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Phil. Trans. R. Soc. Lond. B 1982 **299**, 169-176
doi: 10.1098/rstb.1982.0123

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Phil. Trans. R. Soc. Lond. B **299**, 169–176 (1982)
Printed in Great Britain

Cell–substratum interactions in the adhesion and locomotion of fibroblasts

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[Plates 1 and 2]

The locomotion of primary fibroblasts from explants of embryonic chick heart as well as the subsequent development of focal adhesions by which they ultimately anchor to the substratum in becoming stationary and entering the growth cycle, is shown to involve membrane–substratum interactions in which both exogenous (serum-derived) and endogenous (cell-derived) fibronectins play a part. These conclusions are based on evidence from the influence on cellular functions of changed availability of the two fibronectin species and from the action of specific antisera to them. Immunoelectron microscopy of focal adhesions, intermediate (i.e. microfilament-associated) cell–cell junctions of secondary fibroblasts, and contacts interpreted as stages in the formation (or perhaps dissociation) of both these types of specialization, shows that fibronectin is not detectable when such contacts are fully sealed, thus confirming reports by others; however, it can be detected clearly at other stages, suggesting that it becomes masked (or perhaps removed) late in the condensation of the adhesive structure. Further experiments, in which the expression of cellular fibronectin was suppressed and substrata were coated with laminin (another matrix protein), showed that neither endogenous nor exogenous fibronectin is obligatory for the formation of focal adhesions, thus pointing to a degree of interchangeability of the source and nature of extracellular proteins that mediate cellular contacts. On substrata coated with a new protein spreading factor, we have characterized a stage in the development of adhesions to substratum in which specialized structures are only partly formed and the externalization of cellular matrix products is arrested. These results are discussed in terms of mechanisms of formation of fibroblast adhesion and the roles of adhesion in cellular functions.

1. Introduction

Our contribution to this session entitled ‘What are the basic characteristics of locomotion?’ will be to consider, for fibroblasts, the interactions at areas of membrane contact with substratum – firstly in locomotion itself and then in several other expressions of cell motility. The additional motile functions to be addressed will be: spreading from the rounded state, bracing to the substratum when adhesion stabilizes and the cells become stationary, and the export of matrix materials that then follows.

2. Endogenous and exogenous fibronectin in fibroblast locomotion

A classic system for study of the locomotion and social behaviour of tissue cells has been the migration of fibroblasts from explants of embryonic chick heart (Abercrombie 1980).

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In our earlier work (Couchman & Rees 1979) we showed that these cells undergo a phenotypic change as they gradually become established in culture. Initially the cells are growth-arrested, secrete little matrix material, and move at about 60–80 μm h^{-1}; eventually they divide, secrete extracellular matrix copiously, and are essentially stationary. With these and other changes in cellular activity (Couchman & Rees 1982), relations change between the cytoskeleton, cell membrane and substratum, in that (Couchman & Rees 1979) early cells show diffusely organized actin by immunofluorescence with substratum contacts being broad, unspecialized and dynamic; late cells have actin as bundles terminating at focal adhesions, which are stable and long-lived and with the surrounding cell underside lifted clear from the substratum.

Previously we also showed that, even in the continuous presence of serum fibronectin, the addition of cell-derived fibronectin accelerates conversion of the locomotory to the stationary phenotype (Rees *et al.* 1981; Couchman *et al.* 1982*c*). This implies that cell-derived fibronectin is more effective than serum fibronectin in the cell-surface interactions that support the stationary phenotype. To explore this question further (Couchman *et al.* 1982*c*) we prepared antisera specific for each of the two forms of fibronectin in the system, namely foetal calf serum fibronectin and chick cell fibronectin. It is well known that anti-fibronectin can cause rounding of stationary fibroblasts, presumably by perturbing the interactions between fibronectin and the cell surface (Yamada 1978). *Both* antisera had this type of effect on the stationary chick cells, suggesting that, despite the fact that cell-derived fibronectin is more potent in its positive effects as shown by the experiments described above, both sources of fibronectin are actually incorporated into the relevant cell surface complexes so that antibodies to either can destabilize the spread state.

To examine the role of fibronectins in cell locomotion (Couchman *et al.* 1982*c*) we first studied explants in fibronectin-depleted serum and found that the outward migration of cells was severely impaired. Movement was, however, re-established in the same medium if the coverslip was first treated with serum fibronectin and then washed. Substratum-bound fibronectin therefore appears to be necessary to mediate the membrane contacts for locomotion. This conclusion was confirmed by the action of the antibody specific for serum fibronectin, which caused locomotion to cease and the cells to round up. The antibody to chick fibronectin had a similar effect, suggesting, as for stationary cells, that both endogenous and exogenous fibronectins take part in the necessary cell-surface interactions – even though the locomoting cells (as previously shown (Couchman & Rees 1979)) produce cellular fibronectin at low levels.

