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INTERMEDIATE FILAMENT NETWORKS: ORGANIZATION AND POSSIBLE FUNCTIONS OF A DIVERSE GROUP OF CYTOSKELETAL ELEMENTS

ROBERT D. GOLDMAN*, ANNE E. GOLDMAN, KATHLEEN J. GREEN, JONATHAN C. R. JONES, STEPHANIE M. JONES AND HSI-YUAN YANG

Department of Cell Biology and Anatomy, Northwestern University Medical School, Ward Building, 303 East Chicago Avenue, Chicago, Illinois 60611, USA

SUMMARY

Immunofluorescence and electron microscopic observations demonstrate that intermediate filaments (IF) form cytoplasmic networks between the nucleus and cell surface in several types of cultured cells. Intermediate filaments interact with the nuclear surface, where they appear to terminate at the level of the nuclear envelope. From this region, they radiate towards the cell surface where they are closely associated with the plasma membrane. On the basis of these patterns of IF organization, we suggest that IF represent a cytoskeletal system interconnecting the cell surface with the nucleus. Furthermore, IF also appear to interact with other cytoskeletal components including microtubules and microfilaments. In the former case microtubule–IF interactions are seen in cytoplasmic regions between the nucleus and the cell membrane, whereas microfilament–IF interactions occur in the cortical cytoplasm. IF also appear to be cross-linked to each other; especially in the case of the IF bundles that occur in epithelial cells. In order to determine the molecular and biochemical bases of the organizational state of IF we have developed procedures for obtaining IF-enriched 'cytoskeletons' of cultured cells. In these preparations IF–nuclear and IF–cell surface associations are retained. Thus, these preparations have enabled us to begin to study various IF-associated structures (e.g. desmosomes) and associated proteins (IFAPs) using biochemical and immunological methodologies. To date, the results support the idea that IF and their associated proteins may comprise the cell type specific molecular infrastructure that is involved in transmitting and distributing information amongst the major cellular domains; the cell surface/extracellular matrix, the cytoplasm and the nuclear surface/nuclear matrix.

INTRODUCTION

Intermediate filaments (IF) are cytoskeletal components found in many types of cells. They are usually ≈10 nm in diameter, and contain alpha-type proteins in a coiled-coil configuration. Despite their morphological, physical and chemical similarities, they in fact represent a very large and diverse family of cytoskeletal elements (Zackroff *et al.* 1981; Steinert *et al.* 1984; Wang *et al.* 1985). The diversity of IF is especially evident when their polypeptide composition is compared between different cells from different tissues of the same organism. This has led numerous investigators to attempt to categorize cells on the basis of differences in the immunological, molecular and biochemical properties of the structural subunits of their IF. To date, the largest subgroup of IF proteins to be described are the

*Author for correspondence.

keratins. In mammalian cell systems, there may be 30 or even more keratins (Moll *et al.* 1982; Steinert *et al.* 1984; Sun *et al.* 1986). This large group of IF proteins is distinguished by their relative insolubility, their diversity and their abundance in epithelial cells. The type or types of keratin family members expressed in any individual epithelial cell type varies with the state of cellular differentiation and the location of the cell (Zackroff *et al.* 1981; Steinert *et al.* 1984; Wang *et al.* 1986; Sun *et al.* 1985).

In contrast to these differences within the keratin sub-group, there are also obvious similarities amongst the keratin subunits. For example, there are antibodies that react with a variety of keratins, indicating that they possess antigenic similarities. On comparative biochemical grounds the keratins are known to be serine- and glycine-rich and more recent sequencing data have suggested that these serine + glycine-rich residues are located in terminal domains (Steinert *et al.* 1984; Wang *et al.* 1986).

There are also other types of IF proteins, the most extensively studied being vimentin, which is the major structural protein found in mesenchymal cells such as fibroblasts (Zackroff *et al.* 1981; Steinert *et al.* 1984; Wang *et al.* 1986). In addition, the major building blocks of neuronal IF (the neurofilament triplet proteins), the glial IF proteins, and the major protein of muscle IF, which has been called either desmin or skeletin, have also been studied intensively in recent years (Zackroff *et al.* 1981; Steinert *et al.* 1984; Wang *et al.* 1985).

Besides the major structural subunits comprising IF in different cell types, there is also some evidence for the existence of IF-associated proteins (IFAPs). These are diverse and appear to be cell-and-tissue type specific. For example, filaggrin is a specific IF–IF cross-bridging protein found in skin and related tissues (Steinert *et al.* 1981). Filaggrin is a very basic protein, which may act to cross-link individual keratin-containing IF into bundles (tonofilaments) *via* their serine + glycine-rich terminal domains. Another very different IFAP has been described in BHK cells, which appears to form cross-bridges between neighbouring BHK IF. *In situ*, these bridges appear to be intermittent and may be involved in the formation of cytoplasmic IF networks (Yang *et al.* 1985; Lieska *et al.* 1985). In epidermal cells, there is some evidence that a protein called desmoplakin, a major component of desmosomes, may also be an IFAP (Jones & Goldman, 1985).

Recently, we have demonstrated that there are keratin-like proteins in mesenchymal cells, such as cultured BHK and 3T3 cells, which do not form IF under normal conditions. These proteins appear to be localized at the nuclear surface where they form an insoluble polymer system known as the nuclear lamina (Aaronson & Blobel, 1975; Gerace *et al.* 1978, 1984; Zackroff *et al.* 1984; Goldman *et al.* 1984, 1985, 1986). Furthermore, Linck *et al.* (1985) have suggested that the tektins, components of the outer doublet microtubules of cilia, are also intermediate filament-like proteins. These findings are intriguing, as they demonstrate that not all members of the IF protein family form 10 nm diameter filaments. In the case of the nuclear lamina proteins, they appear to possess several keratin-IF-like properties including their high content of glycine and serine, their alpha-helical content, their

cross-reactivity with antibodies directed against IF-forming epidermal keratins and their primary sequence derived from cDNA clones (Zackroff *et al.* 1984; Goldman *et al.* 1984, 1985, 1986; McKeon *et al.* 1986).

The fact that there is remarkable diversity amongst IF structural and associated proteins, and the fact that there are additional IF protein family members that can form polymeric systems other than IF, must presumably reflect functional significance. In this report, we review some of our work that has been aimed at determining the functions of the IF system.

RESULTS AND DISCUSSION

The cytoplasmic distribution of IF in cultured animal cells

Immunofluorescence observations of various types of cultured cells indicate that IF are generally distributed between the nuclear and cell surfaces in cultured fibroblastic cells, such as mouse 3T3 and baby hamster kidney (BHK). When BHK cells are observed by indirect immunofluorescence, using an antibody preparation directed against the major BHK IF structural protein of 55 000 molecular weight (55 K, termed vimentin), a complex network of fibrils is seen to radiate from a juxtanuclear mass towards the cell surface (Fig. 1) and individual IF frequently appear to approach the cell surface very closely (Green & Goldman, 1986). A more dramatic way in which to show the association between IF and the nuclear and cell surfaces is to observe cells during attachment, spreading and shape formation. Following trypsinization and replating, rounded up BHK cells contain a large juxtanuclear cap enriched in IF structural protein (Fig. 2; and Goldman & Follett, 1970; Goldman & Knipe, 1973). As cell spreading progresses, this cap gives rise to fibres that radiate away from the nucleus towards the cell surface (Fig. 3; and Goldman & Follett, 1970; Goldman & Knipe, 1973). Similar observations have been made on all fibroblasts studied to date, on cultured nerve cells such as neuroblastoma (Fig. 4; and Goldman *et al.* 1981), and on several types of epithelial cells (Goldman *et al.* 1973; Jones *et al.* 1982).

The most extensive and dramatic IF networks that can be resolved in the fluorescence microscope are seen in many types of epithelial cells with polyclonal antibodies directed against epidermal keratin. For example, in cultured mouse keratinocytes, these networks are comprised of tonofibrils (IF bundles). These form a complex cage surrounding the nucleus, from which they radiate towards the cell surface (Fig. 5; and Jones *et al.* 1982). During the early stages of attachment and spreading of such cells, the network of tonofibrils can be seen to surround the nucleus. As cells begin to spread upon a substrate, individual tonofibrils are seen to extend into the expanding regions of cytoplasm from the concentrated juxtanuclear mass and move towards the cell surface (Fig. 6).

We have observed another example of nuclear–cell surface reorganization of tonofibrils in cultures of primary newborn mouse keratinocytes (PME) (Jones *et al.* 1982; Jones & Goldman, 1985). These cells are capable of attaching, spreading and dividing in low Ca^{2+} conditions (Hennings *et al.* 1980; Jones *et al.* 1982; Hennings

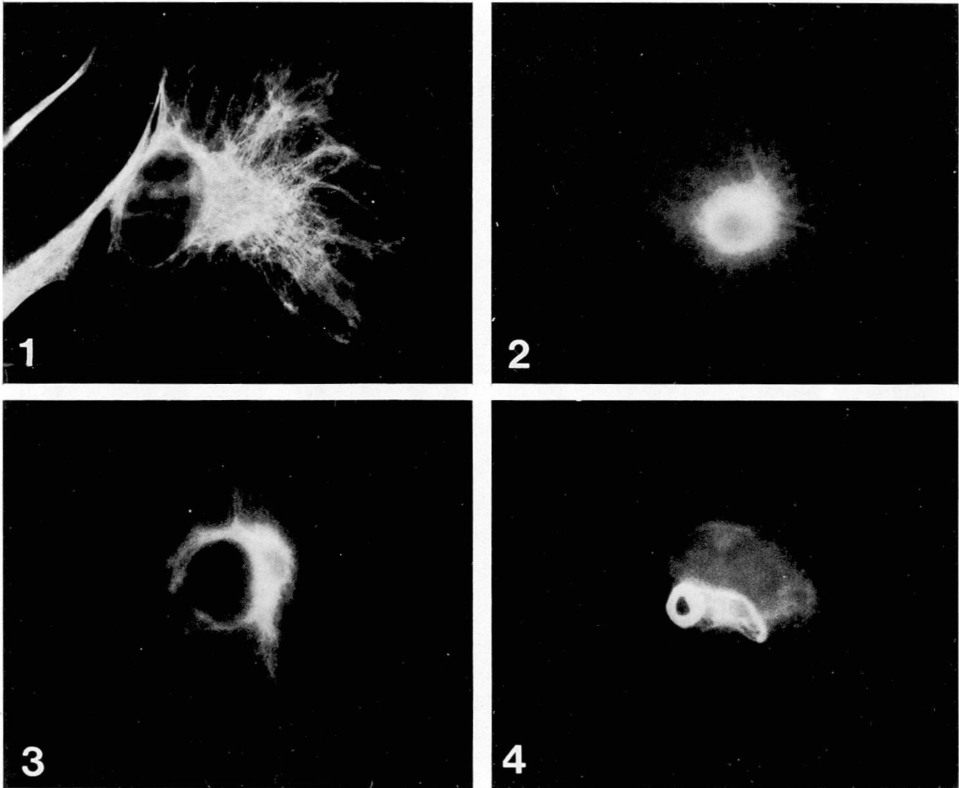


Fig. 1. A BHK cell observed ≈6 h following attachment to a glass coverslip. Note that the IF network is concentrated in a perinuclear region and radiates from this region towards the cell surface. Cells were fixed and processed for indirect immunofluorescence using an antibody directed against the BHK 55 K IF protein (Green & Goldman, 1983). ×750.

Fig. 2. A BHK cell observed ≈30 min following attachment to a glass coverslip. Note the juxtanuclear accumulation of IF as seen by immunofluorescence with the same antibody and preparative procedure described for Fig. 1. ×600.

