PHOSPHORYLATION OF FILAMIN AND OTHER PROTEINS IN CULTURED FIBROBLASTS

P. Davies, Y. Shizuta, K. Olden, M. Gallo and I. Pastan

National Institutes of Health
National Cancer Institute
Laboratory of Molecular Biology
Bethesda, Maryland 20014

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SUMMARY

Incubation of subcellular fractions of fibroblasts with [^{32}P]ATP demonstrated 10 phosphoproteins whose phosphorylation can be increased by cyclic AMP or cyclic AMP-dependent protein kinase. One of these phosphoproteins, MW 240,000, resembles the actin binding protein, filamin, and can be selectively precipitated by antibodies to chicken gizzard filamin. Furthermore chicken gizzard filamin can be phosphorylated by skeletal muscle protein kinase and cyclic AMP stimulates this reaction.

INTRODUCTION

Dibutyryl cyclic AMP and compounds that increase cyclic AMP levels, induce changes in the shape, adhesiveness and motility of cultured fibroblasts (1). Since cyclic AMP-dependent protein kinase appears to mediate most of the actions of cyclic AMP in animal cells (2-4), we have studied protein phosphorylation in normal fibroblasts, and identified those proteins whose phosphorylation may be regulated by cyclic AMP. Here we report that fibroblasts contain at least 10 phosphopeptides whose phosphorylation can be increased by cyclic AMP. Furthermore one of these phosphopeptides, MW 240,000 is very similar to an actin binding protein, filamin, that has been purified from macrophages (5) and smooth muscle (6,7).

MATERIALS AND METHODS

Normal rat kidney fibroblasts (NRK) were grown, harvested, and plasma membranes prepared as previously described (8). The supernatant after

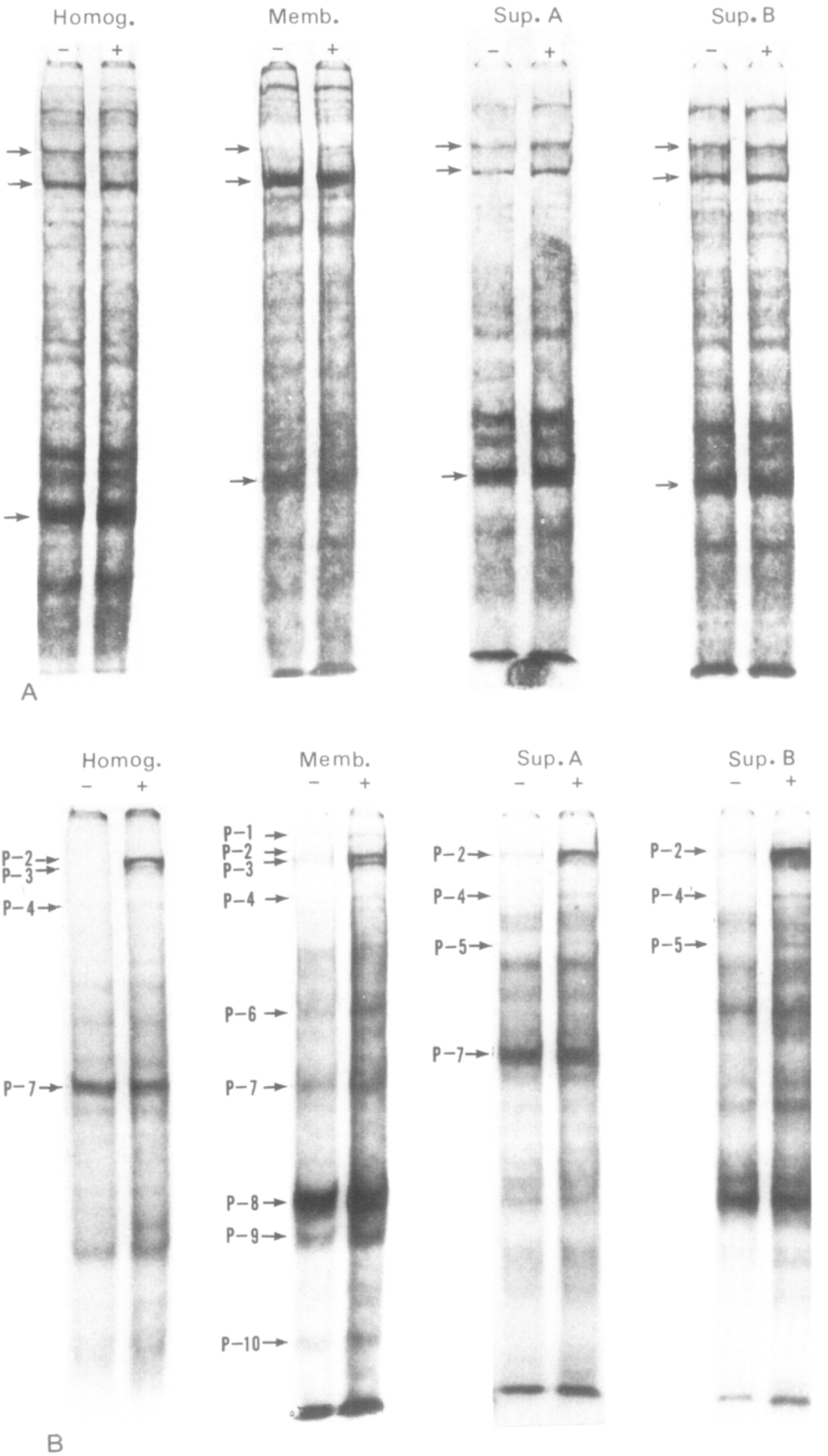
Abbreviations used: cyclic AMP, adenosine 3':5'-cyclic monophosphate; EGTA, ethylene glycol bis(β-aminoethylether)-N,N'-tetraacetic acid; MES, 2-(N-morpholino)ethanesulfonic acid, SDS, sodium dodecyl sulfate; NRK, normal rat kidney fibroblasts.

