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Actin nascent chains are substrates for cyclic AMP-dependent phosphorylation *in vivo*

(two-dimensional gels/DNase I affinity purification/partial proteolysis peptide maps)

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ABSTRACT **Two-dimensional gel electrophoresis of extracts of S49 mouse lymphoma cells labeled with [^{35}S]methionine in the presence of inducers or analogs of cyclic AMP reveals a protein that both affinity purification and peptide mapping show to be a form of nonmuscle actin. This actin species also exhibits cyclic AMP-dependent labeling with [^{32}P]phosphate, and, after acid hydrolysis, ^{32}P label is found associated with phosphoserine. Phosphorylated actin does not appear when cells prelabeled with [^{35}S]methionine are treated with an inducer of cyclic AMP in the presence of emetine, an inhibitor of protein synthesis; this suggests that only the nascent form of actin is a substrate for cyclic AMP-dependent phosphorylation. As well as differing slightly in isoelectric points, β and γ actins are found to yield different partial proteolytic cleavage products with staphylococcal protease. This microheterogeneity in the major cellular actin component is repeated in both the metabolically labile δ/ϵ actin and phosphorylated actin, suggesting that these three forms of actin derive from the same two gene products.**

Protein phosphorylation has been implicated in the regulation of cell morphology, cell movement, cellular secretion, and interaction between cytoskeleton and cell surface proteins by widespread findings that proteins that comprise or interact with cytoskeletal or contractile elements of cells are subject to phosphorylation *in vivo*. Such proteins include tubulin (1, 2), two high molecular weight microtubule-associated proteins (3), myosin light chains (4, 5), troponin (6), tropomyosin (7), spectrin (8), filamin (9), desmin (10), intermediate filament protein (10–12), and human HLA antigens (13). Observations that a number of cell types show alterations in shape upon treatment with analogs or inducers of cyclic AMP (cAMP) (14, 15) have focused interest on cAMP-mediated phosphorylations of cytoskeletal proteins. Evidence has been presented for cAMP-dependent phosphorylations of filamin (9), a microtubule-associated protein (3), and intermediate filament protein (12).

Two-dimensional polyacrylamide gel analysis of S49 mouse lymphoma cell proteins labeled with [^{35}S]methionine in the presence or absence of cAMP reveals about 16 reproducible differences compatible with substrate phosphorylations. These changes in gel pattern are elicited by various analogs and inducers of cAMP, including N^6,$O^{2'}$-dibutyryl cyclic AMP (But$_2$cAMP), 8-bromo cyclic AMP, cholera toxin, and isoproterenol but not by sodium butyrate; variant sublines deficient in catalytic activity of cAMP-dependent protein kinase do not exhibit any of the changes (12). One change involves the appearance of a protein (designated "O") which migrates in the two-dimensional gel system as if it might be a phosphorylated form of nonmuscle actin. In contrast to more "orthodox" products of cAMP-dependent phosphorylation, however, protein O comprises 10% or less of actin labeled in the presence of cAMP and negligible labeling of protein O is observed when cells prelabeled with [^{35}S]methionine are washed free of label before they are treated with But$_2$cAMP (12). This report confirms that protein O is a phosphorylated form of actin and provides evidence suggesting that only nascent chains of actin are subject to this modification.

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MATERIALS AND METHODS

Cells, Media, and Drugs. Wild-type S49 cells (subline 24.3.2) were grown in suspension culture as described (16). Low-methionine and low-phosphate labeling media are described elsewhere (12). But$_2$cAMP, D,L-isoproterenol, and emetine were from Sigma; RO 20-1724 was from H. Shepphard of Hoffmann–LaRoche.

Radiolabeling and Preparation of Cell Extracts. For labeling with [^{35}S]methionine (Amersham; >800 Ci/mmol; 1 Ci = 3.7×10^{10} becquerels), cells were concentrated and preconditioned in low-methionine medium as described (12) before labeling at 37°C as specified in figure legends. For labeling with $^{32}PO_4$, cells were harvested by centrifugation, resuspended in low-phosphate medium for 30 min at 37°C, centrifuged again, and resuspended at 2.5×10^6/ml in low-phosphate medium with or without 1 mM But$_2$cAMP. Labeling was allowed to proceed for 6 hr at 37°C, after addition of 1 mCi of $^{32}PO_4$ (ICN, carrier-free in water) to 1-ml cultures.

