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Ezrin Becomes Restricted to Outer Cells Following

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Mouse Embryo

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During preimplantation development in the mouse, two phenotypically distinct cell populations appear at the 16-cell stage: nonpolarized inner cells that give rise to the inner cell mass and polarized outer cells that give rise mainly to the trophectoderm. The divergence of these two cell lineages is due to asymmetrical cell divisions during the transition from the 8- to the 16 cell stage which can occur following blastomere polarization. During compaction, at the 8-cell stage, cytoplasmic organelles accumulate in the apical domain, a surface pole of microvilli forms, and blastomeres flatten onto one another. During the division from the 8- to the 16-cell stage, the only asymmetrical structure maintained is the pole of microvilli. At the 16-cell stage, only blastomeres inheriting a large part of this apical structure can reestablish a polarized organization. The mechanisms involved in the formation and stabilization of the apical pole of microvilli are still unknown. Ezrin is an actin-associated protein that has been proposed to play a role in the formation of microvillous structures. This led us to study the expression of ezrin during early development of the mouse embryo. We observed that ezrin mRNA and protein are present in the mouse oocyte and throughout preimplantation embryo development, although the amount of protein present decreases continuously during early development, particularly after the 8-cell stage, at the time of compaction. Two isoforms of ezrin phosphorylated on tyrosine residues are present during all of preimplantation development while a third non-tyrosine-phosphorylated isoform appears at the 8-cell stage and its relative amount increases from the 8-cell stage to the blastocyst stage. Before compaction, ezrin is distributed around the cell cortex. However ezrin becomes restricted to the microvilli of the apical pole after compaction. At later stages, ezrin is found in the microvilli of the apical surface of outer cells. Finally, ezrin remains associated with the microvillous pole during the transition from the 8- to 16-cell stage and is found only in the outer cells after division. Thus, ezrin is the first cytocortical protein described that is totally segregated in outer cells at the 16-cell stage after an asymmetrical division. © 1996 Academic Press, Inc.

INTRODUCTION

The preimplantation period in mouse development is characterized by the transformation of a newly fertilized egg to a blastocyst constituted of two populations of cells: the inner cell mass (ICM) and the trophectoderm (TE), which has the features of an epithelium. The ICM will give rise to the embryo and some extraembryonic tissues and the TE will participate in the extraembryonic structures (Gardner, 1983). The formation of these two distinct populations is a crucial process of early mammalian development. Indeed, the trophectoderm is involved in many functions essential for the future development of the embryo, such as the transport of metabolites between the maternal tissues and the ICM or as the proliferative source of cells for placental trophoblast. The mechanisms that enable the formation of these two populations remain unknown but the process of epithelial biogenesis clearly begins at compaction.

The fertilized mouse egg undergoes three cleavage divisions to form an 8-cell embryo in which morphological processes are initiated that lead to the polarization of blastomeres (Johnson and Maro, 1986). Before compaction, all blastomeres are equivalent (in that they are able to form both TE and ICM) and are not polarized. During compaction, the blastomeres flatten upon one another and become polarized along an apical–basal axis. A pole of microvilli forms on the apical surface of the cells and some cytoplasmic components become asymmetrically distributed.

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During the fourth cleavage, the cytoplasmic polarity is lost and only surface polarity is retained (Johnson, 1988). At the 16-cell stage, cytoplasmic polarity is restored in the blastomeres that have inherited a sufficient part of the apical pole. Depending on the orientation of the mitotic spindle with the apical–basal axis of the cells, two types of divisions can occur: a symmetrical division when the spindle axis is orthogonal to the axis of polarity (both daughter cells are polarized) or an asymmetrical division when the spindle axis is parallel to the axis of polarity (one daughter cell is polarized, whereas the other is nonpolarized; Johnson and Maro, 1986). Thus, asymmetrical divisions generate two different cell types: an internal mass of apolar cells which will mostly give rise to the ICM and an outer layer of polar cells which will give rise principally to the trophectoderm (Fleming, 1987).

These observations show that the retention of the pole of microvilli at the apical surface of blastomeres is a critical requirement for the generation of cell diversity by unequal cell division. Experimental evidence suggests that there exists, within the surface membrane or its associated cytocortex, a distinct and relatively stable domain able to direct the formation of the polar axis in interphase cells, causing the cells to enter into the trophectodermal lineage (Johnson *et al.*, 1988). The surface pole is characterized by microvilli containing a cytochalasin D-resistant core of actin filaments (Fleming *et al.*, 1986) and serves as a ''memory'' trace for polarity during mitosis. The stabilization of these microvilli could be explained by the presence of actin-binding proteins able to cross-link elements within the cytocortex. α-actinin (Lehtonen and Badley, 1980) and spectrin-like proteins (Reima and Lehtonen, 1985) have been localized in cortical and cytoplasmic domains of mouse embryo blastomeres, but changes in their distributions have not been detected concurrent with microvillous polarization.

The proteins ezrin, moesin, and radixin, regrouped in the ERM family (Sato *et al.*, 1992), are homologous to the band 4-1 protein which is involved in membrane–cytoskeletal interactions. Their structural similarities suggest that these proteins could play a role by modulating the association of the cortical cytoskeleton with the plasma membrane. This proposed role is supported by the localization of ERM proteins in actin-rich surface structures (Sato *et al.*, 1992) and recently they have been shown to work as molecular linkers between a cell surface glycoprotein, CD44, and the actin-based cytoskeleton (Tsukita *et al.*, 1994). Moreover, ezrin has a binding site for filamentous actin localized in the C-terminal domain (Turunen *et al.*, 1994). Ezrin is also highly concentrated in the apical cortex of many epithelial cell types (Berryman *et al.*, 1993) and is present in actin-rich surface structures such as microvilli, microspikes, and membrane ruffles (Bretscher, 1989). Several observations have suggested that this protein may be involved in cytoskeletal and membrane reorganization. The addition of epidermal growth factor (EGF) to human carcinoma A-431 cells induces a production of peripheral membrane ruffles in which ezrin is recruited and becomes phosphorylated on serine and tyrosine residues. This correlation between the generation of cortical structures containing ezrin and ezrin phosphorylation suggests a functional relationship (Bretscher, 1989). In parietal gastric cells, ezrin is also relocalized and, at the same time, is phosphorylated on serine residues (Hanzel *et al.*, 1991). Furthermore, overexpression of different ezrin domains in insect cells has demonstrated recently that the C-terminal domain possesses a constitutive cell surface extension activity which is inhibited by the N-terminal domain (Martin *et al.*, 1995).