centrifugation at 4° at 20,000 g for 15 min is designated as the cytosol fraction, and contains microsomes but few mitochondria. Endogenous protein phosphorylation was performed as follows: each 0.1 ml reaction contained 2.5 μmoles of N-morpholinoethane sulfonic acid (MES) (pH 7.0), 1 μmole of magnesium acetate, 0.69 nmole of [γ-^{32}P]ATP (S.A. 14.3 μCi/nmole), and 100 μg of cell protein with or without 0.1 nmole of cyclic AMP. When indicated 10 nmoles of EGTA or 3 μg of homogeneous catalytic subunit of beef skeletal muscle cyclic AMP-dependent protein kinase was added. Incubation was for 5 min at 37°. The reaction was stopped by adding 0.5 ml of 20% trichloracetic acid. After standing at 0° for 15 min the precipitate was collected by centrifugation, washed with diethyl ether and dissolved, electrophoresed and radioautographed as described by Yamada et al. (9). For studies on the immunoprecipitation of band P-4 by anti-filamin antisera a different procedure was utilized to maximize the recovery of filamin in the cytosol fraction and to minimize non-specific precipitation of phosphoproteins by the antibody. NRK cells were grown to confluency in 100 mm Falcon culture dishes, washed three times with 10 ml PBS and once with 10 ml 5 mM Tris-HCl (pH 7.8) containing 0.1 mM EDTA and 0.1 mM DTT. Cells were scraped into residual buffer (0.1 ml per dish), allowed to stand 20 min at 0°, homogenized with 10 strokes of a tight-fitting Dounce homogenizer and then centrifuged 500 g at 4° for 5 min. The supernatant was made 0.6 M KCl by addition of 2.4 M KCl. After standing 10 min at 0° the extract was centrifuged at 100,000 g for 30 min. This supernatant (high salt cytosol) was added to a reaction mixture (final volume of 0.1 ml) containing 2.5 μmoles MES (pH 7.0), 1 μmole magnesium acetate, 20 nmoles EGTA, 0.1 nmoles cyclic AMP, 30 μmoles KCl, 4 nmoles of [γ-^{32}P] ATP (S.A. 13.6 μCi/nmole) and 100 g of sample protein. After incubation for 2 min at 30° the reaction was stopped by 50 μl of a solution containing 25 mM EDTA, 5 mM ATP, 5 mM sodium pyrophosphate and 50 mM NaF and immediately brought to 0°. To this mixture was added 0.1 ml of purified anti-filamin antibody (0.73 mg/ml), control antisera (0.73 mg/ml), or buffer (PBS). The mixture was incubated 5 min at 0° and centrifuged at 10,000 g for 5 min. The supernatant was immediately transferred to a tube containing 75 μl of rabbit anti-goat globulin antisera (Miles laboratories, 10 mg/ml) and the mixture incubated 10 min at 0° and then spun at 10,000 g at 4° for 10 min. The pellet was resuspended in 200 mM potassium phosphate (pH 7.0) containing 500 mM KCl and again centrifuged. This pellet was resuspended in 5 mM Tris HCl (pH 7.5), 0.1 mM EDTA (0°) and again sedimented. The pellet was then processed for electrophoresis (9). Chicken gizzard filamin was purified as previously described (7). Phosphorylation of filamin by protein kinase was determined by the filter paper method of Reimann et al. (10). Homogeneous catalytic subunit and holoenzyme of cyclic AMP-dependent protein kinase from beef skeletal muscle were gifts from Dr. Peter J. Bechtel at U. of California, Davis. Antibodies to chicken gizzard filamin were purified by affinity chromatography on a chicken gizzard filamin - Sepharose column. The standard protein markers for electrophoresis were chicken gizzard filamin, 240 K, myosin, 200K; RNA-polymerase β and β', 160 K and 150 K; β-galactosidase, 130 K; phosphorylase a, 95 K; bovine serum albumin, 67 K; egg albumin, 43 K. [γ-^{32}P]ATP was from New England Nuclear. Other chemicals were as described (7).