For the experiments of Figs. 1–4, labeling was terminated by the addition of 2–3 vol of ice-cold phosphate-buffered saline; cells were centrifuged at 4°C and washed once with phosphate-buffered saline. For the experiment of Fig. 1, cells were extracted with gel sample buffer [9.5 M urea/1.6% Ampholines, pH 5–7/0.4% Ampholines, pH 3.5–10/2% (wt/vol) Nonidet P-40/5% (vol/vol) 2-mercaptoethanol (17)]; the extracts were frozen and stored at −70°C. For those of Figs. 2–4, cell pellets were frozen in dry ice and stored for up to 4 days at −70°C before extraction as described (18) for further fractionation. For the experiment of Fig. 5, after labeling, cells were harvested by centrifugation at room temperature for 15 sec at top speed (about $7000 \times g$) in a Fisher microcentrifuge, the medium was aspirated, and the cells were immediately lysed by addition of 25 μl of gel sample buffer; this harvesting and extraction procedure took a total of about 40 sec. Extracts were frozen and stored at −70°C.

DNase I-Agarose Affinity Chromatography. DNase I-agarose was a gift from G. Johnson who prepared it by allowing 20 mg of DNase I to react with about 2 g of Affigel-10 (Bio-Rad) according to the manufacturer's recommendations. Unreacted sites were blocked with ethanolamine. Control agarose was prepared by treating Affigel-10 with ethanolamine alone.

For the [^{35}S]methionine-labeled preparations in Figs. 2–4,

Abbreviations: cAMP, cyclic AMP; But$_2$cAMP, N^6,$O^{2'}$-dibutyryl cyclic AMP.

about 100 μl of extract (200–300 μg of protein) was loaded onto 0.2-ml columns of DNase I-agarose at 4°C; about 15 min was allowed for each portion of the sample to bind to the resin. Columns were washed and eluted as described for cAMP affinity columns (18) except for the omission of washes with buffer containing 1 M NaCl. The control agarose column used for Fig. 2*b* was run identically except that sample size, column bed volume, and wash volumes were scaled down by a factor of 10. For the $^{32}PO_4$-labeled preparations of Fig. 4, 50-μl samples were loaded onto 40-μl DNase I-agarose columns, and the columns were washed and eluted as above but with the 1 M NaCl washing steps.

Two-Dimensional Polyacrylamide Gel Electrophoresis. The two-dimensional gel electrophoresis procedure of O'Farrell (17) was used with the modifications described (12, 16). First-dimension isoelectric focusing gels were run for a total of 7000 V·hr, and second-dimension $NaDodSO_4$ gels were 7.5% in polyacrylamide (except for the proteolysis experiment of Fig. 3; see below). Gels were stained, destained, and dried as described by O'Farrell (17). For Figs. 1, 2, 3, and 5, autoradiographic exposures were at room temperature with Kodak NS-2T film; for Fig. 4, exposure was at −78°C with Kodak XR-2 film with Du Pont Lightning-Plus intensifying screens. All gel patterns are shown with the acidic end of the isoelectric focusing dimension at the right and the low molecular weight region of the second dimension at the bottom. Details of sample radioactivity and autoradiographic exposure times are given in figure legends.

Partial Proteolysis Peptide Mapping. Peptide mapping was by the procedure of Cleveland *et al.* (19) modified as outlined below for use on proteins separated on isoelectric focusing gels. First-dimension isoelectric focusing gels run as above were extruded from their tubes, trimmed to give a 5-cm length with actin near the center, and equilibrated against two changes of $NaDodSO_4$ gel sample buffer as described by O'Farrell (17). The trimmed gels were loaded onto 15% $NaDodSO_4$/polyacrylamide gels by using 0.8 ml of agarose solution and embedding two trimmed isoelectric focusing gels on each second-dimension gel by the procedure of O'Farrell (17). *Staphylococcus aureus* strain V8 protease (Miles) or papain (Worthington) were diluted with 0.125 M Tris, pH 6.8/0.5% $NaDodSO_4$/1 mM EDTA/0.5% 2-mercaptoethanol from 1 mg/ml stock solutions in this buffer (stored frozen at −70°C). The diluted proteinase solutions were kept at 42°C for about 15 min, diluted an additional 1:10 with the same buffer containing 1% agarose (melted, then cooled to 42°C), and then used to overlay the embedded gels (about 0.3 ml of agarose solution per 5 cm of gel). Proteins were electrophoresed into stacking gels (until tracking dye was within 0.5 cm of the lower gel surface), the power was turned off for 30 min, and then electrophoresis was resumed. Gels were stained, destained, and subjected to autoradiography as described above.

