Vimentin: A Phosphoprotein under Hormonal Regulation

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ABSTRACT   Past studies of norepinephrine-stimulated protein phosphorylation in intact C-6 glioma cells had identified a 58,000 molecular weight, 5.7 isoelectric point protein (58K-5.7) as a cyclic AMP-dependent phosphoprotein and had shown that 58K-5.7 was one of the most abundant proteins of the nuclear fraction. Initial experiments of present studies showed that the 58K-5.7 protein remained with the nuclear ghost, or matrix structure, after removal of chromatin. Based on the size, acidity, abundance, nonsolubilization by nonionic detergent and salt, and solubilization by urea, the hypothesis was advanced that the 58K-5.7 protein was the vimentin-type intermediate filament protein. The hypothesis was tested by two types of immunochemical experiments. Antisera against hamster vimentin reacted selectively with only the 58K-5.7 protein in polyacrylamide gels of urea-solubilized cellular residues (i.e., nonionic detergent and 0.6 M salt-insoluble material) as determined by immunoautoradiography. Antisera against the pure 58K-5.7 protein of C-6 cells bound selectively to a fibrous array of cellular material typical of vimentin filaments as determined by indirect immunofluorescence. It is concluded that the 58K-5.7 protein is vimentin.

A number of hormones that interact with receptors on the cell surface have been shown to exert their effects on the cell by raising the intracellular concentration of the second messenger, cyclic AMP. These hormones include norepinephrine (acting at β-adrenergic receptors), glucagon, ACTH, prostaglandins, and others (32). The evidence is mounting that cyclic AMP acts in higher eukaryotes by stimulation of specific protein phosphorylations catalyzed by cyclic AMP-dependent protein kinases (25). When rat C-6 glioma cells are treated with norepinephrine, synthesis of several enzymes is induced (3, 14, 24, 36), the cells change shape (28), nerve growth factor is synthesized and released (37), and glycogenolysis is activated (8). We have investigated the changes in protein phosphorylation in intact C-6 cells after norepinephrine treatment and found that phosphate content in eight polypeptides reproducibly and significantly increases within 5 min (17, 34). One of these polypeptides, previously designated 58K-5.7 for its molecular weight and isoelectric point, is a major protein in the cell and copurifies with nuclei in subcellular fractionation experiments (17). We report here the identification of the 58K-5.7 protein as the vimentin-type intermediate filament protein of the cytoskeleton of these cells.

MATERIALS AND METHODS

Cell Culture

C-6 glioma cells were grown to postconfluent density on the surface of glass roller bottles or glass cover slips in Ham's F-10 medium as described previously (8, 9). Some of the later roller bottle cultures used for 58K-5.7 protein purification were grown in 10% calf serum in place of fetal calf serum with no apparent change in cellular content of the 58K-5.7 protein.

Selective Extraction and Purification of the 58K-5.7 Protein

Surface cultures in roller bottles (2,660 cm² of surface area for two bottles), containing ~100 mg of cellular protein, were washed three times at 0°C with 200 ml/bottle of Ham's F-10 medium and once with 10 mM HEPES, 2 mM MgCl₂, pH 7.4 (HEPES-Mg). Cells were scraped into 100 ml/bottle HEPES-Mg, the bottle was rinsed with 100 ml HEPES-Mg, and pooled material was centrifuged as 10,000g for 10 min at 0°C. Cellular pellets were dispersed by homogenization in 70 ml HEPES-Mg containing 0.5% Nonidet P-40 (NP-40). The homogenate was stored for 10 min on ice and centrifuged for 10 min at 20,000g (standard centrifugation conditions for the remainder of the procedure). The resulting pellets were resuspended at room temperature in 35 ml of HEPES-Mg-NP-40 containing 0.6 M KCl and the protease inhibitors, 0.1 mM phenylmethylsulfonylfluoride and 1 mM sodium tetrathionate. The pellets were immediately homogenized vigorously in a Teflon glass homogenizer and allowed to stand at room temperature for 10 min. This homogenate was centrifuged at room temperature and the KCl extraction was repeated. The pellet was resuspended at room temperature in HEPES-Mg containing 4 M urea at pH 8.0 and dispersed by vigorous homogenization. This suspension was allowed to stand for 30 min and centrifuged. Approximately one-half of the 58K-5.7 protein was released by the 4 M urea treatment. The 4 M urea extract was dialyzed to remove excess urea and lyophilized. That the band present in 4 M urea extract and in the final pellet were the same polypeptide was confirmed by peptide mapping according to Bordier and Crettol-Jarvinen (6) (data not shown).