RESULTS

Effects of Cyclic AMP on Endogeneous Phosphorylation

Homogenates and subcellular fractions of NRK fibroblasts were incubated with [γ-^{32}P]ATP with and without cyclic AMP and the resultant ^{32}P-phosphopeptides fractionated by SDS-gel electrophoresis, stained with Coomassie Blue (Fig. 1A)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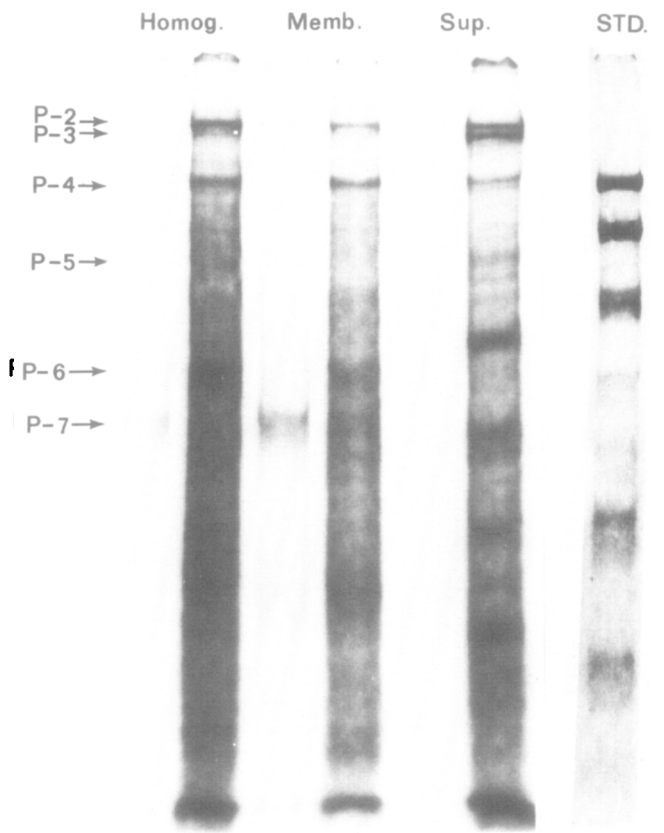

Figure 2. Phosphorylation of subcellular Fractions by Catalytic Subunit of Protein Kinase. After incubation with or without protein kinase (3 µg) and [γ-^{32}P]-ATP (7 µCi/nmole) samples were electrophoresed and analyzed by radioautography for 12 hr.

and radioautographed (Fig. 1B). In the homogenate, cyclic AMP increased ^{32}P incorporation into 4 major phosphopeptides, P-2 (MW 280 K); P-3 (MW 270 K); P-4 (MW 240 K); P-7 (MW 95 K). In the membrane fraction ^{32}P was incorporated into numerous phosphopeptides, 9 of which were enhanced by cyclic AMP. Besides the 4 peptides observed in the homogenate 5 additional bands, labelled P-1 (MW 300 K); P-6 (MW 140 K); P-8 (MW 60 K); P-9 (MW 48 K); P-10 (MW 30 K) had their phosphorylation increased by cyclic AMP. In the cytosol fraction (Sup

Figure 1. Gel Electrophoresis of Subcellular Fractions. After incubation with [^{32}P]-ATP aliquots with 20 µg protein were applied on 5% gels, (A) protein staining by-Coomassie Blue, (B) radioautograph after 48 hr. Arrows on Coomassie Blue stained gel indicate positions of filamin, myosin and actin. Homog., Memb., and Sup. represent crude homogenate, plasma membrane, and cytosol fractions; (+) indicates presence of 10^{-6}M cyclic AMP. Sup. A and Sup. B are experiments with and without 0.1mM EGTA.

