Plectin Sidearms Mediate Interaction of Intermediate Filaments with Microtubules and Other Components of the Cytoskeleton

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Abstract. By immunogold labeling, we demonstrate that "millipede-like" structures seen previously in mammalian cell cytoskeletons after removal of actin by treatment with gelsolin are composed of the cores of vimentin IFs with sidearms containing plectin. These plectin sidearms connect IFs to microtubules, the actin-based cytoskeleton and possibly membrane components. Plectin binding to microtubules was significantly increased in cells from transgenic mice lacking IFs and was reversed by microinjection of exogenous vimentin. These results suggest the existence of a pool of plectin which preferentially associates with IFs but may also be competed for by microtubules. The association of IFs with microtubules did not show a preference for Glu-tubulin. Nor did it depend upon the presence of MAP4 since plectin links were retained after specific immunodepletion of MAP4. The association of IFs with stress fibers survived actin depletion by gelsolin suggesting that myosin II minifilaments or components closely associated with them may play a role as plectin targets. Our results provide direct structural evidence for the hypothesis that plectin cross-links elements of the cytoskeleton thus leading to integration of the cytoplasm.

INTERMEDIATE filaments (IFs)[1] are a family of cytoskeletal fibrils whose structure, biochemistry, and molecular biology have been established in detail but whose function at the cellular level is still poorly understood (for reviews see Albers and Fuchs, 1992; Fuchs and Weber, 1994; Heins and Aebi, 1994; Klymkowsky, 1995). The leading hypothesis proposes a mechanical role for IFs in cells (Fuchs, 1994) based on data showing that neurofilaments participate in the maintenance of axon calibre (Ohara et al., 1993; Eyer and Peterson, 1994) that keratin filaments are required to maintain a strong epidermis (McLean and Lane, 1995), together with a large body of circumstantial evidence. Remarkably, however, the loss of vimentin, which composes the IFs of fibroblasts, has no demonstrable effect on cell function as vimentin-null mice showed no clear phenotype (Colucci-Guyon et al., 1994).

Analysis of IF function may be complex because of the multiplicity of its interaction partners. IFs have been proposed to interact with the plasma membrane, nuclear envelope, mitochondria, microtubules (MTs) (for review see Georgatos and Maison, 1996), and actin filament bundles (Goldman et al., 1986). Interaction of IFs with the plasma membrane occurs at specialized attachment sites, focal adhesions (Bershadsky et al., 1987; Geiger et al., 1987), desmosomes and hemidesmosomes (for review see Schmidt et al., 1994), and a new protein family consisting of desmoplakin, bullous pemphigoid antigen and plectin, is thought to mediate IF association with contact structures at the plasma membrane (Green et al., 1992; Seifert et al., 1992).

The hypothesis of IF-MT interaction was suggested almost three decades ago (Ishikawa et al., 1968), but the mechanism of this interaction has remained essentially unresolved. The basic phenomenon consists in the fact that MT depolymerization induces IF collapse. Structural evidence for the existence of cross-bridges between the two cytoskeletal elements has been provided only for neurofilaments (Hirokawa, 1982) and MAP2 has been proposed as a component of the cross-bridges in vitro (Leterrier et al., 1982; Heiman et al., 1985) and in vivo (Hirokawa et al., 1988). In nonneuronal cells, functional tests by microinjection of specific antibodies causing IF collapse suggested a role for a 95-kD protein colocalizing with IFs (Lin and Feramisco, 1981), kinesin (Gyoeva and Gelfand, 1991), and MT-associated protein p210 (Draberova and Draber, 1993). However, no physical links between MTs and vimentin or keratin IFs have been identified and the mechanism of interaction of these cytoskeletal components remains unclear.

By analogy with MTs and actin filaments which perform their functions with accessory molecules, it is reasonable to expect that IFs also use associated proteins for interactions with their partners. In a recent paper (Svitkina et al., 1995), we showed that when the cytoskeleton of cultured

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1. *Abbreviations used in this paper*: Glu-tubulin, posttranslationally detyrosinated tubulin; IF, intermediate filament; MAP, microtubule-associated protein; MT, microtubule; Tyr-tubulin, tyrosinated tubulin.

fibroblasts is depleted of actin, an unusual "millipede-like" structure is exposed in which numerous sidearms connected a core, IF-like filament, to components of the cytoskeleton. However, the identity of neither the core filament nor the projecting sidearms was established. These images prompted us to search for an IF-associated protein that could be a constituent of the sidearms and mediate IF interactions with other cellular components.

The list of IF-associated proteins (for review see Yang et al., 1990; Foisner and Wiche, 1991) is not as large as that for actin filaments or MTs. A likely candidate was plectin because of its wide distribution among tissues and species (Wiche, 1989; Foisner and Wiche, 1991) and its capability to interact with multiple proteins as well as with itself (Foisner and Wiche, 1987; Foisner et al., 1991*a*). Based on immunolocalization and biochemical data, plectin was proposed to be a versatile cytoplasmic cross-linker connecting IFs to MTs, the actin cytoskeleton, and membrane adhesion sites (Foisner and Wiche, 1991). Recently, studies on the molecular basis of skin blistering disorders associated with muscular dystrophy and neurodegeneration (Gache et al., 1996; McLean et al., 1996; Smith et al., 1996) have identified plectin as a candidate target gene for the disease termed epidermolysis bullosa with muscular dystrophy. The syndrome is consistent with the hypothesis that plectin is a multifunctional protein because loss of this single molecule was associated with structural defects in skin, muscle, and the central nervous system. However, structural evidence for the hypothesis of a multifunctional cross-linking activity of plectin is limited with only one study showing apparent plectin association with the actin cytoskeleton (Foisner et al., 1995).

Here, we identify the core of the newly revealed "millipede" structures as vimentin IFs and the multiple sidearms as plectin molecules, demonstrate specific associations with other cytoskeletal components and test mechanisms of their association. Our structural results suggest specific roles for plectin and IFs in cytoplasmic integrity.

Materials and Methods

Cells

Rat embryo fibroblasts (REF-52), mouse fibroblasts (Swiss 3T3), monkey kidney cells (TC-7), human foreskin fibroblasts (356), and chicken embryo fibroblasts (SL-29) were cultured as previously described (Verkhovsky et al., 1995). A rat kidney epithelial cell line (NRK) was a gift of Dr. D. Fishkind (Department of Biological Sciences, University of Notre Dame, Notre Dame, IN) and was cultured in HAM's F-12K medium supplemented with 10% FBS and antibiotics. A vimentin negative cell line, MFT-16, and a vimentin positive line, MFT-6, were provided by Dr. R. Evans (Department of Pathology, University of Colorado Health Science Center, Denver, CO) and cultured in HAM's F12:DMEM (1:1) medium mixture containing 5% FBS and antibiotics. These cell lines were identically derived from primary embryo fibroblasts of normal or vimentin null mice (see Colucci-Guyon et al., 1994) by transfection with an expression plasmid encoding SV-40 early genes (Evans, R., personal communication). Generally, subconfluent cell cultures (15–20 h) were used, although for some experiments, cells were cultivated longer (2–3 d).

For microinjection, cells were cultured on coverslips coated with gold through a finder grid as described (Verkhovsky and Borisy, 1993). Affinity-purified MAP4 antibody was microinjected into human foreskin fibroblasts (line 356) as described (Wang et al., 1996). Bovine lens vimentin isolated and biotinylated as in Vikstrom et al. (1991) was provided by Dr. V. Rodionov (Laboratory of Molecular Biology, University of Wisconsin, Madison, WI) and microinjected into MFT-16 cells at a needle concentration of 4 mg/ml. Cells were processed for light or electron microscopy 2–3 h after MAP4 antibody injection or 3–19 h after vimentin injection.

Antibodies

Primary antibodies used were (*1*) mouse monoclonal IgM vimentin antibody, clone NT30 (Troyanovsky et al., 1985) provided by Dr. I.S. Tint (University of Illinois, Urbana, IL); (*2*) mouse IgG monoclonal plectin antibody, clone 7A8 (Foisner et al., 1991*b*), (Sigma Chem. Co., St. Louis, MO) which, in our hands, reacted well with rat but not human or mouse cells; (*3*) rabbit polyclonal antibody to tyrosinated (Tyr) tubulin (Gundersen et al., 1984) provided by Dr. J. C. Bulinski (Department of Anatomy and Cell Biology, Columbia University College of Physicians and Surgeons, New York, NY); (*4*) rabbit polyclonal antibody to detyrosinated (Glu) tubulin (Gundersen et al., 1984) provided by Dr. G. Gundersen (Department of Anatomy and Cell Biology, Columbia University College of Physicians and Surgeons); (*5*) mouse monoclonal β-tubulin antibody (Amersham Corp., Arlington Heights, IL); (*6*) goat polyclonal biotin antibody (Sigma); (*7*) rabbit polyclonal brain axon spectrin antibody (Chemicon, Temecula, CA); (*8*) rabbit polyclonal keratin antibody (ICN Biomedicals, Irvine, CA); (*9*) rabbit polyclonal myosin II antibody (Verkhovsky et al., 1987); (*10*) affinity-purified rabbit polyclonal antibody to COOH-terminal domain of MAP4; and (*11*) rat polyclonal antibody to NH_2-terminal domain of MAP4 (Wang et al., 1996). Secondary fluorescein- or rhodamine-conjugated goat antibodies to rat, rabbit, goat, and mouse (IgG- or IgM-specific) immunoglobulins were used for immunofluorescence microscopy. Goat anti–mouse and goat anti–rabbit antibodies conjugated with 10 nm (Sigma) or 18 nm (Jackson ImmunoResearch Laboratories, West Grove, PA) colloidal gold were used for immunoelectron microscopy.

Light Microscopy

For immunofluorescence, coverslips with cells were rinsed with PBS, lysed for 5 min at room temperature with 1% Triton X-100 in a cytoskeleton-stabilizing solution containing 50 mM imidazole, 50 mM KCl, 0.5 mM $MgCl_2$, 0.1 mM EDTA, 1 mM EGTA, and 4% polyethylene glycol, M_r 40,000 (Serva, Heidelberg/New York), as described (Svitkina et al., 1984), briefly washed with the same solution without Triton X-100 and polyethylene glycol, and fixed with 2% glutaraldehyde in sodium-cacodylate buffer followed by sodium borohydride quenching. Fixation with 4% formaldehyde in PBS was used for myosin staining. Glutaraldehyde fixation of intact cells followed by 1% Triton X-100 permeabilization was used to assess whether the distribution of proteins was affected by prior detergent extraction. The major difference observed was that detergent extraction before fixation caused decreased background staining (not shown). The exception was MAP4 where detergent extraction caused partial loss of MAP4 immunostaining, especially in the central cytoplasm. Peripheral MTs, however, retained enough antigen for unmistakable detection. Sequential incubations with primary and secondary antibodies were carried out in PBS for 40 min at room temperature. Stained coverslips were mounted in Aqua-Polymount (Polysciences, Warrington, PA).

