorial accumulation of blastocoelic fluid, occurs progressively over three cell cycles by the acquisition of polarized epithelial features, initiated at compaction in the eight-cell embryo (reviewed in 11, 21). This model system therefore provides a unique opportunity to investigate the processes governing tight junction assembly de novo and to evaluate their relationship with the generation of other epithelial characteristics.

## Materials and Methods

### Embryo Collection, Culture, and Manipulations

MF1 female mice (3–4 wk old, Central Animal Services, Cambridge, UK) were superovulated by intraperitoneal injections (5 IU) of pregnant mares serum gonadotrophin and human chorionic gonadotrophin (hCG; Intervet, Cambridge, UK), 44–48 h apart. Mice were paired overnight with HC-CFLP males (Interfauna, Huntingdon, UK) and checked for copulation plugs the following morning. Embryos at different stages were obtained by flushing oviducts at different times post-hCG into Hepes-buffered medium 2 + 4 mg/ml bovine serum albumin (M2 + BSA; reference 14) and culturing them in medium 16 + 4 mg/ml BSA (M16 + BSA; reference 51) under oil at 37°C in 5% $CO_2$ in air in sterile culture dishes. Unfertilized eggs were obtained at 12 h post-hCG from superovulated but unmated mice. In most cases, embryos were flushed at 48 h post-hCG (late two-cell/early

four-cell stage) and cultured overnight for experiments on eight-cell stage embryos or cell couplets.

Removal of the *zona pellucida* was achieved by 15–30 s incubation in acid Tyrode's solution (36) followed by M2 + BSA washes. Embryos were disaggregated to single cells using a flame-polished micropipette following 15 min incubation in $Ca^{2+}$-free M2 + 6 mg/ml BSA. Cells were reaggregated in phytohemagglutinin (Gibco Laboratories, Grand Island, NY) at 1:20 in M2 + BSA. To obtain synchronous populations of natural cell couplets at the eight-cell stage (2/8 pairs), late four-cell embryos were disaggregated to 1/4 cells, cultured, and examined hourly for division to 2/8 pairs; these were harvested and cultured individually in 10-$\mu$l drops of M16 + BSA until required. All manipulations were carried out on a Wild dissecting microscope fitted with a 37°C heated stage.

### Drugs and Antibody Treatments

Embryos or 2/8 couplets were cultured in (*a*) $Ca^{2+}$-free M16 + 6 mg/ml BSA or in normal M16 + BSA containing monoclonal antibody ECCD-1 (ascites fluid diluted 1:50, reference 24) to neutralize the calcium-dependent uvomorulin (E-cadherin) cell adhesion system; (*b*) nocodazole (Aldrich Chemical Co., Ltd., Gillingham, UK) at 10 $\mu$M in M16 + BSA to depolymerize microtubules (20, 31); (*c*) cytochalasin D (CCD; Sigma Chemical Co., St. Louis, MO) at 0.5 $\mu$g/ml in M16 + BSA to destabilize microfilaments (13, 22); (*d*) aphidicolin (ICI Ltd., Macclesfield, Cheshire, UK) at 2 $\mu$g/ml in M16 + BSA to inhibit DNA polymerase-$\alpha$ activity (41); (*e*) $\alpha$-amanitin (Boehringer Mannheim GmbH, Mannheim, FRG) at 100 $\mu$g/ml in M16 + BSA to inhibit RNA polymerase II activity (3); (*f*) cycloheximide (Sigma Chemical Co.) at 400 $\mu$M in M16 + BSA to inhibit protein synthesis (27; TCA-precipitable incorporation of [$^{35}$S]methionine was reduced to 6% normal level at this concentration). Control specimens for experiments with nocodazole, CCD, and aphidicolin were cultured in the presence of the drugs' solvent, DMSO (Sigma Chemical Co.; 0.5–1 $\mu$l/ml). In all cases where appropriate, drug/antibody treatments were maintained during subsequent lectin labeling and washing (see below), up until the time of fixation.

### Immunocytochemistry

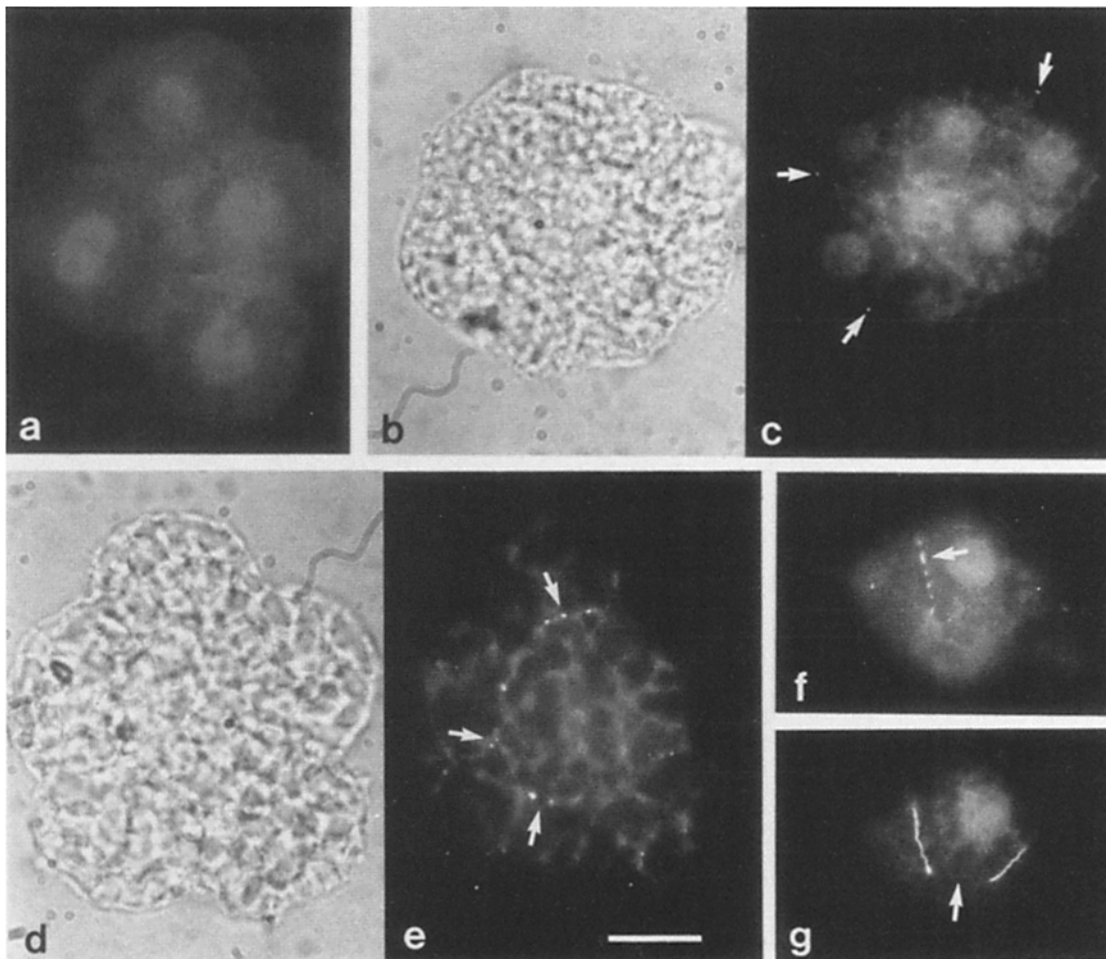Embryos or cell couplets were usually labeled with rhodamine-conjugated

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*Figure 2.* ZO-1 localization in cleavage stage embryos. (*a*) Four-cell embryo negative for ZO-1. (*b* and *c*) Brightfield and corresponding fluorescence midsectional views of compacted eight-cell embryo. ZO-1 is present as spots (*arrows*) localized at the apical extremity of the contact region between blastomeres. (*d* and *e*) Brightfield and fluorescence images of compacted eight-cell embryo viewed tangentially. A series of ZO-1 spots (*arrows*) demarcate the cell border of a blastomere in the center of the field. (*f*) Tangential fluorescence view of 16-cell embryo showing a series of ZO-1 discontinuous lines (*arrows*) at the border between two outside cells. (*g*) Tangential fluorescence view of 16-cell embryo showing outside blastomere with a continuous line of ZO-1 at two regions of the cell border whereas ZO-1 staining is absent from the intervening region (*arrow*). Bar, 10 μm.