The 58K-5.7 protein was purified from the 4 M urea extract and from the final pellet by two cycles of SDS-gradient slab gel electrophoresis. The lyophilized 4 M urea extract was taken up in 0.5 ml of 4 M urea and prepared for electrophoresis (see Electrophoresis Conditions). The final pellet was taken up in 1 ml of water and dispersed by sonication (Branson model 185D sonifier, microtip, 40 s, setting 4 [Branson Sonic Power Co., Danbury, Conn.]) which

reduced the viscosity of the sample. The sonicate was made up to the same final concentration of glycerol, SDS, mercaptoethanol and Tris as the 4 M urea extract. The solubilized samples were then electrophoresed separately. The gels were stained with 0.2% Coomassie Brilliant Blue R in 50 mM Tris buffer, pH 6.8, for 15 min, destained for a few minutes, and the 58K band was cut from each gel. The partially purified material was stored overnight at 0°C, pooled, homogenized in 1 ml of sample buffer, and applied to the top of a second gel and electrophoresed, stained, and cut from the gel as before. The 58K–5.7 protein was electrophoretically eluted from the second gel using an ISCO model 1750 electrophoretic concentrator (Instrumentation Specialties Co., Lincoln, Nebr.). The strip of gel was chopped and placed in a Nylon mesh basket above the small cup of the apparatus. The electrode buffer was 40 mM Tris acetate, 0.2% SDS, 2 mM EDTA, pH 8.0 and the cup buffer was 4 mM Tris acetate, 0.2% SDS, 0.2 mM EDTA, pH 8.0. Elution was performed at 10 mA during two 90-min periods.

Electrophoresis Conditions

Two-dimensional polyacrylamide gel electrophoresis was conducted by electrofocusing in the first dimension and discontinuous SDS gel electrophoresis in the second dimension as described previously (17). A 5–16% log-linear gradient gel was used for the second-dimensional gel. One-dimensional electrophoresis was conducted using essentially the same gradient slab gel as for two-dimensional separations. Samples for one-dimensional separations were made up in the sample buffer of Laemmli (22), by mixing 2:1 with a three-times concentrate of the sample buffer.

Immunoautoradiography

The experimental approach was essentially that of Burridge (10) as used for the intermediate filament protein by Hynes and Destree (18). Briefly, the 58K–5.7 protein was electrophoresed in the gradient SDS gel system used for protein purification and slices of gel were cut along the molecular weight axis. One slice was fixed, stained with 0.2% Coomassie, and destained. The other slices were fixed but not stained and these gels were equilibrated with buffer A (10). The unstained gel slices were then exposed overnight to antivimentin serum diluted 1:10, washed for 36 h, and exposed overnight to ^{125}I-goat anti-rabbit serum (GAR) and again washed. The resulting gel slices were dried against filter paper and subjected to autoradiography using Kodak XB–5 film and a Kodak X-omatic fluorescence enhancement screen. The ^{125}I-GAR was prepared using a lactoperoxidase-glucose oxidase iodination kit with GAR bound to 1 ml of Sepharose-rabbit IgG gel. The Sepharose–IgG was prepared by reacting 5 mg of rabbit IgG fraction with 1 ml of cyanogen bromide-activated Sepharose 4B. The resulting ^{125}I-GAR was essentially pure IgG as indicated by polyacrylamide gel electrophoresis.

Immunization of Rabbits

Rabbits were immunized with ~200 μg of pure 58K–5.7 protein in polyacrylamide gel. The gel was emulsified in Freund's complete adjuvant and injected subcutaneously. At monthly intervals thereafter, booster injections of 50–200 μg of 58K–5.7 were given either in gel or in phosphate-buffered saline, in each case emulsified with Freund's complete adjuvant. Animals were bled 1 wk after each booster injection. Control animals received injections of Freund's complete adjuvant emulsified with either gel or phosphate-buffered saline as appropriate.

