Localization of uvomorulin, fodrin and actin in mouse embryos during compaction, decompaction-recompaction and blastulation

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Compaction, a critical event in the early mouse development, constitutes the beginning of trophoblast differentiation. Since compaction is attributed to the increasing in cell adhesion due to the cadherin uvomorulin and to the organization of cytoskeleton, and since uvomorulin is linked to the cytoskeleton, we examined by confocal microscopy the localization of uvomorulin, fodrin and actin in morulae during normal compaction, during various decompacting-recompacting assays and at the expanded blastocyst state. Decompacting treatments used are cytochalasin D and latrunculin (microfilament inhibitors), EGTA (calcium chelator), verapamil (calcium channel blocker), TFP (calmodulin inhibitor) and TMB-8 (calcium release inhibitor). The results indicate that uvomorulin deregionalizes when embryos are decompacted and that regionalization is recovered when recompaction is allowed; the same holds for fodrin, though to a lesser extent, while actin remains unchanged. Regionalization of uvomorulin by a mechanism involving the cytoskeleton and probably regulated by calcium-calmodulin is discussed.

Key words: *Compaction, cytoskeleton, mouse embryo, uvomorulin.*

INTRODUCTION

During preimplantation development of the mouse, trophoblastic cells differentiate eventually forming an epithelium that gives rise to the placenta, while cells that will give rise to the embryo (inner cell mass cells) remain totipotential (Rossant and Lis, 1979; Fernández and Izquierdo, 1983). Thus, this period of free embryonic life could be analyzed as the establishment of the first epithelium in mammals.

The homogeneity of the fertilized mouse oocyte is lost at first cleavage by the establishment of cell contact at the 2-cell stage, because areas of cell contact and away from contact differ (Izquierdo and Ebensperger, 1982; Izquierdo *et al*, 1980; Schatten *et al*, 1986a; Sepúlveda and Izquierdo, 1990; Sobel, 1983a,b; Sobel and Alliegro, 1985). Later, during compaction of the 8-cell embryo, morphogenetic changes increase this regional heterogeneity.

Compaction can be recognized by the flattening of the cells against each other, thus reducing the intercellular space, and changing the embryonic contour from lobulated to spherical (Ducibella and Anderson, 1975; Mulnard, 1967). Consequences of this morphogenetic process are the disappearance of microvilli on areas of cell contact while persisting at areas of no contact (Sepúlveda *et al*, 1985), the establishment of cytoskeletal connections (Mayor *et al*, 1989), of gap junctions (Goodall and Johnson, 1984; Lee *et al*, 1987; Lo and Gilula, 1979) and

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later of tight junctions between blastomeres (Ducibella and Anderson, 1975, 1979; Fleming and Hay, 1991; Fleming *et al*, 1989), and the subsequent cell polarization (Dizio and Tasca, 1987; Manejwala and Schultz, 1989; Watson and Kidder, 1988; Wiley, 1984; Wiley *et al*, 1990; reviewed by Izquierdo, 1991). This critical event in trophoblast differentiation is mediated by cell adhesion (Ducibella and Anderson, 1979), by the cytoskeleton (Izquierdo *et al*, 1984; Pratt *et al*, 1981, 1982; Sutherland and Calarco-Gillam, 1983) and also by intracellular calcium (Bilozur and Powers, 1982; Pey, Schatten and Izquierdo, unpublished results).

A calcium-dependent cell adhesion molecule, uvomorulin (also called E-cadherin, L-CAM and CAM 120/80), has been reported to play an important role during compaction because this process can be prevented or reversed using antibodies raised against uvomorulin or in a medium devoid of calcium (Ducibella, 1980; Hyafil *et al*, 1981; Peyrieras *et al*, 1983). Uvomorulin appears at the 2-cell stage, but at the 8- cell stage embryo it is localized at the cell contact areas (Vestweber *et al*, 1987) and it is in a phosphorylated state (Sefton *et al*, 1992). Uvomorulin is a transmembrane glycoprotein and its adhesion function is due to the extracellular domain which contains the putative calcium binding sites (Vestweber and Kemler, 1985; Ringwald *et al*, 1987). The intracellular domain associates with catenins that link uvomorulin to cytoskeletal components (Ozawa *et al*, 1989). At least one of the catenins is a vinculin-like protein, which suggests that uvomorulin could be associated to actin fibers through catenin-vinculin association (Herrenknecht *et al*, 1991).