Concanavalin A (Sigma Chemical Co., 120 μg/ml in M2 + BSA, 5 min followed by M2 + BSA wash) to detect surface microvillous polarity, before mild fixation in 0.5% formaldehyde in PBS for 20 min. Specimens were attached to poly-L-lysine-coated coverslips in chambers designed for immunocytochemistry, washed, or stored overnight in PBS, permeabilized in 0.25% Triton X-100 in PBS, and incubated in R26.4 monoclonal antibody to ZO-1 (undiluted culture supernatant, reference 45) for 45–60 min. They were then washed three times in PBS + 0.1% Tween 20 (PBS/Tween), incubated in a combined solution of fluorescein-conjugated goat anti–rat Ig antiserum (Miles Laboratories Inc., Naperville, IL, diluted 1:150) and Hoechst dye 33258 (50 μg/ml) in PBS + Tween for 45–60 min, and washed three times in PBS + Tween. The coverslips were then removed from the chamber and mounted on slides using Citifluor mounting medium (City University, London, UK), and viewed on a Leitz Ortholux II microscope fitted with appropriate filter systems. Photographs were taken on Tri-X film (Eastman Kodak Co., Rochester, NY).

### Western Blotting

Staged embryos were washed three times in M2 + 4 mg/ml polyvinyl pyrollidone (M2 + PVP), boiled in SDS sample buffer, and stored at -70°C until required. The soft upper pellet from liver homogenate in 1 mM NaHCO₃, spun for 10 min at 2,000 *g* (44, 45), served as a positive control. Proteins were separated on 7.5% SDS polyacrylamide gels as described previously (9), electrophoretically transferred (Trans-Blot Cell, Bio-Rad Laboratories, Richmond, CA) onto Hybond C-extra nitrocellulose (Amersham International, Amersham, UK) in 25 mM Tris, pH 7.0, 150 mM glycine, 0.1% SDS, 20% methanol at 0.15 A for 16 h. The nitrocellulose was treated with blocking buffer (BB; 1% dried milk powder, 0.02% Tween 20, 0.01% sodium azide in PBS) for 3 h before incubation in R40.76 ascites fluid diluted 1:100 in BB for 16 h at 4°C. R40.76 recognizes the same domain of ZO-1 as R26.4 and was found to be more sensitive for mouse embryo blots. The nitrocellulose was washed several times in BB, incubated in ¹²⁵I-labeled goat anti–rat Ig (Cappel Laboratories, Cochranville, PA; ≅3 × 10⁵ cpm/ml) in BB for 1 h at 4°C, and finally washed several times in BB for 1 h before drying. Immunoblots were exposed to preflashed Fuji x-ray film for ≅4 d at -70°C before developing and fixing.

## Results

### Time Course of ZO-1 Expression and Localization in Embryos

Immunofluorescent staining of ZO-1 in mid-expanded blastocysts (93–97 h post-hCG; 32–64-cell stage) showed antibody binding at the apicolateral margin between cells of the
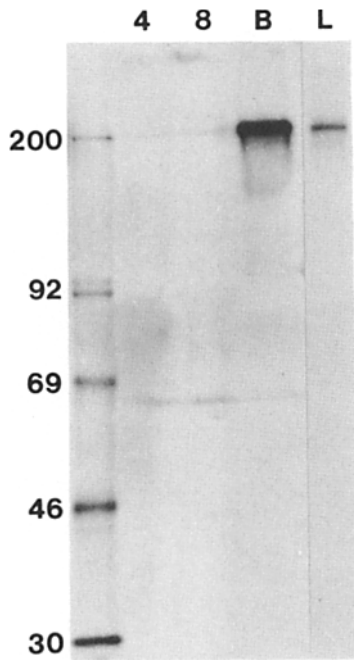
4  8  B  L

200 —

92 —

69 —

46 —

30 —

*Figure 3.* Immunoblot analysis of the relative amounts of ZO-1 detectable in four-cell embryos (*4*), compact eight-cell embryos (*eight*), and expanded blastocysts (*B*). 1,000 embryos were loaded per lane of a 7.5% SDS polyacrylamide gel and, after transfer to nitrocellulose, the sample was incubated with R40.76 monoclonal antibody to ZO-1, using iodinated anti–rat antibody as a detection system (see Materials and Methods). Mouse liver homogenate (*L*) was used as a positive control. Molecular mass standards are shown on the left in kilodaltons. ZO-1 is present as a single band at ≅225 kD in both embryos and liver. Note the increase in levels of ZO-1 with embryo maturation. The band evident at ≅60 kD may represent a breakdown product of ZO-1 at early stages.

outer trophectoderm layer (Fig. 1, *a* and *b*). ZO-1 forms an uninterrupted belt at the perimeter of each cell corresponding to the region where complete (i.e., zonular) tight junctions have been visualized in freeze-fracture and thin section studies (6, 29, 38). Immunolabeling of eggs and early cleavage stages, including early precompact eight-cell embryos, failed to detect any ZO-1 (Fig. 2 *a*). The antigen was first localized in compact or compacting eight-cell embryos as a linear series of spots at the border between free and apposed cell surfaces (Fig. 2, *b–e*). In some late eight-cell embryos and in the outer cell layer of 16-cell morulae, ZO-1 sites consisted of a series of discontinuous lines (Fig. 2, *f* and *g*). In late morulae, a continuous linear pattern was present, as in blastocysts. This transition from focal (eight-cell) to zonular (16–32-cell) ZO-1 staining at the cell periphery occurred over a 12-h period and is consistent with the gradual assembly of tight junctions seen in ultrastructural preparations (7, 29, 38).

Immunoblot analysis of ZO-1 expression in staged preimplantation embryos revealed a polypeptide band at ≅225 kD, of equivalent molecular mass to that derived from mouse liver (Fig. 3). ZO-1 was clearly detectable in blastocysts and was evident in an equivalent number of compact eight-cell embryos, but at markedly lower intensity. A trace of reactivity, lower than in compact embryos, was also found in four-

cell embryos (Fig. 3). ZO-1 total protein therefore increases with time, with synthesis initiating (perhaps for a minority of embryos, see later) slightly in advance of immunocytochemically detectable surface assembly.