In this study, we have, for the first time, characterized ezrin in the mouse embryo. We observed that ezrin protein and mRNA are always present during preimplantation mouse development. The amount of ezrin protein decreases from the oocyte to the blastocyst stage, with a more pronounced drop during compaction. Two isoforms of ezrin protein are present in early stages of development and are phosphorylated on tyrosine residues. A new isoform of ezrin, not phosphorylated on tyrosine, appears at the 8-cell stage at compaction. The localization of ezrin follows the distribution of microvilli and when the embryo is constituted of two cell populations, ezrin is exclusively localized in the outer cells. After an asymmetrical division, ezrin is totally segregated into outer polarized cells.

MATERIALS AND METHODS

Recovery of Oocytes and Embryos

Swiss female mice (8–11 weeks old) were superovulated by intraperitoneal injection of 5 IU of pregnant mare serum gonadotrophin (PMSG, Intervet) and human chorionic gonadotrophin (hCG, Intervet) 48 hr apart.

Oocytes and embryos were recovered in medium 2 containing 4 mg/ml bovine serum albumin (M2 + BSA; Fulton and Whittingham, 1978). Oocytes were recovered at 12–14 hr post-hCG by puncturing the ampullae of oviducts. The cumulus cells were dispersed by brief exposure to 0.1 *M* hyaluronidase (Sigma).

To obtain embryos, females were paired overnight with Swiss males and checked for vaginal plugs the next morning. Under these conditions, fertilization occurs about 12 hr post-hCG. Embryos were collected by flushing oviducts in M2 + BSA and they were then cultured in T6 medium containing BSA (T6 + BSA; Howlett *et al.*, 1987) under paraffin oil in sterile culture dishes at 37°C in 5% CO_2 in air. One-cell stage embryos were recovered from females at 10 hr postfertilization. Two-cell stage embryos were recovered at 36 hr postfertilization. Four-cell stage embryos were collected after about 4 hr of culture (40 hr postfertilization), noncompacted 8-cell stage after 21 hr (53 hr postfertilization), compacted 8-cell stage after 28 hr (60 hr postfertilization), 16-cell stage after 36 hr (68 hr postfertilization), and blastocyst stage after 58 hr of culture (90 hr postfertilization).

When necessary, zonae pellucidae were removed by a brief incubation in acid Tyrode's solution (Nicolson *et al.*, 1975).

Antibody

The rabbit antibody raised against human recombinant ezrin was characterized previously (Andréoli *et al.*, 1994). By immunoblot-

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ting, this antibody recognizes only ezrin in HGT-1 cells that contain the three ERM proteins (ezrine, radixin, and moesin).

Cell Fixation and Immunological Staining

Zona-free embryos were placed in specially designed chambers as described by Maro *et al.* (1984), previously coated with 0.1 mg/ml concanavalin A (Sigma) in phosphate-buffered saline (PBS). The chambers containing samples were centrifuged at 450*g* for 10 min at 37°C. Cells were fixed in 3.7% formaldehyde (BDH) in PBS for 30 min at 37°C, neutralized with 50 m*M* NH_4Cl in PBS for 10 min, and postpermeabilized with 0.25% Triton X-100 in PBS for 30 min.

Immunological staining was performed by incubating the fixed samples in the anti-ezrin antibody diluted 1:400 in PBS/Tween (PBS containing 0.1% Tween 20 and 3% BSA) for 30 min, followed by an incubation in FITC-conjugated anti-rabbit antibody (KPL) diluted 1:150 (in PBS/Tween + BSA) for 20 min. Chromatin was visualized by staining with propidium iodide (5 μg/ml in PBS, Molecular Probes) for 5 min. The samples were examined using a Bio-Rad MRC-600 confocal laser microscope mounted on an Optiphot II Nikon microscope equipped with a 60X objective (Plan Apo; NA 1.4). An argon ion laser adjusted at 488 nm wavelength was used for fluorescein and a helium–neon laser was adjusted at 453 nm for propidium iodide.

Immunoblotting

Eggs and embryos were collected in sample buffer (Laemmli, 1970) and boiled for 5 min. The total embryo protein content was separated in a 10% SDS–polyacrylamide gel and then transferred electrophoretically onto a nitrocellulose membrane. The membranes were blocked in TBS/Tween (150 m*M* NaCl, 10 m*M* Tris, pH 7.4, 0.1% Tween 20) containing 3% (w/v) dry milk powder, followed by an incubation with the anti-ezrin antibody (dilution 1:50 000 in TBS/Tween containing 3% milk) overnight at 4°C. After several washes in TBS/Tween, the membranes were incubated for 1 hr at room temperature with the secondary anti-rabbit immunoglobulin antibody linked to peroxidase (Amersham, dilution 1:1000 in TBS/Tween containing 3% milk). The membranes were revealed using the ECL Western Blotting Detection System (Amersham) according to the manufacturer's instructions.

2D Gel Electrophoresis

Samples of 200 eggs or embryos were stored at −80°C in water. They were diluted (1:1) with a mixture of 9.8 *M* urea, 2% Triton X-100, 2% carrier ampholytes, pH 7–9 (Serva), and 100 m*M* DTT.

Samples were loaded into individual capillary tubes containing 9.2 *M* urea, 4% acrylamide, 2% Triton X-100, 0.6% carrier ampholytes, pH 3–10, 1% carrier ampholytes, pH 4–6.5, and 1.4% carrier ampholytes, pH 5–8 (Pharmacia).

The gels were first run at 400 V for 15 min, then at 750 V for 15 min, and finally at 1000 V for 2.5 hr. After focusing, the gels were removed from the capillary tubes and were placed on the top of a 10% SDS–polyacrylamide gel. Conditions of migration and immunoblotting were the same as described previously. The membranes were blocked in TBS/Tween containing 3% BSA and then incubated in an anti-phosphotyrosine antibody (UBI, dilution 1:1000 in TBS/Tween containing 3% milk) for 2 hr at room temperature. The membranes were revealed using the Vectastain ABC kit (Vector Laboratories) according to the manufacturer's instructions. The membranes were then dehybridized by incubation in 2% SDS, 100 m*M* β-mercaptoethanol, and 70 m*M* Tris, pH 6.8, for 45 min at 60°C. After blocking in TBS/Tween containing 3% milk, they were incubated in the anti-ezrin antibody as described previously.

RT–PCR Experiments

The primers used in our experiments, 5′GTG TCA CAC TTG GCT CTT TAG3′ (5′-specific primer) and 5′TGC TGT CCC AGT GAC AAT GAA3′ (3′-specific primer) were defined using the Amplify software package (v1.2, W.R. Engels, University of Wisconsin, Madison). They define a 253-bp fragment in the ezrin cDNA between positions 1902 and 2152.

Eggs and embryos were collected in H_2O treated with 0.1% diethyl pyrocarbonate (H_2O-DEPC) and stored at −80°C. To reduce the loss of mRNAs, no further purification was undertaken, samples were pretreated with DNase, followed by a standard RT–PCR in the same tube.