Digital fluorescence microscopy and image processing were performed as previously described (Verkhovsky et al., 1995). To obtain ratio images, paired 16-bit images were first aligned and intensity was linearly adjusted before the ratio image was calculated.

Electron Microscopy

Electron microscopy of cytoskeletons was performed as described (Svitkina et al., 1995). Briefly, cells on coverslips were lysed as for light microscopy, treated, if necessary, with recombinant gelsolin NH_2-terminal domain (a gift of Dr. M.L. Greaser, Department of Meat and Animal Science, University of Wisconsin, Madison, WI) as described (Verkhovsky and Borisy, 1993), fixed with glutaraldehyde, tannic acid and uranyl acetate, critical point dried, and coated with platinum and carbon. To preserve MTs, 10 μg/ml of taxol was added to all solutions before fixation. Myosin S1 (Sigma) decoration was performed after cell lysis as described (Svitkina et al., 1995).

Immunostaining with all antibodies except myosin was done after glutaraldehyde fixation as described (Svitkina et al., 1995) and followed by additional fixation with glutaraldehyde. Myosin antibody was applied to lysed, unfixed cells as described (Verkhovsky et al., 1995); cells were then fixed, quenched, incubated with secondary antibody conjugated to colloidal gold, and fixed again. Electron micrographs were obtained as de-

scribed (Svitkina et al., 1995), converted to digital files using a Nikon (Melville, NY) Scantouch digital scanner and processed for presentation using Adobe Photoshop (Adobe Systems Inc., Mountain View, CA) software.

Quantification of Electron Microscopic Data

Plectin sidearm density on IFs or MTs was determined by counting projecting arms along segments of 1 μm length in electron micrographs prepared from gelsolin-treated cells. For analysis of plectin sidearm density on MTs, sidearms were counted along MTs not interacting with IFs, that is, MT-IF distance greater than 0.2 μm. Y-shaped arms were counted as 1 or 2 depending on whether the stem or branches emerged from the filament analyzed. Periodicity of plectin binding on IFs was evaluated by measuring spacing between neighboring projections. Length was measured for plectin sidearms that were smooth, nonbranching, and not obviously folded.

The possible correlation between plectin association with MTs and level of tubulin detyrosination in the same MTs was determined by immunogold staining for Glu-tubulin. Fields were selected if they contained at least one Glu-MT (>20 gold particles per μm). MT segments 1 ± 0.3 μm long having at least one IF within interaction distance (0.2 μm) were picked. Numbers of gold particles and associated plectin sidearms were scored for each MT segment and their densities per μm were calculated.

For each kind of quantification, electron micrographs at the same magnification were first scanned and converted to digital form. After analysis, all calculations and generation of plots were carried out using SigmaPlot (Jandel Scientific, San Rafael, CA) software. All dimensions given are after subtraction of the thickness of the platinum layer, 2 × 2.5 nm (Svitkina et al., 1995).

Results

Intermediate Filaments Bear Sidearms That Are Made of Plectin

Electron microscopy of REF-52 fibroblasts after detergent extraction and depletion of actin by gelsolin treatment revealed abundant filaments with an unusual millipede-like appearance: a continuous core with multiple sidearms (Fig.1 *a*). Sidearms were thin (2–3 nm), long (176 ± 20 nm) for straight, fully extended projections (Fig. 2 *A*), flexible and often branched or anastomosed, making Y-contacts with each other. The density of sidearms along the filaments was highly variable. In peripheral cell regions where individual filaments could be clearly visualized, sidearms were present at a higher density with a mean value of 24 ± 12 per μm (Fig. 2 *B*). To evaluate the possible periodic distribution of sidearms, distances between side projections were measured on filaments with varying sidearm densities (Fig. 2 *C*). The distribution was complex and showed no strong periodicity; however, a weak preference for sites with periods of 20 nm and 65–70 nm is not excluded.

The core filaments varied in apparent thickness depending on the density of sidearms. Filaments with a high density of sidearms had an irregular contour and thickness close to 15–17 nm while filaments with few or no sidearms were smoother and thinner, 10 nm, having the conventional appearance of IFs. Immunogold labeling for vimentin gave heavy decoration along the cores of the millipede-like structures (Fig. 1 *b*), confirming their identity as vimentin IFs.

Because the sidearms had dimensions of the IF-associated protein, plectin, we performed immunogold labeling with a monoclonal antibody reactive with a characterized plectin epitope, namely, the midpoint of the plectin rod (Foisner et al., 1991*b*). The resultant immunostaining showed gold particles associated specifically with the middle of sidearms (Fig. 1 *c*) and not with core filaments or other cytoskeletal structures. Virtually no unlabeled sidearms remained, indicating that most if not all of the sidearms were composed of plectin.

Plectin sidearms are a general property of IFs in mammalian cells. The millipede-like appearance of IFs in actin-depleted cytoskeletons was a characteristic feature for all mammalian cell lines examined (rat, mouse, human, or monkey) but sidearms were sparse on IFs of chicken embryo fibroblasts (not shown). Electron microscopy showed plectin sidearms were present on IFs in epithelial as well as fibroblast cells. Double immunofluorescence staining in the epithelial cell line, NRK, showed colocalization of plectin with keratin IFs (not shown), demonstrating that plectin in vivo interacts not only with vimentin but with keratin IFs as well. Although greatly facilitated by actin removal, observation of millipede-like structures did not depend upon treatment with gelsolin. Careful examination of untreated cells showed similar plectin immunogold labeling of millipedes as indicated by the distribution of gold particles on thin sidearms along core IFs (Fig. 1 *d*).

Plectin Distribution on IFs Varies with Position in the Cell

The variability of sidearm density along IFs was not random but seemed to depend upon position in the cell. Electron micrographs yielded values ranging from 0–10 per μm in central regions to up to 60 per μm in peripheral regions. Because electron microscopy provides only a limited window for analyzing overall distributions of cell structures, light microscopic analysis was also performed. Double immunofluorescence staining for vimentin and plectin showed similar networks for each antigen at low magnification (Fig. 3, *a* and *b*) and detailed coincidence at high magnification (Fig. 3, *d–f*). However, ratiometric imaging (plectin/vimentin) (Fig. 3 *c*) indicated that plectin was more abundant relative to vimentin at the cell periphery, confirming the conclusion drawn from electron micrographs.

The distribution of plectin could reflect variation in the binding affinity of subpopulations of IFs. Alternatively, the affinity of IFs for plectin could be uniform and their differential association reflect a gradient of some cell factor. To distinguish between these possibilities, we induced rearrangement of IFs by depolymerization of MTs, a treatment known to cause collapse of the IF network to a perinuclear location (Ishikawa et al., 1968). If plectin binding reflected the higher affinity of a peripheral IF subpopulation, their collapse to the center should bring the sidearms with them. If, on the other hand, plectin binding reflected a gradient of some cell factor which was low in the central region, sidearms would be predicted to remain sparse in the center.

As expected, vimentin IFs collapsed to the perinuclear region after depolymerization of MTs with nocodazole (1 μg/ml for 18–24 h) (Fig. 4 *a*). However, not all vimentin was eliminated from the peripheral cytoplasm; numerous short fragments of vimentin filaments remained which could be seen after appropriate intensity windowing (insert in Fig. 4 *a*). The immunostaining pattern of plectin was practically identical to vimentin (compare inserts in 4,

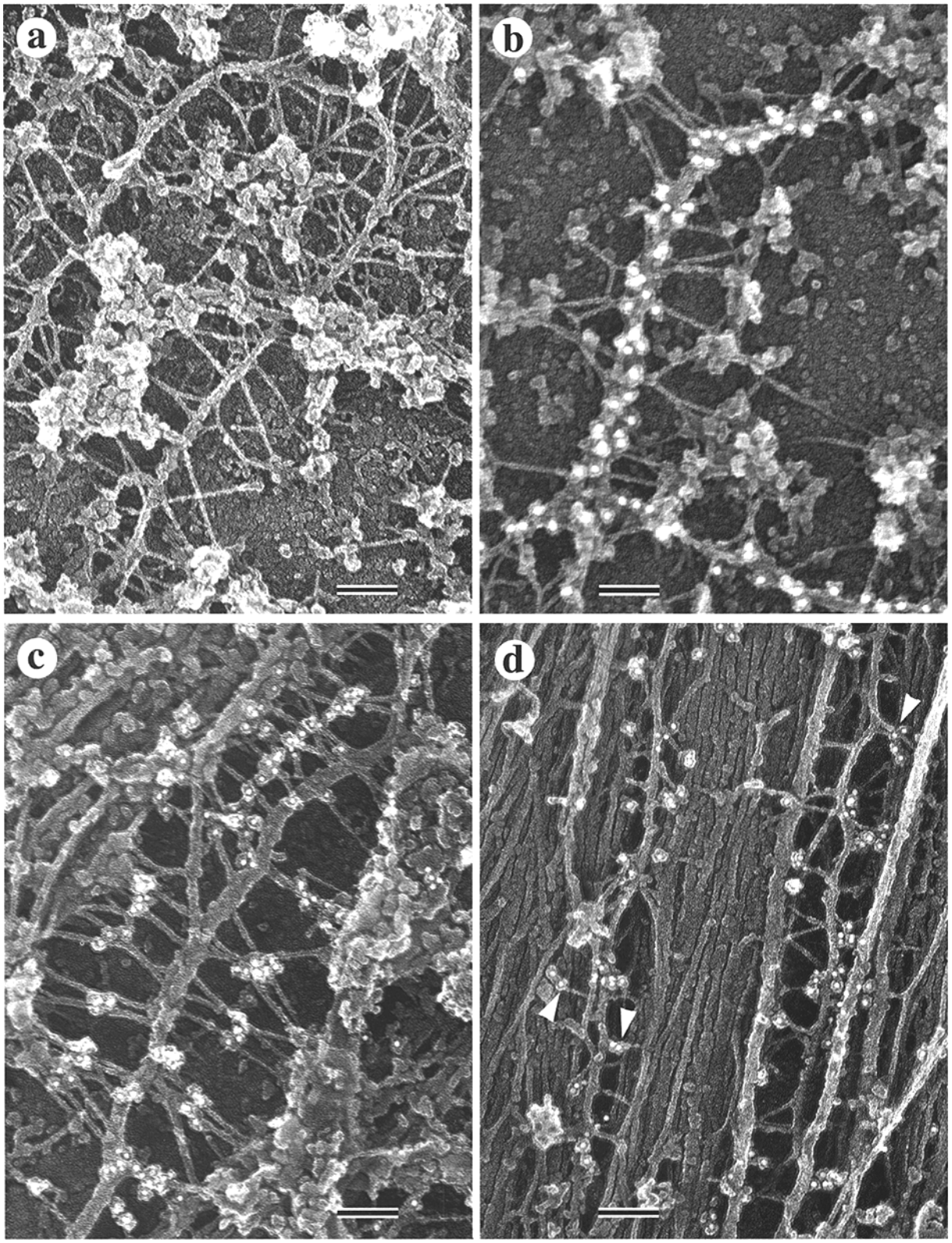

Figure 1. Plectin sidearms on IFs. Electron microscopy of REF-52 cytoskeletons treated with gelsolin to remove actin (*a–c*) or non-treated cytoskeletons (*d*). (*a*) Gelsolin treatment exposes millipede-like structures. (*b*) Vimentin immunogold (18 nm) labeling shows that the core of millipede is vimentin IF. (*c* and *d*) Plectin immunogold (10 nm) labeling shows sidearms are plectin. (*d*) Actin-containing cell; gold particles (10 nm) are distributed along IFs and can be seen on sidearms (*arrowheads*). Bars, 0.1 μm.

