Occurrence and Immunolocalization of Plectin in Tissues

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ABSTRACT Various tissues from rat were examined for the occurrence and cellular localization of plectin, a 300,000-dalton polypeptide component present in intermediate filament-enriched cytoskeletons prepared from cultured cells by treatment with nonionic detergent and high salt solution. The extraction of liver, heart, skeletal muscle, tongue, and urinary bladder with 1% Triton/0.6 M KCl yielded insoluble cell residues that contained polypeptides of M_r 300,000 in variable amounts. These high M_r polypeptide species and a few bands of slightly lower M_r (most likely proteolytic breakdown products) were shown to react with antibodies to rat glioma C_6 cell plectin using immunoautoradiography and/or immunoprecipitation.

By indirect immunofluorescence microscopy using frozen sections (4 μm) of stomach, kidney, small intestine, liver, uterus, urinary bladder, and heart, antigens reacting with antibodies to plectin were found in fibroblast, endothelial, smooth, skeletal, and cardiac muscle, nerve, and epithelial cells of various types. Depending on the cell type, staining was observed either throughout the cytoplasm, or primarily at the periphery of cells, or in both locations. In hepatocytes, besides granular staining at the cell periphery, conspicuous staining of junctions sealing bile canaliculi was seen. In cardiac muscle strong staining was seen at intercalated disks and, as in skeletal muscle, at Z-lines. In cross sections through smooth muscle, most strikingly of urinary bladder, antibodies to plectin specifically decorated regularly spaced, spot-like structures at the cell periphery.

By immunoelectron microscopy using the peroxidase technique, antiplectin-reactive material was found along cell junctions of hepatocytes and was particularly enriched at desmosomal plaques and structures associated with their cytoplasmic surfaces. A specific immunoreaction with desmosomes was also evident in sections through tongue. In cardiac muscle, besides Z-lines, intercalated disks were reactive along almost their entire surface, suggesting that plectin was associated with the fascia adherens, desmosomes, and probably gap junctions. In smooth muscle cells, regularly spaced lateral densities probably representing myofilament attachment sites were immunoreactive with plectin antibodies.

The results show that plectin is of widespread occurrence with regard to tissues and cell types. Furthermore, immunolocalization by light and electron microscopy at junctional sites of various cell types and at attachment sites of cytoplasmic filaments in epithelial and muscle cells suggests that plectin possibly plays a universal role in the formation of cell junctions and the anchorage of cytoplasmic filaments.

Intermediate filament-enriched cytoskeletal residues prepared from various cultured cell lines by treatment with 1% Triton X-100/0.6 M KCl (24) contain considerable amounts of a high M_r (~300,000-dalton) polypeptide component (21), termed plectin (28). In such preparations from fibroblast cells, plectin accounts for up to 30% of total protein and thus is the only other major polypeptide component besides vimentin, the intermediate filament protein subunit (28). In indirect immunofluorescence microscopy elaborate cytoplasmic network arrays are revealed in cultured fibroblasts by antibodies to plectin that are clearly different from the three major cytoskeletal network systems identified so far, the microtubules, the class of intermediate filaments, and the microfilaments (27). The immunolocalization of plectin within a seem-

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ingly highly cross-linked network in cultured cells, its interaction with vimentin filaments in vitro (unpublished data) and the fact that it has some properties in common with high M_r polypeptides considered to be associated with microtubules (21, 26) have prompted our suggestion that plectin plays a role as a cytoplasmic cross-linker (28).

To gain further insight into the function(s) of plectin we have studied its occurrence and subcellular localization in various tissues of rat using gel electrophoresis, immunoautoradiography, immunofluorescence microscopy, and immunoelectron microscopy. It is shown in this report that plectin is of widespread occurrence in diverse tissues and cell types. Its conspicuous association with desmosomes and other junctional complexes in various tissues and with Z-lines of muscle cells suggests that it plays some role in the formation of cell junctions and anchorage of cytoplasmic filaments.

MATERIALS AND METHODS

Tissues and Cells: The following tissues were taken from rat immediately after sacrifice: stomach, liver, kidney, small intestine, uterus, heart, tongue, skeletal muscle, ischiatic nerve, and urinary bladder. For immunolocalization experiments tissues were frozen in isopentane at the temperature of liquid nitrogen. For preparations of cell fractions enriched in plectin, tissues were chilled on ice and further processed with minimal delay. For preparation of dissociated epithelial cells from small intestine and their examination using indirect immunofluorescence microscopy, essentially the procedures of Franke et al. (8, 10) were followed, except that Triton X-100 treatment was omitted. Murine hepatoma cells (line MH_1C_1) obtained from the American Type Culture Collection (Rockville, Maryland) were grown and processed for immunofluorescence microscopy as described previously (11).

Preparation of Cell Fractions: For the preparation of extracts from whole tissues, chilled material was incubated in Laemmli's sample buffer (16) at 95°C for 5 min, followed by homogenization in a glass-teflon homogenizer. Insoluble residues were removed by centrifugation and supernatants were used for analysis.

For the preparation of cell fractions enriched in plectin, chilled tissues were rinsed several times with 6 mM sodium phosphate, pH 7.0, 150 mM sodium chloride (buffer A), minced with scissors, and homogenized in buffer A supplemented with 1% Triton X-100, 0.6 M KCl, 2 mM phenylmethylsulfonyl fluoride, for 20 min. All tissues used, except bladder, were fairly homogeneously dispersed by this treatment. Tissue homogenates were then spun at 30,000 *g* for 30 min at 4°C and the pellets, representing detergent/high salt-resistant residues, were dissolved in Laemmli's sample buffer (16) and stored at −20°C. Intermediate filaments from rat glioma C_6 cells were prepared as described previously (21).

Antibodies: The following antibody preparations were used: (*a*) rabbit antibodies to rat glioma C_6 cell plectin (27); (*b*) guinea pig antibodies to component D of mouse liver cytokeratin (7, 11); (*c*) guinea pig antibodies to chicken gizzard desmin. The latter was prepared following the procedure of Franke et al. (9). In addition, a monospecific antiplectin immunoglobulin-fraction, affinity-purified on plectin-Sepharose columns (kindly provided by E. Briones), was used in some experiments (Fig. 12).

Gel electrophoresis and Immunoautoradiography: SDS PAGE was performed according to Laemmli (16) using gels with a ratio of bisacrylamide to acrylamide of 0.027. Gels were stained with 0.05% Coomassie Blue, 35% isopropanol, 10% acetic acid for 10 min and destained in 7% acetic acid.

For detection of antigens in gels we used Burridge's method (4), modified as described in detail elsewhere (R. Pytela, PhD thesis, University of Vienna). In brief, stained gels were fixed in 50% isopropanol, 10% acetic acid overnight, washed in 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% azide (TBS), incubated with antiserum for 24 h, and washed for several days with TBS. This was followed by a 24 h incubation with tritium-labeled (25) Staphylococcal protein A, and another wash in TBS for 3–5 d. Finally, gels were restained with Coomassie Blue and radioactivity was detected using fluorography (6).

Immunoprecipitation: Triton/high salt-resistant residues from rat tissues were suspended in 200 mM borate, pH 9.0, and tritium-labeled according to (25). The reaction was stopped by adding concentrated SDS sample buffer (16) to a final concentration of 3% SDS, 5% 2-mercaptoethanol, 10% glycerol, 60 mM Tris-HCl, pH 6.8. Mixtures were then heated at 95°C for 5 min. 10-μl aliquots of these samples were diluted 1:30 with 1% Triton X-100, 1% sodium deoxycholate, 1 mM EDTA, 150 mM NaCl, 50 mM Tris-HCl, pH 7.5 (buffer B), centrifuged for 15 min at 12,500 *g*, and incubated with 10 μl of a suspension of formaldehyde-fixed *Staphylococcus aureus* (Bethesda Research Laboratories, Gaithersburg, Maryland) for 30 min. Bacteria were then removed by centrifugation at 12,500 *g* for 30 s, and supernatants were incubated sequentially with 5 μl of antiserum for 2 h at room temperature and 10 μl of *S. aureus* solution for 30 min. Bacteria-bound immunecomplexes were pelleted and washed by centrifugation for 5 min through a cushion of 30% sucrose in buffer B supplemented with 0.1% SDS. This washing step was repeated once using the same buffer without detergents. The final pellets were suspended in 30 μl of sample buffer (16) and heated to 95°C prior to loading onto the gel.

Immunofluorescence Microscopy: Frozen tissue sections cut on a cryotome were treated with acetone at −20°C for 10 min and subsequently incubated with antiserum to plectin diluted 1:50 with PBS for 30 min at room temperature. The sections were then rinsed three times with PBS, and thereafter incubated with fluorescein isothiocyanate-conjugated swine IgG to rabbit IgG (Dako-Immunoglobulins, Copenhagen) diluted 1:15 in PBS for 30 min at room temperature. The sections were again thoroughly rinsed with PBS, briefly immersed in distilled water and 100% ethanol, air dried, and finally mounted in Moviol 4-88 (Hoechst, AG, Frankfurt). The processing for immunofluorescence microscopy of cultured cells grown on coverslips and dissociated cells from tissue adhering to microscope slides was similar (11). For double immunofluorescence microscopy, fixed cells or tissue sections were incubated in sequence with rabbit antiserum to plectin (1:50), tetraethylrhodaminisothiocyanate-conjugated swine IgG to rabbit IgG ([1:15]; Dako-Immunoglobulins), guinea pig antiserum to mouse liver cytokeratin component D (1:80) or guinea pig antiserum to chicken gizzard desmin (1:20), and fluorescein isothiocyanate-conjugated goat IgG to guinea pig IgG (1:15) (Nordic Laboratories, Tilburg). Specimens were viewed in a Reichert Polyvar fluorescence microscope using epi-illumination and photographs were taken with Kodak Tri-X Pan film.

