

Monoclonal Antibody to Intermediate Filament Antigen Cross-React with Higher Plant Cells

PETER J. DAWSON,* JOHN S. HULME,† and CLIVE W. LLOYD*

* Department of Cell Biology, John Innes Institute, Norwich, NR4 7UH United Kingdom; and † Biosciences Division, Unilever Research Colworth Laboratory, Bedford, United Kingdom

ABSTRACT The monoclonal antibody (anti-IFA) raised (Pruss et al., 1981, *Cell* 27:419–428) against an intermediate filament antigen, which is widespread throughout phylogeny, has been shown here to cross-react with higher plants. On immunoblotting, anti-IFA cross-reacted with proteins in homogenates of carrot suspension cells and of meristematic cells from onion root tips. A 50-kD cross-reactive protein was enriched in a fraction that consisted of detergent-insoluble bundles of 7-nm fibrils from carrot protoplasts (Powell et al., 1982, *J. Cell Sci.* 56:319–335). By use of indirect immunofluorescence, anti-IFA stained formaldehyde-fixed onion meristematic cells and carrot protoplasts in patterns approximating those obtained with monoclonal anti-tubulins. That anti-IFA was not recognizing plant tubulins was established by use of immunoblots of two-dimensional gels on which the proteins that comprised isolated fibrillar bundles and taxol-purified carrot tubulins had been separated. The two groups of proteins had different positional coordinates: anti-IFA recognized the fibrillar bundle proteins, and anti-tubulins recognized plant microtubule proteins with no cross-reaction to the heterologous proteins. Likewise, formaldehyde-fixed taxol microtubules from carrot cells could be stained with anti-tubulin but not with anti-IFA. It is concluded that an epitope common to intermediate filaments from animals co-distributes with microtubules in higher plant cells.

Cytoskeletons of plant and animal cells contain similar subunits, and this is now established both for microtubules (MTs)¹ and for the actomyosin network. Phallotoxins, for instance, stain bundles of F-actin microfilaments in plant cells (1, 2) as well as in fibroblasts (3); heavy meromyosin binds to microfilaments in representatives of both kingdoms (see reference 4). MTs also possess characteristics that span phylogenetic barriers: plant tubulins co-polymerize with brain MTs (5), and peptide maps of MTs assembled in the presence of taxol from homogenates of plant tissue culture cells show similarities with maps of brain tubulin (6). Indeed, antibodies raised against brain tubulins have been used for staining MT arrays in higher plant cells (7, 8). The third cytoskeletal system, the intermediate filaments, has not, however, been identified in higher plants.

In previous work (9), we described the detergent-insoluble cytoskeleton of carrot protoplasts. This consisted of cortical MTs, but where the nucleus remained, it was retained by paracrystalline bundles of 7-nm fibrils. By optical diffraction, lack of binding of heavy meromyosin, SDS gel electrophoresis,

and drug sensitivity, the fibrils were found to be unrelated to actin, and in the present study, the possibility is tested that they may be related to intermediate filaments. Intermediate filaments constitute a broad family, but the monoclonal antibody raised against an intermediate filament antigen (anti-IFA) characterized by Pruss et al. (10) recognizes a common antigenic determinant in all five filament classes and binds to proteins from organisms as evolutionarily diverse as marine worms and rats. Subsequently, anti-IFA has been further characterized (11), and the cross-reacting epitope is known to be located within the most conserved region of six different intermediate filament proteins. In this paper, we describe the reactions of this antibody with higher plant cells.

MATERIALS AND METHODS

Protein Samples: Carrot suspension cells can be grown in bulk, and their protoplasts were used as a source of detergent-insoluble 7-nm fibrils for biochemical characterization (9); onion root tip cells were used for immunofluorescence since their anti-tubulin staining characteristics are now well established (2, 8).

