Fibronectin in the Developing Sea Urchin Embryo

EVELYN SPIEGEL, MAX BURGER, and MELVIN SPIEGEL

Department of Biological Sciences, Dartmouth College, Hanover, New Hampshire 03755, and Biocenter, University of Basel, CH 4056 Basel, Switzerland

ABSTRACT   The presence of fibronectin in developing sea urchin embryos was studied using immunofluorescence staining. The fluorescence pattern indicates that fibronectin is found on the cell surfaces and between cells in the blastula and gastrula stages, indicating that it plays a role in cell adhesion. Its presence on invaginating cells also suggests its involvement in morphogenesis during early development.

Fibronectin is a high molecular weight glycoprotein that is found on the cell surfaces of many cultured vertebrate cells and in the basal laminae and connective tissue matrices of vertebrate cells (10, 19, 25, 26). Of great interest has been the observation that the loss of fibronectin from the surfaces of transformed cell lines is associated with tumorigenicity (11, 22). Some of the properties associated with the loss of fibronectin include a decrease in cell adhesion, cell spreading and cell motility, accompanied by an altered cellular morphology. The addition of fibronectin restores these cells to a more normal morphology and behavior (1, 27). These results have suggested that fibronectin may play a major role in cell adhesion and may also be involved in other cell-cell interactions, such as cell recognition and cell communication (21).

To investigate the role of fibronectin in mediating the interactions and subsequent behavior of cells in vivo, the developing embryo provides a model system. In addition to exhibiting changes in cell-cell interactions, these changes can be followed through morphogenesis and differentiation. A few such studies have been initiated using chick and mouse embryos. Fibronectin has been found in the ectoderm of very early chick embryos, and it is suggested that its function is to facilitate certain morphogenetic movements in early development (4). It has also been found on the surface of undifferentiated mesenchyme cells and loose connective tissue of very early chick embryos (14). In the mouse, fibronectin has been seen in the intercellular spaces of the 4-d embryo and as large fibrils that crisscross the surface of the isolated inner cell mass (28). It is also found in the uterine wall and in Reichert's membrane, an extracellular basal lamina-like structure that surrounds the embryo (24).

Recent evidence has shown that fibronectin binds to collagen (2, 6, 8) and can be cross-linked to proteoglycans (17). It may, therefore, have an organizing role in the formation of the extracellular matrix (ECM) (21). This has led to the hypothesis that the matrix, in addition to its structural role, may also contribute to the orderly growth and positioning of cells (21). It has recently been shown that the sea urchin embryo is

surrounded by an ECM containing proteoglycans, glycosaminoglycans, and collagen fibrils (20). The ECM maintains the cohesion and orientation of cells during cleavage stages and through gastrulation, when invagination of the cells occurs (3, 5, 7). This process involves cell motility, as cells migrate and change their positions; changes in cell adhesion, as micromeres detach and move into the blastocoel; changes in the shapes of cells and of the embryo itself; and changes in the appearance and organization of the ECM (7, 20). These analogies in cell behavior between cultured cells and developing embryos, the presence of an ECM in both sea urchin embryos and cultured cells, and the finding of fibronectin in chick and mouse embryos, led us to investigate the possibility that fibronectin might also be present in an invertebrate system such as the sea urchin embryo. Using the indirect immunofluorescence method, we have found fibronectin on the cell surfaces and between the cells of sea urchin embryos in the blastula and early gastrula stages, in both fixed and live embryos.

MATERIALS AND METHODS

Sphaerechinus granularis were obtained from Banyuls-sur-mer, France, and kept in seawater aquaria at 19°C. Gametes were obtained by electrical stimulation (9). Fertilized eggs were cultured at 21°C until they hatched out of the fertilization membrane, which occurs at the early blastula stage. Hatched embryos were used in order to expose their surfaces to the antisera that could not penetrate the fertilization membrane.

In addition to using embryos without the fertilization membrane, it was also necessary to expose the cell surfaces by removing the ECM (or hyaline layer) by treating with calcium- and magnesium-free seawater (CMFSW). The length of treatment required varied with different batches of eggs and also with the season, and ranged from 20 to 50 min. Removal of the ECM was monitored by light microscopy.

