DIFFERENT CYTOSKELETAL DOMAINS IN MURINE FIBROBLASTS

J. H. M. TEMMINK* AND H. SPIELE
The Netherlands Cancer Institute, Division of Cell Biology, 121 Plesmanlaan, 1066 CX Amsterdam, The Netherlands

SUMMARY

The morphology of the cytoskeleton of normal 3T3 fibroblasts was studied in the electron microscope on embedded and sectioned cells as well as on critical-point-dried whole mounts. Four ultrastructurally different cytoplasmic domains could be distinguished within every 3T3 cell, namely: Cytocortex, ruffle area, endoplasm, and stress fibre. However, many transitions between the different domains were also found. In addition, 2 other domains were occasionally encountered in the same cells. Based on the knowledge that actin is present in relatively high concentration throughout non-muscle cells and that it can, under different physiological conditions, assume different morphological appearances, an attempt has been made to formulate a hypothesis that links the ultrastructural domains to different cellular functions. Many experimental approaches are available to test the hypothesis and at the same time increase our knowledge of the cytoskeleton of 3T3 cells.

INTRODUCTION

Over the past few years it has become increasingly clear that many fundamental functions of cells are influenced by or dependent on the action of contractile protein structures in the cytoplasm. Not only attachment and movement of the whole cell (spreading and locomotion) are apparently regulated by these contractile structures (Clarke & Spudich, 1977; Goldman, Schloss & Starger, 1976; Hitchcock, 1977; Pollard & Weihing, 1974), but also the movement of many different cell membrane receptors seems to be partly dependent on them (Nicolson, 1976). In spite of the great number of studies that have resulted from the understandable interest in morphology and function of the cytoskeletal elements, most questions in this connexion have only received tentative answers. This is undoubtedly due to the enormous complexity of the cytoplasmic contractile elements (Porter, 1978) and to the sensitivity of their functioning to small changes in their microenvironment (Pollard, 1976b; Taylor, 1977).

Two different avenues can be followed in attempts to elucidate the function of the contractile and other elements of the cytoskeleton. The first has been successfully taken by several investigators (Condeelis & Taylor, 1977; Pollard, 1976a; Stossel & Hartwig, 1976; Wang & Singer, 1977), who made cytoplasmic preparations in vitro, experimentally manipulated these, and then correlated physical changes in the prep-

* Present address: Agricultural University, Dept. of Toxicology, De Dreijen 12, 6703 BC Wageningen, The Netherlands.
arations with the electron-microscope images after fixation. These valuable studies in which functional changes are secondarily tied to the morphology of the contractile elements can be complemented with results of a reverse approach. In that approach a morphological description of the intracellular cytoskeletal elements under different experimental conditions is linked to the observable macroscopic effect of these conditions upon the living cell. The obvious advantage of the latter approach is that experiments are done with complete and living cells; a clear disadvantage is that treatment of the complete cellular system probably has many different effects at the same time. Nonetheless, we have opted for this primarily morphological approach. Although this has been taken before by several investigators (Goldman et al. 1973; Pollard, 19766; Wessells, Spooner & Ludueña, 1973), additional data can be obtained from cells that have been dried by the critical point method and studied as whole mounts (Buckley, 1975; Wolosewick & Porter, 1976).

The present paper gives an ultrastructural description of 3T3 murine fibroblasts growing under more or less normal conditions, with emphasis on the different areas that can be distinguished in the cytoskeletal system of the cells. Following papers will be concerned with changes of this ‘standard’ morphology as brought about by physical and chemical manipulation of the growing 3T3 cells.

**MATERIAL AND METHODS**

**Cell culture**

Normal Swiss 3T3 fibroblasts were grown as described before (Collard & Temmink, 1976) in Dulbecco’s modified Eagle’s medium with newborn calf serum and antibiotics. For transmission electron microscopy of embedded and sectioned material, cells were grown on carbon-coated coverslips; for transmission electron microscopy of critical-point-dried whole mounts, cells were grown on gold grids coated with Formvar and carbon. Cell cultures were fixed when at or close to confluency.

**Electron microscopy**

For conventional transmission electron microscopy cells were fixed in glutaraldehyde (2.5% in 0.1 M cacodylate buffer, pH 7.2) for 30 min, followed by osmic acid (1% in the same buffer) for 15 min. Sometimes postfixation in osmic acid was reduced (0.025% for 5 min) (Temmink & Spiele, 1978). After several rinses in buffer, the cells were dehydrated in a graded series of ethanol. During dehydration contrast was enhanced by a block staining in uranyl acetate (2.5% in 100% ethanol). After embedding in a mixture of Epon and Araldite, the cells were sectioned almost parallel to the substrate, stained with lead hydroxide, and studied in a Philips EM 300 operating at 60 kV.