The antigen for the second rounding antibody was localized by indirect immunoelectron microscopy with the use of protein A – colloidal gold conjugate, showing small patches on the cell surface, each associated with an electron-dense submembranous plaque and a tuft of wispy, extracellular strands; these structures, which we call 'non-contacting plaque structures', are also seen in secondary fibroblasts (figure 1*a*, for micrographs showing them in locomoting primary cells, see Rees *et al.* (1981) and Couchman *et al.* (1982*c*)). Since they are reminiscent in morphology of focal adhesions and cell-cell junctions of the intermediate type (Zonulae and Fascial adhaerentes), we propose that they are related in origin to these adhesion structures. Because they are observed in cells unable to form adhesions and junctions (Couchman *et al.* 1982*c*), it is unlikely that they represent dissociation products of the latter structures. Rather it is more likely that they are precursors because (i) they correspond in size to the subunits that can be observed in focal adhesions (Badley *et al.* 1980*b*) and not to fully developed

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adhesions or junctions, and (ii) in the temporal sequence of events leading to the formation of focal adhesions, they are seen to develop from small foci staining for α-actinin and vinculin, which are similar in dimension to non-contacting plaque structures (Couchman *et al.* 1982*a*). These plaque structures could therefore lead to labile adhesions for cell locomotion, to stable adhesions for anchorage, or to intercellular junctions, depending on other cellular control mechanisms. The requirements for both cell-derived and substratum-bound fibronectin would suggest an interaction between such fibronectin-bearing specializations on the cell surface with fibronectin bound on the substratum (no doubt with other molecules also involved in the interactions), to unite in a single bonding structure. A similar mechanism could lead to the formation of cell–cell junctions through fibronectin-bearing specializations on apposing cell surfaces. Such a simple-minded model is, however, immediately open to questions and objections, and much of the remainder of this paper will be concerned with examination of these with modification of the hypothesis where necessary.

3. Fine structure of cell adhesions and junctions

A first objection to the hypothesis that adhesions and junctions are formed through the interaction of fibronectin-bearing surfaces could be that fibronectin is often not detected in adhesions and junctions by immunohistochemical methods either at the level of the light microscope (Fox *et al.* 1980; Birchmeier *et al.* 1980) or the electron microscope (Chen & Singer 1980). Reports are, however, conflicting in that others have claimed that fibronectin does co-distribute with focal adhesions (Badley *et al.* 1978) or with markers for them such as the termini of actin cables (Hynes & Destree 1978; Singer 1979) or vinculin foci (Singer 1982). We therefore reinvestigated the fibronectin distribution by immunoelectron microscopy, at and around adhesions and junctions and also in structures with a morphology suggesting that they could represent stages in assembly or disassembly of adhesions and junctions. Rather than use the primary chick fibroblast system for this work we chose cell lines that formed specialized adhesions and junctions clearly recognizable by morphological criteria and which gave minimum production of matrix fibronectin, which might otherwise have confused interpretation.

In cell adhesions that are fully developed by the criterion that their contacting membrane is firmly flattened to substratum with a separation distance of about 15 nm, we confirmed that fibronectin labelling is seen on the surrounding membrane and substratum but cannot be detected within the area of contact itself (figure 1*b*). When separation distances are slightly greater, however (about 18 nm or more), labelling is seen clearly in association with strands of material that bridge to the substratum and have an electron density, width and spacing similar to those in non-contacting plaque structures (figure 1*c*, region marked A). In other examples (such as in figure 1*c*, region marked B), one part of the contacting membrane is pressed firmly onto the substratum with fibronectin labelling excluded, while an adjacent part of the same continuous structure allows the distinct entry of some label. Even in those regions with tightly apposed membrane and that are devoid of label, the characteristic strands can be seen bridging the small gap. Whether they represent focal adhesions in the final stages of formation or early stages of separation, the morphology of these fibronectin-positive contacts resembles that of focal adhesions in the presence of electron-dense submembraneous plaque into which microfilaments can often be seen to be inserted, and in the drawing away of

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membrane from substratum on either side of the area of contact. Similar results were obtained for intercellular junctions. Labelling was absent from structures that were fully formed with an intermembrane separation of less than 20 nm (figure 1*d*), but structures with an increased separation distance but otherwise related morphology showed heavy labelling and the characteristic bridging strands (figure 1*e*).

