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Reconstitution of intermediate filaments from a higher plant

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Immunological studies have shown that plants contain intermediate-filament antigens, but it is not known whether these proteins are capable in themselves of forming filaments. To address this problem, a detergent-resistant and high-salt-insoluble fraction from carrot (*Daucus carota* L.) suspension cells was solubilized with 9 M-urea and then subjected to a two-step dialysis procedure, devised for the reconstitution of animal intermediate filaments. This induced the self-assembly of 10 nm filaments and large bundles of filaments. The predominant components of reconstituted material were polypeptides with apparent molecular masses between 58 and 62 kDa. These polypeptides immunoblotted with two monoclonal antibodies known to show broad cross-reactivity with intermediate filaments across the phylogenetic spectrum. This establishes that the antigens are able to self-assemble into intermediate-sized filaments.

INTRODUCTION

Intermediate filaments (IFs) of 7–12 nm width are a major cytoskeletal component in animal cells (Steinert & Parry, 1985; Geisler & Weber, 1986), but there is only immunological evidence to support their existence in plants (Dawson *et al.*, 1985; Parke *et al.*, 1987; Hargreaves *et al.*, 1989). Anti-IFA, a monoclonal antibody that recognizes an epitope in the conserved α-helical rod domain present in all known IFs (Pruss *et al.*, 1981; Geisler *et al.*, 1983) has been shown by Dawson *et al.* (1985) to immunoblot polypeptides in preparations of carrot (*Daucus carota* L.) fibrillar bundles (FBs). These FBs are composed of fibrils of 7–10 nm width in lateral register (Powell *et al.*, 1982). Since then we have raised a monoclonal antibody against fibrillar bundles (AFB). This antibody stains and immunoblots the type III intermediate filaments of animal cells (Hargreaves *et al.*, 1989), underlining immunological cross-reactivity between the plant and animal kingdoms. FBs therefore represent a convenient source of IF antigens in plants, but it is not known whether these antigens occur in filaments as such. The antigens need not necessarily occur in the filament-forming fraction; they might simply be adventitiously associated with the FBs or cortical microtubules in a non-filamentous form.

A diagnostic feature of IFs is their ability to re-form *in vitro* after solubilization with urea (Franke *et al.*, 1979, 1981; Geisler & Weber, 1981; Steinert, 1981; Ip *et al.*, 1985). Therefore, in the present work, we have studied the de- and re-polymerization of the FBs: (1) to see whether filaments can be reconstituted under conditions used for animal IFs; and (2) to establish that these filaments are composed of the plant IF antigens. It was found that 10 nm filaments and bundles of such filaments could be reconstituted under conditions similar to those used for vimentin (Ip *et al.*, 1985). Reconstituted material was enriched in polypeptides that contained the epitopes recognized by both anti-IFA and AFB, suggesting that FBs are a form of IFs occurring in plants.

MATERIALS AND METHODS

Materials

A non-embryogenic carrot (*Daucus carota* L.) suspension culture (Powell *et al.*, 1982) was used in the work presented here. However, an embryogenic carrot cell line, kindly supplied by Dr. C. Hawes (University of Oxford) gave identical results. Cellulase R-10 and Driselase were purchased from Yakult Honsha Co. (Tokyo, Japan) and Sigma Chemical Co. (Poole, Dorset, U.K.) respectively. Proteinase inhibitors and DNAase I were obtained from Sigma. The monoclonal antibody AFB was produced and purified as described by Hargreaves *et al.* (1989). The cell line TIB 131, producing the universal anti-(intermediate filament) antigen (anti-IFA), was obtained from the American Tissue Culture Collection (Rockville, MD, U.S.A.) and grown in Dulbecco's modified Eagle's medium (Gibco, Paisley, Renfrewshire, Scotland, U.K.). Horseradish-peroxidase-coupled rabbit anti-mouse IgG and goat anti-rat IgM were from Dako (Slough, Berks., U.K.) and International Laboratory Services (London, E.C.1, U.K.) respectively. SDS was supplied by FSA Laboratory Supplies (Loughborough, Leicestershire, U.K.), and all other reagents were of the highest purity available.