The immunofluorescence method used was adapted from Mautner and Hynes (15) with slight modifications for the sea urchin material. Embryos were fixed in 2% glutaraldehyde in 75% CMFSW, pH 7.8, for 1.5 h, rinsed twice gently so as not to dissociate the embryos, and put into 0.1 M glycine in CMFSW for 1 h. The latter treatment binds aldehyde groups in the fixative which might cause nonspecific fluorescence (18). After rinsing three times in CMFSW, the embryos were pipetted onto polylysine-coated coverslips. 20-µl drops of control serum (1: 5 dilution) or antifibronectin serum (1:25 dilution) were added to each coverslip

and incubated in a moist chamber for 45 min at room temperature. The coverslips were rinsed three times in CMFSW and incubated with 20-μl drops of fluorescein-conjugated goat antiserum to rabbit IgG (Miles Laboratories, Inc., Elkhart, Ind.) (1:10 dilution) for 45 min at room temperature. Coverslips were rinsed three times in CMFSW and once in distilled water. They were mounted in 50% glycerol on slides containing two strips of double sticky tape as a support for the coverslips to prevent crushing of the embryos.

Living embryos, after removal of the ECM, were pipetted onto polylysine-coated coverslips and processed as described above, except that they were incubated in the sera for 20 min instead of 45 min and were mounted after the last CMFSW wash in a drop of CMFSW and examined immediately.

Antiserum against fibronectin, provided by Dr. A. Vaheri, was prepared as indicated by Vaheri and Mosher (22) and specified by Mosher (16). It formed a single precipitin band with purified human fibronectin (Collaborative Research Inc., Waltham, Mass.) or whole plasma.

Monospecific rabbit antibodies were prepared with the help of Dr. J. Finne. The immunogen was purified according to Vuento and Vaheri (23) using gelatin-Sepharose and arginine-Sepharose affinity columns. 300 μg in 1 ml of Freund's adjuvant was administered intramuscularly, once a week for 3 wk. The serum was harvested after an intravenous booster given 1 mo after the last injection. Monospecific antibodies were isolated from a human fibronectin-Sepharose 4B column, as described by Jockusch et al. (12).

Controls included omission of the first serum, pre-immune serum, and anti-fibronectin serum pre-absorbed on a fibronectin-Sepharose column using excess antigen (Collaborative Research). Pre-immune IgG was prepared by a 50% ammonium sulphate step and was used in the control specimen in a concentration at least fivefold higher than the pure monospecific antibodies or the antibodies in the antiserum preparation. Immunogen absorbed rabbit antiserum used as a control was prepared by running the serum over a human fibronectin containing Sepharose 4B gel column (23).

Embryos were examined by phase-contrast and epifluorescence illumination in a Zeiss Universal microscope, equipped with filters and dichroic mirrors for viewing fluorescein fluorescence and an Osram HBO-mercury arc lamp as a light source. Photographs were taken with Kodak TriX film.

RESULTS

The distribution of fibronectin, using the immunofluorescent staining method, was similar in both fixed (Fig. 1) and living (Fig. 2) specimens. Representative individuals of large samples are shown in the photographs and are indicative of the general results obtained. Fig. 1 *a* is a phase-contrast picture of a beginning gastrula of *S. granularis*. It shows the flattening of the formerly spherical embryo at the vegetal pole and the beginning of invagination of cells at the blastopore. At this stage, the embryo is composed of a single layer of cells surrounding the fluid-filled blastocoel. The cell size can be seen most clearly in the upper center of Fig. 1 *a* where the cell borders and dark-appearing nuclei are readily apparent. Fig. 1 *b* is the control using pre-immune serum; no fluorescent staining pattern is visible. Fig. 1 *d–f* are of the same embryo, taken at different levels of focus. Fig. 1 *d* is focused at the equator of the embryo and the fluorescent staining pattern indicates the presence of fibronectin on the exterior cell surfaces. The fluorescence is more pronounced at the blastoporal lip where the cells are beginning to migrate inward. In Fig. 1 *e*, focused away from the equator, fluorescence can be seen between the cells as well as around their exterior surfaces. Fig. 1 *f* is focused even farther from the equator and shows a honeycomb pattern of fluorescent staining between the cells. This pattern is directly correlated with the appearance of the cell borders shown in Fig. 1 *a* and gives further evidence that fibronectin is located between the cells of the embryo.