The detection of fibronectin in adhesions and junctions showing slightly increased separation distances cannot be attributed to sampling artefacts, for example by sections being cut along the edge of specialized contacts; such an explanation would require a fortuitous sectioning in the appropriate plane that would be observed only rarely in random sampling.

From these results we conclude that if fibronectin is truly absent from fully formed focal adhesions and from intercellular junctions of the microfilament-associated type, its removal occurs at a very late stage in formation and must somehow proceed without the removal of the stranded material with which it is associated in the non-contacting plaque structures and more open adhesions and junctions. An alternative explanation could be that fibronectin is not in fact removed, but becomes masked – though probably not through simple physical inaccessibility (Chen & Singer 1980) but by a covering up of the antigenic determinants in the final stages of structural condensation. Whether the fibronectin is actually removed or masked, the results are in any case consistent with the hypothesis that adhesions and junctions derive from the fibronectin-bearing structures as precursors.

4. Fibroblast functions on alternative protein substrata

A further objection to the hypothesis that adhesions and junctions form through the interaction of fibronectin-bearing surfaces could be that cells do not require fibronectin since they have been reported to spread on substrata coated with other proteins (Grinnell & Hays 1978; Grinnell 1978; Aplin & Hughes 1981). This spreading leads to characteristic fibroblast morphology of a type believed to be associated with the formation of focal adhesions, although the experiments reported did not establish that such adhesions were in fact formed, and were with plant lectins which are not natural substrates for animal cells. To re-examine this issue

Description of plate 1

Figure 1. Immunoelectron micrograph of thin sections of 16C fibroblasts grown in medium containing 10% foetal calf serum fixed as described below, and stained with anti-cell-derived fibronectin by the indirect method with protein A – colloidal gold. (*a*) Specimen fixed in aqueous paraformaldehyde (30 mg ml^{-1}) in a procedure that causes osmotic disruption of the cells and removal of cytoplasmic material to highlight membrane specialization (Smith *et al.* 1982). Note the non-contacting plaque structures (n.c.p.) associated with extracellular strands labelling for fibronectin and with an electron-dense plaque (e.d.p.). Bar = 0.1 μm. (*b*) Specimen fixed in paraformaldehyde (30 mg ml^{-1}) in phosphate-buffered saline. Note that labelling is seen on the substratum and cell surface but not in the area of contact between the membrane and substratum that is within the focal adhesion structure, *i.e.* within the region bounded by the two vertical arrows. (*c*) Specimen processed as for (*b*) but note that labelling for fibronectin is now detected in the two zones of contact; in the region marked A the separation is increased to 18–20 nm and labelling is seen together with bridging material with a stranded morphology, which seems rather regularly spaced. In the region marked B, the membrane on the left is firmly apposed to the substratum and label is absent, whereas on the right some entry of label into the gap can be clearly detected. (*d*) Specimen processed as for (*b*) and (*c*); this micrograph shows a region of cell–cell contact with a fully sealed junction (j) that fails to label for fibronectin, in contrast to the contact adjacent to it at which label can be clearly seen (f.n.). (*e*) As for (*d*), but note the contact, which is morphologically similar to an intermediate junction except that the intermembrane separation is increased to permit heavy labelling with fibronectin and display of a fine structure of intercellular components, especially regularly spaced strands (st.) and a midline (m.l.).