Immunofluorescence

An indirect immunofluorescence procedure was used which employed the sera resulting from the above immunization as the primary antibody and utilized fluorescein-conjugated GAR–IgG as the indicator antibody. Fixation of C-6 cells, permeabilization, and incubations with antibodies were conducted essentially as described by Hynes and Destree (18), except that the C-6 cells were washed for 30 min at pH 9.5 after the reactions with the rabbit antibody to elute nonspecifically bound protein (4). Cover-slip samples of cells were mounted in 50% glycerol in phosphate-buffered saline and examined in a Zeiss Universal microscope equipped for epifluorescence using FITC filters.

Materials

Rabbit antiserum against hamster NIL–8 cell vimentin-type intermediate filament protein was a gift from Dr. Richard O. Hynes, Massachusetts Institute of Technology. GAR–IgG and fluorescein-conjugated GAR–IgG fraction was obtained from Cappel Laboratories, Cochranville, Pa. Cyanogen bromide activated Sepharose 4B was obtained from Pharmacia Fine Chemicals, Piscataway, N. J. The lactoperoxidase glucose oxidase iodination materials were obtained as a kit from New England Nuclear, Boston, Mass. *S. aureus* strain V₈ protease was obtained from Miles Laboratories, Inc., Elkhart, Ind. ^{32}Pi was obtained from ICN Pharmaceuticals, Irvine, Calif. Materials were obtained as described previously for nuclear subfractionation (33), gel electrophoresis (17), and cell culture (9).

RESULTS

The first step toward establishing the identity of the 58K–5.7 protein was to subfractionate the nonionic detergent-treated nuclear preparation. This was accomplished by micrococcal nuclease treatment and stepwise salt extraction (33). The procedure generates a fraction of soluble nuclear proteins (S0), four structurally different nucleosome subfractions (SS2, SS3, SS4, and SS6), and a residue (P6). Samples from the intact nuclei and the nuclear subfractions were coelectrophoresed with whole cell extracts in two-dimensional polyacrylamide gels (17, 29). The ^{32}P-labeled 58K–5.7 protein is present in the nuclei as indicated by autoradiography (Fig. 1 B) and by increased abundance of the spot in the stained gel (Fig. 1 A). It is essentially absent from S0 and SS2 autoradiograms (Fig. 1 D and F) and the relative intensity of the stained spot in these subfractions is typical of whole cell extract alone. It was also absent from SS3, SS4, and SS6 (not shown). The 58K phosphoprotein was enriched in the P6 fraction (Fig. 1 G and H) which includes the nuclear matrix or ghost structure, depleted of chromatin, but with cytoskeletal elements stable to the nuclear fractionation conditions still attached. For example, vimentin-type intermediate filaments are known to resist both the nonionic detergent and the 0.6 M NaCl extractions of cultured cells (7, 39).

A number of reagents, including detergents, reducing agents, chelating agents, and denaturants were tested as agents for solubilizing the 58K–5.7 protein from the nuclear ghost. Of these, SDS was effective but completely unselective, and most of the other agents were completely ineffective. However, 4 M urea solubilized one-half or more of the 58K–5.7 protein (data not shown). Urea is known to solubilize several intermediate filament proteins including vimentin from the nonionic detergent-treated cellular residues (39). From its abundance, size, isoelectric point, subcellular distribution, and solubilization characteristics, the working hypothesis was advanced that the 58K–5.7 protein was the vimentin-type intermediate filament protein of the C-6 glioma cell (see Discussion).

The 58K–5.7 protein was purified to provide material for further testing of its identity. Cultures were successively extracted with nonionic detergent and salt to solubilize most extraneous cellular proteins. The residues from these extractions were solubilized with urea or SDS and purified by two cycles of SDS-gradient slab gel electrophoresis. The product was pure in that it gave rise to a single spot in a two-dimensional gel at high load (Fig. 2 A). That the pure protein was unaltered during purification was substantiated by coelectrophoresis of the purified material with the 58K–5.7 protein of C-6 cell whole cell extract (Fig. 2 B and C).

