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CYTOSKELETON ARCHITECTURE OF C6 RAT GLIOMA CELL SUBCLONES WHOLE MOUNT ELECTRON MICROSCOPY AND IMMUNOGOLD LABELING

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Abstract

Whole mount electron microscopy of extracted cells combined with immunogold labeling techniques can be used to characterize the cytoskeletal architecture of cultured cells. As shown with subclones of the C6 rat glioma cell line, heavy metal shadowing was suitable for getting basic information concerning the arrangement of the various filament types within the networks. Pure carbon shadowing combined with immunogold double labeling proved to be optimal to identify linkages between filaments, to localize filament associated proteins and to follow the arrangement of filaments in dense arrays such as lamellipodiae and cell margins. Thin connecting filaments which interact with actin as well as with vimentin filaments and can be labeled with antibodies to the intermediate filament associated protein plectin may play a major role in the structural organization of the cytoskeleton of these cells.

Key Words: Connecting filaments, cytoskeleton, immunogold labeling, intermediate filaments, plectin, vimentin, whole mount.

Introduction

Microscopic techniques, most of all immunofluorescence but also electron microscopy have made important contributions to the current image of the cytoskeleton in mammalian cells. Intermediate filaments, microtubules, and actin filaments compose the prominent structures of this cytoskeleton and are arranged in a complex three-dimensional network. In addition, there is a growing number of filament associated proteins, which either control filament assembly and/ or stability or regulate their interaction with cellular constituents, such as proteins which cap or fragment actin filaments [35], proteins which attach to microtubules (MAPs)[21] and those showing affinity for intermediate filament (IF) proteins [12]. There is evidence that the various filament systems do not act independently but are structurally linked [38], either directly or indirectly via crosslinking proteins, and that this interaction is a prerequisite for the cell to properly react to an external stimulus [23]. Thus alterations in structure and organization of one of the interacting components would have a significant influence on the structural arrangement and function of the others, a suggestion which corresponds to observations, that substances which act quite specifically on one filament system simultaneously change the arrangement of others [3]. Similar observations have been made in studies on integrins. By interaction of the transmembrane integrins with the corresponding ligands mechanical force is transmitted into the cell, a process which induces binding of the integrin molecule to submembraneous actin filaments and leads to activation of signal transduction pathways [28, 45]. The interaction of actin filaments with these plasma membrane proteins is accompanied by selection and anchoring of certain cytoplasmic proteins (α -actinin, talin, focal adhesion kinase) into larger complexes [15, 31]. This process of force transmission seems to be dependent on the integrity of all of the major filament systems [23]. Thus, the structural interaction of the major filament systems seems to influence not only the cell shape, but also specific functions, such as the transmission of signals from the extracellular matrix into the cell, or the maintenance of junctional complexes in lateral membrane areas

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of epithelial cells which is essential to stabilize their polarized organization. At the same time, the data point to special arrangements and local differences in the composition of the cytoskeleton in the various cell compartments which is essential for the cell to fulfill specific functions.

Based on the suggestion that a specific structural organization of the cytoskeleton reflects a specific function, a major question concerns the localization and arrangement of the numerous cytoskeletal proteins and how their structural interactions change in complex dynamic processes.

Visualization of Cytoskeletons in Electron Microscopy

Electron microscopy combined with immunocytochemical procedures can give some answers to these questions, with certain limitations [36, 38]. Filaments of the cytoskeleton are arranged in a three-dimensional network. Thus it is reasonable to choose electron microscopic methods which will reveal the three-dimensional orientation. This is realized with whole mount cytoskeletons obtained from cells permeabilized or extracted with a non-ionic detergent and then either quick frozen/deep etched or critical point dried and shadowed with metal and carbon.