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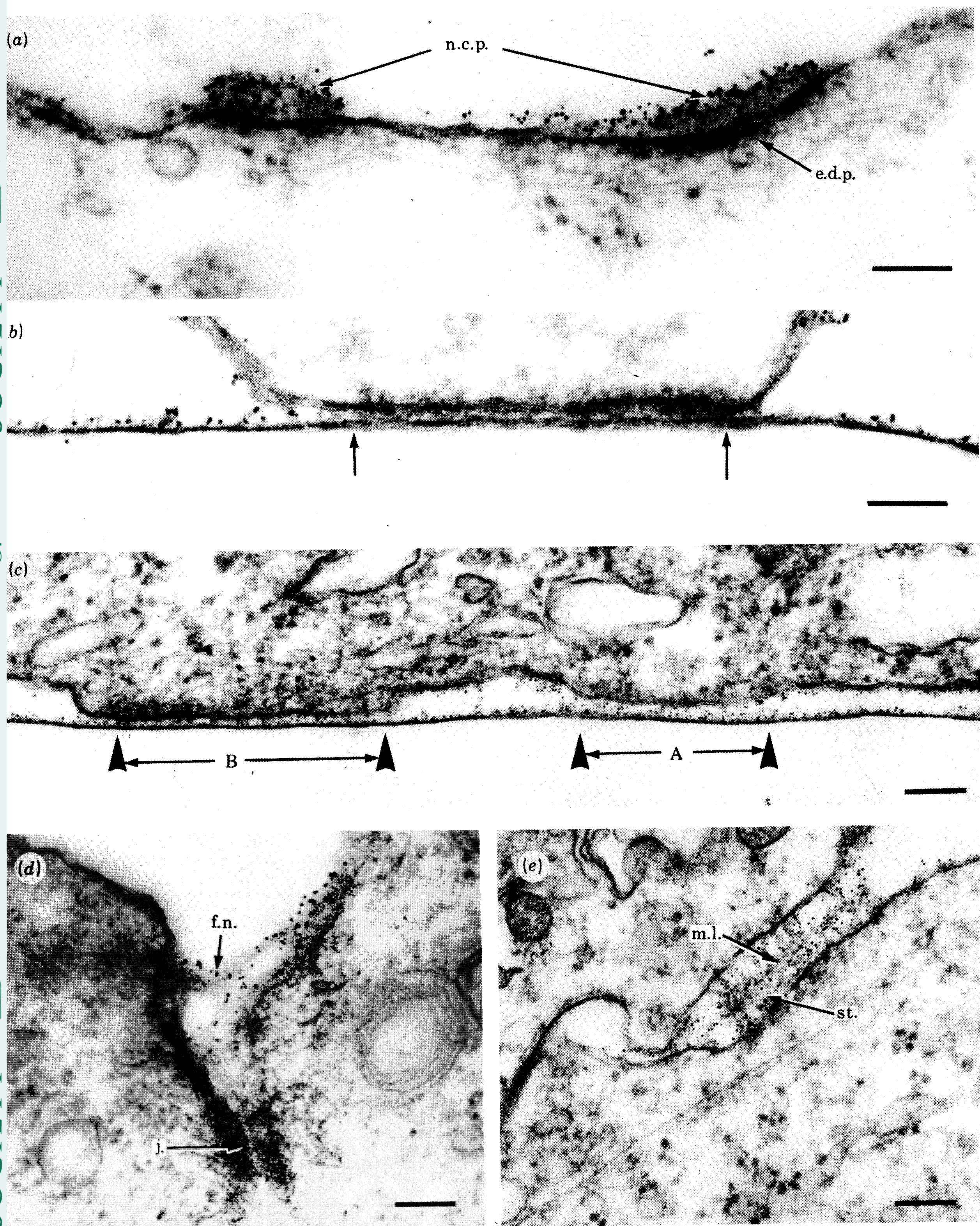

FIGURE 1. For description see opposite.

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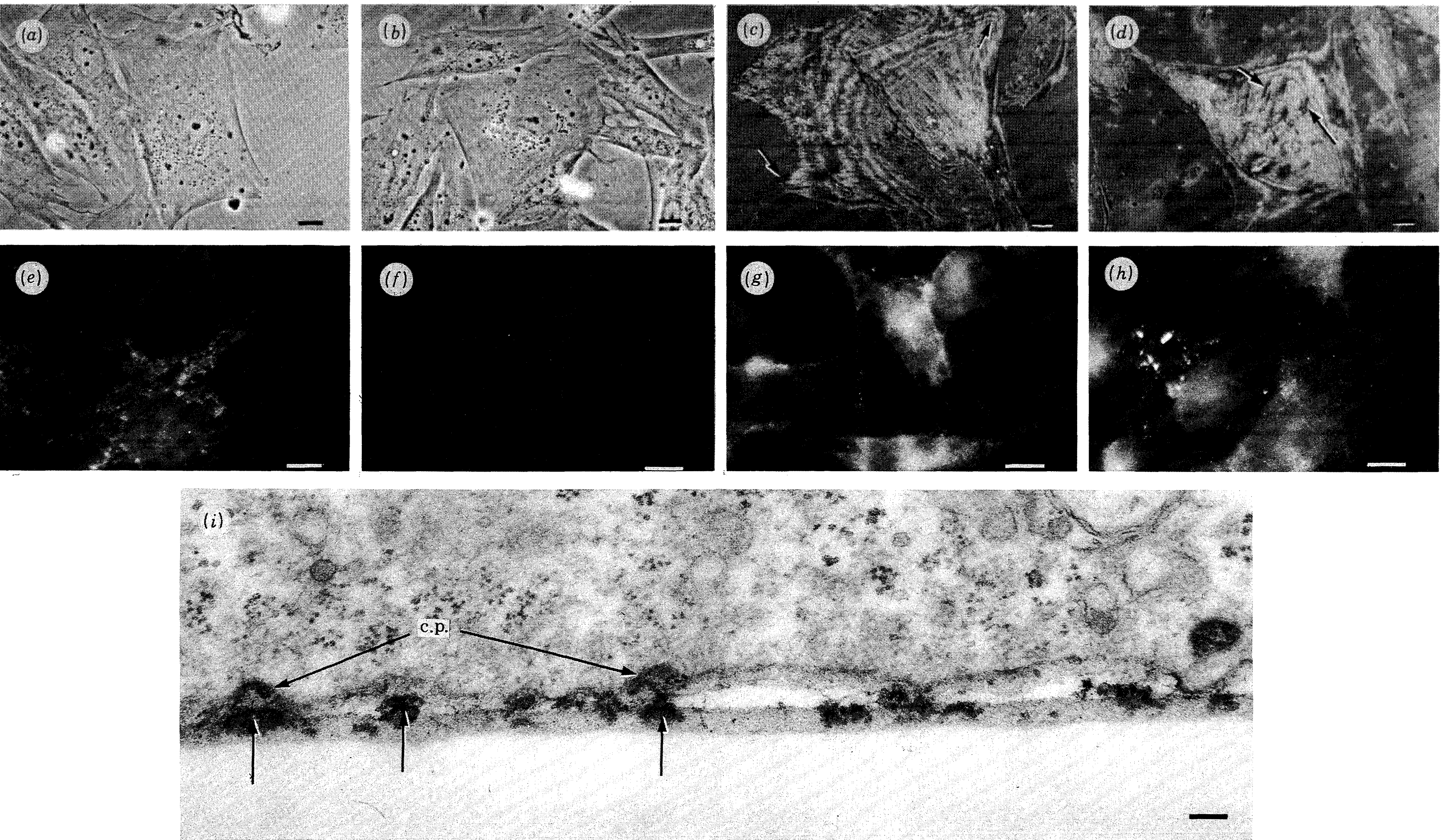