Electron Microscopy: Immunolocalization of plectin on frozen tissue sections (liver, tongue, smooth and cardiac muscle) was carried out using the immunoperoxidase method. Since protocols of detailed procedures have already been published (12, 15), only the principle of the method and details relevant to the work presented shall be mentioned. Tissue sections (6–8 μm) adhering to glass coverslips were incubated with antiserum to plectin (1:20; PBS) and subsequently with peroxidase-conjugated goat IgG to rabbit IgG (1:20) (Dako-Immunoglobulins). Sections were then fixed with 4% paraformaldehyde, washed with PBS, and rinsed with 50 mM Tris/HCl, pH 7.6. Subsequently, 0.05% 3,3′-diaminobenzidinetetrahydrochloride (Fluka AG, Buchs, Switzerland) and 0.015% hydrogen peroxide were added for 5 min to deposit oxidized electron-dense reaction product at sites of antibody binding. Specimens were postfixed with 2% osmium tetroxide, dehydrated, embedded, thin-sectioned, and processed for electron microscopy following standard procedures.

RESULTS

Occurrence of Plectin in Various Tissues

Plectin (M_r ~300,000) and vimentin are the two major polypeptide components found in insoluble residues obtained by extraction of cultured cells with 1% Triton X-100/0.6 M KCl. When whole tissues from rat were extracted similarly, polypeptide components of M_r 300,000 and slightly lower were detected in preparations from liver, urinary bladder, heart, skeletal muscle, and tongue by SDS-PAGE and Coomassie Blue staining of protein bands (Fig. 1*A*). In liver, other major components were bands of ~55,000 and 49,000, most likely cytokeratins (7, 11), a band of M_r ~170,000 and a group of bands in the M_r range of 250,000-210,000. Cytokeratin components were also prominent in tongue. Major components of extracted urinary bladder, heart, skeletal muscle, and tongue were bands in the M_r range of actin and myosin.

To examine whether the high M_r components revealed by Coomassie Blue staining were related to plectin, Triton X-100/high salt-resistant tissue residues were run on SDS polyacrylamide electrophoresis gels and then subjected to immunoautoradiography (4) using specific antibodies to rat glioma C_6 cell plectin (27) and tritium-labeled protein A (Fig. 1*B*). In all cases tested (liver, heart, and urinary bladder), immunoreactive polypeptides of M_r 300,000 were clearly identified

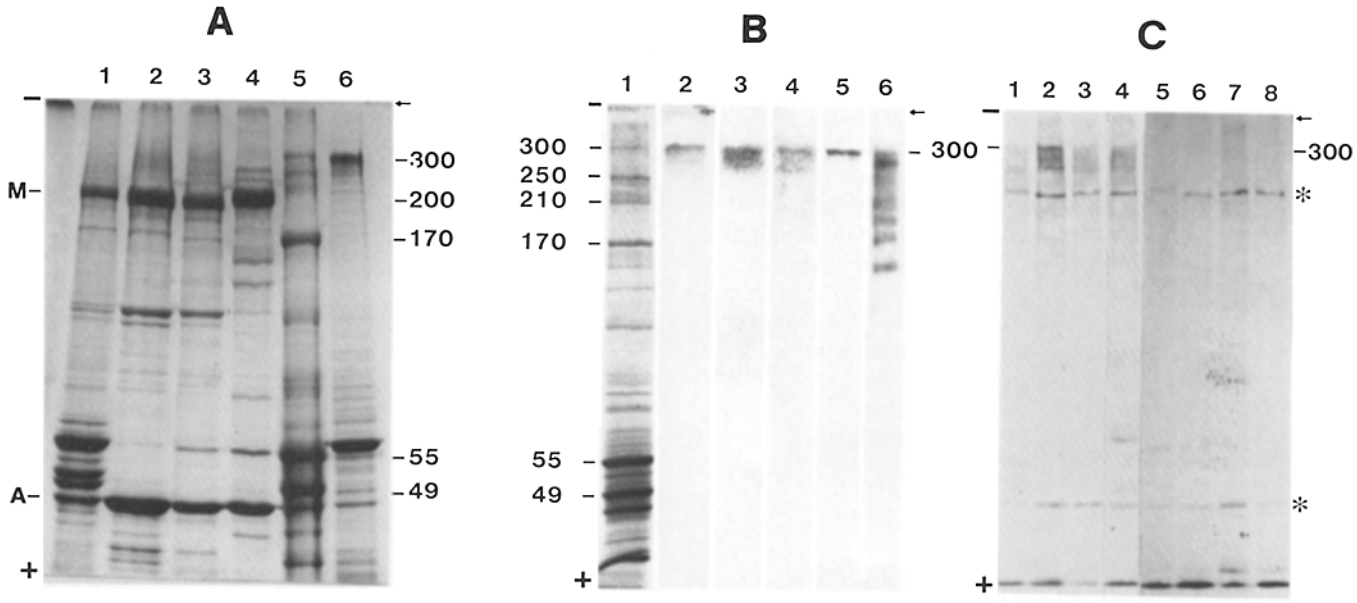


FIGURE 1 Detection and identification of plectin present in Triton X-100/high salt-resistant residues of various rat tissues by SDS PAGE. (*A*) Coomassie Blue staining of 7.5% polyacrylamide gel (10 cm long). *1*, tongue; *2*, skeletal muscle; *3*, heart; *4*, urinary bladder; *5*, liver; *6*, rat glioma C_6 cells. *A*, actin; *M*, myosin. (*B*) Immunoautoradiography (lanes *2–6*). SDS 7.5% polyacrylamide gels (10 cm long) containing separated proteins of Triton X-100/high salt-resistant residues from rat liver (lane *2*), heart (lane 3), urinary bladder (lane *4*), and glioma C_6 cells (lanes *5* and *6*) were incubated with antiserum to plectin diluted 1:40, followed by an incubation with ^{3}H-protein A (see Materials and Methods). Gels were impregnated with 1 M sodium salicylate, dried, and exposed for fluorography. The glioma C_6 cell residues analyzed were prepared in the presence (lane *5*) and in the absence (lane 6) of 2 mM phenylmethylsulfonyl fluoride. Lane *1*, Coomassie Blue staining of the gel shown in lane *2*, performed prior to immunoautoradiography. (*C*) Immunoprecipitation. Detergent/high salt-resistant tissue residues were tritiated by reductive methylation (25), dissolved in 3% SDS, and subjected to immunoprecipitation in 0.1% SDS with the aid of formalin-fixed *Staphylococcus aureus*. Pellets were analyzed on SDS 7.5% polyacrylamide gels, 18 cm long. Lanes *1–4*, immunoprecipitates obtained using antiserum to plectin. Lanes *5–8*, polypeptides precipitated nonspecifically using normal fetal calf serum instead of antiserum (major bands are indicated by asterisks). Lane *1* and *5*, urinary bladder; *2* and *6*, heart; *3* and *7*, skeletal muscle; *4* and *8*, tongue. Arrows, start of resolving gels. Values = molecular weight × 10^3.

(Fig. 1 *B*, lanes *2–4*). Immunoreactive polypeptides of M_r slightly lower than 300,000 (between 300,000 and ~260,000), which were commonly observed (Fig. 1 *B*, lanes *2–4*), most likely represent proteolytic breakdown products of plectin. High sensitivity to proteolysis is characteristic also for plectin from cultured cells (Fig. 1 *B*, lane *6*; [28]). Immunoreactive protein species of M_r 300,000 were identified in detergent-resistant residues of various tissues, including those examined using immunoautoradiography, also by an alternative method, i.e., immunoprecipitation of radioactively labeled antigens followed by electrophoretic analysis and autoradiography (Fig. 1 *C*). In these cases, probably due to long procedures, the presumptive proteolytic breakdown products of plectin in the M_r range between 300,000 and ~260,000 became slightly more prominent than when immunoautoradiography was used[1]. When extracts of several different whole tissues (liver, urinary bladder, heart, tongue, skeletal muscle, small intestine, and kidney) were assayed by immunoautoradiography, immunoreactive polypeptides could not be identified except for urinary bladder, which showed a weak reaction of high M_r components (data not shown). Evidently, the amount of plectin in most of the extracts was too small to permit detection under these conditions.

[1] The differences between breakdown profiles shown in Fig. 1 *B*, lane *6*, and those in Fig. 1 *C*, lanes *1–4*, can be explained assuming that protease activities of distinct specificity were present.

Immunolocalization of Plectin in Tissues

The examination of stomach using indirect immunofluorescence microscopy (Fig. 2, *A* and *B*) revealed strong staining of various cell types, including stratified epithelium of the fore stomach, connective tissue cells (fibroblasts), smooth muscle cells, endothelial cells of vessels, nerve cells, and epithelial cells of the glandular portion of stomach. In stratified epithelium, specific fluorescence was concentrated and most conspicuous in the basal cell layer. There, a mostly diffuse cytoplasmic staining and a concentration of fluorescence at the periphery of cells, especially at the basal region, were observed (Fig. 2 *A*). Fibroblasts, endothelial cells, and parietal cells of the gastric glands showed a diffuse type of staining throughout the cytoplasm, whereas chief cells and epithelial cells lining the foveolae were stained peripherally (Fig. 2 *B*). Smooth muscle cells were decorated in a more granular pattern (Fig. 2, *A* and *B*).

In kidney, various cell types were stained by antibodies to plectin, including cells of glomeruli of interstitial connective tissue and tubular epithelial cells (Fig. 3, *A* and *B*). Within glomeruli, cells of Bowman's capsule as well as mesangial cells were also stained (Fig. 3 *A*).