Preparation and reconstitution of carrot fibrillar-bundle proteins

Carrot suspension cells were grown in Murashige & Skoog's supplemented medium as described by Powell *et al.* (1982). Cells from 10-day-old cultures were converted into protoplasts by incubation for 2–3 h at 25 °C in growth medium containing 0.4 M-mannitol, 2% (w/v) R-10 cellulase and 2% (w/v) Driselase. The enzyme mixture was incubated at 55 °C for 5 min (to inactivate proteinases) and clarified by centrifugation before use. Protoplasts were collected by centrifugation as for cells and washed twice by centrifugation in fresh medium.

Lysis was performed in fresh growth medium (one-

Abbreviations used: FB, fibrillar bundle; AFB, anti-FB monoclonal antibody; anti-IFA, anti-(intermediate filament) antigen; TBS, Tris-buffered saline (140 mM-NaCl/10 mM-Tris, pH 7.4); PMSF, phenylmethanesulphonyl fluoride; PVP, polyvinyl-pyrrolidone; BSA, bovine serum albumin.

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fifth of the original cell culture volume) containing 2% (v/v) Triton X-100, 0.4 M-mannitol, 5% (w/v) solid PVP and a proteinase-inhibitor cocktail [0.5 mM-PMSF, leupeptin (10 μg/ml), benzamidine hydrochloride (10 μg/ml), aprotinin, (10 μg/ml), pepstatin A (10 μg/ml) and phenanthroline (1 μg/ml)], for 45 min at 0–4 °C on a shaking platform. The resultant lysate was filtered through four layers of muslin and then centrifuged at 300 g for 7 min at 4 °C in a bench centrifuge. The supernatant was layered on to a 1 M-sucrose cushion and centrifuged at 1000 g for 20 min at 4 °C. The upper phase was carefully removed and centrifuged at 35000 $g_{av.}$ for 30 min at 4 °C, and the resultant pellet (crude FBs) rinsed with FB wash buffer (10 mM-Tris, pH 8.0, with 5 mM-$MgCl_2$, 150 mM-NaCl and 1% (v/v) Triton X-100). It was then resuspended to a final protein concentration of 1–2 mg/ml in buffer containing DNAase I (100 μg/ml) and the proteinase-inhibitor cocktail. After 45 min incubation on ice, solid KCl was added to a final concentration of 1.5 M and the mixture incubated for a further 45 min at 0–4 °C. It was then centrifuged at 35000 $g_{av.}$ for 30 min at 4 °C. This pellet was then resuspended in FB wash buffer and the DNAase and salt treatments repeated. After a further centrifugation at 35000 $g_{av.}$, the final pellet (washed FBs) was the starting material for reconstitution experiments.

Washed FBs were resuspended in depolymerization buffer [10 mM-Tris (pH 8.0)/50 mM-β-mercaptoethanol and 9 M-urea] to a final protein concentration of 0.1–0.5 mg/ml and incubated overnight at 25 °C. Non-solubilized material was removed by centrifugation at 100000 $g_{av.}$ for 2 h at 20 °C. The proteinase-inhibitor cocktail was added to the solubilized proteins, which were then dialysed for 4–6 h against 100 vol. of dialysis buffer [10 mM-Tris, pH 8.0, containing 10 mM-β-mercaptoethanol, the proteinase-inhibitor cocktail and 0.01% (w/v) NaN_3]. This was followed by overnight dialysis against fresh dialysis buffer containing 150 mM-NaCl and two further changes at 12–16 h intervals. Samples were prepared for electron microscopy as described below, and repolymerized filaments were pelleted by centrifugation at 100000 $g_{av.}$ for 2 h at 4 °C.

Electron microscopy of reconstituted material

For analysis by electron microscopy, a drop of reconstituted material was adsorbed on to a carbon- and Formvar-coated copper grid for 2–3 min at room temperature. Adsorbed material was briefly rinsed with distilled water and then stained with aq. 2% (w/v) uranyl acetate for 5 s. Excess stain was removed by blotting the junction between grid and forceps with uncut filter paper. The specimens were viewed in a JEOL 1200 electron microscope.

Revealing carrot fibrillar bundles *in situ* by indirect immunofluorescence

Cultures (10 days old) of carrot cells were fixed on multiwell slides and permeabilized as described previously (Hargreaves *et al.*, 1989). Permeabilized cells were then incubated with affinity-purified AFB for 2 h at room temperature and then washed extensively with TBS. Fluorescein-conjugated goat anti-rat IgM (diluted 1:100 in TBS) was then applied to the wells and incubated for 1 h at room temperature. Excess secondary antibody was removed by several washes with TBS, and the samples were mounted in anti-fade mountant and viewed in a Zeiss epifluorescence microscope.

