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Norepinephrine-Dependent Protein Phosphorylation in Intact C-6 Glioma Cells

Analysis by Two-Dimensional Gel Electrophoresis

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SUMMARY

GROPPI, V. E., JR., AND E. T. BROWNING. Norepinephrine-dependent protein phosphorylation in intact C-6 glioma cells: Analysis by two-dimensional gel electrophoresis. *Mol. Pharmacol.* **18:** 427–437 (1980).

Norepinephrine rapidly induced phosphorylation of 7 of the more than 200 neutral and acidic phosphoproteins which were resolved by two-dimensional gel electrophoresis of a whole cell extract of C-6 glioma cells. Cellular ATP pools were nearly optimally labeled by $^{32}P_i$ after a 4-hr exposure, and norepinephrine treatment did not modify the specific radioactivity of the γ-phosphate of cellular ATP. That the ^{32}P-labeled spots of two-dimensional gels were indeed phosphopolypeptides was verified by comparing the migration of ^{35}S-methionine-labeled and $^{32}P_i$-labeled samples and by extensive solvent extractions and chemical treatments. Subcellular fractionation resulted in assignment of a subcellular compartment to five of the seven norepinephrine-dependent phosphopolypeptides. These modified proteins were designated the "phosphoprotein domain" of norepinephrine for the C-6 glioma cell and each was provisionally named in terms of molecular weight, isoelectric point, and, where possible, subcellular compartment. Accordingly the proteins were named: 58K-5.7-nuclear; 50K-6.1, 48K-6.8-cytosolic; 38K-6.4-cytosolic; 20K-6.2, 19K-6.6-mitochondrial; and 16.5K-6.3-cytosolic. We have concluded that protein 58K-5.7-nuclear is the intermediate filament protein of the C-6 glioma cell based on similarities of molecular weight, isoelectric point, abundance, subcellular fractionation, nuclear binding, and elution as well as phosphorylation.

INTRODUCTION

Regulatory phosphorylative reactions are catalyzed by protein kinases, which in turn are often regulated by low molecular weight effectors such as cAMP, Ca^{2+}, and possibly cGMP. The effectors in turn generally act as second messengers mediating an extensive variety of hormonal and neurotransmitter actions. Thus protein phosphorylation appears to constitute the final regulatory step in a great variety of endocrinological and neuronal controls. However, the number of second messengers and the number of protein kinases activated by each appear to be few (1, 2), raising the question of how such a broad variety of cellular controls is channeled meaningfully through these few components. Langan has advanced the concept that the requisite degree of specificity is achieved through the selective distribution of protein substrates for the protein kinases among the various types of cells (2). The identification of the individual proteins which undergo hormone-stimulated phosphorylation in each individual cell type, therefore, becomes of general significance to understanding the regulation of cellular function. It is the purpose of the present paper to describe an experimental approach to the identification of the protein substrates of hormone-stimulated protein phosphorylation in intact cells.

The advent of high-resolution two-dimensional gel electrophoresis (3) provides a separatory method capable of resolving more than 1000 polypeptides, thus providing a powerful tool for studying regulatory protein phosphorylation in the presence of the entire complement of cellular proteins. In addition, sample preparation techniques for two-dimensional gel electrophoresis concomitantly solubilize and denature cellular material (3, 4), thereby inactivating degradative cellular enzymes, and

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provide the remaining methodology needed to study covalent protein modification as regulatory events in intact cells. Present research employs these techniques to investigate the spectrum of protein phosphorylation that occurs as a result of norepinephrine action in intact C-6 glioma cells and thereby to define the phosphoproteins which express the action of norepinephrine in these cells.

The present work has been conducted with the C-6 glioma cell line because it is a relatively differentiated clonal cell line which synthesizes S-100 protein and possesses a β-adrenergic receptor. The β-adrenergic receptor–cAMP system of these cells is relatively well understood and is known to be associated with three hormone-controlled output functions—glycogenolysis, enzyme induction, and a change in cellular shape. The present study has identified seven neutral or acidic phosphopolypeptides which are increased in phosphorus content by exposure of the intact cells to norepinephrine and we have characterized these proteins in terms of molecular weight, isoelectric point, and intracellular compartmentation. One basic phosphopolypeptide has been similarly identified and is described separately (5). An abstract of these results has appeared (6).

MATERIALS AND METHODS

Cell culture and growth conditions. Growth conditions for coverslip and roller bottle cultures of C-6 glioma cells have previously been described (7, 8). Miniwell cultures (plastic multiwell trays with 15-mm-diameter wells) were maintained similarly to coverslip cultures; each well received 1 ml of growth medium at each feeding. Cells were grown to postconfluent density in Ham's F-10 medium supplemented with 10% fetal calf serum and were used for experimentation 1 day after feeding. The properties of cells grown on the surface of coverslips, roller bottles, or miniwells were indistinguishable.

Cellular incubations. The general procedure for cellular incubations was to wash the surface cultures of C-6 cells four times with freshly prepared Ham's F-10 medium to remove serum proteins and incubate the cells in low-phosphate (0.2 mM) Ham's for the indicated times at 37°C under an atmosphere of 95% air–5% carbon dioxide.

Subcellular fractionation. Postconfluent roller bottle cultures were washed with low-phosphate Ham's medium and incubated for 4 hr in 30 ml of this medium containing 0.1 mCi $^{32}P_i$/ml. In samples used for analysis of subcellular marker enzyme activities in subcellular fractions, $^{32}P_i$ was omitted. After the 4-hr incubation, the cells were treated with 10 μM norepinephrine for 5 min, washed with 100 ml/bottle low-phosphate Ham's medium containing 10 μM norepinephrine, harvested by scraping into 10 ml of Earle's balanced salt solution per bottle, and centrifuged at 700g for 5 min at 30°C.

The harvested cells were resuspended in one-tenth the original volume of Earle's balanced salt solution containing 10 μM norepinephrine. The subsequent lysis and subcellular fractionation were a modification of the procedure of Jett *et al.* (9). The pelleted cells containing glycerol were hypotonically lysed by the rapid addition of 10 mM Tris–HCl, pH 7.4, 1 mM $MgCl_2$, 1 mM $CaCl_2$ (TMC buffer), in a volume equal to 10% of the original incubation volume, and homogenized with 10 strokes of a Dounce homogenizer using a B pestle. Lysis was judged by phase-contrast microscopy to be greater than 95%. Nuclei were collected by centrifugation at 700g for 10 min. The supernatant was carefully removed and saved, while the crude nuclear pellet was resuspended in TMS buffer equal in volume to the original lysate. Half of the nuclear suspension was made 0.5% in Nonidet P40 (NP-40), and the other half was homogenized with 10 strokes of a Dounce homogenizer as before. The supernatant above the Dounce-homogenized nuclei was removed and combined with that above the NP-40-treated nuclei. Both nuclear fractions were washed with twice the lysate volume of TMC buffer and pooled with the respective previous supernates. For enzyme assays, the isolated nuclei were resuspended in 0.25 M sucrose containing 10 mM Tris–HCl, pH 7.4, 10 mM $MgCl_2$. For two-dimensional gel electrophoresis, the nuclei were solubilized as described below.

