Epinemin: A New Protein Associated with Vimentin Filaments in Non-neural Cells

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ABSTRACT In this report I describe a new protein, defined by a monoclonal antibody, which is associated with vimentin filaments in a variety of cultured cells and in skeletal muscle. By immunofluorescence it is absent in smooth muscle, in cells without vimentin, and in neural vimentin containing cells. This protein has a molecular weight of 44,500, a pI of 5, a two-dimensional tryptic peptide fingerprint pattern different from vimentin, is unrelated to actin by Cleveland peptide analysis and by light and electron microscopy, and is not recognized by either a polyclonal antivimentin antibody (Frank, E. D., and L. Warren, 1981, *Proc. Natl. Acad. Sci. USA*, 78:3020–3024) or a monoclonal antibody against all classes of intermediate filaments (Pruss, R. M., R. Mirsky, M. C. Raff, R. Thorpe, A. J. Dowding, and B. H. Anderton, 1981, *Cell*, 27:419–428).

The protein is resistant to nonionic detergent extraction, is soluble in high salt and can thus be removed from vimentin filaments, but fragments with vimentin in either low salt or anionic detergent and collapses with vimentin in colchicine-treated cells.

By light microscopy, the distribution of the protein is indistinguishable from vimentin filaments and appears uniform along them. In contrast, immunoferritin electron microscopy reveals that the molecule is distributed in an intermittent pattern on vimentin filaments. Adopting the terminology of Granger and Lazarides (1980, *Cell*, 30:263–275), the molecule is called epinemin, meaning "upon filaments."

Most animal cells contain three major fibrous systems that can be distinguished biochemically (10, 12, 28, 29, 38, 40) and by light and electron microscopy (2, 6, 11, 19, 39, 41, 42, 45, 46, 50). These filament systems are actin-like microfilaments of 6-nm diameter, microtubules of 25-nm diameter, and intermediate filaments of 10-nm diameter.

Intermediate filaments can be divided into five major classes by means of their cell type specificity and subunit structure (28, 29). These classes are the following: (*a*) keratin (with molecular weights from 40,000–65,000), (*b*) desmin filaments (with molecular weights ranging from 50,000 to 54,000), (*c*) vimentin filaments (with molecular weights of 52,000–58,000), (*d*) neurofilaments (with molecular weights of 68,000–200,000) found only in neurones, and (*e*) glial filaments (with a molecular weight of 50,000) found in astrocytes and some other cells of glial origin.

These various filamentous systems are most easily seen by using specific antibodies coupled to fluorochromes, a technique which reveals that they form complex and interconnecting networks (16, 23, 24, 30). In the case of vimentin, these networks are particularly easily studied since most cell types grown in tissue culture contain this class of intermediate filament (28, 29)—and some contain only vimentin (49). Treatment of a variety of cultured cells with nonionic detergent leaves an insoluble cytoskeleton consisting of actin filaments, cell nuclei, microtubules (under certain conditions), and intermediate filaments of the vimentin class (19, 28, 29). Extraction with potassium iodide or chloride removes actin but leaves vimentin filaments (9, 19), while the reverse holds true with the ionic detergent sarkosyl (3) or the use of low ionic strength buffers (3). Further manipulation of vimentin filaments can be performed by exposing tissue culture cells to colcemid, which causes the aggregation of their vimentin filaments (and associated proteins) into nuclear associated bundles (21) but does not affect actin-like microfilaments or keratin filaments (28, 29). Intermediate filaments can also be collapsed by microinjecting antibodies to vimentin or associated polypeptides (23, 31). Biochemically, vimentin has been peptide mapped (9) and is easily identified on two-dimensional gels as are its family of proteolytically resistant breakdown products that are more acidic and have a molecular weight of 10,000–15,000 less than vimentin itself (9, 13, 34).

A cytoskeletal role for vimentin filaments has not yet been clearly defined but it has been suggested that they may provide

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mechanical support for the nucleus (29). They do not seem to be involved in a variety of cell functions (23, 31).

Recently, several proteins have been found in association with vimentin filaments: these are plectin, molecular weight 300,000 (51), synemin (14–16) molecular weight 230,000, paranemin (29) molecular weight 280,000, two molecules of molecular weights 95,000 and 210,000 (30), and a messenger RNA cap binding protein of 50,000 (56).

Polyclonal anti-sera against purified cytoskeletal components have been extensively used as probes for the structure and function of these and other defined cytoskeletal components. However, it seems likely that many of the accessory molecules involved in cytoskeletal interactions may be present in small amounts (as is the case for synemin and paranemin), and may be nonimmunogenic, and/or lost during purification protocols. I have therefore used the approach of raising monoclonal antibodies, in this case against partially purified smooth muscle preparations, to describe similar molecules associated with the cytoskeleton. This broad spectrum immunogen was chosen in the hope of obtaining antibodies to as yet unknown actin associated proteins (7). This, however, did not happen since the monoclonal antibody obtained recognizes intermediate filaments.

In this paper I describe a new vimentin-associated protein, epinemin, identified by a monoclonal antibody, which has a distribution indistinguishable from vimentin at the light microscope level, but is seen to be intermittent along vimentin filaments by electron microscopy. By biochemical immunofluorescence and ultrastructural criteria the molecule is unrelated to vimentin or actin.

MATERIALS AND METHODS

Immunogen

This was prepared according to the method of Feramisco and Burridge (7). Fresh chicken gizzard was homogenized in a Waring blender with 3 × 10-s bursts at top speed in 10 vol of deionized water (4°C) plus 0.5 mM phenylmethylsulfonyl fluoride. The suspension was centrifuged at 5,000 *g* for 10 min, the supernatant discarded, the pellet resuspended as above and blended for 10 s at low setting. The centrifugation was repeated and the pellet resuspended at 37°C in 10 vol of 2 mM Tris, 1 mM EGTA, 0.5 mM phenylmethylsulfonyl fluoride (pH 9 at room temperature), and stirred for 30 min at 37°C. The centrifugation was repeated and the supernatant adjusted to pH 7–7.2 with 0.5 M acetic acid. Molar $MgCl_2$ was added till the suspension contained 10 mM $MgCl_2$. After stirring for 15 min at room temperature the suspension was centrifuged as above and the supernatant used as the immunogen.

Immunization

BALB/c mice were immunized, as follows, with 100 μg of the immunogen per injection: day 1: subcutaneous in complete Freund's adjuvant; days 14, 63, and 77: intraperitoneal in incomplete Freund's adjuvant; day 81: tail bleed and test for antibody response; days 84, 85, and 86: intravenous injection; day 87: fusion.

Fusion and Hybridoma Production

This was performed essentially as described by Pruss et al. (37), except that the myeloma cell line used was the nonsecretor SP2 (25) and the culture medium was Dulbecco's modified Eagle's medium (DME).[1]

Screening and Cloning Hybridomas

When the hybridoma wells were two thirds confluent they were tested by immunofluorescence on formaldehyde fixed methanol permeabilized 3T3 cells (see below). Antibody producing wells were cloned by limiting dilution into 96 well Linbro round bottom microtitre plates with a feeder layer of adherent BALB/c macrophages at a concentration of 5×10^3 cells/ml. Cloning was carried out twice and cells were frozen in 5% DMSO, 95% fetal calf serum at −27°C for 10 min, and then stored in liquid nitrogen. Ascites was raised by injecting nu/nu mice with 1×10^7 cells and harvesting the ascitic fluid 10 d later.

Purification of Monoclonal Antibody

As a partial purification (25), supernatant (but not ascites) was precipitated with 50% saturated ammonium sulphate in phosphate-buffered saline (PBS) plus 1% glycerol overnight at 4°C, spun at 2,000 *g* for 15 min at 4°C, resuspended in PBS (see below) plus 0.5% glycerol, and dialyzed into the same buffer. The class of antibody was IgM (see below) and therefore further purification was either on a lentil lectin column (Pharmacia Fine Chemicals, Piscataway, NJ) and elution with 0.1 M α-methyl glucopyranoside (25) or, in the case of ascites, diethylaminoethyl cellulose (DEAE) fractionation followed by sucrose gradient centrifugation (56).

Cell Lines and Primary Cultures

3T3, African green monkey kidney cells (BSC-1), and PtK1 cells were adjusted to 15×10^3 cells/ml in DME plus 10% heat inactivated fetal calf serum (FCS) plated in 0.5-ml aliquots on to 13-mm diameter glass coverslips in 24 well Linbro plates and used for immunofluorescence screening of hybridomas (and other experiments) the following day.

Immunofluorescence

HYBRIDOMA SCREENING: 3T3 cells were rinsed in PBS (NaCl 137 mM, KCl 2.6 mM, Na_2HPO_4 8 mM, KH_2PO_4 1.5 mM, pH 7.2), fixed in 2% stabilized formaldehyde (British Drug House Dagenham, Essex, United Kingdom) for 10 min at room temperature, rinsed twice in PBS, permeabilized in methanol at −20°C for 10 min, rinsed six times in PBS and once in PBS plus 1% bovine serum albumin (BSA) (Sigma fraction V), and then labeled with hybridoma supernatant for 30 min at room temperature. The coverslips were rinsed as above and incubated in goat anti-mouse IgG coupled to tetramethyl rhodamine (diluted 1:50 in PBS + 1% BSA) for 30 min at room temperature. This conjugate was an IgG fraction which had been passed over DEAE after rhodamine coupling to remove overcoupled IgG and was a kind gift from Stefanello de Petris (Zoology Department, University College, London). Following this incubation, the coverslips were mounted in Gelvatol (polyvinyl alcohol; Monsanto Polymers and Petrochemicals, St. Louis, MO) in PBS plus 16 mM NaN_3. All immunofluorescence photographs were taken on a Zeiss microscope using a 63× lens and equipped for phase-contrast, epifluorescence and selective filters (including interference filter BP470) for fluorescein and rhodamine. No staining was seen when hybridoma supernatant was omitted or when supernatant from non secreting clones was used. The class of antiepinemin was found to be IgM by Ouchterlony immunodiffusion (20) using rabbit anti-mouse class specific antisera (Miles Laboratories, Inc., Elkhart, IN) to IgA, IgG1, IgG2a, IgG2b, IgG3, and IgM.