The reactivity of the 58K–5.7 protein with antisera to the vimentin-type intermediate filaments of cultured hamster NIL–8 cells (18) was tested using the immunoautoradiographic procedure of Burridge (10). Fig. 3 shows stained polyacrylamide gel strips and autoradiograms of separate slices from the same gels that have been incubated with the rabbit antiserum against vimentin and subsequently with ^{125}I-GAR. Panels *A–C* show the 4 M urea extract of the cells and panels *D* and *E* show the final purification gel of a separate experiment. The ^{125}I-GAR second antibody showed some reactivity with the low molecular weight proteins of the gel (*A*) but when antibody
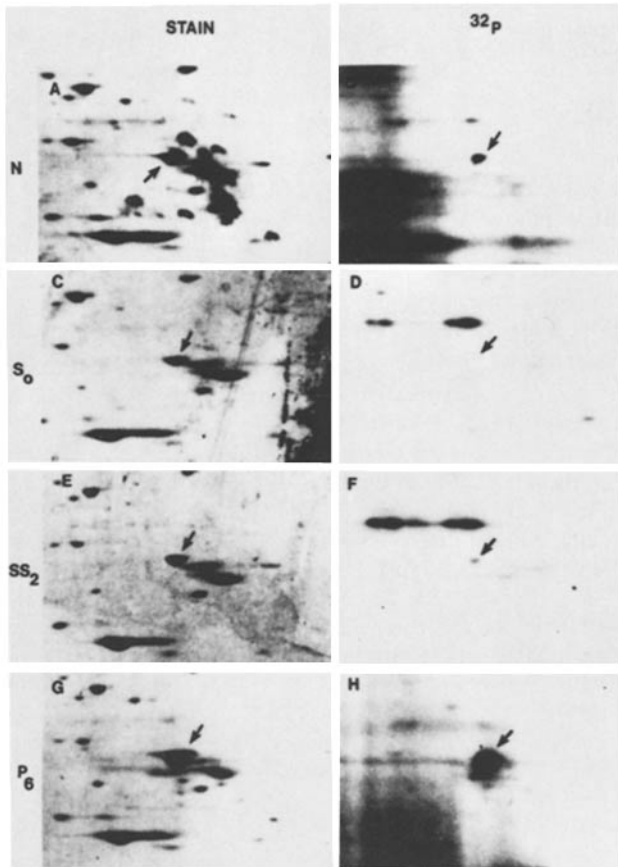
FIGURE 1   Salt fractionation of nuclease-digested nuclei to establish nuclear location of the 58K–5.7 protein. A roller bottle culture (230 cm² of surface) of C-6 glioma cells was washed with 100 ml of Ham's F-10 medium followed by 100 ml Ham's F-10 containing one-sixth the normal amount of phosphate (low phosphate Ham's medium) containing 6 mCi (1 Ci = 3.7 × 10¹⁰ bequerels) ³²Pi for 4 h at 37°C. At the end of the incubation, the medium was aspirated and the cells were scraped from the bottle surface into 10 ml of Earle's balanced salt solution. They were collected by centrifugation at 1,000 rpm at room temperature, loaded with glycerol and lysed by hypotonic shock (19) in 1 mM Tris, 25 M KCl, 0.9 M MgCl₂, 0.9 mM CaCl₂, 0.14 mM spermidine, pH 7.6 (C buffer). Nuclei were collected by centrifugation at 3,000 rpm for 10 min at 4°C in a Sorvall HB-4 rotor. They were washed once with C buffer containing 0.5% NP-40 and resuspended in 0.5 ml of C buffer. After digestion with 100 U of micrococcal nuclease for 5 min, the digested nuclei were centrifuged as before and extracted stepwise with C buffer containing 0.2, 0.3, 0.4, and finally 0.6 M NaCl (33). Samples of the whole nuclear digest (N), each of the solubilized subfractions (designated S_0, SS_2, SS_4, and SS_6 with the subscript indicating the NaCl concentration at which the fraction was solubilized) and the residue remaining after all the nucleosomes had been eluted ($P6$) were precipitated with 2 vol of ethanol at −20°C for 16 h and solubilized (17) for gel electrophoresis.