Mitochondria were prepared from the combined nuclear supernatants from the Dounce-homogenized material. The sample was first centrifuged, 700g, for 10 min to remove any contaminating nuclei. The pellet was discarded and the mitochondria were collected at 4000g for 10 min. The mitochondrial pellet was resuspended in 225 mM mannitol, 75 mM sucrose, 1 mM EDTA and centrifuged at 7000g for 10 min. The pellet was resuspended in a small volume of 225 mM mannitol, 75 mM sucrose, 1 mM EDTA for enzyme assays or solubilized as described below for electrophoresis.

The plasma membrane fraction was prepared from the postmitochondrial supernate essentially according to Jett *et al.* (9). The plasma membranes were resuspended in a small volume of TMC buffer for enzymic analyses or solubilized as described below. In some experiments, the cytosol was concentrated in a 3-ml Amicon ultrafiltration apparatus fitted with a UM-2 membrane.

Enzyme assays and protein were as described by Jett *et al.* (9) except for succinic dehydrogenase, which was measured according to Kuff and Schneider (10).

Sample preparation for gel electrophoresis. Intact cells of miniwell cultures and pelleted particulate fractions from the subcellular fractionation experiment were solubilized directly in 10 M urea, 0.2% SDS, 20 mM lysine, and 2.5 mM $ZnCl_2$ (pH 4.8) (S_1A_1 buffer) at room temperature, a modification of the procedure of Peterson and McConkey (4). Cytosol- and NP-40-solubilized nuclei were prepared by adding urea, SDS, lysine, and $ZnCl_2$ to achieve the final concentrations cited above.

The nucleic acid in the viscous extracts was digested with 70 units of phosphoprotein phosphatase-free S_1 nuclease (see below) per A_{260} at 45°C for 5 min (4). Nuclease incubations were terminated with the addition of 0.5 M Tris–HCl, pH 7.4, 16% NP-40 (S_1 C buffer), equal in volume to one-fifth the amount of S_1A_1 solubilization buffer, at room temperature. Samples were either immediately electrofocused or stored at −70°C.

Commercial preparations of S_1 nuclease have a contaminating phosphoprotein phosphatase activity. The nuclease and phosphoprotein phosphatase activities

could be separated by electrofocusing, but the procedure was time-consuming and the yields were low. An alternative procedure for the elimination of the contaminating phosphoprotein phosphatase activity took advantage of the instability of the contaminant at pH 4.8. Immediately prior to use the S_1 nuclease was diluted 1:5 in 0.03 M sodium acetate, pH 4.8, 0.05 M NaCl, 1 mM $ZnCl_2$, and 5% glycerol and incubated at 45°C for 20 min. Under these conditions, the nuclease activity was unchanged, while the phosphoprotein phosphatase activity was inactivated. Autoradiograms of two-dimensional gels from samples prepared with either phosphatase-free S_1 nuclease or phosphatase-inactivated S_1 nuclease were identical. ^{32}P-phosphorylase *a* which was added to S_1A_1 buffer-solubilized nonradioactive cell extracts and incubated with phosphatase-inactivated S_1 nuclease showed no loss of ^{32}P from protein during the sample preparations procedure.

Two-dimensional gel electrophoresis. Two-dimensional gel electrophoresis was performed according to the method of O'Farrell (3), with the following modifications: The first-dimension electrofocusing gels contained the following Ampholines (LKB): 0.4% pH range 3.5–10, 0.4% pH range 4–6, 0.4% pH range 6–8, and 0.8% pH range 5–7. Samples containing equal amounts of protein were made 10 M in urea, 2% in Ampholines (pH 5–7), and 5% in mercaptoethanol, loaded on top of prefocused gels, and overlaid with 10 μl of overlay solution (5) containing 0.4 mg/ml Evan's blue dye (p*I* = 4.0) (11).

Samples were electrofocused at room temperature for 15 h at 400 V followed by 2 h at 800 V. The gels were extruded from the tubes and equilibrated with three changes of 5 ml of SDS sample buffer (3) for 45 min and stored at −70°C until electrophoresis in the second dimension. The pH gradient was obtained by standard procedures on slices of gel, always using a gel run with a sample comparable to others of the experiment.

Discontinuous gradient SDS slab gels were used to separate proteins by molecular weight in the second dimension (3). A linear-exponential polyacrylamide gradient gel formed with an LKB Ultrograd electronic gradient mixer was used as the separation gel. A 5 to 16% linear-exponential gradient produced the best overall separation of proteins from a whole cell extract. A double slab gel apparatus was used, similar to the single apparatus used by O'Farrell (3).

Staining, drying, and autoradiography. Following electrophoresis in the second dimension, the slab gels were stained in 0.1% Coomassie brilliant blue, 50% methanol, 10% acetic acid for 15 min and destained in 5% methanol, 10% acetic acid overnight. Gels were dried onto a Whatman 3 MM filter paper backing using a Bio-Rad gel drier. Dried gels were exposed to Kodak XR-1 or SB-5 X-ray film for the times indicated in the figure legends. X-Ray film was processed by the standard procedures.

Densitometry. Norepinephrine-dependent ^{32}P-phosphoprotein changes in autoradiograms from matched hormone-treated and control samples were identified by visual inspection. Autoradiograms were overlaid and compared over a light box. Visual interpretations were verified and quantitated with a Joyce–Loebl 3CS microdensitometer equipped with an integrator. The instrument was set up to scan the entire ^{32}P-phosphoprotein spot of interest. Each spot was scanned and integrated repeatedly and the reproducibility of an integrated area of any protein spot was ±5%.

Preparation of purified proteins. Phosphorylase *b* was prepared from rabbit skeletal muscle by the method of Fischer and Krebs (12). Twice-crystallized phosphorylase *b* migrated as a single band on an SDS gradient gel (data not shown).

Tubulin from C-6 cells was prepared as described by Wiche and Cole (13) for these cells. C-6 tubulin purified through two rounds of polymerization and depolymerization migrated as three bands on an SDS gradient gel, while comparable brain tubulin migrated as two bands (data not shown).

Materials. Tissue culture supplies and many other materials were obtained as described (8). Supplies for two-dimensional gel electrophoresis were generally obtained from Bio-Rad Laboratories, Richmond, California, except for Nonidet P40 (NP-40) which was obtained from Particle Data Laboratories, Ltd., Elmhurst, Illinois. Nuclease S_1 from *Aspergillus oryzae* was obtained from Sigma Chemical Company, St. Louis, Missouri. Urea was "ultrapure" from Schwarz/Mann, Orangeburg, New York. Multiwell tissue culture trays were obtained from Linbro Scientific, Hamden, Connecticut. The C-6 glioma cell line was obtained from the American Type Culture Collection, Rockville, Maryland, Cat. No. CCL-107. $^{32}P_i$ was obtained from ICN, Irvine, California; L-[^{35}S]methionine was obtained from New England Nuclear Corp., Boston, Massachusetts. All other chemicals were reagent grade or the highest grade available.