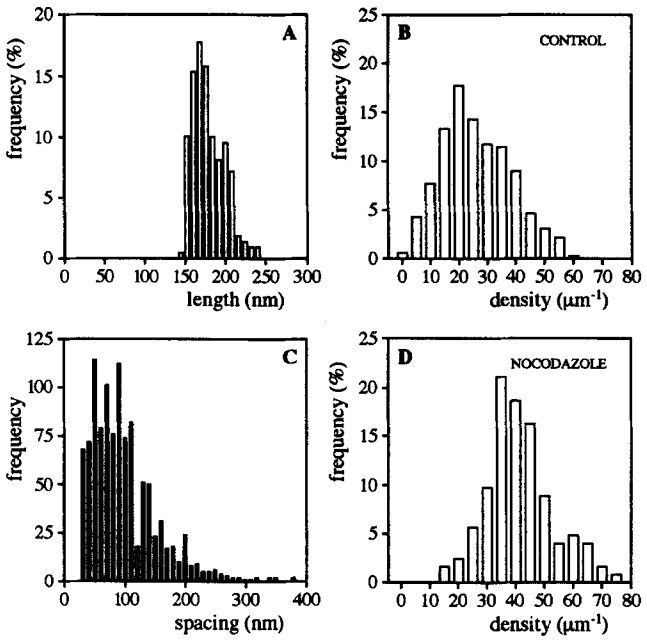

Figure 2. Quantitative characteristics of plectin binding to IFs. Frequency histograms showing length of sidearms (*A*), density of sidearms along IFs (*B* and *D*) and spacing between neighboring sidearms along IFs (*C*) in control- (*A–C*) and nocodazole-treated (*D*) cells. Only fully extended nonbranching sidearms were measured in *A*. IFs with very high density of sidearms (≥50/μm) were not included in the spacing measurements (*C*) because sidearms partially overlapped making it difficult to determine distances. Density of sidearms on peripheral IFs increased after nocodazole treatment (*D*) as compared to untreated control (*B*). Spacing histogram (*C*) shows no clear periodicity.

a and *b*), but the relative intensity of plectin was clearly higher in the periphery (Fig. 4 *b*). Ratiometric imaging confirmed that the plectin/vimentin ratio was higher in the periphery as compared to the perinuclear region (not shown). Another difference was the higher level of diffuse plectin fluorescence in the nocodazole-treated cells suggesting that some proportion of the plectin was no longer bound to IFs. Electron microscopy of the nocodazole-treated cells confirmed that the peripheral cytoplasm contained short vimentin IFs associated with numerous plectin sidearms (Fig. 4 *c*) whereas IFs in perinuclear bundles were basically smooth and contained low levels of plectin (Fig. 4 *d*). Compared to control cells, the density of plectin sidearms on peripheral IFs in nocodazole-treated cells was increased (mean value 39 ± 12 per μm) (Fig. 2 *D*) and plectin molecules not associated with IFs were also evident (not shown; also see Fig. 9 *e*). These results do not support the idea of subpopulations of IFs with different affinities for plectin but, rather, are consistent with a gradient of some cell factor.

Plectin Mediates Interaction of IFs with MTs

Although interaction between IFs and MTs has long been postulated, direct evidence for a structural association has been lacking. Electron microscopy of gelsolin-treated REF-52 or NRK cells demonstrated that plectin often formed bridges between IFs and MTs (Fig. 5). The constituents of these complexes were identified morphologically and immunochemically. In unstained specimens, MTs (22–25-nm thick, indefinite length, slightly curved), IFs (8–10 nm thick, indefinite length, highly curved) and plectin filaments (2–3-nm thick, less than 200 nm length) were easily distinguished by their dimensions and appearance. Immunogold staining for plectin (Fig. 5 *a*), vimentin (not shown) or tubulin (Fig. 5, *b* and *c*) confirmed the results of morphological identification. Individual IFs were not necessarily colinear with interacting MTs and plectin connections were often observed at sites where IFs crossed MTs at angles (see Fig 7 *i*). Plectin can apparently interact with MTs without the participation of IFs (Fig. 5 *d*). Such examples were more common for leading lamella of locomoting cells, where MTs were abundant and IFs sparse. In these cases plectin bridges cross-linked adjacent MTs or bound them to other cytoskeletal material. Plectin bridges between IFs and MTs were not sensitive to ATP as they were retained in gelsolin-treated cytoskeletons further treated with 1 mM ATP in extraction buffer lacking Triton X-100 and polyethylene glycol (not shown).

The structural evidence for interaction of MTs with IFs raised the question of whether a subpopulation of MTs bound plectin preferentially. Subpopulations of MTs have been distinguished by whether tyrosine is present (Tyr-tubulin) or absent (Glu-tubulin) at the COOH terminus of α-tubulin (Gundersen et al., 1984). We tested whether these subpopulations showed preferential association with plectin sidearms. Subpopulations of MTs were identified with antibodies specific for Tyr-tubulin (Fig. 5 *b*) or Glu-tubulin (Fig. 5 *c*). The distribution of Tyr and Glu MTs was examined by double immunofluorescence staining for β-tubulin and Tyr- or Glu-tubulin (not shown) and immunogold staining for either Tyr- or Glu-tubulin. Staining for Tyr-tubulin confirmed that in REF-52 cells, as in most other cells studied, Tyr-MTs form the bulk population (Gundersen et al., 1984). Staining for Glu-tubulin demonstrated that while some MTs were heavily labeled, other MTs were completely unlabeled and many MTs had intermediate levels of staining (see also Svitkina et al., 1995).

Plectin sidearms were detected bridging IFs to MTs heavily labeled with antibody to Tyr-tubulin (Fig. 5 *b*) or to Glu-tubulin (Fig. 5 *c*) and to unlabeled or intermediately labeled MTs in preparations stained for Glu-tubulin (not shown). In cases of intermediately labeled MTs, plectin connections did not correlate with sites of gold deposition. Quantification of the density of plectin cross-bridges between IFs and MTs with respect to the level of anti–Glu-tubulin labeling (Fig. 6) showed no correlation between these two parameters. These results do not support the idea that IF-MT association is determined solely by detyrosination.

Since electron microscopy permits analysis of only a small sample of cytoplasm in a limited number of cells, we analyzed plectin-MT associations by double label immunofluorescence. In two cell lines examined, REF-52 fibroblasts and NRK epithelial cells, plectin and tubulin had an overlapping overall distribution, i.e., regions with high density of MTs generally also contained more plectin with the exception of some regions of leading lamella (Fig. 7, *a* and *b*). High resolution imaging showed some plectin fibrils were colinear with MTs (Fig. 7, *c–e*), whereas others crossed MTs at angles or were colinear over only short do-

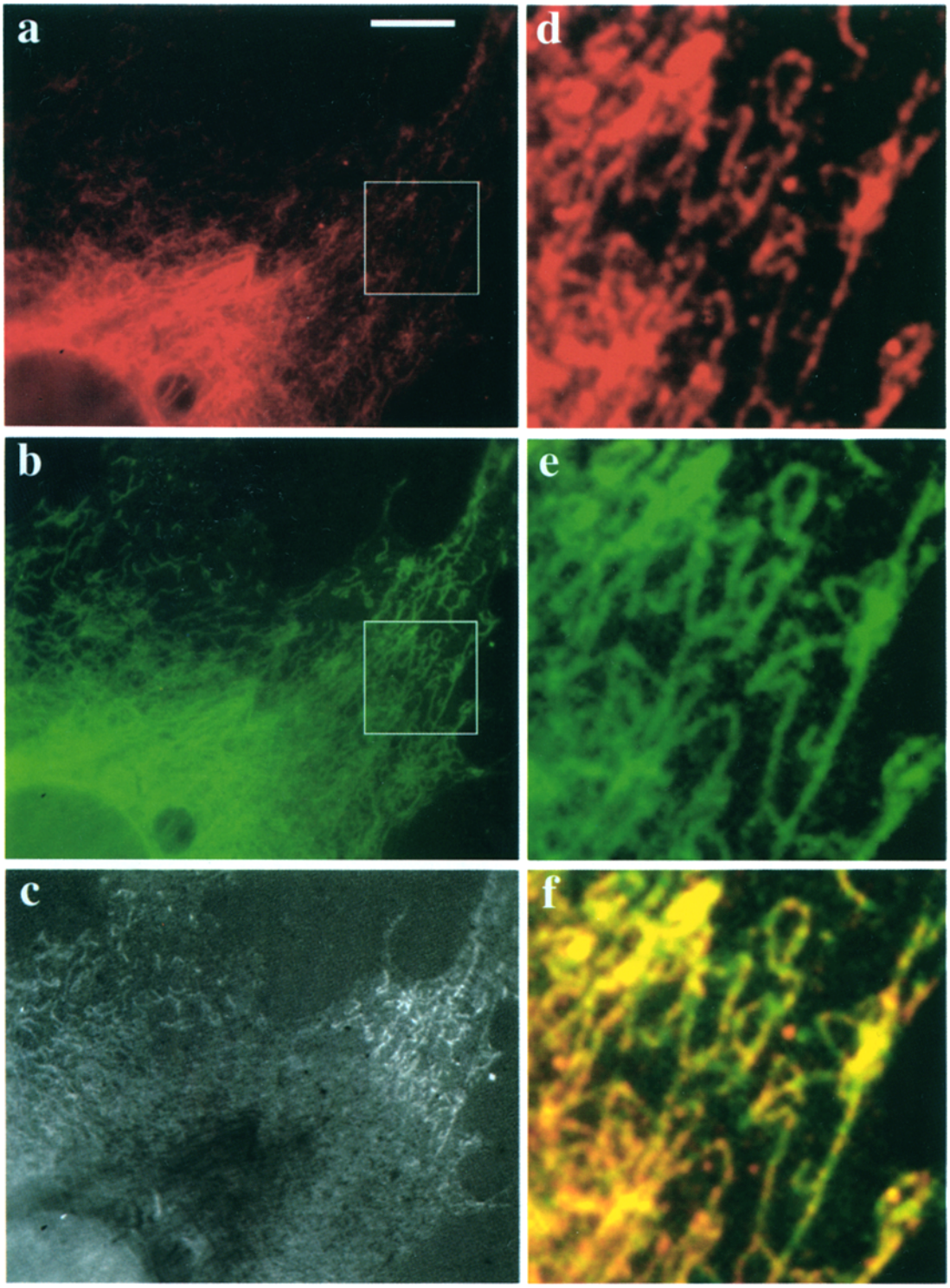

Figure 3. Relative distribution of vimentin and plectin. Double immunofluorescence for vimentin (*red*) and plectin (*green*) in REF-52 cells shows very similar distribution of the two antigens. Boxed regions from *a* and *b*, enlarged in *d* and *e*, respectively, are overlapped in *f* showing coincidence. Plectin/vimentin ratio image (*c*) shows higher plectin content (*lighter shades*) on peripheral filaments and lower content (*darker shades*) in cell center. Bar, 10 μm.

mains (Fig. 7, *f–h*). Electron microscopy revealed that plectin connected IFs and MTs at points of crossing even where no obvious colinearity existed (Fig 7 *i*). These data support the concept of flexible IFs interacting with multiple MTs by means of plectin sidearms.