Subfractions of ³²P-labeled C-6 nuclei were coelectrophoresed in two dimensions with unlabeled whole cell extracts as described in Materials and Methods. The gels were stained, dried, and autoradiograms were prepared. The arrow indicates the known position of the 58K–5.7 protein. Stained gels: whole cell extract plus intact nuclei (A), subfractions S_0 (C), SS_2 (E), and $P6$ (G). Autoradiograms: nuclei (B), S_0 (D), SS_2 (F), and P_6 (H).

against vimentin had been present (*B*), only one additional band was labeled. This antivimentin antibody-reactive band was the 58K–5.7 band. Binding of the rabbit antivimentin
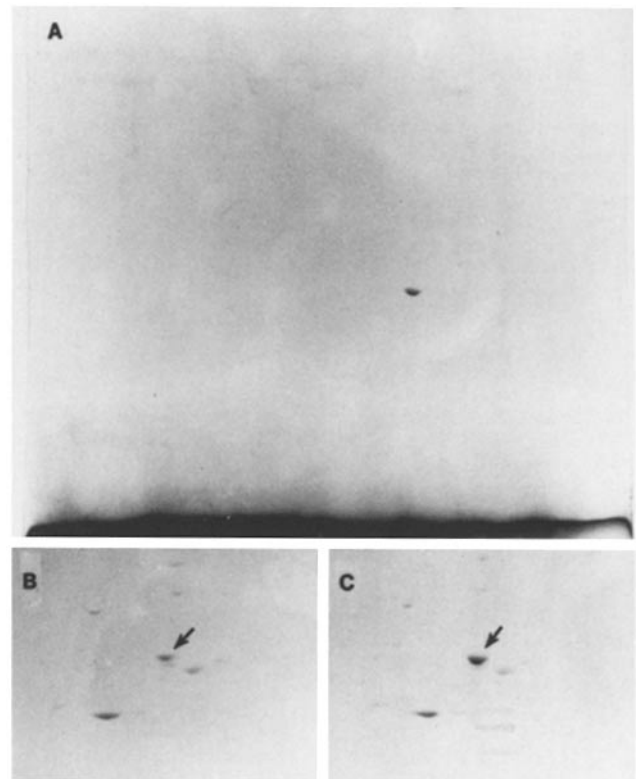
FIGURE 2   Purity of the 58K–5.7 following selective extraction and purification in polyacrylamide gels. The purified product was electrophoresed in two-dimensional gels as described elsewhere (17) either alone (panel *A*) or mixed with whole cell extract (*C*). A sample of whole cell extract alone at equivalent load is presented in panel *B*. The arrow indicates the 58K–5.7 protein in panels *B* and *C*.

serum to 58K–5.7 was also observed in the final purification gel (*D*) where the 58K–5.7 protein band was pure. Therefore, antifilament antibody reacted selectively with the 58K–5.7 protein. This result strongly supports the conclusion that the 58K–5.7 protein is vimentin.