In MDCK cells, uvomorulin induces the polarization of Na/K ATPase and fodrin (McNeil *et al*, 1990) and in epithelia, colocalization of cadherins and cortical actin of intermediate junctions (Boller *et al*, 1985) and of actin bundles (Hirano *et al*, 1987) have been observed. In mouse embryos the presence and distribution of cytoskeletal components like fodrin, myosin, actin and microtubules have been reported (Houliston *et al*, 1987; Johnson and Maro, 1984; Lehtonen and Bradley, 1980; Schatten *et al*, 1986a; Sobel, 1983a,b; Sobel and Alliegro, 1985). Since regionalization of uvomorulin is important for compaction and could be directed by the cytoskeleton, we have examined by confocal microscopy the localization of uvomorulin, fodrin and actin in morulae during normal compaction, during various decompacting-recompacting assays and in the expanded blastocyst. Here we show that uvomorulin deregionalizes when embryos are decompacted and that regionalization is recovered when recompaction is allowed; the same holds for fodrin, though to a lesser extent, while actin remains unchanged. We also show the different locations of these molecules in the inner cell mass and trophoblastic cells in the expanded blastocyst.

MATERIALS AND METHODS

Collection and culture of embryos

Mouse embryos were obtained from CF1 or ICR males mated with females of the same strain that had been induced to superovulate by intraperitoneal injections with 5 IU pregnant mares serum (Sigma) and 44 h later with 4 IU human chorionic gonadotropin (hCG; Sigma). Eight-cell embryos were flushed, at 68 h post-hCG, from the oviduct with M 2 medium (Hogan *et al*, 1986) supplemented with 4 mg/ml bovine serum albumin (BSA; Sigma). They were cultured for different periods in M 16 supplemented medium (Hogan *et al*, 1986) under mineral oil in an atmosphere of 5% CO_2 in humid air at 37° C until the required stage of development was reached (until 72 h post-hCG for non-compacted morula, 76 h post-hCG for compacted morula and 98 h post-hCG for expanded blastocyst).

Decompacting-recompacting treatments

Batches of morulae, monitored by light microscopy, showed asynchrony at the onset of compaction. In order to synchronize development, only embryos that compacted within one hour were used for each decompacting assay. Synchronized compacted morulae were exposed to treatments and monitored under the light microscope every 10 min to determine the minimum time to

reach decompaction. After washing, normal fresh medium was added and recompaction monitored. EGTA treatment medium contained 0.5 mM EGTA (Sigma) in M 16 medium in which sodium replaced calcium. Verapamil treatment medium contained 0.3 mM verapamil (Sigma) in M 16 medium in which calcium concentration had been reduced to 0.171 mM; reduction of calcium concentration to 0.171 mM does not itself affect compaction. CCD, latrunculin, TFP and TMB-8 treatment media contained 0.5 μg/ml CCD (Sigma) or 10 μM latrunculin (red sea sponge toxin, prepared by Dr I Spector; Schatten *et al*, 1986b) or 10 mM TFP (Sigma) or 100 mg/ml TMB-8 (Sigma) in M 16 normal medium.