Alternatively proteins may be identified by immunolabeling on ultrathin sections of non-extracted cells (post-embedding labeling). However, with non-muscle cells localization of cytoskeletal proteins on ultrathin sections is made difficult by the anisotropic organization of the cytoskeletal network and the limited thickness of the section. In addition, labeling is largely confined to the surface of the section. Labeling on resinless sections seems to be an alternative approach, but it is still limited by the fact that only a very small area of the cell can be visualized [46].

When applying the whole mount technique one has to be aware of significant losses of proteins during extraction and possible rearrangements. To make the findings obtained with extracted cells more reliable different approaches may be followed. First, the extraction procedures can be varied, providing a better definition of the conditions leading to the appearance of certain structures. Secondly, the cell system can be manipulated, for instance with certain cytoskeleton specific drugs. This may show that a specific cytoskeletal organization depends on specific physiological state of the cell. Thirdly, the residual cytoskeleton itself can be manipulated to define conditions that maintain a typical structural arrangement at this stage. In addition, the selection of cell mutants that are deficient in certain cytoskeletal proteins has proven to be a useful approach

to understand the function and dynamics of individual cytoskeletal proteins [1].

Whole Mount Cytoskeletons

Three main aspects have to be considered in the whole mount technique when it is applied to the cytoskeleton, namely detergent solubilization, fixation, and shadowing. An excellent survey of the various methods in existence has been given by Hartwig [16].

Cytoskeletons of C6 cells grown on glass coverslips were either obtained by solubilization with the non-ionic detergent NP40 (1%) in a KCl (80 mM) imidazole (20 mM) buffer at pH 7.0, supplemented with MgCl₂ (2 mM) and the calcium chelating agent ethylene glycol-bis-(β -aminoethylether)N,N,N',N'tetra-acetic acid (EGTA) (2 mM) at 40°C [6]. The remaining cytoskeleton consists mainly of actin, intermediate filaments and microtubules.

Stabilization of the cytoskeleton with a chemical agent is an essential point, when immunolabeling procedures will be applied that enclose prolonged incubations with antibodies and repeated steps of washing. The cytoskeleton of C6 cells was fixed with a mixture of freshly prepared paraformaldehyde (1%) and a low concentration of glutaraldehyde (0.025%) prior to labeling. This low concentration of glutaraldehyde proved to be sufficient to stabilize the structure and had the advantage of minimizing the fixative dependent background staining. Preservation of ultrastructure was not improved at this stage by increasing the glutaraldehyde concentration. The samples were postfixed with 0.5% glutaraldehyde after labeling. Higher concentrations of glutaraldehyde may disrupt microfilaments, as does also osmium tetroxide [8, 26, 37].

After fixation the C6 cytoskeleton was dehydrated through a graded series of ethanol and critical point (CP) dried. Alternatively, the cytoskeletons may be subjected to quick freezing and deep etching, which has been shown to give superior images of cytoskeleton structures at high resolution [9, 17, 20, 24, 27]. CP-drying, which was used throughout in our studies on C6 cytoskeletons, has also been applied with success [10, 11, 29, 32]. The reliability of both methods has been discussed extensively. In some studies a higher variation in filament thickness was noted in CP dried probes as compared with rapidly frozen samples [18]. Others indicated that both methods provide a similar cytoskeletal ultrastructure and when properly handled CP drying was considered to be a reliable method yielding consistent results [7]. In critical point drying, problems seem to arise when residual water is present in CO₂, which leads to a lateral aggregation of filaments [7].

After drying or deep etching the cytoskeletons are

normally shadowed with a layer of heavy metal. Cytoskeletons of C6 cells were either rotary shadowed with platinum/carbon or with pure carbon. For platinum/carbon shadowing the probes were mounted on a stage cooled with liquid nitrogen and shadowing was done at low pressure (10^{-7} bar) to improve the resolution of the metal film [22]. Immunogold-labeled probes were generally shadowed with pure carbon which provided better identification of the label because of increased contrast between the gold label and the cytoskeletal structure. In order not to decrease the filament contrast itself in these probes the cytoskeletons were generally mounted on grids without a supporting film [22].