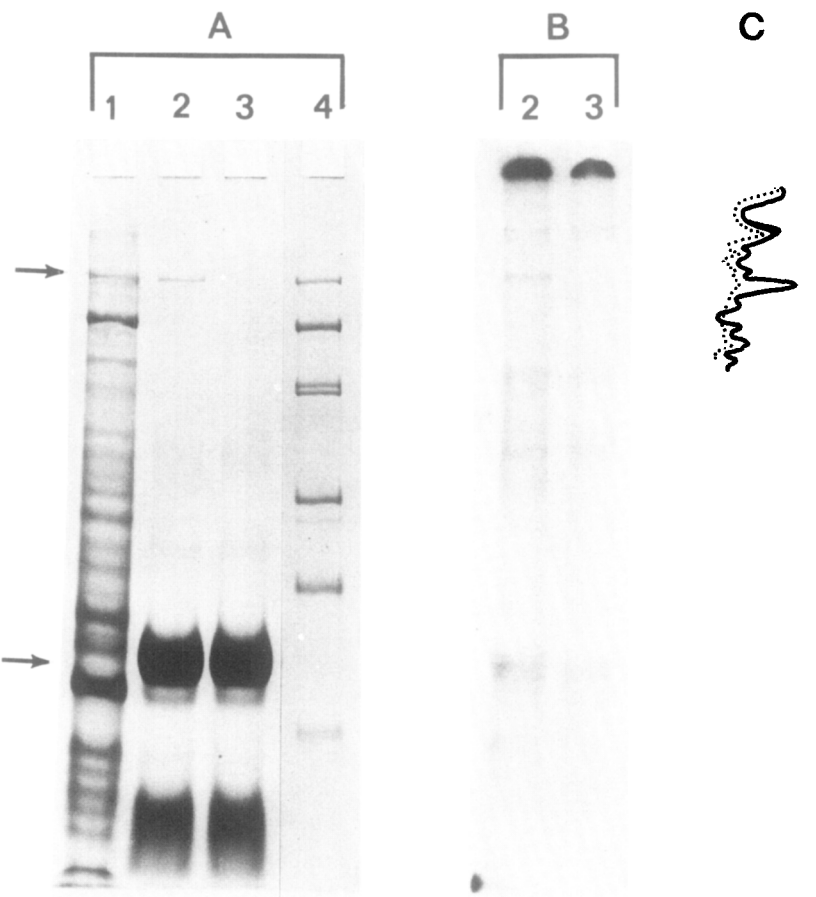

Figure 3. Precipitation of Band P-4 by Anti-Filamin Antibodies. Cytosol was incubated with [^{32}P]ATP and anti-filamin antibody or control goat globulin added as described in Experimental Procedure. (A) Protein staining by Coomassie Blue, (B) radioautograph after 72 hr, (C) Densitometric scan of radioautograph of anti-filamin antibody precipitate (solid line) and control precipitate (dashed line, offset). Lane (1) cytosol, (2) anti-filamin antibody precipitate, (3) control globulin precipitate, (4) protein standards. Arrows indicate P-4 and the heavy chain of IgG respectively.

A - Fig. 1B) cyclic AMP increased the phosphorylation of 4 bands P-2, P-4, P-5, and P-7. The addition of EGTA (Fig. 1B - Sup B) eliminated phosphorylation of band P-7.