The onset of ZO-1 assembly during the eight-cell stage at compaction suggests a close correlation between tight junction formation and other features of epithelial biogenesis that are initiated at that time, namely cell polarization and increased intercellular adhesion. To analyze the interrelationship between these events more precisely during the fourth cell cycle, natural 2/8 couplets (derived from the division in culture of isolated 1/4 blastomeres) were examined at different times postdivision for the presence of cell flattening (mediated by basolateral, uvomorulin-mediated cell adhesion; references 24, 49), apical microvillous polarity (assessed by Con A staining) and ZO-1 assembly. In preliminary experiments, the incidence and pattern of ZO-1 labeling in 2/8 pairs was shown to be unaffected by prior staining with lectin (data not shown). ZO-1 labeling in 2/8 pairs correlated with the staining patterns observed in intact embryos in that the antigen was nearly always localized to the periphery of the contact zone between cells, either as a series of punctate spots (Fig. 4, *c* and *d*), a series of discontinuous lines (Fig. 4, *e* and *f*), or rarely as a continuous zonular line (Fig. 4, *g* and *h*). In a minority of pairs (7% of ZO-1-positive couplets), ZO-1 was not contact localized, but occurred as randomly distributed spots at both free and contact regions of the cell surface (Fig. 4, *a* and *b*). The time course study showed that the expression of ZO-1 (*Z* in Fig. 5) tended to occur slightly later than both intercellular flattening (*F* in Fig. 5) and cell polarization (*P* in Fig. 5). The random ZO-1 distribution pattern may represent an aberrant situation, because 40% couplets stained in this way at 8- and 10-h postdivision had failed to flatten and polarize.

## Cell Contact and ZO-1 Assembly

To investigate whether intercellular adhesion is required for the expression and organization of ZO-1 assembly, 2/8 pairs were cultured for 10 h from division under conditions in which normal cell contacts were perturbed. In pairs cultured in normal medium for 10 h, but subsequently incubated in $Ca^{2+}$-free medium for 15 min to reverse flattening just before fixation, the incidence of cell polarity and contact-localized ZO-1 staining was comparable with controls (∼90% pairs; $n = 67$); however, ZO-1 at the contact zone was contracted in appearance, corresponding with the reduced area of contact in these pairs (Fig. 6, *a* and *b*). In addition, 50% pairs also possessed spotlike ZO-1 sites distributed apparently randomly at the cell surface (Fig. 6, *a* and *b*), indicating a partial breakdown of the normal contact-related pattern. In pairs cultured for 10 h in $Ca^{2+}$-free medium ($n = 60$), or in single eight-cell blastomeres cultured in normal medium ($n = 120$), the incidence of both cell polarity and positive ZO-1 staining was reduced to ∼50% of the control value. Moreover, cell polarity in the pairs was oriented apparently randomly with respect to the contact site (i.e., poles occurred in roughly equal proportions on the cell halves opposite to and adjacent to the contact site) and, in both groups, ZO-1 sites at the cell surface were randomly distributed spots (e.g., Fig. 6, *e* and *f*). These results confirm our earlier observations that the process of cell adhesion catalyzes the expres-
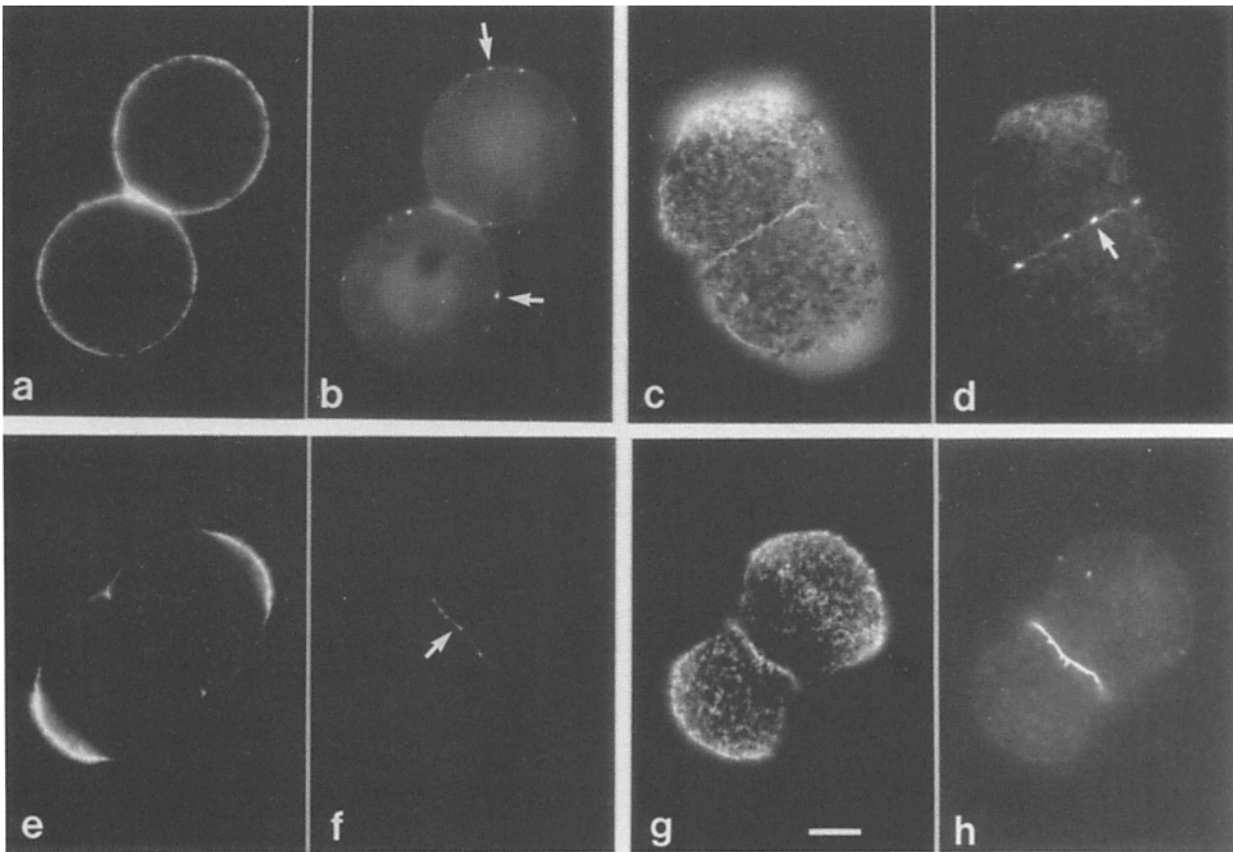
*Figure 4.* Corresponding Con A (*a*, *c*, *e*, *g*) and ZO-1 (*b*, *d*, *f*, *h*) fluorescence staining of 2/8 couplets at 8 h (*a–f*) or 10 h (*g*, *h*) postdivision from 1/4 blastomeres. (*a* and *b*) Couplet in which cells have failed to flatten against each other and showing nonpolar Con A staining (*a*); ZO-1 is present as randomly distributed spots at cell surfaces (*arrows*, *b*). (*c* and *d*) Couplet viewed tangentially in which cell flattening has occurred and ZO-1 is distributed as a series of spots at the periphery of the contact zone (*arrow*, *d*); the cells have a polarized Con A staining pattern (*c*) although this is only evident in the upper cell due to the plane of section. (*e* and *f*) Flattened couplet showing cell polarity after Con A staining viewed in midsectional plane (*e*) and ZO-1 labeling in the form of a series of discontinuous lines in a tangential view at the periphery of the contact zone (*arrow*, *f*). (*g* and *h*) Flattened couplet viewed tangentially showing cell polarity (*g*) and a continuous linear pattern of ZO-1 labeling (*h*). Bar, 10 μm.
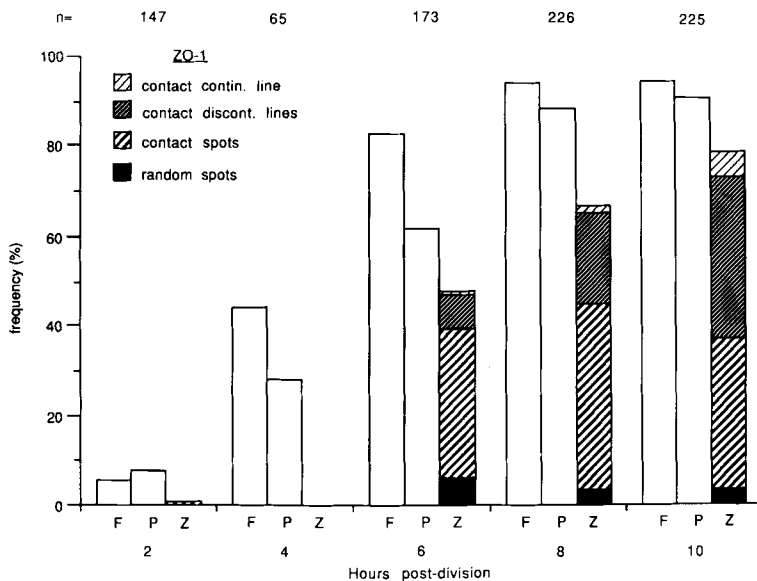