IF-Protein Expression in C6 Rat Glioma Cells

As mentioned above cytoskeletons obtained by extraction of cells mainly consist of actin filaments, microtubules and intermediate filaments. In mammalian cells the proteins of intermediate filaments (IFs) normally represent a major cytoskeleton component, of which the expression is tissue-specific and developmentally regulated. The cytoplasmic IF-proteins probably originate from nuclear lamins as a common ancestor [25]. Those present in vertebrate cells can be divided into four groups, the vimentin-like and the neuronal IF-proteins, the cytokeratins, and the lens IF-proteins. Vimentin is the characteristic IF-protein found in terminally differentiated cells of mesenchymal origin. However, due to unknown reasons, cells frequently start to express vimentin in addition to their tissue specific IF-protein when explanted from tissue, indicating that vimentin expression is dependent on environmental conditions. Thus vimentin is the most frequent IF-protein in cultured cells and has been used as a model system to characterize the biology of IF-proteins. However, despite extensive knowledge of its molecular structure it is still a matter of question if it fulfills a specific function [41]. Vimentin seems to be linked to the nuclear lamina, and probably interacts with the microtubule and actin filament system, and with components in the plasma membrane [25]. Biochemical data indicated that the intermediate filament associated protein plectin binds to vimentin and may be involved in crosslinking cytoplasmic filament systems [12].

In contrast to the actin filament system and microtubules the presence of cytoplasmic IF-proteins is not obligatory, which is well documented by the existence of some IF-protein deficient cell lines [19, 40, 42]. IF-deficient cells have been used in studies on vimentin network formation performed by immunofluorescence either after transfection with an appropriate vector system or after injection of purified vimentin proteins. Absence of cytoplasmic IF-proteins is a rather rare event

and it has proved to be difficult to establish subclones with stable phenotypes from these adherent cell lines.

The C6 rat glioma cell line, which was cloned from a chemically induced rat brain tumor, was found to consist of a mixed population of cells which either contain vimentin (80% of the cells) or completely lacked any cytoplasmic IF-proteins [34]. These latter cells showed a normal content of lamins A, B, and C [30]. In IF-deficient cells the absence of vimentin was found to result from a block at the transcriptional level. As stable subclones could be established with both phenotypes, this cell system seemed to be useful not only to follow IF-protein assembly, but to use IF-protein expression in these cells as a tool for analyzing principal mechanisms in the organization of the cytoskeleton by use of ultrastructural and immunocytochemical methods.

Cytoskeleton of C6 Cells

The cytoskeleton of the C6 subclone containing the intermediate filament protein vimentin revealed a wide spaced filament network (Fig. 1A). The prominent filaments measured 10 nm and 13 nm in width, which corresponds to that of actin and intermediate filaments taking into account a 3 nm metal layer. In between individual thin connecting filaments of about 6 nm were detectable. Similar filament types were visible in carbon shadowed cytoskeletons (Fig. 1B). In addition filaments of about 30 nm were present corresponding in size to microtubules. In IF-deficient C6 cells filament bundles and thin connecting filaments were the prominent structures (Fig. 1C). The cytoskeleton of IF-deficient cells was more fragile and more difficult to handle than that of the vimentin containing cells. To unequivocally identify the arrangement of vimentin and actin in this cytoskeleton and to localize sites of interaction we identified these filament types by immunogold labeling [4].