PREPARATION OF CYTOSKELETONS: For all other immunofluorescence, cells were extracted in 0.15% Triton X-100 in 0.1 M PIPES plus 1.5 mM $MgCl_2$, 1 mM EGTA, 5 mM NaN_3, pH 6.9 for 1 to 1.5 min at 37°C, fixed in 1% stabilized formaldehyde (British Drug House) in PIPES for 5 min at room temperature, rinsed twice in PIPES, placed in −20°C methanol for 10 min, and rinsed in PBS as in the section immediately above, then labeled.

SALT AND SARKOSYL EXTRACTIONS: Following Triton X-100 extraction as above, actin and proteins of similar solubility were removed by treating cells with either (*a*) 0.3 M KI in PIPES for 45 min to 3 h at 4°C (19), (*b*) a cycle of low, high, low ionic strength buffers as described by Schliwa and van Blerkom (41), or (*c*) extracting cells in 0.5% Triton X-100 in PIPES plus 0.6 M KCl (14) for 1 min at 37°C. During *b* and treatments c, microtubules were, when necessary, stabilized by adding 1–2 μg/ml of taxol (38) (supplied by Natural Products Branch, Division of Cancer Treatment, National Institutes of Health) to the Triton X-100. For experiments comparing the distribution of microtubules and epinemin, 4 M glycerol was added to PIPES. To remove intermediate filaments, we added sarkosyl (3) in PIPES to Triton X-100 extracted cytoskeletons for 2.5 min at room temperature. Here, too, taxol was added to the Triton X-100 and in addition actin filaments were stabilized with Phalloidin (52), 10 μg/ml, for 3 min prior to the addition of sarkosyl (41).

CYTOCHALASIN AND COLCHICINE: Cells were incubated either in 3.3 μg/ml of cytochalasin D (Sigma Chemical Co., St. Louis, MO) for 7–15 min or 1 μM demicolchicine (Sigma Chemical Co.) for 30 min to 1.5 h. Both reagents were diluted in DME and used at 37°C before processing as in Preparation of Cytoskeletons.

[1] *Abbreviations used in this paper:* BSA, bovine serum albumin; DME, Dulbecco's modified Eagle's medium; FT, ferritin; Hb, hemoglobin; PBS, phosphate-buffered saline.

FROZEN SECTIONS: Rat proximal colon was processed and incubated with antisera as described (54). Antibody concentrations were as used in the next section below. Sections were cut by Rhona Mirsky (Anatomy Department, University College, London), to whom I am very grateful.

IMMUNOFLUORESCENCE REAGENTS: Following hybridoma screening, these studies were carried out on cells prepared as in the above section, Preparation of Cytoskeletons, using CAPPEL reagents (Dynatech, Billingshurst, Sussex, UK), a class specific goat anti-mouse IgM coupled to rhodamine (G anti-MIg-IgM-RHO) and a goat anti-rabbit IgG fluorescein (G anti-RIg-FL). These conjugates were absorbed on columns of Affigel 10 (Bio-Rad Laboratories, Richmond, CA) coupled to either normal mouse or rabbit IgG. A total of 1 mg of rabbit IgG was used to remove anti-rabbit activity from the G anti-MIg-IgM-RHO and 0.5 mg of mouse IgG in the case of G anti-RIg-FL. After these absorptions the anti-rabbit Ig did not recognize mouse Ig and vice versa (not shown). Conjugates were used at dilutions of 1:50 in PBS/1% BSA. For immunofluorescence, antiepinemin supernatant purified on a lentil lectin column was used at a protein concentration of 50–200 μg/ml.

Polyclonal Antisera

Rabbit antiactin was raised and affinity purified as described (22) using pure skeletal muscle actin (Sigma Chemical Co.) as immunogen. The actin used for affinity column purification was a kind gift from Karen Price. For immunofluorescence, affinity purified antiactin was used at 10 μg/ml. This antisera is specific for actin (see Results). Rabbit antivimentin antisera were a kind gift from Evan Frank (8) and Richard Hynes (21), and rabbit antitubulin was a kind gift from Peter Sheterline (44). All have been characterized (8, 21, 44) and were used for immunofluorescence at sera dilutions of 1:50 to 1:100.

Biochemistry

PREPARATION OF CELLS: Nearly confluent 3T3 cells were trypsinized off Falcon flasks, the trypsin blocked by fresh DME plus 10% FCS and the cells pelleted, washed three times in PBS (20 ml each wash), resuspended in 1 ml of PBS, and pelleted in a Beckman microfuge (Beckman Instruments, Palo Alto, CA) at 12,000 *g* for 1 min. The supernatant was removed and the cells solubilized by the addition of "cell-dissolving buffer," 2 mM Tris, 2% (vol/vol) SDS, 5% (vol/vol) 2-mercaptoethanol, 2 mM EDTA, final pH 6.5. The cells were immediately boiled for 3 min, spun at 100,000 *g* for 20 min at 4°C and the supernatant placed at 0°C. No difference was found between spun and nonspun preparations. Protease inhibitors were not routinely added, but when used were 1 mM PMSF plus 10 μg/ml chymostatin both in cell-dissolving buffer.

GEL ELECTROPHORESIS; ISOELECTRIC FOCUSSING; CLEVELAND PEPTIDE MAPPING: SDS PAGE was carried out by the method of Laemmli (26). Routinely, 10% slab gels were used for all one-dimensional gels and for the second dimension of isoelectric focussing which was performed as described by O'Farrell (35), except that 4–6.5 pI gradients (Pharmacia Fine Chemicals, Piscataway, NJ) were used and the tube gel diameter was 4 mm. The residue from approximately 2×10^6 cells or 7 μg of pure skeletal muscle actin (Sigma Chemical Co.) was loaded per slot on slab gels and a maximum of 250 μg of protein in the tube gels. Samples of actin, vimentin, and epinemin for Cleveland peptide analysis (4) were purified by isoelectric focussing (from cells solubilized in cell-dissolving buffer) followed by SDS PAGE, then briefly stained with Coomassie Blue and excised, run on 15–17.5% SDS gradient gels and digested by 0.1 ng of papain. These gels were stained with silver nitrate (32).

TRYPTIC PEPTIDE MAPPING: 4×10^6 3T3 cells were trypsinized and replated in 20 ml of DME plus 10% FCS. 5 h later the medium was removed and the cells rinsed with DME minus leucine and lysine plus 2% FCS and then incubated in the same medium for 30 min. Fresh medium (18 ml) was then added plus 5 mCi [^{3}H]leucine (TRK 683) and 5 mCi [^{3}H]lysine (TRK 520, both from Amersham Corp., Arlington Heights, IL) in a volume of 2 ml, the cells incubated for 16 h, rinsed in cold medium and then processed as in the sections just above. Vimentin and epinemin were isolated from two-dimensional gels that had been stained briefly by Coomassie Blue, digested with trypsin (Worthington Biochemicals, Freehold, NJ), analysed, and processed as described by Elder et al. (5). I am very grateful to M. Hayman (Imperial Cancer Research Fund) for carrying out the thin-layer chromatography.

PROTEIN TRANSFER TO NITROCELLULOSE: SDS PAGE gels were blotted on to 0.45-μm pore size nitrocellulose (Schleicher and Schuell GmbH Dassel, Federal Republic of Germany) at 600 mA for 2–3 h at room temperature (47) in an electroblot apparatus (E-C Apparatus Corp., St. Petersburg, FL). Following transfer, the nitrocellulose was incubated in 5% (wt/vol) hemoglobin (Hb) in PBS plus 0.1% (wt/vol) NaN_3 for 1 h or overnight, then labeled with the first antibody. Monoclonal antiepinemin purified by DEAE and sucrose gradient centrifugation (56) was used at 50 μg/ml and a monoclonal antibody against all classes of intermediate filaments—anti-IFA—(37) at 25 μg/ml, both diluted in 5–25 ml of 1% Hb in PBS/NaN_3 for 3 h or overnight. The nitrocellulose strips (cut with pinking shears to facilitate any realignment) were then washed for 1 h in 10 changes of 1% Hb/PBS/NaN_3, incubated for 2 h in 20 ml of the same medium containing 250×10^3 counts per minute (cpm)/ml of rabbit anti-mouse IgG-Fab$_2$ coupled to ^{125}I by chloramine T (17), washed for 30 min in at least six changes of PBS, dried, and autoradiographed as described previously (27). Affinity-purified rabbit antiactin was used at 4 μg/ml followed by ^{125}I-Protein A and 5 μl of rabbit antivimentin sera (8) in 20 ml of 1% Hb/PBS/NaN_3 was used with affinity-purified sheep anti-rabbit ^{125}I-IgG as the second layer (a kind gift from Janet Winter, Zoology Department, University College, London). For experiments where nitrocellulose labeled with antiepinemin and anti-IFA were restained by rabbit antivimentin, the stored nitrocellulose was simply rehydrated (48) in 1% Hb/PBS for 1 h and processed as above. This technique considerably enhances the usefulness of gel blotting.

Electron Microscopy

3T3 cells were cultured overnight at a concentration of 20×10^3 cells per ml. Coverslips were rinsed in PIPES (as in the section, Preparation of Cytoskeletons, above), extracted in 0.05% Triton X-100 in PIPES plus 2 μg/ml taxol (included at all stages until cells were in OsO_4) at 37°C for 1 min, rinsed in PIPES, fixed for 15 min in 1% formaldehyde (British Drug Houses) in PIPES, rinsed in 0.1 M lysine in PIPES, rinsed in PIPES then in PBS plus 1% BSA. Coverslips were then incubated with 50 μl of either DEAE/sucrose gradient purified epinemin at a concentration of 3 mg/ml or rabbit antivimentin serum (8) diluted 1:10 in PBS/1% BSA both for 30 min at room temperature. The cells were then fixed in 0.1% glutaraldehyde in PIPES for 15 s, rinsed in 0.1 M lysine in PIPES and then in PBS. Coverslips labeled with antiepinemin were incubated for 30 min with 50 μl of affinity-purified rabbit anti-mouse IgG coupled to ferritin (FT) by a two-step glutaraldehyde method (36), (1 mg/ml IgG conjugated) while those labeled with rabbit antivimentin were labeled with goat anti-rabbit IgG coupled to FT by a single-step glutaraldehyde method (36). After this incubation, cells were rinsed, fixed in 2% gluatraldehyde in PIPES for 1 h at room temperature, rinsed, and placed in 0.5% OsO_4 in PIPES for 3 min at 4°C, then rinsed again, block stained in 1% aqueous uranyl acetate for 1 h at 4°C, rapidly dehydrated, and embedded in EPON 812. Coverslips were removed and areas of high cell density cut out, reorientated, glued on to EPON moulds and 30–50-nm sections cut parallel to the plane of the coverslip. Sections were stained with uranyl acetate followed by lead citrate and photographs taken on a JEOL 100CX operating at 80 kV.