Whole mounts of 3T3 cells received the same fixation, block staining, and dehydration procedure as embedded cells. They were, however, dried by the critical-point method, coated with a layer of carbon and studied in a Philips EM 300 operating at 100 kV. For stereo microscopy, specimens were photographed at tilt angles between 6° and 12° from the horizontal axis.
Different cytoskeletal domains

Fig. 1. Low-magnification image of the anterior part of a critical-point-dried, whole-mounted 3T3 cell. Roman numbers in boxed areas refer to the domains as described in the text: I-cytocortex; II-ruffle area; III-endoplasm; IV-stress fibre. The white part at bottom left is a bar of the supporting grid. A network of ER strands is seen throughout the endoplasm (arrowheads). × 7500; bar, 2 μm.
OBSERVATIONS

Fig. 1 is a low-magnification image of part of a critical-point-dried 3T3 whole cell mount. It shows a number of clearly distinguishable domains that have been given numbers.

Domain I will be designated **cytocortex** (**cc**). Although areas of different width with this ultrastructure occur all around the cell just underneath the plasmalemma, it is most prominent in the advancing lamellipodia. For that reason it has also been called lamelloplasm (Vasiliev & Gelfand, 1977). It is characterized by a high concentration of ground substance and a complete exclusion of all cellular organelles. The only disturbance of its uniform ultrastructure occurs when a ruffle is formed (Fig. 1) or when it is traversed by stress fibres (**sf**) or microtubules (**mt**) that run into filopodia (Fig. 2). At higher magnification the ground substance in domain I is seen to consist of very short, interconnected threads and ovoid-to-round electron-dense globules, forming a relatively fine-mazed network (Fig. 2). Once recognized, this network can also be seen in thin sections of embedded cells (Fig. 3), although much less clearly (Temmink & Spiele, 1978). In whole mounts it has been described before as the subplasmalemal region (Buckley, 1975).

Domain II in Fig. 1 will be called **ruffle area** (**ra**). Although we assume that it is a sudden functional adaptation within a local part of domain I, we prefer to distinguish it from domain I on the basis of its clearly different ultrastructure. In scanning micrographs it is very apparent that a ruffle is part of the cellular lamellipodia that is lifted above the plain of the substrate and retracted (Vasiliev & Gelfand, 1977). How this phenomenon is linked with ultrastructural changes can be seen in the stereo pair of Fig. 4. The upper part of the cytocortical network of fine filaments seems to condense into a few electron-opaque bands of cytoplasmic material. In the dense core of the ruffle area no separate filaments can be discerned any longer, but the laterally trailing parts clearly consist of parallel oriented, thick fibres. A similar, vertical gradient of morphological changes is superimposed over and combined with the lateral one. Together they seem to tighten and roll back the upper cell membrane.

Domain III in Fig. 1 will be labelled **endoplasm** (**ep**) (Vasiliev & Gelfand, 1977). It consists of a lattice of interconnected microfilaments that has been described as a microtrabecular system (Wolosewick & Porter, 1976). In contrast to the filamentous network of domain I, the threads of the lattice in domain III are longer and the mazes of the lattice are much wider (Fig. 5). In addition, the whole system seems only to form the ground substance of an area that harbours all common cellular organelles.
Different cytoskeletal domains
J. H. M. Temmink and H. Spieles

like nucleus, mitochondria, lysosomes, and clustered polysomes. A network of ER also intertwines with the microfilamentous lattice (Fig. 1, arrowheads). Through the lattice of the endoplasm run a number of microtubules (Fig. 5), that are sometimes oriented in roughly the same direction, but sometimes seem to have a random course.

It should be stressed at this point that there is no clear separation between the network of domain I and that of domain III in whole mounts. The microfilaments are interconnected throughout the cell and the transition in density of the lattice is gradual. Nevertheless, we prefer to make a distinction between the 2 forms of the system. Partly for functional reasons that are admittedly hypothetical at this stage (see Discussion), partly because of morphological differences in embedded cells. As mentioned before (Temmink & Spieles, 1978), the lattice of the cell body is not well preserved in embedded specimens, resulting in a relatively ‘empty’ background for the well known cell organelles in this material (Fig. 6). On the other hand, the embedded specimens clearly illustrate that another filamentous system, the intermediate filaments (IF), traverses the cell body. This system cannot be clearly recognized in whole mounts of the fibroblasts (Temmink & Spieles, 1978), perhaps because it consists in part of the same actin filaments (Buckley, Raju & Stewart, 1978).

