

# Changes in the Distribution of a Spectrin-like Protein during Development of the Preimplantation Mouse Embryo

J. SABINA SOBEL and MARY ANNE ALLIEGRO

Department of Anatomical Sciences, State University of New York at Buffalo, Buffalo, New York 14214

**ABSTRACT** The mouse blastocyst expresses a 240,000-mol-wt polypeptide that cross-reacts with antibody to avian erythrocyte  $\alpha$ -spectrin. Immunofluorescence localization showed striking changes in the distribution of the putative embryonic spectrin during preimplantation and early postimplantation development. There was no detectable spectrin in either the unfertilized or fertilized egg. The first positive reaction was observed in the early 2-cell stage when a bright band of fluorescence delimited the region of cell-cell contact. The blastomeres subsequently developed continuous cortical layers of spectrin and this distribution was maintained throughout the cleavage stages. A significant reduction in fluorescence intensity occurred before implantation in the apical region of the mural trophoblast and the trophoblast outgrowths developed linear arrays of spectrin spots that were oriented in the direction of spreading. In contrast to the alterations that take place in the periphery of the embryo, spectrin was consistently present in the cortical cytoplasm underlying regions of contact between the blastomeres and between cells of the inner cell mass. The results suggest a possible role for spectrin in cell-cell interactions during early development.

The cortical cytoskeleton of the preimplantation mouse embryo contains actin microfilaments and microtubules (7), intermediate filaments of the cytokeratin type (15), myosin (31), and  $\alpha$ -actinin (20). A developmental function for these components has been inferred from the changes in cortical organization and from the effects of cytoskeletal disrupting agents on major developmental transitions during preimplantation development (7, 27, 35, 36). Essentially nothing is known, however, about the actual mechanisms whereby the cytoskeleton influences cell shape and polarization, locomotion, and contact interaction between the cells.

The recent discovery of spectrin-like proteins in many nonerythroid cells and the possibility that nonerythroid spectrin may function in a manner analogous to erythroid spectrin (for reviews see references 6, 8, 12, 18) is of potential significance for an understanding of developmental processes because spectrin, in association with actin and other components of the membrane cytoskeleton, is a major determinant of cell shape, deformability and fusogenicity, and the mobility of membrane proteins (1, 3, 23, 25). The present study was undertaken to see when spectrin appears during embryonic development. Using immunoblotting procedures, a 240,000-mol-wt polypeptide was identified in the mouse blastocyst that cross-reacts with antibody to avian erythrocyte  $\alpha$ -spectrin. Immunofluorescence localization, moreover, shows changes in the distribution of the putative embryonic spectrin that suggest a role for spectrin in cell-cell interactions during preimplantation development.

## MATERIALS AND METHODS

**Collection of Embryos:** Preimplantation mouse embryos were obtained from 5–7-wk-old Institute for Cancer Research (ICR) females (West Seneca Breeding Facility, Roswell Park Memorial Institute, Buffalo, NY). The animals were superovulated with intraperitoneal injections of 5 IU each of pregnant mare's serum (Gestyl, Diosynth, Inc., Chicago, IL), followed 48 h later by injection of human chorionic gonadotropin (HCG)<sup>1</sup> (Pregnyl, Organon Diagnostics, West Orange, NJ) and mated with CB6F<sub>1</sub>/J males (Jackson Labs, Bar Harbor, Maine). Unfertilized and fertilized eggs were recovered 12 h post-HCG; 2-cell embryos 40–48 h post-HCG; morulae, 72 h post-HCG; and blastocysts 96 h post-HCG by flushing oviducts or uteri with modified Hank's balanced salt solution (11). Blastocysts were cultured on coverslips in modified Eagle's medium (34). Protein analyses were performed on embryos that had been washed in bovine serum albumin-free balanced salt solution and stored at  $-70^{\circ}\text{C}$ .

Cerebelli were obtained from 12-d embryos derived from natural matings of ICR females and CB6F<sub>1</sub>/J males.