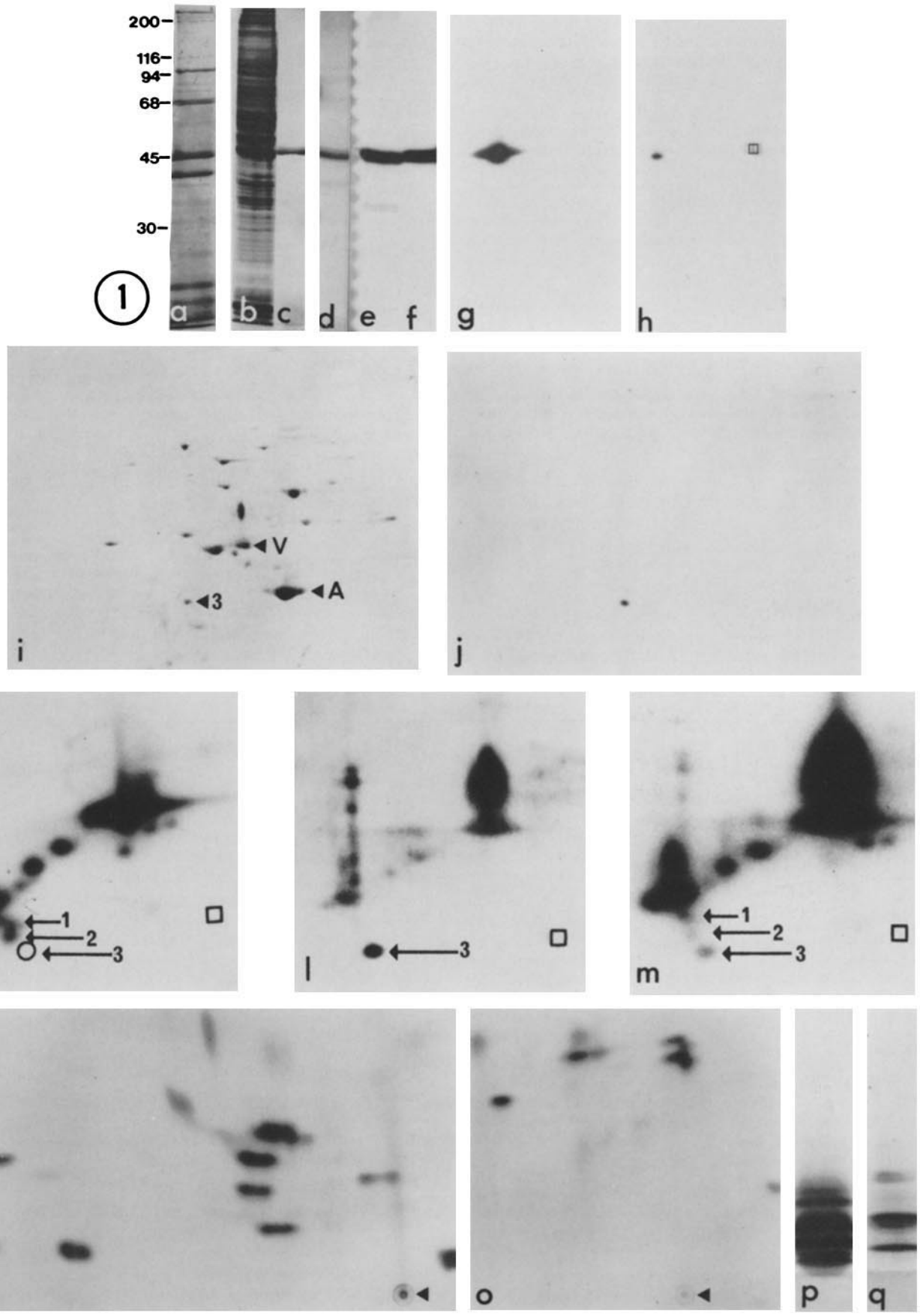
RESULTS

Preliminary experiments showed that the monoclonal antibody, antiepinemin described in Materials and Methods, stained, by immunofluorescence, a fibrous structure in Triton X-100 extracted cells (see below) and labeled a single band in SDS PAGE. The relationship of this protein, epinemin, to other known cytoskeletal molecules was therefore examined by biochemical methods and light and electron microscopy.

Biochemistry

Comparison of Actin/Epinemin

Fig. 1 *a* shows a gel of the immunogen, supernatant from a 10 mM $MgCl_2$ precipitation, showing bands at 45,000 and 95,000 mol wt, which correspond to actin and α-actinin, respectively (see also reference 7). The identity of the other bands is unknown. Little desmin (55,000 mol wt) is seen in this preparation (see also reference 7). One-dimensional SDS PAGE of SDS solubilized 3T3 cells (Fig. 1 *b*), pure skeletal muscle actin (Fig. 1 *c*), crude chicken gizzard (not shown), or $MgCl_2$ supernatant from chicken gizzard (not shown) followed by protein transfer to nitrocellulose, incubation with antiepinemin then Fab$_2$ fragments of rabbit anti-mouse IgG ^{125}I revealed a band of apparent molecular weight of 45,000 comigrating with purified actin and present only in solubilized 3T3 cells (Fig. 1 *d*), not gizzard or skeletal muscle actin (not shown). The position of actin was shown by labeling identical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tracks from the same gel with affinity-purified rabbit antiactin followed by Protein A coupled to ^{125}I and revealed that the antiactin stained gizzard and 3T3 cells (Fig. 1, *e* and *f*). However, when SDS-solubilized 3T3 cells were separated by isoelectric focussing, followed by SDS PAGE (Fig. 1 *i*), transferred to nitrocellulose and labeled with either affinity-purified rabbit antiactin or antiepinemin, then ^{125}I second layers as above, a clear and reproducible difference was found between the position of actin (Fig. 1 *g*) and epinemin (Fig. 1 *h*). The position of epinemin relative to actin and the vimentin family (see below) on all two-dimensional gels (Fig. 1 *i*) was independent of protease inhibitors, the iodinated second antibody (compare fig. 1 *k* and *l*, both labeled with the same rabbit anti-mouse Fab_2 ^{125}I), length of time in 9 M urea, and amount of protein loaded in the first dimension. The affinity-purified antiactin only labeled actin (Fig. 1 *g*), never recognized epinemin (Fig. 1 *h*) nor did an IgG fraction from the same antisera (not shown). To minimize variations between gels when comparing actin and epinemin, we ran two first-dimension tube gels on a large second-dimension slab gel that was subsequently processed as detailed in Materials and Methods.

These experiments show that epinemin has a molecular weight of 44,500, unchanged in nonreducing conditions (not shown) and a pI of 5 compared with the molecular weight of 45,000 and pI of 5.4 found for actin in these gels. The amount and distribution of epinemin did not appreciably change when Triton X-100-insoluble 3T3 cytoskeletons instead of total cells were run on two-dimensional gels and stained by Coomassie Blue (not shown). In all these experiments, the antiepinemin supernatant used was concentrated by ammonium sulphate (see Materials and Methods). Identical results were obtained with antiepinemin purified by DEAE followed by differential sucrose gradient centrifugation (Fig. 1 *j*).

Comparison of Vimentin/Epinemin

Similar experiments were carried out, also on SDS-solubilized 3T3 cells, to investigate whether or not epinemin was a breakdown product of vimentin. Gel *k* in Fig. 1 was incubated with a monoclonal antibody (anti-IFA) that has been shown to interact with all classes of intermediate filaments (37), followed by Fab_2 fragments of rabbit anti-mouse IgG ^{125}I. After being autoradiographed (not shown), the nitrocellulose was reincubated with a polyclonal rabbit antivimentin (8) followed by affinity purified sheep anti-rabbit ^{125}I and subsequently re-autoradiographed (Fig. 1 *k*). The result showed that the monoclonal anti-IFA recognized the same number of vimentin breakdown products, approximately eight in these gels, as the broad specificity polyclonal antivimentin. Note in particular the breakdown products marked 1 and 2 in Fig. 1 *k*. For comparison, gel *l* of Fig. 1 was incubated with monoclonal anti-IFA and monoclonal antiepinemin simultaneously, followed by Fab_2 fragments of rabbit anti-mouse IgG ^{125}I. When autoradiographs of gels *k* and *l* in Fig. 1 were aligned, they showed that epinemin is not a breakdown product of vimentin, since its position does not coincide with any of the breakdown products recognized by either the anti-IFA or the polyclonal antivimentin antibody. The breakdown products marked 1 and 2 in Fig. 1 *k* are not visible in Fig. 1 *l* (see below). Further proof of this observation was obtained by reincubating Fig. 1's gel *l* (after a delay of 4 mo to allow most of the original radioactivity to decay) with the same polyclonal rabbit antivimentin as Fig. 1 *k*, followed by affinity-purified sheep anti-rabbit IgG ^{125}I and then re-autoradiographing (Fig. 1 *m*). Fig. 1 *k*, *l*, and *m* (all at the same magnification) can now be more easily compared since the vimentin breakdown products marked 1 and 2 in Fig. 1 *k* are