Apical meristems were cut from roots of 4-d onion seedlings and, as for carrot suspension cells (7), were homogenized in liquid nitrogen before they were boiled in Laemmli (12) SDS sample buffer. Fibrillar bundles were prepared from carrot suspension cells as previously described (9). Because of their relative insolubility, they were prepared for gel electrophoresis by freeze-thawing in

¹ *Abbreviations used in this paper:* anti-IFA, the monoclonal antibody raised against an intermediate filament antigen; MT(s), microtubule(s).

liquid nitrogen in the presence of 9.5 M urea. Sub-confluent BHK₂₁ and 3T3 fibroblasts were scraped, pelleted, and extracted in detergent, and the intermediate filament component of their cytoskeletons was used as a positive control for anti-IFA.

Plant tubulin was prepared from carrot suspension cells by the addition of taxol to a homogenate which had been passed through a DEAE Sephadex column according to modifications (13) of a method (6) for the isolation of taxol-MTs from rose suspension cells. These taxol-MTs from carrot were seen in the electron microscope to have a regular, tubular appearance. By peptide mapping and by Western blotting with monoclonal antibodies to α - or β -tubulins (Amersham International plc, Bucks, UK), they were confirmed to contain only α - and β -subunits (Dawson, P. J., and C. W. Lloyd, unpublished observations).

Immunoblotting: Immunoblotting was performed with minor modifications of the method of Towbin et al. (14). Proteins separated on 10% polyacrylamide gels were transferred to nitrocellulose by use of a Bio-Rad transblot apparatus (Bio-Rad Laboratories, Watford, UK). Transfer was monitored by staining the nitrocellulose with amido black. Blots were incubated for 2 h at room temperature with affinity-purified anti-IFA (kindly provided by Dr. Durward Lawson, Department of Zoology, University College, London, UK), then with ¹²⁵I-labeled anti-mouse IgG F(ab) at 5 μ Ci per blot (Amersham International plc). As a control, the second antibody was added alone. Dried blots were autoradiographed using Kodak X-omat X-ray film. ¹⁴C-labeled standards were supplied by Amersham International plc.

Two-dimensional gels were prepared essentially according to the method of O'Farrell (15) using the modifications of Burland et al. (16). Nitrocellulose blots were prepared as described for one-dimensional gels and were blocked by the method of Hawkes et al. (17). Cross-reaction between transferred proteins and anti-IFA was detected by use of peroxidase-conjugated anti-mouse IgG (Miles UK Ltd.) which, in turn, was visualized with 4-chloro-1-naphthol (Sigma Chemical Co., London, UK) and hydrogen peroxide as substrate.

Indirect Immunofluorescence Microscopy: Onion root tip meristematic cells were fixed in freshly prepared formaldehyde using a PIPES-containing MT-stabilizing buffer (2) as a modification of the method of Wick et al. (8). Affinity-purified mouse anti-IFA was counterstained with fluorescein isothiocyanate-conjugated, affinity-isolated F(ab) sheep anti-mouse IgG (Sigma Chemical Co.). Rat monoclonal antibody YOL 1/34 to yeast tubulin (gift of Dr. John Kilmartin, Laboratory of Molecular Biology, Cambridge, UK) was stained with fluorescein isothiocyanate-conjugated rabbit anti-rat IgG antibodies (Miles). Chromatin was routinely stained by the addition of propidium iodide.

A pellet of carrot taxol-MTs was resuspended in 0.1 M PIPES, 2 mM EGTA, 1 mM MgSO₄, 0.1 mM EDTA, pH 6.9 at 2 mg protein/ml and then placed on polylysine-coated coverslips. These were fixed and stained with anti-IFA or anti-tubulin by use of the routine method for the immunofluorescence of cells (2).

Carrot protoplasts were prepared according to the method of Lloyd et al. (7), settled onto polylysine-coated coverslips, and fixed in culture medium supplemented with 0.25 M sorbitol that contained 4% freshly prepared formaldehyde. After 15 min, these were washed in MT-stabilizing buffer that contained 0.25 M sorbitol and lysed by the addition of 2.5% (vol/vol) Triton X-100. Detergent-extracted protoplasts were then stained with antibodies according to the method of Clayton and Lloyd (2).