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(Couchman *et al.* 1982*b*) we used substrata coated with laminin, a characteristic protein of basement membranes, which has not previously been studied as a possible substrate for fibroblast spreading. To avoid complications in the interpretation we used medium from which fibronectin had been removed by affinity chromatography and cells in which protein synthesis was suppressed by cycloheximide so that they did not produce intracellular or extracellular fibronectin as monitored by immunofluorescence. For control experiments, substrata were coated separately with fibronectin or bovine serum albumin, the latter being known (Grinnell *et al.* 1977) to be ineffective in promoting cell spreading.

Attachment assays showed that laminin is comparable with fibronectin as a substrate for cell adhesion, but (as expected) serum albumin is not (Couchman *et al.* 1982*b*). The cells spread to similar shapes on fibronectin and laminin (figure 2*a*, *b*) with microfilament bundles visible as phase-dense fibres (figure 2*a*, *b*) and by immunofluorescence (result not shown). Clear patterns of focal adhesions were visible by interference reflexion (figure 2*c*, *d*). Any doubt that exogenous laminin was the genuine substrate for this behaviour, rather than small amounts of endogenous or exogenous fibronectin that we had failed to remove, could be eliminated by control experiments with specific antisera. The response of cells to laminin was blocked by including anti-laminin in the culture medium from the time of seeding or after spreading but was unaffected by anti-fibronectin; conversely, the response of cells to fibronectin was blocked by anti-fibronectin but not by anti-laminin (results not shown, see Couchman *et al.* (1982*b*)).

Further experiments showed not only that laminin-coated substrata can support spreading and specialized adhesions but, when the block on protein synthesis was lifted by removal of cycloheximide from the culture medium, cell growth occurred and a further cellular activity was expressed, namely the secretion of matrix materials onto the substratum. Immunofluorescence staining with anti-type III procollagen and anti-fibronectin showed the deposition of a fibrillar matrix comparable in its state of organization with that developed on a fibronectin substratum (results not shown, see Couchman *et al.* 1982*b*).

These results call for a significant qualification to the hypothesis for the origin of focal adhesions in its simple form as proposed in §§2 and 3. Even if these adhesions do normally arise by interaction between fibronectin-bearing surfaces, it is now apparent (i) that endogenous