In cryostat sections of small intestine, antibodies to plectin specifically decorated the columnar cells lining the crypts and villi (Fig. 4, *A* and *B*) with a concentration of fluorescence at subapical and basal cell regions (Fig. 4 *B*). Connective tissue and smooth muscle cells were also stained in a diffuse (fibro-

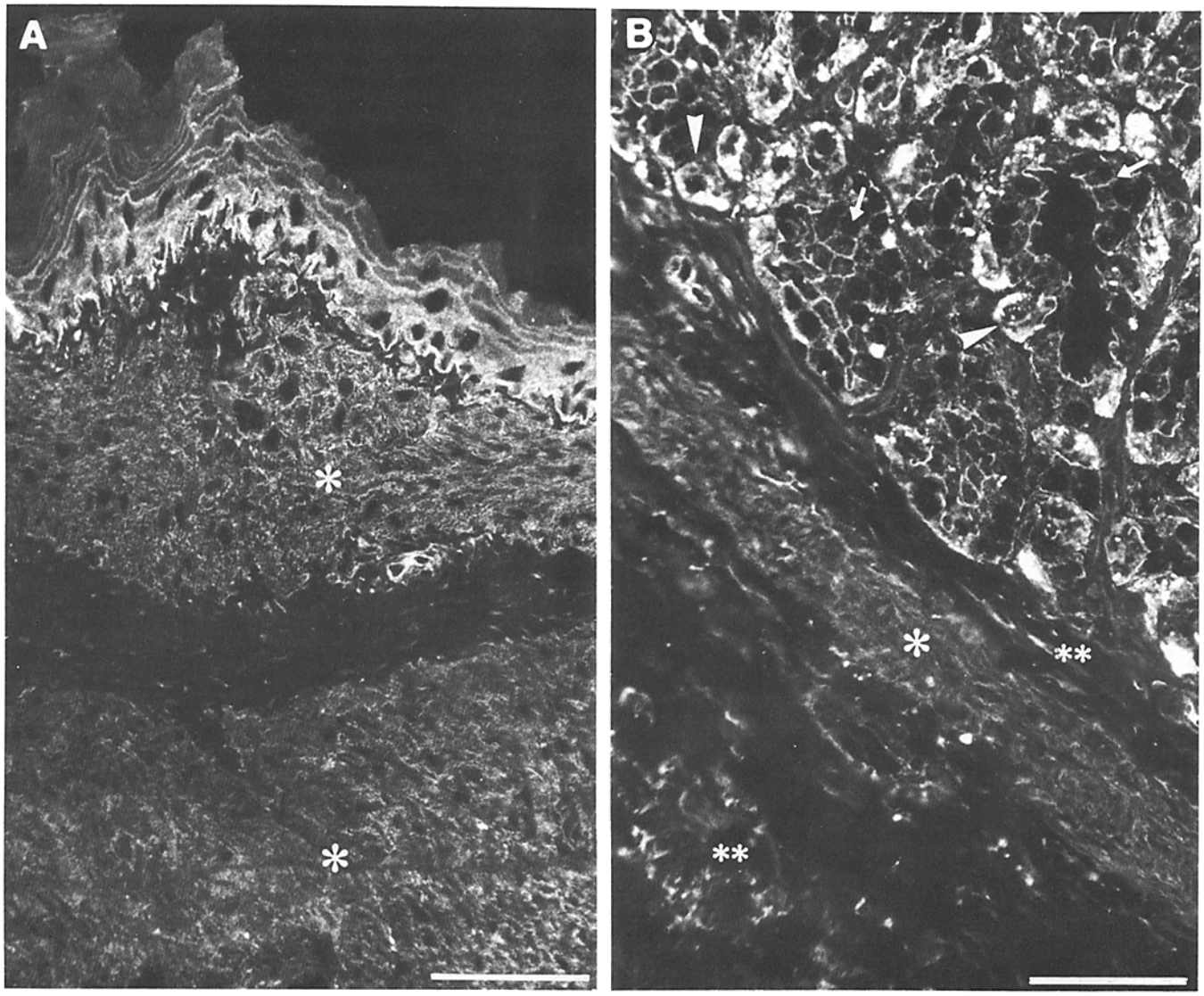


FIGURE 2 Immunofluorescence microscopy of frozen sections of stomach using antibodies to plectin. (*A*) Section through fore stomach showing specific decoration of stratified epithelium, connective tissue cells, and cells of muscular layers (asterisks). Note diffuse cytoplasmic staining and concentration of fluorescence at cell peripheries of epithelial basal cell layer cells. (*B*) Section through glandular portion showing mostly peripheral staining of epithelial cell lining the deep gastric glands (arrows), more diffuse staining of parietal cells (arrowheads), and specific decoration of cells of the muscularis mucosae (asterisk), and mesenchymal cells of the lamina propria and submucosa (two asterisks). Bar, 50 μm.

blasts) and granular (smooth muscle cells) pattern (Fig. 4*A*). Inspection of individual, isolated absorptive cells from rat small intestine showed a similar distribution of fluorescence with high staining intensity in the subapical and basal zones but also throughout the cytoplasm (Fig. 4*C*). As demonstrated using double immunofluorescence microscopy, this distribution pattern roughly resembled that obtained with an antibody preparation to liver cytokeratin (Fig. 4*D*), although the cytoplasmic structures stained by plectin-antibodies appeared more dense and less filamentous than those stained by antibodies to cytokeratin.

In sections through liver, hepatocytes were almost exclusively stained at their periphery (Fig. 5*A*). The staining was granular rather than continuous, which was best appreciated in grazing sections. As shown using double immunofluorescence microscopy, this staining pattern differed significantly from that observed with antiserum to cytokeratin, where, in addition to peripheral staining, a complex cytoplasmic filamentous meshwork was seen (Fig. 5*B*). The most striking feature of the immunostaining with antiserum to plectin was the clear, seemingly uninterrupted outlining of bile canaliculi, which, in longitudinal sections, was apparent over relatively long distances (Fig. 5*A*; arrows). In perpendicular sections of canaliculi, two fluorescent dots localized exactly opposite to each other were seen (Fig. 5, *A* and *C*; arrows), suggesting that plectin was specifically associated with junctions between hepatocytes. In contrast, bile canaliculi were outlined along their entire circumference by antibodies directed against liver cytokeratin D (Fig. 5*D*; arrows). In portal tract areas of liver, plectin-antibodies conspicuously reacted with bile duct epithelia, connective tissue cells, and cells of vessel walls (endothelial cells, cells of tunica media) (Fig. 5*E*). As shown using double immunofluorescence microscopy this was in clear contrast to the staining specificity of cytokeratin antibodies, which reacted exclusively with hepatocytes and bile duct epithelia (Fig. 5*F*). However, the staining of bile duct epithelia

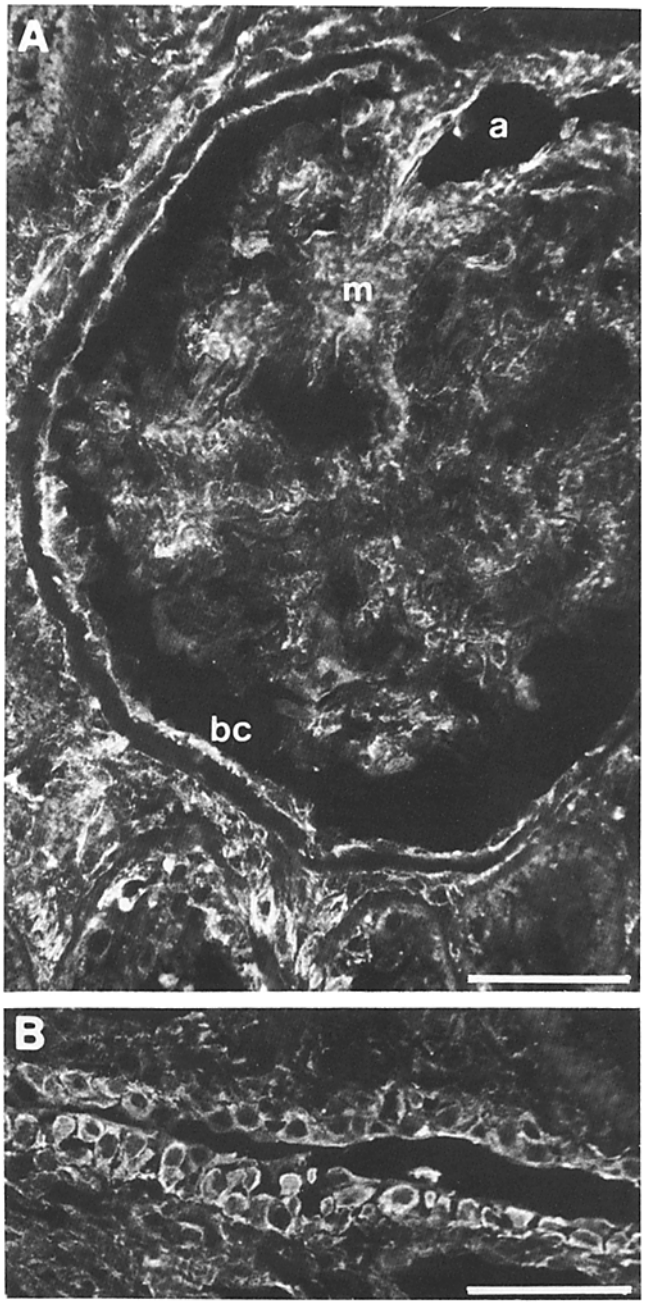


FIGURE 3 Immunofluorescence microscopy showing frozen sections through kidney after reaction with antibody to plectin. (*A*) Staining of mesangial cells in the glomerulum (*m*), cells of the Bowman's capsule (*bc*), and of endothelial cells of the afferent artery (*a*). (*B*) Staining of tubular epithelium. Bar, 50 μm.

by both antisera was similar, indicating a cytoplasmic localization of plectin in this type of epithelium.

The immunolocalization of plectin at hepatocyte junctions sealing canaliculi and in other regions of close contact between hepatocytes suggested a possible association of plectin with desmosomes and other junctional structures. To investigate this possibility at the ultrastructural level, immunoelectron microscopy of thin sections of liver was performed using the immunoperoxidase technique. In agreement with the immunofluorescence microscopy data, the electron-dense reaction product was found to be deposited primarily at the cell periphery (Fig. 6*A*). Controls run with preimmunserum instead of antiserum to plectin were negative (Fig. 6*B*). At higher magnification the association of plectin with desmosomal plaques became apparent (Fig. 6, *C* and *D*). In addition, structures positively reacting with the antiserum seemed to extend into the cytoplasmic space for distances of up to 0.3 μm.