RESULTS

Indirect Immunofluorescence

MTs form four different assemblies during the cell cycle: transverse cortical arrays during interphase, the pre-prophase band, the mitotic spindle, and the cytokinetic phragmoplast.

This has now been described for squashes of formaldehyde-fixed onion root tip cells (2, 8) and was used as a basis for comparison of the staining pattern produced with anti-IFA.

During interphase, anti-IFA (but not the second antibody alone) produced a diffuse perinuclear staining not seen with anti-tubulin (Fig. 1*a*). The fixed onion cells are not flat but retain their cuboidal shape, and it is consequently difficult to record the fine fibrogranular staining that occurs throughout the body of the perinuclear region. However, by focusing at the cell cortex, another pattern was discernible: here, anti-IFA stained transverse elements—reminiscent in general terms of the anti-tubulin staining pattern—but in a discontinuous, linear-punctate manner that we have never seen using an anti-tubulin (Fig. 1*b*).

The pre-prophase band could be stained with anti-tubulin as well as with anti-IFA (Fig. 1*c*). With the latter antibody only, other more-or-less linear, coarse elements could be seen which connect the nucleus to the cortex (Fig. 1*d*).

The mitotic apparatus was clearly stained with anti-IFA (Fig. 1*e*) as was the phragmoplast at early (Fig. 1*f*) and late (Fig. 1*g*) stages of its centrifugal development. In Fig. 1*h*, a phragmoplast, still attached to one of the daughter nuclei, has been inadvertently isolated during squashing. Fig. 1*h* also demonstrates that anti-IFA produces a granular staining of this structure.

After cytokinesis, the interphase MT array is restored by MTs radiating between nucleus and cortex. At this stage, anti-IFA does not stain linear elements but does stain condensed patches of material that surround the nucleus. In Fig. 1*i*, these patches of fluorescence define the sister nuclei, but at this stage do not extend out into the cytoplasm.