Description of plate 2

Figure 2. Light and electron micrographs of fibroblasts spread on alternative protein substrata. (*a*)–(*d*) Spreading of human embryonic skin fibroblasts in medium containing cycloheximide and fibronectin-depleted calf serum; for full experimental details, see Couchman *et al.* (1982*b*). Note that fibroblasts seeded on fibronectin and laminin ((*a*) and (*b*) respectively) showed no qualitative difference in spread shape under phase optics, both leading to angular morphology with phase-dense fibres visible in the cytoplasm; and that interference reflection microscopy showed focal adhesions (examples are arrowed) with no difference over a population of cells in the size or number of focal adhesions on fibronectin compared with laminin ((*c*) and (*d*) respectively). (*e*–*h*) Fibronectin distribution in 16C fibroblasts by immunofluorescence with and without permeabilization of the cells; for experimental details, see Smith *et al.* (1982). Note that cells spread on SF-140 in serum-free medium show strong punctate staining after permeabilization (*e*) but no staining without permeabilization (*f*), whereas when spread in serum-containing medium the pattern is similar with or without permeabilization ((*g*) and (*h*) respectively; equivalent results are obtained on a fibronectin substratum in serum-free medium). (*j*) Electron micrograph of vertical thin section through a 16C fibroblast spread on SF-140-coated substratum in serum-free medium, then fixed in paraformaldehyde (35 mg ml^{-1}) in phosphate-buffered saline and stained with ruthenium red and anti-cell-derived fibronectin by the indirect method with protein A – colloidal gold. Note the coated pits (c.p.) and the globules of amorphous material (arrows) usually associated with both ruthenium red and antibody.

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fibronectin is not an obligatory component, because specialized adhesions can form when its synthesis is suppressed, and (ii) that neither is exogenous fibronectin obligatory, as shown by experiments on laminin. A third and related observation (Grinnell 1978; Thom *et al.* 1979) with certain cell types or appropriate culture conditions, or both, is that exogenous protein spreading factors may not be required apparently because substratum contacts can be mediated instead by cell-derived fibronectin that has been externalized. All this would imply some degree of interchangeability of function between the origin (i.e. endogenous versus exogenous) and nature (e.g. fibronectin versus laminin) of the extracellular components involved in setting up specialized cell adhesions.

5. Cell spreading can be uncoupled from focal adhesions and microfilament bundles

The properties of the locomotory chick cells (see §2) already illustrate that fibroblasts can spread to perform specialized functions without necessarily developing focal adhesions and microfilament bundles. In this system, the behaviour is under cellular control mechanisms believed to be activated by a process analogous to a tissue wounding response (Couchman & Rees 1979). We now show that related cellular behaviour can be evoked by variation of the protein that coats the contacting substratum, by using a spreading factor from chick serum described earlier (Thom *et al.* 1979) and having a molecular mass of 140 kDa. This protein, referred to here as SF-140, has already been shown to promote the spreading of fibroblasts in serum-free medium to angular morphology with areas of adhesion that are broad and unspecialized at the level of the light microscope (Thom *et al.* 1979) and without formation of actin cables (Badley *et al.* 1980*a*). In contrast, the same cells in the same medium contacting substratum-bound fibronectin, develop both focal adhesions and microfilament bundles. Cells spread on SF-140 can, however, be stimulated by addition of serum (which may be fibronectin-depleted) or certain growth factors to develop focal adhesions and microfilament bundles, and monitoring of this by time-lapse video recording with interference reflexion optics shows (Smith *et al.* 1982) that the broad substratum contacts ('close contacts', which show as grey areas of the image) constantly fluctuate and contain only transitory focal contacts before serum stimulation. Serum addition causes gradual stabilization of focal contacts to long-lived focal adhesions as surrounding membrane areas lift from the substratum and cell margins draw in to concave outlines. Although the original areas of close contact appear to be unspecialized at the level of the light microscope, electron microscopy reveals that almost all the coated pits that can be located around the cell surface are in fact concentrated in them. Each pit is associated with a bridging deposit of matrix material which, having a globular, amorphous form, is quite different in fine structure from the stranded morphology characterizing the extracellular components of focal adhesions, and yet is similar in composition because it labels with anti-fibronectin and ruthenium red (figure 2*i*). Because focal adhesions are also associated with extensive coated membrane (Heuser 1980; Maupin & Pollard 1981; Smith *et al.* 1982) and, as described above, are observed to form within the close contact areas, it would seem that the plasma-membrane organization induced by contact with SF-140 could represent a stage towards the formation of focal adhesions, coupled to a spread shape which, however, is not stabilized by microfilament bundles. The impression that cellular response is arrested when SF-140 is used instead of fibronectin is confirmed by a study of the progress of matrix deposition

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as the cells spread. On a fibronectin substratum, granular cytoplasmic concentrations of matrix can be visualized by immunofluorescence with anti-fibronectin, which are gradually externalized as spreading proceeds to deposit eventually on the substratum; on SF-140, however, the granular components remain within the cytoplasm, concentrated against the ventral surface, and are not externalized (figure 2*e–i*). We conclude that the externalization process is coupled to fully developed adhesion status, and is arrested when the specialization of adhesion is arrested.