Fig. 1 *c* shows an early gastrula that has developed slightly beyond the beginning gastrula shown in the other figures. This specimen was also focused at the equator and shows the fluorescent pattern migrating farther inward than in *d*, as the cells continue to migrate inward at the blastopore.

Fig. 2 *a*, is a phase-contrast picture of a living blastula stage embryo. In Fig. 2 *b*, the same embryo, shown with fluorescence

optics, is surrounded by an outer layer of fibronectin. In certain areas near the surface, it can be seen that fibronectin is located between the cells as well as on the exterior cell surface. Fig. 2 *c* is a living blastula that shows some of the cells loosening from their attachment to the embryo as a result of CMFSW treatment. In Fig. 2 *d*, the same embryo, with fluorescence optics, shows the honeycomb pattern of fibronectin between some of the cells. In the area where the cells were coming loose from the embryo, very little fluorescence is seen. Too long an exposure to CMSFW was found to lessen fluorescent staining, probably because of the loss of fibronectin in this area of the embryo.

Fig. 2 *e* is a control specimen in which the first serum was omitted. Fig. 2 *f* is a control specimen that was incubated with pre-immune serum. In both controls, no fluorescent staining pattern is observed. In a further control with antigen-absorbed serum, no fluorescein-staining structures could be seen. A preparation of monospecific, i.e., affinity purified rabbit antibodies, did confirm both location and intensity of the fluorescence patterns seen with the antiserum.

DISCUSSION

This study has presented evidence that fibronectin is present on the outer cell surfaces and between cells of the sea urchin embryo during early development. In Fig. 1 *d*, the exterior cell surfaces show fluorescent staining. In Fig. 1 *e*, fluorescent staining is seen between the cells as well as on their exterior surfaces. In Fig. 1 *f*, the honeycomb pattern of staining of the cell borders is further evidence for the presence of fibronectin between the cells. That these are indeed the cell borders that are stained is borne out by comparison with the cell outlines seen in Fig. 1 *a*. Additional support is given in Fig. 2 *d*, where the honeycomb pattern of fluorescent staining is seen between the cells of the living embryo.

During gastrulation, there appears to be a decrease in fibronectin on the outer cell surfaces as indicated by the narrower rim of fluorescence in Fig. 1 *c* as compared to Fig. 1 *d*. Because the cells in the ectodermal layer are elongating as they migrate inward at the blastopore, it could mean that the fibronectin layer is also elongating or stretching to cover the increased surface area. The fluorescence is much brighter in the invaginating area, which could indicate an increase in fibronectin as the cells migrate inward. It could, however, be caused by the multiple layer of fluorescent cells at the blastopore which causes a brighter glow than does a single layer of cells. The presence of fibronectin on invaginating cells suggests its involvement in morphogenesis during early development.