RESULTS

$^{32}P_i$ Incorporation into C-6 cellular ATP. Coverslip cultures at postconfluent density were equilibrated with $^{32}P_i$ for varying time periods from 30 min to 6 h. The rate of $^{32}P_i$ incorporation into ATP was linear for the first 2 h, then decreased, approaching a plateau by 6 h. Therefore an equilibration time of 4 h for $^{32}P_i$ incorporation into cellular ATP was chosen for subsequent experiments. The effect of norepinephrine on the specific radioactivity of the γ-phosphate of cellular ATP was analyzed after a 4-h equilibration of cells with $^{32}P_i$. Norepinephrine (10 μM) treatment of the cells for 5 min did not significantly alter this specific radioactivity (111 ± 0.4 vs 112 ± 2 cpm/pmol ATP). Therefore, there was no basis for attributing changes in the phosphate content of proteins in C-6 cells treated with 10 μM norepinephrine for 5 min to an alteration in the specific radioactivity of cellular ATP.

In the above experiments, C-6 cells were labeled with $^{32}P_i$ using low-phosphate Ham's medium to increase the specific radioactivity of $^{32}P_i$. Since it was important to know if an incubation in low phosphate affected the high-energy phosphate compounds of C-6 cells, ATP and creatine phosphate concentrations were examined. Cells incubated for 4 h in a low-phosphate Ham's medium had only slightly lower concentrations of ATP (24 vs 28 nmol/mg protein) and creatine phosphate (20 vs 25 nmol/mg

protein) than did cells incubated in complete Ham's F-10, and these values were not altered by norepinephrine treatment. These values are well within the range of previous values obtained for C-6 cells (7).

Norepinephrine-dependent protein phosphorylation and two-dimensional gel electrophoresis of ^{32}P-labeled proteins. Cells were incubated for 4 h in $^{32}P_i$, treated with or without 10 μM norepinephrine for 5 min, then extracted, and the polypeptides were resolved by two-dimensional gel electrophoresis. The cellular extractions were accomplished by directly solubilizing intact cells in a denaturing solution containing SDS, urea, lysine, and zinc at pH 4.8, and therefore avoided proteolysis (4) and phosphate ester hydrolysis (Materials and Methods). Any manipulation that might produce charge modification of protein was avoided so that proteins resolved by two-dimensional gel electrophoresis were representative of polypeptides which existed in the cell just prior to solubilization.

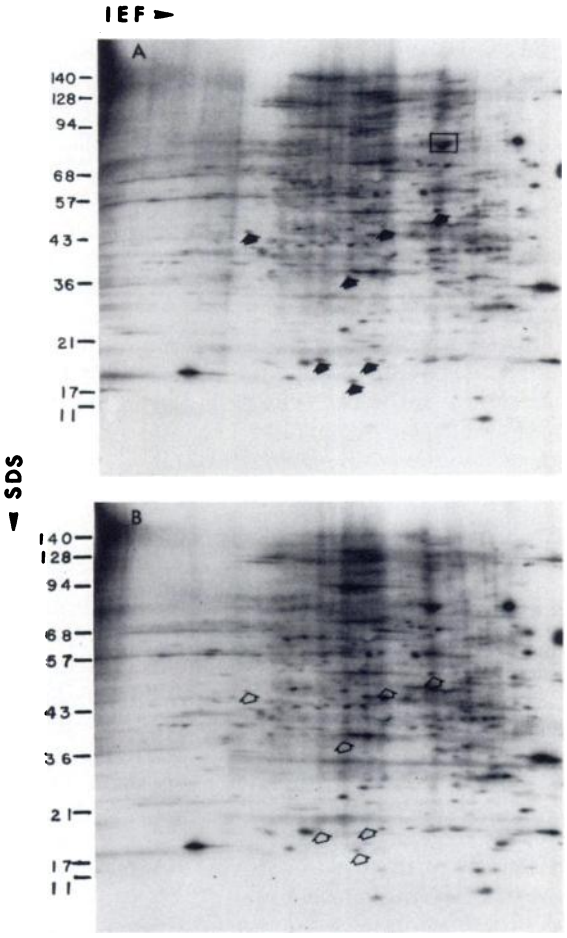

FIG. 1. *Effect of norepinephrine on the ^{32}P content of cellular proteins*

Postconfluent cultures in miniwells were incubated for 4 h in 0.5 ml of low-phosphate Ham's medium containing 0.2 mCi/ml $^{32}P_i$. After labeling, the cells were treated either with or without 10 μM norepinephrine for 5 min and the incubation was terminated by solubilizing the cells in 10 M urea, 0.2% SDS. Samples containing 780,000 cpm were prepared as described in Materials and Methods and electrophoresed in two dimensions. The second dimension was a 5–10% linear/exponential polyacrylamide gradient gel. The dried gels were exposed to Kodak SB-5 X-ray film for 9 days. Proteins that increase in ^{32}P content after norepinephrine treatment are indicated by filled arrows in autoradiogram A. The corresponding phosphopolypeptides in control samples are indicated by open arrows in autoradiogram B.

TABLE 1

Properties of polypeptides which increase in ^{32}P content after norepinephrine treatment of intact C-6 glioma cells

The polypeptides increased in ^{32}P content after norepinephrine treatment (Fig. 1) are listed in order of decreasing molecular weight. The integrated areas of each of the indicated phosphopolypeptide spots in autoradiograms from four sets of matched norepinephrine-treated and control samples equivalent to those shown in Fig. 1 were determined by microdensitometry. The increase in ^{32}P content of each polypeptide spot is expressed as the ratio of the integrated area of the ^{32}P-phosphopolypeptide spot in norepinephrine-treated samples to the integrated area of the ^{32}P-phosphopolypeptide spot in the control samples. The subcellular compartment designation below was derived from experiments shown in Figs. 3 and 4.

MW	pI	^{32}P ratio: treated/control	Subcellular compartment
58,000	5.7	1.9	Nuclear
50,000	6.1	3.3	
48,000	6.8	2.1	Cytosolic
38,000	6.4	2.2	Cytosolic
20,000	6.6	>20	
19,000	6.6	5.0	Mitochondrial
16,500	6.3	2.5	Cytosolic

More than 200 ^{32}P-phosphoproteins were detected in autoradiograms from the present two-dimensional gels (pH 4.3–7) (Fig. 1). If the ampholyte composition was modified to electrofocus more acidic components, only a few additional stained or ^{32}P-labeled polypeptides were detected, but most spots seen in Fig. 1 were less well resolved (data not shown). Basic proteins, resolved in the first dimension by nonequilibrium pH electrophoresis (14), included 100 or more additional ^{32}P-labeled polypeptides (5). In the present report, only phosphoproteins with isoelectric points between pH 7 and pH 4.2 will be considered in detail.