Samples corresponding to 20 eggs or embryos were lysed by freezing and thawing. The genomic DNA was digested by adding 2 units of RQ1 DNase (Promega) in 40 μl of the reverse transcriptase mix (10 m*M* Tris–HCl, pH 8.3, 5 m*M* $MgCl_2$, 50 m*M* KCl, 1 m*M* each dNTPs (Pharmacia), 40 units of RNase inhibitor (RNasin, Promega) and incubating at 37°C for 30 min. The reaction was stopped by heating to 85°C for 5 min. Then, 2.5 μM random hexamer (pd(N)6, Pharmacia) was added and the samples were divided into two parts. One was supplemented with 50 units of MMuLV reverse transcriptase (Gibco BRL) to synthesize the cDNA. After 1 hr incubation at 37°C, the reaction was stopped by heating to 95°C for 5 min. The same reaction was carried out on the other part of the template without reverse transcriptase to control for false-positive PCR amplification of contaminating DNA. PCR amplification was performed in the same tube in a final volume of 50 μl containing 10 m*M* Tris–HCl, pH 8.3, 2 m*M* $MgCl_2$, 50 m*M* KCl, 1 μM of the 5′-specific primer, 1 μM of the 3′-specific primer, and 2.5 units of *Taq* polymerase (Cetus). Samples were denatured at 94°C for 3 min and then subjected to 40 cycles of amplification (94°C, 1 min; 57°C, 1 min; 72°C, 1 min). Five microliters of the amplified mixture corresponding to 1 egg or 1 embryo was run on 1.2% agarose gels. The gels were stained with BET (5 μg/ml) and photographed under UV illumination.

Culture in the Presence of Phosphorotiorate Oligonucleotides

The antisense (5′CCG GAC GTT GAT TGG CTT GGG CAT3′) and sense (5′ATG CCC AAG CCA ATC AAC GTC CGG3′) phosphorothiorate oligonucleotides complementary to position 1–24 of the ezrin cDNA were identical to the ones used by Takeuchi *et al.* (1994) and were synthetized by Eurogentec (Seraing, Belgium).

The oligonucleotides were added to the culture media at a concentration of 10 μM. Newly formed 4-cell stage embryos were cultured in media containing either antisense or sense oligonucleotides. We used this experimental protocol because Brice *et al.* (1993) demonstrated that antisense EGF receptor oligonucleotides added to the culture media were active in preimplantation mouse embryos. The culture media were replaced with new medium containing freshly solubilized oligonucleotides every 4 hr. At these times, each group of embryos was scored for their developmental stage (8-cell stage formation, compaction, and cavitation). After 44

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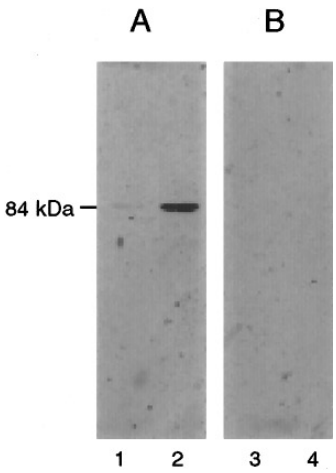

FIG. 1. Characterization of the anti-ezrin antibody. Immunoblotting with the anti-ezrin antibody on 1-cell stage mouse embryos. Lanes 1, 3: 10 1-cell stage embryos. Lanes 2, 4: 20 1-cell stage embryos. Lanes 3, 4: anti-ezrin antibody preincubated with the immunogenic protein.

hr of continuous culture in oligonucleotides, the embryos were collected for immunoblotting.

Electron Microscopy

Oocytes or embryos were prefixed in 0.3% Triton X-100 and 4% paraformaldehyde in PHEM buffer (60 m*M* Pipes, 25 m*M* Hepes, 10 m*M* EGTA, 2 m*M* $MgCl_2$, 0.6 μM taxol) for 15 min. Samples were then fixed with PHEM buffer containing 4% paraformaldehyde for 15 min and extracted in PHEM containing 0.5% Triton X-100. Then the samples were incubated first with the anti-ezrin antibody (dilution 1:400 in 5% calf fetal serum/PBS) for 1 hr, washed, and then incubated with 10-nm gold-labeled anti-rabbit immunoglobulin antibodies. The samples were postfixed first in Sørensen buffer (0.1 *M* Na_2HPO_4, 0.1 *M* NaH_2PO_4) containing 5% sucrose, 4% paraformaldehyde, and 0.2% tannic acid for 20 min and then in Sørensen containing 0.5% osmic acid on ice for 4 min. They were stained with 2% uranyl acetate for 15 min. After dehydratation in ethanol, samples were contrasted with 3% uranyl acetate for 10 min and 1% lead citrate for 90 sec. They were observed on a Philips EM 410 electron microscope at 80 kV.

RESULTS

The Anti-ezrin Antibody Recognizes a Single Polypeptide of 84 kDa in Mouse Embryos

First we tested the ability of an antibody raised against human ezrin to recognize ezrin in mouse embryos. Sequence analysis shows that the murine ezrin shares 96.2% identity to the human (Funayama *et al.*, 1991).

The antibody directed against human ezrin recognized a single band in 1-cell stage embryos (Fig. 1). The use of molecular mass markers allowed us to evaluate the molecular mass of this protein at 84 kDa which is consistent with the molecular mass found in other cell types (Sato *et al.*, 1992). This band was not detected if the antibody was preincubated with the immunogenic protein.

These results show that the antibody raised against human ezrin is also able to recognize specifically ezrin in 1-cell stage mouse embryos.

Ezrin Is Present during Early Development

To investigate the pattern of expression of the ezrin protein during preimplantation development, immunoblots were performed using the total protein content of embryos collected at different stages of development (Fig. 2). A single band of 84 kDa was recognized by the anti-ezrin antibody at all stages of development from the 1-cell-stage to blastocyst. However, the amount of ezrin present in the embryos decreased from 1-cell to blastocyst stage.

To analyze this more precisely, we measured the amount of ezrin present on the immunoblots throughout all stages of preimplantation development. A range of oocytes was used to verify the proportionality between the intensity of the band and the amount of protein. Quantitative analysis confirmed a drop in ezrin content from the 1-cell to blastocyst stage: blastocysts contained approximately 50% less ezrin than 1-cell stage embryos. Most of this reduction occurred after compaction at the 8-cell stage.