As mentioned in Materials and Methods, there was great variability in the amount of time required to remove the ECM (hyaline layer) with CMFSW. A longer treatment was required at the beginning of the spawning season than later in the season. There was also occasionally some variability in the ease of removing the ECM between different egg batches at the same time of the season. In addition, as shown in Fig. 2 *c* and *d*, there was even, at times, some variability within the same embryo in its response to the CMFSW treatment. This could indicate localized differences in both the ECM and fibronectin layers surrounding the developing embryo. Fluorescence was not observed if the ECM was not removed before proceeding with the immunofluorescence procedure. The antisera apparently cannot penetrate the ECM to obtain access to the cell surface, nor does staining of the intact ECM occur. These results indicate that although the ECM and fibronectin layers
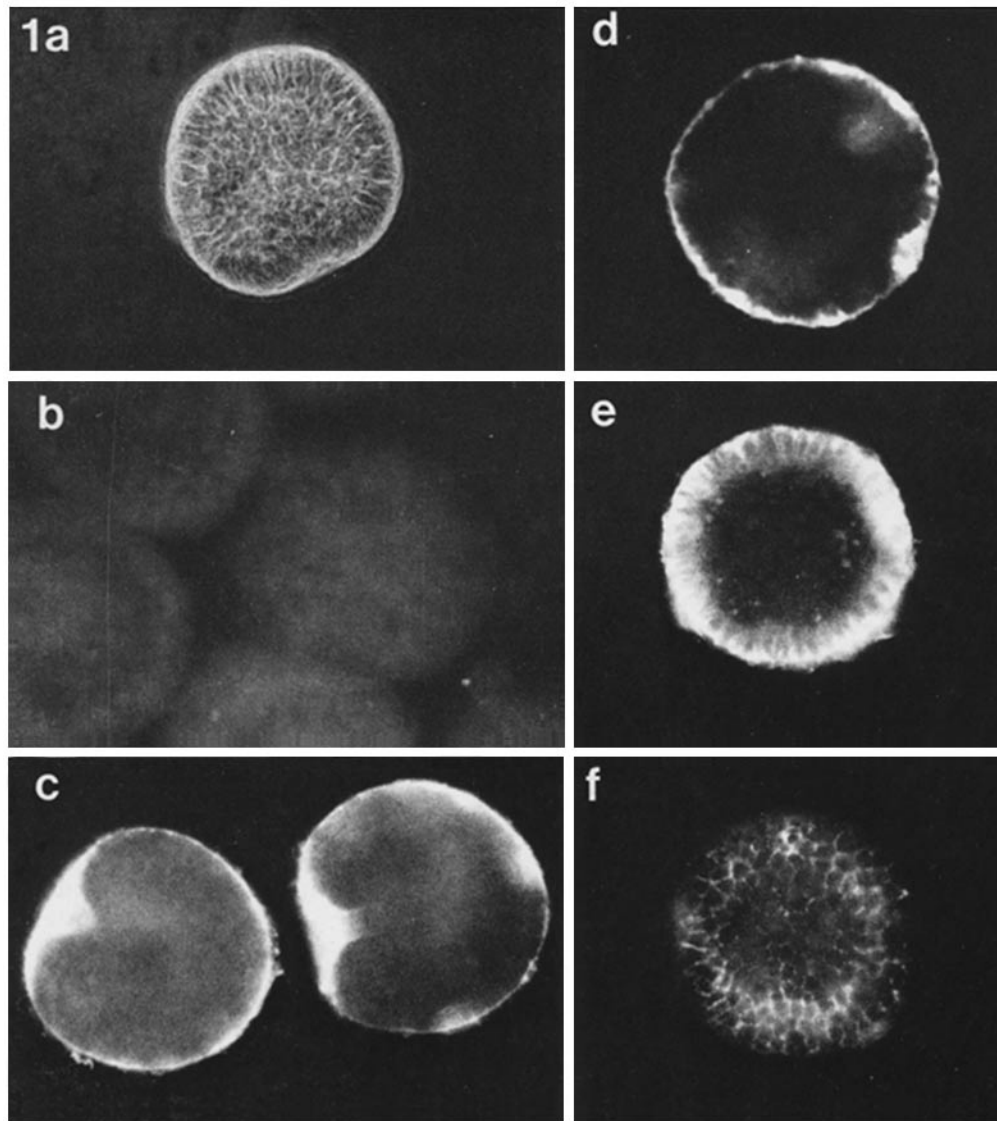
FIGURE 1 Distribution of fibronectin in fixed specimens of S. granularis embryos at the beginning and early gastrula stages. (a) Phase contrast of beginning gastrula. Note cell borders surrounding dark-appearing nuclei in center of embryo. (b) Control embryo incubated with pre-immune serum and showing no fluorescent staining. (c) Early gastrulae showing fluorescent pattern migrating further inward as the cells continue to migrate inward at the blastopore. (d) Beginning gastrula, focused at equator of embryo, showing fluorescent staining pattern of fibronectin on the exterior cell surfaces. Fluorescence is more pronounced at the blastoporal lip where the cells are beginning to migrate inward (at 4 o'clock). (e) Same embryo as in d, focused slightly away from equator, showing fluorescent staining between cells in addition to exterior cell surface staining. (f) Same embryo as in d and e, focused further away from equator. This shows a honeycomb pattern of fluorescent staining indicating the presence of fibronectin between the cells. × 350 (approx.).

are closely apposed and probably adherent to each other, the attachment can be disrupted and the two layers separated. Some fibronectin may also be present in the ECM layer, but it is shielded from access to the antibodies by other macromolecules.