Immunofluorescence

Embryos were fixed for 1 h at room temperature (RT) in 3.7% formaldehyde in M 2 culture medium. After washing, fixed embryos were treated for 1 h with 0.1 M glycine in PBS with 0.3 mg/ml BSA (PBS-BSA). Cells were permeabilized by incubation for 15 min at RT in 0.1% Triton X-100 in PBS. After washing, specimens were incubated for 1 h at 37° C in each first and second antibodies or with Rhodamine-phalloidin. Washing steps between each incubation were 5 times for 10 min. Dilutions in PBS-BSA were rat monoclonal anti-uvomorulin (DECMA-1, Sigma) 1:100, rabbit polyclonal anti-bovine fodrin (prepared by Dr R Cheney) 1:40, biotin conjugated goat anti-rat IgG (Sigma) 1:40, TRITC conjugated goat anti-rabbit IgG (Sigma) 1:40, FITC conjugated extravidin (Sigma) 1:40, rhodamine conjugated phalloidin (Molecular Probes, Inc.) 1:20. Controls of second antibodies are shown in Figure 13. In order to determine the cell number, all samples were finally DNA stained with DAPI (Sigma) 5 μg/ml in PBS-BSA for 10 min at RT. Zona pellucida (zp) was removed mechanically by gently pipeting (no differences in fluorescence patterns were observed when zona pellucida were removed with acid medium instead of mechanically before incubations). After washing in distilled water, specimens were mounted on slides using DABCO (Aldrich). Specimens were viewed in a BIORAD confocal laser scanning microscope MRC-600 mounted over a Nikon Optiphot upright microscope (this equipment is located in the Integrated Microscopy Resource, University of Wisconsin, Madison). Photographs were taken directly from the monitor using Kodak Ektachrome 400 ASA film.

RESULTS

Decompaction-recompaction

In order to determine the precise conditions for decompaction, various treatments were assayed and the minimum time for maximum decompaction of batches of embryos was measured. Complete decompaction is obtained using cytochalasin D (CCD) and latrunculin (microfilament inhibitors), medium devoid of calcium with EGTA, or verapamil (a calcium channel blocker). Semidecompaction is obtained with TMB-8 (an inhibitor of intracellular calcium release) and with TFP (a calmodulin inhibitor). It is possible to recover compaction by adding fresh normal medium after the above treatments (Table I).

Localization of uvomorulin, fodrin and actin during compaction

In early 8-cell stage embryos (72 h post hCG), that have not yet undergone compaction, uvomorulin is dispersed (Fig 1a) and fodrin (Fig 1b), as well as actin, are homo-

TABLE I

Time of decompaction by various treatments

Treatment		Decompaction	t (min) decompaction	t (min) recompaction
CCD	0.5 μg/ml	+	45	30
Latrunculin	10 μM	+	25	25
EGTA	0.5 mM	+	10	120
Verapamil	0.3 mM	+	20	90
TFP	10 μM	+/-	60	ND
TMB-8	100 μg/ml	+/-	60	ND

ND, not determined.

geneously detected in the cortex (Fig 1c). A few hours later, when the morula compacts (76 h post hCG), a colocalized regionalization of uvomorulin and fodrin, restricted to the extended apposed plasma membrane areas, is observed (Fig 2a, b). Actin did not regionalize during compaction and remained unchanged all over the cortex (Fig 2c).

Localization of uvomorulin, fodrin and actin during experimental decompaction-recompaction

To determine if the various decompacting treatments defined above (Table I) modify the localization of uvomorulin, fodrin and actin, embryos were double stained for uvomorulin and fodrin or uvomorulin and actin immediately after treatment (decompacted embryos) or after recovery of compaction (recompacted embryos). Decompaction induced by EGTA resulted in redistribution of uvomorulin all over the embryo without any preferred region (Fig 3a), while fodrin partly redistributes from contact regions (Fig 3b). After EGTA treatment, morulae recompact completely and regionalization of uvomorulin and fodrin at cell contacts is also recovered (Fig 4). Similar results were obtained with verapamil treatment (Figs 5, 6) and to a lesser extent with CCD and latrunculin treatments (Figs 7 and 8, respectively, recompaction not shown). Treatments that induce semide-