Seven of the more than 200 phosphopolypeptides in these gels were found to be reproducibly increased in ^{32}P content after norepinephrine treatment (Fig. 1). Polypeptides in the gels were identified by molecular weight and isoelectric point in the presence of urea. The polypeptides that increased in ^{32}P content after norepinephrine treatment were designated "58K-5.7," "50K-6.1," "48K-6.8," "38K-6.4," "20K-6.2," "19K-6.6," and "16K-6.3," the first component of the designation reflecting the molecular weight and the second component the isoelectric point. Later the subcellular compartment of the polypeptide will be added to the designation.

Microdensitometry of autoradiograms from hormone-treated and control samples was used to quantitate the effect of norepinephrine on polypeptide phosphorylation. For those proteins for which a control spot was detectable, increases in ^{32}P ranged from two- to fivefold (Table 1). For one phosphopolypeptide, 20K-6.2, the density of the ^{32}P-spot for the untreated sample was not detectable above background. Densitometry of several spots which did not visually appear to be changing with norepinephrine confirmed that these spots were the same in control and treated samples and justified the visual inspection for qualitative interpretation. Although a few proteins

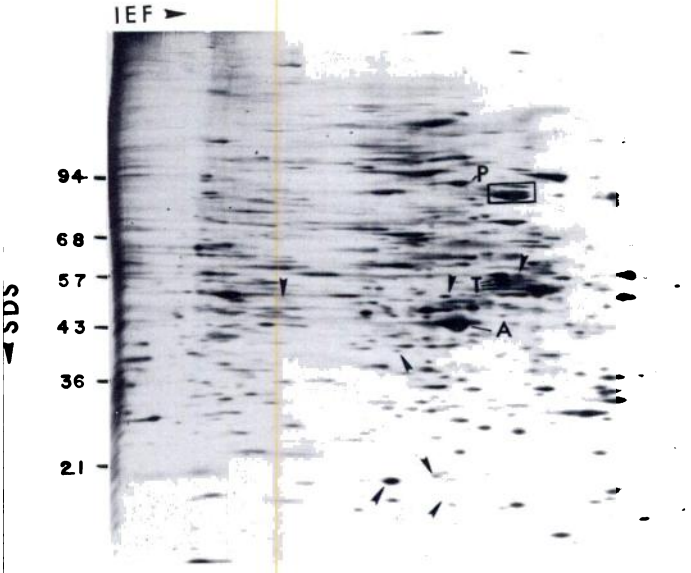

FIG. 2. *Two-dimensional gel electrophoresis of ^{35}S-methionine-labeled cellular proteins*

Postconfluent miniwell cultures were incubated for 4 h in 0.5 ml Ham's medium containing a decreased methionine concentration (1 μM) and 0.1 mCi ^{35}S-methionine. After labeling, cells were treated either with or without 10 μM norepinephrine for 5 min. Incubations were terminated by quickly washing the cells with low-methionine Ham's followed by solubilization of cellular material as described in detail in Materials and Methods. Samples of whole cell extract for electrophoresis contained 480,000 cpm. The second-dimensional gel was an 8–16% linear/exponential polyacrylamide gradient gel. The dried gel was exposed to Kodak XR-1 X-ray film for 4 days. Arrows indicate the positions of ^{35}S-labeled spots corresponding to ^{32}P-labeled spots (incubated in parallel as described in the legend to Fig. 1) which were increased by norepinephrine treatment. The positions of the protein spots comigrating with purified C-6 tubulin (T) and rabbit muscle phosphorylase *b* (P) are indicated, as is the position of actin (A). The migrations of tubulin and phosphorylase *b* were determined by coelectrophoresis of the respective purified protein with whole cell extract. The purified protein increased the size and density of the respective spot(s) in the stained gel compared to the whole cell extract alone. The position of actin was determined from its size (43,000 daltons), high abundance, and approximate isoelectric point which placed it near the center of the gel on the p*I* axis. This identification was confirmed by the presence of a known (15) actin contamination of the tubulin preparation which comigrated with the indicated actin spot.

were seen to vary in ^{32}P content irreproducibly and have not been included in the present report, the overriding result of the present study was that most of the more than 200 phosphoproteins apparent in these gels were unaltered by the norepinephrine treatment. In that norepinephrine changed the ^{32}P content of only seven phosphopolypeptides, the hormone clearly had a specific effect on a discrete set of polypeptides.

In experiments not shown, the β-adrenergic agonist, isoproterenol (1 μM), was used in place of norepinephrine. Isoproterenol produced the same phosphoprotein changes as were observed with norepinephrine. In other experiments, sodium azide (200 nM), which elevates cGMP in this cell line,[2] was used in place of norepinephrine; the phosphoprotein change observed with norepinephrine was not observed.

[2] B. McL. Breckenridge, personal communication.

Two-dimensional gel electrophoresis of ^{35}S-methionine-labeled proteins. To test the polypeptide nature of ^{32}P-labeled spots, cellular protein was labeled with ^{35}S-methionine. Approximately 900 ^{35}S-methionine-labeled polypeptides with isoelectric points between pH 7.0 and pH 4.2 were detected by a 4-day autoradiographic exposure of a two-dimensional gel (Fig. 3). When autoradiograms of ^{32}P-phosphopolypeptides were aligned with autoradiograms of ^{35}S-polypeptides, most, if not all, of the ^{32}P-autoradiographic spots could be identified as ^{35}S-methionine-labeled polypeptides. In particular, all of the ^{32}P-autoradiographic spots corresponding to the ^{32}P-phosphopolypeptides which increased in ^{32}P content after norepinephrine treatment could be identified in the autoradiogram of ^{35}S-methionine-labeled polypeptides shown in Fig. 2.

Interestingly, there were two classes of phosphopolypeptides in C-6 cells. In the first class, the ^{32}P-autoradiographic spot encompassed the entire stained or ^{35}S-autoradiographic spot, suggesting that most of the copies of the polypeptide were modified by phosphorylation. The array of polypeptides designated "90K-5.6" in Figs. 1 and 2 is an example of this class of phosphopolypeptide. In the second class of phosphoproteins the ^{32}P-spot appeared as a satellite spot of low abundance on the acidic side of the stained or ^{35}S-spot. The top of the ^{32}P-autoradiographic spot and the top of the stained spot were at the same molecular weight position, while the bottom of the stained spot extended below the ^{32}P-autoradiographic spot. It was concluded that only a small proportion of the copies of these polypeptides was phosphorylated. The phosphorylated form was rarely abundant enough to stain, but could be discerned in gels of ^{35}S-methionine-labeled polypeptides (Fig. 2). The phosphopolypeptide designated 58K-5.7 (Fig. 1) is an example of the second class of phosphopolypeptides. Recently Steinberg and Coffino have exploited the charge shifts occurring as a result of phosphorylation to study cAMP-stimulated protein phosphorylation in ^{35}S-methionine-labeled S49 lymphoma cells (15).