As domain IV we consider the stress fibre (SF) (Fig. 1). They have been described extensively in whole-mount cells (Heath & Dunn, 1978; Wolosewick & Porter, 1977), as well as in sectioned material (Goldman et al. 1973; Wessells et al. 1973). Most of them occur close to the cytocortex of the under and lateral sides of the cell, just inside the cell body. The microfilaments in a stress fibre are continuous with the microfilaments forming the lattice of domain III. As a matter of fact, here too the transition between the 2 domains is gradual. Towards the centre of a stress fibre the microfilaments assume an increasingly parallel course and the remaining cross-connexions become increasingly shorter, thus decreasing the distance between the parallel oriented microfilaments (Figs. 6, 7). Local densities can be recognized occasionally in whole mounts (Fig. 7, arrow), but are more prominent in sectioned and stained material (Fig. 6, arrows). In most fully spread cells the course of the stress fibres is generally parallel to one another and in the direction of the anterior–posterior polarization of the cell. However, in less well spread cells stress fibres radiate out from a core area of increased electron density (Figs. 8, 9).

The 4 domains described above can be found in every 3T3 cell that is well spread and attached to the substrate. For that reason they may be considered as normal differentiations of the cytoplasm. Occasionally, however, 2 other domains could be...
Different cytoskeletal domains
found in the whole mounted, critical-point-dried cell. Without implying that they are abnormal, we suggest that they apparently form as a result of more specific inducing stimuli or are more transient in character. The first of these domains (a) is illustrated in Fig. 7 and in the stereo pair of Fig. 10. They are generally found underneath or right behind a ruffle and consist essentially of a network of profiles that may, to a greater or lesser extent, be broken up into vesicular structures. The ground substance is not different from that in the surrounding areas of the cytocortex, although it seems less dense. The profiles and vesicles are very close to the upper or lower plasmalemma (Fig. 10) and often the vesicles apparently are extruded from the cell (Fig. 7).

The second domain (b) that is found in some critical-point-dried cells also appears as a specialization in or near the cytocortex (Fig. 11). It is characterized by a distinct increase in electron density without a concomitant structural differentiation. The higher electron density apparently results from an increased number of the ovoid-to-round globules present in the cytocortex.

DISCUSSION

The rather dramatic difference between images of critical-point-dried cells and images of embedded and sectioned cells raises the question of artefact induction by the methods used and of the possibility of comparing the results. This subject has been treated in previous papers (Porter, 1978; Temmink & Spiele, 1978), and the present authors maintain that both methods facilitate electron-microscopic imaging of essentially the same cellular elements and that the results therefore are mutually complementary.

As has been stated explicitly a number of times, none of the domains described above can be unequivocally delimited from neighbouring domains in the cell. However, in their 'purest' form they are distinctly different from each other and this morphological distinction may well be indicative of functional differentiations. The ubiquity of transitional areas between the domains gives proof of the fact that the domains are all part of one dynamic cytoplasmic system with a high degree of flexibility. This system is known to consist in large part of contractile proteins and its flexibility is illustrated by the many cellular functions in which it is known or assumed to play an important role.

Fig. 6. Part of embedded and sectioned 3T3 cell showing a stress fibre (sf) in an area of endoplasm with microtubules (mt) and intermediate filaments (if). Note that endoplasm looks rather empty and that stress fibre has some areas of increased electron density (arrows). × 32000; bar, 0.5 μm.

Fig. 7. Anterior part of critical-point-dried 3T3 cell with relatively wide cytocortex (cc) and endoplasm (ep) with network of ER profiles. Note that distinction between endoplasm and the developing stress fibre (sf) is less clear in this instance. An electron-dense area in the stress fibre is marked (small arrow). The boxed area behind the collapsed ruffle indicates domain a (see text) with its high concentration of smooth vesicles and profiles. Some of these seem to be extruded from the cell (big arrow). × 14000; bar, 1 μm.
Different cytoskeletal domains
Figs. 8, 9. Sections through less well spread parts of embedded 3T3 cells, showing cores (arrow) with microfilaments radiating from them in all directions. ×36000; bar, 0.5 μm.

Thanks to several studies on other cell systems (Condeelis & Taylor, 1977; Condeelis, Taylor, Moore & Allen, 1976; Stossel & Hartwig, 1976; Taylor, 1977), something is known about the mechanism that may enable the cell to adapt so flexibly to changing chemical and physical conditions or environmental requirements and to maintain a dynamic but integrated identity. We will tentatively link some of these mechanisms to structures found in our 3T3 cells, thus creating a framework for future experiments. It is based on 2 assumptions. The first is that the mechanochemical system encountered in completely different cell systems, will in many respects be the same in murine fibroblasts. This seems reasonable because of the presence of the same cytoskeletal elements in these cells and the detection of many similar proteins of the contractile system (Pollard & Weihing, 1974). The second assumption is that different states of the system do have a recognizable morphological correlate. It should be corroborated by the present and future work.