*Figure 5.* Time course of the generation of intercellular flattening (*F*), cell surface polarity (*P*) and ZO-1 assembly (*Z*) in 2/8 couplets synchronized from division of 1/4 blastomeres. The relative frequencies of different patterns of ZO-1 cell surface labeling in couplets are indicated at each time point, distinguishing randomly distributed spots from staining localized at the periphery of the contact zone, as a series of spots, discontinuous lines or a continuous belt. See Fig. 4 for examples. *n*, number of couplets examined at each time point.
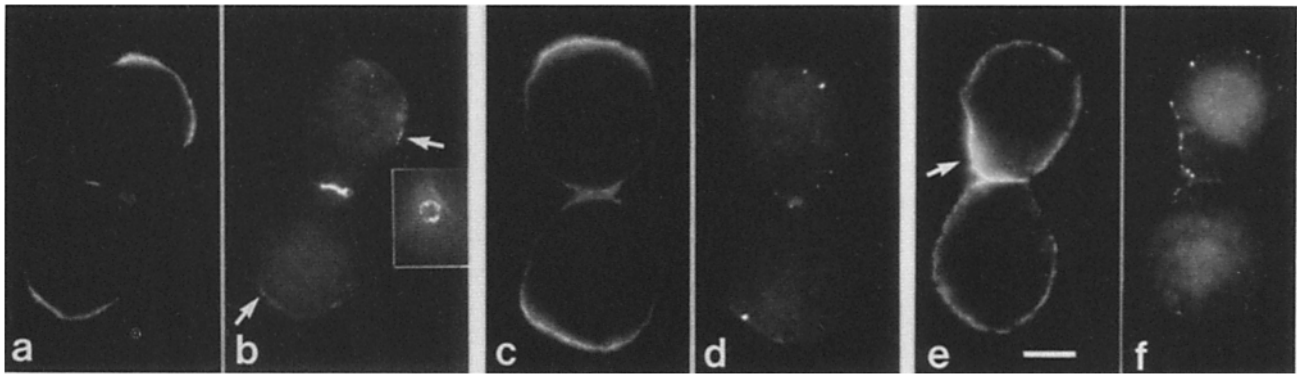
*Figure 6.* Con A (*a*, *c*, *e*) and ZO-1 (*b*, *d*, *f*) staining in 2/8 couplets (10 h postdivision) after inhibition of cell contact. (*a* and *b*) Couplet cultured in normal medium but then treated with $Ca^{2+}$-free medium for 15 min to reverse flattening; cells have polarized Con A staining opposite the contact point (*a*) and ZO-1 localized mainly at the contact point but also in spots distributed randomly at the cell surface (*arrows*, *b*). The inset shows the contracted nature of the contact-localized ZO-1 labeling when viewed in cross-section at the level of the contact zone. (*c* and *d*) Couplet cultured from division in medium containing ECCD-1 showing cell polarity opposite the point of contact (*c*) and ZO-1 in randomly distributed spots at the cell surface (*d*). (*e* and *f*) Couplet cultured in $Ca^{2+}$-free medium from division with upper cell showing Con A pole (positioned slightly out of the plane of focus) that is off-axis and close to the contact point (*arrow*, *e*) and ZO-1 distributed as spots at random surface sites (*f*). Bar, 10 μm.
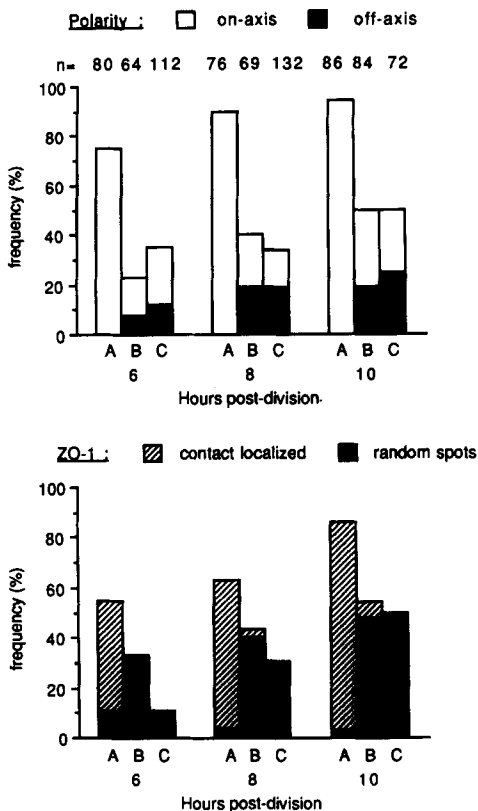


*Figure 7.* Time course of the generation of cell surface polarity (upper histogram) and ZO-1 assembly (lower histogram) in synchronized 2/8 couplets cultured in (*A*) control medium, (*B*) medium containing monoclonal antibody ECCD-1, (*C*) $Ca^{2+}$-free medium. The relative frequencies of surface polarity being on-axis (occurring opposite the contact point) or off-axis (occurring adjacent to the contact point), and of ZO-1 surface assembly being random spots or contact-localized (including both spot and linear patterns), are indicated. *n*, number of couplets examined.

sion of cell polarity and orients the axis of polarity perpendicular to the point of contact (24). They also indicate a comparable catalytic and localizing influence of the cell adhesion system on the assembly of ZO-1.