**Preparation of Erythrocyte Membranes:** Chicken erythrocyte membranes were isolated according to a modification of the method of Granger et al. (14). Blood was collected in 0.02% heparin, 155 mM choline chloride, and 5 mM HEPES (pH 7.4). Erythrocytes were then washed three times in the same buffer without heparin, and the buffy coats were removed by aspiration after each centrifugation. This and the subsequent procedures were carried out at  $0^{\circ}\text{C}$ . The cells were lysed in hypotonic buffer (28) and enucleated by sonication. Sonication (Ultrasonic Decontaminator, Nuclear Associates, Inc., Carle Place, NY) was carried out for 5–7 min or until examination by phase-contrast microscopy indicated at least 90% naked nuclei. Aliquots of the membrane pellet were then suspended in sample buffer that contained 2% SDS, 1.5% dithiothreitol, M Tris (pH 6.8), 10% glycerol, and 0.007% Bromphenol Blue and stored at  $-70^{\circ}\text{C}$ .

<sup>1</sup> *Abbreviation used in this paper:* HCG, human chorionic gonadotropin.

**Electrophoresis:** Protein samples were analyzed by SDS PAGE using the Tris-glycine buffer system of Laemmli (17). The running gel contained 10% acrylamide and 0.13% *N,N'*-methylene bis acrylamide; the stacking gel contained 5% acrylamide and 0.13% *N,N'*-methylene bis acrylamide.

**Antibody Production:** Antiserum directed against erythroid  $\alpha$ -spectrin was raised in rabbits using methods previously described (28). Erythrocyte membranes were run on one-dimensional SDS gels and the  $\alpha$ -spectrin band excised and homogenized in a motor-driven homogenizer. The first injection was emulsified with Freund's complete adjuvant; subsequent injections contained no additives. The rabbit was injected at 25-d intervals and blood was collected 10 d after the third and subsequent injections. IgG fractions were partially purified by fractionation with  $\text{NH}_4\text{SO}_4$  at 50% saturation.

**Immunological Detection of Spectrin on Nitrocellulose:** Protein subjected to SDS PAGE was stained with Coomassie Blue or electrophoretically transferred to nitrocellulose sheets according to Towbin et al. (38). The blotting buffer contained 25 mM Tris, 192 mM glycine, 20% methanol, and 0.1% SDS (9). Blots were washed in 10 mM sodium phosphate, 0.9% NaCl (pH 7.5) in PBS with 0.05% Tween 20 (TPBS) for 30 min, incubated with antibody (1:80 dilution) in TPBS, and washed three times in TPBS. Antibody binding was detected by immunoperoxidase staining using the Vectastain ABC Kit (Vector Laboratories, Inc., Burlingame, CA) according to the manufacturer's instructions. This procedure employs a biotinylated anti-rabbit IgG followed by an avidin biotinylated horseradish peroxidase complex that binds to the biotinylated secondary antibody. Antigen is then localized by incubation in a peroxidase substrate solution. Because of the large number of embryos required to obtain visible bands, only immunoblots and no Coomassie Blue-stained gels were prepared for the preimplantation stages.

**Immunofluorescent Localization of Spectrin in Mouse Embryos:** A minimum of 20 embryos in at least two experiments were examined for each stage of development. Embryo cytoskeletons were prepared as described previously (31). The embryos were rinsed in stabilization buffer, permeabilized with 0.5% Triton X-100 in permeabilization buffer for 60 s at 37°C, rinsed in stabilization buffer, and fixed for 5 min in methanol at -20°C. Embryos were then washed in phosphate-buffered saline and incubated with antispectrin IgG (1:20) for 20 min. After rinsing, the embryos were treated with rhodamine-conjugated goat anti-rabbit IgG (Cappel Laboratories, Cochranville, PA) that had been presorbed with mouse tissues and diluted 300-fold for 15 min and washed in phosphate-buffered saline. Embryos were examined in a Zeiss Photomicroscope 11 and photographed with Kodak Tri-X film. Controls for the specificity of the staining reaction were the omission and preabsorption of the first antibody. Antibody was preabsorbed with avian  $\alpha$ -spectrin that had been electrophoretically eluted from excised gels, dialyzed against  $\text{H}_2\text{O}$ , and lyophilized.

## RESULTS

### *Immunological Cross-reaction of Mouse Embryonic Spectrin with Avian Erythrocyte Spectrin*

Fig. 1 (lanes *a* and *b*) illustrates the co-migration of erythroid  $\alpha$ -spectrin (molecular weight, 240,000) with a weakly staining doublet in embryonic cerebellum. Immunoblots (lanes *c-e*) show that antibody that is specific for erythroid  $\alpha$ -spectrin reacts with a single cerebellar polypeptide, presumably the  $\alpha$ -chain of brain spectrin (fodrin) (2, 13, 22), as previously shown (28), and a co-migrating polypeptide in the mouse blastocyst.