Immunogold-Labeling of Filaments

As shown above, the samples obtained after extraction of cells with non-ionic detergents represent a filamentous network which mainly consists of actin filaments, intermediate filaments, and microtubules which can be distinguished on the basis of their substructure and width. These filaments represent structures built of identical subunits assembled in a specific way. Antibodies developed against these proteins are directed against these subunits. Thus, in labeling experiments, one would expect a continuous labeling along these filaments as was demonstrated with antibodies to tubulin [2] and is shown with antibodies to actin and vimentin in

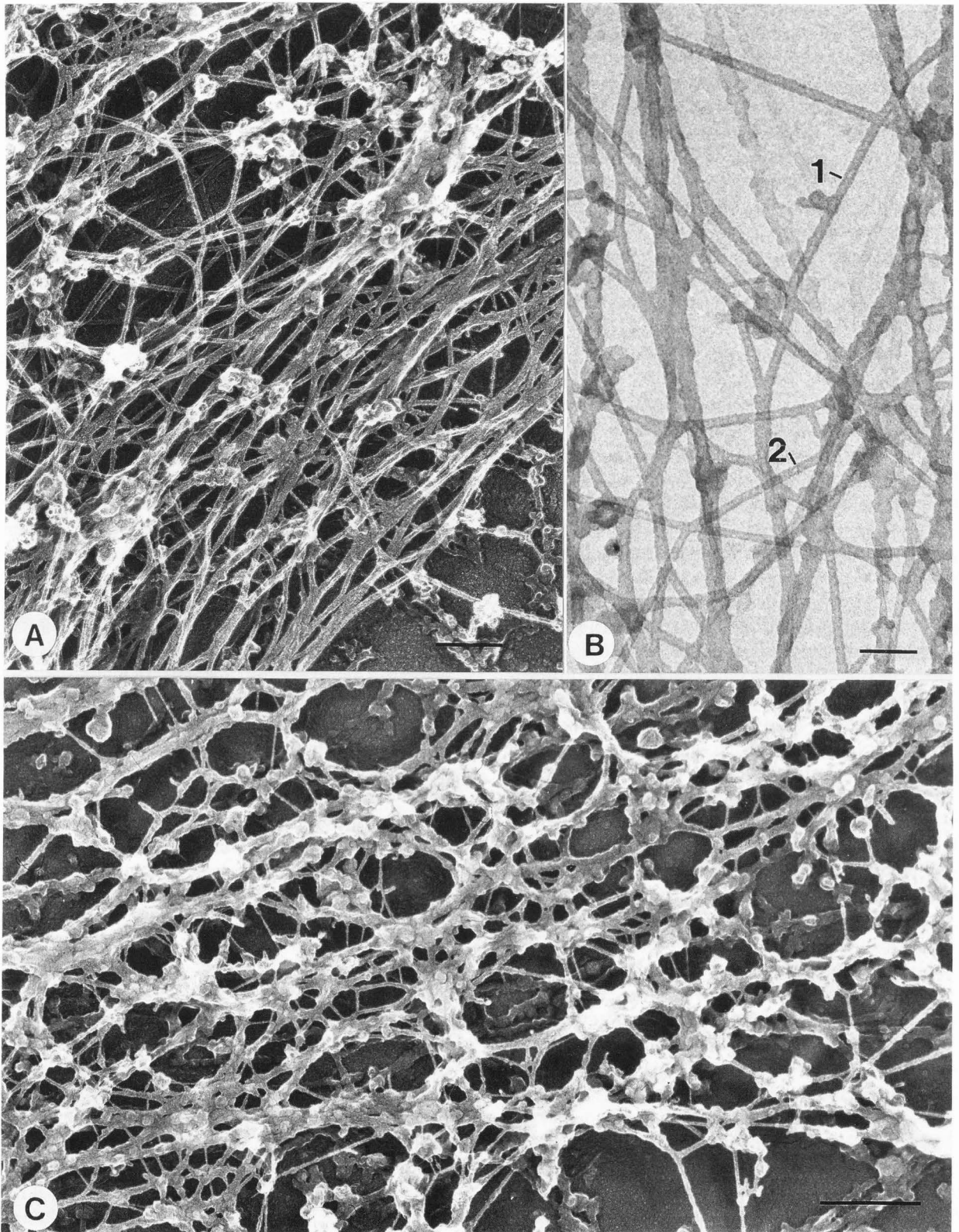


Figure 1: Cytoskeletons of C6 subclones. (A and B) Cytoskeletons of vimentin positive C6 cells; (C) Cytoskeleton of an intermediate filament deficient C6 cell. (A and C) rotary shadowing with platinum/carbon; (B) rotary shadowing with carbon. Bars in (A) = $0.2\mu\text{m}$; (B) = $0.1\mu\text{m}$; (C) = $0.5\mu\text{m}$.