This interpretation was confirmed by time course experiments on the expression of polarity and ZO-1 assembly in synchronized 2/8 pairs cultured in $Ca^{2+}$-free medium or in medium containing the monoclonal antibody ECCD-1 (24) to neutralize specifically uvomorulin-mediated adhesion (Fig. 7). During 6–10 h postdivision, the incidence of cell polarity and ZO-1 staining increased but at levels well below those of control pairs. Moreover, cell polarity appeared randomly oriented and ZO-1 assembly was predominantly randomly distributed spots (for ECCD-1 effect, see Fig. 6, *c* and *d*), whereas in controls both features were organized normally (Fig. 7). The perturbations induced by inhibition of cell contact are reversible. Thus, pairs of 2/8 blastomeres maintained in $Ca^{2+}$-free medium or medium containing ECCD-1 for 8 h postdivision showed, on restoration of intercellular flattening by their return to normal medium, a gradual reorganization of polarity and ZO-1 assembly over a 2-h period to normal orientation and localization patterns, respectively, and at levels comparable with controls (Fig. 8).

The reestablishment of a typical ZO-1 staining pattern is slower for pairs previously cultured in $Ca^{2+}$-free medium than for those cultured in medium containing ECCD-1. This distinction is borne out in long-term (24–30 h) incubations of eight-cell embryos; those cultured in the presence of ECCD-1 eventually compact, cavitate and express a zonular distribution of ZO-1 in trophectoderm cells, whereas those cultured in $Ca^{2+}$-free medium remain as a noncompact cell cluster expressing ZO-1 at both random and contact-localized sites on cell surfaces (data not shown).

## The Cytoskeleton and ZO-1 Assembly

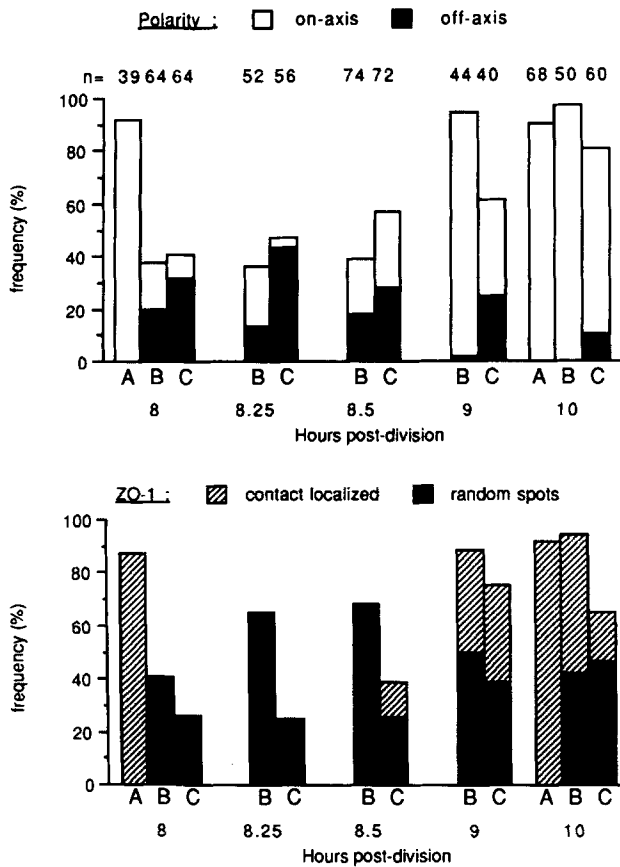To assess the influence of the cytoskeleton on ZO-1 assembly,

Figure 8. Time course of frequency of cell polarity (upper histogram) and ZO-1 assembly (lower histogram) in synchronized 2/8 couplets returned to control medium for up to 2 h after culture from 0-8 h postdivision in (A) control medium, (B) medium containing ECCD-1, (C) Ca²⁺-free medium. n, number of couplets examined.

synchronized 2/8 pairs were cultured from division in medium containing either nocodazole at a concentration (10 μM) shown previously to depolymerize microtubules (20, 31), or cytochalasin D (CCD; 0.5 μg/ml) to disrupt microfilaments (22). Nocodazole treatment did not lead to a detectable change in the time course and distribution pattern of ZO-1 surface assembly when compared with controls (data not shown).

Similarly, intercellular flattening and cell polarity were not inhibited by nocodazole, as shown previously (10, 23, 31). Treatment of 2/8 couplets for up to 10 h from division with CCD prevented intercellular flattening and delayed the expression of cell polarity, which was oriented randomly with respect to the contact point, as shown previously (10, 23; Table I). CCD treatment also led to a slower rate of assembly of ZO-1 compared with controls; in most labeled cells, the protein was present as spots either distributed randomly at the cell surface or localized preferentially to the position of the surface pole (Table I, Fig. 9, c-f). In a minority of cells, ZO-1 was localized as spots in the perinuclear cytoplasm, either exclusively or in addition to surface sites (Table I, Fig. 9, a and b).

## Temporal Control of ZO-1 Assembly

The means by which ZO-1 synthesis and assembly is regulated temporally was investigated using drugs to perturb cell cycling and protein processing activities. Inhibition of DNA synthesis during the third and fourth cell cycles by prolonged incubation of newly formed three- or four-cell embryos in aphidicolin (2 μg/ml) did not influence the pattern or timing of intercellular flattening, cell polarization, or ZO-1 assembly when compared with controls, although treated embryos remain as four-cells due to a requirement for DNA synthesis to accomplish division. Thus, after 16, 20, and 24 h incubation, ZO-1 was present at contact sites in approximately 17, 40, and 78%, respectively, of control (eight-cell) and aphidicolin-treated (four-cell) embryos (n = 38-46 embryos per treatment). These results for flattening and polarity confirm those reported earlier (41); they also show that the time of ZO-1 assembly is similarly not regulated by the number of rounds of cytokinesis or DNA replication preceding expression.

Treatment of 2/8 pairs with α-amanitin (100 μg/ml) for 10 h from division to block transcription did not affect the development of cell flattening, cell polarity, or ZO-1 assembly, all of these features being present in normal patterns in over 80% couplets (n = 115). Transcription of mRNA necessary for normal ZO-1 assembly and cell polarity is therefore accomplished before the eight-cell stage, as has been shown previously to be the case for intercellular flattening (26). When α-amanitin treatment of intact embryos was extended from the early four-cell stage to the late eight-cell

Table 1. Influence of Cytochalasin D (0.5 μg/ml) on Compaction and ZO-1 Assembly in 2/8 Couplets after Drug Treatment from Division of 1/4 Blastomeres