Depending on physical and chemical conditions and on the association with other protein molecules, actin can occur in a ‘gel’-state and in a ‘sol’-state (Condeelis & Taylor, 1977). Transition from one to the other can be induced in vitro and is thought

Fig. 10. Stereo image of domain a (see text) behind and underneath a ruffle. Vesicles are abundant in this domain. ×23000; bar, 1 μm.

Fig. 11. Cytocortex (cc) in a critical-point-dried cell with a specialized area (domain b; see text) of increased electron density and stress fibres (sf) running into a (broken) filopodium (fp). ×14000; bar, 1 μm.
Different cytoskeletal domains
to be possible in vivo. Since actin has been shown to be present throughout the cell (Lazarides, 1976; Weber & Groeschel-Stewart, 1974; Webster, Henderson, Osborn & Weber, 1978), it is the component that probably is responsible for the continuity underneath the domains that we have distinguished.

We, therefore, hypothesize that the main morphological difference between the cytocortex (domain I) and the endoplasm (domain III) is due to the fact that the cytocortex consists of actin (and several actin-binding proteins) in the gel-state, whereas the endoplasm contains a network of actin in the sol-state. The gel supposedly consists of a fibrous aggregate of F-actin cross-linked by actin-binding proteins. Under certain conditions (filopodia formation, ruffling) these cross-linked actin filaments might align into parallel bundles (Heggeness, Wang & Singer, 1977). In both forms the gel would serve a cytoskeletal function. Towards the centre of the cell (part of) the actin would get solated and lose (part of) its actin-binding proteins, thus facilitating the formation of bundles (stress fibres) that contained myosin and would show ATP-dependent contraction. The general difference between the 2 domains may result from a gradient in pH or Ca concentration, maintained by the cell (Condeelis et al. 1976; Taylor, 1977). This hypothesis can be verified by manipulating these parameters by drugs and micro-injection experiments.

Whether the formation of stress fibres is triggered by extracellular stimuli, like contact phenomena, is unknown. One possible mechanism would be that stress fibre formation is induced in areas where the cell makes contact with the substrate (Goldman et al. 1976). If the area illustrated in Figs. 8 and 9 is such an attachment site (Abercrombie & Dunn, 1975; Badley et al. 1978; Heath & Dunn, 1978), the radially developing microfilament bundles might result from the contact. This induction hypothesis is congruent with the notion that transformed cells have much less contact areas with the substrate and also a much less developed cytoskeleton (Goldman, Yerna & Schloss, 1976; McNutt, Culp & Black, 1973). The formation of ruffles (domain II) and their ultrastructure fits into this general framework. Ruffles are retracting parts of the lamellipodia (Fig. 4). They require the formation of fibres as a prerequisite for the development of a contractile system. Bundle formation may be induced again by extracellular contact (Trinkaus, Betchaku & Krulikowsky, 1971; Heggeness et al. 1977) and contraction in the ruffle may induce bundle formation and cellular retraction in areas where myosin is present (Condeelis & Taylor, 1977). Immunofluorescence microscopy has shown that this is the case (Weber & Groeschel-Stewart, 1974).

The function of domain a, the blister-like structure, is unclear. The occurrence near ruffles suggests that it might have a function in re-integrating or extruding the access surface membranes after retraction of the ruffle. However, it cannot be excluded that the structure is a fixation artefact (Hasty & Hay, 1978), occurring in the most vulnerable areas of the cells. Domain b may, judging by its preferent location behind ruffles, be the ultrastructural equivalent of the myosin-free, filamin-containing area found by Heggeness et al. (1977) in their fluorescent study of NRK fibroblasts. A more detailed investigation of this area is needed to corroborate this suggestion.
Different cytoskeletal domains

The main purpose of the hypothesis presented here is to connect a number of ultrastructural observations into a tentative framework. Many of the underlying assumptions are amenable to experimental verification. Subsequent results will undoubtedly necessitate modifications of the hypothesis, but in the process our understanding of the complex cytoskeleton of 3T3 cells and its role in fundamental cellular functions will have increased.

The authors are grateful to Dr. J. G. Collard for his willingness to grow the 3T3 cells, to Mr. N. Ong for printing the photographs, and to Mrs. M. A. van Halem for typing the manuscript.

REFERENCES


J. H. M. Temmink and H. Spiele


(Received 11 July 1979)