Organization of actin filaments in early chick embryo ectoderm: an ultrastructural and immunocytochemical study

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SUMMARY

By combining transmission electron microscopy (TEM), scanning electron microscopy (SEM) and immunocytochemistry at both light and TEM levels the organization of actin in early chick ectoderm has been demonstrated. Every cell is encircled by a ring of actin filaments which are associated with apically situated intercellular junctions. In this way the actin ring of each individual cell is connected to that of surrounding cells and a continuous network is formed. As clearly shown by immunofluorescence and SEM studies on intact and Triton X-100 treated whole-mount preparations this network extends throughout the entire layer, interrupted only at the blastoderm margins and the primitive streak. A more diffuse arrangement was sometimes seen at cell bases. It is suggested that the network extending throughout the ectoderm gives it stability and cohesion and is important in gross morphogenetic movements involving the entire layer. The less-organized arrangement at cell bases is associated more with movements of individual cells.

INTRODUCTION

Epithelial cells in vitro show two major behaviour patterns which are not generally seen in fibroblasts. They form extensive and longstanding lateral adhesions with their neighbours and the continuous sheets thus created move as a unit with only the marginal cells undergoing active locomotion (Di Pasquale, 1975). Such united movements of whole sheets of cells implies the existence of some co-ordinating mechanism. Albrecht-Buehler (1979) has demonstrated that when PtKI cells form cohesive epithelial sheets in vitro, actin microfilament bundles appear to continue in direction across cellular boundaries thus providing a ‘tissue’ level of cytomuscular organisation and the possibility of a mechanical form of communication between cells. Meittenen, Virtanen & Linder (1978) describe a similar situation for sheets of rat hepatocytes in vitro. The ectoderm of the early chick embryo is a single relatively flat layer of

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epithelial cells which ultimately participates in a wide range of morphogenetic movements, and whose general ultrastructure is relatively well known (Trelstad, Hay & Revel (1967), Bancroft & Bellairs (1975). The emphasis of the present study was on the arrangement of actin filaments both within individual cells and throughout the layer as a whole.

This was of interest partly from the point of view of using the ectoderm as a generalized and easily obtained isolated example of an epithelium in vivo, and partly because the contraction of actomyosin filaments has been widely implicated in many developmental processes in which epithelial deformation occurs (Wessels et al. 1971; Karfunkel, 1972; Burnside, 1971; Burgess & Schroeder, 1979). Normal embryos were examined by scanning (SEM) and transmission (TEM) electron microscopy and immunocytochemistry at light (LM) and EM levels. Triton X-100 extracted preparations, as frequently used for cultured cells, (Brown, Levinson & Spudich, 1976; Small, Isenberg & Celis, 1978; Bell, Miller, Carraway & Revel, 1978; Ip & Fischman, 1979) were made of whole embryos and also examined by SEM and TEM. It was hoped that in this way any physical continuity of the cytoskeleton from cell to cell might be more clearly visible, unobscured by other cellular components.

MATERIALS AND METHODS

White Leghorn eggs were incubated at 37 °C to produce embryos at stages 3–8 (Hamburger & Hamilton, 1951).

Five normal embryos each for scanning (SEM) or transmission electron microscopy (TEM) were dissected from the eggs into Pannet & Compton
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saline (1924) washed free of adherent yolk then fixed overnight in Karnovsky’s (1965) fixative. Fixed embryos were washed in 0.2 M-sodium cacodylate buffer pH 7.3, post-fixed in 1% cacodylate-buffered osmium tetroxide and dehydrated in a graded ethanol series. For TEM embryos were transferred from absolute alcohol to propylene oxide then embedded in Araldite. For SEM embryos were transferred to acetone then critical-point dried after replacing acetone with liquid CO₂ in a Polaron apparatus. Dried specimens were mounted on aluminium stubs with double-sided Sellotape and coated with 20 nm of gold in an Edwards S150 sputter coating unit.