Functions previously ascribed to the ECM, such as cell recognition, cell adhesion, cell motility, and the mediation of other cell-cell interactions, may now be more appropriately ascribed to either layer. In the present study, the role of fibronectin in cell adhesion is supported by the immunofluorescent staining pattern of the sea urchin embryo. Additional support for its role in cell adhesion is shown in Fig. 2c and d. In Fig. 2c, some cells became loose from the embryo upon treatment with CMFSW, and these loose cells did not exhibit fluorescent staining, whereas their still-adhering neighbor cells

did, as shown in Fig. 2d. This indicates that there may be localized differences in the ECM or the fibronectin layer or both. Further study is required, therefore, to ascertain which roles are played by fibronectin and by the ECM, separately and together, during early development and morphogenesis.

The presence of a fibronectin-like protein has been reported in one other invertebrate species, the freshwater sponge, Ephydatia mulleri, during reaggregation of dissociated cells (13). The antifibronectin sera used by these authors and also in our experiments were raised against human plasma fibronectin. This suggests a strong conservation of at least some of the structural features of fibronectin all through phylogenesis (13). Our unpublished observations suggest that fibronectin is also present on the cell surfaces of a number of invertebrate phyla between sea urchins and sponges. It appears, therefore, that
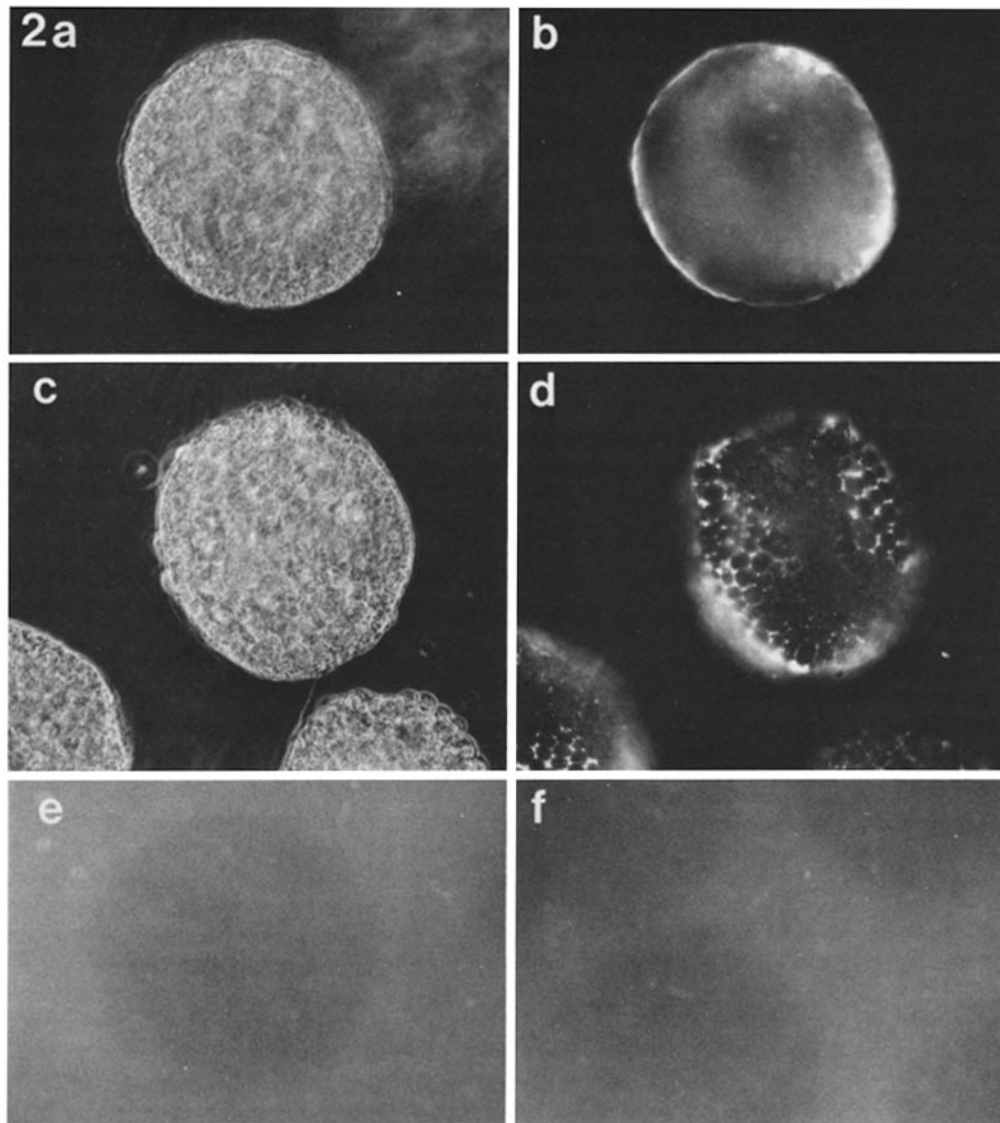
FIGURE 2 Distribution of fibronectin in living specimens of S. granularis embryos at the blastula stage. (a) Phase contrast of living blastula stage. (b) Same embryo as in a, showing fluorescent staining pattern of fibronectin on exterior cell surfaces. In some areas, the staining pattern indicates the presence of fibronectin between the cells. (c) Phase contrast of living blastula showing slight loosening of some cells from their attachment to the embryo due to CMFSW. (d) Same embryo as in c showing honeycomb pattern of fluorescent staining which indicates presence of fibronectin between the cells. No fluorescence is seen where cells are losing their attachment to the embryo. (e) Control embryo in which first serum has been omitted; no fluorescent staining pattern is seen. (f) control embryo treated with pre-immune serum; no fluorescent staining pattern is seen. × 350 (approx.).