Two Isoforms of Ezrin Are Present throughout Preimplantation Development and a Third Isoform Appears at the 8-Cell Stage

The quantitative change in the amount of ezrin protein were associated with qualitative changes that we observed on

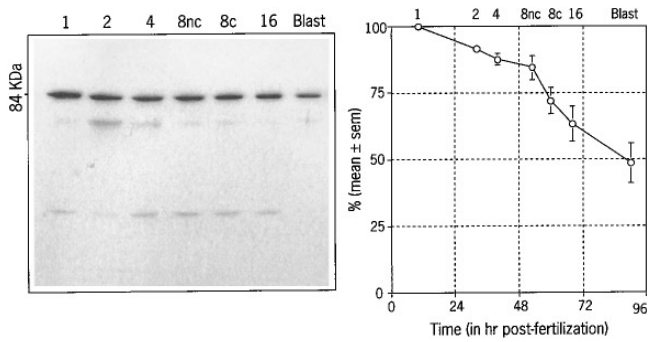

FIG. 2. Quantification of ezrin protein during mouse preimplantation development. Blots were performed using samples containing 40 embryos selected at different stages of development (1, 2, 4, 8nc, 8c, 16, and Blast correspond to 1-cell embryos, 2-cells, 4-cells, 8-cells noncompacted, 8-cells compacted, 16-cells, and blastocysts, respectively. The membranes were incubated with the anti-ezrin antibody, revealed by chemiluminescence, and quantified (a range of oocytes was used to verify the proportionality between the intensity of the band and the amount of protein; data not shown). The bars show the means ± SEM (n = 4).

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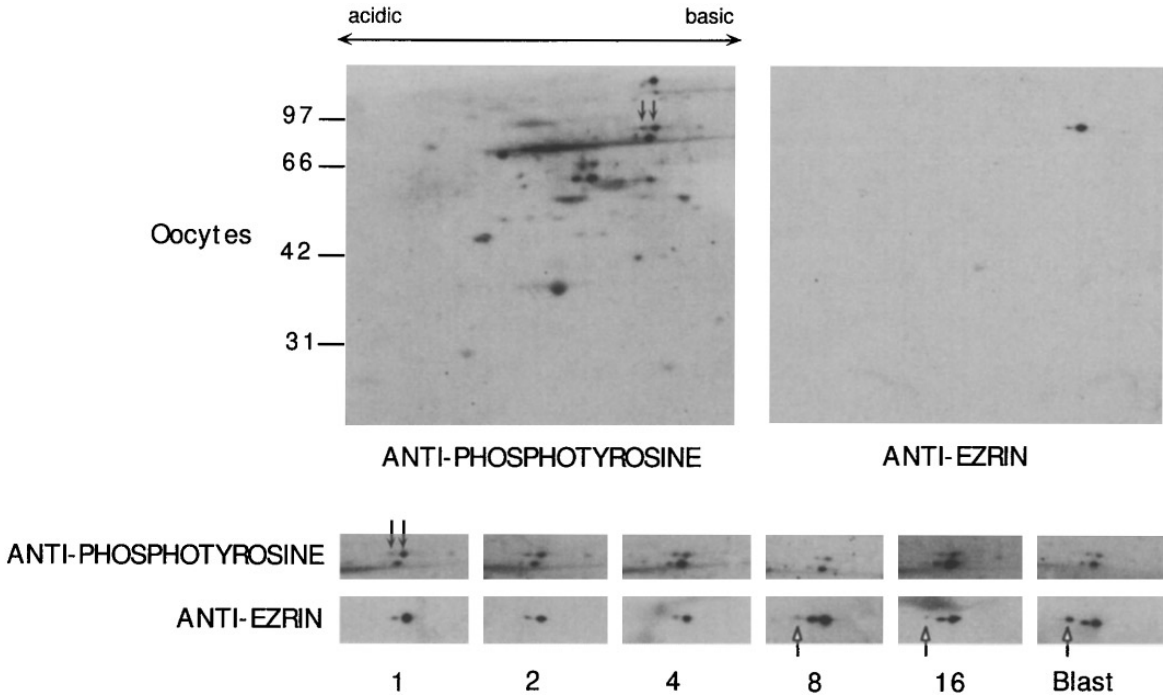

FIG. 3. Identification of different isoforms of ezrin in mouse embryos. Two-dimensional IEF/SDS–PAGE separation of mouse embryo proteins at different stages of development (see Fig. 2 for abbreviations). After electrophoresis, gels were blotted and revealed with an anti-phosphotyrosine antibody, dehybridized, and restained with the anti-ezrin antibody. Black arrows indicate the spots corresponding to ezrin present in all stages of development and phosphorylated on tyrosine; white arrows indicate the third spot of ezrin.

immunoblots performed after two-dimensional gel electrophoresis of samples collected at various stages of development (Fig. 3). Two spots were recognized by the anti-ezrin antibody throughout development which showed the same molecular weight, but a different isoelectric point. From the 8-cell stage to the blastocyst stage, the relative intensity of the more acidic isoform increased and a third isoform, even more acidic, was observed. The intensity of this new isoform of the protein increased also with the stage of development.

Previous experiments have shown that some morphological changes of certain cell types are correlated with a phosphorylation of ezrin on tyrosine, serine, or threonine residues. Since we were not able to immunoprecipitate the minute amount of ezrin present in mouse embryos with two different anti-ezrin antibodies, we decided to investigate the possible tyrosine phosphorylation of ezrin during mouse preimplantation development by immunoblotting after two-dimensional gel electrophoresis. The membranes were developed using first an anti-phosphotyrosine antibody and then, to identify the spots corresponding to ezrin, they were developed with the anti-ezrin antibody (Fig. 3). These experiments showed that the two isoforms of ezrin present throughout preimplantation development were phosphorylated on tyrosine residues. However, the third isoform of ezrin detected from the 8-cell stage was not phosphorylated on tyrosine residues.

The Ezrin mRNA Is Present at All Stages of Preimplantation Development

The presence of ezrin protein in mouse oocytes before fertilization indicates that ezrin is maternally expressed. Moreover, the decrease in the amount of protein during early development may suggest that the gene is not transcribed following activation of the embryonic genome at the late 2-cell stage (Flach *et al.*, 1982). Since we were not able to immunoprecipitate ezrin, to investigate whether the ezrin present at later stages could be of embryonic origin, we looked for the presence of ezrin mRNA by RT–PCR in eggs and embryos at different stages of development. In the mouse, almost all maternal mRNAs are destroyed upon activation of the embryonic genome (Flach *et al.*, 1982). A band migrating at the expected size (253 bp) was detected at all stages of development (Fig. 4A). We checked that this fragment was indeed an amplification product derived from the ezrin cDNA. After total digestion with *Hae*III, we observed the three expected fragments (135, 82, and 36 bp) corresponding to cutting at the two *Hae*III sites located at positions 82 and 217 (Fig. 4B). Thus, the ezrin mRNA is present in mouse embryos from the oocyte to the blastocyst stage, suggesting that the ezrin protein pool observed in the blastocyst is derived from both maternal and zygotic transcripts.