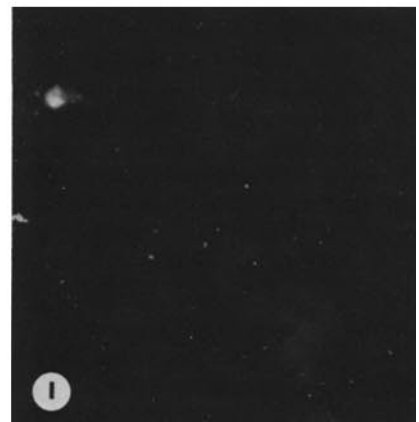
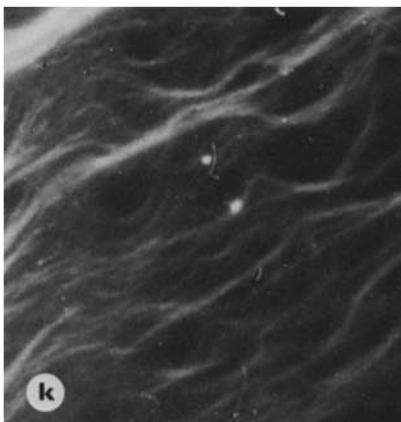
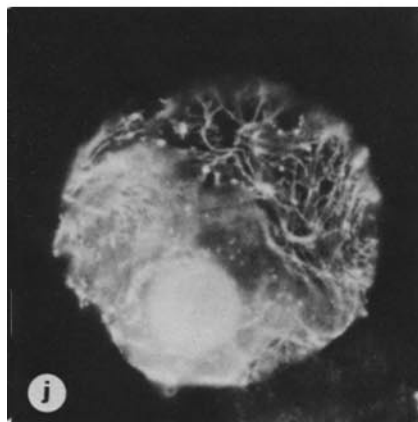
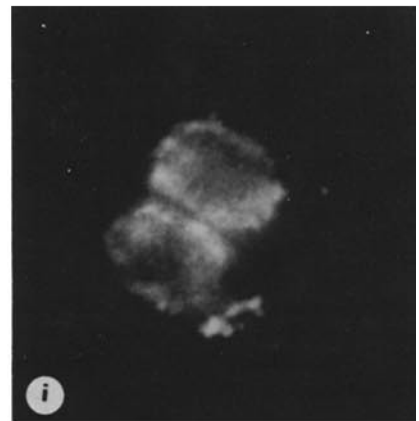
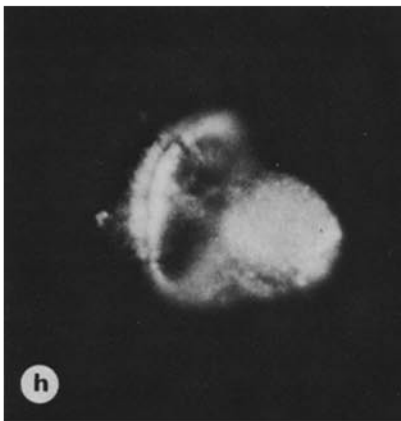
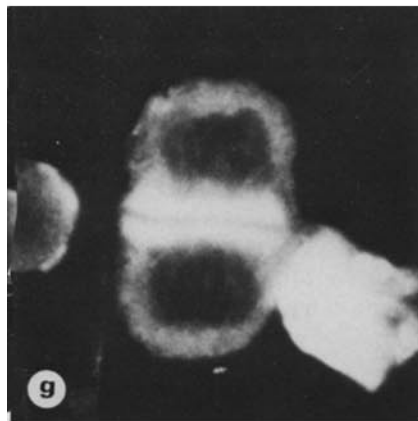
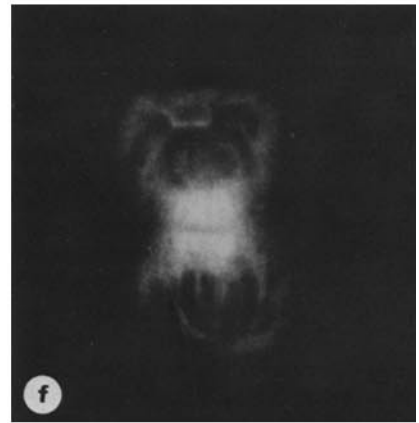
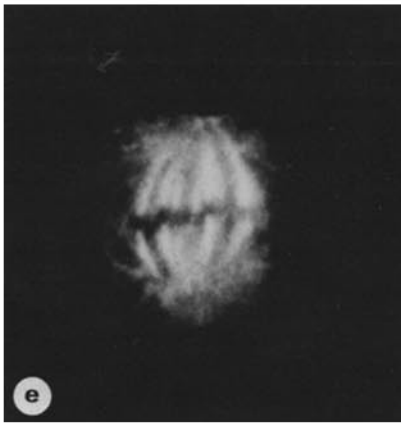
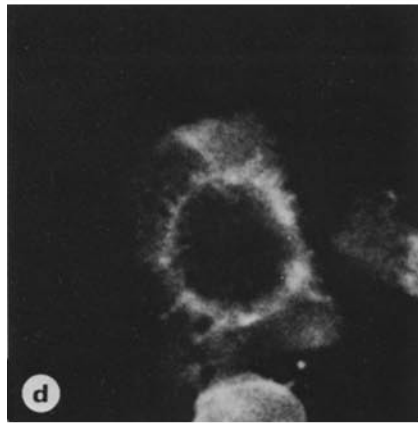
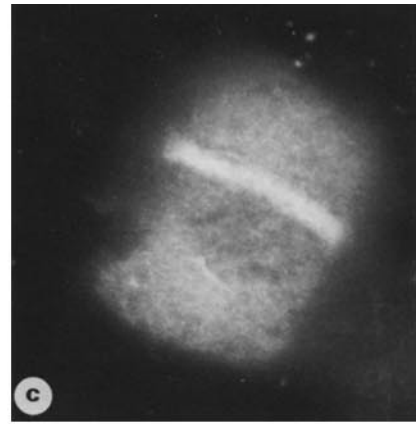
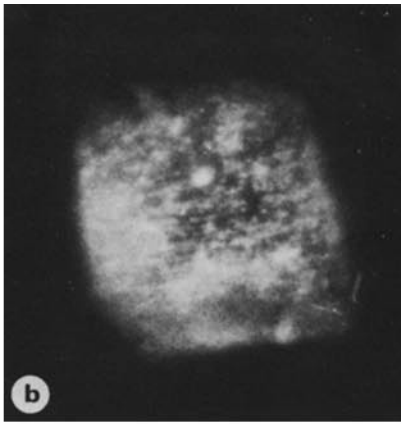
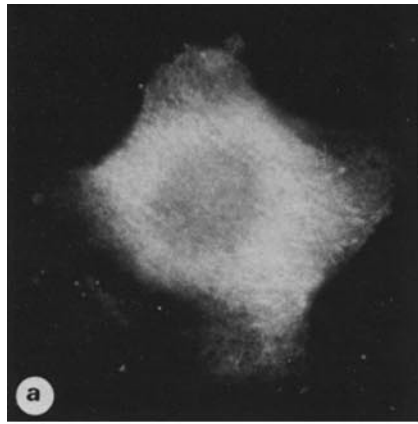
Using protoplasts derived from interphase carrot suspension cells, anti-IFA stains coarse, linear, cortical elements (Fig. 1*j*).