Double immunofluorescence microscopy of hepatoma cells in culture (rat MH_1C_1 cells; for similarities with respect to freshly dissociated hepatocytes, see 11), using antisera to plectin and to cytokeratin, showed a similar cellular distribution of both antigens (Fig. 7), in contrast to the results obtained with hepatocytes in tissue sections. However, whereas keratin was clearly affiliated with cytoplasmic filaments (Fig. 7*B*), the nature of plectin-positive structures was less clearly defined (Fig. 7*A*). The staining pattern was reminiscent of network arrays consisting of short, irregularly oriented filaments and lines of dots, resembling the appearance of plectin arrays in cultured fibroblast cells (27). Thus it appeared that plectin had undergone cellular rearrangement from peripheral into cytoplasmic regions during the transition from tissue to cultured cells. Alternatively, plectin might have been localized within cytoplasmic network arrays underneath the plasma membrane that were fully revealed using immunofluorescence microscopy of cultured cells but only partially seen in tissue sections.

Immunostaining with plectin-antibodies in sections through tongue revealed the decoration of a diversity of cell types (Fig. 12, *H*, *I*, *K*, *L*). The stratified epithelium was clearly decorated, whereby the basal cell layer showed a higher fluorescence intensity than the more superficial layers. In general, these epithelial cells were clearly outlined in a suggested granular pattern of immunostaining, although some cytoplasmic fluorescence, particularly evident at higher magnifications (data not shown), was present too. Skeletal muscle cells showed prominent cross-striations probably due to the staining of Z-lines (Fig. 12, *K* and *L*). Connective tissue cells and cells of vessel walls were also specifically decorated by plectin-antibodies (data not shown). Electron microscopy using the immunoperoxidase method showed that in stratified epithelia of tongue, as in liver, the immunoreactive material was specifically localized at desmosomal plaques and in their immediate neighborhood (Fig. 8).

In sections through uterus, epithelial cells at the surface and lining the endometrial glands were stained by plectin-antiserum throughout their cytoplasm with a concentration of fluorescence at the subapical and basal regions. Cytoplasmic staining was also seen in fibroblasts, endometrial stromal cells, and cells of vessel walls. The staining of smooth muscle cells was less conspicuous than that of other cells, though a faint granular staining was visible (data not shown).

The specific decoration of regularly spaced lines (in grazing sections) and of dots (in cross-sections) by antiserum to plectin at the periphery of smooth muscle cells was particularly striking in sections through urinary bladder (Fig. 9, *A* and *B*). It seems likely that these brightly stained areas represent intracellular attachment zones of myofilaments cross-sectioned at different angles. No appreciable staining was seen in the center of smooth muscle cells. Among nonmuscle cells of urinary bladder, the urothelium was stained, with a predominance of fluorescence in the basal cell layer (Fig. 9*C*). In the surrounding lamina propria, fibroblasts were also specifically decorated.

The peripheral localization of plectin in smooth muscle

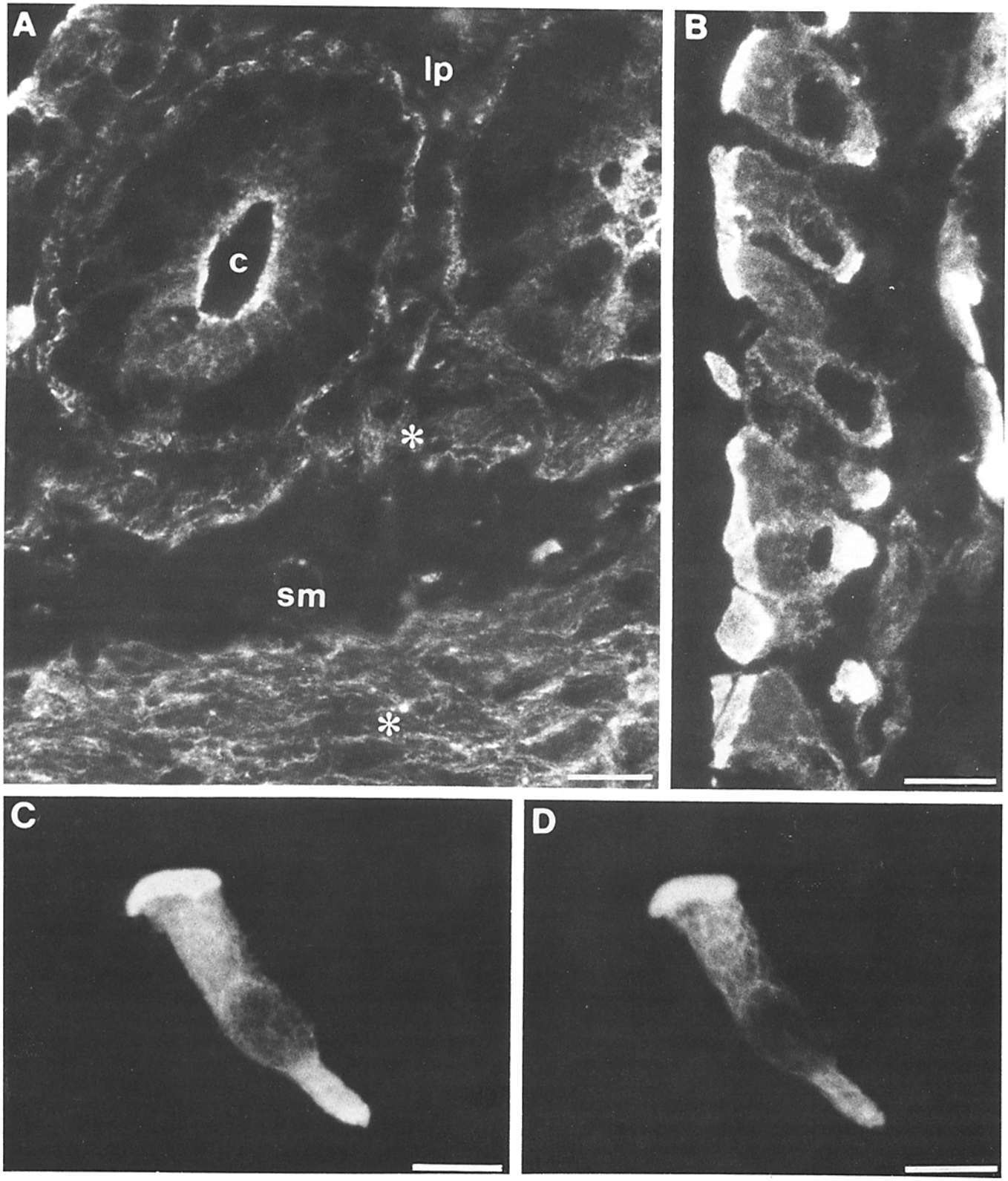


FIGURE 4 Immunofluorescence microscopy showing reaction of antibodies to plectin with frozen sections (*A* and *B*) and isolated epithelial cells (*C* and *D*) of small intestine. (*A*) Section demonstrating the presence of antigens in columnar cells lining crypts (*c*), in fibroblasts of the lamina propria of the gut (*lp*), and the submucosa (*sm*), and in cells of the muscular layers (asterisks). (*B*) Partly detached epithelial cells of a villus showing concentration of antigen at the subapical and the basal region. (*C* and *D*) Double immunofluorescence microscopy of isolated epithelial cells from small intestine using antibodies to plectin and to liver cytokeratin component D. (*C*) Rhodamine optics (plectin-specific). (*D*) Fluoresceine optics (cytokeratin-specific). Note indistinct cytoplasmic staining in addition to prominent staining of subapical and basal cell regions in C and decoration of cytoplasmic structures, clearly filamentous in nature, in *D*. Bar, 10 μm.

cells of bladder became clearly evident also at the electron microscopic level (Fig. 10, *A* and *B*). Using the peroxidase method considerable deposition of the reaction product was observed mostly along the cell margins with a conspicuous accentuation of regularly spaced regions (Fig. 10*A*). This mode of deposition of reaction product (Fig. 10*A*, arrows) probably indicated the presence of plectin at lateral cytoplasmic densities corresponding to Z-line structures of skeletal and cardiac muscle cells (1), probably representing myofilament attachment zones (for ultrastructure of smooth muscle cells, see 1 and 14).

In cardiac muscle, immunofluorescence microscopy using antibodies to plectin revealed a highly organized and specific distribution of immunoreactive material. Fluorescence was concentrated at cell junctions, known as intercalated disks, and at intracellular striations (Fig. 11*A*). As shown using double immunofluorescence microscopy, the antiplectin reactive striations were also stained by an antiserum to desmin (Fig. 11*B*), permitting the identification of the striations as Z-lines (17). The immunolocalization of plectin in cardiac muscle at the light microscopic level was confirmed by immunoelectron microscopy. Deposition of the electron-dense reaction product was primarily observed in regions of intercalated disks and at Z-lines. At intercalated disks, antiplectin-reactive material appeared to be present along stretches of their cytoplasmic surfaces, with high local concentrations at certain shorter regions (data not shown). This suggests that plectin was present in regions of desmosome-like junctional complexes as well as fasciae adherentes, i.e., filament attachment sites.

Fig. 12 summarizes a series of immunofluorescence microscopy control experiments, performed on frozen sections through liver, urinary bladder, and tongue. When preimmunserum was used instead of antiserum to plectin, no staining was observed with any of these tissues (Fig. 12, *A*, *D*, *G*, and *J*). In similar trials stomach, kidney, small intestine, and heart were also negative (data not shown). The characteristics of specific staining were fully retained when tissues were extracted with Triton X-100/high salt prior to the application of the primary antibody (Fig. 12, *B*, *E*, *H*, and *K*). Tissue sections pretreated in this way can be considered a direct pendant to the detergent/high salt-resistant cell residues that were tested for the occurrence of plectin using immunoautoradiography and immunoprecipitation (Fig. 1, *B* and *C*). Finally, the staining patterns revealed with affinity-purified antiserum to plectin were indistinguishable from those generated with dilutions of whole serum (Fig. 12, *C*, *F*, *I*, and *L*).

DISCUSSION

Using antibodies raised to plectin isolated from rat glioma C_6 cells we have identified cross-reacting antigens in detergent-resistant residues of rat liver, heart, urinary bladder, tongue, and skeletal muscle using immunoautoradiography of electrophoretically separated polypeptides and/or immunoprecipitation of radioactively labeled antigens followed by electrophoretic analysis. By both methods polypeptides co-migrating with the immunogen from C_6 cells (300,000) and of slightly lower M_r (most likely proteolytic breakdown products) were shown to be the cross-reacting protein species. We conclude, therefore, that proteins closely related to or even identical with plectin from cultured cells occur in a wide variety of tissues.