^{32}P Label as a covalent modification of polypeptides. That the ^{32}P-autoradiographic spots were in fact phosphorylated polypeptides was tested by several procedures. First, the spots were associated with ^{35}S-methionine-labeled spots (previous section). Second, the majority of phosphoproteins apparent in Fig. 1 was also seen, though less well resolved, in two-dimensional gels run with samples which had been extracted with trichloroacetic acid to remove acid-soluble compounds. Third, extraction of the trichloroacetic acid-insoluble material with either chloroform/methanol (2:1, v/v) or ether/ethanol (1:1, v/v) to remove phospholipids and digestion with DNAse or RNAse to degrade nucleic acids did not perturb the ^{32}P-spots of the gels. In other experiments neutralized perchloric acid extracts and chloroform/methanol (2:1, v/v) extracts from ^{32}P-labeled cells were lyophilized and the mixed residue was combined with nonradioactive solubilized whole cell extracts. Electrophoresis of these mixed extracts gave rise to autoradiograms which contained no detectable ^{32}P-labeled compounds. Therefore, binding of low molecular weight acid- or lipid-soluble material to denatured proteins of the

TABLE 2

Subcellular fractionation of C-6 glioma cells: Distribution of marker enzymes

Postconfluent cultures on the surface of roller bottles were incubated for 4 h in low-phosphate Ham's medium and treated for 5 min with 10 μM norepinephrine. Cells were then scraped from the surface of the bottle, fractionated, and assayed as described in Materials and Methods. Numbers in parentheses in the enzyme activity columns represent the ratio of specific activity of the appropriate fraction relative to the whole cell extract.

Fraction	Volume of fraction	Protein	Total protein	SDH	LDH	Thym-5'-PDE
	ml	*mg/ml*			*μmol/min·mg protein*	
Whole cells	10	3.018	30.18 (100%)	0.011	3.516	0.042
Dounce nuclei	1	6.12	6.12	0.013 (1.18)	0.074 (0.023)	0.027 (0.64)
NP-40 nuclei	1	4.26	4.26 (14%)	0.003 (0.27)	0.016 (0.005)	0.001
Mitochondria	1	1.72	1.72 (5.6%)	0.097 (8.82)	0.342 (0.097)	0.001
Plasmalemma	2.8	0.579	1.621 (5.3%)	0.012 (1.09)	0.983 (0.28)	0.187 (4.45)
Cytosol	29	0.508	14.74 (50%)	0.002 (0.182)	7.120 (2.03)	0.004 (0.09)
Recovery			(94%)			

whole cell extract was not a detectable problem in the present procedure.

As a further proof that no acid-soluble phosphoryl compound or phospholipid was contributing to the spots detected by autoradiography, two-dimensional gels equivalent to those in Fig. 2 were either kept in 5% methanol–10% acetic acid for a week, changing the solution daily, or extracted with chloroform/methanol (2:1, v/v) and ether/ethanol (1:1, v/v) before drying and autoradiography. All the ^{32}P-autoradiographic spots seen in Fig. 1 remained after the above treatments.

The putative ^{32}P-phosphoprotein spots were found to be stable when hydrated two-dimensional gels were treated with 10% trichloroacetic acid for 15 min at 90°C to digest nucleic acids. In other experiments, two-dimensional gels were overloaded for protein and stained with ethidium bromide to identify any contaminating nucleic acid. No stained spots were observed. Based on all of this evidence, it was concluded that the ^{32}P-autoradiographic spots represented phosphopolypeptides.

Subcellular fractionation. The subcellular distribution of the proteins which were increased in ^{32}P content by norepinephrine treatment was determined. The fractionation procedure was designed to isolate nuclei, mitochondria, plasma membranes, and cytosol from a single sample of lysed cells. The purity of each fraction was determined by following the distribution of marker enzymes (Table 2). In addition, each fraction was analyzed by two-dimensional gel electrophoresis. Each subcellular fraction produced a unique and distinctive two-dimensional stained (Fig. 3) and ^{32}P-autoradiographic (Fig. 4) polypeptide pattern, providing further evidence that the subcellular fractionation procedure was effective. An interesting exception to this generalization was actin, which was present in every subcellular fraction and provided a useful, internal reference point for comparing gels.

To identify polypeptides of a subcellular fraction as the corresponding spots in a whole cell protein sample, an aliquot of the starting whole cell extract was combined with an aliquot of each subcellular fraction and the mixture was electrophoresed. The resultant two-dimensional gels possessed the polypeptides of the subcellular fraction, shown in Figs. 3 and 4, enriched in the mixture of the fraction and whole cell extract. The positions of the polypeptides of each subcellular fraction could then be identified as spots in a whole cell extract by their positions relative to the pattern of other easily identifiable polypeptide spots.

Nuclei, isolated by differential centrifugation from the original lysate, were contaminated with clumped particulate debris. Dounce homogenization was only partially effective in removing the debris. The addition of 0.5% NP-40, a nonionic detergent, was sufficient to remove most of the nonnuclear particulate material as judged by phase-contrast microscopy, two-dimensional gel electrophoresis, and marker enzyme distribution (Table 2). However, NP-40-insoluble, cold-stable cytoskeletal elements such as the intermediate filament protein may remain with the nuclei. The recovery of DNA in the nuclear fraction washed with 0.5% NP-40 approached 100% (data not shown), indicating that the nuclei were not significantly disrupted by this procedure.

The NP-40-washed nuclear fraction from C-6 cells contained approximately 30% of the protein of the whole cell. Two-dimensional gels of these nuclei are shown in Figs. 3 and 4. The stained and ^{32}P-autoradiographic polypeptide pattern of nuclei was distinctive and reproducible. It was apparent that, compared to whole cells, nuclei possess relatively few polypeptides and fewer phosphopolypeptides with isoelectric points between 7.0 and 4.2.

The phosphopolypeptide designated 58K-5.7 was found to be associated with the nuclear fraction (Figs. 3 and 4). It was apparent by visual inspection of the stained two-dimensional gel of the nuclear fraction (Fig. 3B) that the phosphopolypeptide "58K-5.7-nuclear" was one of the most abundant polypeptides in the nuclear fraction. Phosphopolypeptide 58K-5.7-nuclear and the α-subunit of tubulin appeared quite similar in isoelectric point and