Immunoblotting

Immunoblots of homogenates of onion root tip cells or of carrot suspension cells show that anti-IFA binds to only few proteins, mainly at 68 and 50 kD (Fig. 2, *a* and *b*). This was the case whether bound anti-IFA was detected with ¹²⁵I-labeled mouse antibodies or (in pilot studies) with ¹²⁵I-labeled protein A. The cross-reaction was specific to anti-IFA since no reactivity due to iodinated detecting agents was found when anti-IFA was omitted in controls.

In previous work (9), we identified nucleus-associated bundles of 7 nm fibrils in detergent-extracted cytoskeletons of carrot protoplasts. A fraction can be isolated which, by electron microscopy, contains fibrillar bundles and no MTs. In the present study, it was this fraction which, after solubiliza-

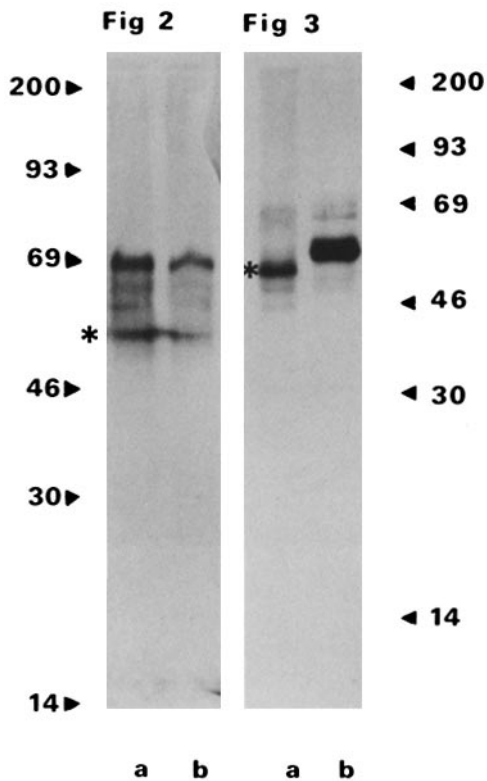
FIGURE 1 (a–i) Fixed onion meristematic cells, all stained with anti-IFA. (a and b) Interphase cells contain cortical, transverse elements that stain in a broken, linear-punctate manner unlike the continuous staining produced with anti-tubulin under these conditions (2). (c) A pre-prophase band. (d) In addition to the pre-prophase band, anti-IFA produces another, but less well defined, staining pattern. The stained structures span the nucleus and cortex. (e) The metaphase spindle. (f) An early phragmoplast. (g) A later phragmoplast stained with anti-IFA. (h) A late phragmoplast accidentally isolated during cell squashing, showing more clearly the stained elements and the association between nucleus and phragmoplast. (i) Two recently divided cells. At this stage, MTs radiate between nucleus and cortex, but anti-IFA stains patches of material around the nucleus, rather than linear structures. (j) A carrot protoplast stained with anti-IFA, focused at the cell cortex. (k) Taxol-MTs prepared from carrot suspension cells fixed in formaldehyde and then stained with anti-tubulin (YOL 1/34). (l) Same as k, but stained with anti-IFA. a, \times 1,600; b, c, d, and e, \times 1,250; f, g, h, and i, \times 1,000; j, \times 800; k and l, \times 590.



tion in 9.5 M urea, could be shown to be enriched in the 50-kD cross-reactive species (Fig. 3a).