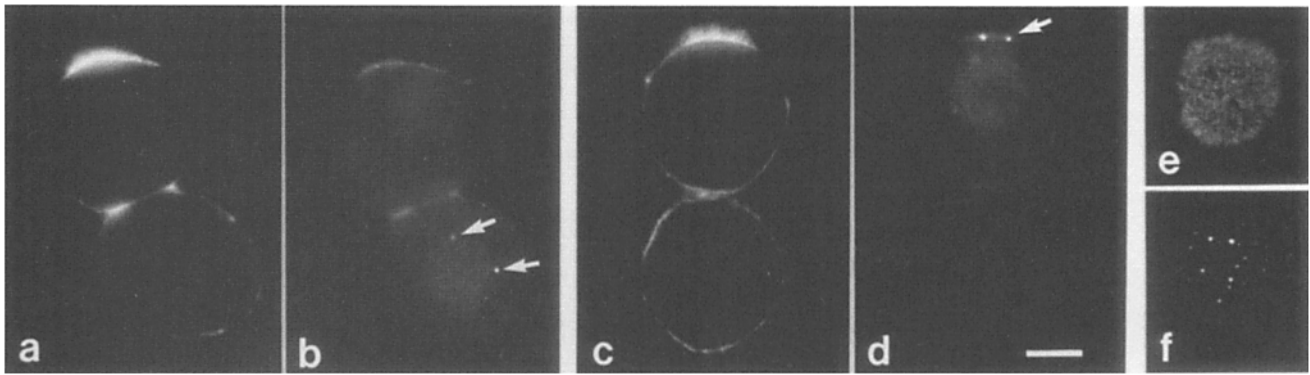| | | | Percent of couplets showing | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Polarity | | | ZO-1 | | | |
| Treatment | Culture time | Sample No. | Flattening | Total | on-axis | off-axis | Total | Contact localized | Random surface sites | Pole localized | Cytoplasmic |
| | h | | | | | | | | | | |
| Control | 6 | 51 | 86 | 74.5 | 74.5 | 0 | 51 | 51 | 0 | 0 | 0 |
| | 8 | 64 | 100 | 97 | 94 | 3 | 81 | 78 | 3 | 0 | 0 |
| | 10 | 46 | 100 | 100 | 100 | 0 | 83 | 83 | 0 | 0 | 0 |
| Cytochalasin D | 6 | 48 | 0 | 25 | 8 | 17 | 29 | 0 | 12.5 | 15 | 2 |
| | 8 | 45 | 0 | 60 | 13 | 47 | 49 | 0 | 18 | 20 | 15.5 |
| | 10 | 69 | 0 | 74 | 35 | 39 | 74 | 0 | 30 | 43.5 | 13 |

*Figure 9.* Con A (*a, c, e*) and ZO-1 (*b, d, f*) staining in 2/8 couplets treated with cytochalasin D for 8 h postdivision. (*a* and *b*) Couplet showing upper cell polarized opposite the contact point (*a*) and lower cell with perinuclear cytoplasmic spot-like sites of ZO-1 labeling (*arrows, b*). (*c* and *d*) Couplet with upper cell polarized opposite the contact point (*c*) and ZO-1 spots restricted to this region of the cell surface (*arrow; d*). (*e* and *f*) Polarized membrane region from another couplet viewed en face (*e*) showing the distribution of ZO-1 spots in this region (*f*). Bar, 10 μm.

stage, weak ZO-1 reactivity was present in only 10% of embryos (*n* = 30), although most (70%) were partially or fully compact and contained polarized cells. This result could suggest that transcriptional events in the four-cell stage are involved in ZO-1 expression.

Inhibition of protein synthesis in 2/8 pairs for up to 10 h from division using cycloheximide (400 μM) did not prevent intercellular flattening or cell polarization, as shown previously (27). However, this treatment resulted in a substantial reduction in the incidence of ZO-1 staining (Fig. 10), indicating a contemporary translational requirement for the expression and assembly of the protein. In experiments where cycloheximide was included for varying periods at the start or end of the total 10 h culture time, the incidence of ZO-1 assembly was consistently lower than controls and reflected the time and duration of drug treatment (Table II). These results suggest that the necessary translation is occurring throughout the cell cycle from division onwards. Extending cycloheximide treatment to include the terminal 2-h period of the third cell cycle as well as 9 h after entry into the fourth cycle, further reduced the incidence of ZO-1 staining to below 5% and led to the complete absence of ZO-1 reactivity in all couplets (*n* = 65) when the predivision drug treatment was extended to 3 h duration. This suggests that translational activity in the final period of the four-cell stage may also contribute to ZO-1 assembly in some couplets.

### ZO-1 Assembly in Asynchronous Cell Aggregates

A previous study has shown that 1/8 blastomeres can flatten against and polarize opposite to the contact point with 1/4 blastomeres (25). Because surface assembly of ZO-1 is not detectable in four-cell blastomeres (see above), asynchronous aggregations of newly formed 1/4 + 1/8 couplets were constructed and cultured for 8 h to assay the influence of the stage of the companion cell on ZO-1 assembly. In such constructs, the incidence of flattening and polarity of the 1/8 cells was equivalent to that of synchronous (1/8 + 1/8) control pairs. However, the incidence of ZO-1 assembly in 1/8s of asynchronous pairs was reduced to ~50% of the control value and in most cases ZO-1 staining appeared as randomly distributed spots at the cell surface rather than showing the

normal contact-related pattern (Figs. 11 and 12, *a* and *b*). ZO-1 staining was not observed in 1/4 cells.

These results suggest that contact-localized ZO-1 assembly depends upon a mutual state of competence to form junctions being present in companion cells, perhaps relating to the ability to assemble ZO-1 itself. However, because the level of ZO-1 expression was clearly reduced in a 1/8 cell paired with a 1/4 cell, it is possible that the failure to localize ZO-1 to the contact site was a secondary phenomenon. To test this possibility, cell triplets were constructed comprising two newly formed 1/8 blastomeres and one newly formed 1/4 blastomere in an attempt to promote ZO-1 expression in the 1/8 cells (induced by contact between them, see earlier) above a putative threshold that might exist before assembly could occur at the margins of contact with the 1/4 cell. In these constructs, flattening and polarity occurred in the eight-cells, with the polar axis respecting the contact point with both the four-cell and the companion eight-cell as described previously (25; Fig. 12 *c*). ZO-1 assembly occurred normally at the contact periphery between the eight-cells but no assembly was observed at contact points with the four-cell (Figs. 11 and 12 *d*). This result suggests that the failure to assemble ZO-1 at the boundary with the four-cell is not due to an inadequate level of ZO-1 synthesis in eight-cells but rather may reflect some deficiency, such as the absence of ZO-1 integral membrane binding sites, in the four-cell.

### Discussion

We have examined various aspects of ZO-1 expression and assembly de novo in mouse blastomeres during the maturation of the trophectodermal epithelium of the blastocyst. We have shown that the protein becomes associated with the apicolateral site of tight junction formation between cells from the time of compaction onwards. The ZO-1 staining pattern matures from a linear series of spots to a zonular distribution during the early morula period, corresponding to the time that tight junction freeze-fracture organization alters from a focal to a zonular state (6, 7, 29, 38).

ZO-1 assembly at compaction occurs when other fundamental changes in cell organization underlying the epithelial-
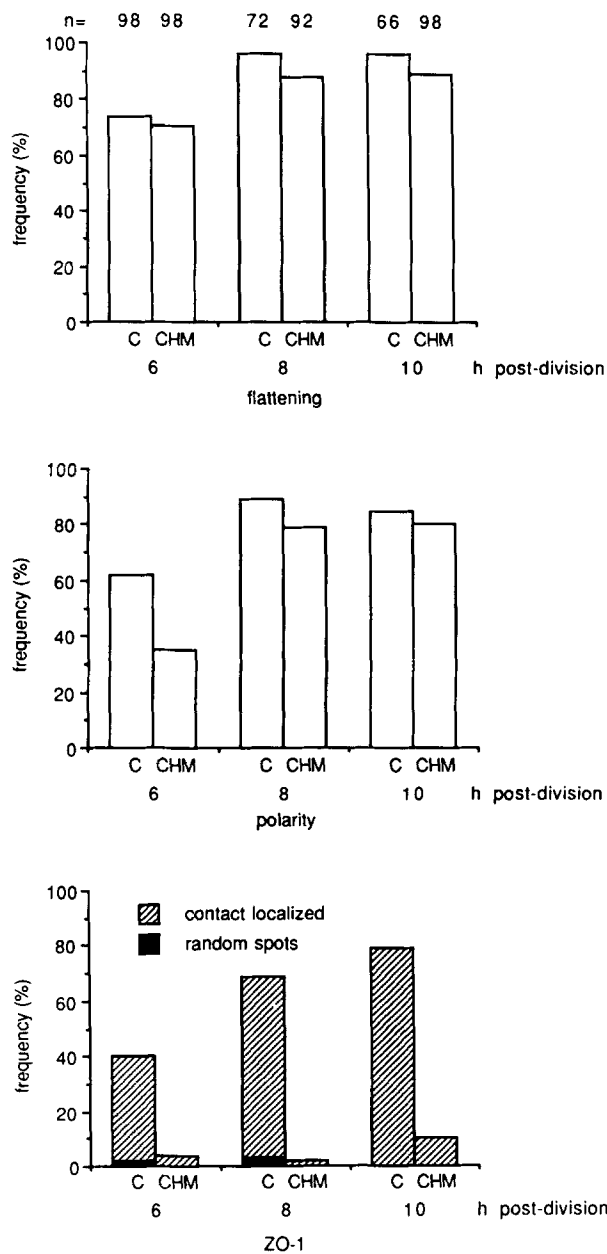
Figure 10. Time course of the generation of intercellular flattening (upper histogram), cell polarity (middle histogram), and ZO-1 assembly (lower histogram) in synchronized 2/8 couplets cultured from division in control medium (*C*), or in medium containing cycloheximide (*CHM*) to inhibit protein synthesis. *n*, number of couplets examined.