Fig. 3. A BHK cell observed ≈2 h following attachment to a glass coverslip. Indirect immunofluorescence was carried out as described in Fig. 1. Note that IF are beginning to become redistributed into the spreading regions of cytoplasm. ×690.

Fig. 4. A mouse neuroblastoma (Nb2a) cell observed ≈2 h after attachment and processed for indirect immunofluorescence as described in Fig. 1. Note the juxtanuclear ring apparently connected to some IF-containing fibrils. ×625.

& Holbrook, 1983; Jones & Goldman, 1985). However, even though the cells are extensively spread under these culture conditions, tonofibrils are seen primarily in large perinuclear accumulations (Fig. 7; and Jones *et al.* 1982; Jones & Goldman, 1985). When the Ca^{2+} concentration is increased to normal levels, the tonofibrils move rapidly towards regions of the cell surface in contact with other cells (Fig. 8; and Jones *et al.* 1982; Jones & Goldman, 1985). When IF associate with the cell surface, desmosome assembly takes place. Desmosomes can be distinguished in this culture system by using immunofluorescence methods with antibodies directed

against desmosomal components, such as desmoplakin (Figs 9, 10; and Mueller & Franke, 1982; Jones *et al.* 1985).

The IF patterns seen in the different cell types described above have some general features in common, including the fact that they appear to have radiating networks between the nuclear and cell surfaces. These observations with the light microscope are readily confirmed at higher resolution with the electron microscope. In the case of fibroblasts such as BHK or 3T3 cells, arrays of 10 nm diameter IF can be seen in the region immediately adjacent to the nuclear surface. These frequently appear to terminate at the nuclear surface (Fig. 11; Goldman *et al.* 1984, 1985, 1986). In mouse epidermal cells an elaborate cage composed of IF bundles can be seen to surround the nucleus, which readily accounts for the images resolved by light microscopy described above (Fig. 12; and Jones *et al.* 1985). Frequently, IF can be seen apparently terminating in close proximity to the nuclear pore complexes (Jones *et al.* 1982, 1985; Goldman *et al.* 1985).

At the level of the cell surface there are numerous types of IF–plasma membrane interactions as revealed by electron-microscopic observations. IF can be found in close association with the cell surface in cultured BHK and chicken embryo (CEF) fibroblasts (Fig. 13; and Green & Goldman, 1986). In the case of CEF, plasma membrane-associated IF are frequently seen in regions in which the outer cell surface contains an accumulation of extracellular matrix material, which by immunofluorescence and immunogold-labelling criteria contains fibronectin (Green & Goldman, 1986). IF are also seen in close proximity to the microfilament bundles that accumulate subjacent to the plasma membrane in the specialized regions known as the 'fibronexus' (Fig. 14; and Green & Goldman, 1986). These latter regions are

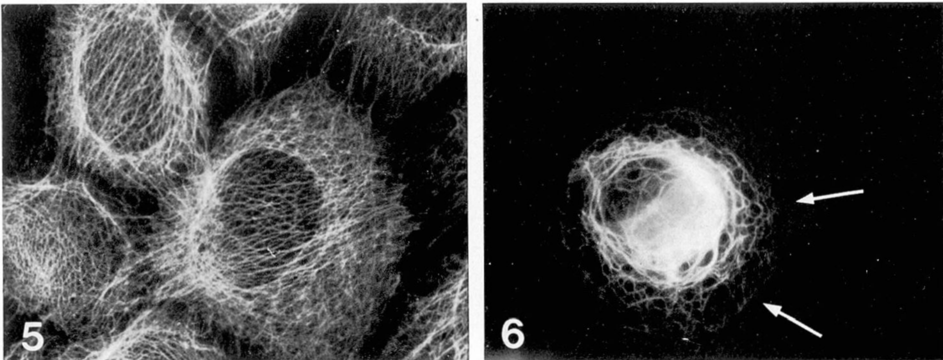


Fig. 5. The overall pattern of tonofibrils in well-spread cells obtained from a mouse skin epidermis cell line (PAM) is most readily observed by indirect immunofluorescence using, in this instance, a monoclonal antibody preparation directed against mouse keratin (Jones *et al.* 1985). Note the network of tonofibrils surrounding the nucleus and extending to the cell surface. ×1000.

Fig. 6. An early stage in the spreading process (≈2 h after attachment to a glass coverslip) of a PAM cell prepared as described for Fig. 5. Note that most tonofibrils surround the nucleus and some are beginning to move into the spreading cytoplasm. Arrows mark the position of the edge of the cell. ×960.

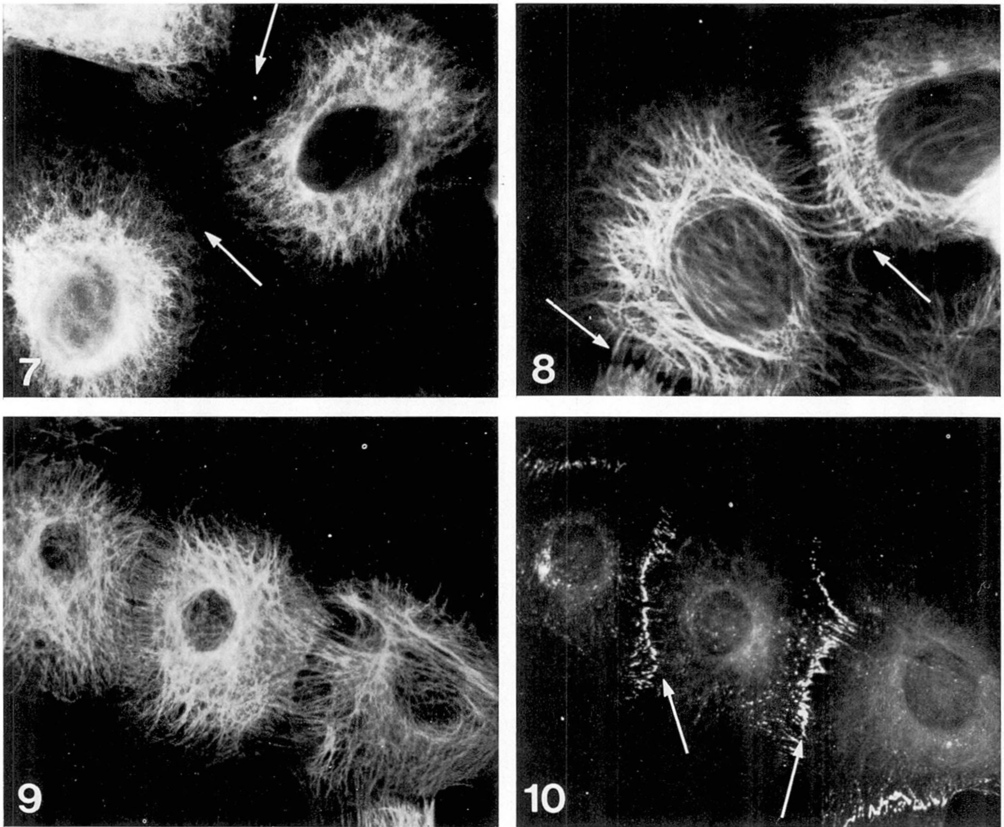


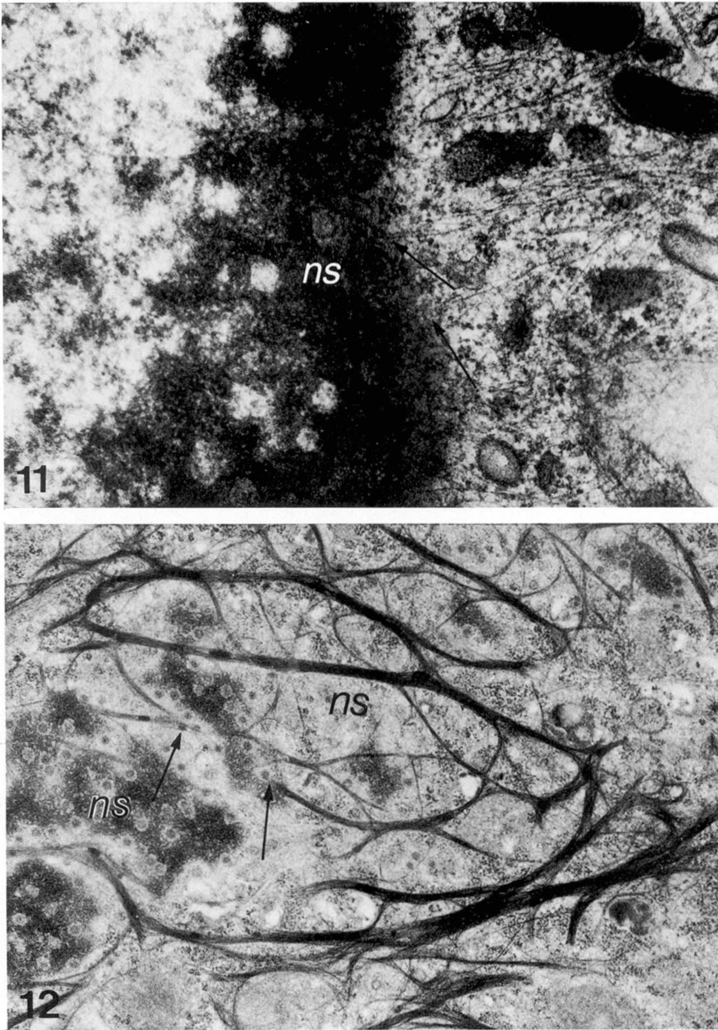
Fig. 7. Indirect immunofluorescence of primary mouse epidermal (PME) cells using the keratin antibody preparation as described for Fig. 5. These cells have been maintained in low Ca^{2+}-containing medium for 48 h and have established cell–cell contacts (arrows). Note that tonofibrils are concentrated around the nucleus and have not extended to the cell surface. ×700.

Fig. 8. A similar preparation of PME cells as described for Fig. 7, except that they have been exposed to normal Ca^{2+} levels for ≈3 h. Note that tonofibrils have moved to the cell surface, especially in areas of cell–cell contact (arrows). ×850.

Figs 9,10. Double indirect immunofluorescence micrographs of PME cells maintained in normal levels of Ca^{2+} for ≈4 h to show keratin-containing tonofibrils (Fig. 9) and desmosomes (arrows) (Fig. 10) (see Jones *et al.* 1984). ×675.

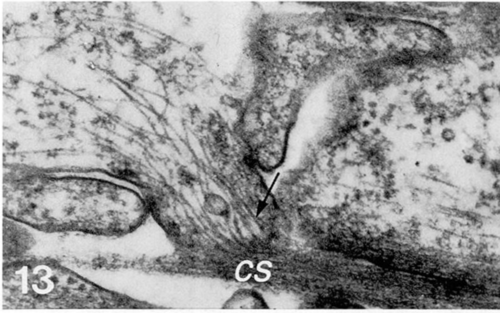
specialized membrane domains that are thought to represent sites of interactions between submembranous microfilament bundles and extracellular matrix components, especially fibronectin (Singer, 1979).