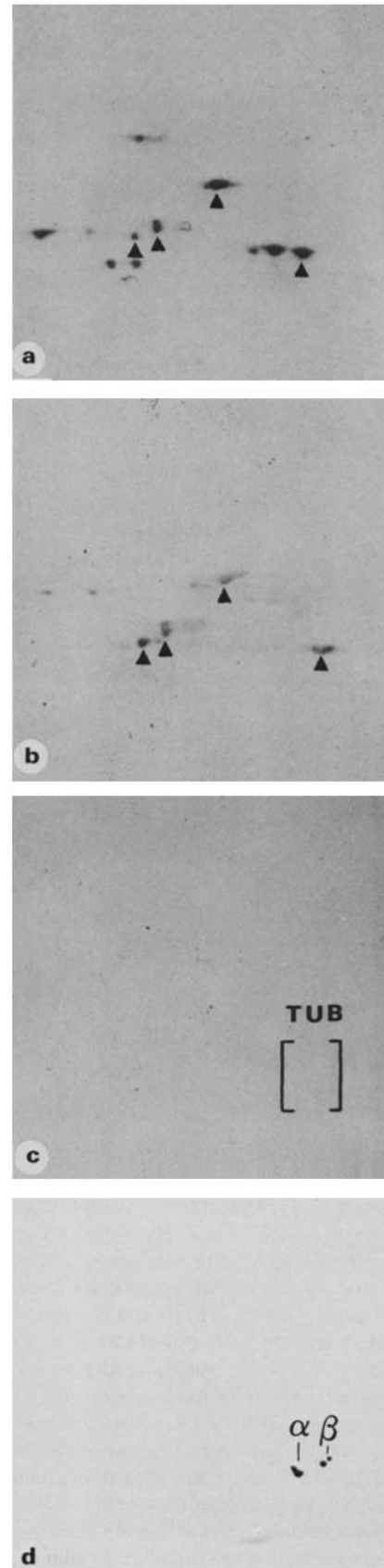
Because anti-IFA stains structures in cells known to contain MTs, it is conceivable that the 50-kD cross-reacting protein is a plant tubulin. To test this possibility, taxol-MTs were prepared from carrot cells (Dawson, P. J., and C. W. Lloyd, manuscript in preparation). These plant MT proteins and the purified fibrillar bundle proteins were then analyzed by two-dimensional gel electrophoresis and immunoblotting (Fig. 4). Anti-IFA cross-reacts (Fig. 4b) with some, not all, of the constituent proteins of fibrillar bundles revealed by amido black staining (Fig. 4a). However, anti-IFA does not cross-

react (therefore not shown) with any of the separated tubulin polypeptides. By contrast, monoclonal anti- α - and anti- β -tubulin cross-react with all of the blotted carrot tubulins (Fig. 4d) present in taxol-MTs, but they do not recognize any of the fibrillar bundle proteins (Fig. 4c). In particular, the 50-



FIGURES 2 and 3 (Fig. 2) Immunoblots of whole-cell homogenates of onion root tips (a) and carrot suspension cells (b) stained with anti-IFA and 125 I-labeled second antibody. (Fig. 3) Immunoblots of detergent-insoluble bundles of 7-nm fibrils purified from carrot suspension cells (a) stained with anti-IFA and 125 I-labeled second antibody. Compared with whole cells (Fig. 2b), the fibrillar bundles show an enrichment of the 50-kD cross-reacting protein (asterisks). Detergent-insoluble cytoskeletons of BHK₂₁ fibroblasts (b) as a control. ◀, molecular weight markers in kilodaltons.