Phosphorylation by the Catalytic Subunit of Beef Skeletal Muscle Protein Kinase

Crude homogenate, membrane and cytosol fractions were incubated with added protein kinase under conditions where endogenous phosphorylation was very

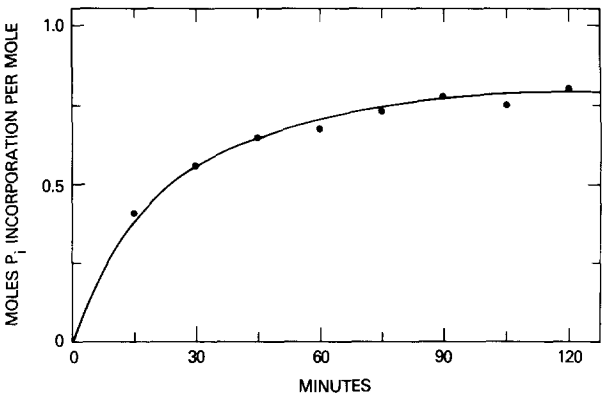

Figure 4. Time Course of Filamin Phosphorylation. Purified chicken gizzard filamin (300 µg) was incubated with 50 µg of homogenous catalytic subunit of bovine skeletal muscle protein kinase in a vol. of 0.5 ml with 25 µmoles MES (pH 7.0), 5 µmoles magnesium acetate, 0.5 µmoles EDTA, 0.5 µmoles [γ-^{32}P]-ATP (specific activity 0.04 µCi/nmole) at 37°. At the indicated times duplicate aliquots (25 µl) were applied to filter paper (8).

low (see legend Fig. 2), overall phosphorylation was markedly increased; prominent among the peptides phosphorylated were P-2, P-3, P-4, P-5 and P-7.

Precipitation of P-4 by Anti-Filamin Antibodies

A protein with the same molecular weight as P-4 (MW 240,000) has been purified from chicken gizzard (6, 7) and macrophages (5) and detected in fibroblasts by immunological methods (6). To determine if P-4 was filamin, cytosol labelled with [^{32}P]ATP was treated with antibodies to chicken gizzard filamin. Several phosphoproteins were non-specifically precipitated by immune or non-immune globulin but band P-4 was the only ^{32}P containing protein selectively precipitated by anti-chicken gizzard filamin antibody (Fig. 3).

Phosphorylation of Chicken Gizzard Filamin

To determine if chicken gizzard filamin could be phosphorylated, it was incubated with [γ -^{32}P]ATP and the catalytic subunit of protein kinase (Fig. 4). The time course of ^{32}P incorporation into filamin is shown in Fig. 4. After 120 min about 0.8 moles of phosphate per mole of filamin subunit were incorporated. To determine the effect of cyclic AMP, purified filamin was also incubated with skeletal muscle protein kinase holoenzyme. Phosphorylation

TABLE 1: REQUIREMENTS FOR THE PHOSPHORYLATION OF FILAMIN

Components	^{32}P incorporated (cpm)
Complete system	26,720
- Cyclic AMP	3,580
- Mg^{2+}	0
- Protein kinase	0
- Filamin	0

The complete reaction mixture contained 5 µmol of MES buffer (pH 7.0), 1 µmol of magnesium acetate, 0.1 µmol of EDTA, 13 nmol of [γ-^{32}P]ATP (S.A. 760 cpm/pmol), 0.25 mg filamin, 0.1 nmol cyclic AMP and 10 µg beef cyclic AMP-dependent protein kinase holoenzyme in a total volume of 0.1 ml. After incubation at 30° for 45 min aliquots (20 µl) were applied to filter paper and radioactivity incorporated into filamin was determined (10).

of filamin was stimulated 7 fold by cyclic AMP (Table 1). These results indicate that filamin is a protein which can be phosphorylated by cyclic AMP-dependent protein kinase.

DISCUSSION

We have examined the protein phosphorylation patterns of cultured fibroblasts to see which proteins can mediate the action of cyclic AMP through their phosphorylation by cyclic AMP-dependent protein kinase. We find there are at least 10 phosphopeptides whose phosphorylation can be stimulated by cyclic AMP. Among these phosphoproteins P-2 (MW 280 K) and P-3 (270 K) have the same mobility as the microtubule associated proteins studied by Sloboda *et al.* (11). The phosphorylation of band P-7 (MW 95 K) unlike the other cyclic AMP dependent phosphorylations was inhibited by EGTA suggesting a role for a Ca^{+2} dependent protein kinase in mediating this reaction. Since Band P-7 has approximately the same molecular weight as a subunit of glycogen phosphorylase, our results suggest that P-7 may be the phosphorylated subunit of glycogen phosphorylase.

Of particular interest is the observation that band P-4 (MW 240 K) appears

to be the actin binding protein, filamin. Direct phosphorylation of purified filamin with purified cyclic AMP-dependent protein kinase and immunoprecipitation of phosphorylated fibroblast filamin by anti-filamin anti-sera supports this notion. What effect phosphorylation of filamin may have on its function within the cell is under investigation.

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