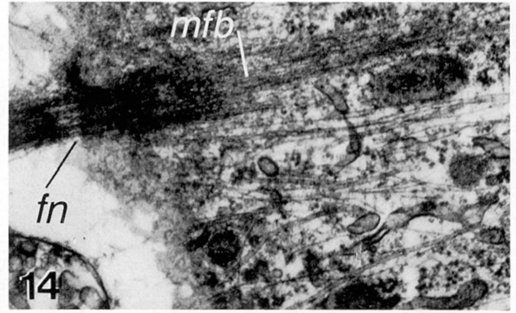
In addition to these ultrastructural observations, double-label immunofluorescence studies have been carried out in CEF, which indicate that, although there is not an extensive degree of overlap between the patterns generated by antibodies directed against CEF IF structural protein and fibronectin, there are regions in every cell in which a close association is seen between the two patterns (Figs 15, 16; and

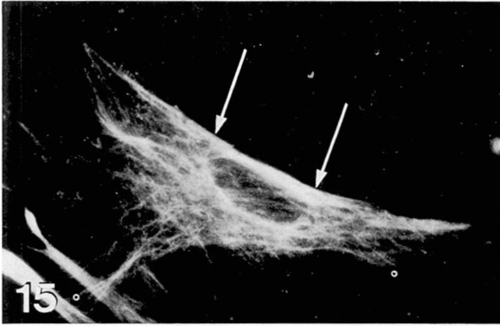


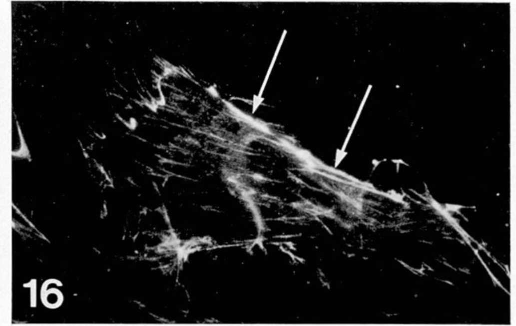
Figs 11, 12. Electron micrographs of thin sections of a BHK cell (Fig. 11) and a PME cell (Fig. 12) fixed and prepared as described (Starger *et al.* 1978). Note the close association between individual IF (arrows in Fig. 11) or IF bundles (arrows in Fig. 12) with the nuclear surface (*ns*). Fig. 11, ×28 000; Fig. 12, ×10 500.

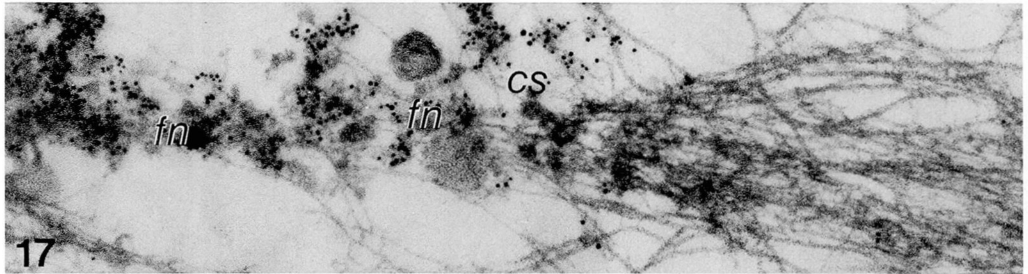
Green & Goldman, 1986). Similar observations have been made using fibronectin antibody in conjunction with the immunogold-labelling procedure (Fig. 17; and Green & Goldman, 1986). Additional evidence to support the possibility that IF associate with a 'cell membrane complex', which acts in some unknown fashion to link IF with the outer cell surface, stems from the observation that trypsinization of cells, which removes surface-bound fibronectin, induces the retraction of IF away from the plasma membrane. However, when CEF are removed from their growth substrates in the absence of trypsin, IF–cell surface associations are retained (Green & Goldman, 1986).

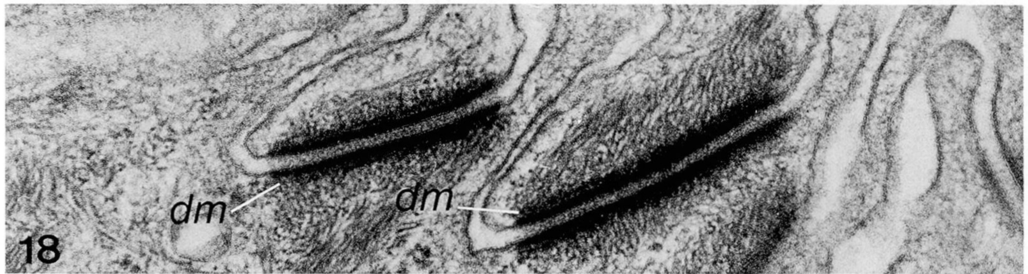










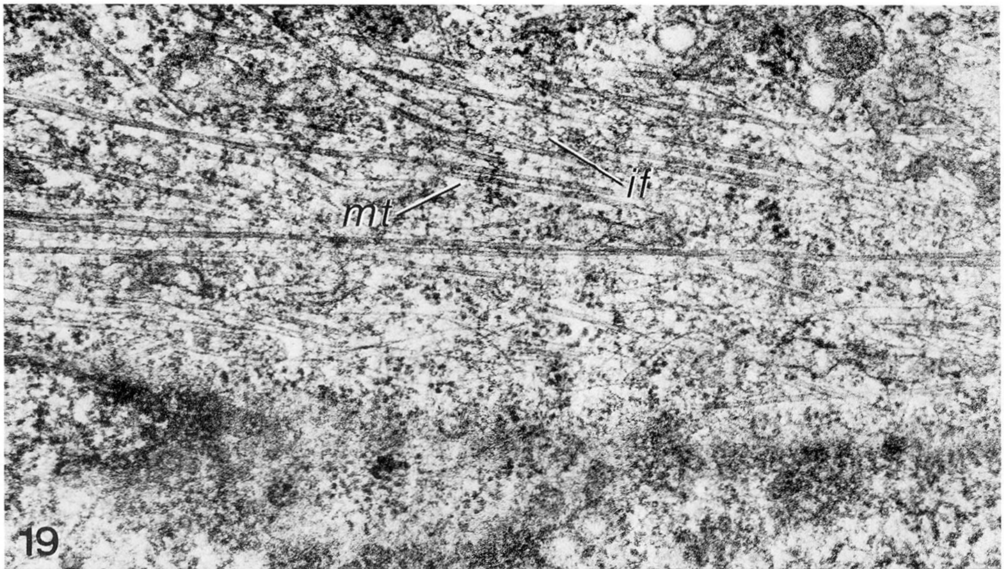


Figs 13, 14. Electron micrographs of thin sections of chicken embryo fibroblasts (CEF) cells to show IF associations (arrows) with the cell surface (*cs*; Fig. 13) and cell surface-associated microfilament bundles (*mfb*) in the region of the fibronexus (*fn*; Fig. 14). Fig. 13, ×55 000; Fig. 14, ×35 750.

Figs 15, 16. Double-label immunofluorescence micrographs of the same CEF cell showing IF organization (Fig. 15) and fibronectin distribution (Fig. 16), prepared as described by Green & Goldman (1986). Regions of close association between the two patterns are indicated by arrows. ×450.

Fig. 17. An electron micrograph of a region at the surface of a CEF cell prepared for indirect immunogold localization using fibronectin antibody as described by Green & Goldman (1986). Note the IF that approach the cell surface zone (*cs*) and the gold labelling in the extracellular fibres containing fibronectin (*fn*). ×87 500.

Fig. 18. An electron micrograph showing two desmosomes separating epithelial cells comprising the oral mucosa of bovine tongue (Jones *et al.* 1987). Note the close proximity of IF and desmosomal plaque material (*dm*). ×60 000.



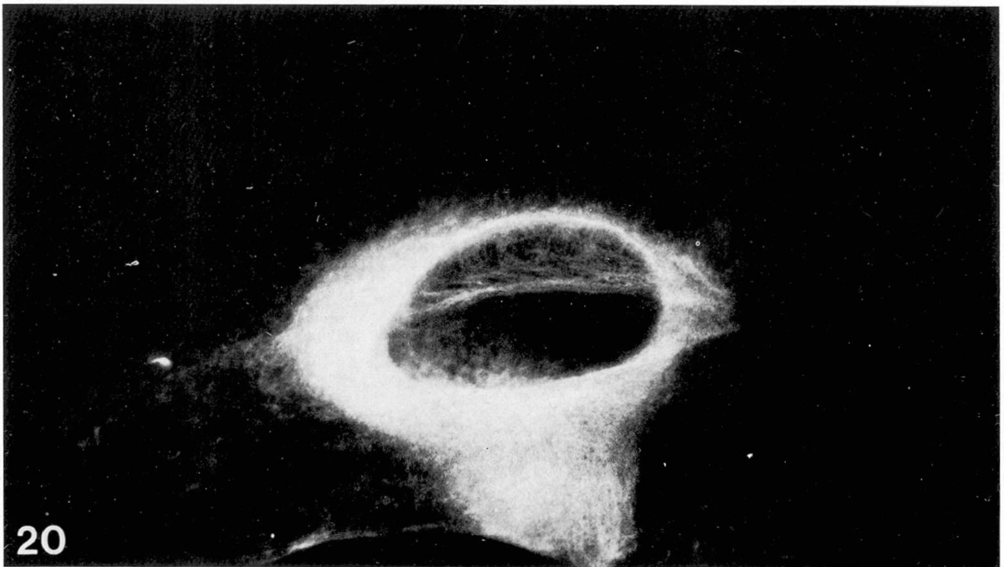


Fig. 19. Parallel arrays of IF (*if*) and MT (*mt*) seen in the cytoplasmic region between the nuclear and cell surfaces in BHK cells prepared for thin-section electron microscopy. ×40 000.

Fig. 20. A colchicine-treated ($10\,\mu g\,ml^{-1}$ in culture medium) BHK cell prepared for indirect immunofluorescence using an antibody directed against the BHK 55 K (vimentin) subunit. Note the large juxtanuclear fluorescent cap. ×1100.

seen coursing parallel to stress fibres in fully spread CEF by immunofluorescence (Green & Goldman, 1986). Electron-microscopic examination of the nodules supports the light-microscopic findings; they appear as an outpocketing of a microfilament bundle that contains electron-dense fine fibrillar material closely associated

Epithelial cells also exhibit obvious IF–cell surface interactions at the level of the desmosome – a double-membrane-containing structure, each half of which is associated with a cell–cell adhesion site (Arnn & Staehelin, 1981). Desmosomes are especially abundant in epidermal cells, where they are associated with keratin-containing IF. Indeed, IF appear to be attached to the innermost region of the submembranous plaque, which is associated with the cytoplasmic face of desmosomes (Fig. 18; and Arnn & Staehelin, 1981; Jones *et al.* 1987). As the bundles of IF approach the desmosomal plaque they appear to loop in and out of the fine fibrillar material associated with the plaque (Kelly, 1966). Recently, we have provided evidence that suggests that at least one of the desmosomal plaque proteins, desmoplakin 1, is associated with IF prior to the formation of desmosomes (Jones & Goldman, 1985).

The results of these morphological studies support the hypothesis that the IF system forms a cytoskeletal network that links the nuclear surface with the cell surface in cultured fibroblasts and epithelial cells (Goldman *et al.* 1985). We have also been able to show that IF interact with the two other major cytoskeletal elements, microtubules and microfilaments.

IF interactions with other cytoskeletal components: microtubules and microfilaments

One of the most obvious cases of IF interaction with microtubules (MT) can be seen in the cytoplasmic region between the nucleus and the cell surface of cultured fibroblasts that have been permitted to flatten on a solid substrate, such as a plastic Petri dish or a glass coverslip. Numerous IF can be seen in this region by electron-microscopic examination of thin-sectioned cells. In these areas, parallel arrays of IF and MT can be found (Fig. 19). Possible cross-bridging elements between these two cytoskeletal components have been reported elsewhere (Goldman & Knipe, 1973). Further support for this interaction also stems from the finding that in the presence of microtubule disrupting agents such as vinblastine or colchicine, the majority but not all of the IF visualized by immunofluorescence collapse around the nuclear surface (Fig. 20; and Starger *et al.* 1978; Green & Goldman, 1983, 1986). These latter arrays of IF are similar to those seen in cells observed during the early stages of attachment and spreading (see Figs 2, 3, above). Similar MT–IF configurations have been reported in nerve cells (Goldman *et al.* 1981).