Gel electrophoresis and immunotransblotting of plant intermediate-filament antigens

Crude FBs and reconstituted material pelleted by centrifugation as described above were solubilized by boiling for 5 min in electrophoresis sample buffer, which contained 62.5 mM-Tris, pH 6.8, 2% (w/v) SDS, 10% (v/v) glycerol, 5% (v/v) β-mercaptoethanol and 0.001% (w/v) Bromophenol Blue. Electrophoresis was then performed in the presence of SDS by using a 7.5%-(w/v)-polyacrylamide running gel overlaid with a 4% stacking gel (Laemmli, 1970). Where appropriate, proteins were stained with Coomassie Blue.

Alternatively, separated proteins were transferred electrophoretically on to nitrocellulose membrane filters (Towbin *et al.*, 1979). The filters were then blocked with 3% BSA (w/v) in TBS for 2 h at room temperature, followed by a similar incubation with TBS containing 0.05% (v/v) Tween-20. They were then probed with either affinity-purified AFB or anti-IFA culture supernatant (undiluted) overnight at 4 °C. Excess primary antibody was removed by several washes with TBS/Tween-20. This was followed by incubation with horseradish-peroxidase-conjugated secondary antibodies and chloronaphthol reagent as described previously (Dawson *et al.*, 1985; Hargreaves *et al.*, 1989).

RESULTS

Analysis of reconstituted filaments by electron microscopy

In the present study we have devised an improved method for FB purification based on the procedure of Powell *et al.* (1982), but using Triton X-100 (instead of Nonidet P40), PVP (to remove phenolic compounds) and a proteinase-inhibitor cocktail during protoplast lysis. The detergent-resistant cytoskeleton was then washed with DNAase and high salt as used for animal IFs (Franke *et al.*, 1979, 1981; Geisler & Weber, 1981; Steinert, 1981; Walter & Biessmann 1984; Ip *et al.*, 1985). The resulting preparations were enriched in FBs, composed of filaments of about 7–10 nm width.

Although IFs are characterized by their unusually high resistance to treatments with detergent and high salt, their denatured polypeptides also have the unique ability to reconstitute filaments *in vitro* which are indistinguishable from native IFs (Franke *et al.*, 1979, 1981; Geisler & Weber, 1981; Steinert, 1981; Ip *et al.*, 1985). Therefore a similar approach was applied to purified FBs to examine their relationship to IFs in more detail. In this case we chose reassembly conditions similar to those described for vimentin (Steinert, 1981; Ip *et al.*, 1985), since AFB (raised against FBs) cross-reacts with vimentin (as well as desmin and glial fibrillary acidic protein type III IFs). Best results were obtained after depolymerization of FBs in a buffer containing 9 M-urea. After a high-speed centrifugation to remove any particulate material, the urea-solubilized fraction was reconstituted by two-step dialysis, first in the absence of salt and then in the presence of 150 mM-NaCl. Individual 10 nm filaments, as well as bundles of such filaments, were found after extensive dialysis of the urea-solubilized FBs (Figs. 1*a* and 1*b*). Reconstituted filaments had a tendency to associate laterally and to form bundles that