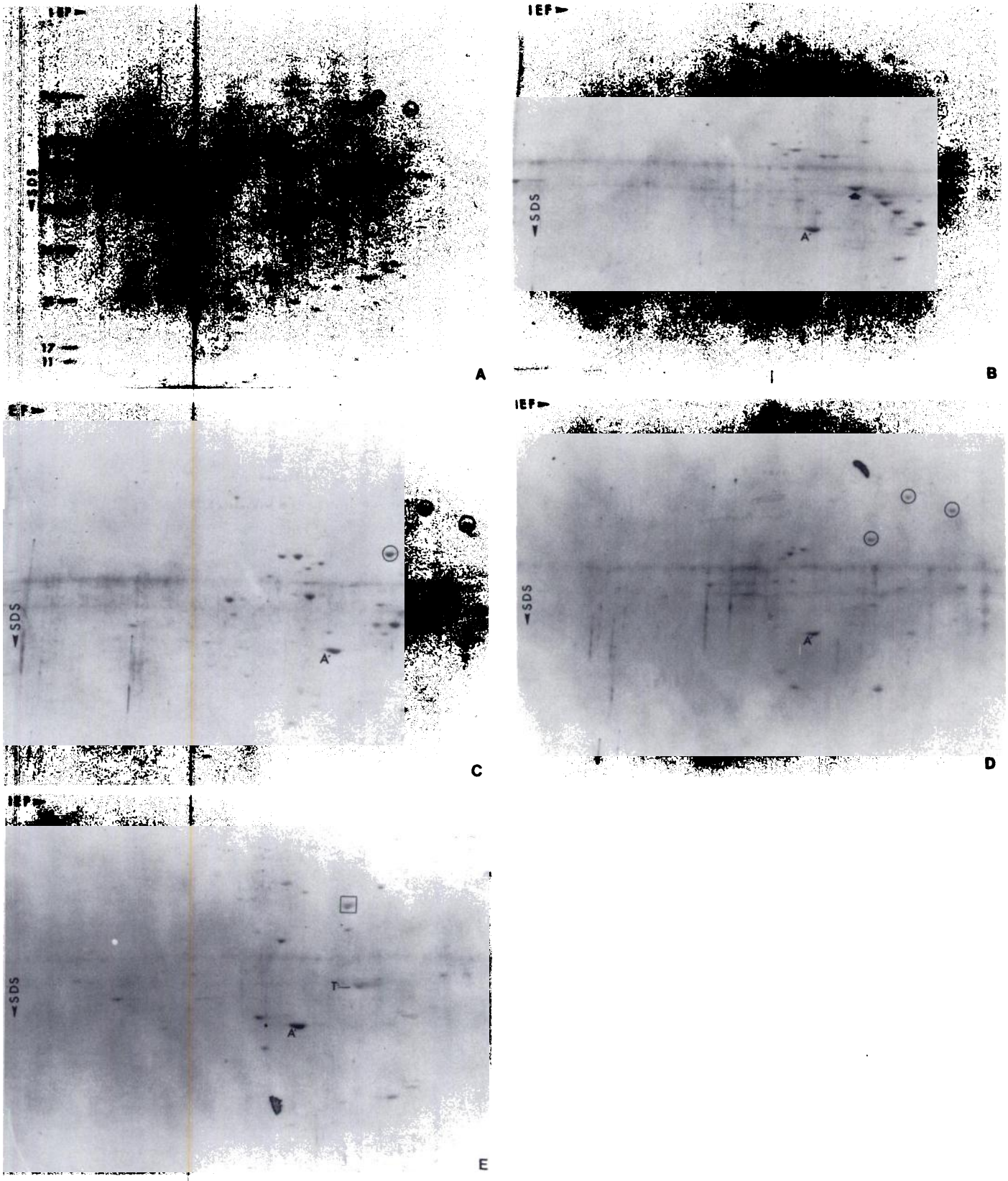

FIG. 3. *Stained two-dimensional gels of whole cell extract and isolated subcellular fractions*

Whole cell extract and isolated subcellular fractions were subjected to two-dimensional gel electrophoresis as described in the text. The conditions of first-dimension electrofocusing and second-dimension electrophoresis are as in Fig. 1. (A) Whole cell extract (40 μg of protein); (B) NP-40-washed nuclei (60 μg of protein); (C) mitochondria (20 μg of protein); (D) plasma membrane (8 μg of protein); (E) cytosol (10 μg of protein). Molecular weight marker proteins prepared according to Ames (16) are present on the left side of gel A. These were rabbit skeletal muscle phosphorylase *b* (94,000), bovine serum albumin (68,000), pyruvate kinase (57,000), ovalbumin (43,000), lactate dehydrogenase (36,000), myokinase (21,000), myoglobin (17,000), and cytochrome *c* (11,500). The positions of the polypeptide spots comigrating with tubulin (T) and actin (A) are indicated. Circles enclose polypeptide spots in high abundance in both the mitochondrial and the plasma membrane fractions (see Figs. 3C and D and 4C and D and the Results). Squares enclose the array of polypeptides designated "90K-5.6" in cytosol (E). The position of the phosphopolypeptide designated "58K-5.7" (see Table 1) is indicated by an arrow in A and B.