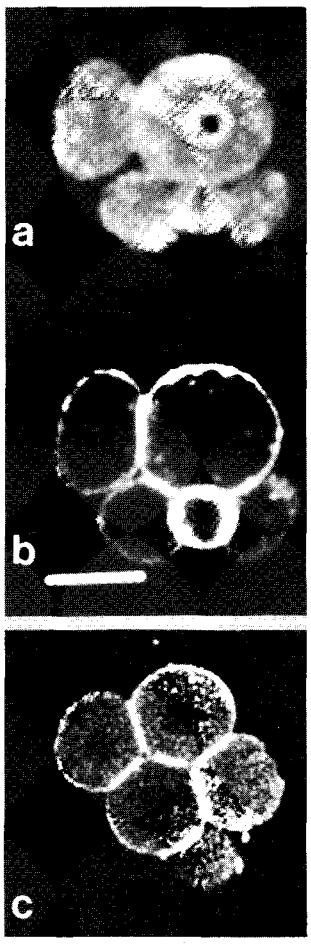

Fig 1. Early not compacted morula (72 h post hCG). (a) Anti uvomorulin staining (FITC). (b) actin staining (Rhodamine). (c) anti fodrin staining (TRITC). Uvomorulin is dispersed and fodrin, as well as actin, is detected in the cortex. Bar = 25 μm.

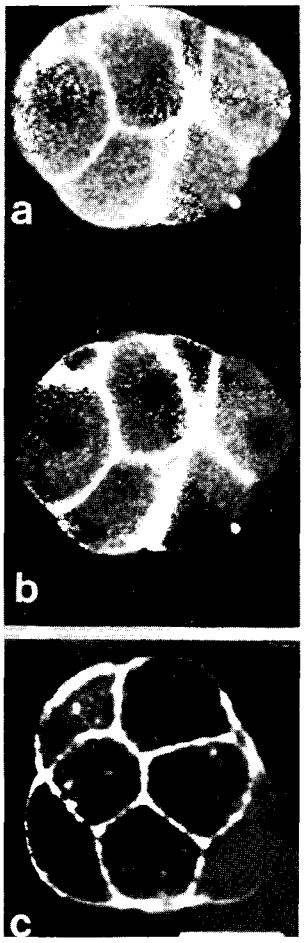

Fig 2. Compacted morula (76 h post hCG). (a) anti uvomorulin staining (FITC). (b) anti fodrin staining (TRITC). (c) actin staining (Rhodamine). A colocalized regionalization of uvomorulin and fodrin is observed while actin remained all over the cortex. Bar = 25 μm.

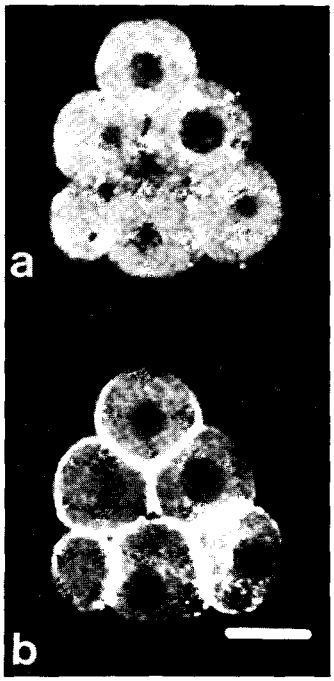

Fig 3. Decompacted morula after 10 min treatment with EGTA medium. (a) anti uvomorulin staining (FITC). (b) anti fodrin staining (TRITC). Uvomorulin and fodrin are dispersed. Bar = 25 μm.

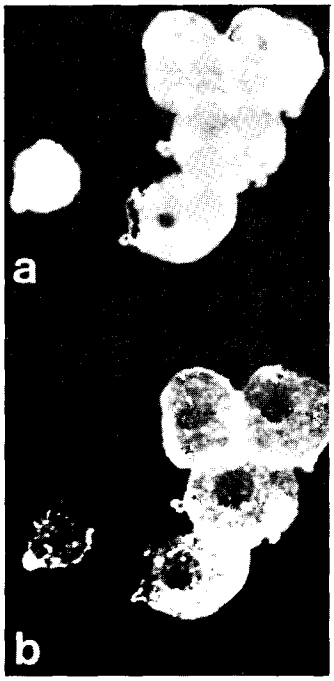

Fig 5. Decompacted morula after 20 min treatment with verapamil medium. (a) anti uvomorulin staining (FITC). (b) anti fodrin staining (TRITC). Uvomorulin and fodrin are dispersed. Bar = 25 μm.