A second type of immunochemical experiment consisted of raising antibody to pure 58K–5.7 from C-6 cells and conducting an indirect immunofluorescence evaluation of its reaction with fixed C-6 cells adherent to cover slips. Fluorescence micrographs shown in Fig. 4 (*A* and *B*) indicate that the antiserum to 58K–5.7 reacted with a fibrous extranuclear network that formed a "halo" or swirl around the nucleus and pervaded the cytoplasm of the cell. Preimmune serum (Fig. 4 *C*) gave a more general fluorescent reaction with only a slight reaction with cytoskeletal fibers. Reaction of the cells to fluorescein-labeled GAR alone produced only a low level of generalized fluorescence (Fig. 4 *D*). The fibrous fluorescent pattern seen with the antiserum to 58K–5.7 remained after a 2-h incubation of cells at 0°C before fixation, indicating that the fibers were not cold sensitive and therefore were probably not microtubules (Fig. 4 *E*). In experiments not shown, treatment of the cells with cytocholasin B or colcemid caused the fluorescent structures to condense into aggregated cablelike structures. Comparison of the fluorescence pattern with that seen with conventional phase-contrast microscopy indicated that the fluorescent structures were often identified with phase-dark elements of the cytoplasm, often particularly easily identified near the margins of the cell (Fig. 4 *F*, *G*, arrows). This phase-dark material
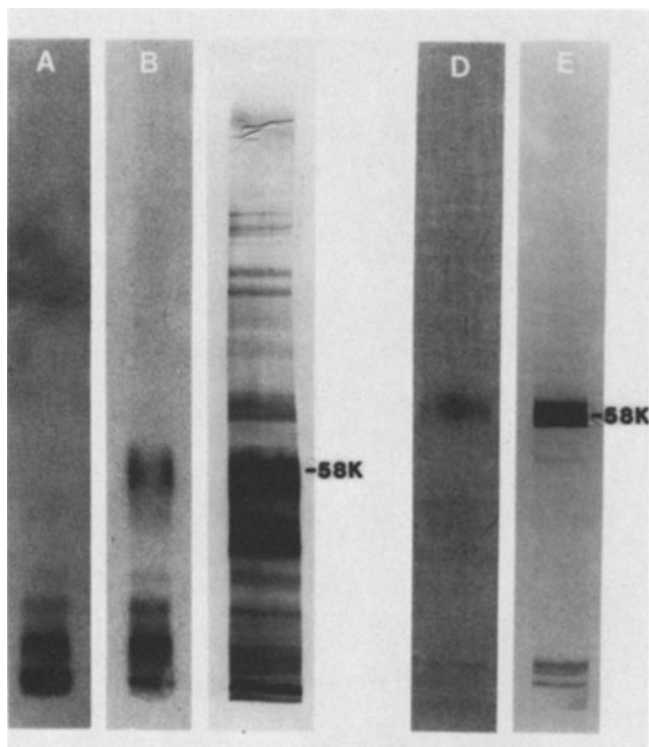
FIGURE 3 Cross-reaction of antiserum to hamster vimentin with 58K-5.7 protein of rat C-6 cells. Strips of slab gels that resolved either the 4 M urea-solubilized residues (A-C) or the "one cycle" purified material (D, E) were cut and either stained with Coomassie brilliant blue (C, E) or exposed to antisera as described in Materials and Methods. A is an autoradiogram showing the reaction of ^{125}I-GAR alone with proteins of the gel. B and D are autoradiograms showing reactions of the antivimentin serum followed by exposure to ^{125}I-GAR.

accounts for a significant fraction of the internal fibrous material of the cells visible by phase-contrast microscopy. Comparison of visual fields using phase and fluorescence optics indicated that most, if not all, cells contained the fluorescent structures. It is concluded that the fibrous fluorescence pattern was due to the 58K-5.7 protein and that the protein is a constituent of a fibrous cytoskeletal network that has been described for vimentin-type intermediate filaments (15, 16, 18, 40).

DISCUSSION

Present work demonstrates that a 58,000-dalton, norepinephrine-dependent phosphoprotein (58K-5.7) of intact C-6 glioma cells is the vimentin-type intermediate filament protein of the cytoskeleton of these cells. The evidence for this conclusion is of three types. First, the two proteins share a number of physical properties. Vimentin is a 54–58,000-dalton polypeptide (4, 15, 18, 39) as is 58K-5.7 and both proteins are acidic (7, 17). The 58K-5.7 protein is one of the 10 or so most abundant proteins in C-6 cells; vimentin is also a highly abundant protein in other cell types (7). Vimentin resists solubilization from cellular material by nonionic detergents (7) and by 0.6 M salt (4, 39) but is solubilized by urea or SDS (39). We showed previously that the C-6 58K-5.7 protein resisted solubilization by nonionic detergents and remained associated with the nuclear fraction after this treatment (17). Present experiments show that the protein is resistant to 0.6 M KCl

extraction of the nuclear fraction (Fig. 1) but is partially solubilized by urea at low ionic strength and completely solubilized by SDS (Materials and Methods). It was previously established that the protein is distinct from tubulin (17).

The second type of evidence that the two proteins are related is that antiserum against vimentin of hamster cells cross-reacts with the 58K-5.7 protein. The vimentin antiboy reacted not only with the 58K-5.7 protein in purified form but also selectively reacted with the 58K-5.7 protein in the gel that resolved the 4 M urea-solubilized material.

The third line of evidence that the 58K-5.7 protein was the intermediate filament subunit came from indirect immunofluorescence studies. Rabbits were immunized with pure 58K-5.7 from C-6 cells and the resulting antiserum was used to test for localization of 58K-5.7 protein within C-6 cells. The fluorescence pattern was a halo of fibrous material surrounding the nucleus and a fibrous network that spread throughout the cytoplasm of the cell. The nuclei were essentially unstained. This pattern of fluorescence was typical of that seen with the vimentin-type intermediate filaments using similar techniques in other cell types (15, 16, 18, 40).