In Vivo Competition between MTs and IFs for Plectin

The structural association of plectin with MTs visualized in cytoskeletons suggested that IFs are not the only binding partners for plectin. The possibility that plectin can

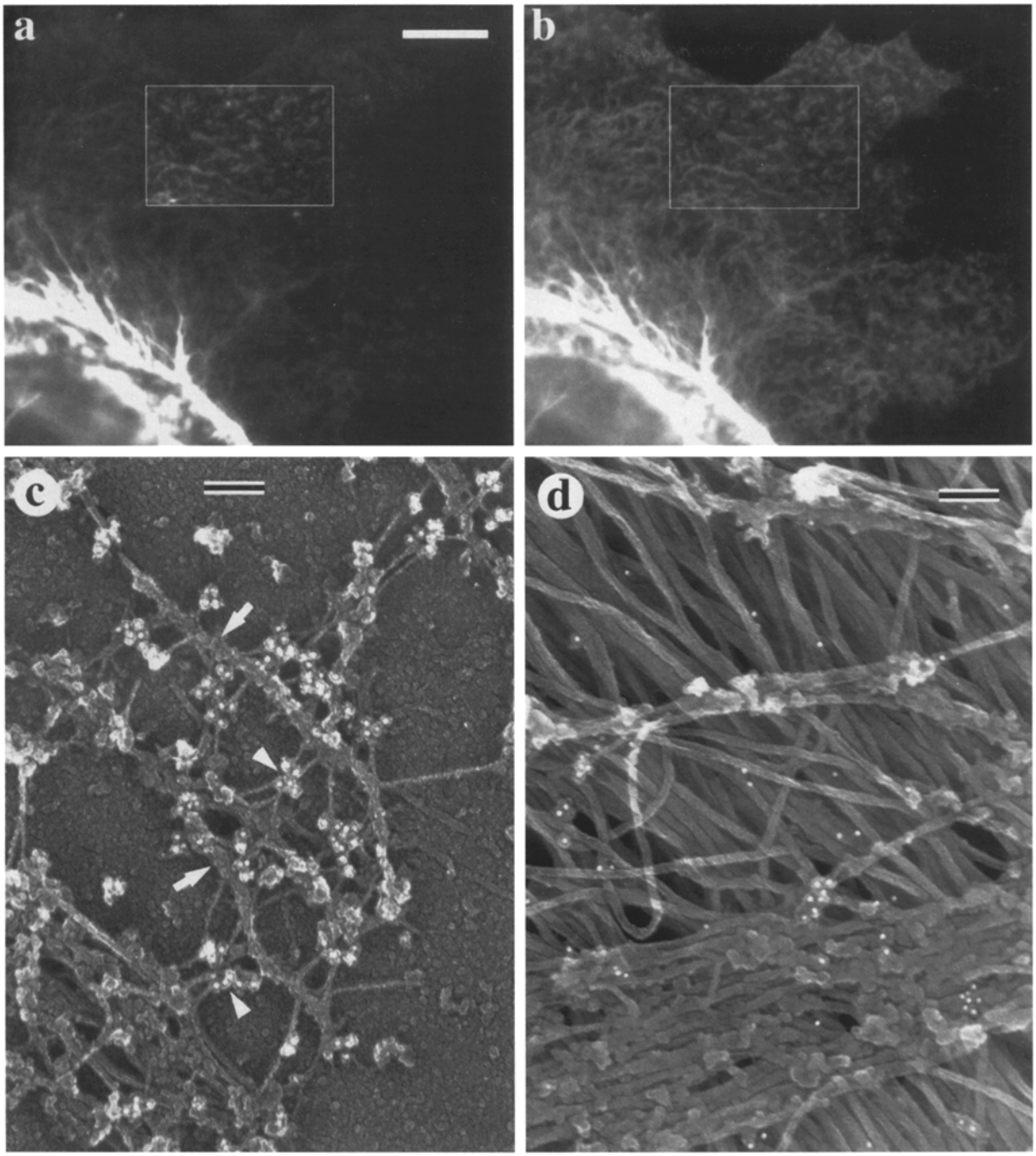

Figure 4. Preferential distribution of plectin in peripheral regions. REF-52 cells were treated with nocodazole to depolymerize MTs and induce collapse of most IFs to the cell center. (*a* and *b*) Double immunofluorescence for vimentin (*a*) and plectin (*b*) shows similar pattern but different intensity distribution for the two antigens. Boxed region in *a* was intensity adjusted to allow visualization of residual peripheral IFs. Corresponding region in *b* shows coincident plectin features. Bar, 10 μm. (*c* and *d*) Electron microscopy of peripheral (*c*) and perinuclear (*d*) cell regions after gelsolin treatment and immunogold (10 nm) labeling for plectin. Short IFs (*arrows*) at the periphery (*c*) have numerous plectin sidearms (*arrowheads*), while perinuclear IF bundles (*d*) are smooth and contain little plectin. Bars, 0.1 μm.

bind in vivo to MTs, as well as IFs, implies the existence of a pool of soluble plectin and suggests that a competition should exist between these two binding partners for plectin. A prediction is that in the absence of IFs, the frequency of plectin binding to MTs should increase. To test this prediction, we determined the distribution of plectin in a cell line (MFT-16) obtained from vimentin-null mice (Colucci-Guyon et al., 1994) and lacking IFs of any kind (Evans, R.M., personal communication). A cell line (MFT-6) obtained in the same way from normal mice was used as a control.

Immunofluorescence staining for vimentin was negative for MFT-16 cells and revealed a normal IF network in MFT-6 cells (not shown). Electron microscopy of MFT-6

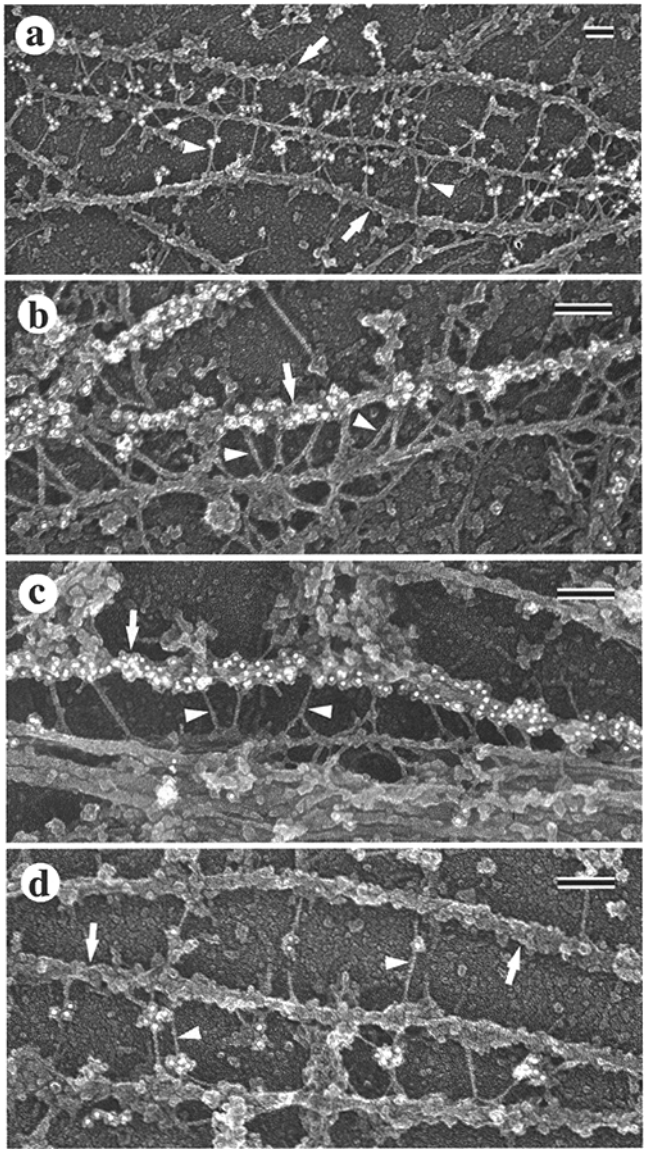

Figure 5. Association of plectin with MTs. Electron microscopy of gelsolin-treated REF-52 cells after immunogold (10 nm) labeling for plectin (*a* and *d*), Tyr-tubulin (*b*), or Glu-tubulin (*c*). Plectin forms bridges (*arrowheads*) between MTs (*arrows*) and IFs (*a–c*) or between two MTs (*d*). MTs composed predominantly of Tyr-tubulin (*b*) or Glu-tubulin (*c*) interact with IFs via plectin. Bars, 0.1 μm.

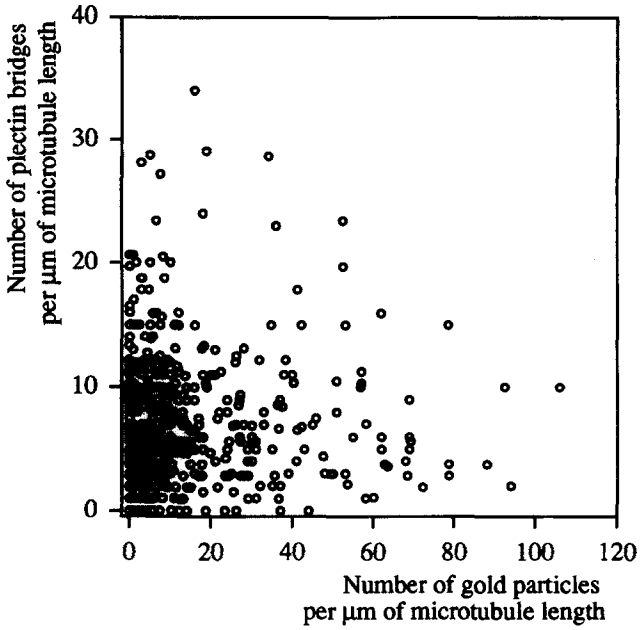

Figure 6. Plectin shows no preference for Glu- over Tyr-tubulin. Gelsolin-treated REF-52 cells were immunogold labeled with antibody to Glu-tubulin and quantified as described in Materials and Methods. Number of plectin sidearms associated with an MT and number of gold particles on the same MT was determined. Scatter plot shows no apparent relationship between the two parameters.

cells revealed the same distribution of plectin as in REF-52 fibroblasts: numerous sidearms on IFs often making junctions with MTs and occasional plectin molecules directly bridging MTs to each other (not shown). However, in MFT-16 cells, numerous sidearms were associated with MTs (Fig. 8 *a*) rendering them an unusual "hairy" appearance. Quantitative electron microscopy showed that, compared to control cells, the density of plectin sidearms on MTs was highly increased (Table I).