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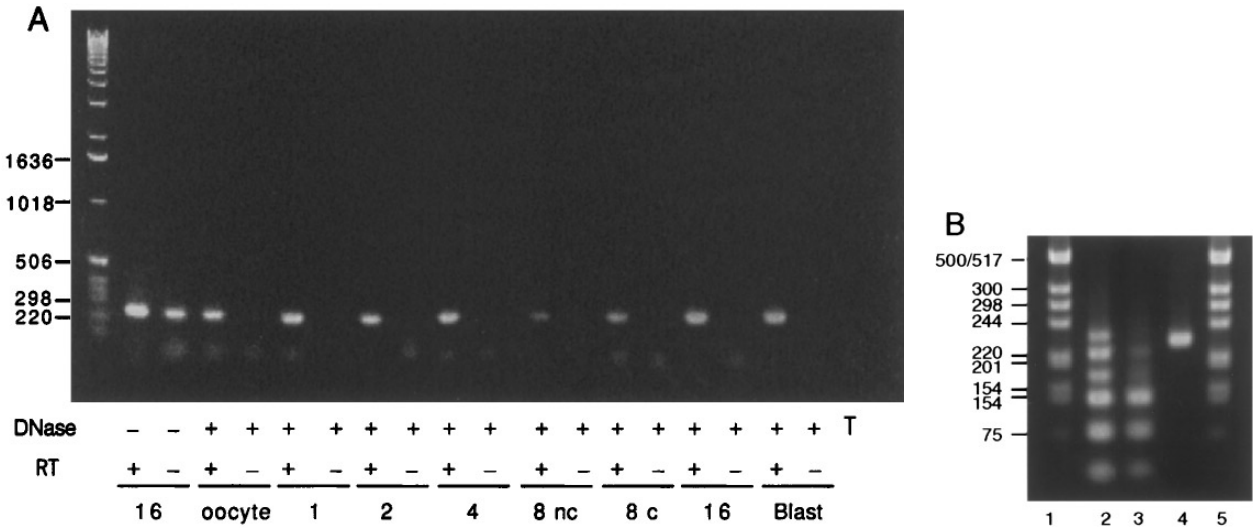

FIG. 4. Detection of ezrin mRNA in mouse embryos. (A) 10 mouse embryos at different stages of development (see Fig. 2 for abbreviations) were subjected to RT–PCR to detect ezrin mRNA following a treatment with (+) or without (−) DNase and with (+) or without (−) RT. An amplified mixture corresponding to 1 egg or 1 embryo was run on the agarose gel. T, template with no embryo. Left, molecular size markers (base pairs). (B) Digestion of the RT–PCR product by *Hae*III. Lanes: 1 and 5, molecular size markers; 2, partial digestion; 3, complete digestion; 4, no digestion.

Effect of Ezrin Antisense Oligonucleotides on Mouse Preimplantation Development

To determine whether newly synthesized ezrin plays a role during mouse preimplantation development, we cultured embryos in ezrin antisense phosphorothiorate oligonucleotides from the beginning of the 4-cell stage to the blastocyst stage. After 44 hr of culture, antisense oligonucleotides had had no effect on the morphological changes observed in preimplantation embryos.

We then examined the effect of these oligonucleotides on ezrin expression by immunoblotting. After probing the membrane with the ezrin antibody, it was reprobed using a monoclonal β-tubulin antibody (Amersham, dilution 1:1000). We quantified the amount of ezrin present on immunoblots for embryos cultured in T6 (control medium), antisense, or sense oligonucleotides and compared this value with the amount of β-tubulin to compensate for any loss of total protein content during the experiment (Table 1). In the presence of antisense oligonucleotides we detected an inhibition of ezrin expression of 14% when compared to the T6 group and 11% when compared to the sense oligonucleotide group. This would suggest that there is only a slight zygotic contribution to the ezrin pool in blastocysts.

Ezrin Is Localized in the Cortex of Microvilli-Containing Membrane Domains

To investigate the cellular distribution of ezrin in eggs and embryos, an immunofluorescence study was performed on whole-mounted samples with a confocal microscope (Fig. 5). Anti-ezrin antibody staining was observed at all stages examined, confirming the presence of ezrin throughout preimplantation development. When the anti-ezrin antibody was preincubated with the immunogenic protein, no staining was observed.

In eggs, ezrin was localized around the cell cortex apart from the region overlying the meiotic spindle. In embryos from the 2-cell stage to the noncompacted 8-cell stage, ezrin was distributed all over the cell periphery, including cell contact areas. The distribution of ezrin changed after compaction when only the apical pole of blastomeres was stained by the antibody in compacted 8-cell stage and 16-cell stage embryos. At the blastocyst stage, ezrin was localized to the external surface of the embryo. No staining was observed in the cytoplasm of the cells and from the 16-cell stage, ezrin was restricted at the cortex of the external

TABLE 1
Effect of Antisense Oligonucleotides on Ezrin Expression in Mouse Blastocysts

Treatment[a]	Ezrin (in AU)[b]	Tubulin (in AU)	Ezrin/tubulin
Antisense	22003	17040	1.29
Sense	22012	15069	1.46
Control	20068	13309	1.51

[a] 44 hr culture. See Materials and Methods for the experimental procedure.
[b] AU, Arbitrary unit.