Fig. 2A. Despite the fact that in our labeling studies antibodies were used in excess, the amount of background label was very low. There was no random distribution of gold particles at the surface of the glass coverslip and the thin connecting filaments as well as structures corresponding to microtubules were clearly unlabeled. On the other hand the labeling density along the actin and vimentin filaments was very high and individual vimentin filaments could easily be followed in the dense filamentous networks in lamellipodia (Fig. 2 C and D). Lower density of gold label along these filaments or a labeling gap may indicate a change in the composition of the filament as it may result from the presence of filament associated proteins. In the IF-deficient subclone antibodies to actin labeled the filament bundles but were absent on the thin connecting filaments (Fig. 2B).

In contrast to the major cytoskeletal proteins, other proteins do not assemble into filaments of repetitive subunits but are present as individual molecules or oligomeric structures. In principle, they cannot easily be identified on the basis of a typical morphology as it is possible to do with the major filament systems. Their localization relies even more on the use of immunocytochemical techniques. The proteins may be randomly distributed throughout the network. Consequently, the label may be dispersed throughout the network. This for instance is shown in immunogold labeling studies on membrane skeletons of platelets [18]. After labeling with antibodies to spectrin, gold conjugates were randomly associated with thin connecting filaments in these skeletons, and the gold labeling of actin-binding-protein complexes was also randomly distributed and could not be assigned to a specific ultrastructure. Similar problems arose when localizing plectin in the cytoskeleton of C6 subclones.

Plectin Localization in C6 Subclones

Plectin is a 466 KD protein, which originally has been identified as a major component of the salt resistant fraction of detergent solubilized C6 cells [43]. The protein contains a N-terminal globular domain, a central α -helical coiled-coil, and a C-terminal globular domain with a prominent sixfold tandem repeat organization [44]. Rotary shadowed probes of purified plectin show dumb-bell shaped (probably homotetrameric) molecules consisting of 9 nm globular domains linked by a 200 nm rod of 2 nm width [13, 14]. The high affinity of plectin for intermediate filament proteins, fodrin, MAPs and its widespread occurrence suggested that this protein plays a major role in linking cytoskeletal structures [12] mainly in interaction with the intermediate filament system. Based on these data, we investigated how plectin

behaves in cells lacking cytoplasmic intermediate filament proteins. Plectin was labeled with antibodies which recognize an epitope in the rod domain of the dumb-bell shaped molecule [13]. In vimentin containing cells the plectin label was present throughout the filament network and frequently found in association with thin connecting filaments (Fig. 3A). Double labeling with antibodies to plectin and actin verified that these thin connecting filaments, the filaments with which the plectin label was associated, did not consist of actin (Fig. 3B). The label was localized at sites where these filaments made contact with intermediate filaments or in the center of these connecting filaments. Obviously, the intermediate filaments changed their direction at sites that were in contact with the thin connecting filaments. Plectin was also present in the IF-deficient subclone bound to actin containing structures. Again, the plectin label was also found in association with thin connecting filaments (Fig. 3C). The association with actin in vimentin negative cells could be substantiated by the fact that the plectin label was lost from these but not from the vimentin containing cytoskeletons after treatment with heavy meromyosin which specifically associated with actin filaments. These data support the suggestion that plectin plays a major role in crosslinking cytoskeletal filament systems and probably in forming thin connecting filaments [33].