To determine whether the density of plectin sidearms on MTs was specifically dependent on the lack of vimentin, we attempted to reverse the plectin binding by injecting exogenous vimentin. In MFT-16 cells injected with vimentin, IFs of variable length were formed within 3 h after injection and persisted for at least 19 h but seemed to comprise less than the normal complement of IFs in MFT-6 cells. The IFs had a very high density of associated plectin (Fig. 8 *b*), suggesting that a shortage of IFs results in a higher level of plectin occupancy (48 ± 28 sidearms per μm vs 16 ± 10 in control MFT-6 cells). Nevertheless, MTs in the vimentin-injected cells lost most of their plectin sidearms (Fig. 8 *b*, Table I), suggesting that even low or moderate amounts of IFs are able to shift the plectin sidearms away from the MTs. These results indicate the existence of a cellular pool of plectin and that MTs and IFs compete for plectin in the living cell. Although IFs are the preferred binding partner, specific interaction of plectin with MTs can clearly be seen in the absence of IFs.

Plectin Association with MTs Is Independent of MAP4

The plectin binding to MTs might be mediated by some accessory proteins interacting with MTs. The major MT-associated protein in cultured human fibroblasts is MAP4 (Bulinski and Borisy, 1980; Bulinski, 1994). To test the possibility that MAP4 mediates plectin binding to MTs, we immunodepleted MAP4 from human 356 fibroblasts by microinjecting an antibody to the COOH-terminal domain (Wang et al., 1996). Antibody to the NH_2-terminal domain of MAP4 was used for immunochemical localization of MAP4 in control and injected cells.

In uninjected cells that were permeabilized after fixation, immunostaining for MAP4 revealed MTs (Fig. 9 *a*, *upper cell*, *arrowhead*). In contrast, microinjected cells lacked the MT staining. Instead, they contained large anti-

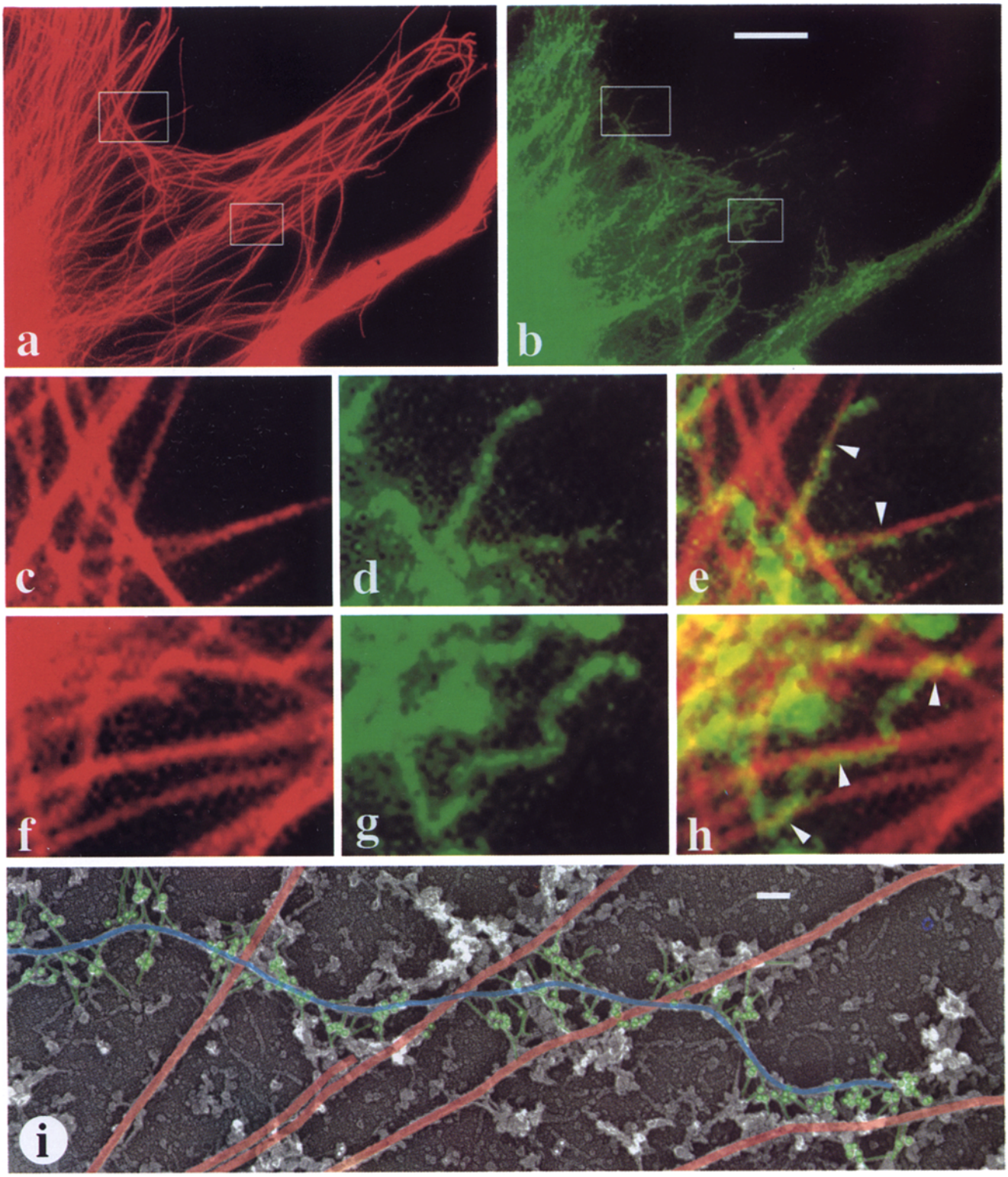

Figure 7. Relative distribution of MTs and plectin. Double immunofluorescence (*a–h*) for Tyr-tubulin (*red*) and plectin (*green*) in REF-52 cells shows nonidentical but locally coincident distribution of the two antigens. Boxed regions from *a* enlarged in *c* and *f* and corresponding regions from *b* enlarged in *d* and *g*, are overlapped in *e* and *h*. Examples of local plectin and tubulin alignment are shown by arrowheads. Digitally colorized electron micrograph (*i*) shows gold-labeled plectin (*green*) connecting an IF (*blue*) to MTs (*red*) when they cross each other at different angles. Bars: (*a–h*) 10 μm, (*i*) 0.1 μm.

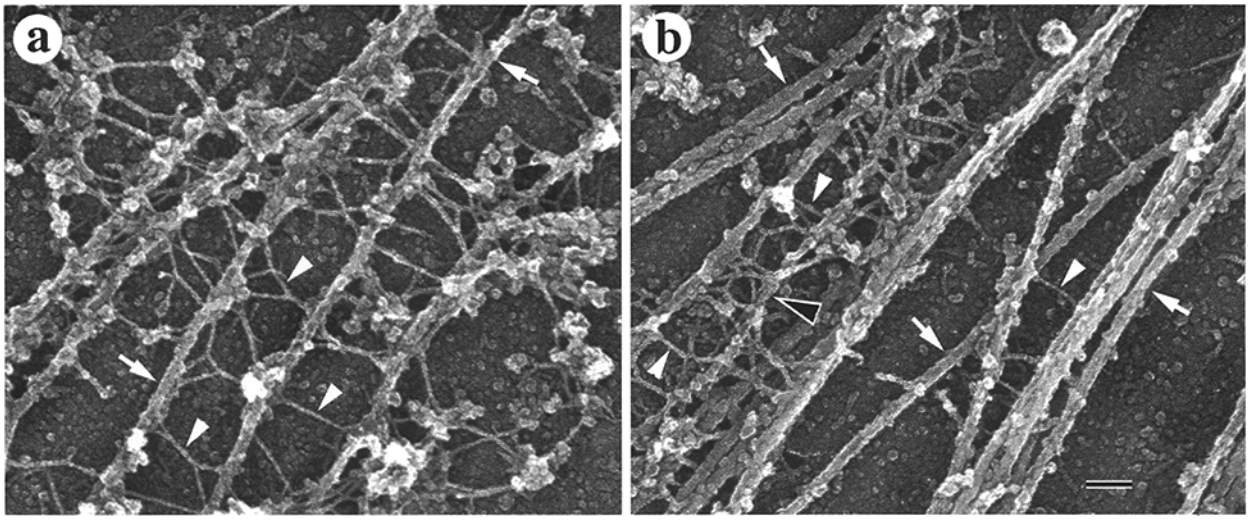

Figure 8. Plectin association to MTs in IF-minus cells and redistribution back to IFs after microinjection of vimentin. Electron microscopy of mouse MFT-16 cells before (*a*) and 3 h after (*b*) microinjection of bovine vimentin. (*a*) MTs (*arrows*) in a noninjected cell bear numerous plectin sidearms (*arrowheads*). (*b*) An IF (*black arrowhead*) in an injected cell has a high density of associated plectin (*arrowheads*), whereas MTs (*arrows*) bear just a few sidearms. Bar, 0.1 μm.

MAP4 immunostained aggregates in perinuclear cytoplasm and smaller ones at the cell periphery (Fig. 9 *a*, *lower cell, arrow*). A similar pattern of anti-MAP4 immunolabeling was demonstrated by electron microscopy. All MTs in control cells had a distinctive decoration with anti-MAP4 gold particles (Fig. 9 *c*). In injected cells, heavily decorated amorphous aggregates were found in the perinuclear cytoplasm (not shown), but MTs were completely unlabeled (Fig. 9 *d*).

Immunofluorescence with antibody to vimentin revealed normal IF networks in both control and injected cells (Fig. 9 *b*). By electron microscopy, IFs in control 356 fibroblasts had, on average, less side projections than REF-52 or NRK cells. However, as in other cell lines, sidearms often bridged IFs to MTs (Fig. 9 *c*), and, the salient point for this study, sidearms were also seen bridging IFs to MTs in MAP4 depleted cells (Fig. 9 *d*). No correlation was observed between plectin attachment sites and localization of anti-MAP4 labeling on MTs. No changes in the number of IF sidearms or extent of their binding to MTs was found after MAP4 removal from MTs. These results indicate that MAP4 is not essential for plectin binding to MTs.