fibronectin is a molecule that could be widely distributed over all of the animal kingdom, in adult organisms ranging from humans to sponges, and in their embryos and larvae.

REFERENCES

1. Ali, I. U., V. Mautner, R. Lanza, and R. O. Hynes. 1977. Restoration of normal morphology, adhesion and cytoskeleton in transformed cells by addition of a transformation-sensitive protein. Cell. 11:115-126.
2. Balian, G., E. M. Click, E. Crouch, J. M. Davidson, and P. Bornstein. 1979. Isolation of a collagen-binding fragment from fibronectin and cold-insoluble globulin. J. Biol. Chem. 254:1429-1432.
3. Citkowitz, E. 1971. The hyaline layer: Its isolation and role in echinoderm development. Dev. Biol. 24:348-362.
4. Critchley, D. R., M. A. England, J. Wakely, and R. O. Hynes. 1979. Distribution of fibronectin in the ectoderm of gastrulating chick embryos. Nature (Lond.). 280:498-500.
5. Dan, K. 1960. Cytoembryology of echinoderms and amphibia. Int. Rev. Cytol. 9:321-367.
6. Engvall, E., and Ruoslahti. 1977. Binding of soluble form of fibroblast surface protein, fibronectin, to collagen. Int. J. Cancer. 20:1-5.
7. Gustafson, T., and L. Wolpert. 1967. Cellular movement and contact in sea urchin morphogenesis. Biol. Rev. Camb. Phil. Soc. 42:442-498.
8. Hahn, L. E., and K.M. Yamada. 1979. Identification and isolation of a collagen-binding fragment of the adhesive glycoprotein fibronectin. Proc. Natl. Acad. Sci. U. S. A. 76:1160-1163.
9. Harvey, E. B. 1956. The American Arbacia and Other Sea Urchins. Princeton University Press, Princeton, N. J. 49.
10. Hynes, R. O. 1973. Alteration of cell surface proteins by viral transformation and by proteolysis. Proc. Natl. Acad. Sci. U. S. A. 70:3170-3174.
11. Hynes, R. O. 1976. Cell surface proteins and malignant transformation. Biochim. Biophys.