FIGURE 4 Two dimensional gel electrophoresis and immunoblotting of purified fibrillar bundles and taxol-MTs from carrot suspension cells. (a) Purified, detergent-insoluble fibrillar bundles stained with amido black. (b) Immunoblot which shows cross-reaction of anti-IFA with fibrillar bundle proteins. (c) Immunoblot which shows lack of cross-reactivity of monoclonal anti- α (YOL 1/34) and anti- β tubulin antibodies with fibrillar bundle proteins. In particular, those proteins that migrate towards the acidic region of the gel ([]) where carrot tubulins also migrate (compare with d) fail to cross-react with these anti-tubulins. (d) The two monoclonal anti-tubulins employed in c show a positive cross-reaction with purified carrot MT proteins. None of these proteins cross-reacted with anti-IFA (not shown).



kD fibrillar bundle polypeptides that migrate in the vicinity of the carrot tubulins (bracketed in Fig. 4c) do not cross-react with the anti-tubulins.

To check the possibility that anti-IFA might be binding to an epitope in formaldehyde-fixed MTs but which becomes inactivated by methods leading to its presentation upon nitrocellulose, we fixed taxol-MTs from carrot cells by the standard method used for cells. The fixed MTs, attached to coverslips, could be stained with monoclonal anti-tubulin antibodies but not with anti-IFA (Figs. 1, k and l).

DISCUSSION

The monoclonal antibody, anti-IFA, was originally isolated by Pruss et al. (10) from mice immunized with glial fibrillary acidic protein. In addition to this immunogen, the antibody recognizes desmin, vimentin, several cytokeratins, the three vertebrate neurofilament proteins, and neurofilament proteins of the marine worm *Myxicola*. Anti-IFA also cross-reacts with a 66-kD protein found in various preparations of intermediate filaments. These authors concluded that anti-IFA recognizes part of a highly conserved domain common to most, if not all, intermediate filaments, a conclusion borne out by Geisler et al. (11) who have mapped the epitope to within the last 20 amino acid residues of the conserved rod region in six distinct intermediate filament proteins.

In a previous work (9), detergent-insoluble bundles of 7-nm fibrils were shown to be associated with the nucleus in cytoskeletons prepared from carrot protoplasts. By several criteria, they were found to be unlike F-actin microfilaments but in their detergent insolubility, dimensions, and sub-unit molecular weight, these fibrils share some of the characteristics of intermediate filaments. For this reason, we tested the cross-reaction between anti-IFA and the carrot fibrils. The present study now demonstrates that anti-IFA does recognize epitopes present in plant cells, especially a 50-kD protein enriched by purification of the fibrillar bundles.

For immunofluorescence studies, fixed onion root tip cells have certain advantages over carrot protoplasts: their shape is not distorted by the enzyme treatment used for making protoplasts, and, since plant cells are not easily synchronized, meristematic cells are a better source of different stages of the cell cycle (8). Because the immunoblot profiles of carrot and onion cells against anti-IFA are very similar (compare *a* and *b* of Fig. 2), we therefore concentrated upon onion cells for immunofluorescence.

Anti-IFA produces staining patterns which, to varying degrees, co-distribute with the four microtubule assemblies (2, 8) in onion root tip cells. Monoclonal antibodies directed against either α - or β -tubulins stain interphase MTs crisply (2, 8), but with anti-IFA the cortical elements are stained in a linear-punctate and a more inconsistent manner. The pre-prophase band, the metaphase spindle, and the phragmoplast all contain closely grouped MTs and are stained adequately with anti-IFA. In cells with pre-prophase bands, coarse elements which run between nucleus and cortex are sometimes stained with anti-IFA. This has been seen using two different second antibodies which do not stain by themselves. During early interphase, anti-IFA produces an indistinct perinuclear staining of the kind not seen with anti-tubulins. It is our impression that anti-IFA stains a general, fuzzy meshwork that, in places, co-distributes with MTs. The staining patterns produced with anti-IFA are therefore similar in parts but not