ization process also commence. These events include the polarization of cytoplasmic constituents (microtubules, microfilaments, and specific membraneous organelles) apically (12, 20, 22, 30), and the regionalization of the cytocortex (surface membrane and underlying skeleton) into basolateral adhesive and apical microvillous domains (11, 24, 49). The present study supports the view that during compaction the cytocortex actually differentiates into three regions, with the emergence of a ZO-1-enriched junctional band also characterized by an integral membrane substructure typical of tight junctions (7, 38) and underlying sites where certain actin-

Table II. Influence of Cycloheximide (400 μM) on ZO-1 Assembly in 2/8 Couplets after Treatment for Varying Periods up to 9 h Postdivision

| Treatment | | Sample No. | Pairs positive for ZO-1 |
|---|---|---|---|
| *h* | *h* | | % |
| Cycloheximide →control medium | | | |
| 0 | 9 | 50 | 82 |
| 2 | 7 | 38 | 79 |
| 4 | 5 | 53 | 41 |
| 6 | 3 | 60 | 28 |
| 9 | 0 | 54 | 13 |
| Control Medium →cycloheximide | | | |
| 2 | 7 | 55 | 16 |
| 4 | 5 | 54 | 70 |
| 6 | 3 | 44 | 66 |

binding proteins are reported to associate preferentially (see 11). Although in intact embryos the junctional zone might appear to be localized at the interface between apical and basolateral domains, examination of the distribution of ZO-1 in 2/8 couplets suggests that it is in fact a subdomain of the basolateral cytocortex. In these constructs, the region of cell contact is reduced compared with that in whole embryos so that part of the basolateral surface, surrounding the apical microvillous pole, lies on the exposed face of the couplet (e.g., Fig. 4 *e*). ZO-1 assembly does not therefore localize at the apical-basolateral boundary but rather at the border of a contact-dependent zone on the basolateral surface.

Taken together, our data from immunoblotting and from fluorescence staining after protein synthesis inhibition by cycloheximide suggest that ZO-1 assembly at compaction is preceded by synthesis of the protein that in some blastomeres begins late in the four-cell stage but in most blastomeres is initiated at the eight-cell stage. Thus, treatment of 2/8 pairs throughout the cell cycle with cycloheximide reduced substantially the proportion of couplets with assembled ZO-1 (Fig. 10). When the drug was administered only for the initial period of the cell cycle before return of couplets to normal medium, the proportion showing assembled ZO-1 decreased with increasing duration of cycloheximide treatment (Table II), suggesting that the onset of ZO-1 synthesis within the synchronized cell population is staggered. The fact that ZO-1 assembly in couplets is not blocked in the entire population unless cycloheximide treatment is extended to include the terminal period of the four-cell stage, further supports the view that synthesis is not linked rigorously with the cell cycle. The ZO-1 trace detectable in the four-cell immunoblot (Fig. 3) may therefore represent a minor group of embryos in which synthesis of the protein is precocious. A clearer understanding of the relationship between ZO-1 synthesis and assembly will require more specific protocols than those employed here. Analysis of synthesis rates at different stages by immunoprecipitation and more extensive immunoblotting is planned and will be reported separately.

Our earlier studies have indicated that the apical cytocortical pole, characterized by microvilli and generated at compaction, is stable upon cell isolation, and that this stability is instructive for the concomitant reorganization of the cyto-
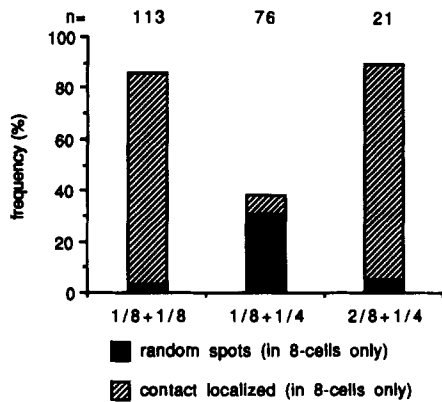
*Figure 11.* Frequency of ZO-1 assembly in constructs of asynchronous stages cultured for 8 h postdivision. *n*, number of constructs examined.

plasm and necessary for preserving cellular asymmetries into later cell cycles (reviewed in 11, 21). Therefore, to assess the relevance of junctional maturation for the overall construction of the trophectoderm epithelium requires ZO-1 assembly characteristics to be compared directly with the processes governing cytocortical differentiation. We have
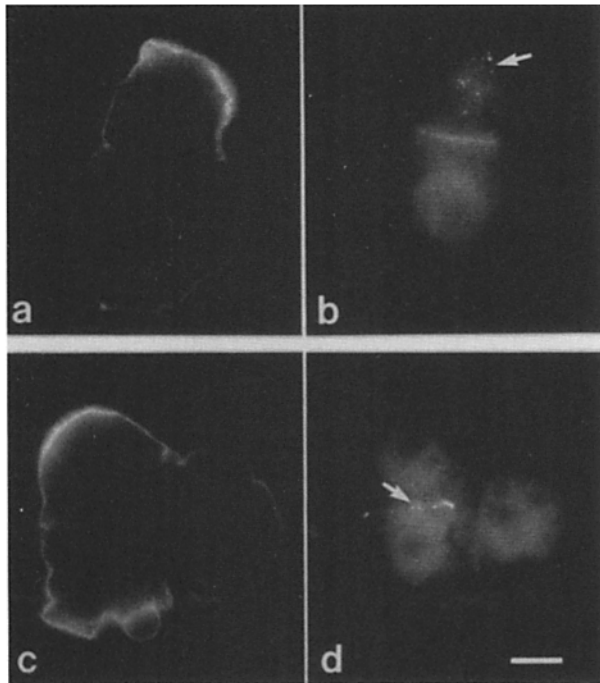