### *Immunofluorescent Localization of $\alpha$ -Spectrin in the Preimplantation Mouse Embryo*

Antibody to erythroid  $\alpha$ -spectrin was utilized for immunofluorescence localization in cytoskeletal preparations of the preimplantation mouse embryo and blastocyst cultures (Fig. 2). Embryonic spectrin was not detectable in either the unfertilized (Fig. 2*a*) or fertilized (Fig. 2*b*) eggs. It was first detected in regions of blastomere apposition in the early 2-cell embryo (Fig. 2*c*), and then appeared in continuous cortical layers at the later 2-cell stage (Fig. 2*d*). A similar cortical staining pattern characterized later cleavage stage blastomeres (Fig.

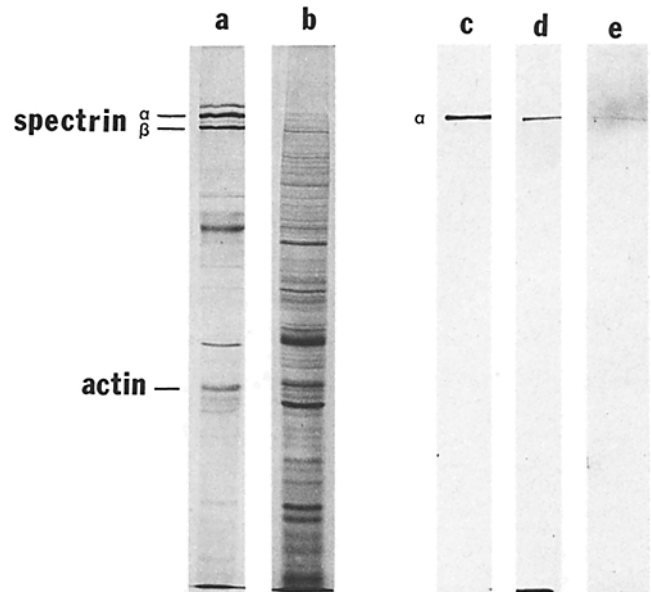


FIGURE 1 Detection of a spectrin-like protein in embryonic mouse cerebellum and blastocysts. Coomassie Blue staining pattern of avian erythrocyte membranes (*a*) and mouse-embryonic cerebellum (*b*) separated in a 10% gel by SDS PAGE. Polypeptides transferred to nitrocellulose sheets (Western blots) that reacted with antibody to avian  $\alpha$ -spectrin were visualized by immunoperoxidase staining. Erythrocyte membranes (*c*) exhibit a single intensely stained band that co-migrates with  $\alpha$ -spectrin. Mouse cerebellum (*d*) and blastocysts (*e*) exhibit a single band with the same mobility as  $\alpha$ -spectrin. Lane *e* is derived from 575 embryos.

2*e*). Polarization of spectrin distribution occurred in the advanced blastocyst. At this stage the mural trophoblast continued to exhibit cortical spectrin at the inner and lateral cell borders while at the same time fluorescence was lost from the apical regions of the cells (Fig. 2*f*). Fig. 2*g* is a view of the dorsal surface of the embryo (apical region of the trophoblast) at higher magnification. Spectrin is again seen to be concentrated in the lateral contacted regions of the cells and appears to be absent from the apical regions. This particular embryo was attached to a coverslip and was at the stage just preceding outgrowth of the trophoblast. The cortical layer in the region where outgrowth would occur is seen to be breaking up into rod-shaped spots. In the spreading trophoblast the spots were arranged in linear arrays that are oriented in the direction of spreading (Fig. 1*h*). At the same time the cells of the inner cell mass continued to exhibit continuous cortical layers of fluorescence (Fig. 2*h*). Paraformaldehyde fixed embryos that had not been permeabilized with Triton X-100 did not stain, indicating an absence of reactive sites on the outer side of the plasma membrane (not illustrated). The specificity of the staining reaction was demonstrated by an absence of fluorescence when the embryos were treated with the preabsorbed first antibody or with only the second antibody (not illustrated).