Ten embryos for Triton X-100 extraction were taken from the eggs and washed in Hank’s solution, then transferred to 0.5% Triton X-100 in Hank’s solution at room temperature for 10–15 min. They were then fixed and prepared for either SEM (5 embryos) or TEM (5 embryos) as above.

Antisera were prepared against chicken gizzard actin in New Zealand white rabbits previously screened for low pre-immune activity against acetone-extracted fibroblasts. Serum collected from these rabbits after five injections was found to show antiactin activity. Gamma globulin fractions were collected from this serum.

Antibodies were further purified by affinity chromatography on columns of sepharose 4B to which actin had been coupled (1–10 mg/g wet weight) using cyanogen bromide.

The immunoglobulin was then eluted with 4 mM MgCl₂ at pH 6 dialysed against phosphate-buffered saline. 200 μg/ml solutions were stored at 4°C in the presence of 0.02% sodium azide.

Antibody specificity was checked by comparing the staining activity of the prepared antibody with preimmune antibody preparations and with absorbed antibody, using rat embryo fibroblasts and chicken gizzard myofibrils as test samples.

Embryos for immunofluorescence were fixed in 3% formaldehyde in phosphate-buffered saline (PBS), for 15–30 min. They were washed overnight in PBS before staining and as much as possible of the endoderm and mesoderm dissected off. Staining was carried out on 30 embryos, processed on slides with the embryos lying ectoderm-side uppermost. Of these embryos 8 were control incubations performed either with absorbed antiserum (3) or without antiactin IgG (5). Details of the staining procedure and the control incubations performed were as described by Badley, Lloyd & Woods (1978). Immunoperoxidase staining was performed using the method of Couchman et al. (1979). Treated embryos and controls were examined as whole-mounts (3 treated, 3 control), after alcohol dehydration and xylene clearing or, after Araldite embedding, by LM and TEM in sections (4 treated, 4 control). The same pattern of staining was obtained each time the procedure was carried out. The only variations obtained were regional, as described in the results.

Sections were cut on a Porter Blum MTI Ultramicrotome. For LM 1 μm
sections were stained with 1% Toluidine blue in 1% borax. For T.E.M. some
cytochemical and all normal and Triton X-100 treated specimens were stained
after sectioning with uranyl acetate and lead citrate. Some immunocytochemical
material was examined unstained.

S.E.M. images were recorded on a ISI 60 Scanning Electron Microscope;
T.E.M. material was examined on a JEOL 100S and fluorescent and other
light microscope studies were made on a Zeiss Universal Microscope with an
epifluorescence u.v. illumination system, photographed on Kodak Ektachrome
400 or Ilford HP5 film.

RESULTS

Light, scanning and transmission electron microscopy – untreated material

The ectoderm cells varied in shape from a flat squamous epithelium in the
peripheral (area opaca) parts of the embryo to a low columnar form in the
central (area pellucida) regions (Figs. 1–3).

In all areas ectoderm sectioned in the plane of the epithelium formed a
tightly packed epithelial sheet (Fig. 4). Each cell was attached to surrounding
cells by junctions situated a short distance below the cell apex. Most of these
junctions were simple in structure, consisting of a plaque-like thickening of
the apposed cell membranes, with little narrowing of the intercellular gap.
Under the plasma membrane a concentration of fibrillar material was present,
coextensive with the membrane thickening. Sometimes a little electron-dense
material was seen in the intercellular gap at a junction but true desmosomes
were very rare. Occasional small junctions showing close membrane apposition
were also seen. Their structure was not investigated further to identify them
positively as gap or tight junctions. At the level of the junctions each cell was
ringed by a bundle of (8 nm) microfilaments, lying immediately beneath the
plasma membrane. The filament bundle appeared to be connected to the
fibrillar material associated with cell junctions (Fig. 5). No filament bundles
were seen other than in association with apical cell junctions. Intermediate

Fig. 2. 1 μm Araldite section of anterior area pellucida ectoderm stained with
toluidine blue. The cells form a pseudostratified columnar epithelium. The basal
surface (arrowed) of the cells has artificially separated from the mesoderm. Some
basal cell processes (p) can be seen. Stage-4 embryo.