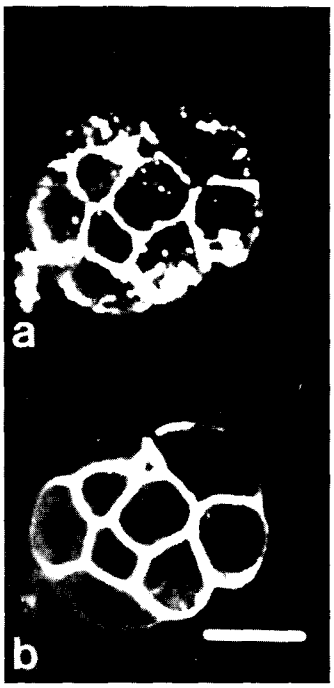

Fig 4. Recompacted morula in fresh normal medium after EGTA treatment. (a) anti uvomorulin staining (FITC). (b) anti fodrin staining (TRITC). Regionalization of uvomorulin and fodrin is recovered. Bar = 25 μm.

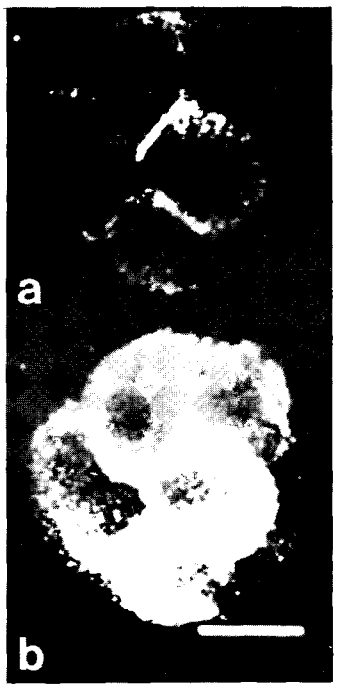

Fig 6. Recompacted morula in fresh normal medium after verapamil treatment. (a) anti uvomorulin staining (FITC). (b) anti fodrin staining (TRITC). Regionalization of uvomorulin and fodrin is partly recovered. Bar = 25 μm.

compaction, TFP and TMB-8, affect only in part the regionalization of uvomorulin (Figs 9a, 10a) while localization of fodrin remains unmodified (Figs 9b, 10b). Actin localization is only affected by microfilament inhibitors, *i.e.*, CCD (not shown) and latrunculin treatments. In both cases a punctuate pattern appears at cell contact regions (arrows in Fig 8b).

Localization of uvomorulin, fodrin and actin in the expanded blastocyst

In the expanded blastocyst (98 h post hCG), actin was still observed all over the cortex in both inner cell mass and trophoblastic cells (Figs 11c,d); uvomorulin is found only in cell contacts and it is excluded from the basal and apical faces of the trophoblast cells

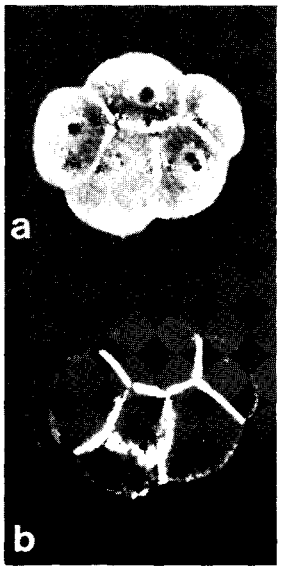

Fig 7. Decompacted morula after 45 min treatment with CCD medium. (a) anti uvomorulin staining (FITC). (b) anti fodrin staining (TRITC). Uvomorulin is dispersed but fodrin remained at cell contacts. Bar = 25 μm.