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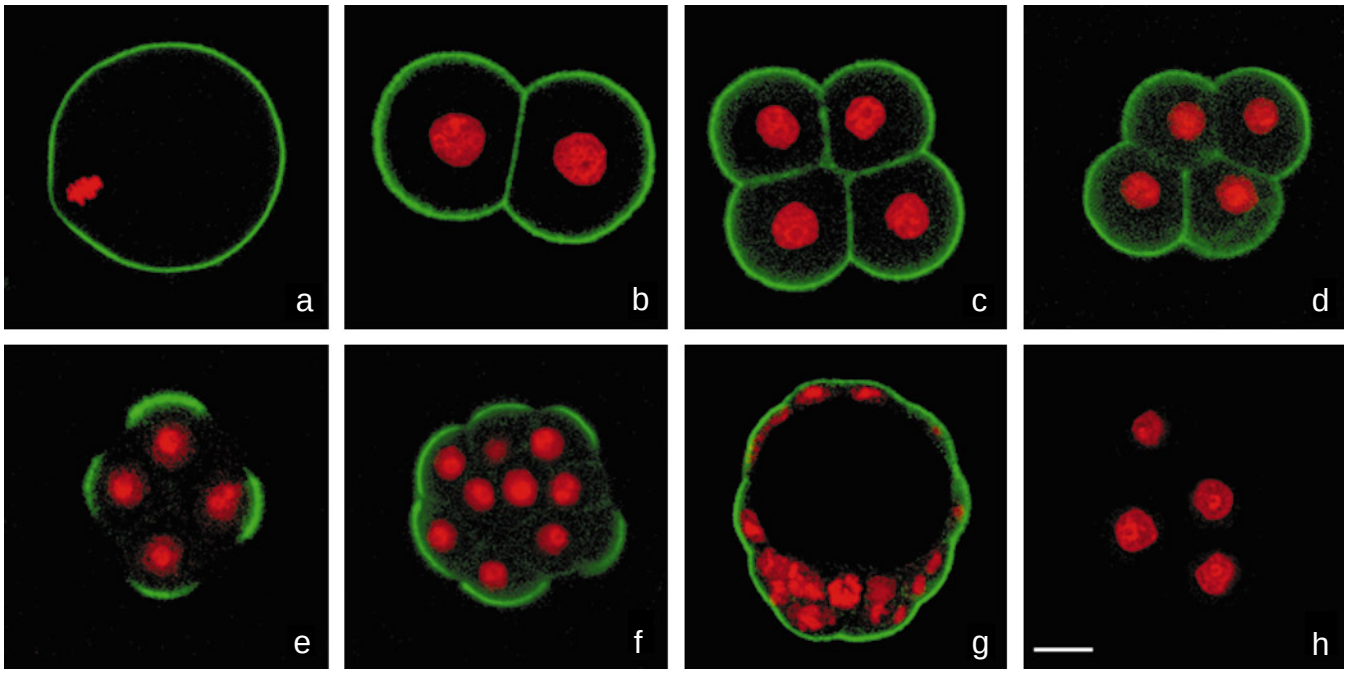

FIG. 5. Localization of ezrin in mouse preimplantation embryos. Indirect immunofluorescence of mouse embryos at different stages showing the distribution of ezrin (green) and nuclei (red). Single confocal sections. (a) Oocyte; (b) 2-cell stage; (c) 4-cell stage; (d) non-compacted 8-cell stage; (e) compacted 8-cell stage; (f) 16-cell stage; (g) blastocyst stage; (h) 8-cell stage embryo stained with the anti-ezrin antibody in the presence of the immunogenic protein. Bar, 20 μm.

blastomeres. Thus, the localization of ezrin followed the distribution of microvilli during development. Oocytes are covered with microvilli except in the area overlying the meiotic spindle (Johnson *et al.*, 1975). In the embryo before compaction, the microvilli are present over the whole surface of the blastomeres, whereas at compaction, the microvilli undergo a reorganization and become restricted to the apical pole of the cells (Reeve and Ziomek, 1981).

We confirmed the localization of ezrin in the microvilli at the ultrastructural level (Fig. 6). Immunogold staining with the anti-ezrin antibody in compacted 8-cell embryos showed that gold particles were concentrated in the microvilli. Little or no staining was associated with nonmicrovillar regions of the plasma membrane and no staining was observed in cytoplasm.

Ezrin Remains Associated with the Microvillous Pole during Mitosis

In the previous experiments, the localization of ezrin was performed on interphase cells. It was also important to analyze the behavior of ezrin during mitosis, especially during the transition between the 8- and the 16-cell stages, when symmetrical and asymmetrical divisions can take place. A population of compacted late 8-cell stage embryos were followed at hourly intervals to identify those entering into mitosis, signified by the decompaction of some of the blastomeres (Goodall and Maro, 1986). The embryos entering into mitosis were fixed immediately and double stained with the anti-ezrin antibody and propidium iodide to stain the chromatin and determine their cell cycle stage (Fig. 7).

Most of the mitotic cells examined were found to be in metaphase and some in anaphase or telophase. The results showed that ezrin was localized at the apical pole of both interphase and metaphase cells. Thus, ezrin remains associated with the microvillous pole throughout the division process. In telophase cells undergoing asymmetrical division, ezrin is found only in the external polarized cells. Ezrin appears therefore to be the first cytoskeletal protein described that is totally segregated in outer cells at the 16-cell stage after an asymmetrical division.

FIG. 7. Localization of ezrin in mouse embryos during the transition 8- to 16-cell stage. (Left) Indirect immunofluorescence of mouse embryos during the transition from 8- to 16-cell stage showing the distribution of ezrin (green) and chromatin (red). Observation under a confocal microscope. Projection of optical sections. (Right) Scheme showing the expected type of division according to the position of the spindle with the respect to the axis of the cell polarity. P, polar cell. A, apolar cell. Note in the last picture the total segregation of ezrin into the outer daughter cell. Bar, 20 μm.