IF also appear to be closely associated with submembranous microfilaments. In fibroblasts, we have been able to demonstrate, utilizing both immunofluorescence and electron microscopy, two types of MF–IF associations. One of these is apparent in the early stages of spreading in cultured CEF. In about 50 % of these cells, one or more phase-dense nodules are apparent within the cytoplasm in close association with stress fibres (microfilament bundles). These structures stain intensely with rhodamine-labelled phalloidin, indicating the presence of F-actin. When double stained with an antibody preparation directed against the CEF IF structural protein, these actin-rich nodules appear as focal centres from which IF-containing fibres radiate (Figs 21, 22; and Green & Goldman, 1986). In addition, numerous IF can be

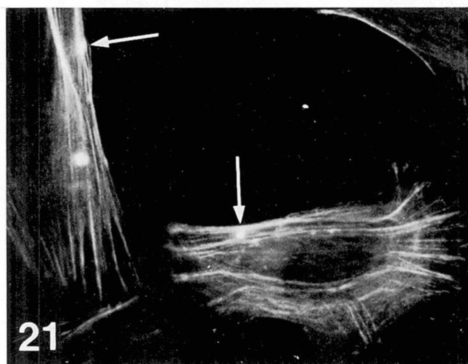
with IF (Figs 23, 24; and Green & Goldman, 1986). The fine fibrillar material probably represents the F-actin-enriched region of the nodule as seen by fluorescence microscopy. In addition, as mentioned above for fluorescence, we have seen close associations between MF bundles and IF by electron microscopy in spread CEF (Fig. 25; and Green & Goldman, 1986).

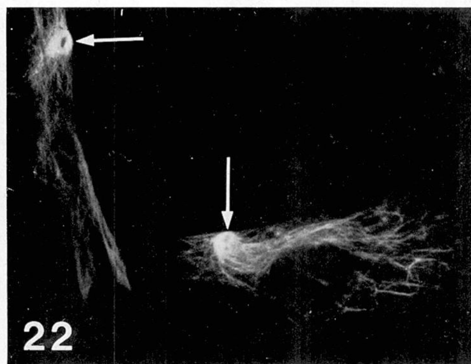
IF also interact with IF as determined ultrastructurally. For example, parallel arrays of IF are seen both in the juxtanuclear caps in the early stages of spreading in BHK cells and, during spreading, in the cytoplasm between the nucleus and cell surface (Figs 26, 27; and Goldman & Follett, 1970; Goldman & Knipe, 1973; Goldman *et al.* 1973). In epithelial cells, IF bundles can be found that form the tonofibrils resolved by light optical methods (Jones *et al.* 1982). These consist of tightly packed parallel arrays of IF (Fig. 28). In the case of epidermal cells, these latter arrays are cross-linked by a protein called filaggrin (Steinert *et al.* 1981).

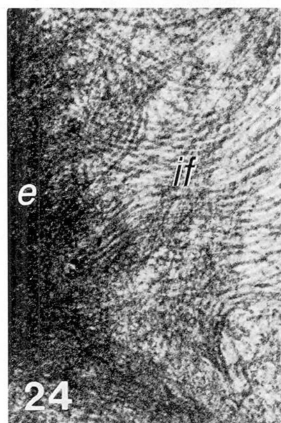
The preparation of IF-enriched cytoskeletons: their morphological, biochemical and immunological properties

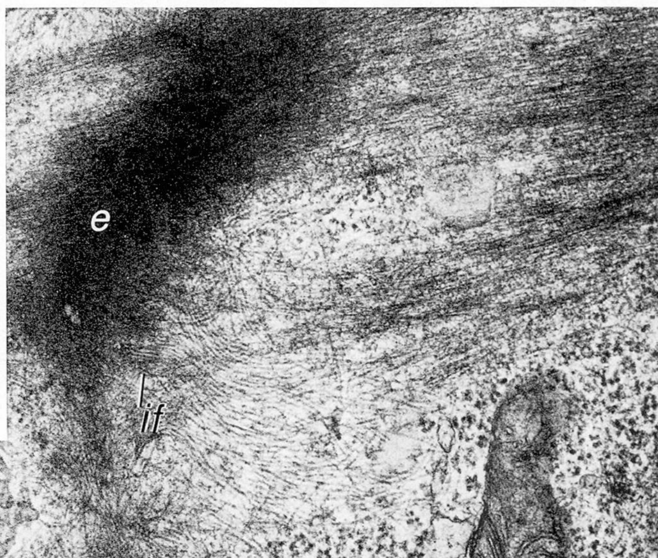
When cultured cells are extracted with high ionic strength detergent-containing solutions (0·6 M-KCl, 1% Triton X-100 in phosphate-buffered saline) and subsequently treated with DNase I (Starger & Goldman, 1977; Zackroff & Goldman, 1979), a so-called cytoskeletal preparation is formed that is greatly enriched in IF. Despite these rather drastic lysis conditions, the IF-enriched cytoskeletons appear to retain their overall shape in both fibroblasts and epithelial cells, as determined by whole-mount electron-microscopic observations (Figs 29, 30; Jones *et al.* 1982; Green & Goldman, 1986). In addition, these cytoskeletons are virtually devoid of other major cytoplasmic structural proteins such as tubulin and actin. In the case of fibroblast cytoskeletons, elements of the extracellular matrix can also be seen in their normal location (Fig. 30; and Green & Goldman, 1986). In the cell centre lies a nuclear remnant and between this latter structure and the cell surface there are large numbers of fibrous components that represent IF networks (Figs 29, 30; and Jones *et al.* 1982; Green & Goldman, 1986).

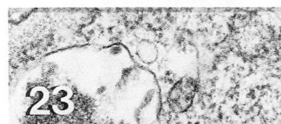
When such preparations are fixed and embedded for conventional electron-microscopic observations, more detailed views of the morphological properties of these cytoskeletons can be obtained. Beginning at the level of the nuclear surface, an obvious 'nuclear ghost' is present that is decorated on its cytoplasmic face with large numbers of IF (Figs 31–34; and Jones *et al.* 1982; Goldman *et al.* 1985). The nuclear ghost contains fine fibrillar material that represents the nuclear lamina region (Figs 31, 32, 34; Jones *et al.* 1982; Goldman *et al.* 1985). Nuclear pore complexes are frequently seen embedded in this latter region (Figs 31, 32, 34; and Jones *et al.* 1982; Goldman *et al.* 1985). In the case of fibroblast-derived cytoskeletons, IF at the cytoplasmic surface of the nuclear ghost appear to be anchored to a network of finely fibrous material (Figs 31, 32). In epidermal cell preparations, IFB are frequently seen to splay apart as they approximate the nuclear ghost and individual IF appear to terminate at the level of the nuclear pore complexes (Fig. 34; and Jones *et al.* 1982). The cytoplasmic networks of IF appear to be in their usual organizational states.









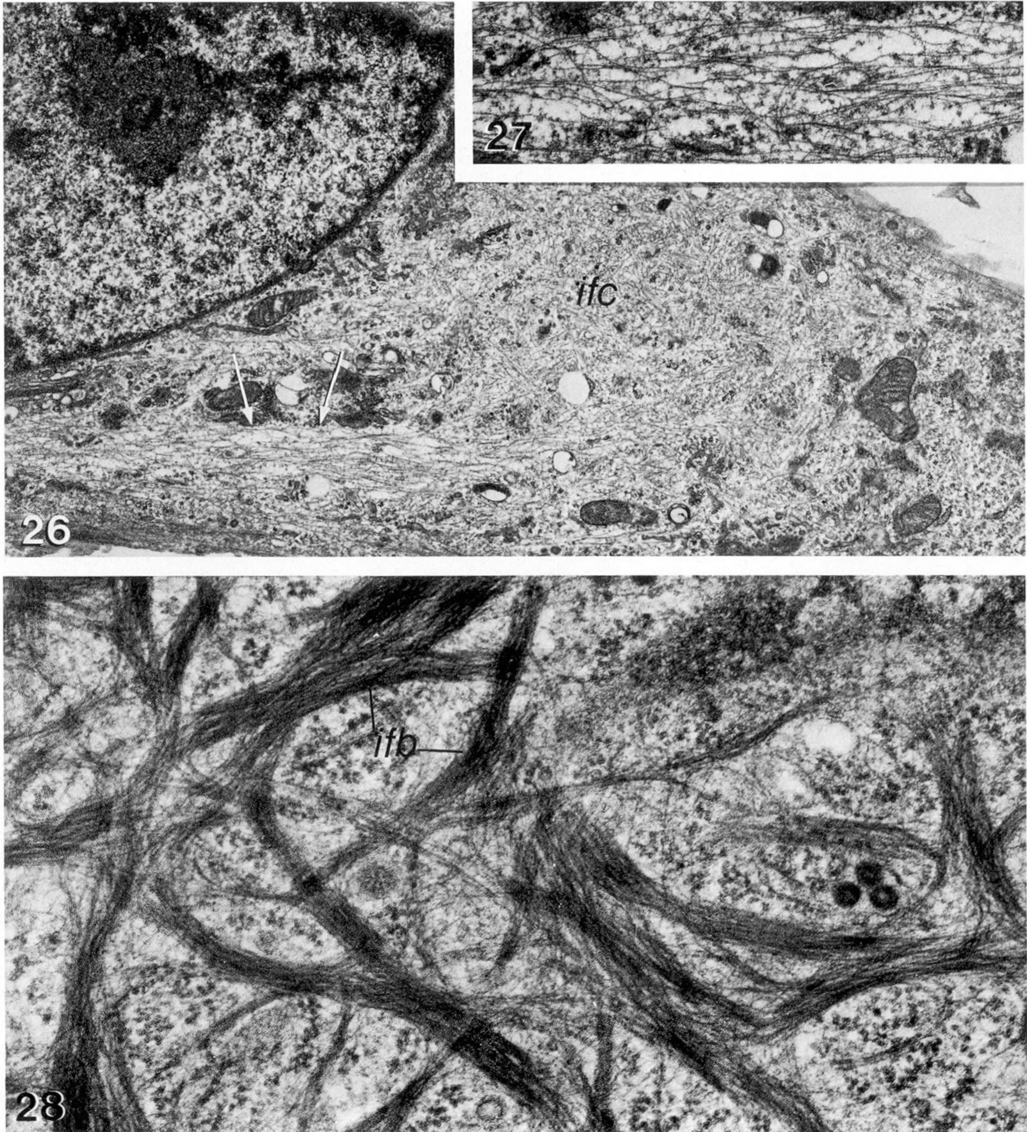


Figs 21, 22. Double-label fluorescence observations of CEF cells showing the distribution of F-actin, visualized using rhodamine-labelled phalloidin (Fig. 21), and IF (Fig. 22) (Green *et al.* 1986). Note the presence of actin-rich nodules or focal centres that are closely associated with IF (arrows). ×1200.

Figs 23, 24. Electron micrographs of a thin section through a 'nodule' of a spreading CEF cell. Note the electron-dense fibrillar material (*e*) and associated IF (*if*). Fig. 24 is a region taken from Fig. 23, but at higher magnification. Fig. 23, ×36 000; Fig. 24, ×51 500.

Fig. 25. An electron micrograph of a thin section through a submembranous region of a spread CEF cell showing a microfilament bundle (*mfb*) and associated IF (*if*). ×50 000.