The localization of plectin in frozen sections of various tissues using indirect immunofluorescence microscopy revealed the occurrence of plectin in almost every cell type, including fibroblasts, stratified and nonstratified epithelia, endothelial cells, nerve cells, as well as striated, smooth, and cardiac muscle cells. The pattern of cellular staining, however, varied among cell types. In some cases, such as fibroblasts of all tissues tested, endothelial cells of vessel, and epithelia of bile duct, small intestine, uterus, urinary bladder, and stomach, staining was observed throughout the whole cytoplasm of cells. In contrast, hepatocytes and smooth muscle cells were primarily stained at their periphery. Epithelial cells of tongue and cardiac muscle cells showed cytoplasmic as well as accentuated peripheral staining. In cardiac muscle the cytoplasmic staining was confined to Z-lines.

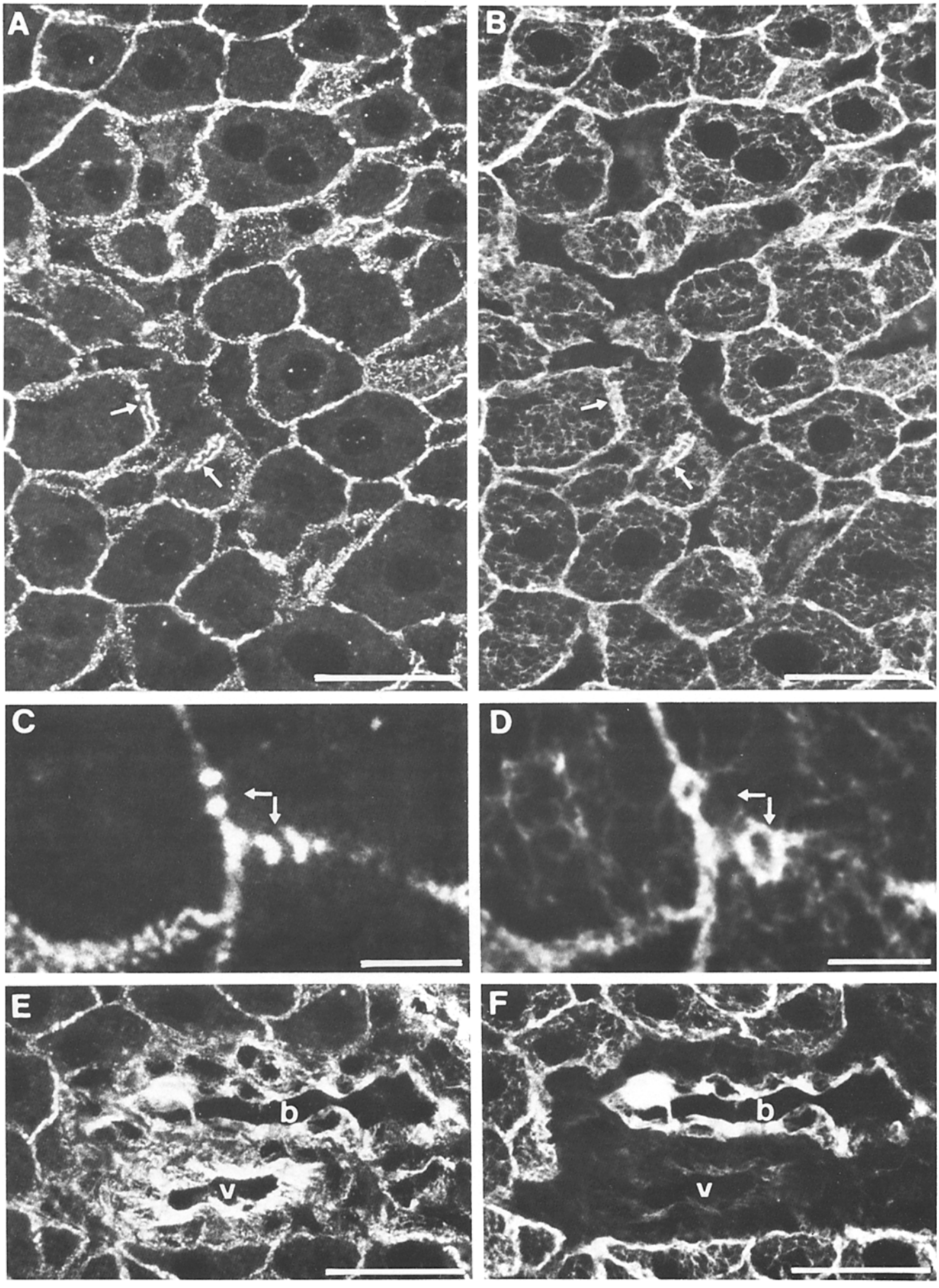
The nature of the structures stained in the cytoplasm of nonmuscle cells in tissue sections is as yet unclear. In general, the cytoplasm was stained rather densely, preventing the distinction of individual structural elements. This might reflect a high concentration of relatively insoluble cytoplasmic plectin. Even when cells were dissociated and viewed individually, as in the case of absorptive epithelial cells from small intestine, it was hard to distinguish structural details. Nevertheless, using this system, it was possible to show, by double immunofluorescence microscopy with antiserum to keratin employed in addition, that plectin-positive structures were not typically filamentous. Instead, they were reminiscent of dense network-type arrays consisting of short fibers and lines of dots. Very similar staining patterns were seen previously in cultured fibroblasts and HeLa cells (27), recently also in PtK_2[2] cells, and, as reported here, in cultured hepatoma cells (MH_1C_1).

The immunolocalization of plectin by fluorescence microscopy at the periphery of various cell types suggested its association with junctional complexes. Four major classes of junctions are distinguishable in vertebrates: desmosomes (macula adherens), tight junctions (zonula occludens), gap junctions (nexus), and the intermediate junctions (zonula adherens, puncta adherens, fascia adherens). By immunoelectron microscopy using the immunoperoxidase method, plectin was clearly identified as a constituent of desmosomal structures in liver, tongue, and cardiac muscle. In all cases the reaction product was found specifically associated with desmosomal dense plaques as well as with adjacent material extending into the cytoplasm, possibly filaments.

The conspicuous outlining of bile canaliculi by immunofluorescence microscopy suggests that plectin was localized along hepatocyte junctions sealing canaliculi. It is possible, therefore, that plectin was also present in tight junctions (zonula occludens), which are known to form large parts of this areas.

The striking, seemingly uninterrupted decoration of intercalated disks observed in heart muscle using both immunofluorescence and immunoelectron microscopy suggests that plectin is a constituent of desmosomes as well as of the other major junctional complex found in this region, the fascia adherens. Plectin may also be localized in regions of gap

[2] In our original paper on the immunofluorescent localization of plectin in cultured cells, we reported that antiserum to plectin did not stain rat kangaroo PtK_2 cells, an epithelial derived cell type, although a positive reaction of 300,000-dalton polypeptide components present in intermediate filaments prepared from these cells was observed using immunoblotting techniques (27). Meanwhile, probably due to improved fixation, plectin networks could be visualized also in this cell type.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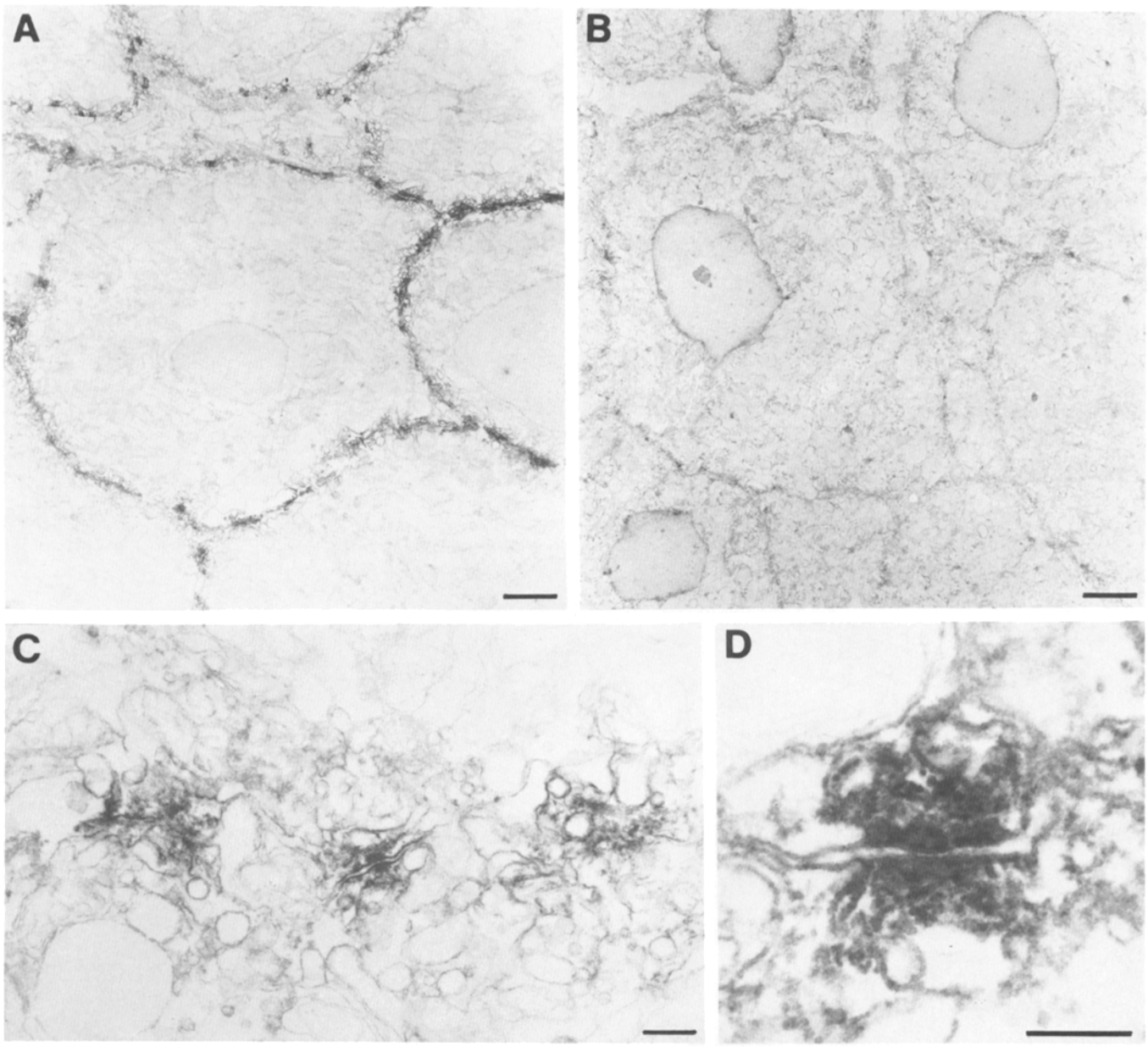