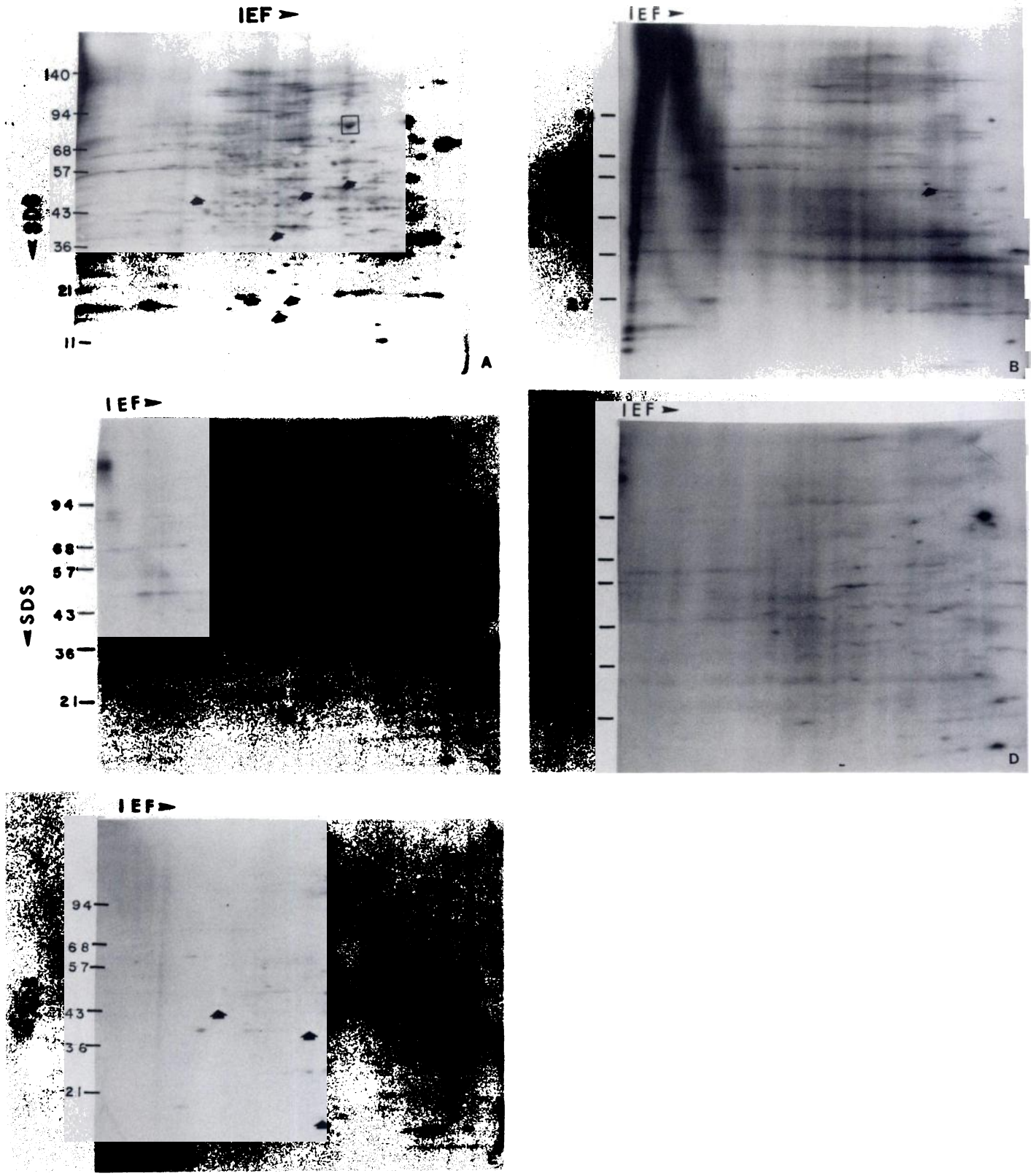

FIG. 4. *Two-dimensional gel autoradiograms of whole cell extract and isolated subcellular fractions*

A–E represent autoradiograms of the stained two-dimensional gels shown in Fig. 3 (A, whole cell extract; B, NP-40-washed nuclei; C, mitochondria; D, plasma membranes; E, cytosol). The arrows in A indicate the positions of the seven ^{32}P-phosphopolypeptides in a two-dimensional gel autoradiogram of a whole cell extract which increases in ^{32}P content after norepinephrine treatment. The arrows in B–E indicate the positions of the ^{32}P-phosphopolypeptides described in A in the isolated subcellular fractions as follows: B, "58K-5.7"; C, "19K-6.6"; E, "48K-6.9," "38K-6.9," and "16K-6.3." Exposure of the dried gels to Kodak SB-5 X-ray film is as follows: A, 6 days; B, 62 h; C, 13 days; D, 18 days; E, 18 days.

size and near-equimolar in amounts in the whole cell extract (Fig. 3A); however, the polypeptides fractionated differently. In contrast to 58K-5.7-nuclear, tubulin fractionated with the cytosol, presumably because the fractionation was conducted at 0°C (Fig. 3E), with only trace amounts in the nuclear fraction. Subsequent evidence suggests that the 58K-5.7-nuclear protein is the major subunit of the intermediate (10-nm) filament protein (see Discussion).

The mitochondrial fraction typically contained 5% of the protein and 34% of the succinate dehydrogenase activity of whole cells (Table 2). The specific activity of succinate dehydrogenase in the mitochondrial fraction was enriched approximately nine times relative to that in whole cells. Plasma membrane (thymidine-5′-phosphodiesterase) and cytosolic (lactate dehydrogenase) contaminations were low (Table 2). Two-dimensional gels from mitochondria/whole cell extract mixing experiments showed that the phosphopolypeptide designated 19K-6.6 was in the mitochondrial fraction (Fig. 4C).

The plasma membrane fraction was collected from the supernatant above the mitochondrial fraction by high-speed centrifugation onto a 38% sucrose cushion. The recovery of thymidine-5′-phosphodiesterase, the plasma membrane marker enzyme, was approximately 20% with a four- to fivefold increase in specific activity (Table 2). Low recovery of the plasma membrane and mitochondrial fractions was attributable to incomplete disruption by Dounce homogenization.

The plasma membrane fraction was analyzed by two-dimensional gel electrophoresis and the results are shown in Figs. 3D and 4D. The stained and ^{32}P-autoradiographic pattern of plasma membrane polypeptides differed from that of the other fractions, although three polypeptides enriched in the mitochondrial fraction were also present in plasma membranes. These polypeptides are circled in Figs. 3A, C, and D. It was unlikely that the three polypeptides common to both fractions were the result of cross-contamination since such contamination was judged to be minimal by marker enzyme analysis. None of the polypeptides in the whole cell extract possessing an increased ^{32}P content after norepinephrine treatment was found in the plasma membrane fraction.

The cytosolic fraction was relatively free from nuclear, mitochondrial, and plasma membrane contamination and typically contained 50% of the protein and nearly 100% of the lactate dehydrogenase activity of the initial extract (Table 2). Two-dimensional gel electrophoresis of the cytosolic fraction is shown in Figs. 3E and 4E, and the positions of the polypeptide spots comigrating with C-6 tubulin are indicated. A distinctive cluster of five abundant phosphopolypeptide spots was detected in the two-dimensional gels of the cytosolic fraction and designated "90K-5.6-cytosolic." An array of polypeptides similar to 90K-5.6-cytosolic can be observed in previously published two-dimensional gels of rat hepatoma (17) and S49 mouse lymphoma cells (18), indicating that these polypeptides are expressed in several eukaryotic cell types. Three of the phosphopolypeptides that increased in ^{32}P content after norepinephrine treatment were detected in autoradiograms of two-dimensional gels of the cytosolic fraction and were designated "48K-6.9-cytosolic," "38K-6.4-cytosolic," and "16K-6.3-cytosolic."

Of the seven phosphopolypeptides listed in Table 1, only 50K-6.1 and 20K-6.2 were not detected in any of the two-dimensional gel autoradiograms of the subcellular fractions. It is presently unclear whether these two polypeptides are localized in subcellular fractions not recovered by the present procedure or were otherwise lost or degraded during fractionation.

DISCUSSION

The present experiments show that intact C-6 glioma cells respond to norepinephrine by specifically phosphorylating 7 of the more than 200 phosphopolypeptides resolvable by two-dimensional gel electrophoresis. The proteins were further defined in terms of subunit molecular weight, isoelectric point, and, in the case of 5 of the 7 proteins, subcellular localization. Accordingly the proteins were named 58K-5.7-nuclear; 50K-6.1, 48K-6.8-cytosolic; 38K-6.4-cytosolic; 20K-6.2, 19K-6.6-mitochondrial; and 16.5K-6.3-cytosolic. In the case of the 2 proteins for which no subcellular compartment could be assigned, it is unclear whether these proteins reside in compartments not recovered by our procedure such as ribosomes or were otherwise lost or degraded during subcellular fractionation. In experiments not shown, polypeptides which were more acidic or more basic than those shown here were resolved; only one additional protein was found to undergo norepinephrine-dependent phosphorylation. This component was a very basic histone H1-like protein which was increased in ^{32}P content after a 60-min hormone treatment. This protein is described in more detail along with a procedure which has been developed for resolving histones and other very basic proteins of the cell (5).

Tomkins has introduced the term "domain" to describe the specific set of cellular functions regulated by a specific hormone in an individual cell type (19). The set of phosphoproteins which undergo increased phosphorylation in response to norepinephrine may be considered to represent the "phosphoprotein domain" of the action of norepinephrine.