Very few other cytoplasmic constituents can be detected (Figs 35, 36; and Jones *et al.* 1982; Jones & Goldman, 1985; Green & Goldman, 1986). At the level of the cell surface, close associations between IF and the extracellular matrix are retained (Fig. 35; and Green & Goldman, 1986). Frequently, in these regions, there appear

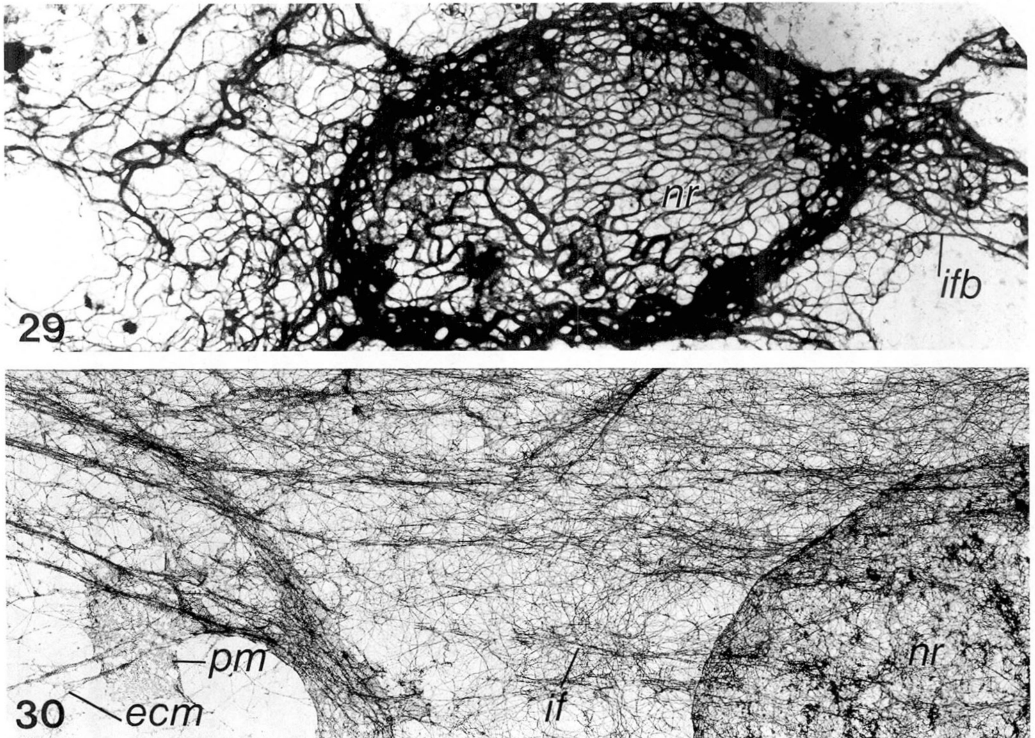


Figs 26, 27. Electron micrographs of a thin section taken through a region of a juxtanuclear accumulation of IF (*ifc*) with an associated loosely packed parallel array of IF (arrows). Fig. 27 is a higher-magnification view of the parallel array seen in Fig. 26. Fig. 26, ×11 650; Fig. 27, ×20 000.

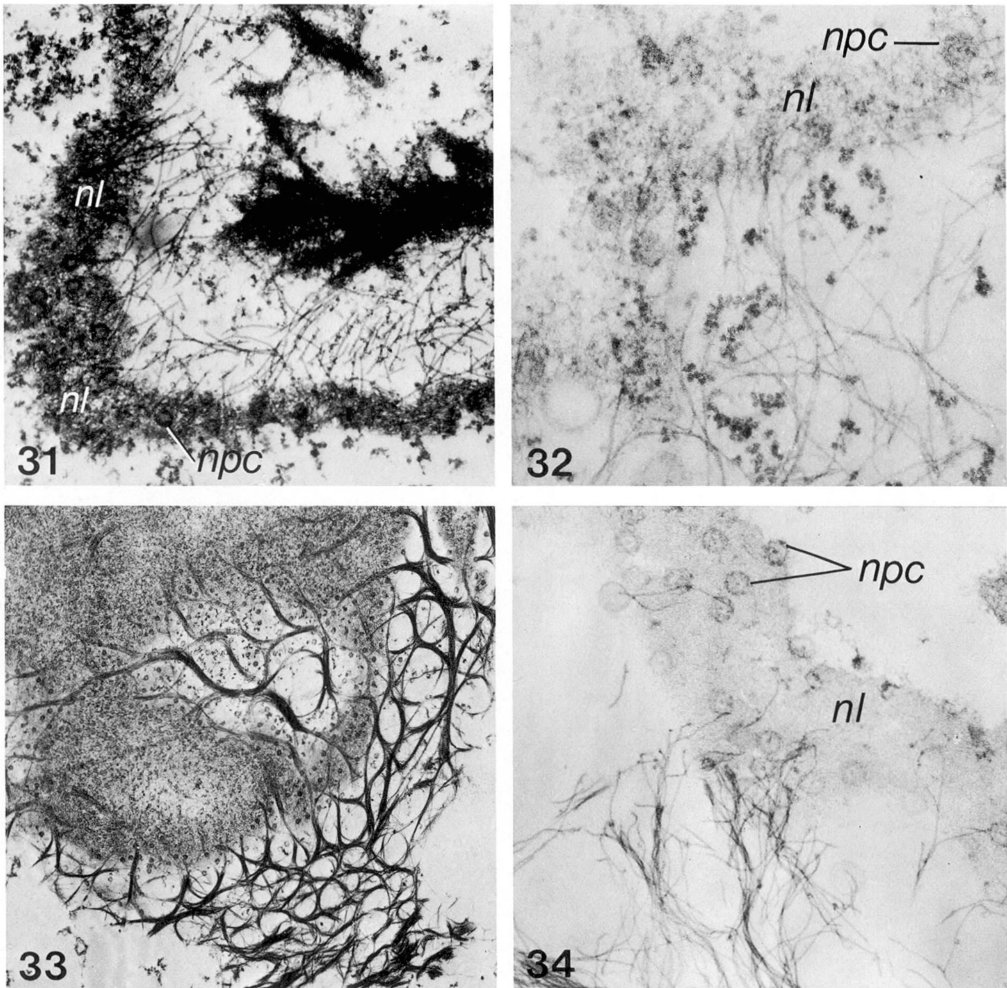
Fig. 28. An electron micrograph of a thin section cut through the cytoplasm of a spread PME cell to show IF bundles (*ifb*). ×42 600.

to be residual elements of the plasma membrane (membrane ghost material) (Fig. 30; and Green & Goldman, 1986). In the case of cultured keratinocytes, desmosomes as well as their connections with IF are retained (Fig. 36; and Jones *et al.* 1982; Jones & Goldman, 1985).

The biochemical properties of these IF-enriched cytoskeletal preparations have been analysed in some detail (e.g. see Starger & Goldman, 1977; Zackroff & Goldman, 1979; Steinert *et al.* 1982; Jones *et al.* 1982). SDS–PAGE (sodium dodecyl sulphate–polyacrylamide gel electrophoresis) analyses indicate the presence of relatively few polypeptides; the major components being the IF structural proteins. Since these vary from cell type to cell type, this is reflected in differences in the major proteins comprising their cytoskeletons. For example, in BHK preparations, two major polypeptides are apparent, of molecular weights 54 K and 55 K (the 54 K component is frequently termed desmin and the 55 K, vimentin) (Fig. 37; and Starger & Goldman, 1977), whereas in the case of PME cells, several bands of the keratin-type of IF structural protein are resolved (Fig. 38; and Jones *et al.* 1982; Jones & Goldman, 1985).



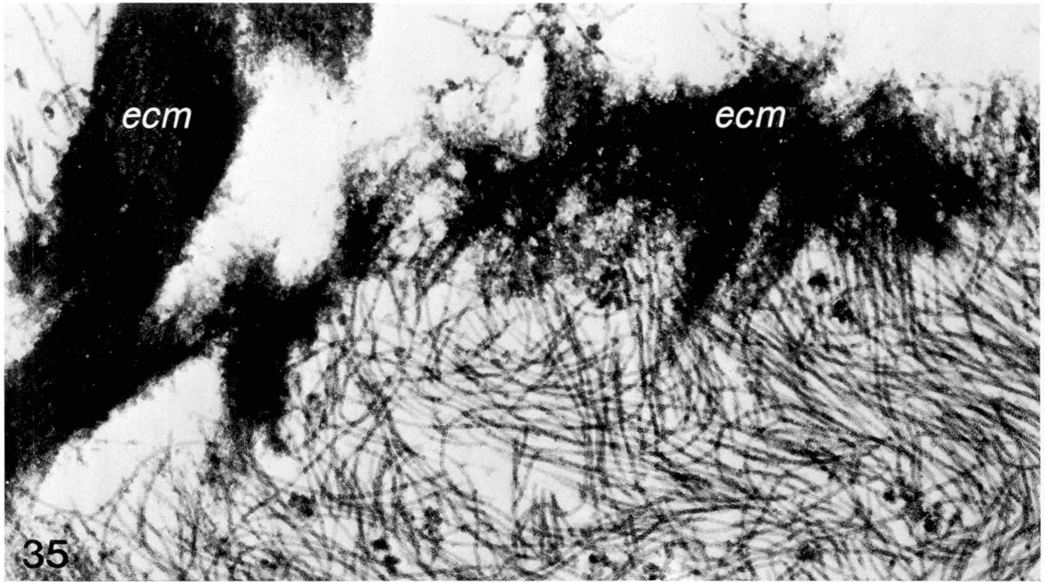
Figs 29, 30. Electron micrographs of cells grown and lysed on electron-microscope grids. Fig. 29 shows the IF bundle (*ifb*) network including the juxtanuclear IF cage, which remains when mouse epidermal cells are prepared according to Jones *et al.* (1982). Fig. 30 shows a region of a CEF prepared as described by Green & Goldman (1986). Note the nuclear remnant (*nr*), IF (*if*), elements of the extracellular matrix (*ecm*), and remnants of the plasma membrane (*pm*). Fig. 29, ×3500; Fig. 30, ×9100.



Figs 31–34. Electron micrographs of thin sections of the nuclear regions of IF-enriched cytoskeletons of CEF (Fig. 31), BHK (Fig. 32) and PME (Figs 33, 34) cells. Note the close associations between IF and the nuclear surface and in some cases nuclear pore complexes (*npc*). The nuclear lamin region is also evident (*nl*). Fig. 31, ×23 800; Fig. 32, ×48 000; Fig. 33, ×3700; Fig. 34, ×37 000.