FIGURE 6 Electron microscopic immunolocalization of plectin on frozen sections through liver. (*A*) Survey showing specificity of immunoperoxidase reaction along periphery of cells. × 4,100. (*B*) Control using preimmunserum instead of antiserum to plectin. × 4,100. (*C*) Higher magnification showing preferential deposition of the reaction product at desmosomal regions. × 25,900. (*D*) Detail demonstrating specificity of reaction product at junctional area and associated ill-defined filamentous structures extending into the cytoplasm. × 68400. Bar, 2 μm (*A* and *B*); 0.25 μm (*B* and *C*).

FIGURE 5 Double immunofluorescence microscopy showing the localization of plectin and cytokeratin component D on frozen sections of liver. *A*, *C*, and *E*, Plectin-staining; *B*, *D*, and *F*, cytokeratin component D-staining. (*A*) Note granularity of fluorescence outlining hepatocytes, particularly evident in grazing sections, the distinct outline of bile canaliculi in longitudinal sections (arrows), and the lack of appreciable cytoplasmic staining; Kupffer cells also remained unstained. (*B*) Filamentous anticytokeratin reactive meshwork concentrated at cell peripheries and around bile canaliculi (arrows) of hepatocytes is shown. (*C* and *D*) High magnification of transverse sections through bile canaliculi (arrows) showing the confinement of plectin-specific staining to junctional complexes sealing bile canaliculi (*C*), and the staining along the circumference of canaliculi by antibodies to cytokeratin (*D*). (*E* and *F*) Portal tract area of liver. Note conspicuous reaction of antibodies to plectin with connective tissue cells and cells of vessel walls (*E*; *v*), unreactive with antibodies to cytokeratin (*F*). Bile duct epithelia (*E*, *F*; *b*) are stained by both antibodies. For controls regarding the specificity of staining with antiserum to plectin see Fig. 12. Bar in *A*, *B*, *E*, and *F*, 20 μm; Bar in *C* and *D*, 5 μm.

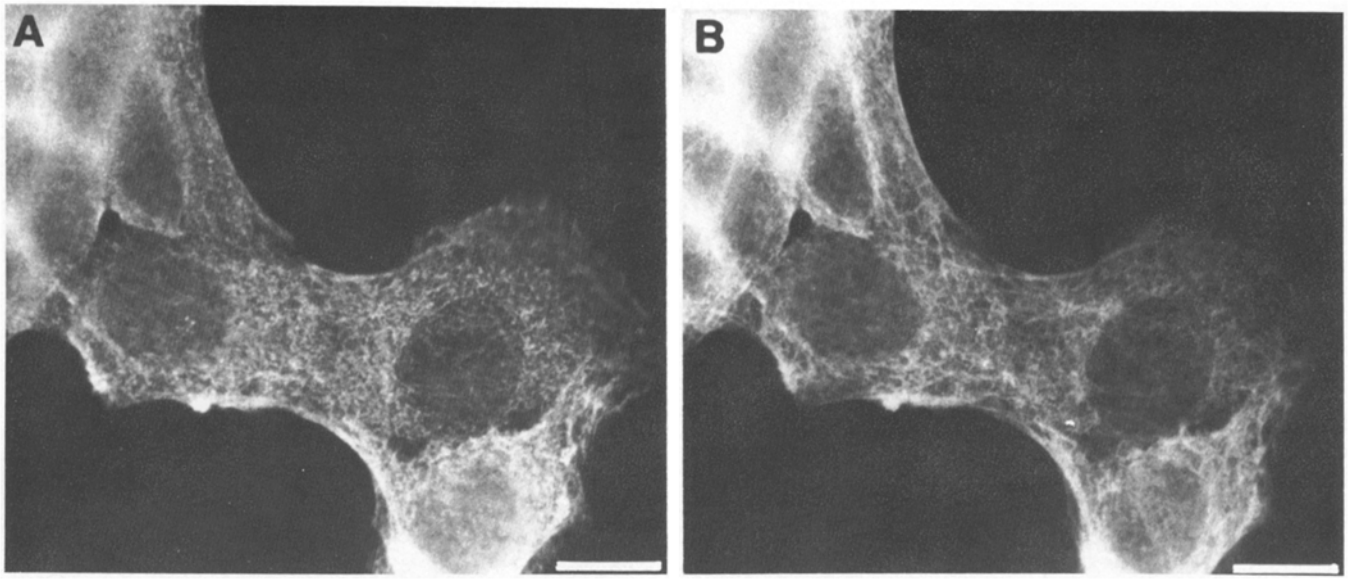


FIGURE 7 Double immunofluorescence microscopy showing the distribution of plectin (*A*) and cytokeratin (*B*) in cultured hepatoma (MH_1C_1) cells. Note staining of typical filaments in *B* and of granular structures, which are possibly short filaments extending to cell margins in *A*. Bar, 10 μm.

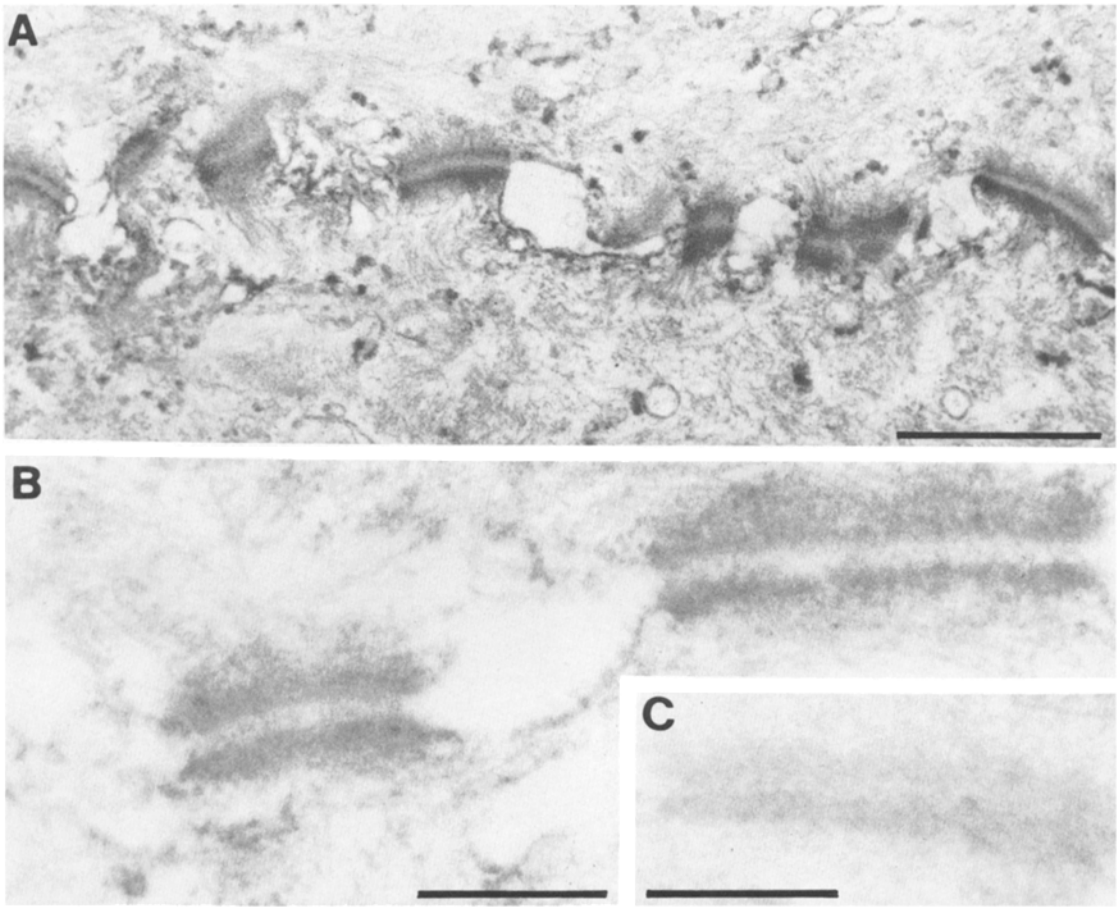


FIGURE 8 Immunolocalization of antigens present in tongue after reaction with antibodies to plectin visualized using electron microscopy. (*A*) Survey showing the deposition of reaction product using the peroxidase technique. Note the specific decoration of numerous desmosome-like structures. × 30,000. (*B*) Higher magnification revealing the specific decoration of desmosomal plaques. × 101,700. (*C*) Control using preimmune serum instead of antiserum to plectin. × 101,700. Bars, 1 μm (*A*) and 0.25 μm (*B* and *C*).