FIGURE 1 Localization of epinemin and cytoskeletal proteins by 1 and two-dimensional SDS PAGE. (*a*) Supernatant (used as immunogen) from 10 mM $MgCl_2$ precipitation. Molecular weights, $\times 10^{-3}$. (*b*) Coomassie-Blue-stained lane of solubilized 3T3 cells. (*c*) Coomassie-Blue-stained lane of pure skeletal muscle actin. (*d*) Autoradiograph of lane *b* transferred to nitrocellulose and incubated with antiepinemin showing one band at 45,000 mol wt. (*e*) Autoradiograph of solubilized chicken gizzard transferred to nitrocellulose and incubated with affinity purified antiactin. The major band is at 45,000 mol wt. (*f*) Autoradiograph of solubilized 3T3 cells transferred to nitrocellulose and incubated with affinity purified antiactin showing one band at 45,000 mol wt. (*g*) Two-dimensional gel of solubilized 3T3 cells transferred to nitrocellulose and incubated with affinity purified antiactin. Only one spot stains at the position of actin, *A* in gel *i*. Isoelectric focussing is from left to right. (*h*) As gel *g* except that the nitrocellulose was incubated with antiepinemin. Only one spot labels, numbered *3* in gel *i* and its position relative to actin, marked with a square, never varies. Isoelectric focussing is from right to left in this photograph, which is a mirror image of gel *g*. Gels *g* and *h* were run on the same second dimension slab gel. (*i*) Coomassie-Blue-stained two-dimensional gel of solubilized 3T3 cells with the positions of actin (*A*), vimentin (*V*), and epinemin (*3*) marked. No vimentin breakdown products can be seen. Isoelectric focussing is from right to left. (*j*) Similar gel to gel *i* transferred to nitrocellulose and incubated with purified antiepinemin showing that only one spot ever labels, in position *3* in gels *i*, *l*, and *m*. Magnification here is the same as in *i*, the entire gel is shown, and isoelectric focussing is from right to left. (*k*) Autoradiograph from a two-dimensional gel of solubilized 3T3 cells transferred to nitrocellulose and incubated with monoclonal anti-IFA and then a polyclonal antivimentin (see Results). Neither recognizes epinemin, marked *3*. Vimentin is the largest spot at the top right hand side and its breakdown products (eight in number) are lower in molecular weight and run in a diagonal from right to left. Note the position of the breakdown products numbered one and two and their proximity to epinemin. Isoelectric focussing is from right to left in gels *k*, *l*, and *m*, all are at the same magnification, and have the position of actin marked with a square. (*l*) Autoradiograph of a two-dimensional gel of solubilized 3T3 cells transferred to nitrocellulose and incubated simultaneously using the same monoclonal anti-IFA as in gel *k* and antiepinemin, which now stains strongly at position *3*. Compare with *k*. Vimentin and its breakdown products are labeled by the anti-IFA, but not those at positions *1* and *2*. (*m*) Autoradiograph from the same nitrocellulose as gel *l* incubated after 4 mo using the same polyclonal antivimentin as in gel *k*. The staining of vimentin and its breakdown products is considerably enhanced and numbers one and two are now visible, close to but clearly separate from epinemin (number *3*), whose position is revealed by residual radioactivity from the previous experiment in gel *l*. (*n*) Tryptic peptide fingerprint of vimentin. The origin is marked with an arrowhead. Gels *n* and *o* are at the same magnification to allow comparison between their peptide distribution. (*o*) Tryptic peptide fingerprint of epinemin with the origin marked by an arrowhead. No other peptides were visible on this autoradiograph. (*p*) Cleveland peptide digest of actin showing no apparent homology with epinemin in gel *q*. (*q*) Cleveland peptide digest of epinemin from the same gel as gel *p*.

visible in Fig. 1 *m*, possibly because the greater number of determinants seen by the polyclonal antivimentin facilitates subsequent autoradiographic detection in some gels. It is clear, however, that there is an additional spot, marked 3, visible in Fig. 1 *m*, which is not present in *k*, even after labeling with both mono- and polyclonal antisera to intermediate filaments. This third dot is epinemin which, as seen in Fig. 1 *m* is close to, but clearly separate from, the nearest vimentin breakdown product.

Tryptic Peptide Map

The proximity of epinemin with the nearest vimentin breakdown product (labeled 2 in Fig. 1, *k* and *m*) and the possibility that epinemin was not recognized as an additional vimentin breakdown product simply because of its denaturation by SDS dictated that the primary structure of the two molecules be examined. Two-dimensional separation of complete tryptic digests of [^{3}H]leucine + lysine-labeled vimentin (Fig. 1 *n*) and epinemin (Fig. 1 *o*) clearly reveals that the two proteins have a completely dissimilar peptide distribution pattern with no apparent homologous peptides. This can be verified by measuring from the origin (arrowhead) since both gels are at the same magnification. The tryptic peptides of epinemin are restricted to the area shown.

Cleveland Peptide Digest

In agreement with the results above little homology was found between actin (Fig. 1 *p*) and epinemin (Fig. 1 *q*) following their isolation from Coomassie Blue-stained two-dimensional gels and digestion with papain.

Immunofluorescence

Several detergent extraction/fixation protocols were tried before using the one described in Materials and Methods, which seemed to give the best preservation/visualization of the cytoskeleton. The distribution of the epinemin network does not appear to be artifactual since it remained essentially unchanged if either methanol or formaldehyde/glutaraldehyde fixation was left out of the protocol or if cells were treated with methanol alone.

Immunofluorescent Staining and Cell Type Distribution of Epinemin

The distribution of epinemin in well spread subconfluent or fully confluent populations of 3T3 or BSC-1 cells is a fine branching nonperiodic wavy filigree-like network, strongly nuclear associated and also extending towards the cell periphery (Fig. 2, *b* and *d*, and Fig. 3 *b*). No diminution or noticeable alteration in staining was seen in fibroblasts extracted with 0.5% Triton X-100 for 2 min followed by 1% Triton X-100 for 4 min (not shown) or in macrophages extracted with 0.15% Triton X-100 for 1 h at room temperature (not shown). This network has been found in all cultured cell types looked at that have vimentin filaments: these are in embryonic dorsal root ganglion neurones, Schwann cells, macrophages, avian fibroblasts, PtK1, at the Z line and neuromuscular junction of skeletal muscle (not shown), in 3T3 cells (Fig. 3 *b*), and BSC-1 cells (Fig. 2, *b*, *d*, and *f*). This latter cell line, although of epithelial origin, had negligible staining for keratin (not shown).

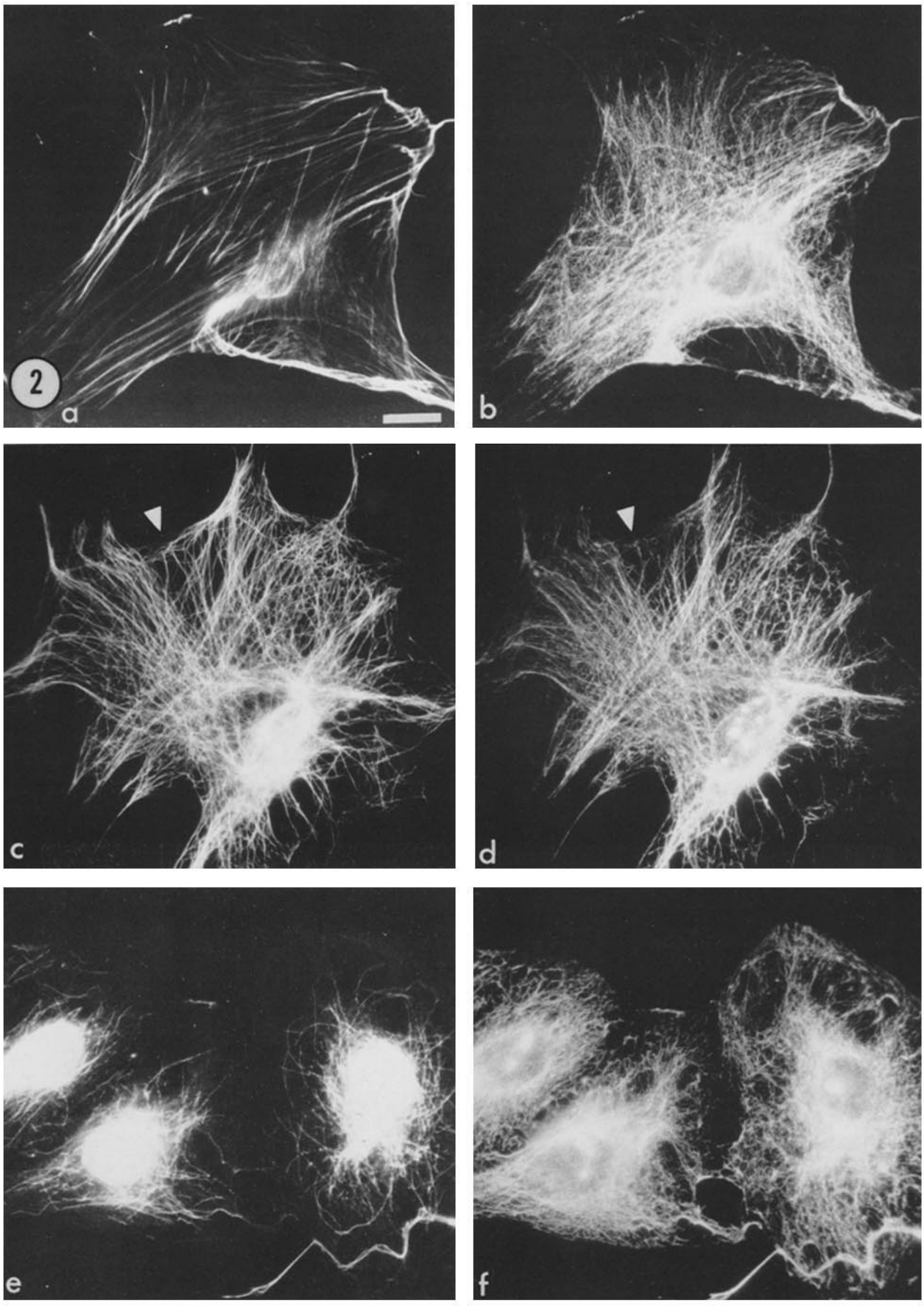
By frozen sections epinemin has not as yet been found in cells that contain only, or predominantly, nonvimentin subunits. Examples looked at were Purkinje cells (neurofilaments), grey matter astrocytes (glial fibrillary acidic protein), central nervous system neurones (neurofilaments), gut epithelia (keratin) (all not shown), and visceral smooth muscle (desmin). Here, frozen sections of rat proximal colon are positive by immunofluorescence for anti-IFA (Fig. 4 *a*) that has previously been shown to react strongly with desmin (37) and negative (Fig. 4 *b*) for polyclonal antivimentin (21) and antiepinemin (Fig. 4 *c*). Identical results have been found for smooth muscle from chicken gizzard (not shown). In addition epinemin has not been found in frozen sections of those glial elements that have been shown to contain vimentin, white matter astrocytes, and Bergmann glia (55) (R. Mirsky and D. Lawson, unpublished observations).