Besides the major building-block proteins that make up the walls of IF, there are numerous other proteins that are consistently found in these IF-enriched cytoskeletons. In the majority of such preparations studied to date, which include several types of fibroblasts, epithelial cells and nerve cells, we have identified several proteins in the 60–70 K range (Fig. 37). In the case of BHK cytoskeletal preparations, there are four polypeptides of 60 K, 65 K, 67 K and 70 K (Fig. 37; and Zackroff *et al.* 1984). We have been able to purify these proteins as paracrystalline arrays (Figs 39, 40) and to demonstrate their keratin-like nature on the basis of their amino acid composition, their alpha-helix content and their cross-reactivity with

antibodies directed against keratin (Zackroff *et al.* 1984; Goldman *et al.* 1986). Antibodies directed against these proteins have been prepared and when used in indirect immunofluorescence they generate a bright ring around the edge of the



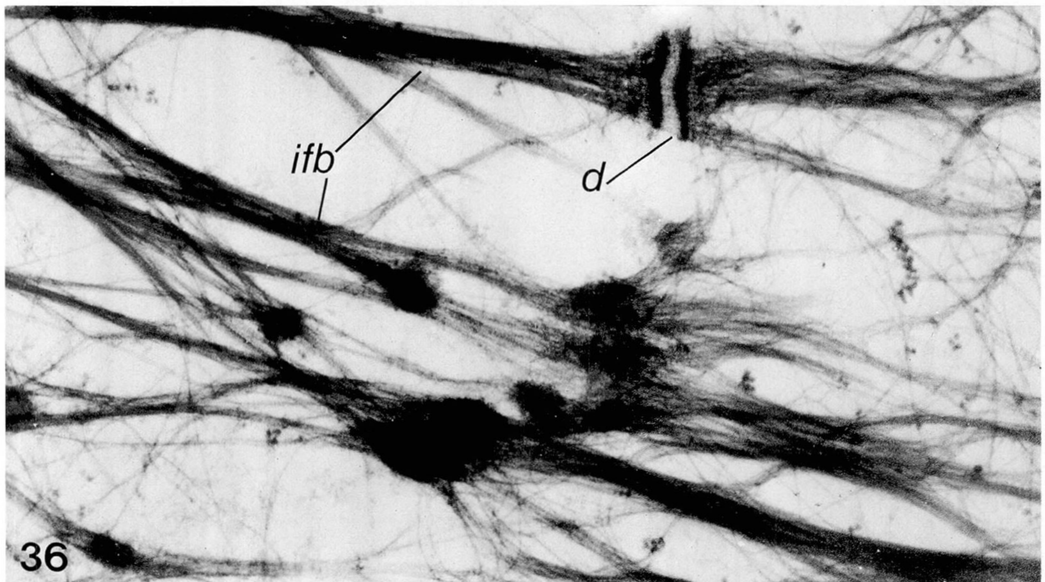


Fig. 35. An electron micrograph of a thin section through a region of the surface of a CEF IF-enriched cytoskeleton showing IF and fibronectin-enriched structures in the ECM (*ecm*). ×43 000.

Fig. 36. An electron micrograph of a thin section through a region of the surface of contacting PME cell cytoskeletons showing IF bundles (*ifb*, arrows) and a desmosome (*d*). ×45 000.

— 300 K

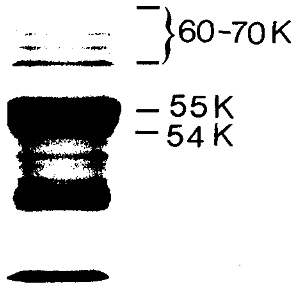


Fig. 37. SDS–PAGE analysis of a BHK cytoskeletal preparation containing the major 54 K (desmin) and 55 K (vimentin) IF structural proteins as well as the 60–70 K and 300 K components.

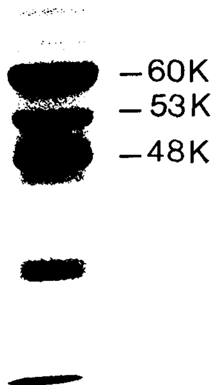


Fig. 38. SDS–PAGE analysis of a cytoskeletal preparation of PME cells. Note the major keratin IF structural proteins of 48, 53 and 60 K.

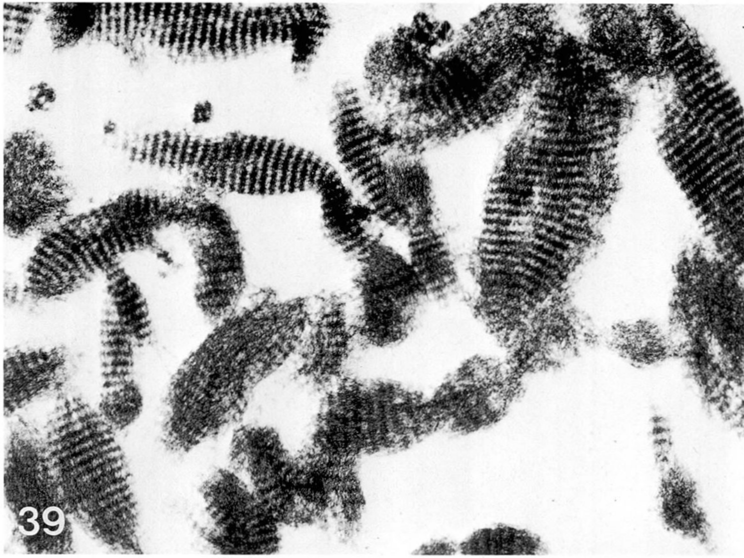


Fig. 39. An electron micrograph of a thin section through a pellet of paracrystals prepared from BHK IF-enriched cytoskeletons as described by Goldman *et al.* (1986). ×40 500.

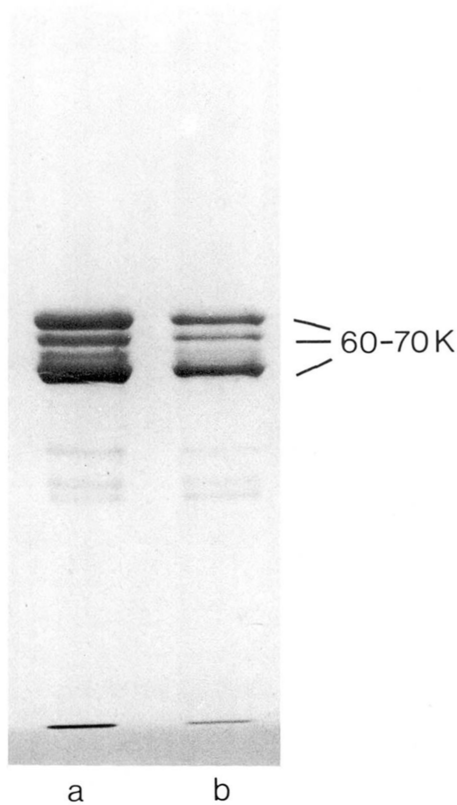


Fig. 40. SDS–PAGE analysis of paracrystals as prepared from BHK cytoskeletons (lane a) and a fraction of morphologically identical paracrystals prepared from *bona fide* nuclear lamins isolated from BHK nuclei (lane b) (Goldman *et al.* 1986). Note the enrichment of the four 60–70 K proteins seen in Fig. 37.

nucleus and diffuse fluorescence throughout the nuclear matrix (Fig. 41; and Goldman *et al.* 1985, 1986), indicating that this set of IF-like proteins is concentrated at the nuclear surface. From their size and cellular location, these proteins appear to be very similar to a set of nuclear matrix-derived proteins, which have been termed the nuclear lamins (Gerace *et al.* 1984), and which are thought to comprise the nuclear lamina. Indeed, we have been able to demonstrate that *bona fide* nuclear lamins derived from isolated BHK nuclei (Fig. 40) form identical paracrystalline arrays as described for the 60–70 K proteins that co-isolate in the IF-enriched cytoskeletal preparations (Goldman *et al.* 1986). Furthermore, antibodies directed against the nuclear lamins recognize the 60–70 K cytoskeletal polypeptides and *vice versa* (Goldman *et al.* 1986). Moreover, it has recently been shown that nuclear lamin sequences derived from cDNA clones are homologous to keratin sequences (McKeon *et al.* 1986).

Therefore it appears that the peripheral region of the nucleus contains a set of IF-like proteins. This latter term is used to describe these proteins, which have numerous biochemical and immunological properties indicating their similarities to the IF protein family, yet they appear to be incapable of forming IF either *in situ* or *in vitro*. These 60–70 K keratin-like proteins are probably major structural constituents of the fine fibrillar material associated with the ends of the IF as seen in Fig. 32. We find these results intriguing as they suggest, but by no means prove, that the 60–70 K proteins may comprise a binding domain to which the cytoskeletal IF may be anchored, albeit indirectly (see below; Goldman *et al.* 1985, 1986). These results are even more provocative if one considers the possibility that the nuclear lamina and its constituent proteins are thought to be involved in the attachment of chromatin to the nuclear surface, and in this fashion may be involved in regulating gene activity (Gerace *et al.* 1984). It is conceivable, therefore, that an insoluble, relatively amorphous IF-like protein system localized at the nuclear surface may interact with the cytoplasmic IF system, providing a skeletal continuum involved in nuclear–cytoplasmic communication. However, it should be emphasized that this is speculative as there are no known molecular connecting links between cytoplasmic IF and the nuclear IF-like system. If such connecting links exist, they would probably be in the form of complex transmembranous elements or transnuclear pore-complex components (Goldman *et al.* 1986).

There are also higher molecular weight components in the IF-enriched cytoskeletal preparations, some of which have been investigated using biochemical and immunochemical approaches. For example, in fibroblasts such as BHK and CEF there is a doublet or triplet of polypeptides at approximately 220 K, which by immunoblotting analyses are recognized by fibronectin antibody preparations (Fig. 42; and Green & Goldman, 1986). Furthermore, fibronectin is absent in the cytoskeletal preparations if they are prepared from freshly trypsinized cells, lending support to the idea that IF are part of a 'cell membrane complex', as mentioned above (Green & Goldman, 1986).

In the case of BHK cytoskeletal preparations we have initiated studies on a 300 K protein, which we have termed the 300 K IFAP (IF-associated protein) (Yang *et al.*

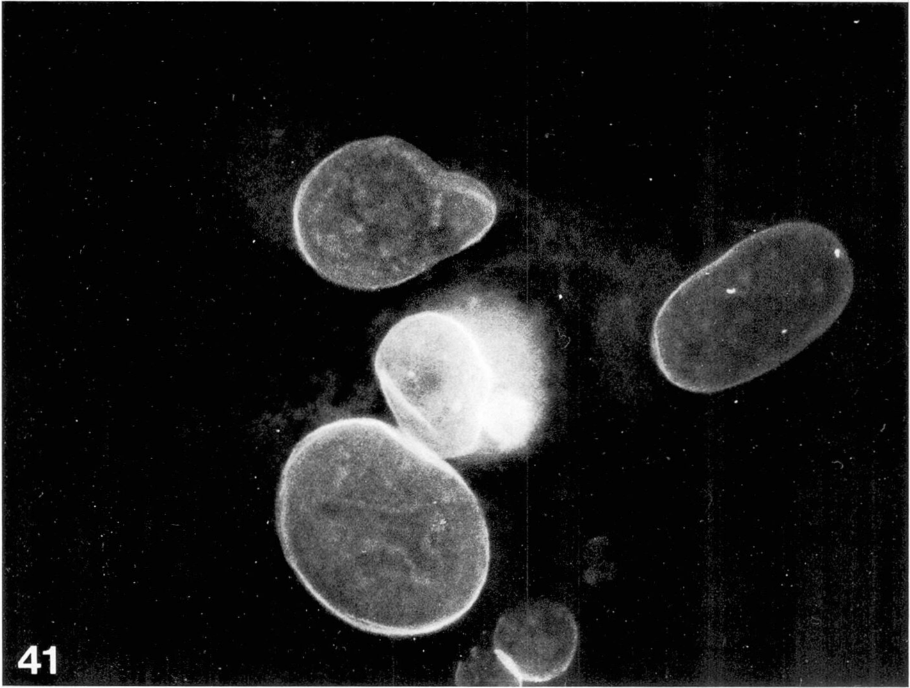


Fig. 41. An indirect immunofluorescence micrograph of BHK cells fixed and reacted with a monoclonal antibody that reacts with the 60–70 K proteins present in the IF-enriched cytoskeletons. Note the ring of fluorescence at the edge of the nucleus. ×1100.