Acta. 458:73–107.

12. Jockusch, B. M., K. H. Kelley, R. K. Meyer, and M. M. Burger. 1978. An efficient method to produce specific anti-actin. *Histochemistry.* 55:177–184.

13. Labat-Robert, J., M. Ravans de Ceccatty, L. Robert, C. Auger, C. Lethias, and R. Garrone. 1979. Surface glycoproteins of sponge cells: presence of a fibronectin-like protein on differentiated sponge cell membranes; its role in cell aggregation. *In* Glycoconjugates, Proc. 5th Internatl. Symp. Kiel. R. Schauer, P. Boer, E. Buddecke, M. F. Kramer, J. F. G. Vliegenthart, and H. Weigandt, editors. Georg Thieme Publishers, Stuttgart, W. Germany. 431–432.

14. Linder, E., A. Vaheri, E. Ruoslahti, J. Wartiovaara. 1975. Distribution of fibroblast surface antigen in the developing chick embryo. *J. Exp. Med.* 142:41–49.

15. Mautner, A., and R. O. Hynes. 1977. Surface distribution of LETS protein in relation to the cytoskeleton of normal and transformed cells. *J. Cell Biol.* 75:743–768.

16. Mosher, D. F. 1975. Cross-linking of cold insoluble globulin by fibrin-stabilizing factor. *J. Biol. Chem.* 250:6614–6621.

17. Perkins, M. E., T. H. Ji, and R. O. Hynes. 1979. Cross-linking of fibronectin to sulfated proteoglycans at the cell surface. *Cell.* 16:941–952.

18. Peters, K., and F. M. Richards. 1977. Chemical cross-linking: Reagents and problems in studies of membrane structure. *Annu. Rev. Biochem.* 46:523–551.

19. Ruoslahti, E., A. Vaheri, P. Kuusela, and Linder. 1973. Fibroblast surface antigen: A new serum protein. *Biochim. Biophys. Acta.* 322:352–358.

20. Spiegel, E., and M. Spiegel. 1979. The hyaline layer is a collagen-containing extracellular matrix in sea urchin embryos and reaggregating cells. *Exp. Cell Res.* 123:434–441.

21. Vaheri, A., K. Alitala, K. Hedman, J. Kiski-Oja, M. Kurkinen, and J. Wartiovaara. 1978. Fibronectin and the pericellular matrix of normal and transformed adherent cells. *Ann. N. Y. Acad. Sci.* 312:343–353.

22. Vaheri, A., and D. F. Mosher. 1978. High molecular weight, cell surface-associated glycoprotein (fibronectin) lost in malignant transformation. *Biochim. Biophys. Acta.* 516:1–25.

23. Vuento, M., and A. Vaheri. 1979. Purification of fibronectin from human plasma by affinity chromatography under non-denaturing conditions. *Biochem. J.* 183:331–337.

24. Wartiovaara, J., I. Leivo, I. Virtanen, and A. Vaheri. 1978. Cell surface and extracellular matrix glycoprotein fibronectin: expression in embryogenesis and in teratocarcinoma differentiation. *Ann. N. Y. Acad. Sci.* 312:132–141.

25. Yamada, K. M., and K. Olden. 1978. Fibronectins—adhesive glycoproteins of cell surface and blood. *Nature (Lond.).* 275:179–184.

26. Yamada, K. M., and J. A. Weston. 1974. Isolation of a major cell surface glycoprotein from fibroblasts. *Proc. Natl. Acad. Sci. U. S. A.* 71:3492–3496.

27. Yamada, K. M., S. S. Yamada, and I. Pastan. 1976. Cell surface protein partially restores morphology, adhesiveness and contact inhibition of movement to transformed fibroblasts. *Proc. Natl. Acad. Sci. U. S. A.* 73:1217–1221.

28. Zetter, B. R., and G. R. Martin. 1978. Expression of a high molecular weight cell surface glycoprotein (LETS protein) by preimplantation mouse embryos and teratocarcinoma stem cells. *Proc. Natl. Acad. Sci. U. S. A.* 75:2324–2328.