*Figure 12.* Con A (*a, c*) and ZO-1 (*b, d*) staining of asynchronous cell aggregates cultured for 8 h before analysis. (*a* and *b*) Couplet of 1/8 (*upper*) and 1/4 (*lower*) cells. The 1/8 cell has flattened on the 1/4 cell and has polarized opposite the contact point (*a*); spot-like ZO-1 sites are only evident in the 1/8 cell and, although appearing to be cytoplasmic due to the tangential plane of focus, are in fact distributed randomly at the cell surface (*arrow, b*). (*c* and *d*) Triplet of two 1/8 cells (*upper and lower left*) and 1/4 cell (*right*). The cells have flattened upon each other and the 1/8 cells have polarized opposite the collective contact zone (*c*); ZO-1 is evident only at the contact zone between the 1/8 cells (*arrow, d*). Bar, 10 μm.

used Con A as a marker for cytocortical differentiation so that double labeling with ZO-1 can be achieved. Although lectin receptors appear uniformly distributed on the cell surface, once cell polarization of microvilli occurs, Con A preferentially, but not exclusively, labels the pole due to the excess of membrane at this domain (19). A marker exclusive for the cytocortical pole is unfortunately not available at present. However, our results show that cell polarization and ZO-1 appearance are both initiated at approximately the same time in development. Does this temporal coincidence imply a common underlying control mechanism? Whereas some of the experimental manipulations that we have applied to the early embryo do reveal similarities between polarization and ZO-1 expression (discussed next), it seems likely from our overall analysis that each event is in fact regulated differently (discussed later).

Depriving cells of uvomorulin-mediated contact did not prevent the expression of either cell polarity or ZO-1 but did delay the time of their appearance and randomized both the axis of polarization and the localization of ZO-1 (see also reference 24). Although inhibiting contact between mature cultured epithelial cells by calcium removal both prevents and reverses tight junction sealing (reviewed in 16, 43), the effect has been thought to be indirect, resulting from the failure to assemble (or maintain) the *zonular adherens* junction that provides a structured linkage between cells (17, 44). Recently, uvomorulin neutralization in Madin–Darby canine kidney (MDCK) cells was shown to inhibit the assembly of ZO-1 at putative tight junction sites during the initial period of epithelium formation after calcium switching (18). However, in these experiments, desmosome and *zonula adherens* junctions were similarly perturbed, suggesting that uvomorulin adhesion may be involved directly in the formation of all junction types (18). Whether the randomizing of ZO-1 assembly in blastomeres in the absence of cell adhesion results from direct or indirect means awaits details of when *zonula adherens* maturation is initiated. The failure to establish contact-localized ZO-1 in couplets denied basolateral adhesion from their formation (Fig. 6, *d* and *f*) suggests that adhesion is necessary to instigate the focusing of the putative cell surface binding site for the ZO-1 protein to the contact periphery. However, once tight junction assembly has commenced, neutralization of uvomorulin is much less effective at randomizing the ZO-1 from the margins of the contact zone (Fig. 6 *b*), suggesting that the ordered distribution of the ZO-1 binding site is maintained, at least temporarily, when adhesion is abolished. This distinction implies that focusing of ZO-1 binding sites, and their subsequent stabilization, may be mediated by their intercellular interaction. Whether stabilization of binding sites leads secondarily to the preservation of bound ZO-1 (cf. fodrin turnover in contact-modulated MDCK cells, 35), thereby explaining the catalytic effect of adhesion on the time of appearance of ZO-1, awaits biochemical analysis.

Both ZO-1 assembly and the development of surface polarity are generated and oriented correctly after microtubule depolymerization (see also references 20, 31), whereas disruption of microfilaments with CCD modified the expression of both features in ways that are mostly (but not exclusively) explicable as a secondary result of the drug's interference with the cell adhesion system. The cytoplasmic localization of ZO-1 in some cells cultured in CCD suggests a role for mi-

crofilaments either in the transport of the protein to the cell surface or in its anchorage to the membrane. The finding, in many CCD-treated cells, of surface ZO-1 localized preferentially at the microvillous pole is curious and further demonstrates the distinct nature of the cytocortex at this region which has been shown previously to contain CCD-resistant microfilaments (e.g., reference 13). The relative stability of this domain may therefore be responsible for the trapping of ZO-1 binding sites unable to associate at the contact zone in the absence of cell flattening. These effects of cytoskeletal disruption on ZO-1 assembly are consistent with published data on mature epithelia showing tight junction integrity to be relatively unaffected by microtubule depolymerization (32) but susceptible to microfilament disorganization which appears to interfere with the correct positioning of intramembraneous junctional elements at the cell surface (32). Thus, ZO-1 junctional localization has been shown to be altered in confluent MDCK monolayers after CCD treatment (43), indicating also that maintenance as well as assembly of tight junctions is microfilament-dependent.

Although cell contact and the cytoskeleton influence the expression of cytocortical polarity and ZO-1 assembly in similar ways, our data on the molecular level at which production of ZO-1 is regulated indicate considerable differences. The use of aphidicolin suggests that the timing of ZO-1 expression is independent of DNA replication in the third and fourth cell cycles, as is also the case for cell polarization (41, this study). However, ZO-1 expression is reduced substantially when transcription is inhibited (by α-amanitin) from the third cell cycle and when translation is inhibited (by cycloheximide) from early in the fourth cell cycle. In contrast, neither of these biosynthetic inhibitors prevent the generation of apical microvillous polarity or basolateral cell adhesion (26, 27, this study). The formation of an asymmetric cytocortex, essential for subsequent epithelial differentiation (11), can therefore take place in the absence of the complete assembly of all tight junction components; fully assembled tight junctions cannot therefore provide an obligatory part of the positional signal that orients polarity appropriately. It will be important to establish, using freeze-fracture techniques, whether tight junction substructure is also absent in blastomeres under these conditions.

Further evidence of the independence of cytocortical polarization from ZO-1 localization comes from the experiments in which eight-cell blastomeres were aggregated with four-cell blastomeres. Despite successful development of intercellular adhesion and surface polarity, ZO-1 was not able to assemble in a contact-localized pattern. The implication from these experiments is that either ZO-1 or a putative ZO-1 binding protein participating in junction elaboration is absent or deficient in the four-cell, limiting contact-localized assembly in the neighboring eight-cell. This argues that the tight junction assembly process is a cascade reaction (cf. zonula adherens assembly; 15) and that the formation of intercellular linkages, involving as yet undefined integral membrane components (fibril particles?), is permissive and necessary for the assembly of downstream molecules, including ZO-1. Because flattening occurs between asynchronous blastomeres, activation of uvomorulin-mediated adhesion alone would appear to be insufficient for initiating assembly, but can promote and focus assembly (see earlier) once the full complement of junctional molecules are being synthesized.

In conclusion, our results show that ZO-1 expression and surface assembly in mouse embryos is a temporally regulated component of the epithelialization program leading to trophectoderm formation. The localized distribution of the protein at contact sites is influenced by the cell adhesion system, the microfilament cytoskeleton, and the state of differentiation of neighboring cells. Although certain features of the control of ZO-1 assembly imply a close relationship with the establishment of distinct cytocortical domains fundamental to trophectoderm generation, the ability to inhibit ZO-1 specifically under certain conditions suggests that tight junction formation represents an elaborative rather than a causal event during epithelial biogenesis.

Such a conclusion is consistent with the role of tight junction integrity during the terminal phase of trophectoderm maturation, contributing to the maintenance of membrane polarity of basolateral ATPases (50) involved in vectorial fluid transport at cavitation, as well as the paracellular barrier to leakage.

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