## DISCUSSION

This study has demonstrated that the preimplantation mouse embryo expresses a 240,000-mol-wt polypeptide that cross-reacts with antibody against avian erythrocyte  $\alpha$ -spectrin. Immunofluorescence localization in cytoskeletal preparations of the embryos showed striking alterations in detectable spectrin in the periphery of the embryo whereas spectrin was

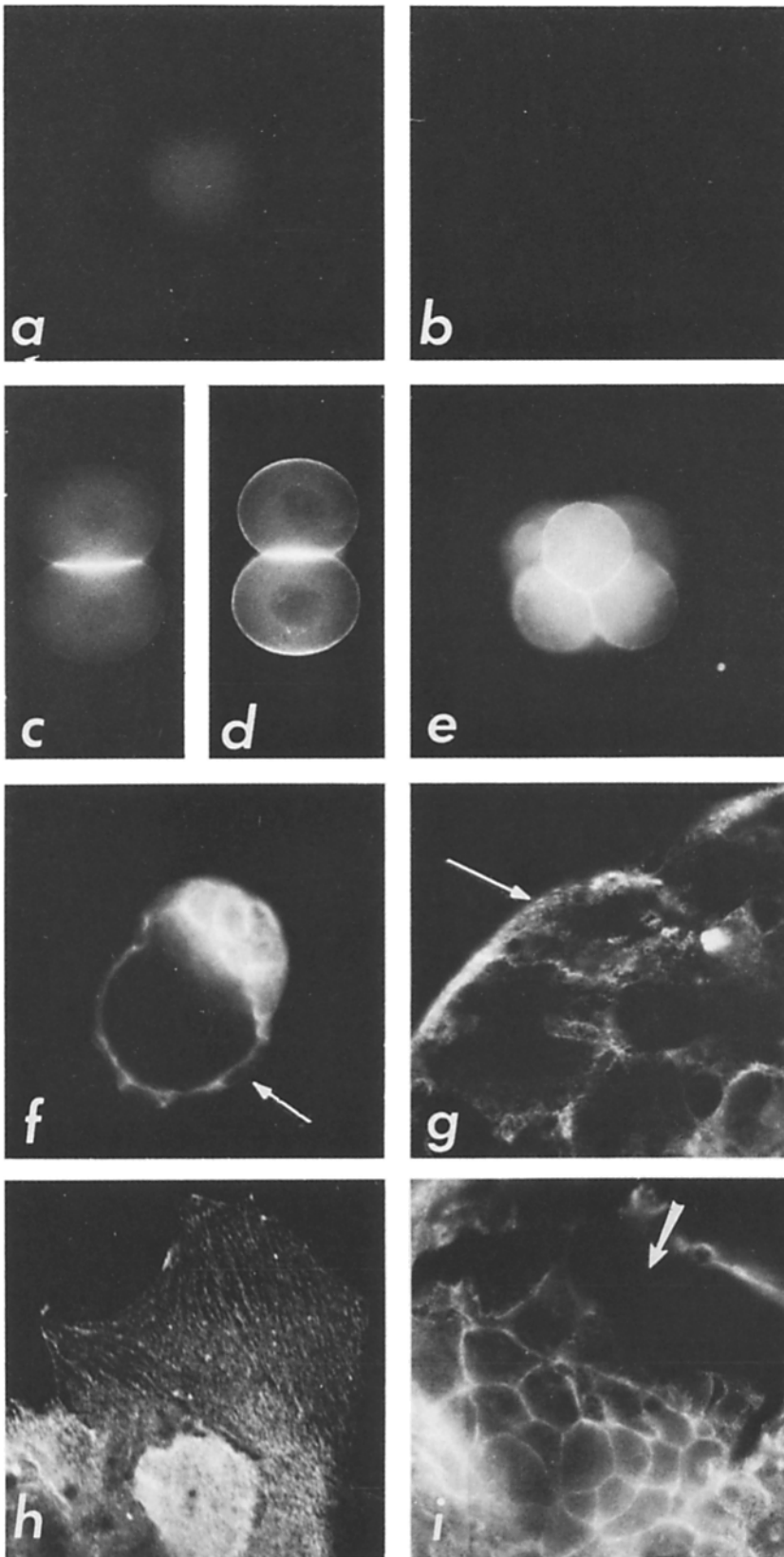


FIGURE 2 Immunofluorescence localization of  $\alpha$ -spectrin in cytoskeletal preparations of preimplantation mouse embryos and trophoblast outgrowths. The unfertilized egg (a) and fertilized egg (b) show almost no detectable fluorescence. A bright band of fluorescence appears in the contact region of the early 2-cell stage (c) and becomes a continuous cortical layer in the later 2-cell stage (d). Continuous cortical bands of fluorescence also characterize the 8-cell embryo (e). The blastocyst (f) exhibits barely detectable fluorescence in the apical border of the mural trophoblast (arrow). A dorsal view of a blastocyst attached to a coverslip (g) illustrates the breakup of the cortical fluorescent layer in the region where spreading begins (arrow). Cultured blastocysts show a radial orientation of fluorescent spots in the trophoblast outgrowth (h) and continuous cortical layers of fluorescence in the inner cell mass (i). The arrow points to the remnant of the blastocoel.

consistently present in the cortical cytoplasm underlying regions of cell contact.

Spectrin was first detected in the region of cell-cell contact in the 2-cell embryo and continued to be associated with contacted regions of the blastomeres during the subsequent

cleavage stages. The receptors mediating contact between the blastomeres seem to be cross-linked to the underlying cytoskeleton since the contacts are resistant to disruption by the nonionic detergent Triton X-100 (32). Spectrin would appear to be a good candidate for the cross-linking agent (19).

Loss of the immunoreactive form of spectrin characterized the periphery of the embryo at two stages of development. The first was in the egg stage when the cortical layer failed to show any detectable fluorescence. A similar reaction has also been reported in sperm (28). Loss of detectable spectrin occurred in the embryo periphery for a second time in the blastocyst stage when the mural trophoblast cells underwent a significant reduction in fluorescence intensity in their apical borders. These are the cells that attach and form junctional complexes with the uterine epithelium (30, 37). Thus, the stages of preimplantation development that are characterized by absent or reduced levels of detectable peripheral spectrin are also the stages when the embryo is preparing to form contact relations with other cells. It should be emphasized that loss of detectable spectrin in the periphery of these embryos does not necessarily reflect loss of the protein. It could also indicate conversion to a soluble form, a masking of antigenic sites, or replacement by an immunologically altered  $\alpha$ -chain (10) at these stages of development.