Hormone-dependent phosphorylation of vimentin has not previously been observed in intact cells. That vimentin was phosphorylated on a small fraction of its copies was described for two cell types (12, 26) but modulation of phosphorus content by cyclic AMP was not demonstrated. Our observation of the norepinephrine-stimulated phosphorylation showed a twofold increase in ^{32}P content of vimentin following hormone treatment (17). The change due to norepinephrine can be increased to four to fivefold by the addition of actinomycin D to the incubation fluid along with the ^{32}Pi; actinomycin D decreases the ^{32}P incorporation into vimentin during the ^{32}P equilibration.[1]

A report of in vitro studies has appeared (27) that showed phosphorylation of vimentin by two kinases that seemed to be the cAMP-dependent protein kinases. However, interpretation of the in vitro findings are difficult. The catalytic subunit of cyclic AMP-dependent protein kinase is a rather nonspecific enzyme that phosphorylates many exposed serine or threonine residues which are 2–3 amino acid residues to the carboxyl terminal side of two adjacent basic amino acids (21). Because many such sequences become exposed during protein denaturation (11), in vitro demonstration alone of phosphorylation by cyclic AMP-dependent protein kinase may be of limited significance. For example, histone H2b is extensively phosphorylated in vitro by cyclic AMP-dependent protein kinases but most cell types contain no detectable phosphorylated H2b in vivo (11, 20, 34). Also, the pattern of phosphorylation of erythrocyte membrane that occurs in the intact cell is quite different from that which occurs in vitro (41). Present findings in intact cells are not inconsistent with the in vitro results, but designation of vimentin as a substrate for cyclic AMP-dependent protein kinase in vivo must await characterization of the phosphorylation site. Phosphorylation by another protein kinase that is activated indirectly by norepinephrine is in no way excluded.

The glial origin of the C-6 cell line raises the consideration of another fibrillary protein, the glial fibrillary acidic protein (GFA), with respect to the identity of the 58K-5.7 protein. Both GFA and vimentin form 10-nm filaments in glial cells (30, 42), and the two proteins have similar solubility properties
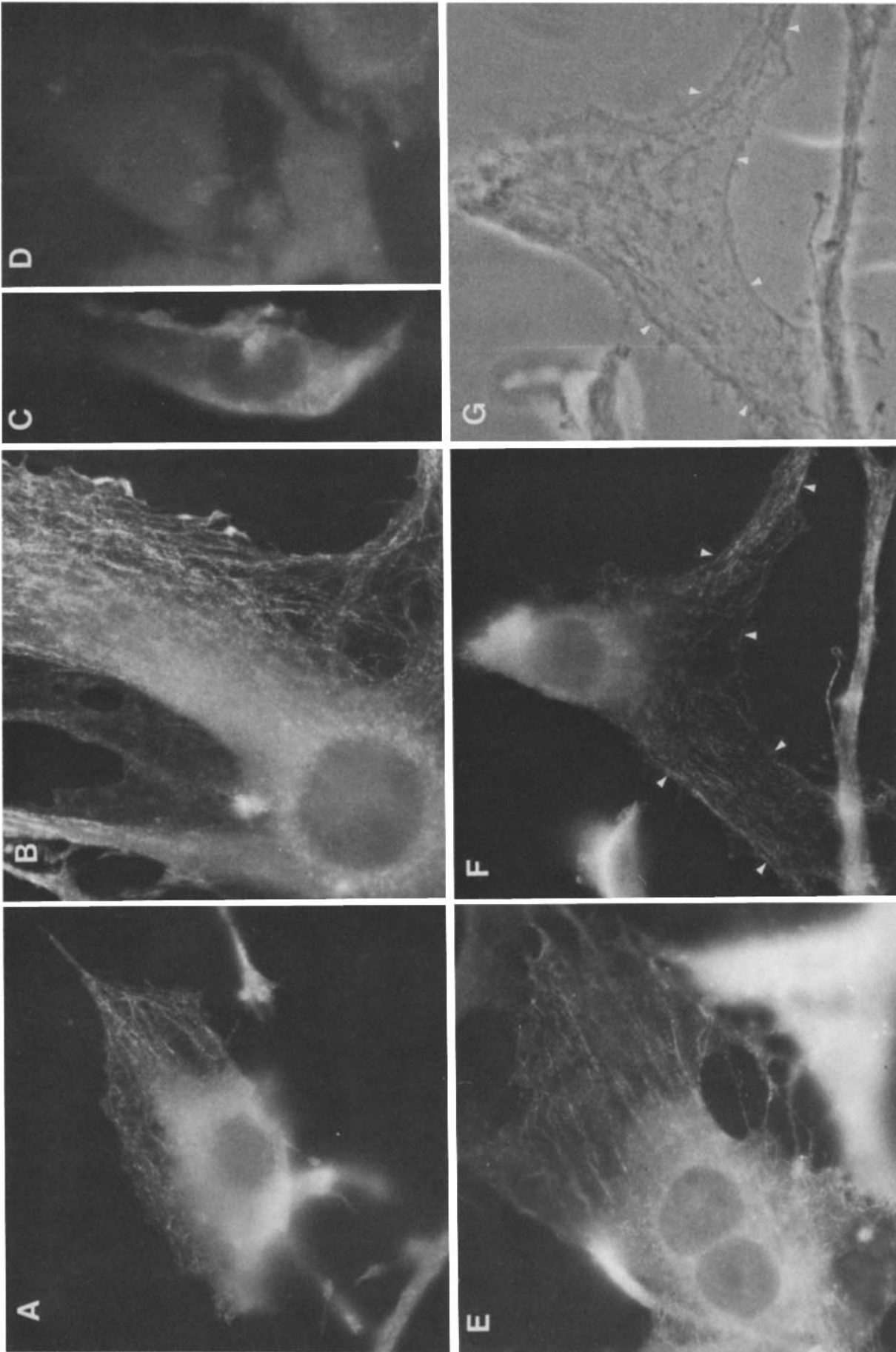
FIGURE 4 Immunofluorescence of C-6 glioma cells treated with anti-58K–5.7 serum. Details of the indirect immunofluorescence procedure are presented in Materials and Methods. Panels A and B show the reaction of antiserum against 58K–5.7 protein with C-6 cells during log-phase growth. Panel C shows the reaction of cells with preimmune serum and panel D the reaction with fluorescein-conjugated GAR alone. Panel E shows the fluorescence pattern observed after incubation of the cells at 4°C for 2 h before fixation. Panels F and G are fluorescence (F) and phase micrographs (G) of a field observed through the same optical system.