Double Label Immunofluorescent Patterns of Cytoskeletons

ACTIN/EPINEMIN: Double immunofluorescence to compare the distribution of actin microfilaments with epinemin showed that the two have very different labeling patterns. The antiactin labeled stress fibers very clearly in both 3T3 and BSC-1 cells (Fig. 2 *a*) and never the meshwork of filaments recognized by antiepinemin (Fig. 2 *b*). Preincubation of detergent extracted cells with either the S1 head of heavy meromyosin or polyclonal antiactin did not block the binding of antiepinemin and later experiments at the ultrastructural level showed that any apparent association of epinemin with actin stress fiber filaments was due to the inability of the light microscope to resolve different types of filaments running closely together (see below).

TUBULIN/EPINEMIN: When cells were detergent extracted in the presence of 4 M glycerol or taxol to stabilize microtubules it was apparent that some homology exists between the microtubules (Fig. 2 *c*) and the epinemin network (Fig. 2 *d*). However, the tubule network extends further to the cell periphery (arrowheads Fig. 2, *c* and *d*) and is much straighter than the more filigree-like epinemin network. Preincubation of extracted cytoskeletons with antitubulin did not affect the binding of epinemin. The absence of 4 M glycerol in the extraction buffer led to ~50% destruction of microtubules (Fig. 2 *e*) and while it was apparent that the epinemin staining was not so extensively affected it was also slightly

FIGURE 2 Double immunofluorescence on Triton X-100–extracted BSC-1 cells comparing the distribution of (*a*) actin and (*b*) epinemin on the same cells. Little homology exists between the two networks. (*c*) Tubulin and (*d*) epinemin on the same cell Triton X-100 extracted in the presence of 4 M glycerol to preserve microtubules. The two networks follow each other closely but the microtubules extend much further to the periphery (arrowheads), and are less branching. (*e*) Tubulin and (*f*) epinemin on the same cell Triton X-100 extracted in the absence of 4 M glycerol. Microtubules have largely disappeared except for a few radiating from the nucleus in e. Antiepinemin staining is only slightly fragmented. Bar, 20 μm. × 500.


2
a
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fragmented and disrupted (Fig. 2 *f*). This experiment clearly shows the difference between the two networks. Conversely the addition of 4 M glycerol or taxol led to the preservation of microtubules and to the best overall preservation of the epinemin (and vimentin) networks (compare Fig. 2, *d* and *f*). Actin appeared unaffected, at the light microscope level, by the presence or absence of 4 M glycerol or taxol (not shown).

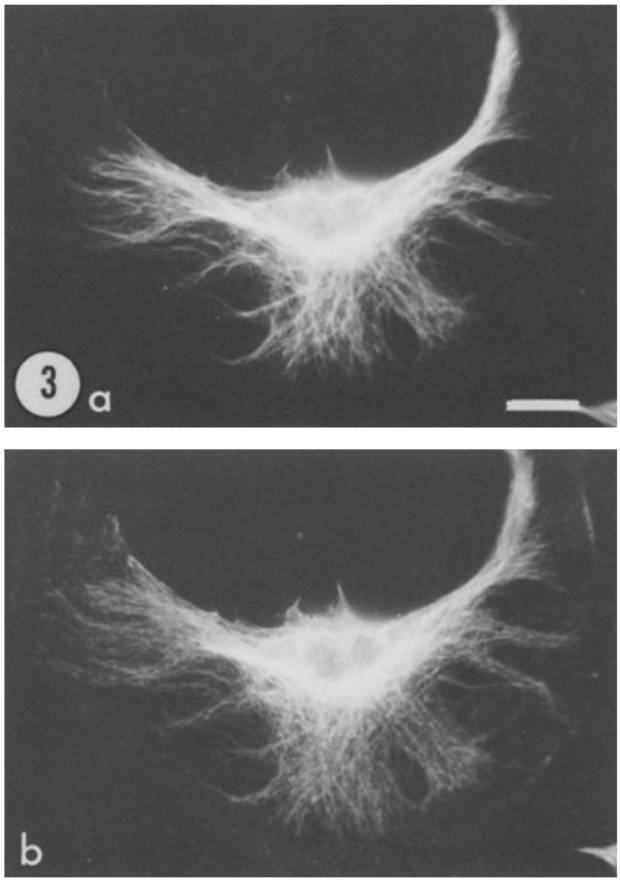

FIGURE 3 Double immunofluorescence on 3T3 cells comparing the distribution of (*a*) vimentin and (*b*) epinemin. The distribution of the two networks is identical. Bar, 20 μm. × 500.

VIMENTIN/EPINEMIN: The staining patterns of vimentin and epinemin were almost indistinguishable in the cell types looked at by using both the monoclonal anti-IFA (not shown) and polyclonal antisera to vimentin (Fig. 3 *a*). Preincubation of detergent extracted cells with the polyclonal antivimentins did slightly reduce the staining intensity of antiepinemin at the dilution of sera normally used for immunofluorescence (1:100), (Fig. 3 *b* and compare with Figs. 2, *b* and *d*). However, when the antivimentin was used as for electron microscopy (1:10), antiepinemin staining was almost completely blocked (not shown). No differences in distribution between vimentin/epinemin could be found in motile, confluent, dividing, or resettling cells.

Salt or Sarkosyl Extraction of Cytoskeletons

EFFECT OF LOW-HIGH-LOW SALT OR SINGLE TREATMENT WITH HIGH OR LOW SALT: When coverslips of 3T3 or BSC-1 cells were extracted in 0.15% Triton X-100 in the presence of taxol for 1.5 min, subjected to a cycle of low-high-low salt, and then labeled with various antibodies, clear differences were noted in their cytoskeletal architecture. Vimentin distribution, as visualized by monoclonal and polyclonal antisera was to a large extent undisturbed by this treatment (Fig. 5 *a*). In contrast, actin microfilaments (Fig. 5 *b*) and the meshwork delineated by antiepinemin (Fig. 5 *c*) were extensively fragmented and both networks were reduced to a series of juxtanuclear dots.

Similar results on the vimentin and epinemin networks were seen when cells were extracted for 1 min in 0.5% Triton X-100 plus 0.6 M KCl (or 0.3 M KI) (not shown). The distribution of the antibodies mentioned above was unaffected in cells Triton X-100-extracted as above, but without high salt (not shown).

In contrast cells extracted in 0.15% Triton X-100 for 1.5

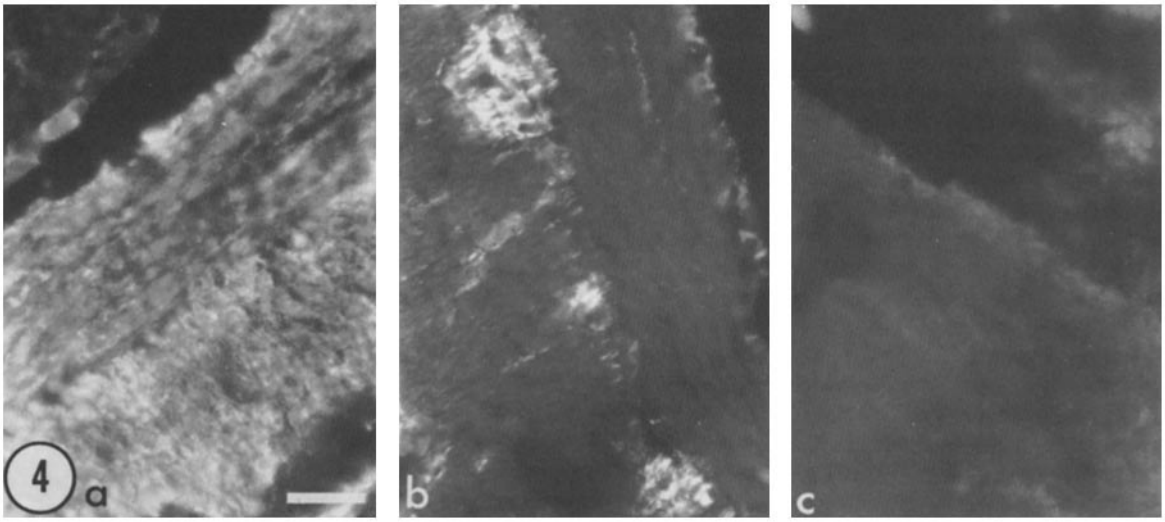

FIGURE 4 (*a*) Frozen section of anti-IFA staining longitudinal and transverse smooth muscle, the serosal layer and Auerbach's plexus in rat proximal colon. (*b*) Frozen section of rat proximal colon showing the absence of rabbit antivimentin staining in longitudinal and transverse smooth muscle. Staining is present in the glial cells of Auerbach's plexus, connective tissue, and the serosa. (*c*) Frozen section of rat proximal colon incubated with antiepinemin. No staining is seen in longitudinal and transverse smooth muscle or Auerbach's plexus. Faint staining of the serosal layer can be seen. Bar, 20 μm. × 500.

min and then incubated in 2 mM Tris, pH 6.9, for 1 h at 4°C had fragmented vimentin as well as actin, and epinemin (not shown). Control cells, similarly treated, but plus 100 mM KCl, had intact filaments (not shown).

EFFECT OF SARKOSYL: Before sarkosyl treatment, it was found necessary to treat cells with taxol and phalloidin to stabilize their microtubule and actin filament networks respectively, otherwise no cytoskeletons remained on the coverslip.

Incubation of the stabilized cells in 0.05% sarkosyl in PIPES for 2.5 min almost totally removed vimentin and epinemin staining (Fig. 5, *d* and *f*). Actin filaments were disrupted but not to such an extent (Fig. 5 *e*).

Cytochalasin D or Colchicine

EFFECT OF CYTOCHALASIN D: BSC-1 or 3T3 cells were incubated in 3.3 μg/ml of cytochalasin D for 5–15 min, a dose and time that has been shown to cause minimal arborization of the cells (39), but had significant effects on their cytoskeletons. After detergent extraction most of the actin in these cells was found to be collapsed, mainly from the center outwards, with the concomitant appearance of many foci, all over the cell, staining strongly for actin (Fig. 6 *a*).