6. Conclusions

We have considered the roles of proteins that interact between the cell surface and the substratum when motile functions of fibroblasts are expressed. Being rather remote from the plasma membrane, these proteins are termed cell matrix or pericellular components. Proteins and glycoproteins integral to the plasma membrane itself must of course also perform key mediating functions, but the consideration of these and their roles is a topic for separate consideration.

Both endogenous and exogenous matrix proteins can be important in setting up the motile functions of fibroblasts, as illustrated by the function of fibronectins in primary cell locomotion (§2), in the spreading of rounded cells to develop focal adhesions and actin cables (§§4 and 5), in promoting further matrix secretion (§§4 and 5) and, as described elsewhere (Couchman *et al.* 1982*c*), in accelerating entry into the growth cycle. Under the usual conditions of culture it would seem likely that specialized adhesions and junctions may arise from contacts between separate fibronectin-bearing structures on the cell surface and either on the substratum or a cell partner respectively (§3). However, under other conditions there may be a degree of interchangeability of function between different matrix components – specifically between exogenous fibronectin and laminin and between endogenous and exogenous fibronectin (§4).

Experiments with a new spreading factor suggest that the development of specialized adhesions can be a multi-step process, susceptible to interruption when extracellular interactions are incomplete (§5). This protein ('SF-140') can support the spread shape and the redistribution of cell surface structures such as coated pits and glycocalyx, but lacks the ability of fibronectin to promote further steps leading to fully specialized adhesions, actin cables and effective externalization of matrix components. Further study of such systems should allow us to dissect out the separate stages in the mediation of motile responses by extracellular contacts.

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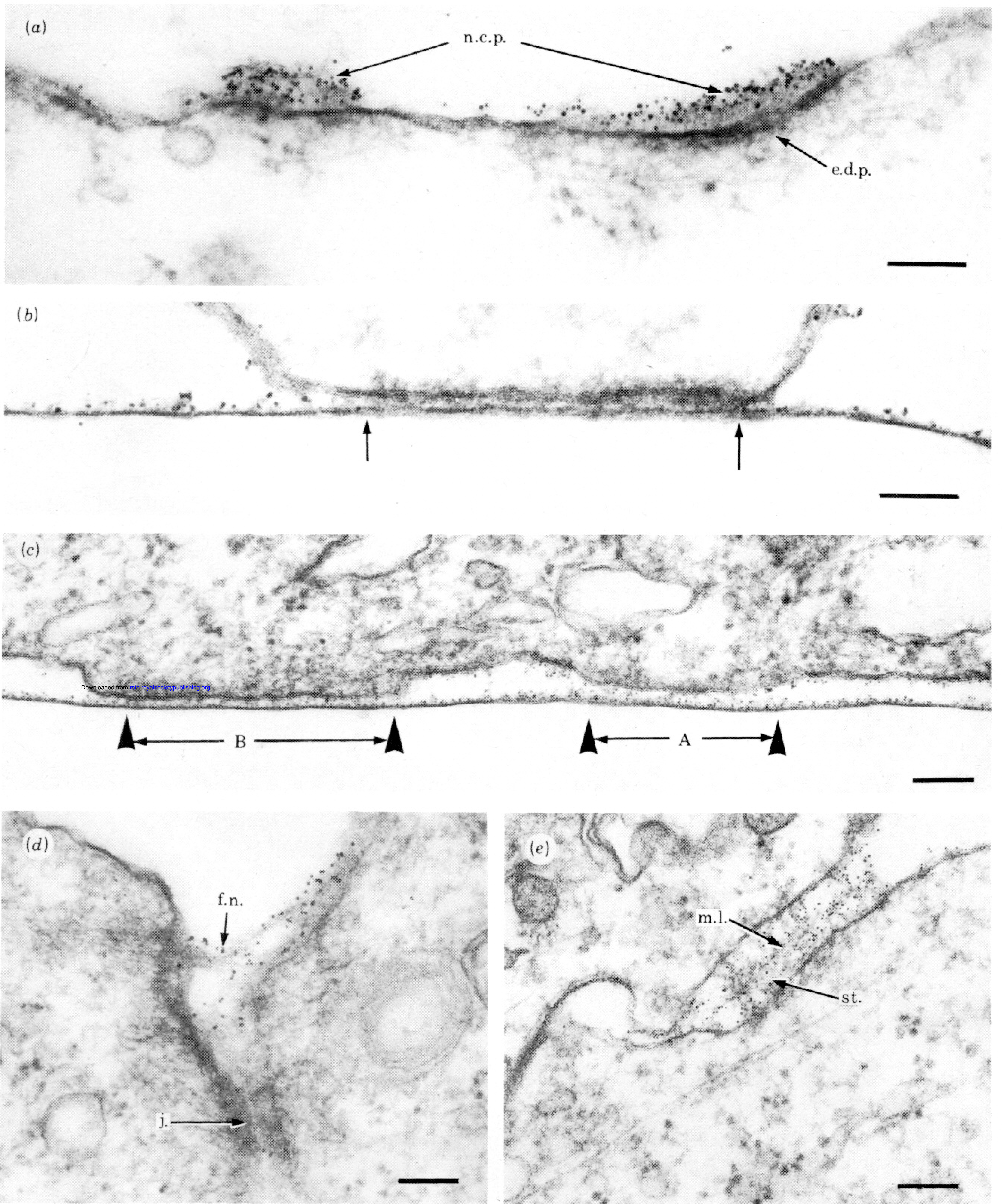