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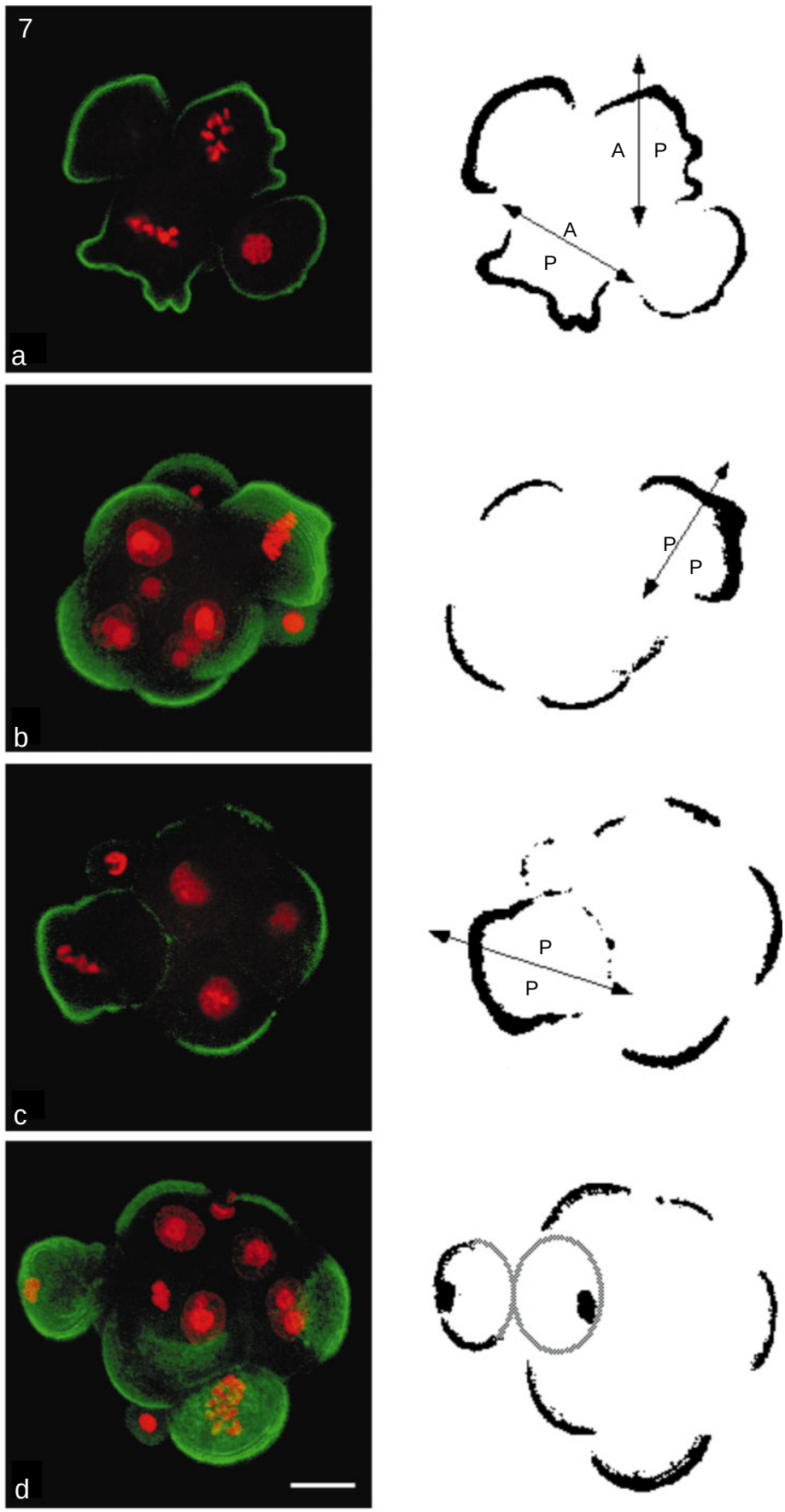
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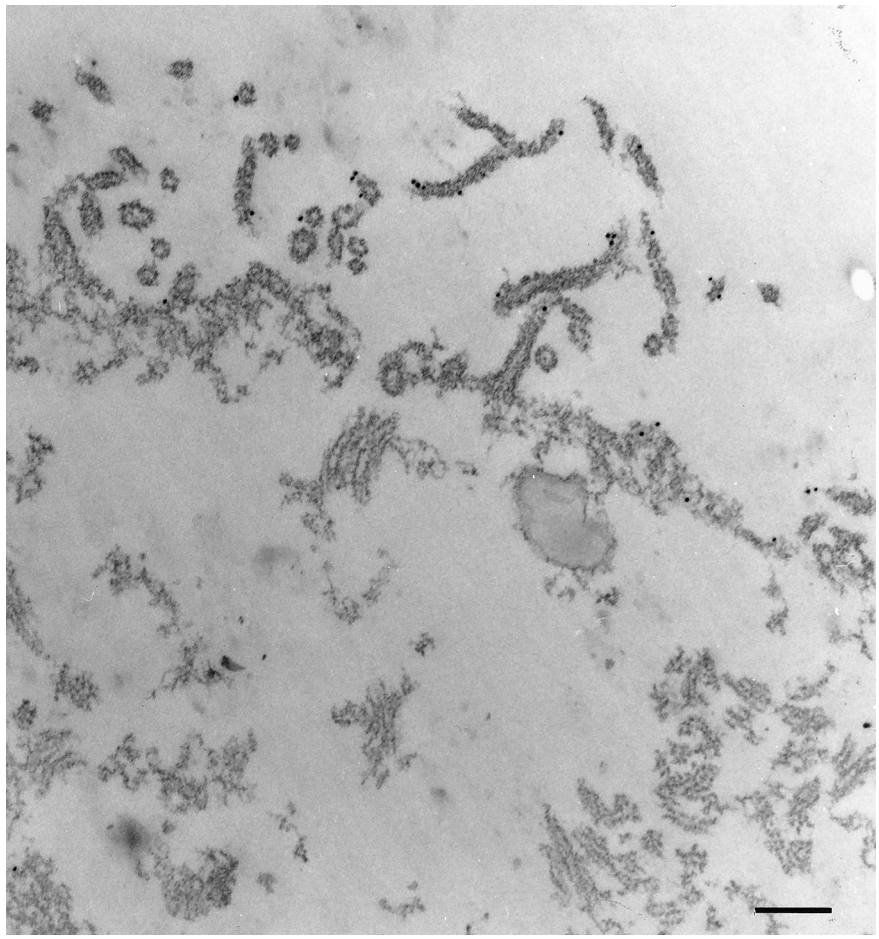

FIG. 6. Ezrin is concentrated in microvilli. Immunogold localization of ezrin in compacted 8-cell stage mouse embryo. Bar, 0.25 μm.

DISCUSSION

During the preimplantation development of the mouse embryo, cell polarization permits asymmetrical cell division to occur that can generate the two cell populations present in the blastocyst: the trophectoderm and the inner cell mass. Some elements of the cytoskeleton, like the microtubules and the actin filaments, are involved in the formation and stabilization of cell polarization (Gueth-Hallonet and Maro, 1992). However, these elements are distributed throughout the cell and are not specific to the surface pole domain. To understand the mechanisms involved in these processes, the molecules that segregate specifically in the surface pole of microvilli must be identified. Among actin-associated proteins, myosin, α-actinin, and vinculin are present in the mouse embryo. Vinculin exhibits a diffuse cytoplasmic distribution. It forms a subplasmalemmal layer that accumulates in the areas of intercellular contact. After compaction, vinculin is concentrated in areas of cell contact (Lehtonen and Reima, 1986). α-actinin is also concentrated in the regions of intercellular contact (Lehtonen and Badley, 1980). Myosin forms a continuous cortical band in apical borders and is never detected in the regions of cell contact, but no clear polarization is observed (Sobel, 1983). So far, none of the actin-binding proteins described in the mouse embryo follows exactly the distribution of microvilli and none seems to be involved in the triggering of polarization. In this study, we have attempted to determine if ezrin, a protein involved in the interactions between the actin cytoskeleton and the plasma membrane, could be involved in the formation and stabilization of the microvillous pole in preimplantation mouse embryos.

Due to the 75% sequence identity among ezrin, moesin, and radixin, it is important to address the issue of antibody specificity. Sato *et al.* (1992) have described several antibodies that cross-react with all three proteins. In mouse embryos, using an antibody directed against human ezrin, we have detected a single band migrating at 84 kDa and which was not detected if the anti-ezrin antibody was preincubated with the immunogenic protein. Moreover, on two-dimen-

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sional immunoblots, this antibody recognized only two to three closely related spots. These results allowed us to conclude that the antibody recognizes specifically murine ezrin and does not cross-react with other ERM family members potentially present in the mouse embryo. With this specific antibody directed against ezrin, we are able to characterize, for the first time, ezrin in the preimplantation mouse embryos.