Fig. 3. 1 μm Araldite section of the same embryo as Figure 2, showing the cells
of the area opaca ectoderm (eo) and the large yolky cells of the endoderm (en).

Fig. 4. Scanning electron micrograph of mid-area pellucida ectoderm of stage-5
embryo. The epithelial cells are closely packed. The visible apical surfaces show
long and short microvilli (mv); and beaded threads (t).

Fig. 5. Transmission electron micrograph of tangentially sectioned ectoderm of
stage-4 embryo, passing through the level of the junctions. Deep to the opposing
cell membranes is a zone containing many microfilaments (mf). At junctions a
more electron-dense fibrous mat may be seen (arrow).
filaments (10 nm) were not evident. Microtubules were present, crossing the cell apex in all directions, not obviously aggregated into bundles or preferentially associated with any other organelle.

Deeper into the cell, at the level of the nucleus, the peripheral ring of microfilaments disappeared and no specialized cellular junctions were seen (Fig. 6).

The base of the cells rested on a thin basement membrane. This was thicker and more compact in appearance in samples taken from the area pellucida than from the area opaca. It was also better developed in the older embryos in the series. The plasma membrane of the cell was separated by a gap 10–30 nm wide from the basement membrane. No plaques or other specialized contacts between the plasma and basement membranes were observed. Contacts between adjacent cell bases were complex, although no membrane specializations were seen. Many cells formed processes, which either extended for a short distance under the flat base of the neighbouring cell, partly separating it from its portion of the basement membrane (Fig. 7), or interlocked with a similar process from a neighbouring cell. These specializations were particularly common in the area pellucida near the primitive streak. Near such cell contacts and in the processes fine bundles of microfilaments were sometimes seen in the cytoplasm immediately next to the plasma membranes (Fig. 7). In many places, however, the general cytoplasmic features of free ribosomes, intracellular yolk, mitochondria, and microtubules extended right down to the membrane.

Scanning and transmission electron microscopy – Triton X-100-treated material

Cell ultrastructure was grossly disrupted. Nuclei and large intracellular yolk droplets were still recognisable but most other organelles including microtubules were seen by TEM to have dispersed during treatment. In general plasma membranes had also disappeared, except for the junctional sites and a small membrane area immediately surrounding the junction. Apical microfilament bundles were clearly seen. It was clear that whole microfilament bundles did not insert end-on into the junctions but passed alongside them and were linked by the fibrillar mat to the plasma membrane (Fig. 8). It was not possible to ascertain whether individual filaments terminated at the junctions. Sections below the cell apex did not show this organization.

In maximally extracted embryos viewed by SEM the entire sheet of ectoderm

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**Fig. 6.** Stage-4 ectoderm sectioned at the level of the nuclei (*n*). No microfilament bundles can be seen.

**Fig. 7.** Vertically sectioned ectoderm cells, showing the base of the cell and the basement membrane (*bm*). The cell on the left sends a process under the cell on the right (*p*). The process and the cell which it underlaps contain microfilaments (*mf*).
Fig. 8. Transmission electron micrograph of Triton X-100-treated ectoderm. The cytoplasm has largely disappeared, leaving yolk particles (y) and other debris (d). Surviving portions of the plasma membrane (m) show junctions (j) associated with a fibrillar mat (f) and bundles of microfilaments (mf).