The appearance of the vimentin and epinemin network was less altered by cytochalasin D, but there were notable areas of disruption in many cells with again the formation of foci, distributed all over the cell. These foci contained vimentin (Fig. 6 *b*) and epinemin (Fig. 6 *c*). The disruption of the vimentin/epinemin network was slower than actin but became more complete with longer incubation times in cytochalasin D (not shown).

EFFECT OF COLCHICINE: Incubation of 3T3, BSC-1, or PtK1 cells with 1 μm colchicine for 30 min before detergent extraction caused most microtubules to collapse into the perinuclear area (Fig. 6 *d*). Vimentin (Fig. 6 *e*) and epinemin (Fig. 6 *f*) were also present in these areas with little remaining immunofluorescence over the rest of the cell. Actin filaments were unaffected (not shown) as were keratin filaments in PtK1 cells, which had no detectable antiepinemin staining (not shown).

Immunoferritin Electron Microscopy

For these experiments, 3T3 cells were extracted with the minimal concentration of Triton X-100 compatible with antibody/FT penetration since this was found to best preserve their cytoskeletons. Taxol (38) was added during extraction and subsequent labeling and dramatically enhanced the overall cytoskeletal integrity. Such cells had few broken intermediate filaments and many more 2–3-nm diameter filaments than cells extracted in the absence of taxol. Actin filaments appeared unaffected by the presence or absence of taxol.

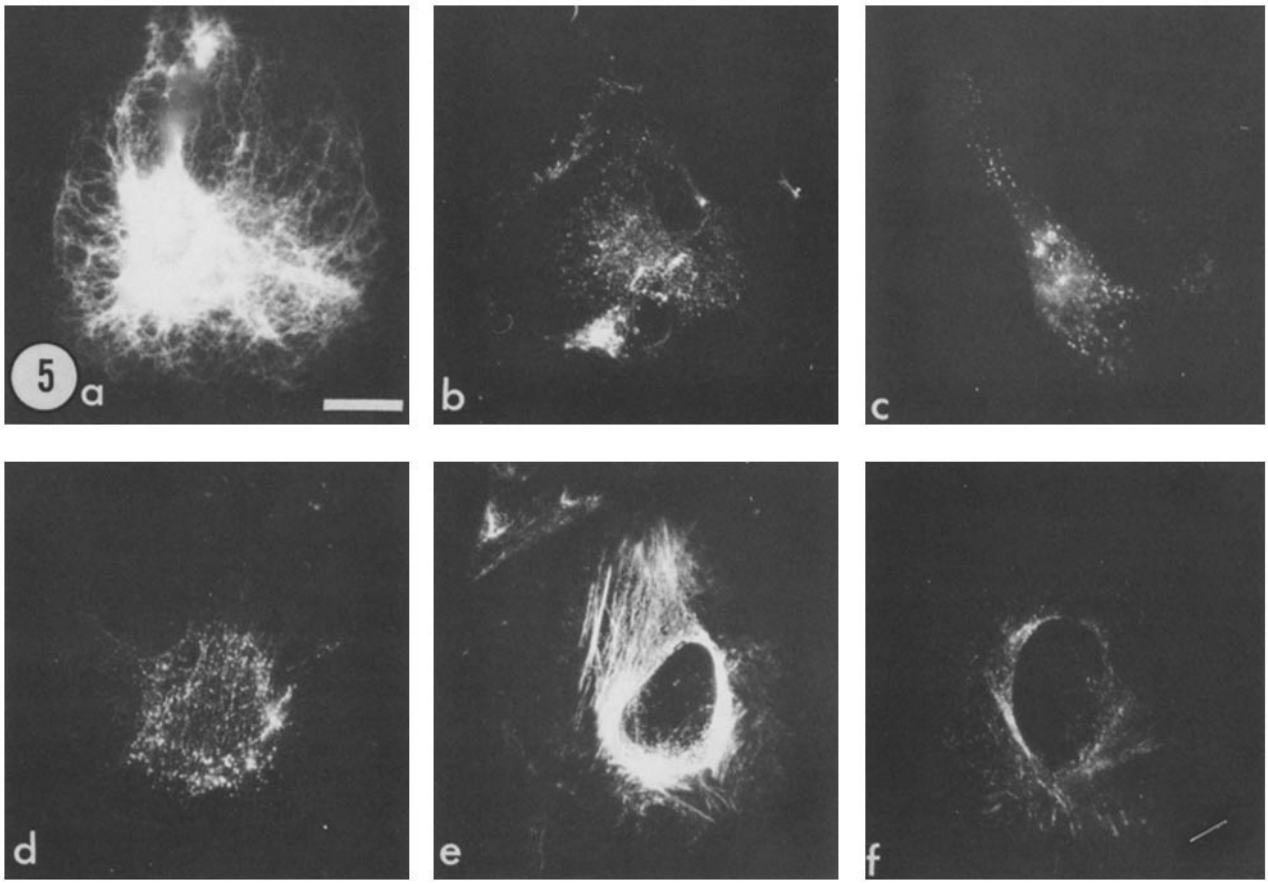

FIGURE 5 BSC-1 cells extracted in low high and low salt (*a*, *b*, and c), or sarkosyl (*d*, e, and *f*). (a) Vimentin network is unaltered. Compare with Fig. 3 *a*. (*b*) Actin network reduced to a series of dots—compare with Fig. 2 *a*. (c) Epinemin network has almost disappeared apart from a few dots round the nucleus. Compare with Fig. 2, *b*, *d*, and *f*, and Fig. 3 *b*. (*d*) Vimentin network removed by sarkosyl. (e) Actin network largely intact after treatment with sarkosyl. (*f*) Epinemin network removed by sarkosyl. Bar, 20 μM. × 500.

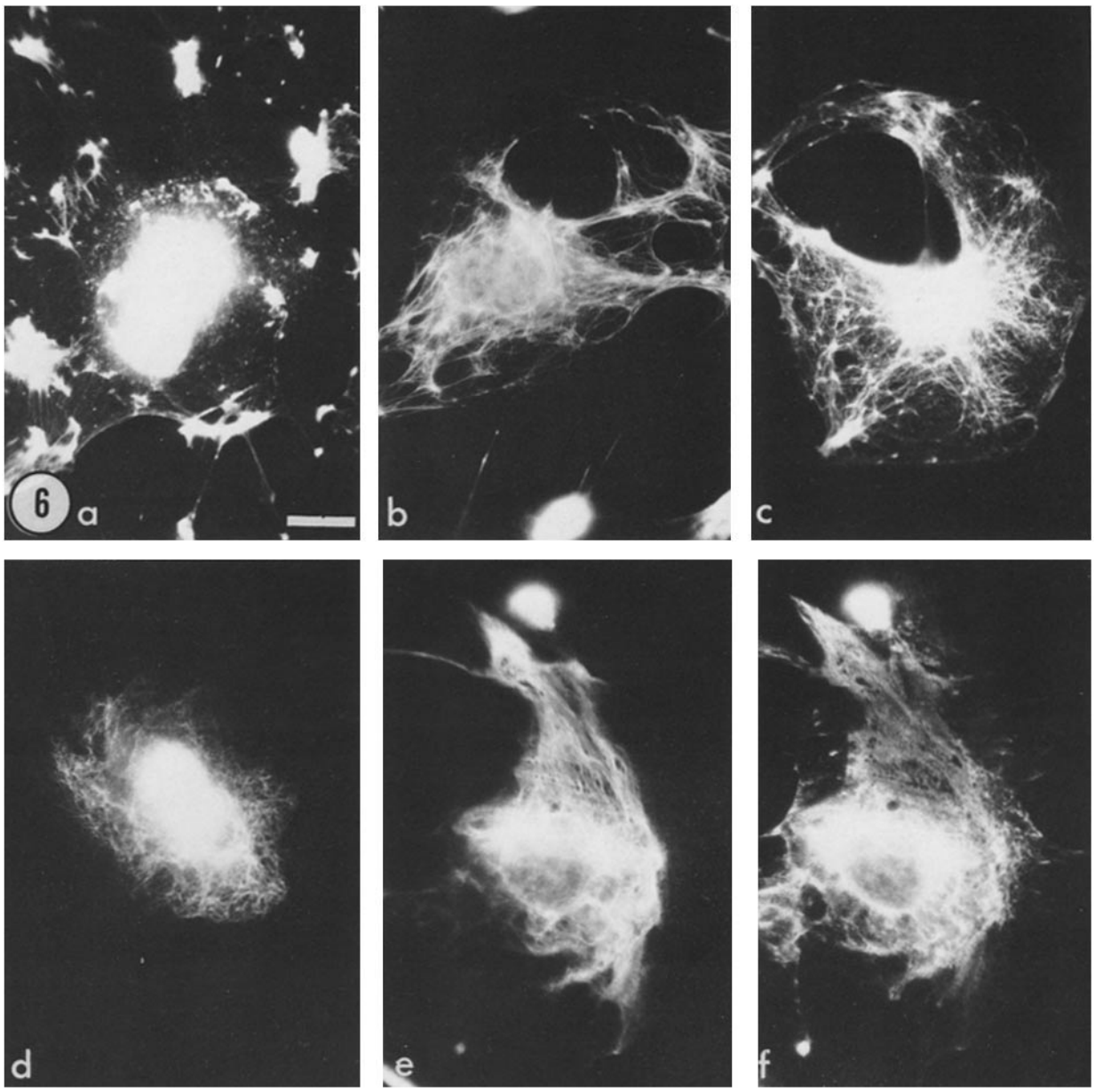

FIGURE 6 BSC-1 cells incubated in cytochalasin D (*a*, *b*, and c) or colchicine (*d*, e, and *f*). (a) Actin network destroyed apart from a few foci. (*b*) Vimentin network disrupted but not so extensively. Small foci can be seen at the cell periphery. (c) Epinemin network. Affected to the same extent as vimentin in the previous figure. Small foci are visible. (*d*) Microtubules, shown here partially collapsed after colchicine treatment. (e) Vimentin and (*f*) epinemin collapsed in a similar manner by colchicine on the same cell. Bar, 20 μM. × 500.