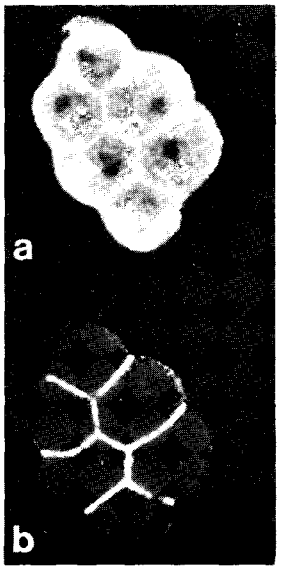

Fig 9. Semidecompacted morula after 60 min treatment with TFP. (a) anti uvomorulin staining (FITC). (b) anti fodrin staining (TRITC). Uvomorulin is partly dispersed. Bar = 25 μm.

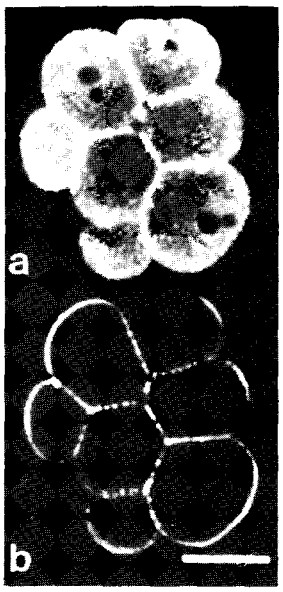

Fig 8. Decompacted morula after 25 min treatment with latrunculin medium. (a) anti uvomorulin staining (FITC). (b) actin staining (Rhodamine). A punctated pattern of actin at cell contact regions appears. Bar = 25 μm.

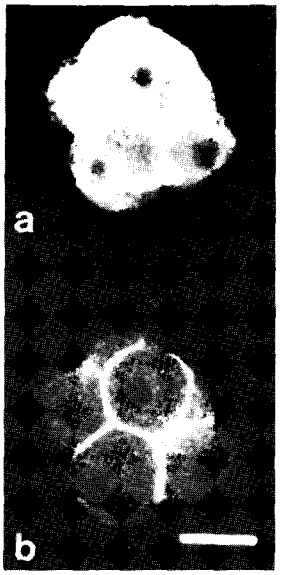

Fig 10. Semidecompated morula after 60 min treatment with TMB-8 medium. (a) anti uvomorulin staining (FITC). (b) anti fodrin staining (TRITC). Uvomorulin is partly dispersed. Bar = 25 μm.

(Figs 11a,b; 12a,b); fodrin, also found at contact areas, is excluded only from the apical but not from the basal cortex of trophoblastic cells (Figs 12c,d).

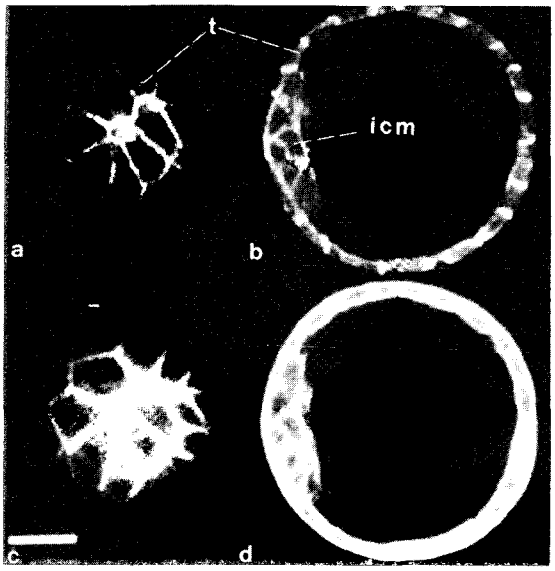

Fig 11. Expanded blastocyst (98 h post hCG). A tangential plane (a,c) and an equatorial plane (b,d) are shown. (a,b) anti uvomorulin staining (FITC). (c,d) actin staining (Rhodamine). Uvomorulin is observed only in cell contact regions and is excluded from apical and basal faces of trophoblastic cells. Bar = 25 μm.