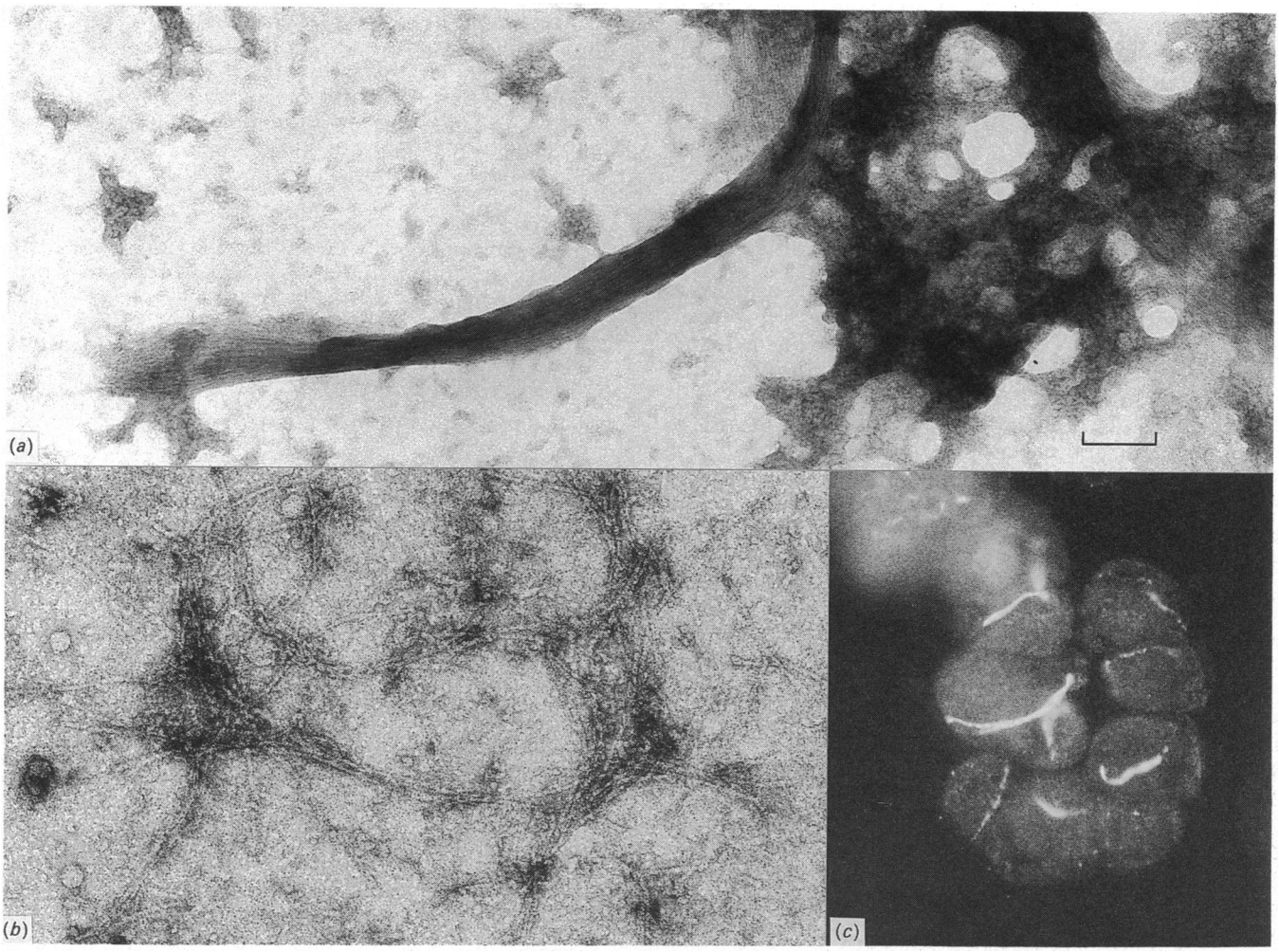

Fig. 1. Ultrastructural characterization of reconstituted 10 nm filaments and filament bundles

Shown are electron micrographs of a reconstituted filament bundle (*a*) and reconstituted 10 nm filaments (*b*), together with indirect immunofluorescence of carrot cells showing native fibrillar bundles stained with AFB followed by a fluorescein-conjugated goat anti-rat IgM (*c*). The bar (shown only in *a*) represents 100 nm in (*a*) and (*b*) and 10 μm in (*c*).

often reached lengths of several micrometres, similar to those stained in intact cells (Fig. 1*c*). Particularly during the early stages of dialysis, filaments and filament bundles were both associated with short rods arranged in stacks or aggregates. Such rod-like structures have been described in other studies involving the reconstitution of animal IFs (Steinert, 1981; Ip *et al.*, 1985; Steinert & Parry, 1985) and have been taken to represent intermediates in IF assembly. Zackroff & Goldman (1979, 1980) indicated that similar aggregates or 'dense bodies' may act as nucleation sites for IF assembly.