Plectin Mediates Interaction of IFs with Myosin II

Electron microscopy of REF-52 cytoskeletons showed that IFs were often bound through plectin bridges to actin filament bundles (also designated as "stress-fibers") (Fig. 1 *d*; see also Fig. 6 *b* in Svitkina et al., 1995) or their remnants after gelsolin treatment (Fig. 1 *c*) and to focal contacts (not shown). Since stress-fibers are multiprotein complexes (for review see Bershadsky and Vasiliev, 1988; Burridge et al., 1988), it was not clear whether plectin binds directly to actin or to other constituent(s) of stress fibers. For unambiguous identification of actin filaments, we decorated them with subfragment-1 (S1) of muscle myosin and searched for clear plectin-actin junctions. Gold-labeled plectin bridges associated with S1-decorated stress-fibers or smaller groups of actin filaments were clearly seen (Fig. 10 *a*). However, such examples did not preclude the participation of other proteins in plectin-actin association. Plectin molecules bound to single actin filaments were rare and could be accounted for by incidental superposition.

Whether actin was necessary for plectin binding to stress

Table I. Plectin Binding to MTs Is Competed by Vimentin IFs

	Vimentin-postitve MFT-6 cells	Vimentin-negative MFT-16 cells	Vimentin-injected MFT-16 cells
Plectin density*	1.6 ± 1.5	5.8 ± 3.5	2.1 ± 1.9
Number of microtubule segments scored‡	1802	4061	1759
Number of cells§	9	12	9

Vimentin-positive (MFT-6), vimentin-negative (MFT-16), and vimentin-injected MFT-16 cells were assayed for density of sidearms on microtubules by electron microscopy. Sidearm density was higher in vimentin-negative cells as compared to vimentin-positive cells, but decreased after microjection of vimentin into vimentin-negative cells. Differences between mean plectin densities were highly significant as determined by *t* test ($P < 0.001$).

*mean ± SD of a number of plectin sidearms per μm microtubule length; standard errors of means were 0.03–0.05 arms/μm.

‡microtubules scored in 1-μm segments.

§3–17 (8 in average) electron microscopic fields in peripheral cytoplasm cytoplasm (at 15,000 magnification) were scored per cell.

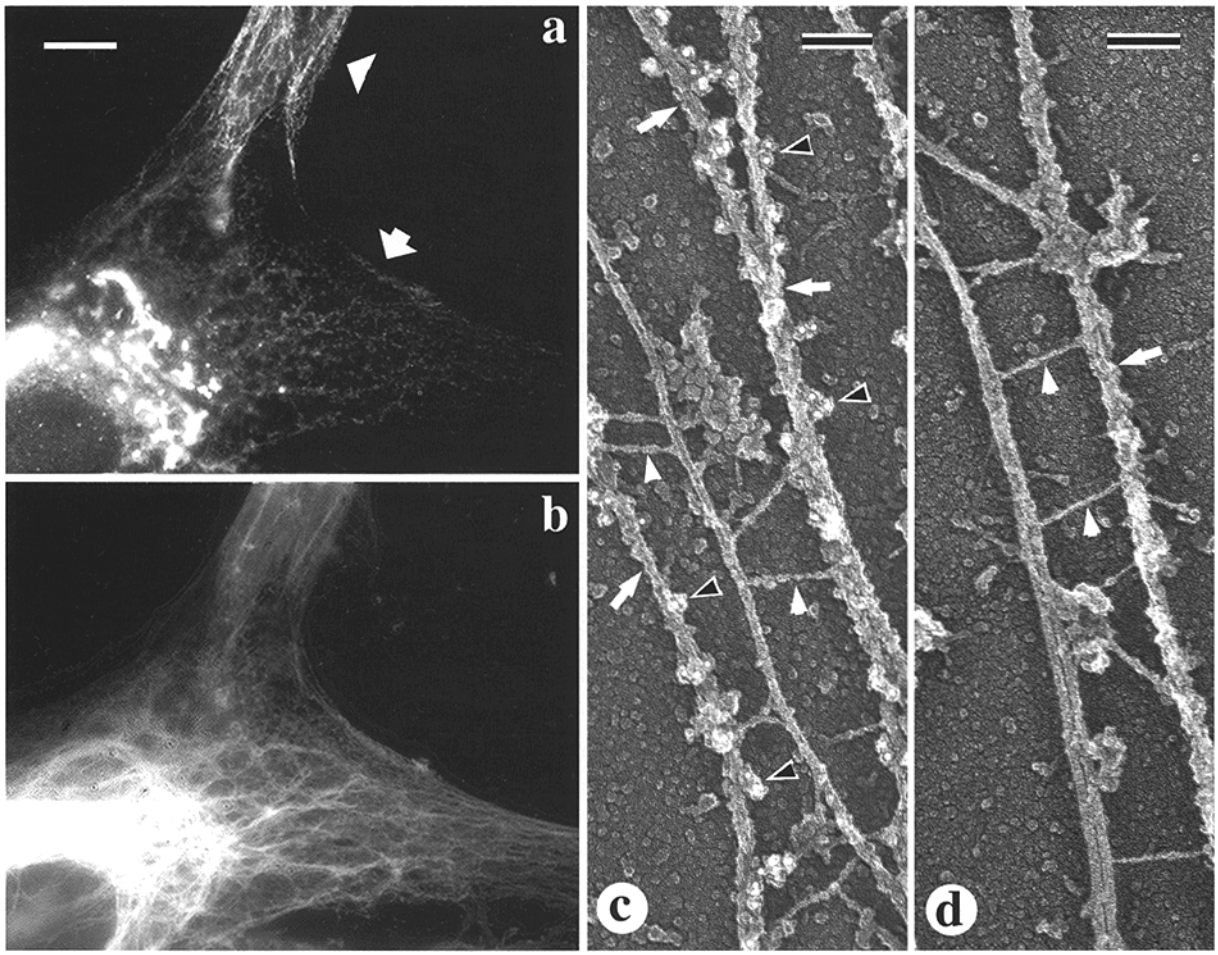

Figure 9. MAP4 is not required for plectin binding to MTs. Human 356 fibroblasts were microinjected with antibody to COOH-terminal domain of MAP4 to remove MAP4 from MTs. (*a*, *b*) Double immunofluorescence for MAP4 (antibody to MAP4 NH_2-terminal domain) (*a*) and vimentin (*b*) of a cell microinjected with antibody to MAP4 COOH-terminal domain (*lower cell, arrow*) and noninjected cell (lamellar fragment showing in upper part of image, *arrowhead*). Antibody to MAP4 stains MTs in the control cell and micro immunoprecipitates in the injected cell. Vimentin IFs (*b*) remain distributed throughout cytoplasm of both cells. Bar, 10 μm. (*c* and *d*) Electron microscopy of control (*c*) and MAP4 immunodepleted (*d*) cells after staining with antibody to NH_2 terminus of MAP4. 10-nm gold particles (*black arrowheads*) decorate MTs in control cells, but are absent in injected cells. Plectin forms bridges (*white arrowheads*) between IFs and MTs (*arrows*) in both cells. Bars, 0.1 μm.

fibers was tested by depletion of actin by treatment with gelsolin. Gelsolin treatment also removes the actin-binding proteins, α-actinin and vinculin, (Verkhovsky et al., 1987), leaving a structure composed primarily of myosin II minifilaments that can be easily identified by their characteristic bipolar shape (Svitkina et al., 1989; Verkhovsky and Borisy, 1993; Verkhovsky et al., 1995). IFs in gelsolin-treated cells remained structurally associated with the residual stress-fibers through plectin sidearms (Fig. 1, *c* and 10 *b*). Plectin arms usually terminated at myosin filament assemblies in the former actin bundles. Definitive identification of the plectin target in stress-fibers, however, was not possible because of dense aggregation of myosin filaments. In lamellar regions, where myosin filaments were distributed more loosely and could be easily identified by their bipolar shape (Fig. 10, *c* and *e*) or by antibody labeling (Fig. 10 *d*), direct binding between plectin and myosin filaments was observed. Plectin molecules not associated with IFs, which became especially abundant after nocodazole treatment (see above), cross-linked myosin filaments to each other or to other cytoskeletal material (Fig. 10 *e*). Numerous plectin arms were also observed bound to myosin filaments in vimentin negative cells (MFT-16) (not shown), further supporting the conclusion that myosin II could serve as an independent plectin binding partner. Plectin molecules usually terminated at the head regions of myosin filaments; however, their occasional binding to the central rod of myosin filaments was also observed.

Plectin-myosin junctions were visualized by electron microscopy only in actin-depleted cytoskeletons. To test if plectin-myosin association existed before depletion of actin and to analyze their relationship over broader areas of the cell, we performed double immunofluorescent localization of the two proteins in intact cytoskeletons (Fig. 11). The data showed no overall colocalization of the two antigens at low magnification (Fig. 11, *a* and *b*); however, at high resolution, their mutual arrangement was clearly nonrandom. In the vicinity of stress-fibers, plectin fibrils