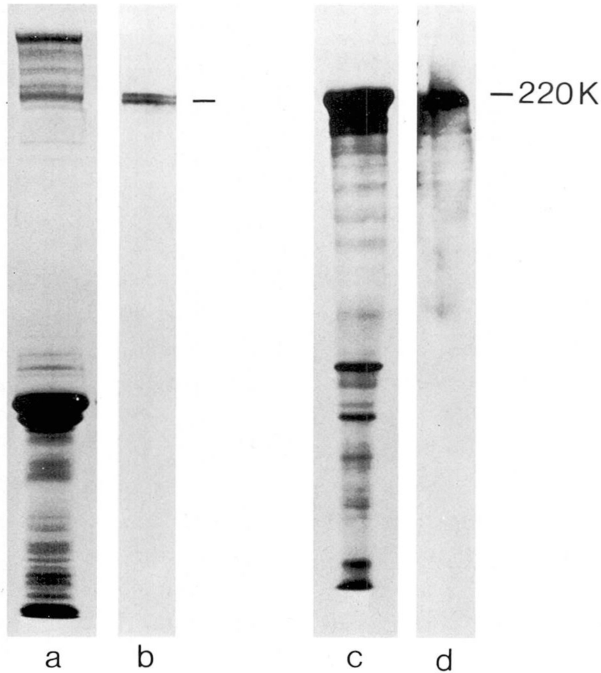
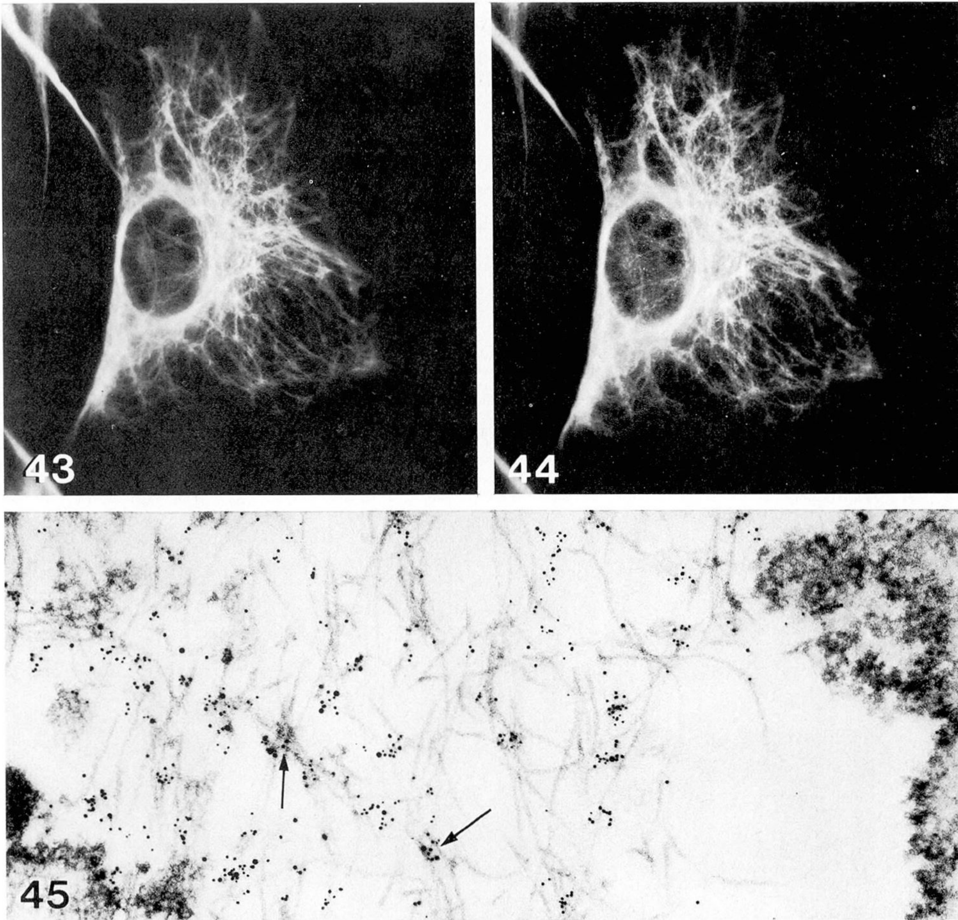


Fig. 42. Western immunoblotting analyses to demonstrate the presence of fibronectin in the IF-enriched preparations obtained from BHK (lanes a, b) and CEF (lanes c, d) cells. Lanes a, c, Amido Black-stained preparations of the polypeptides transferred to nitrocellulose. Lanes b, d, immunoblots using an antibody preparation directed against fibronectin. Note that in both preparations polypeptides of ≈220 K are recognized by the antibody preparation.



Figs 43, 44. Double-label immunofluorescence of BHK cells using an antibody preparation directed against the 55 K (vimentin) IF subunit (Fig. 37) and a monoclonal antibody preparation directed against the 300 K IFAP (Fig. 43). Note the coincidence of the patterns generated by these antibody preparations. ×750.

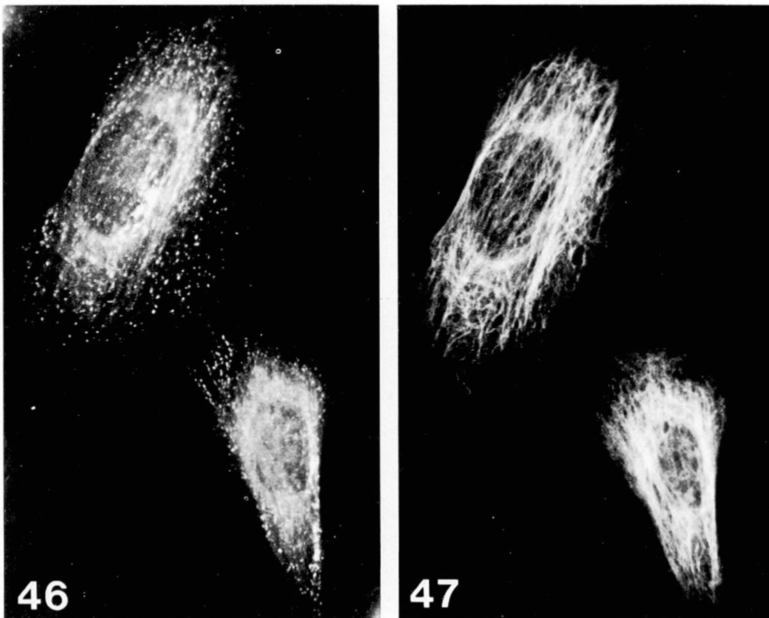
Fig. 45. An electron micrograph of a thin section of a BHK cell prepared for indirect immunogold localization using a monoclonal antibody preparation directed against the 300 K IFAP as described by Yang *et al.* (1985). Note that gold particles are present mainly in areas where IF appear to associate with each other (arrows). ×71 500.

1985). A monoclonal antibody has been prepared that reacts specifically with this protein and in double-label immunofluorescence assays, it appears to co-localize with IF (Figs 43, 44; and Yang *et al.* 1985). Observations made using the immunogold method at the ultrastructural level, indicate that this protein is localized in areas of close association between IF, indicating an involvement in cross-linking IF to IF (Fig. 45). More recently, we have been able to purify this protein and to demonstrate that it forms complexes with *in vitro*-reconstituted IF (Lieska *et al.* 1985).

We have also spent considerable time studying some of the high molecular weight proteins associated with IF in cultured epidermal cells (Jones & Goldman, 1985).

One of these turns out to be desmoplakin (Franke *et al.* 1983), a proposed component of the desmosomal plaque (see above). Indeed, the possibility that desmoplakin might represent another species of IFAP is suggested by its association with bundles of IF found in juxtanuclear complexes long before desmosome assembly occurs in PME cells grown in medium containing low levels of calcium (Figs 46, 47; and Jones & Goldman, 1985). This association has also been shown by immunogold localization (Jones & Goldman, 1985) and by immunoblotting analyses of IF-enriched cytoskeletal preparations made before and at various times after the initiation of desmosome assembly, following the switch to higher levels of calcium in the growth medium (Fig. 48; and Jones & Goldman, 1985). Moreover, once desmosome assembly has occurred and normal IF–desmosome complexes have formed along the borders of contiguous epidermal cells, cytoskeletons can be prepared using the method described above. We have found that the cytoskeletal preparations of these cells retain their intercellular desmosomal contacts by morphological criteria (see Fig. 36; and Jones *et al.* 1982; Jones & Goldman, 1985), and that desmoplakins 1 and 2 are both major constituents of the IF-enriched cytoskeletons (Fig. 48; and Jones & Goldman, 1985).

At this juncture it is worth reviewing briefly some of our work on the desmosomal system, specifically with regard to its subfractionation into constituent cytoplasmic and cell surface domains. With regard to the cell surface domains, we have been able



Figs 46, 47. Double-label indirect immunofluorescence of PME cells using a desmoplakin antibody preparation (Fig. 46) and a keratin antibody preparation (Fig. 47). The cells were maintained in medium containing low levels of Ca^{2+} for 48 h prior to fixation. Note that under these conditions desmoplakin-staining bodies (Fig. 46) appear concentrated in a juxtanuclear region where IF bundles (Fig. 47) are also found. ×900.

Immunofluorescence analyses have indicated that at least some of the antibodies found in the serum of patients with this autoimmune disease are directed against desmosomal components (Figs 49, 50; Jones *et al.* 1984, 1986*a*), and that these sera induce the separation of adherent keratinocytes in culture (Jones *et al.* 1984). Furthermore, immunoblotting analyses using cell-free preparations of bovine desmosomes (Fig. 51; and Jones *et al.* 1986*a*) indicate that there are several major antigens with which pemphigus serum samples react. In the case of pemphigus vulgaris, the majority of patients exhibit a reaction with a 140 K glycoprotein found primarily in bovine tongue mucosa desmosomes (Jones *et al.* 1986*a*), while in some pemphigus foliaceus patients, the most obvious consistent reaction is with a doublet of proteins in the 160–165 K range (Fig. 51; and Koulu *et al.* 1984; Jones *et al.* 1986*a*). The pemphigus vulgaris reaction is not detectable in desmosomes derived from a closely related stratified squamous epithelial tissue obtained from the epidermis of cow snouts (Jones *et al.* 1986*a*). Since these initial observations, we have prepared rabbit antibodies directed against this 140 K protein. We have been able to determine that this antibody preparation induces cell separation in living cultured mouse keratinocytes possessing desmosomal junctions, and furthermore recognizes a cell surface component (Jones *et al.* 1986*b*). More recently we have been able to demonstrate that this glycoprotein is present in membrane fractions obtained from bovine tongue desmosomes following extraction with urea (Fig. 52; and Jones *et al.* unpublished). Indeed, the 140 K protein possesses the characteristics of a cell adhesion molecule, i.e. it is a glycoprotein located on the cell surface, and an antibody directed against it disrupts cell–cell interactions (Edelman, 1982).

We have also been attempting to determine the nature of the cytoplasmic domains of isolated desmosomes (Fig. 53). In the urea-soluble fraction of desmosome preparations there is enrichment for desmoplakin and several polypeptides in the 50–65 K range that react with keratin antibodies. However, we have been unable to assemble IF from this urea-soluble fraction when it is placed into keratin assembly buffers. Indeed, under these conditions, a precipitate is formed that contains fibrillar material with no obvious IF (Fig. 54; and Jones *et al.* unpublished). This material appears to be morphologically similar to the fine fibrillar region that comprises the innermost (cytoplasmic) portion of the desmosome to which IF appear to attach *in situ* (Jones *et al.* 1986*b*). We are tempted to speculate that the interaction of desmoplakin with these keratins may reflect a mechanism by which IF associate with the desmosomal plaque. On the basis of these observations we hope to be in a position to study the 'chain' of molecules that is involved in interactions between the IF bundles of neighbouring cells *via* their desmosomal junctions.