Fig. 9. Scanning electron micrograph of Triton X-100-treated ectoderm. The epithelial organisation is still apparent, as a basket-like framework of Triton-resistant cytoskeletal material (arrows) outlining former cell boundaries, in which nuclei (n) and yolk (y) are suspended.
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cells was represented by a basket-like meshwork of linked filaments. Deep to the meshwork regularly spaced nuclei were seen, sometimes suspended by sparse fine filaments. Some spherical yolk droplets were also identified. Examination of specimens showing varying degrees of disruption by the detergent showed that the Triton X-100-resistant filaments followed cell perimeters so that each unit in the meshwork represented an encircling band of filaments associated with one cell. At high magnifications the filaments of adjacent cells appeared to be separated by a narrow space representing the position of the cells’ membranes (Fig. 9). Apart from variation in size, the area pellucida cells being smaller in diameter than those of the area opaca, there were no regional or age differences.

Immunochemistry

Because of the thickness of whole-mount specimens background fluorescence was invariably high in immunofluorescent-stained embryos although endoderm removal helped to some extent to reduce this problem. Against the background a patterned distribution of specific fluorescence was seen. This was not present in control embryos processed in the absence of antiactin IgG, or with the use of absorbed antisera.

Fluorescence associated with the presence of actin outlined the perimeter of every cell throughout the area pellucida and area opaca of the embryos. Thus a continuous fluorescent network was formed extending throughout the ectoderm almost without interruption (Fig. 10). The pattern changed at the blastoderm margins where the regular network was replaced by irregular fluorescent strands and at the primitive streak where increased thickness of the specimen made it impossible to resolve cell outlines clearly but the general impression was that the pattern was destroyed. Differential focussing showed that the fluorescent network was situated at the cell apices. Focussing deeper into the cells usually failed to demonstrate discrete patterns of fluorescence at other levels in the cytoplasm. However a more diffuse fluorescence could be detected nearer to the base of the cells and occasionally short fluorescent strands were seen more clearly in the flattest cells from the area opaca, where the fluorescent image was less obscured by an overlying thickness of cytoplasm than in the area pellucida (Fig. 11).

Immunoperoxidase staining examined by light microscopy in whole-mount embryos demonstrated a pattern very similar to that seen by immunofluorescence. A band of dark reaction product outlined the apex of every cell, giving the epithelium as a whole a net-like appearance. The pattern was not seen in control embryos from which the specific antiactin antibody alone, or both that and the peroxidase-labelled goat anti-rabbit serum were omitted from the staining procedure. By T.E.M. electron-dense deposits were seen associated with bands of filaments passing alongside apical cell junctions. They were not seen on other cellular organelles or in control embryos. (Figs. 12, 13).
DISCUSSION

The combination of techniques used in this investigation has demonstrated the presence of a band of actin filaments encircling every cell in the ectoderm of gastrulating chick embryos and attached tangentially to the junctions linking each cell to its neighbours. Thus, as clearly demonstrated by S.E.M. of detergent-extracted material, the peripheral ring of actin in each individual cell is linked to those of the surrounding cells. In effect there is a coherent network of actin forming a ‘skeleton’ to the entire epithelium, broken only where the epithelial organization of the ectoderm is itself destroyed; at the primitive streak and at the margins. The arrangement of actin and junctions in chick ectoderm is similar to that described by Perry (1975) for amphibian embryonic ectoderm and quite different from that seen by Albrecht-Buehler (1979) & Meittenen et al. (1978) in cultured epithelial sheets where bundles of filaments stretch across the cell apex from junction to junction. Retinal pigment epithelium in vitro shows an intermediate situation with filaments both encircling and crossing the cell apex (Crawford, 1979). The intercellular junctions are also of a type common in developing epithelia (Perry, 1975; Sanders, 1973; Mobbs & MacMillan, 1979). The nearest adult equivalent is the zonula adherens Farquhar & Palade, 1963) associated with actin in cultured cells (Miettenen et al. 1978).