Regarding the 58K-5.7-nuclear protein, subcellular fractionation revealed this protein to be one of the most abundant polypeptides of the nuclear fraction. Recent experiments in this laboratory (Sanders and Browning, to be published) indicate that 58K-5.7-nuclear remains associated with the nuclear ghost (P_6 fraction) upon micrococcal nuclease digestion and 0.6 M salt extraction of the NP-40-washed nuclear fraction (20). The intermediate filament protein of several cultured cell lines is an abundant protein with a molecular weight of approximately 58K (21, 22) and an isoelectric point slightly more acidic than actin (23). In addition, the intermediate filament protein, though cytoskeletal, remains associated with nuclei upon extraction with nonionic detergents (21–23) as does 58K-5.7-nuclear, and neither protein is solubilized from the nuclear structure by 0.6 M NaCl (21). We have recently observed 58K-5.7-nuclear to be extracted from the nuclear ghost by 2 M urea (Browning and Sanders, to be published) as has been reported for

the intermediate filament protein of baby hamster kidney cells (22). In addition, a protein which cross-reacts with antibody to the intermediate filament protein of Chinese hamster ovary cells is present in NP-40-insoluble material from C-6 glioma cells (Groppi and Coffino, unpublished). Interestingly, Lazarides and colleagues have recently shown that the intermediate filament protein of chick myogenic cultures exists as a phosphoprotein but that only a small percentage of the monomers is phosphorylated (24), as is true for 58K-5.7-nuclear. These workers were also able to demonstrate phosphorylation of the protein by cAMP-dependent protein kinase (25). Based on the above evidence of similar molecular weight, isoelectric point, abundance, subcellular fractionation, nuclear binding, and elution as well as phosphorylation, we tentatively conclude that protein 58K-5.7-nuclear is the major subunit of the intermediate filament protein of the C-6 glioma cell.

The set of hormone-dependent phosphoproteins described in this paper include one that fractionated with the nuclei, one with mitochondria, three with cytosol, and one that was thought to be an H1 histone. This subcellular distribution raises the question of the subcellular compartmentation of the protein kinase(s) which catalyzes these phosphorylations. However, the present evidence is not sufficient to require a "noncytosolic" localization of the protein kinase(s). First, we feel that the 58K-5.7 protein is attached to, rather than located within, the nucleus of the cell and is therefore exposed to the cytosol. Second, the external surfaces of the mitochondria are exposed to the cytosolic compartment of the cell and the outer mitochondrial membrane is permeable to relatively high molecular weight materials. Finally, the histone phosphorylation was not increased after short (5-min) hormone treatments and therefore there was adequate time (1 hr) for either the histone substrate or the relevant kinase to migrate after cAMP became elevated within the cytosol.

It is also possible that phosphoprotein phosphatases play a role in the present results. Phosphorylase phosphatase has been observed to undergo inactivation when incubated with cAMP-dependent protein kinase, cAMP, and ATP-Mg^{2+} (26). Should such a phenomenon occur in intact C-6 cells, the result would tend to increase the changes in ^{32}P content induced by norepinephrine treatment.

In C-6 cells, cAMP has been implicated thus far in the regulation of three classes of cellular events: regulation of glycogen metabolism, induction of enzymes, and alteration of morphology. Conditions that increase the intracellular cAMP convert glycogen phosphorylase from a *b* form which was dependent on AMP for activity to an *a* form which appeared to be slightly stimulated by AMP (7) and also converted glycogen synthetase *a* to *b* (27). By analogy to rabbit skeletal muscle, it was assumed that the regulation of glycogen metabolism in C-6 cells was mediated by the activation of cyclic AMP-dependent protein kinase. Enigmatically, the phosphoprotein spot in the two-dimensional gels presented herein, which was tentatively identified as glycogen phosphorylase by coelectrophoresing 2× crystallized rabbit skeletal muscle phosphorylase, did not significantly increase in phosphate content after norepinephrine treatment. Further studies are needed to test whether this protein in fact represents the C-6 cell glycogen phosphorylase. Resolution of purified samples of phosphorylase kinase and glycogen synthetase from skeletal muscle in the two-dimensional gel system has thus far not yielded well-defined spots.

The second type of cellular event in which cAMP has been implicated in C-6 cells is the induction of four enzymes. β-Adrenergic stimulation or analogues of cAMP produced an induction of lactate dehydrogenase (28), ornithine decarboxylase (29), cAMP phosphodiesterase (8), and 2′,3′-cyclic nucleotide-3′-phosphohydrolyase (30). The induction of these enzymes was shown to be blocked by both cycloheximide and actinomycin D, indicating that the transcription and translation were required for the cAMP action. In S49 lymphoma cells, the induction of cAMP phosphodiesterase by cAMP was shown to require the catalytic subunit of cAMP-dependent protein kinase (31). It would appear likely that one or more of the presently described phosphoproteins may mediate these enzyme inductions in C-6 cells.

The final cellular event known to be controlled by cAMP in C-6 cells is the alteration in morphology (33). In the absence of β-stimulation or dibutyryl cAMP, C-6 cells possess an irregular flattened shape. Increase in the intracellular concentration of cAMP caused the cells to retract their cytoplasm toward the nucleus and to extend multiple cellular processes (32). The process did not require nuclear control, in that cells enucleated with cytocholasin B responded similarly (32). Evidence for a role for phosphorylation in both microtubule assembly (33) and contraction of nonmuscle actomyosin systems (34) has been presented. Of possible significance is the above-noted phosphorylation of the intermediate filament protein as well as the fact that the myosin system of C-6 cells contain a light chain of 20,000 molecular weight (1) which corresponds to a substrate for myosin light-chain kinase in other cell types (34).

We have recently prepared myosin from C-6 cells according to Ash (35) and electrophoresed the product in the absence and presence of ^{32}P-labeled whole cell extracts. We find that the product does migrate with a component of our whole cell extract but one which is more acidic than those whose phosphorylation was stimulated by norepinephrine. This is consistent with the recent observation that myosin light-chain kinase activity is *decreased* upon cAMP-dependent phosphorylation (36). Whether or not the myosin light chain undergoes a reproducible decrease in phosphorylation upon elevation of cAMP in C-6 cells remains to be determined. Likewise, we do not know enough about the myosin light-chain kinase of C-6 cells to presently elevate its presence in our gel system.

The results of the present research demonstrate that hormonal control of protein phosphorylation can be investigated in intact cells. Because protein phosphorylation has been established as the major mechanism for mediation of cAMP action in eukaryotic cells, the determination of the phosphoprotein domain of a hormone provides a molecular description of the action of that hormone in terms of the phosphoprotein effectors of

cellular function. With this approach one may study the coordinate control of diverse aspects of cAMP-mediated cellular function and gain more perspective concerning the significance of individual events. In addition, the two-dimensional gel resolution of whole cell extracts provides a two-dimensional map of cellular proteins which may be used, through mixing experiments, to evaluate hypotheses of the role of individual purified components in cellular function.

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