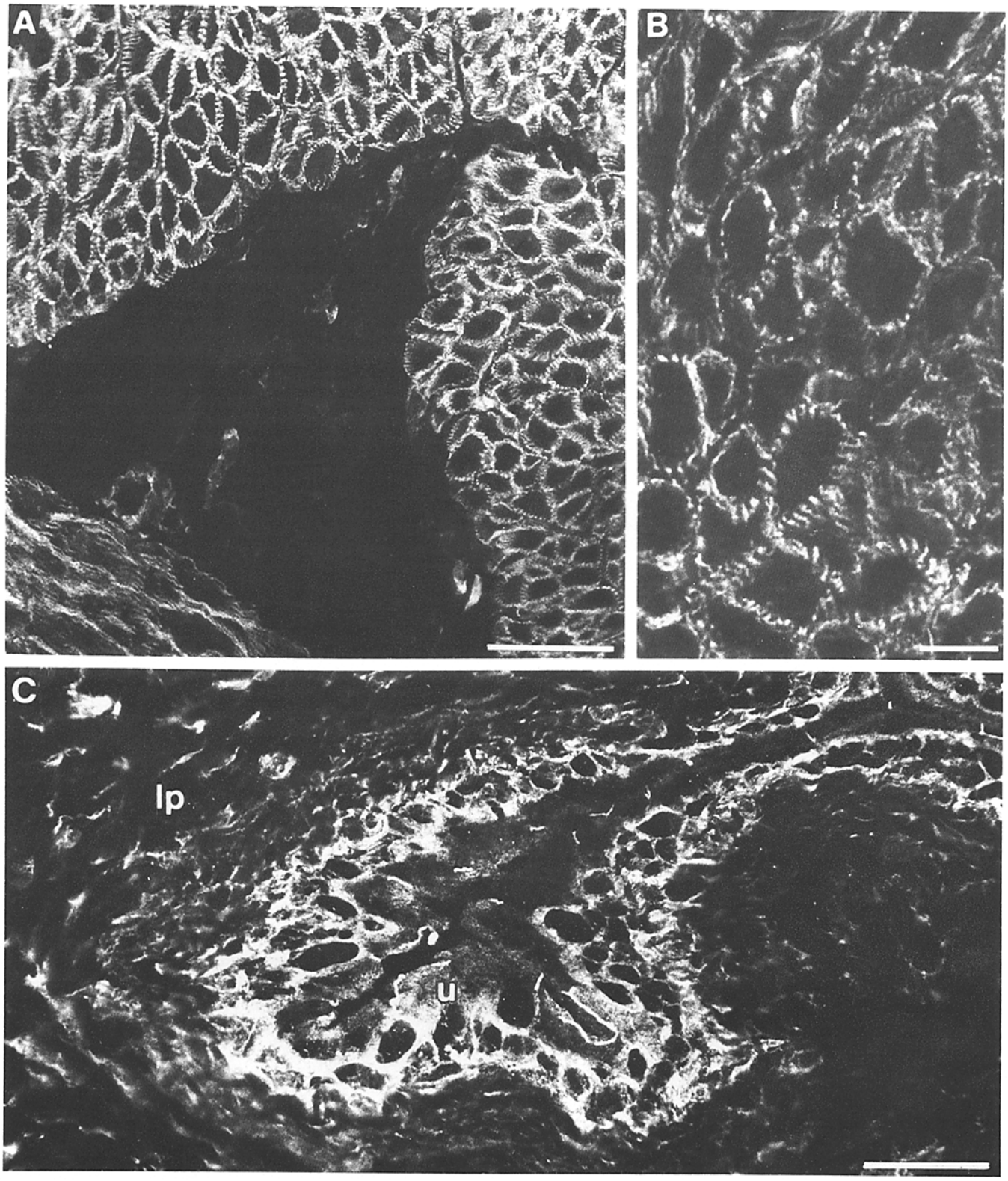
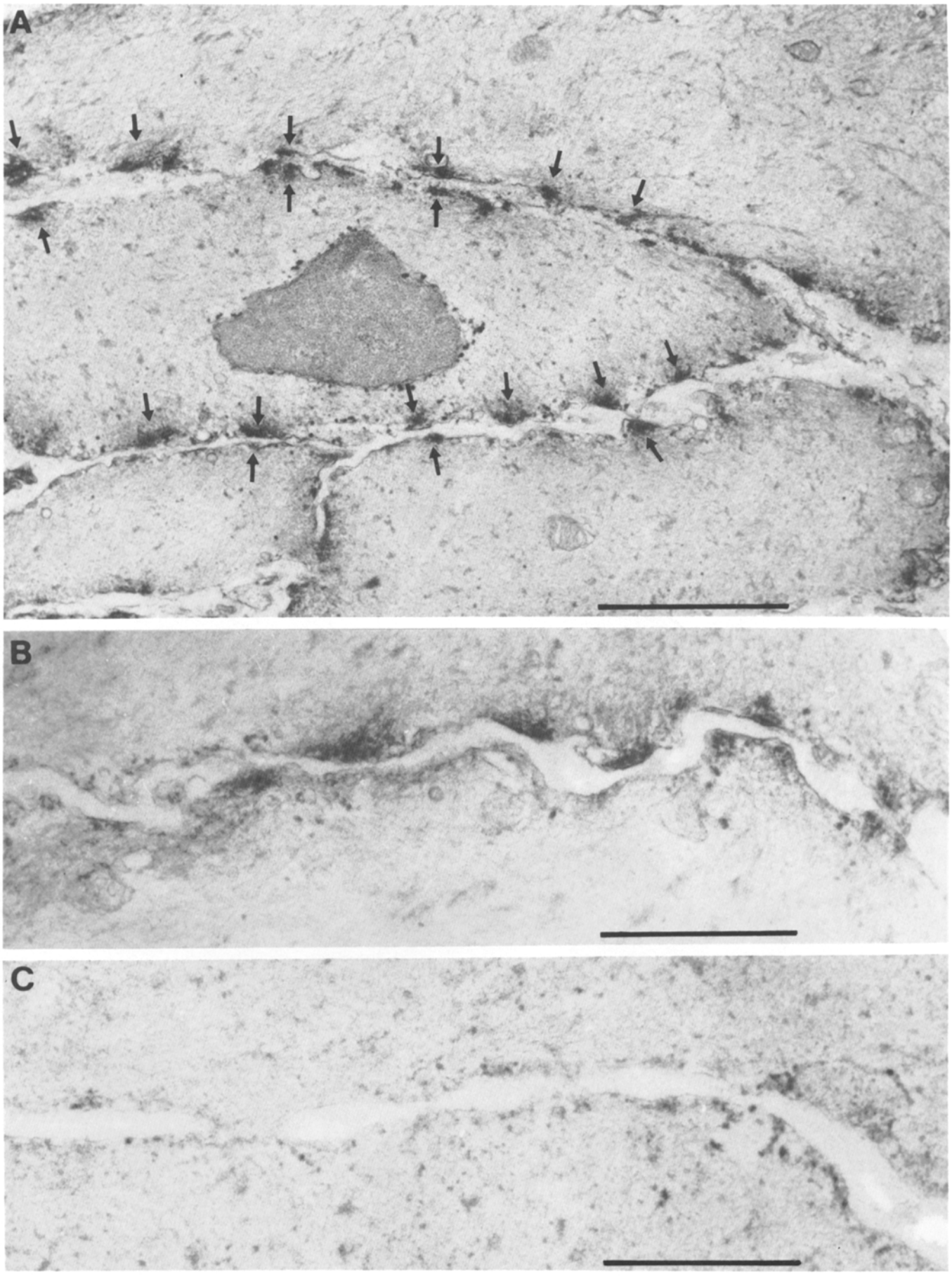


FIGURE 9 Localization of plectin on frozen sections through urinary bladder by immunofluorescence microscopy. (*A*) Decoration of regularly spaced dots and short lines along the periphery of smooth muscle cells. Note appearance of wavy fluorescent lines in longitudinal sections (lower left-hand corner). (*B*) Higher magnification showing decorated short lines and dots at the periphery of smooth muscle cells. (*C*) Staining of urothelium (*u*), predominantly of basal layer cells, and fibroblasts of surrounding lamina propria (*lp*). For control experiments regarding the specificity of the immunostaining with antiserum to plectin see Fig. 12. Bar, 40 μm (*A* and *C*); 10 μm (*B*).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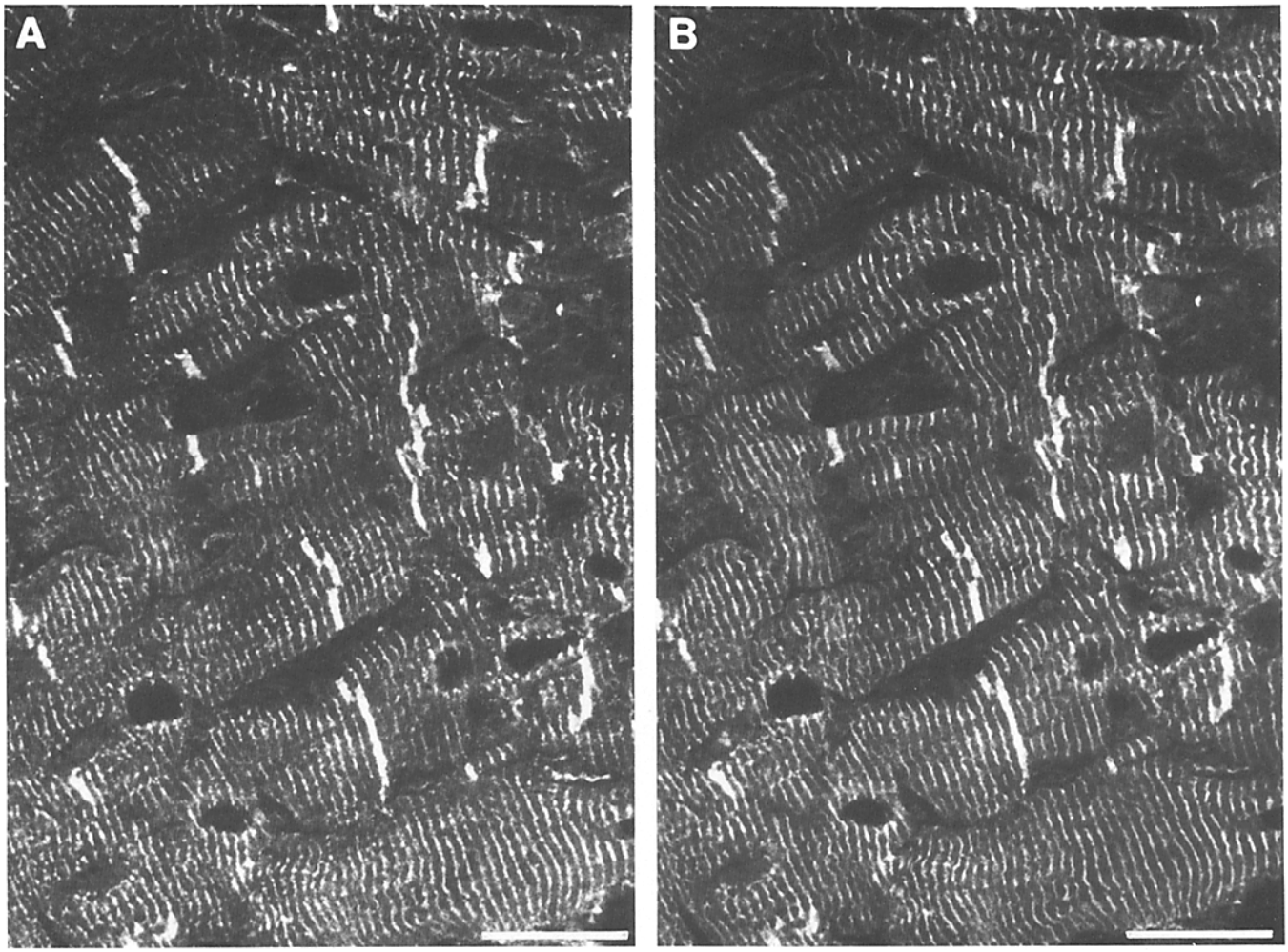