In single non-muscle cells cytoplasmic actin forms part of an actomyosin contractile system, which is involved both in the generation of movement and the stabilization of cell shape (Fleischer & Wohlfarth-Bottermann, 1975; Pollard, 1976; Isenberg et al. 1976; Adelstein, Scordilis & Trotter, 1979); Epithelia in vitro characteristically behave as sheets rather than as individual cells (Di Pasquale, 1975). Perry (1975) suggested that in amphibian embryos a similar ‘tissue level’ organization of actin filaments to that which we have seen in the chick might act as a strengthening framework for the ectoderm during gastrulation. In the gastrulating chick embryo the ectoderm appears to

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Fig. 10. Immunofluorescence micrograph of part of the inner area opaca ectoderm of a stage-5 embryo, (antiactin staining). A peripheral band of fluorescence surrounds every cell.

Fig. 11. Immunofluorescence micrograph of outer area opaca ectoderm of stage-5 embryo. As well as the peripheral localization of actin small strands are faintly discernible nearer to the centre of the cell (arrows).

Fig. 12. Transmission electron micrograph of junctional region of Triton X-100 extracted ectoderm cells studied by the immunoperoxidase method to demonstrate actin. Bundles of filaments (arrowed) associated with the junctions (j) show an overall electron density and larger electron dense deposits. These features are absent from the control specimen. Fig. 13. From which the specific antiactin antibody was omitted. In neither case was any heavy metal staining applied to the sections.
be in a state of tension. Ectodermal wounds immediately gape into round holes (Stanisstreet, Wakely & England, 1980). Cells round the wound margins bulge as though released from tension. Ectodermal tension is at least partly caused by blastoderm expansion (New, 1959; Bellairs, Bromham & Wylie, 1967) and also by an opposite flow of cells towards and into the primitive streak (Nicolet, 1971; Rosenquist, 1966). Cytoskeletal continuity could give the epithelium necessary cohesion to resist being disrupted by this internal tension. The actin net may itself contribute to the tension by participating in an isometric contractile process (Isenberg et al. 1976). Also forces generated by cell-shape changes in one part of the embryo may be transmitted beyond the actively deforming cells so producing morphogenetic changes in whole areas of tissue.

Electron microscopy reveals some sparse filaments at cell bases, some forming a sheet and some forming more organised fibre bundles often associated with cellular processes. The arrangement parallels the general background and short strands seen at cell bases by immunofluorescence. It is reminiscent of that seen by Sanders & Prasad (1979) in the endodermal cells of chick embryos of a comparable age in vitro and Di Pasquale (1975) and Crawford (1979) in cultured epithelial cells. The association of organized microfilament bundles with cell-substrate contacts is also a consistent feature of fibroblasts growing on substrates in culture (Izzard & Lockner, 1976; Badley, Woods, Smith & Rees, 1980; Heath & Dunn, 1978). Similarities such as these suggest an association with cell locomotion. The basal processes and their associated microfilament bundles in ectoderm cells are much smaller and less highly organized than the sole plate of a fibroblast and there are no focal contacts, as judged by the lack of cytoplasmic dense plaques with associated actin filaments. The similarity does, however, suggest a similar functional association between the cells and their substrate (the basement membrane) and that therefore during morphogenetic movements, for example, the migration of ectoderm towards the primitive streak, the cells could migrate over their own basement membrane, until they reach the primitive streak, where the membrane is broken down and epithelial arrangement is lost as the cells join the mesoderm. The morphology of cell bases did not vary regionally within the ectoderm, suggesting that all cells were capable of some movement of this nature.

In summary, then, actin filaments in chick ectoderm appear to be organized in two patterns. The majority of the protein forms an apical band surrounding every cell and linked to that of neighbouring cells via cell junctions. A second smaller system is situated near the basement membrane and associated with basal cytoplasmic processes and less closely linked to that of neighbouring cells. The two structural arrangements reflect two different functions performed by actin in the ectoderm. The basal concentrations of actin are perhaps more directly associated with active locomotion, with the basement membrane as substrate, than the association of actin and cell junctions at the cell apices
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which gives the epithelium as a whole cohesion and stability to enable it to perform complex morphogenetic movements involving large numbers of cells, while at the same time retaining its epithelial integrity.

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