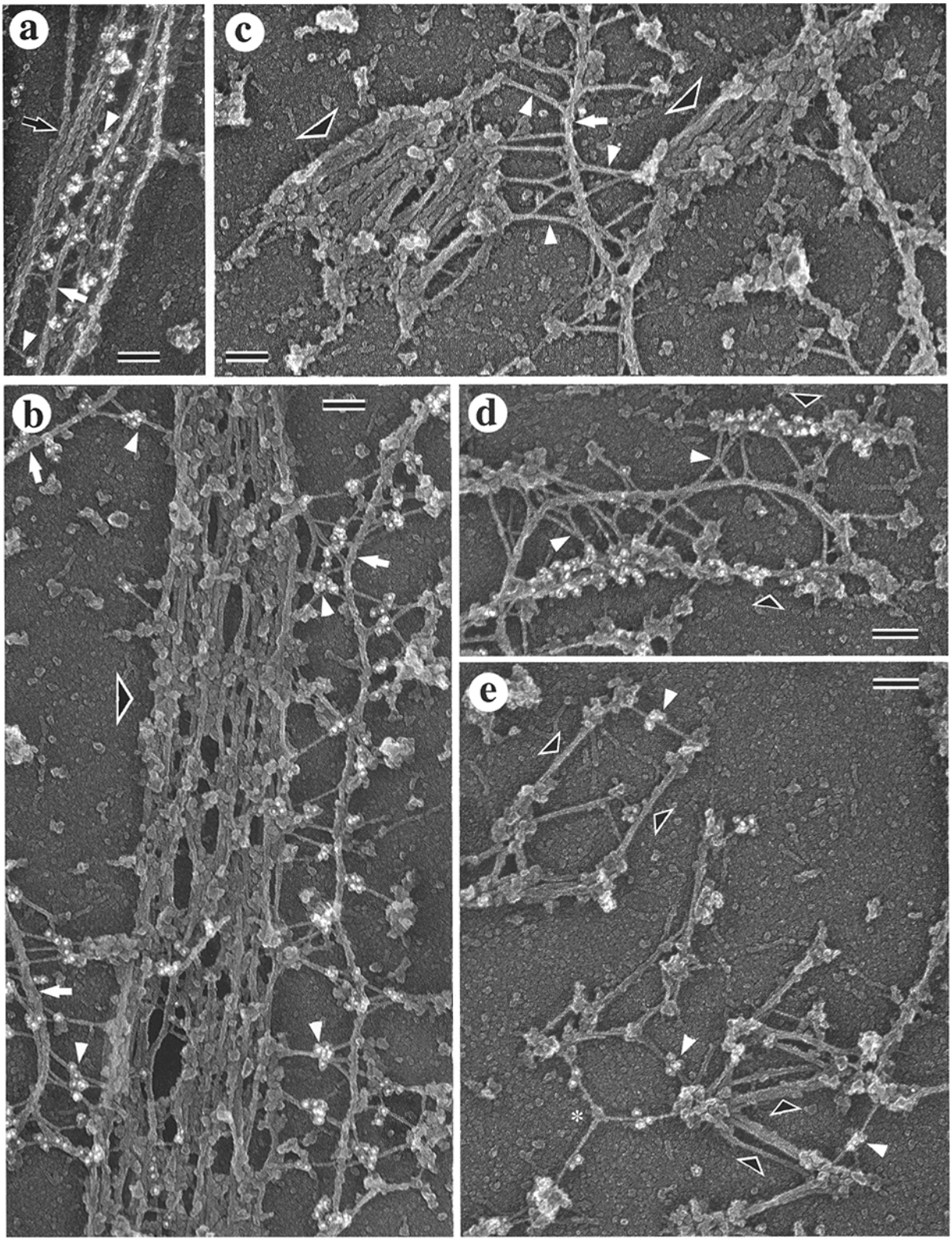

Figure 10. Association of plectin with myosin II. (*a*) REF-52 cytoskeleton with actin filaments decorated by myosin S1 (*black arrow*). An IF (*white arrow*) associates with an actin filament bundle via plectin sidearms (10-nm immunogold labeling, *white arrowheads*). (*b–e*) Gelsolin-treated cells immunogold labeled for plectin (*b* and *e*) or myosin II (*d*). After actin depletion, IFs (*white arrows*) with plectin sidearms (*white arrowheads*) remain associated with myosin-rich remnants of stress fibers (*b*, *black arrowhead*), and also bind to clusters of myosin filaments (*c*, *black arrowheads*) and individual myosin bipolar filaments (*d*, *black arrowheads*). (*e*) Nocodazole-treated cell with actin depleted by gelsolin. Individual plectin molecules in lamella (*white arrowheads*) cross-link myosin filaments (*black arrowheads*) and their clusters. Some plectin molecules bind to each other (*asterisk*). Bars, 0.1 μm.

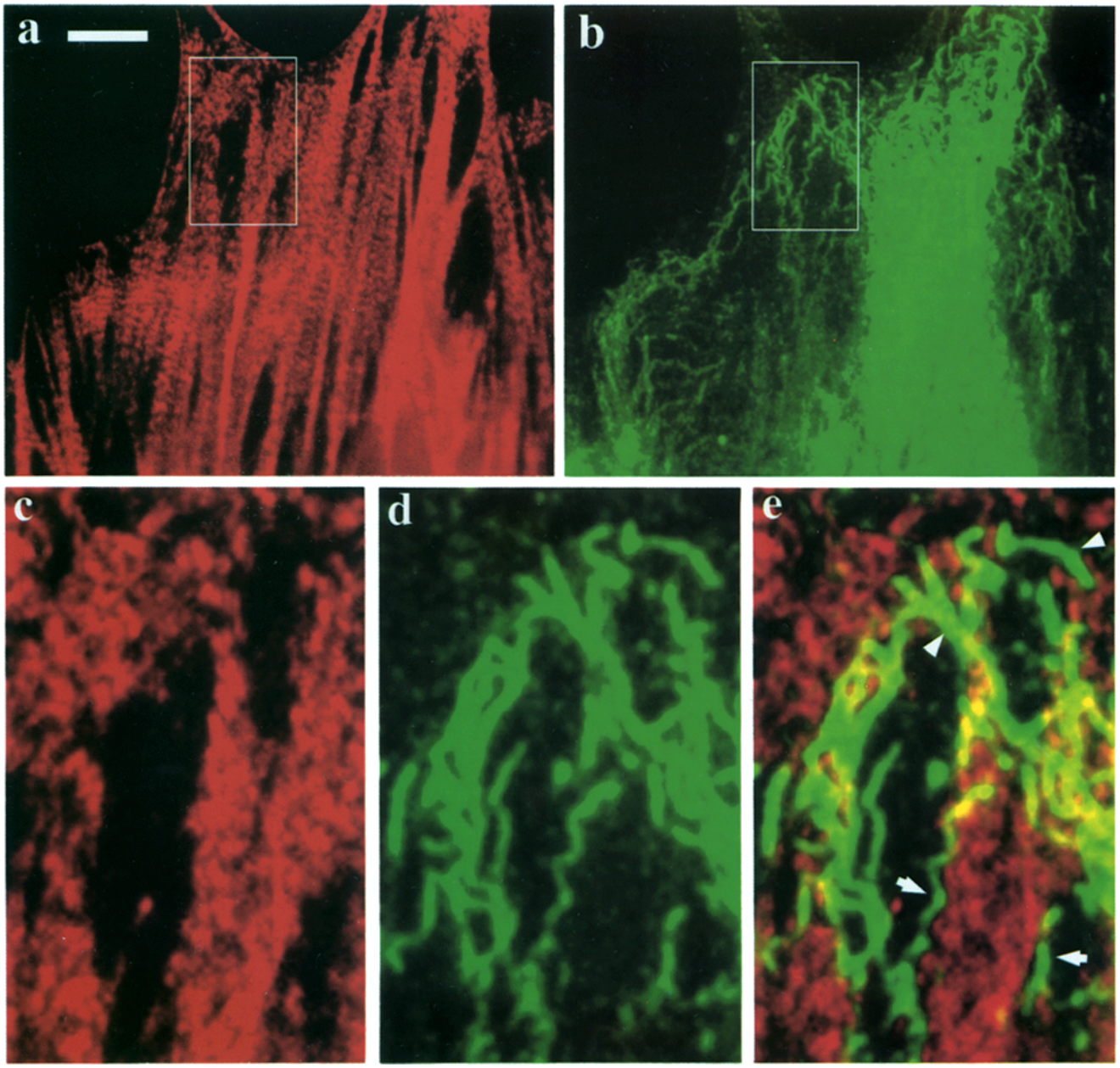

Figure 11. Relative distribution of plectin and myosin. Double immunofluorescence for nonmuscle myosin II (*red*) and plectin (*green*) in REF-52 cells demonstrates different general distribution of two antigens at low magnification (*a* and *b*). Boxed region from *a* enlarged in *c* and corresponding region from *b* enlarged in *d* are overlapped in *e*, showing plectin-containing fibrils outlining stress fibers (*arrows*) or filling spaces between individual myosin features (*arrowheads*). Bar, 10 μm.

tended to run parallel to their side edges, to overlap the width of stress-fibers, or to be inserted in between individual myosin features (Fig. 11, *c–e*). These results are consistent with the idea that IF interaction with the actin-based cytoskeleton is mediated by association of plectin with myosin II or a myosin II–associated component.

Plectin Bridges between IFs and Putative Membrane Components

Another morphological target for plectin binding was amorphous material scattered throughout the cytoplasm (see Fig. 1, *a* and *b*). This material was clearly distinct from cytoskeletal filaments and remained unlabeled after application of antibodies to tubulin, vimentin, myosin, or α-actinin (not shown).

Since one of plectin's binding partners according to solid-phase in vitro assay is spectrin (Herrmann and Wiche, 1987), we considered whether the amorphous material represented remnants of the membrane skeleton and contained spectrin. Double label immunofluorescence gave a finely particulate distribution of spectrin in REF-52 cells with no obvious indication of plectin-spectrin codistribution at the light microscopic level (not shown). Elec-

tron microscopy after immunogold staining with spectrin antibody demonstrated partial labeling of amorphous material (not shown), but no correlation was found between sites of spectrin immunolabeling of amorphous material and its association with plectin.

Plectin may nevertheless have membrane-associated partners. In focal contacts, which were recognized by their location distal to myosin-containing remnants of stress-fibers, plectin associated with IFs and clusters of granular material was often observed (not shown). IFs formed an intricate web, which extended to the myosin-containing parts of stress-fibers. The fraction of focal contacts enriched in IFs and plectin increased in long term cultures in agreement with the data of Seifert et al. (1992).

Discussion

In this study we have identified sidearms of IFs as plectin, a molecule first characterized by Pytela and Wiche (1980) as an IF-associated protein and subsequently proposed as a "versatile cytoskeletal cross-linker" (Foisner and Wiche, 1991). We also visualized at high resolution the organization of plectin in situ and demonstrated that it has multiple binding partners. Based on frequency of association, plectin binds preferably to IFs, but MTs are independent partners, as well as the actin-based cytoskeleton, and, possibly, membrane components (Fig. 12).

Because our structural evidence for plectin associations comes primarily from electron microscopy of detergent-extracted cells in which actin was removed by treatment with gelsolin, it is important to consider the possibility that these procedures introduce artifacts of plectin redistribution or adventitious superposition onto other cytoskeletal components. Several considerations suggest that the observed plectin associations are not artifactual. First, similar plectin distribution was observed by immunofluorescence in detergent-extracted and nonextracted cells suggesting that, at least at the light microscopic level, plectin is not affected by extraction. Second, by correlative light and electron microscopy, we previously showed that gelsolin treatment following detergent extraction did not perturb the in vivo organization of a highly dynamic cytoskeletal component, myosin II (Verkhovsky and Borisy, 1993; Verkhovsky et al., 1995). Third, a physiological test demonstrating redistribution of plectin sidearms to MTs in vimentin-minus cells and away from MTs by introduced IFs indicates that plectin's association with MTs is specific, that is, it depends on the presence or absence of vimentin IFs. Thus, we believe our structural results fairly reflect the organization of plectin in situ.

Interaction of Plectin with IFs

Plectin association with IFs was seen as a striking millipede-like structure with an IF core and plectin sidearms. Thin and short connecting filaments were described many times in the literature (Webster et al., 1978; Schliwa and van Blerkom, 1981; Svitkina et al., 1984; Bridgman and Reese, 1984, Bohn et al., 1993). Recently, Foisner et al. (1995) demonstrated the existence in cytoskeletal preparations of some cross-linking filaments made of plectin. However, this study was not able to provide high resolution

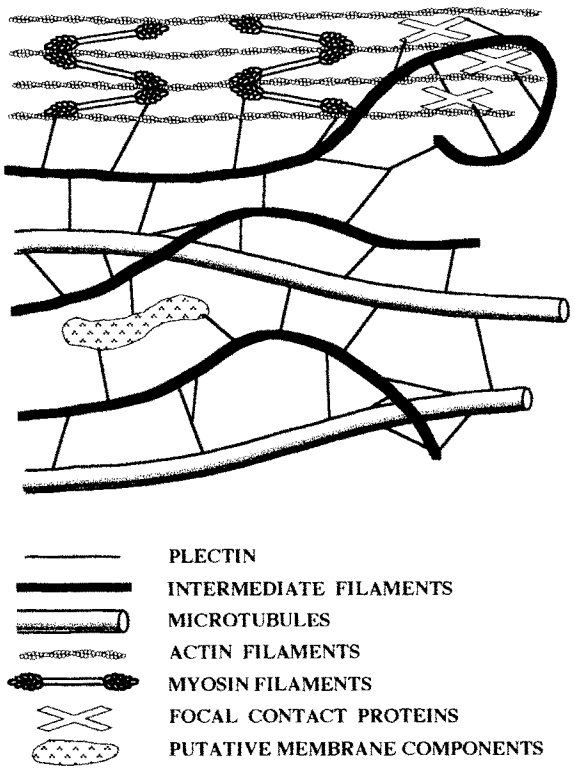

Figure 12. A model for plectin-mediated integration of cytoplasm. Plectin sidearms on IFs link them to MTs, myosin II minifilaments in stress-fibers, and putative membrane components, thus mechanically connecting various cellular structures.

structural information on the organization of plectin because of obscuring actin filaments.