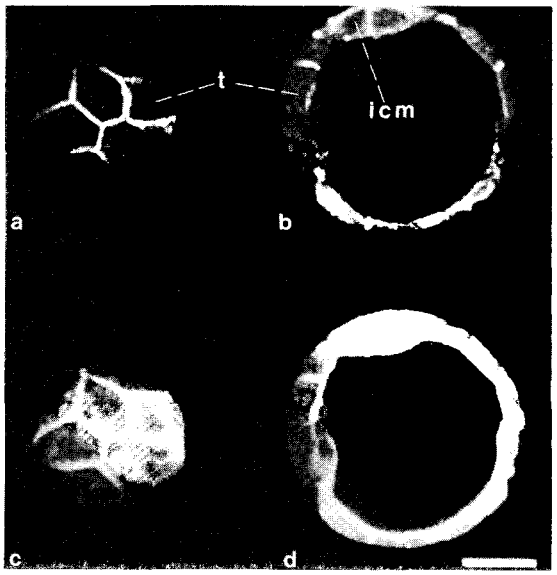

Fig 12. Expanded blastocyst (98 h post hCG). A tangential plane (a,c) and an equatorial plane (b,d) are shown. (a,b) anti uvomorulin staining (FITC). (c,d) anti fodrin staining (TRITC). Fodrin is excluded only from the apical faces of trophoblastic cells. Bar = 25 μm.

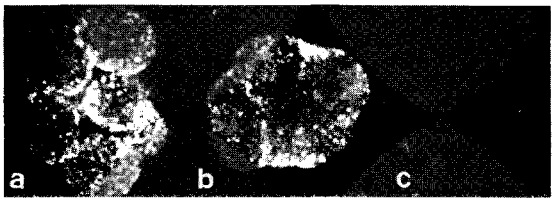

Fig 13. Controls of second antibodies (first antibodies are omitted). (a) biotin conjugated antibody and FITC conjugated extravidin. (b) FITC conjugated extravidin. (c) TRITC conjugated antibody. Bar = 25 μm.

DISCUSSION

The morphogenetic process of compaction has mostly been studied by means of inhibition assays. These have allowed to establish that compaction is mainly mediated by calcium-dependent cellular adhesion and by the cytoskeleton (Ducibella and Anderson, 1979; Pratt *et al*, 1981; 1982). So far, however, no model of compaction integrating these two components has been proposed, even after knowing that in epithelia the adhesion molecule uvomorulin is connected to the cytoskeleton via catenins (Ozawa *et al*, 1990). In the present study we have observed the effects of decompaction-recompaction on the cellular location of uvomorulin, an adhesion molecule, and fodrin and actin, components of the cytoskeleton. The working hypothesis is that treatments affecting various cellular functions resulting in decompaction modify the localization of uvomorulin.

The decompacting-recompacting assay was chosen first, because it allows to work with homogeneous batches of embryos as regards to compaction; secondly, because these processes are faster than compaction; and finally, because even though experimentally induced in these cases, a spontaneous decompaction-recompaction occurs in blastomeres undergoing mitosis before blastulation.

We have defined the conditions under which maximal decompaction is reached with each treatment. Neither decompaction induced by CCD and latrunculin, given the role of cytoskeleton in compaction, nor the effect of EGTA, given that extracellular calcium plays a role in cellular adhesion, are unexpected, but the decompaction induced

by verapamil and the semidecompaction induced by TMB-8 suggest that compaction depends also on intracellular calcium. Also, TFP induced decompaction supports the involvement of calmodulin in compaction, as suggested by Yamamura and Spindle (1988).

In the decompacting-recompacting experiments, we found a relationship between the change in cell shape and the modified location of uvomorulin. Moreover, when a premature compaction is caused in 4-cell embryos (Winkel *et al*, 1990) and 2-cell embryos (Yamamura *et al*, 1987; Sepúlveda and Izquierdo, unpublished results) by activating PKC with phorbol ester or diacylglycerides, there is, simultaneously with the induced compaction, regionalization of uvomorulin in the contact areas.