Conclusion

The data substantiate the usefulness of whole mount electron microscopy combined with immunogold labeling for the identification of the structural arrangement of proteins within the cytoskeleton. With antibodies to actin and vimentin the immunogold conjugates continuously decorated the corresponding filaments without giving any background labeling. Any presence of gold particles at the surface of the glass coverslip outside the cytoskeleton would either indicate inappropriate labeling conditions. Alternatively the protein to be identified may be lost from the cytoskeleton during extraction and dispersed throughout the network. In this case it would be uncertain if gold particles present on specific structures reflect the original localization of this protein.

Absence of background label especially is a prerequisite for a reliable interpretation of those labeling patterns which do not follow the orientation of specific structures such as filaments. In the case of plectin the reliability of the labeling pattern is substantiated by the fact that it was associated with thin connecting filaments.

On labeled probes the structural resolution is deteriorated by the attached antibodies and immunogold conjugates. Thus shadowing with carbon proved to be sufficient for stabilization of the delicate structures and

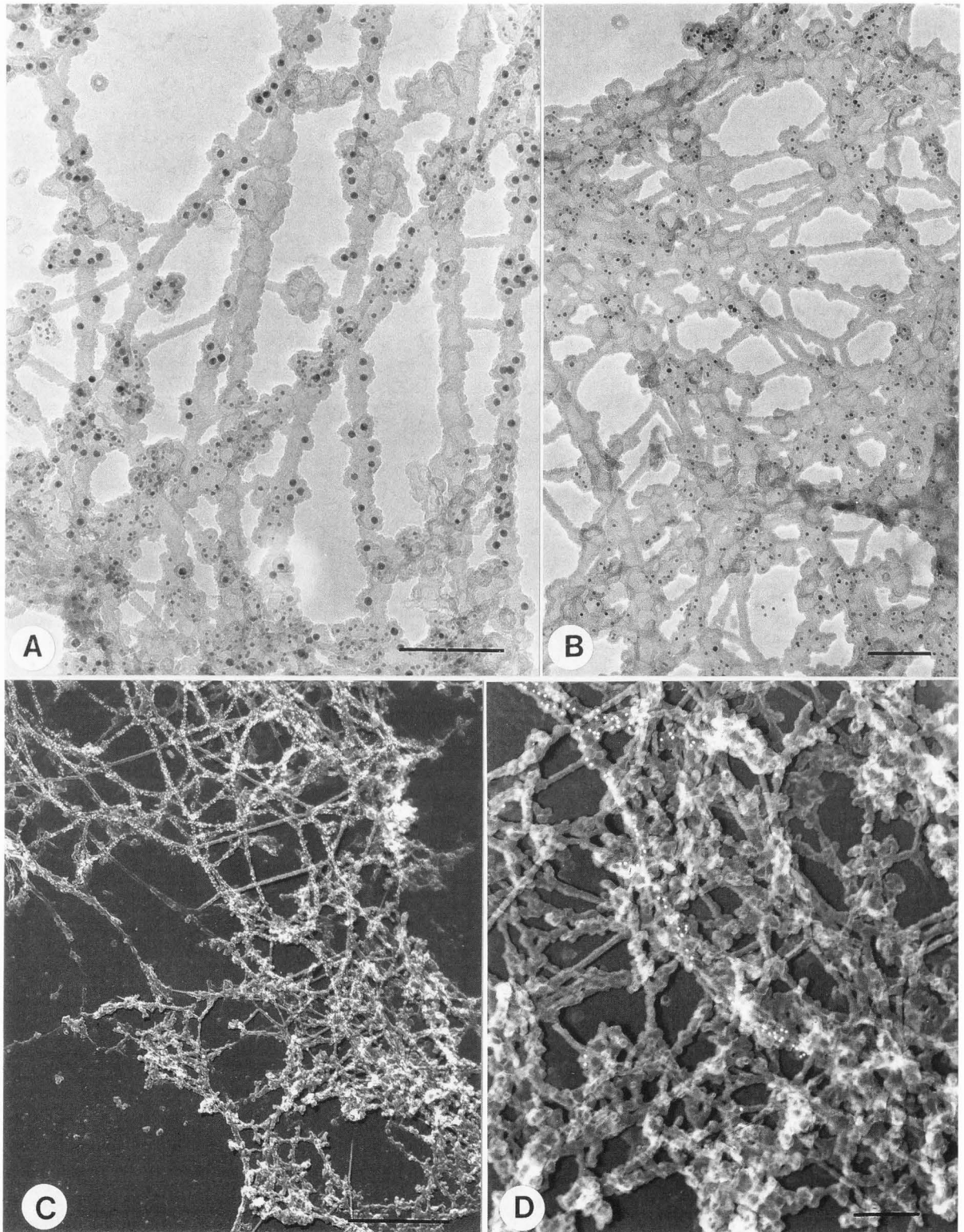


Figure 2: (A) C6 cytoskeleton labeled with rabbit antibodies to actin and a goat anti-rabbit (5nm) immunogold conjugate and a monoclonal antibody to vimentin and a goat anti mouse (10nm) gold conjugate. (B) Cytoskeleton of an intermediate filament deficient cell labeled with antibodies to actin as in (A). (C) and (D) C6 cytoskeleton labeled with a monoclonal antibody to vimentin as in (A). (D) Enlarged detail of (C). Bars: (A) and (B) = 0.1 μ m; (C) = 1 μ m; (D) = 0.2 μ m.