Phosphoserine Determination. The phosphorylated actin spot was excised from the dried gel of Fig. 4*b* by using a tracing of the autoradiogram as a guide; 0.5 ml of 2M HCl was added and the sample was hydrolyzed for 5.5 hr at 110°C. The hydrolyzate was dried under reduced pressure, dissolved in 25 μl of water (less than 15% of a total of about 40 cpm was left in the gel residue), and subjected to paper electrophoresis for 2.5 hr at 5000 V in an acetic acid/formic acid buffer system at pH 1.85. Unlabeled phosphoserine and phosphothreonine samples were run in lanes adjacent to the labeled sample and were separated by about 8 cm in distance migrated; a sample of $^{32}PO_4$ migrated off the electropherogram. Radioactive spots were visualized by autoradiography on Kodak XR-2 film with a Du Pont Lightning-Plus intensifying screen and exposure for 14 days at −78°C.

RESULTS AND DISCUSSION

Fig. 1*b* shows the appearance of protein O in a two-dimensional gel pattern from S49 cells labeled with [^{35}S]methionine in the presence of But_2cAMP; Fig. 1*a* shows the corresponding pattern from cells labeled in the absence of the cAMP analog. The intensely labeled spot to the left of protein O is the predominant cellular form of actin as revealed by staining with Coomassie blue (not shown). Protein O had a mobility identical to that actin in the $NaDodSO_4$ second-dimension gel and was separated from actin in the isoelectric focusing dimension by a distance corresponding to about 1 charge unit (18).

By the criterion of binding to DNase I, an affinity reagent for actin (20), protein O is a form of actin (Fig. 2). Fig. 2*a* shows the actin region from a two-dimensional pattern of crude extract proteins from cells labeled with [^{35}S]methionine in the presence of But_2cAMP; Fig. 2*b* shows the same region from a gel of material bound to a control agarose column from this extract; and Fig. 2*c* shows this region from a gel of the material that bound to DNase I-agarose. Actin is one of very few proteins specifically enriched by DNase I affinity chromatography, and protein O copurified with actin in this procedure. The copurifying material on the basic side of the major actin spot was metabolically labile (unpublished results) and corresponded in mobility and labeling behavior to an unstable form of actin observed in other mammalian cells (ref. 21; also see below).

Fig. 3 confirms by partial proteolysis mapping (19) that protein O is indeed a form of actin. Cells were labeled with [^{35}S]methionine in the presence or absence of But_2cAMP, extracts were prepared and subjected to DNase I-agarose purification, and the purified material was subjected to isoelectric focusing gel electrophoresis. Portions of these gels containing actin were equilibrated, loaded onto 15% acrylamide/$NaDodSO_4$ gels, and then overlayered with an agarose solution containing proteases as indicated in the figure legend. Proteolysis was then carried out and the peptides were resolved. The metabolically labile form of actin, the major form of actin, and protein O gave identical peptide patterns, within the limits of detection, in *d*, *f*, and *g*. The patterns from control cells (*c* and *e*) confirm that the peptides at the position of O were indeed derived from this protein.

In the staphylococcal protease digests (Fig. 3 *c* and *d*), several actin peptides (indicated with arrowheads in *d*) were displaced

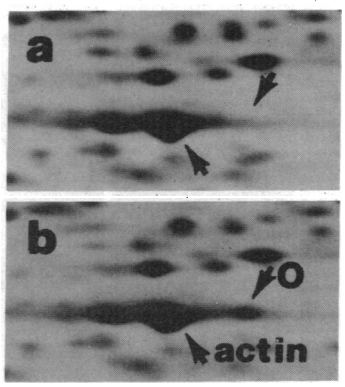

FIG. 1. cAMP-dependent appearance of protein O in [^{35}S]methionine-labeling patterns from S49 cells. Cells were preincubated for 6 hr in low-methionine medium (without drugs) before 0.5-ml samples were labeled for 15 min with [^{35}S]methionine (250 μCi/ml) in the absence (*a*) or presence (*b*) of 1 mM But_2cAMP and 30 μM RO 20-1724, an inhibitor of cAMP phosphodiesterase. Cells were harvested and extracted, and 10^6 acid-precipitable cpm from each extract was subjected to two-dimensional gel electrophoresis. Portions of the gel patterns containing actin are shown from autoradiograms exposed for 4 days. The positions of actin and protein O are designated with arrows.