We have shown that ezrin is present throughout preimplantation development in the mouse embryo. A quantitative analysis of ezrin protein has shown that the amount of ezrin decreases during this period. We observed that a blastocyst contains approximately 50% less ezrin than a 1-cell stage embryo. Brinster (1967) studied the total protein content of mouse embryos and observed approximately a 25% drop from the 1-cell stage to the blastocyst stage. The protein content falls constantly throughout this period. However, the drop in ezrin content speeds up after the 8-cell stage. Between the 8-cell stage and the blastocyst stage 35% of ezrin disappears, while the total protein content only decreases by 4%. Thus, the pattern of ezrin protein loss is different to that observed for proteins in total. This drop could be explained by a difference between the synthesis and the degradation of ezrin, because of either a decrease in ezrin synthesis with a constant rate of degradation or an elevation of the rate of degradation at compaction with a constant rate of synthesis. We were not able to distinguish between these two possibilities since we were unable to assess the synthesis of ezrin by immunoprecipitation after biosynthetic labeling. It is important to note that the increase in ezrin loss begins at the moment of compaction, when the blastomeres undergo a reorganization of their cytoskeleton, cytoplasm, cytocortex, and surface. The decrease in the amount of ezrin could thus be either a consequence or a cause of this reorganization and could depend upon the interaction of ezrin with the cytoskeleton and/or the membrane. Tsukita *et al.* (1994) have shown that ezrin and the other ERM proteins interact with the plasma membrane via an associated protein which is the hyaluronan receptor CD44. It seems that part of the protein is associated with an 85-kDa CD44 isoform in an unstable and dynamic manner and the other part is stably associated with a 140-kDa CD44 isoform. The amount of ezrin could also be regulated by controlling its interaction with CD44 isoforms. The major sites of expression of CD44 in adult murine tissues are spleen, thymus, liver, intestine, and uterus (Kennel *et al.*, 1993), and, although CD44 has not been characterized in preimplantation mouse embryos, it was observed at the surface of all cells during preimplantation human development (Campbell *et al.,* 1995).

The mRNA coding for ezrin is present from the oocyte to the blastocyst stage. In the mouse embryo, the zygotic genome is activated during the 2-cell stage. Thus, the mRNAs detected before this activation are provided maternally, since the mouse oocyte inherits a large supply of maternal mRNA. However, the bulk of this mRNA is eliminated in the 2-cell embryo (Flach *et al.,* 1982; Piko and Clegg, 1982). The presence of ezrin mRNA at all stage suggests that the zygotic gene is rapidly transcribed after destruction of the maternal mRNA. However, the small decrease in the amount of ezrin observed in mouse embryos cultured in antisense oligonucleotides compared to those embryos cultured in T6 alone or in the presence of sense oligonucleotides shows that only a small percentage (about 10%) of the ezrin found in the blastocyst is of zygotic origin. This decrease demonstrates that the antisense oligonucleotides had penetrated the plasma membrane and were active in embryonic cells. Moreover, when we treated embryos during interphase of the third or the fourth cell cycle with puromycin, an inhibitor of protein synthesis, no significant decrease in ezrin content could be observed when compared to controls (data not shown; it was not possible to perform longer incubations since they would lead to the arrest of cell divisions and development). Finally, our experiments suggest that the maternally inherited ezrin protein is sufficient for preimplantation development of the mouse embryo. This is reminiscent of the observations that E-cadherin −/− embryos compact normally because of the maternal store of E-cadherin (Larue *et al.,* 1994; Riethmacher *et al.,* 1995).

Before compaction, ezrin is homogeneously distributed at the cell cortex. At compaction, ezrin distribution becomes polarized. In late 8-cell embryos, it is restricted to the apical pole and at later stages it is found exclusively in the apical domain of the plasma membrane of outer polarized cells. This localization is not due to the fixation procedure used in our experiments since two proteins localized in the basolateral domain, E-cadherin and connexin 43, could be observed using the same experimental protocol (Aghion *et al.,* 1994). These results are also consistent with the localization of ezrin in epithelial cell (Berryman *et al.,* 1993), in which it is preferentially associated with the plasma membrane in microvilli. Indeed, immunoelectron microscopy on compacted 8-cell revealed that ezrin was associated only with microvilli. Ezrin is therefore the first actin-binding protein localized exclusively to the cortical domain of mouse embryo blastomeres that follows exactly the distribution of microvilli. Until now, the only molecule identified as a polarization marker of mouse blastomeres was a membrane protein, the Na+/glucose cotransport system, the distribution of which was enriched in the apical domain (Wiley *et al.,* 1991). Thus, ezrin appears to be the first described marker of surface polarity directly associated with the microvillous pole. The changes in ezrin distribution were detected concurrently with the polarization of microvilli, suggesting a possible role of ezrin in surface polarization. This role is further supported by the continued presence of ezrin at the apical pole during mitosis and by its complete segregation in outer cells after an asymmetrical division.

The association of ezrin with the cytoskeleton has been suggested to be dependent on phosphorylation. In A-431 cells, the redistribution of ezrin to the newly formed microvilli after treatment with EGF is concomitant with a phos-

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phorylation on tyrosine residues and an increase in phosphorylation on serine residues (Bretscher, 1989). In mouse embryos before the 8-cell stage, two isoforms of ezrin are detected. We have shown that they are both phosphorylated on tyrosine residues. Two tyrosine residues have been identified on the ezrin protein as phosphorylation sites *in vitro* as well as *in vivo* (Krieg and Hunter, 1992). Tyrosine 145 is found in the N-terminal domain and tyrosine 353 is present in the α-helix of the C-terminal domain. The two tyrosine-phosphorylated isoforms of ezrin identified in mouse embryos could correspond to different degrees of phosphorylation on the tyrosine residues. An interesting result is the appearance of a third isoform of ezrin at the 8-cell stage. The amount of this third isoform increases at later stages of preimplantation development and it is more acidic and slightly heavier, consistent with an hyperphosphorylation, although it is not phosphorylated on tyrosine residues. However, it is known that ezrin can also be phosphorylated on other residues. For example, the relocalization of ezrin in the microvilli of gastric parietal cells is accompanied by an increase in phosphorylation on serine residues and a increased association of ezrin with the cytoskeleton (Hanzel *et al.,* 1991). In A-431 resting cells, ezrin is also phosphorylated on serine and threonine residues (Gould *et al.,* 1986). It is possible that the third isoform of ezrin detected from the 8-cell stage corresponds to a phosphorylation on serine and/or threonine residues.

It is important to underline the concommitance between the appearance of the third isoform and the timing of polarization. This result could suggest that this isoform is involved in the stabilization of ezrin within the microvillous pole at compaction. Another important observation is the coincidence of the drop in the amount of ezrin and compaction. This destruction of part of the ezrin pool during compaction could facilitate the disappearance of microvilli in the basolateral domain. We are currently designing experiments to test these hypotheses.

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