Finally, while in these experiments a high concentration of antiepinemin (3 mg/ml) was used to saturate all available binding sites, similar results were obtained with 0.39 mg/ml of antiepinemin.

When cells were labeled with the polyclonal rabbit antivimentin used in Fig. 1, *k* and *m*, followed by a goat anti-rabbit IgG-FT a distinctive pattern of FT distribution was observed. Only intermediate filaments were labeled (Fig. 7 *a*) and the FT molecules were closely packed along their entire length (Fig. 7 *a*, and *inset*). The majority of intermediate filaments were so labeled regardless of their positon inside the cell.

In contrast, similarly extracted cells labeled with anti-epinemin followed by an affinity purified rabbit anti-mouse IgG-FT had a very different FT distribution. Again, intermediate filaments were labeled but here the antibody distribution was clearly intermittent with no obvious periodicity (Figs. 7 *b* and 8) and the foci were separated by a minimum distance of 80–100 nm. Some intermediate filaments had little or no label on areas of them (Fig. 7 *b*). While antiepinemin was found on intermediate filaments well inside the cell those most heavily labeled, but still with an intermittent distribution of FT, were found at the cell periphery (Fig. 8), often coursing through and beside stress fiber bundles of actin microfilaments (Fig. 7 *b*). On antiepinemin labeled filaments, approximately equal amounts of two different cluster sizes of FT molecules were seen; those containing up to 10 FT (Figs. 7 *b* and 8) and others of 10 to 25 FT (Fig. 8, and *inset*). These FT clusters were either adhering to a fuzz of antibody on a

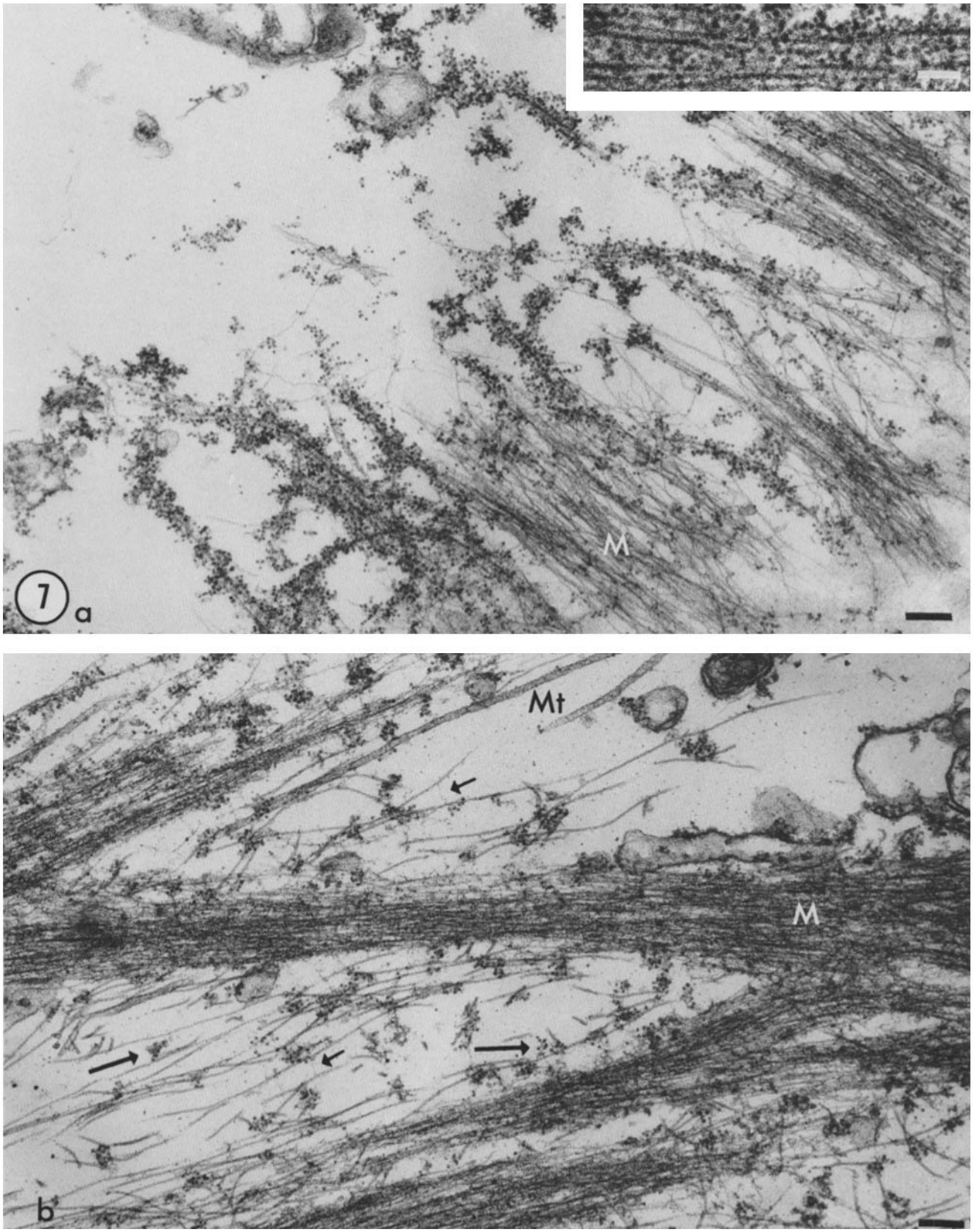

FIGURE 7 (*a*) Extracted 3T3 cell labeled with rabbit antivimentin followed by goat anti-rabbit-FT. Only intermediate filaments are labeled and the distribution of FT is uniform on them. Actin microfilaments (*M*) are unlabeled as are the membrane fragments at the top of the micrograph. Bar, 100 nm. × 75,600. (*Inset*) Intermediate filaments labeled as above showing close packing of the antibody molecules with no clear areas on the filaments. The spaces between them are filled by antibody-FT complexes. Bar, 50 nm. × 144,000. (*b*) Extracted 3T3 cell labeled with antiepinemin followed by affinity purified rabbit anti-mouse-FT. Only intermediate filaments are labeled but here with a discontinuous distribution. Microtubules (*Mt*) and actin microfilaments (*M*) are unlabeled as are membrane fragments and intermediate filament crossover points. Clusters of less than 10 FT molecules (long arrows) can be seen as can short 2–3-nm diameter filaments bridging between adjacent intermediate filaments (short arrows). Bar, 100 nm. × 75,600.

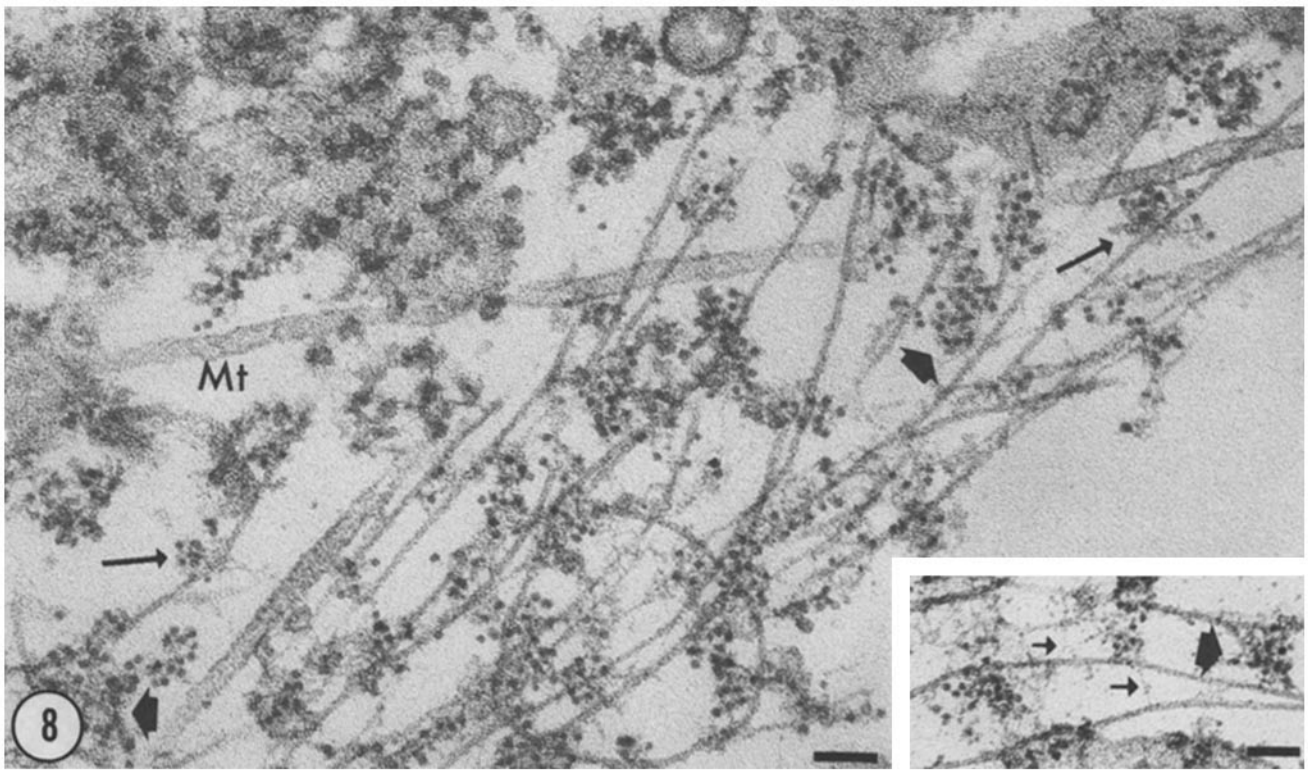

FIGURE 8 Extracted 3T3 cell labeled with antiepinemin as in Fig. 7 *b*. Intermediate filaments are heavily labeled with antiepinemin in a random pattern. Microtubules (*Mt*) cytoplasmic and membrane remnants, intermediate filament/microtubules cross-over points and intermediate filament cross-over points are all unlabeled. Clusters of up to 25 FT molecules are seen between adjacent intermediate filaments (large arrows) while those clusters of 10 or less FT molecules are mainly found on less closely apposed filaments (long arrows). Bar, 50 nm. × 158,400. (*Inset*) Antiepinemin antibody was very often found in areas containing small 2–3-nm filaments (short arrows) that are undecorated. Bar, 50 nm. × 133,200.

single filament (Fig. 8) or were often seen between adjacent intermediate filaments. Here the larger cluster size of FT was usually found (Fig. 8, *inset*).