The dotted pattern of actin in contact areas, observed on decompaction induced by microfilament inhibition with CCD and latrunculin, suggests that not all microfilaments are involved in the maintenance of compaction and that those that remain undisturbed could be irrelevant for this process. The permanence of the actin cortex on decompaction with the other treatments does not rule out its association with uvomorulin; it is possible that the decompaction treatments employed affect the binding of actin to fodrin, which is calcium dependent (Fowler and Taylor, 1980), while having a reduced effect on a possible binding of fodrin to uvomorulin in these cells.

The adhesive function of uvomorulin occurs in the presence of calcium (Ringwald *et al*, 1987). However, in our experiments we have induced the partial or total deregionalization of this molecule by decompacting at a normal calcium concentration (CCD, latrunculin, TFP, TMB-8), at a decreased calcium concentration (verapamil, 0.171 mM calcium) or in the absence of calcium (EGTA). Therefore, the observed deregionalization cannot be ascribed to the loss of the adhesive function. Rather, we consider it very likely that uvomorulin deregionalization results from disturbing the cytoskeleton, either directly by drugs or by disturbing the many functions and relationships of the cytoskeleton which depend on calmodulin and intracellular calcium levels.

A possible role for the cytoskeleton in compaction seems to be the location of molecules, such as uvomorulin, in the correct position, and this could be the result of the contraction-relaxation function of actin microfilaments in regions of the cells, and forces exerted by microtubules, as suggested by Maro and Pickering (1984) when they observed that nocodazole (a drug that causes depolymerization of tubulin) produces a more rapid compaction. In addition, calmodulin interacts with tubulin indirectly through the microtubule-associated proteins MAP 2 and tau (Lee and Wolff, 1984a,b). Besides promoting microtubule polymerization, MAPs bind by the same site to actin, thus interfering with microtubules or calmodulin causing the release of MAPs, could stimulate microfilament contraction (Correas *et al*, 1990; Kotani *et al*, 1985; Matus, 1991; Schliwa, 1976; Schliwa *et al*, 1981) in wrong places of the blastomeres, inducing the observed decompaction. Furthermore, it must be taken into account that fodrin has a calmodulin-binding subunit (Davies and Klee, 1981) and also that a catenin is a vinculin-like protein (Herrenknecht *et al*, 1991) and vinculin is an actin-cross-linking protein (Jockusch and Isenberg, 1981).

If early mammal development is analyzed as the differentiation process of the trophoblast, regionalization and later compaction appear as the beginning of the establishment of this epithelium. From this point of view uvomorulin regionalization in the areas of cellular contact during compaction (Vestweber *et al*, 1987) could induce the regionalization of other molecules, as shown here for fodrin and as occurs with Na/K ATPase and fodrin in mRNA uvomorulin transfected fibroblasts (McNeil *et at*, 1990). Also, the localization of uvomorulin and fodrin observed in compacted morulae suggests that fodrin could be associated with the link between cadherins and the cytoskeleton, without ruling out the participation of other molecules. This proposition is supported by the observation that both molecules deregionalize on decompaction and again colocalize on recompaction.

In the blastocyst, when the trophoblast is already differentiated and the epithelium established, different patterns of distribution

of the studied molecules are observed between inner cell mass cells and trophoblastic cells. In the former, the distribution of uvomorulin, fodrin and actin is similar and no polarization is observed. In the latter, uvomorulin is not present all over the cortex but it is restricted to the contact areas and fodrin is excluded only from the apical faces, thus a complete colocalization is lost. The observation of uvomorulin exclusively in the area of contact between the trophoblast cells does not agree with the observations reported by Slager *et al* (1991) who detected uvomorulin also in the basal face. We believe that this difference is due to the fact that their observations were carried out in early blastocysts and not at the expanded blastocyst stage (98 h post hCG) with trophoblastic cells at a more advanced differentiation stage. In murine trophoblastic epithelium, uvomorulin could have a different role as it has in cell fusion in human trophoblast (Coutifaris *et al*, 1991).

The interactions between extracellular adhesion molecules and cytoskeletal elements lead to fundamental changes in cell fate and polarity; these events are critical for normal development.

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