Our results on the organization of plectin in situ are in agreement with previous data on the structure and binding of plectin in vitro. The dimensions of the sidearms (up to 200 nm long and 2–3-nm wide) matches the size of plectin molecules (Foisner and Wiche, 1987), the mode of antibody staining (in the middle of projections) corresponds to the location of the antigenic site on plectin molecules (Foisner et al., 1991*b*), and the terminal association with IFs fits the mapping of IF-binding sites to globular COOH-terminal domains of the plectin molecule (Wiche et al., 1993). These results indicate that side projections are made of individual plectin molecules or a few of them in register. The frequent appearance of Y-shaped sidearms and some variability in their thickness suggest that more than one plectin molecule may form an individual projection. The capability of plectin to bind to similar structures (e.g., two IFs) with both ends suggests that the ends are equal in binding properties. This accords with the idea that the plectin molecule is a homotetramer composed of two parallel dimers arranged antiparallel and overlapping by their entire length (Wiche et al., 1991). Plectin-IF complexes have been produced as a result of in vitro reconstitution from purified proteins (Foisner et al., 1988). In vitro, however, plectin-IF interaction produced an irregular network instead of "millipedes," perhaps, because of the absence of interaction with other structures.

Although plectin clearly has specific domains for inter-

action with IFs (Wiche et al., 1993), the existence of specific sites on IFs for plectin binding is unclear. Periodic binding of plectin to IFs would be evidence for site-specific association. However, our results showed neither clear periodicity nor definitely aperiodic distribution. A possible interpretation is that the density of plectin binding sites on IFs is high but some sites are more favorable than others.

The quantitative characteristics of plectin-vimentin complexes presented here correlate well with the reported data on plectin/vimentin mass ratios. For rodent fibroblast cell lines this value is 0.3 (Wiche, 1989), equivalent to 1 plectin tetramer per 70 vimentin polypeptide chains. Assuming that 32 vimentin monomers contribute to the cross-section of an IF and the length of vimentin rod domain is ~45 nm (Fuchs and Weber, 1994; Heins and Aebi, 1994), the calculated average density is 10 plectin arms per μm of IF length. Since plectin association with IFs varies with position in the cell, being higher in the periphery than central regions, the observed densities on peripheral IFs of 24 ± 12 arms/μm for REF-52 and 16 ± 10 for MFT-6 cells are consistent with the biochemical data.

Interaction of Plectin with MTs

Our data provide evidence for the existence of cross-bridges between IFs and MTs and demonstrate that these bridges are composed of plectin. Plectin also formed cross-bridges between two MTs or an MT and some other structure indicating that its affinity for MTs was not dependent on first binding to an IF. Most strikingly, plectin formed side projections on MTs in a cell line derived from vimentin negative mice. These results, taken together, establish MTs as independent binding partners of plectin. However, the affinity of plectin for MTs is apparently lower than its affinity for binding to IFs. This conclusion derives from the observation that plectin is normally found associated primarily with IFs, and that vimentin injected into vimentin-negative cells competes plectin away from MTs. Thus, the interaction of IFs with MTs may result from a high affinity interaction of plectin with IFs and a lesser affinity interaction with MTs.

The biochemical basis of plectin binding to MTs is not clear. The simplest possibility is that plectin binds directly to tubulin. Although plectin-tubulin interaction assayed in vitro by a blot overlay procedure was negative (Herrmann and Wiche, 1987), plectin may recognize only polymerized tubulin, or a modified tubulin species. The latter possibility would be consistent with results of Gurland and Gundersen (1995) who provided evidence that stable MTs enriched in Glu-tubulin play a role in the intracellular localization of IFs. These data prompted us to examine whether plectin binds to a specific or a random set of MTs. We failed to find any correlation between plectin association and Glu-tubulin labeling, but the disparity between our results and those of Gurland and Gundersen (1995) could be accounted for if plectin interaction with MTs is regulated and is modulated differently in cells rapidly moving into a wound (Gurland and Gundersen, 1995) and in subconfluent cultures (our experiments).

Plectin binding to MTs may be mediated by MT-associated proteins. In vitro blot overlay assays suggested that plectin binds to MAP1 and MAP2 (Herrmann and Wiche, 1987). Nonneuronal cells do not contain MAP1 or 2, but rather, they have MAP4 (Bulinski and Borisy, 1980; reviewed by Bulinski, 1994) as a major MT-associated protein. MAP4 thus seemed a likely candidate for mediating IF-MT association. However, in our experiments, immunodepletion of MAP4 from MTs in human fibroblasts failed to prevent plectin binding to MTs and to redistribute IFs. This result demonstrates that MAP4 does not serve as an essential link between plectin and tubulin.

Since microinjection of anti-kinesin head antibody induced the collapse of IFs in cultured fibroblasts (Gyoeva and Gelfand, 1991), a member of the kinesin motor family could assist in plectin binding of MTs and IFs. This possibility, though not completely excluded, seems unlikely because plectin attachments were retained after ATP treatment (see Results) which causes kinesin to dissociate from MTs. It seems more reasonable to assume independent but complementary roles for plectin and kinesin in the IF–MT interaction: a kinesin-like protein may provide the driving force for IF extension and plectin may anchor them at the achieved position. However, the MT component responsible for plectin binding remains to be identified.

Interaction of Plectin with the Actin Cytoskeleton

Many plectin projections apparently connect IFs to the actin cytoskeleton. This association is not unexpected because IFs tend to associate with stress-fibers (Goldman et al., 1986) and focal contacts (Bershadsky et al., 1987; Geiger et al., 1987). Plectin is enriched in focal contacts and colocalizes with stress-fibers in cells after long-term cultivation (Seifert et al., 1992). The most recent evidence has been provided by Foisner et al. (1995), who described numerous plectin bridges apparently connecting actin-containing structures in cells lacking IFs. However, plectin association with the actin cytoskeleton, while suggestive, is essentially an unresolved problem for several reasons. First, actin filaments are the most abundant cytoskeletal component in mammalian cells in culture and there is a high probability of adventitious superposition that could be mistaken for a specific interaction. Second, the components of the actin cytoskeleton are multiprotein complexes and the identity of the plectin binding partner(s) remains to be established.

Our results provide structural evidence for plectin mediating the interaction of IFs and the actin cytoskeleton but not necessarily through actin itself. Application of gelsolin to cytoskeletons removes actin (Verkhovsky and Borisy, 1993) and some actin-binding proteins, α-actinin, and vinculin (Verkhovsky et al., 1987). But plectin crossbridges remained associated with the major component of residual stress-fibers, myosin II bipolar minifilaments. Although we cannot exclude a possibility that a few actin molecules remain after gelsolin treatment and mediate plectin-myosin association immunofluorescence data showing nonrandom codistribution of myosin and plectin in cells not treated with gelsolin suggest a specific interaction of the two proteins, directly or through another molecule that survives gelsolin treatment. So far, there are no data that distinguish between these possibilities. Also undetermined at this point is where the putative association with myosin

II fits on the hierarchy of plectin associations. The affinity of plectin for myosin II is probably weaker than for IFs but its strength relative to plectin association with MTs is unknown. Finally, our structural results, although suggesting an association with myosin II filaments, do not preclude an additional association of plectin with actin, for example, via the predicted actin-binding domain in the plectin polypeptide sequence (McLean et al., 1996).

Interaction of Plectin with Putative Membrane Components

Another association partner of plectin seen in our cytoskeleton preparations consists of material with irregular granular and fibrillar substructure. This material, as yet unidentified, may represent protein remnants of membrane structures, such as plasma membrane or membrane organelles. For example, we often observed accumulation of granular material in the area of focal contacts which are known to contain structural and regulatory proteins linked to the substratum through integral membrane proteins (for review see Burridge et al., 1988; Turner and Burridge, 1991; Hitt and Luna, 1994). Experiments to test whether spectrin was a plectin binding partner were inconclusive and evaluation of other membrane markers was beyond the scope of this study.

Role of IFs and Plectin in the Integration of Cytoplasm

A mechanical function has been proposed for IFs (Fuchs, 1994; Klymkowsky, 1995) and an integrating function for plectin (Foisner and Wiche, 1991), but, in the absence of structural information, no clear schema could be constructed for how these functions might be achieved. The in situ organization of plectin is consistent with the idea that IF-plectin complexes comprise an extensive cross-linking of cellular components (Fig. 12) and provide a structural framework for the integration of cytoplasm. In this model, IFs provide the core while plectin forms peripheral linkers that connect to MTs, the actin-based cytoskeleton and membrane structures.

However, plectin and IFs do not seem to be equal partners in performing their function. Though plectin prefers to associate with IFs, it is capable to cross-link cytoskeletal structures independently of IFs. The ability of plectin to act in an IF-independent manner may explain the absence of obvious phenotype in vimentin-null mice (Colucci-Guyon et al., 1994). Plectin may provide sufficient cross-linking of cytoplasm on its own, especially if it is upregulated in vimentin-deficient conditions. In contrast, IFs may be nonfunctional or less functional in cross-linking cytoplasm without plectin. A key role for plectin in maintaining tissue integrity has been demonstrated by analysis of a hereditary disorder, epidermolysis bullosa with muscular dystrophy. Mapping, cloning, and sequencing of the human plectin gene and identification of mutations in the affected individuals establish plectin deficiency as the molecular basis of the disease (McLean et al., 1996; Smith et al., 1996). Skin blistering may be accounted for by lack of plectin to attach keratin IFs to hemidesmosomes. Muscle degeneration may result from deficiencies in desmin IF attachment to either the membrane or the contractile machinery. The role of IFs in cells may be to facilitate plectin cross-linking capability. The limited length of plectin molecules restricts their interactions to a local scale whereas IFs, because of their indefinite length, dramatically extend the potential range of cytoplasmic integration permitting long distance connections.

An unusual feature of plectin-IF complexes is that the stoichiometry of their association depends on location within the cell: more plectin is bound to IFs at the cell periphery than at the center. This relationship remains even after IF redistribution in the course of nocodazole treatment. The mechanism of preferential association of plectin with peripheral IFs is not clear. However, a cellular factor or mechanism favoring association at the periphery would seem reasonable to ensure that the IF network was distributed throughout the cytoplasm.

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