Short 2–3-mm diameter filaments (39, 41, 46, 50) were noticeable in taxol stabilized preparations. These small 2–3-nm diameter filaments were most often observed cross-linking adjacent intermediate filaments (Fig. 7 *b* and *inset* to Fig. 8), to a lesser extent intermediate filaments to microtubules and seldom actin filaments. While many antiepinemin/FT complexes were found decorating areas where a meshwork of small 2–3-nm diameter filaments were cross-linking adjacent intermediate filaments (*inset*, Fig. 8), there was no unequivocal evidence, in this study, of any label on the 2–3-nm filaments themselves.

Antiepinemin was never seen in association with actin microfilaments (Fig. 7 *b*) or any of the many membrane fragments and remnants of intracellular organelles that remained after the light detergent extraction protocol used (Figs. 7 *b* and 8). This was also true for the antivimentin antisera (Fig. 7 *a*) and clearly demonstrates the specificity of FT label for both antisera. Finally, although antiepinemin antibody was occasionally found in areas where intermediate filaments were seen diagonally crossing and in close association with microtubules (Fig. 8), it was seldom found on microtubules themselves (Fig. 8), or at intermediate filament crossover points (Figs. 7 *b* and 8).

DISCUSSION

These studies identify a new protein, called epinemin, which is recognized by a monoclonal antibody and associated only with vimentin filaments in non-neural cells and skeletal muscle. Antiepinemin is almost certainly an autoantibody, since it does not recognize the immunogen, chicken gizzard, either by autoradiography or by immunofluorescence in fresh frozen sections. In addition, smooth muscle from another source, rat proximal colon, was negative by immunofluorescence.

Biochemical Characterization of Epinemin and Comparison with Actin, Vimentin, and Desmin

The results show that epinemin has a molecular weight of 44,500, and a pI of 5. These parameters did not vary under a number of experimental conditions, thus making it unlikely that epinemin is a breakdown product of a higher molecular weight component or that its position in isoelectric focussing gels was induced either by the amount of protein loaded or by deamination due to length of time in 9 M urea. Epinemin is unlikely to be a cytoplasmic molecule nonspecifically associated with vimentin filaments since it is not removed even after 4 min in 1% Triton X-100.

Epinemin/Actin

When actin and epinemin were compared by two-dimensional gel electrophoresis, it was found that affinity-purified antiactin never recognized epinemin nor did a crude IgG fraction of the same antisera. Cleveland peptide mapping reinforced these results, actin and epinemin having a different sequence of peptides, and therefore it seems clear that epine-

min is not a degradation product of actin and is unlikely to be a separate actin species as described by others (10).

Epinemin/Vimentin/Desmin

The molecular weight of epinemin is very different from vimentin (58,000), the ubiquitous species of intermediate filament found in nearly all cultured cells (28, 29) and recently in some neural cells looked at by frozen sections (55). However, vimentin has a well documented (9, 13, 34) family of breakdown products which have molecular weights ranging between 58,000 to 45,000, the lower having a pI of approximately 5. Epinemin is close in both molecular weight and pI to the lower of these vimentin breakdown products, and while the experiments in this report were carried out in conditions that have been shown to minimize the breakdown of vimentin (9, 33), it was still crucial to find whether epinemin was simply another fragment derived from the vimentin parent molecule. The experiments carried out in this study indicate that this is not the case, since neither a monoclonal antibody that interacts with all classes of intermediate filaments (37) nor, and more importantly, a broad spectrum polyclonal antivimentin (8) recognize epinemin. Both these antisera label the identical number and position of vimentin breakdown products, all of which share extensive peptide homology with the parent molecule (34). For these antisera not to recognize epinemin as a breakdown product of vimentin, epinemin must have lost all its antigenic determinants and then gained new ones. This seems unlikely with only 22% difference in molecular weight between epinemin and vimentin and as little as 2–5% between the nearest vimentin breakdown product and epinemin. In addition, an entirely different breakdown product must be produced by a different vimentin-associated protease, and so far only one protease has been described (33). Further unequivocal evidence for the independent nature of epinemin comes from tryptic peptide mapping that shows that epinemin and vimentin do not share any primary peptide homology and are clearly different molecules. It should also be noted that epinemin is present in cells extracted for long periods in high Triton X-100 concentrations. This has been shown to remove vimentin breakdown products in fibroblasts (34).

Desmin also has a family of degradation products (13) produced by a similar, if not identical, protease, but it seems unlikely that antiepinemin is an antidesmin for the following reasons: (*a*) antiepinemin does not recognize smooth muscle, a rich source of desmin (29), by either immunoautoradiography or immunofluorescence, (*b*) desmin is not present in 3T3 cells (29, 49); (*c*) the monoclonal anti-IFA used in these studies which has been shown to recognize desmin strongly (37) does not recognize epinemin.

Immunofluorescence

Immunofluorescence studies showed that epinemin is present in all cultured cells expressing vimentin, including cultured neural cells, and has a distribution coincident with vimentin under many experimental conditions. The opposite was found in frozen sections of neural tissue where those glial elements that have been shown to possess vimentin (55) are negative for epinemin, a finding that possibly reflects an as yet unknown role for epinemin as may its presence in skeletal muscle.

In addition, those cell types that do not contain vimentin (28, 29) but are a source of other intermediate filament types, neurofilaments, glial fibrillar acidic protein, keratin, and desmin (28, 29) were found to be negative for epinemin by immunofluorescence on frozen sections indicating that the molecule does not appear to be associated with other intermediate filament subunit types. Particularly important in this respect was the absence of staining in rat proximal colon smooth muscle, a rich source of desmin (29).

Salt or Sarkosyl Extraction: Effects of Cytochalasin D and Colchicine

To further characterize epinemin, its solubility characteristics were used as a third parameter in this study. Cells extracted in Triton X-100 and subjected to either a cycle of low-high-low salt (41), a single treatment with high salt plus high Triton X-100 concentration (3, 14) or with potassium iodide (19) showed that epinemin has very different solubility characteristics from vimentin. It is well established that vimentin is resistant to high salt plus Triton X-100 extraction (14, 29) and the homology in peptides within the vimentin family (34) makes it likely that its breakdown products are similarly endowed by the mild Triton X-100 extraction employed for this experiment. The results of Granger and Lazarides (14) show that desmin is similarly resistant. However, epinemin is almost completely removed under these conditions as was actin. Not unexpectedly, treatment with low salt, which has been shown to disrupt vimentin (3) similarly fragmented epinemin and actin.

Much more dramatic, however, was the effect of the anionic detergent sarkosyl that has been shown to remove intermediate filaments from the cytoskeleton, (3) since treatment of extracted cells with this detergent completely abrogated epinemin and vimentin staining. These results indicate the considerable extent of interweaving existing within the cytoskeleton since the removal of intermediate filaments partially destroyed the actin network even after stabilization with phalloidin. That epinemin is associated with intermediate filaments, not actin, was shown by the use of cytochalasin D, which destroyed most of the actin but left much of the vimentin/epinemin network intact. The opposite was found after treatment with colchicine, which caused the collapse (well documented by others [21, 29]) not only of microtubules and vimentin filaments but also of epinemin.

Immunoferritin Electron Microscopy

This revealed that vimentin and epinemin have very different distributions on intermediate filaments. The antivimentin used in these experiments had a close-packed, uniform distribution with no visible gaps in the fuzz of antibody coating each filament. Only intermediate filaments were so labeled and few FT molecules were found associated with other filaments or cell organelles.

In contrast, the pattern of antiepinemin label was intermittent along intermediate filaments with again no label on actin filaments or cell organelles. This type of distribution could have been caused by less efficient penetration of antiepinemin into cells due to the size of the IgM antibody (~30 nm diameter [1]) but it seems unlikely because (*a*) cells extracted in high Triton X-100 (0.5% for 3 min) showed the same discrete distribution (not shown) and (*b*) peripheral intermediate filaments in areas devoid of plasma membrane had the same distribution as those deeper inside the cell. The high

concentration of antibody used (3 mg/ml) makes it unlikely that the discrete pattern of label was caused by lack of saturation of available binding sites. The possibilities of the redistribution or removal of antiepinemin by cross-linking due to the anti-mouse–FT second antibody were excluded by lightly aldehyde fixing cells before and after labeling with antiepinemin. It seems likely therefore that the discrete ultrastructural distribution of antiepinemin into numerous foci is correct, and not induced.

The number of FT molecules seen (maximum cluster size of about 25 FT) indicate that at most two to three IgM molecules are found at each of these foci since only three to four FT-IgG molecules can bind to one IgE (D. Lawson, unpublished observation), a much smaller molecule than IgM (20). It is not clear from these experiments whether the epinemin foci have any radial symmetry, are at the sides of intermediate filaments like synemin (15), or have the helical distribution seen for both another IgM monoclonal that labels vimentin filaments with a repeat distance of 31 nm (1) and the 200,000 mol wt subunit of neurofilaments which has a repeat distance of 140 nm (43, 53). In contrast, the distance between epinemin foci varied considerably in these experimental conditions suggesting that epinemin labels a polypeptide whose distribution is not simply related to the vimentin core polymer (18). These observations, taken in conjunction with the Coomassie-Blue-stained gels, show that the amount of epinemin in cells is small (~5–10% by densitometry) compared with vimentin and make it unlikely that epinemin is a major component of the vimentin core.

While some antiepinemin foci were found apparently bridging between adjacent intermediate filaments, it seems probable that this is due to the coincident apposition of FT label on adjacent filaments rather than any specific labelling of a filament-bridging structure such as 2–3-nm filaments that are known to bridge intermediate filaments (39, 41). Furthermore, the molecular weight of epinemin (44,500) argues against it forming filament side arms over any appreciable distance, as has been suggested for the high molecular weight protein synemin (15) and the 200,000-mol-wt subunit of neurofilaments (11, 43, 53). A functional role for epinemin is at present being investigated by microinjection and the three dimensional relationship of epinemin to other cytoskeletal components by antibody labelled helium cooled deep etched cytoskeletons.

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