(31). 58K–5.7 coincides in molecular weight with vimentin (54–58,000) but is slightly larger than GFA (13). GFA is present in only a small fraction of C-6 cells especially in subconfluent surface cultures (5, 23); however, antibody against 58K–5.7 reacted with filaments in essentially all of the cells of such cultures (present work), suggesting that 58K–5.7 is different from GFA. Finally, antibody against vimentin reacted with the 58K–5.7 protein of the 4 M urea extract of C-6 cell residues (present work). These observations support the view that the 58K–5.7 protein is distinct from the GFA protein.

Hormone-dependent phosphorylation of vimentin in the intact cell carries with it the implication of a functional role for these structures in the mediation of norepinephrine effects on C-6 cells. That the protein is phosphorylated on only a small fraction of its copies may provide an additional clue to the role of phosphorylation of these structures. Clearly, the phosphorylation relates to the role played by only a selected few copies, at least at any one point in time. Further understanding of the localization of the phosphorylated copies within the filament might help in the understanding of the significance of phosphorylation of this cytoskeletal element.

Two classes of physiological effects of cyclic AMP elevation within C-6 cells with which vimentin phosphorylation may be associated are the induction of several enzymes (3, 14, 24, 36) and a change in cellular shape toward one with more prominent processes (28, 35). The induction of cyclic nucleotide phosphodiesterase in C-6 cells is inhibited by vinblastine (38) which disrupts microtubules and causes vimentin filaments to condense into cables and perinuclear caps (30). Possibly, the integrity of the vimentin filament and its phosphorylation are required for the phosphodiesterase induction because protein phosphorylation appears to be the major mechanism of cyclic AMP action in mammalian cells (25). An involvement of vimentin phosphorylation in cyclic AMP effects on cellular shape may also occur, possibly in concert with phosphorylative controls of other cytoskeletal elements such as myosin light-chain kinase phosphorylation by cyclic AMP-dependent protein kinase (1).

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