FIGURE 11 Double immunofluorescence microscopy showing frozen sections through cardiac muscle stained by antibodies to plectin (*A*) and desmin (*B*). Note coincidence of striations (Z-lines) stained in *A* and *B*, and prominent staining of intercalated disks by both antisera. Bar, 20 μm.

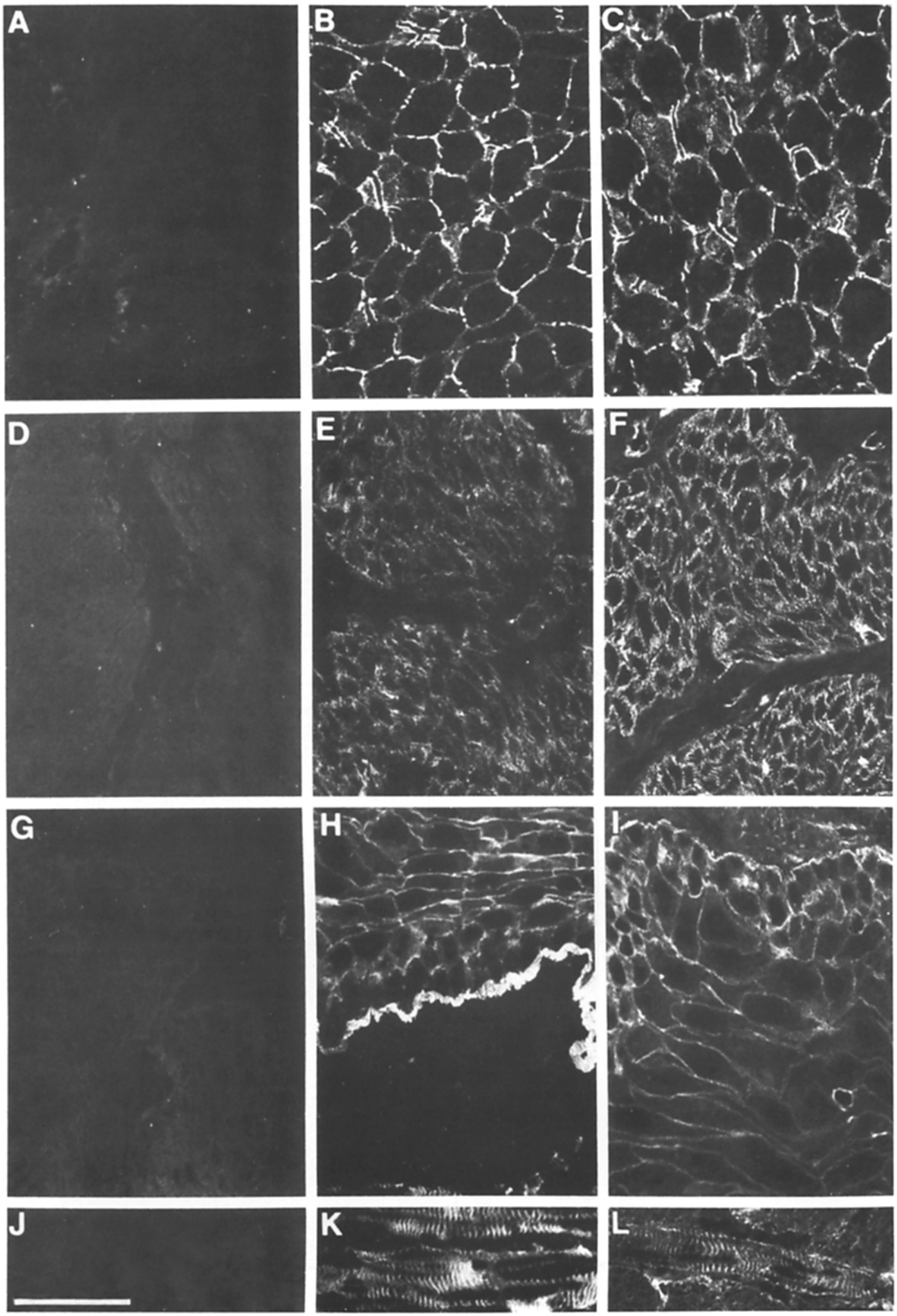
junctions, tight cellular connections of low electric resistance. The localization of plectin in zonula adherens-type junctions was also suggested by the immunostaining of smooth muscle (urinary bladder) cells, which were immunoreactive particularly at dense patches spaced in regular intervals along their periphery.

The predominant localization of polypeptides reacting with antiserum to plectin at sites of cell junctions as well as at intracellular attachment sites, such as Z-lines of cardiac and skeletal muscle and possibly focal densities of smooth muscle, suggests that plectin or at least immunologically closely related proteins have important functions in the structural organization and mechanical adhesiveness of cells and tissues.

Being found in desmosomal plaques and in their immediate vicinity on the interior side of epithelial cells, structures composed of plectin might function in the anchorage of intermediate filaments (tonofilaments) whose focal attachment to desmosomal plaques is highly characteristic. Considering the similarity of intermediate filaments in terms of structure and possibly function (18), our recent finding (E. Leichtfried and G. Wiche, unpublished results) of interactions between plectin and intermediate filaments of the vimentin type in vitro would be consistent with this idea. Furthermore, as a constituent of intercalated disks and Z-lines of striated muscle and of related structures in smooth muscle cells, plectin might also play a role in the spatial organization of myofilaments.

Polypeptides with M_r similar to that of plectin have been found previously in various cells and tissues. These include desmoplakins (M_r 250,000–215,000; [10, 13]), projectin (M_r 360,000; [23]), paranemin (M_r 280,000 [2]) and spectrin-like polypeptides ($M_r \approx 250{,}000$; [for a brief review see 19]). However, a scrutiny of published immunological as well as biochemical data reveals that none of these proteins seems to be closely related to plectin. Spectrin-like proteins, e.g., though resembling plectin with regard to their widespread occurrence

FIGURE 10 Electron microscopic immunolocalization of plectin on frozen sections through smooth muscle of urinary bladder using the immunoperoxidase technique. (*A*) Survey showing preferential deposition of reaction product at regularly spaced regions (arrows) along the periphery of cells. × 12,600. (*B*) Higher magnification of peripheral area. × 36,500. (*C*) Control using preimmunserum instead of antiserum to plectin. × 36,500. Bar, 2 μm (*A*) and 1 μm (*B*, *C*).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in tissues and cells, differ biochemically from plectin in electrophoretic mobilities, isoelectric points (22, and unpublished data), and amino acid composition (20, and unpublished data). Paranemin, on the other hand, possesses a very similar isoelectric point, but can clearly be distinguished from plectin by its different immunolocalization in tissues and cells (2). The biochemical and structural relationship between plectin and the high M_r microtubule-associated proteins from mammalian brain, MAP-1 and MAP-2, has still to be clarified. However, by immunological criteria, such as cross-reactivity with specific antisera and immunolocalization of antigens in cultured cells (27, and unpublished data), no relationship was found. Finally, the assessment of whether the polypeptide doublet of 325,000 and 300,000 recently identified as junctional element of rabbit skeletal muscle (3, 5) is related to plectin has to await the biochemical and immunological characterization of these polypeptides.

The widespread occurrence of plectin in the cytoplasm of cells, such as of fibroblasts, endothelial cells, and some epithelial cell types, as well as at the periphery of cells in zones of cell junctions, particularly of muscle and certain epithelial cells, suggests that this protein might have a number of diverse functions. To characterize these more precisely, further biochemical as well as ultrastructural studies seem to be mandatory. Microinjection of antibodies to plectin into living cells might also be a promising approach to the problem. While experiments along these lines are in progress, on the basis of the data reported here and previously, it might be justified to speculate that plectin plays a rather universal role in the anchorage of cytoplasmic filaments and the formation of cell junctions.

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FIGURE 12 Immunofluorescence microscopy control experiments. Frozen sections through liver (*A–C*), muscular layer of urinary bladder (*D–F*), squamous epithelium of tongue (*G–I*), or skeletal muscle of tongue (*J–L*) were stained with preimmunserum (*A, D, G,* and *J*), 1/50 dilutions of antiserum to plectin (*B, E, H,* and *K*), or affinity-purified antibodies (0.05 mg protein/ml) to plectin (C, F, I, and *L*). Frozen sections shown in *B, E, H,* and *K* were incubated in buffer A, supplemented with 1 % Triton X-100, 0.6 M KCl, 2 mM phenylmethylsulfonyl fluoride at 37°C for 20 min prior to processing for immunofluorescence microscopy. Magnification is the same for all pictures. Bar, 40 μm.