SUMMARY AND CONCLUSIONS

The data reviewed in this paper provide support for the hypothesis that the IF system and its associated proteins may represent a chain of molecular connecting links between the nucleus and the cell surface. To date, we believe that we have characterized a number of links in this chain that has suggested to us that the IF

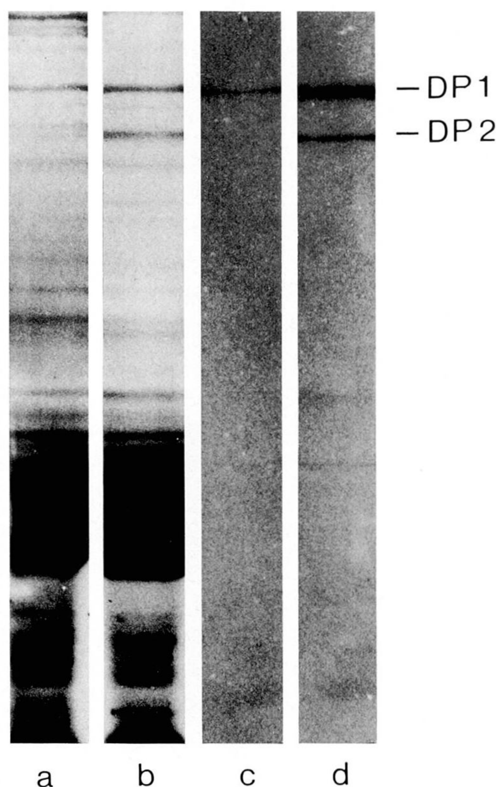
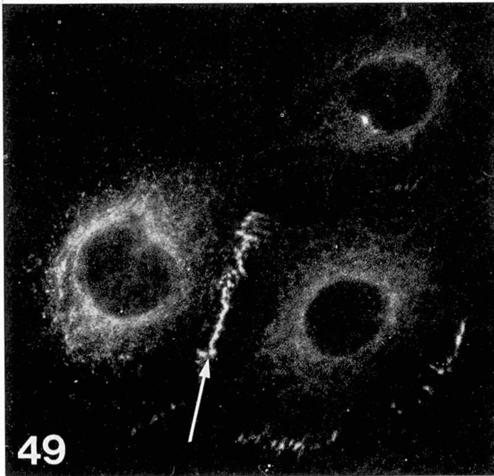
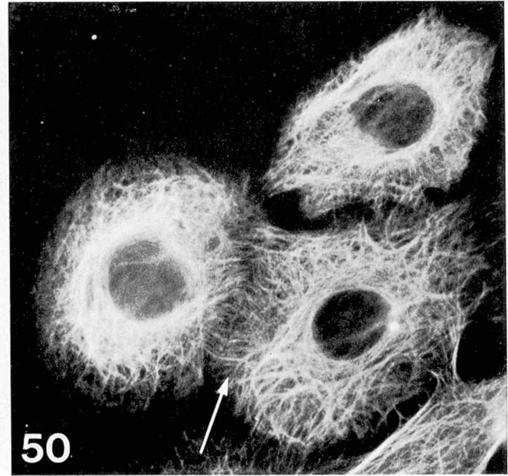


Fig. 48. Western immunoblotting analyses of PME cytoskeletal preparations isolated from cells maintained in low levels of Ca^{2+} (lanes a, c) or from PME cells 24 h following the initiation of desmosome assembly by increasing the extracellular Ca^{2+} concentration (lanes b, d). Lanes a, c, Amido Black-staining of polypeptides in these preparations. Lanes c, d, immunoblots using an antibody preparation directed against desmoplakin. Both cytoskeletal preparations possess high molecular weight polypeptides that are recognized by desmoplakin antibodies. In the case of the cytoskeletons isolated from PME cells maintained in low Ca^{2+} medium, a 250 K polypeptide (desmoplakin, DP1) appears present. However, both desmoplakins 1 and 2 (DP1 and DP2) are present in the cytoskeletal preparations isolated from PME cells 24 h following the initiation of desmosome assembly.

to determine that there are glycoproteins present that represent cell-type-specific adhesion molecules. The first clues that suggested that desmosomes might contain specific cell–cell adhesion (CAM) molecules came from studies of human auto-antibodies found in patients with pemphigus, a blistering disease of the skin (Beutner *et al.* 1970; Lever & Schaumberg-Lever, 1983). In this disease, blister formation is coincident with a loss of cell–cell adhesion in the epidermis, followed by cell death (Beutner *et al.* 1970; Lever & Schaumberg-Lever, 1983). This process is known as acantholysis. The acantholytic process takes place either in the suprabasal cell layer or in the upper cell layers of the epidermis. In the former case, the disease subclass is termed pemphigus vulgaris, while in the latter case it is called pemphigus foliaceus (Beutner *et al.* 1970; Lever & Schaumberg-Lever, 1983).





Figs 49, 50. Desmosome possessing PME cells prepared for double-label indirect immunofluorescence using a pemphigus vulgaris serum sample (Fig. 48) and a keratin antibody preparation (Fig. 49). Note that the pemphigus autoantibodies generate a staining pattern along areas of cell–cell contact where IF bundles of neighbouring cells associate and where desmosomes are located (arrows). ×800.

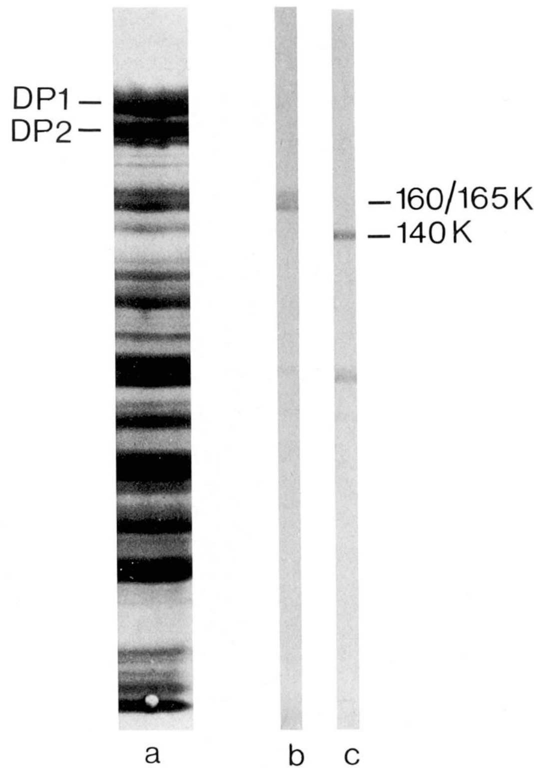


Fig. 51. Western immunoblotting analyses of two pemphigus serum samples using a bovine tongue mucosa desmosome-enriched fraction as a substrate (Jones *et al.* 1986*a*). Lane a, Amido Black staining of the polypeptides present in the desmosome-enriched fraction. Autoantibodies present in the serum of the pemphigus foliaceus patient recognize 160/165 K polypeptides in the desmosome fraction (lane b), while autoantibodies in the serum from a pemphigus patient with pemphigus vulgaris recognize a 140 K polypeptide in the same preparation. The desmoplakins are also indicated (DP1 and DP2 in lane a).

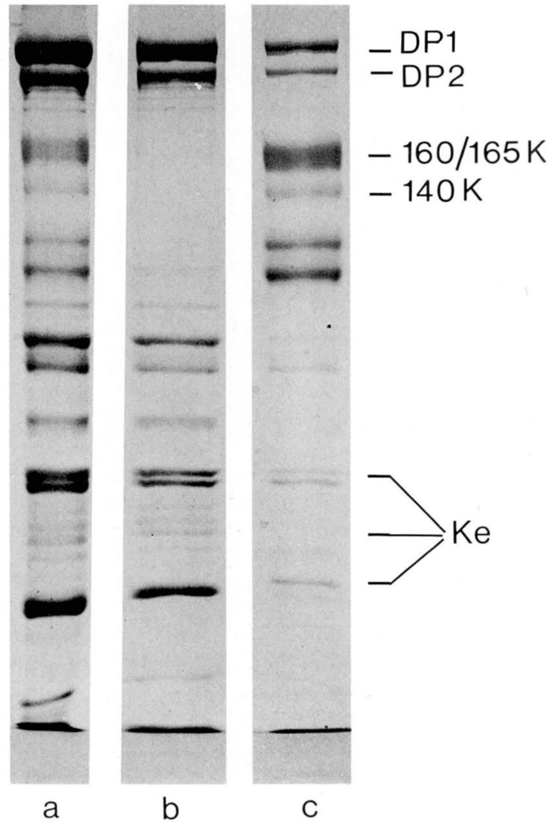
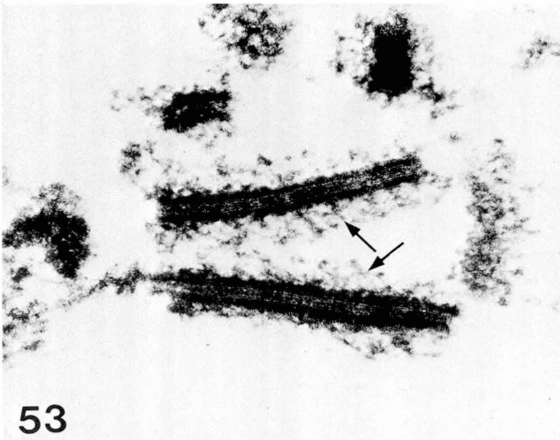
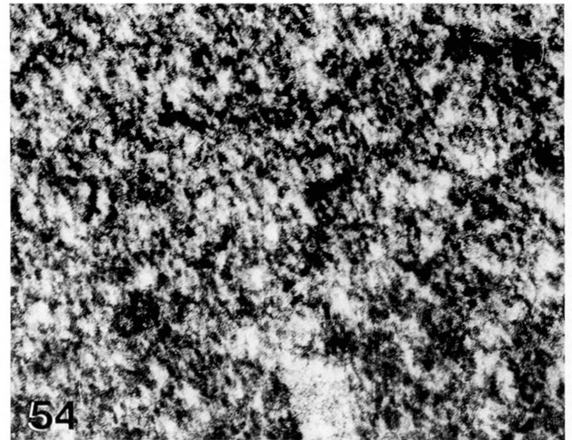


Fig. 52. SDS–PAGE analyses of a bovine mucosa desmosome-enriched preparation (lane a) and the 9·5 M-urea-soluble (lane b) and -insoluble (lane c) fractions of the same desmosome preparation (Jones *et al.* 1986). Note the enrichment of the 160/165 K and 140 K polypeptides in the urea-insoluble fraction (lane c). The soluble fraction (lane b) is enriched in desmoplakin 1 and 2 (DP1 and DP2) and certain keratin-like polypeptides (ke).





Figs 53, 54. Electron micrographs of an enriched preparation of bovine mucosa desmosomes (Fig. 53) and fibrillar material reconstituted from the 9·5 M-urea-soluble component of such a preparation (Fig. 54) (Jones *et al.* 1986). Note the similarity between the fibrillar material associated with the desmosome plaque (Fig. 53) (arrows) and the 9·5 M-urea-soluble reconstituted fibrillar material (Fig. 54). ×61 500.

networks in cells may represent a type of variable cytoplasmic infrastructure upon which other reactions or interactions take place. These may include interactions with the plasma membrane–extracellular matrix, interactions with other cytoskeletal components such as microtubules and microfilaments and the nuclear surface–nuclear matrix. Such a system may function in many aspects of cell surface–nuclear interactions, cell–cell adhesion and intercellular cytoskeletal interactions that are important in tissue structure and homeostasis.

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