identical to those produced with anti-tubulins. But because of this similarity, it is conceivable that anti-IFA is cross-reacting with a plant tubulin which, from immunoblotting, could be migrating on SDS gels with an apparent molecular weight of 50 kD. Pruss et al. (10) reported that anti-IFA does not cross-react with brain tubulin that is cycled three times, and we (unpublished) have confirmed this by using phosphocellulose-purified brain tubulin. However, plant tubulins may not necessarily be identical to those of vertebrate brains and rather than rely on animal proteins as standards, taxol-MTs were prepared from carrot cells (Dawson, P. J., and C. W. Lloyd, manuscript in preparation). All subunits of these MTs, detectable on gels by sensitive silver staining, could also be detected on immunoblots with different monoclonal antibodies specific to α - or β -tubulin. On two-dimensional gels, the plant tubulins had positional coordinates usually distinct from those of the isolated fibrillar bundles and, on immunoblots of these gels, anti-tubulins reacted positively with plant MT proteins but not with the other proteins. Conversely, immunoblotting showed that anti-IFA cross-reacted only with proteins derived from isolated fibrillar bundles and not with the tubulin polypeptides. Polypeptides from detergent-insoluble fibrillar bundles that migrate nearest to the plant tubulins on two dimensional gels would, if tubulins themselves, most likely be β subunits, but negative results of immunoblotting with subunit-specific monoclonal antibodies rule out this possibility.

These data suggest that anti-IFA does not recognize epitopes present on plant MTs, but the possibility remains that such epitopes might have been rendered inactive during immunoblotting. Taxol-MTs from carrot cells form bundles (as has been reported for brain MTs; see reference 18) which, after fixation in formaldehyde, can be stained by indirect immunofluorescence using anti-tubulin antibodies. Anti-IFA does not stain such MTs, yet it stains cells and protoplasts treated with the same fixative and solutions. It is therefore concluded that anti-IFA recognizes a protein or proteins that co-distribute(s) with plant MTs but is not tubulin.

There is gathering evidence that intermediate filaments and MTs may be interlinked, and there are at least three groups of evidence relating to this. First, Geiger and Singer (19), for instance, show extensive but not complete superposition of anti-desmin and anti-tubulin staining filaments in interphase chicken gizzard cells. Other studies (20) illustrate that a similarly extensive co-distribution of MTs and anti-vimentin-staining intermediate filaments exists for a variety of tissue culture fibroblasts in interphase. This association is broken by shifting normal rat kidney fibroblasts, which are infected with a temperature sensitive Rous sarcoma virus, to the permissive temperature whereupon the intermediate filaments alone recoil to the nucleus. These studies imply linkage between the two cytoskeletal systems; this is supported by Bloom and Vallee's (21) finding that the MT-associated protein MAP 2 co-localizes with MTs and vimentin filaments but re-distributes with the vimentin-containing perinuclear cables when MTs are depolymerized with vinblastine.

Second, Pruss et al. (10) found that anti-IFA reacted with a 66-kD protein present in all organisms tested. In preliminary reports (22, 23), a 66-kD protein from spinal cords is said to bind avidly to core filament proteins of at least four classes of intermediate filaments. Antibodies to this protein stained a colchicine-sensitive, intermediate filament-like cytoskeleton in cultured fibroblasts (see also reference 24).

Third, and relevant to the previous point, thermin A is a 68-kD heat shock protein (25) indistinguishable from similar-sized proteins of nervous tissue, and is known to associate with either intermediate filaments (24) or MTs of non-nerve cells (26). There are precedents, therefore, for an association between MTs and either intermediate filaments, intermediate filament-associated proteins, or heat shock proteins. Each of these possibilities could account for our observations that anti-IFA produces staining patterns approximating those of plant MTs.

However, since monoclonal antibodies can cross-react with antigenic sites present on unrelated molecules (e.g., reference 27) we are cautious in drawing the conclusion that intermediate filaments exist in plants. For this reason, we are raising polyclonal antibodies against each of the four major proteins of fibrillar bundles that were recognized by anti-IFA on two-dimensional immunoblots.

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