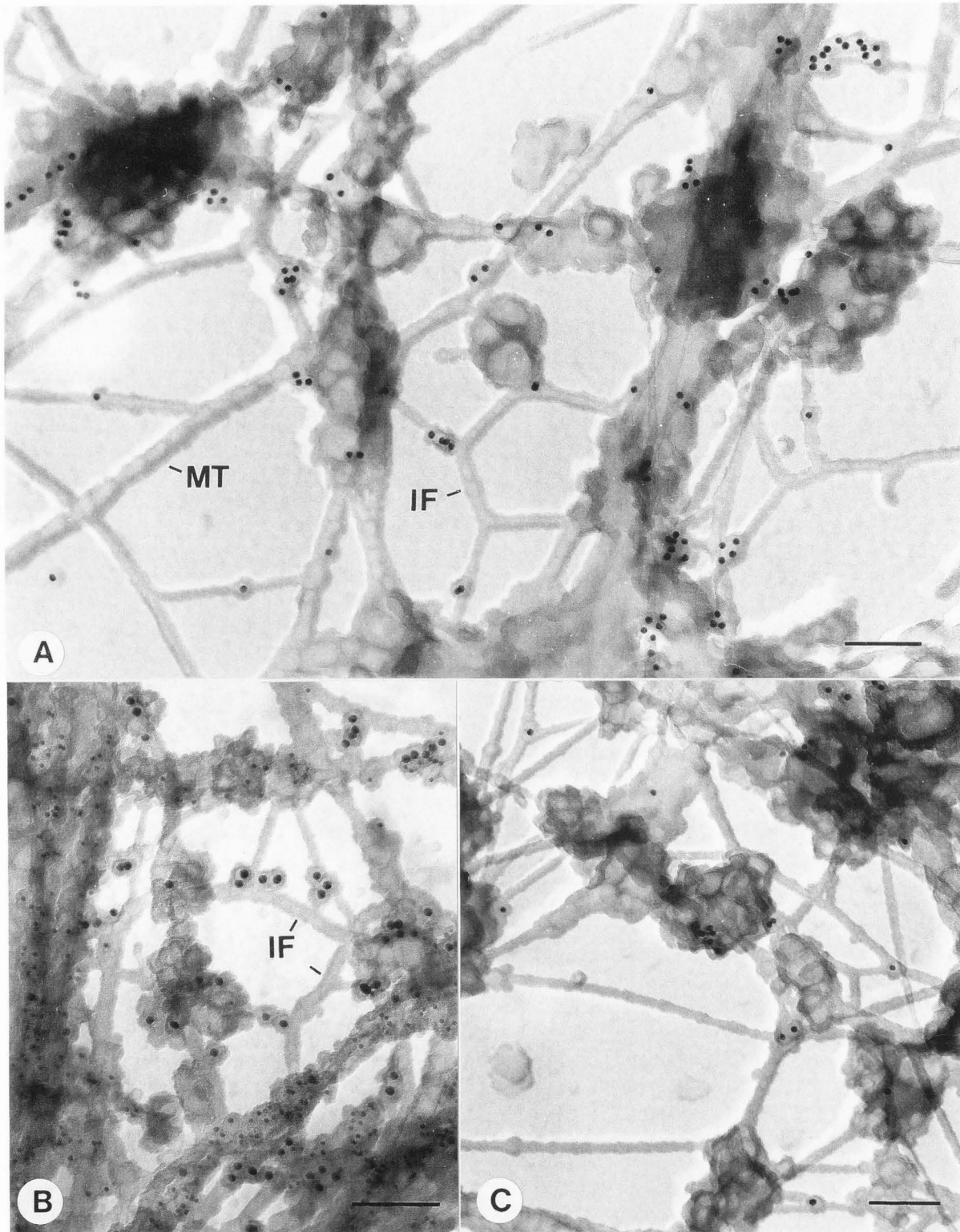


Figure 3: (A) Cytoskeleton of a vimentin positive cell labeled with a monoclonal antibody to plectin and a goat anti-mouse (10nm) immunogold conjugate. (B) Cytoskeleton of a vimentin positive cell labeled with an antibody to plectin (as in A) and antibodies to actin (as in Figure 2). (C) Cytoskeleton of a intermediate filament deficient cell labeled with an antibody to plectin as in (A). Bars in (A), (B), and (C) = 0.1 μ m.

had also the advantage to retain the contrast between the gold label and the biological structure.

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Discussion with Reviewers

P. Bell: Considering that your extraction is done under conditions that do not stabilize most of the components of the cytoskeleton, it is not surprising that your preparations are very heavily extracted. Therefore, I question your use of the term "cytoskeleton whole mounts" to refer to your preparations. Would not the term "detergent-extracted whole mount" be more appropriate since most of the cytoskeleton is gone?

Authors: In general, cytoskeletons which were obtained with the extraction procedure described were very dense (see Fig. 1A), but also showed differences in composition dependent on the subcellular compartment. The arrangement of immunogold labeled cytoskeletal structures, especially that of plectin, could best be demonstrated with micrographs showing areas of lower density. This was the basis for selecting the micrographs. Biochemical studies concerning the solubility of plectin in vimentin positive versus vimentin deficient cells

substantiated the microscopical data [47].

P. Bell: Have you tried any of the methods that can improve the preservation of cytoskeletal components, such as protein crosslinking and stabilizing buffers?

Authors: So far we did not include stabilizing or crosslinking substances in our extraction procedure. We admit that especially the use of crosslinking agents as suggested by you and your colleagues has to be tested to determine if structures which connect the various filament systems can better be maintained. At least the thin connecting filaments seemed to be very sensitive to mechanical stress.

P. Bell: Did you encounter any problems with collapse or destruction of structures when you mounted preparations on uncoated grids?

Authors: Head over drying of the grid attached cytoskeletons minimized mechanical stress during drying.

P. Bell: Under the best of circumstances there are variations in the diameter (thickness) of filamentous structures coated evaporatively with carbon or metal. Can you provide quantitative and statistically relevant data to support your statement that actin and intermediate filaments can be distinguished on the basis of size alone.

Authors: In individual preparations the various filament types can roughly be identified on basis of relative differences in thickness. However, we agree that the thickness of the carbon coat varies considerably in successive experiments. That is why we introduced immunogold labeling procedures to unequivocally follow the arrangement of individual filaments and to identify structural linkages between the various filament types.

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