Gel electrophoresis and immunblotting of crude and reconstituted fibrillar-bundle proteins

To establish that the reconstituted filamentous material contained the IF antigens, it was analysed by gel electrophoresis and immunoblotting. After reconstitution there was, compared with the crude FB fraction, an enrichment of polypeptides in the 58–62 kDa region of Coomassie Blue-stained gels (Figs. 2*a* and 2*b*). This cluster of bands has been previously shown to be a major feature of crude FBs (Hargreaves *et al.*, 1989) and is the major component of reconstituted material. When

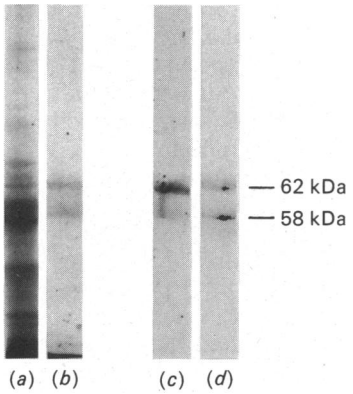

Fig. 2. Comparison of crude FBs with reconstituted proteins by gel electrophoresis and Western blotting

Coomassie Blue-stained gels of approx. 40 μg of crude FB polypeptides (*a*) and 10 μg of reconstituted material (*b*), together with the Western blots of reconstituted proteins with AFB (*c*) and anti-IFA (*d*). These demonstrate that major polypeptides enriched after reconstitution, and belonging to the filament-forming fraction, contain two epitopes in common with animal IFs.

subjected to immunoblotting, the 58–62 kDa bands of the reconstituted fraction reacted positively with both AFB and anti-IFA, the upper band giving the stronger reaction with both antibodies (Figs. 2*c* and 2*d*). This clearly demonstrates that the IF-related epitopes occur in polypeptides which are the predominant components of the filament-forming fraction.

DISCUSSION

IFs are characterized as such by their size, resistance to high salt and detergent, relatedness to other members of the IF family (in terms of primary sequence and antigenicity) and their ability to reconstitute after solubilization in urea.

Carrot FBs were already known to be composed of filaments of the appropriate size (Powell *et al.*, 1982) and to contain polypeptides that were antigenically related to animal IFs (Dawson *et al.*, 1985; Hargreaves *et al.*, 1989). In the present study we demonstrate the ability of the major FB polypeptides to re-form filaments after solubilization with urea. Although this, in itself, is a diagnostic feature of IFs, it also serves to establish that the IF-related epitopes in carrot occur in polypeptides capable of forming 10 nm filaments. Not all IF antigens occur in filamentous form (Franke *et al.*, 1982; Walter & Biessmann, 1987), and it is clear from our other studies (Goodbody *et al.*, 1989) that, in addition to the FBs, IF antigens can also exist in an apparently non-filamentous form that co-distributes with plant microtubules. The reconstitution data demonstrate that the major FB polypeptides belong to the filament-forming class. Furthermore, both the 58 and 62 kDa bands immunoblot with AFB and anti-IFA, the latter reaction confirming the presence of the α-helix-rich sequence necessary for filament formation (Geisler *et al.*, 1983). A 68 kDa FB polypeptide, previously shown to cross-react with anti-IFA (Dawson *et al.*, 1985), was absent from the reconstituted fraction, suggesting that not all IF antigens are necessarily selected by our procedure.

The presence of two major bands after reconstitution indicates that plant IFs may form obligate heteropolymers, as is known for the cytokeratins (Franke *et al.*, 1979; Hatzfeld & Franke, 1985). On the other hand, the lower-molecular-mass polypeptide could represent a proteolytic cleavage product that retains its ability to copolymerize with intact IFs, as described for a degradation product of desmin (Kaufmann *et al.*, 1985). If this were the case, the larger polypeptide should be capable of forming homopolymers, as is known for vimentin (Steinert, 1981; Ip *et al.*, 1985). Further work is needed to resolve these questions.

The tendency of plant IFs to form large paracrystals both *in vivo* and, now, when reconstituted *in vitro*, is intriguing. The fact that the reconstituted fraction is composed almost entirely of polypeptides cross-reacting with AFB and anti-IFA encourages the idea that the ability to form bundles resides within these IF antigens themselves. It seems unlikely that bundling could be due to an IF-associated ('bundling') protein, since such a protein is apparently not detected under the present electrophoresis conditions. However, this possibility should not be ruled out.

We conclude that carrot IF antigens can be reconstituted into 10 nm filaments and filament bundles *in vitro* and that the cytoplasmic FBs are a form of intermediate filament occurring in plants.

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