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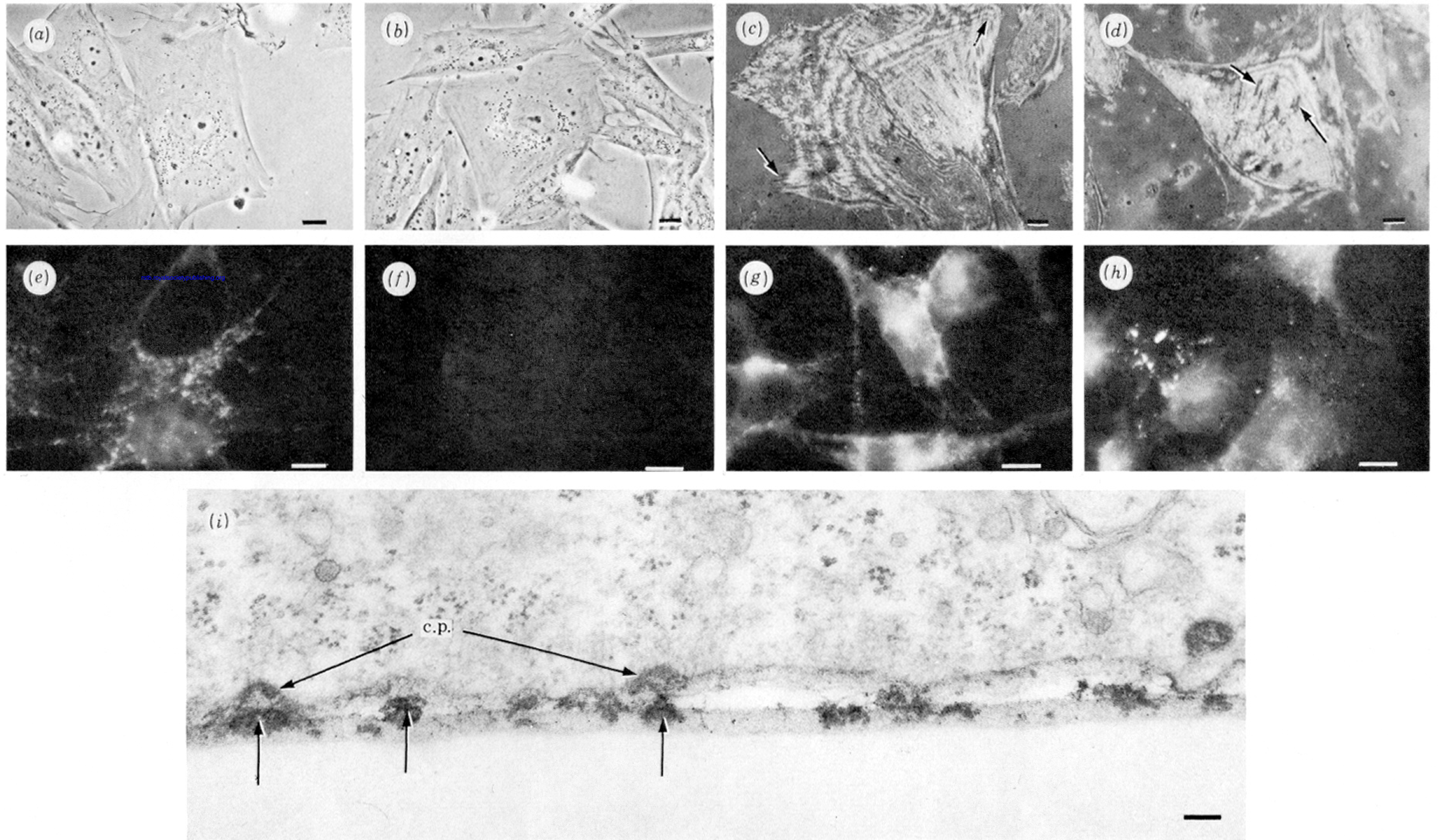

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