The pattern of change in the distribution of the putative embryonic spectrin leads us to propose that spectrin may function in cell-cell contact interaction. In such a role a reduction in the level of detectable spectrin may be related to an enhanced potential for cell contact interaction. This change in state could be manifested by cell fusion or by the formation of cell contacts. The presence of detectable spectrin, on the other hand, may be associated with stabilization of these contacts. A seeming contradiction to this hypothesis is the failure to detect redistribution of spectrin during compaction when the blastomeres flatten against each other and form junctional complexes (7, 24). It is quite possible, however, that such alterations were missed due to the low probability of detecting transient events that take place in limited domains of the cell cortex (in contrast to the extensive and long-term changes in the embryo periphery). Moreover, a partial reduction in fluorescence intensity in contacted regions may not be detected because of the nonlinear response of photographic films and prints (5). Present experiments are designed to overcome these limitations by using a model system for compaction that permits continuous monitoring of spectrin distribution in an en face view of the contact region (33).

An anchoring role for spectrin would also appear to be at variance with the suggested motile function of spectrin in the capping of lymphocyte surface receptors (22, 26) and axonal transport of proteins and organelles (21, 22). One explanation for a dual role for spectrin is suggested by the organization of the cortical cytoskeleton of the blastomeres where myosin is specifically lost from the region of cell contact (32, 33). Insofar as the interaction of actin and myosin provides the motive force for movement of membrane proteins (16, 29), a spectrin-mediated linkage of receptors to a myosin-depleted cytoskeleton could immobilize the receptors and restrict them to the contact region. On the other hand, spectrin-mediated linkage to a myosin-containing cytoskeleton (as in lymphocytes [16] and perhaps axons [4]) could involve spectrin in motile activities.

We thank Dr. E. Repasky and Dr. E. Koenig for critical readings of the manuscript.

This study was supported by March of Dimes Birth Defects Foundation Grant 1-812.

Received for publication 7 August 1984, and in revised form 15 October 1984.

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