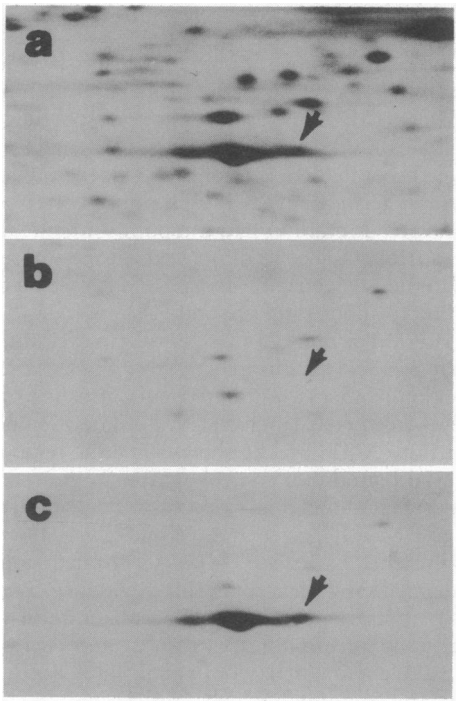

FIG. 2. Purification of protein O by DNase I-agarose affinity chromatography. Cells labeled in the presence of 1 mM But$_2$cAMP for 4 hr with [^{35}S]methionine (40 μCi/ml) were extracted and the extract was fractionated on columns containing either control agarose or DNase I-agarose. The figure shows portions of two-dimensional gel patterns of the unfractionated cell extract (*a*), material bound to control agarose (*b*), and material retained by DNase I-agarose (*c*). The position of protein O is indicated by arrows. The load was 10^6 acid-precipitable cpm for *a* and 10^5 cpm for *b* and *c*. Autoradiograms were exposed for 2 days.

slightly to the basic side of the major actin peptides; corresponding "off-line" peptides were also seen under the other actin forms. Off-line peptides were not seen in the papain digests (*e*, *f*, and *g*). Previous studies, using shallower pH gradients and decreased equilibration of isoelectric focusing gels, have resolved the major form of nonmuscle actin into two species, designated β and γ (21, 22); the metabolically labile form has also been resolved into two species, designated δ and ϵ (21). The correspondence of these species with the forms resolved here is indicated in Fig. 3*b*. Reports by Hunter and Garrels (23) and Vandekerckhove and Weber (24) suggest that β and γ actins are derived from two gene products differing primarily at their amino termini by the substitution of three glutamates in γ for aspartates in β; δ and ϵ appear to be nonacetylated forms, respectively, of β and γ actins (23). The appearance of off-line peptides as a result of digestion with staphylococcal protease is apparently a reflection of the β/γ and δ/ϵ heterogeneity and can be rationalized by the specificity of this protease for glutamoyl bonds (25). Furthermore, the microheterogeneity of protein O suggests that both β and γ actins are subject to cAMP-dependent modification.

To determine whether or not the cAMP-dependent modification of actin is a phosphorylation, cells were labeled for 6 hr with [^{32}P]phosphate in the presence or absence of But$_2$cAMP. Extracts were prepared and purified on DNase I-agarose, and the purified material was subjected to two-dimensional gel electrophoresis. Fig. 4*a* shows a portion of the gel pattern from material labeled in the absence of But$_2$cAMP and Fig. 4*b* shows a similar portion of the gel pattern from material labeled in the presence of this analog. One labeled spot (designated with an arrow) shows strong cAMP dependence in its intensity of labeling; neither this spot nor the spot immediately to its left was retained by an agarose column containing N^6-(2-aminoethyl)-cAMP instead of DNase I (not shown). Fig. 4*d* shows that the position of the cAMP-enhanced spot of Fig. 4*b* is identical to that of protein O in an [^{35}S]methionine-labeled preparation; the ^{35}S pattern is shown by itself in Fig. 4*c* for comparison. The cAMP-dependence, DNase I binding, and comigration of protein O and the ^{32}P-labeled species provide strong evidence that protein O is phosphorylated actin. This conclusion was further supported by peptide mapping of the ^{32}P-labeled species by using the method illustrated in Fig. 3 (not shown). After autoradiography the phosphorylated actin spot was ex-

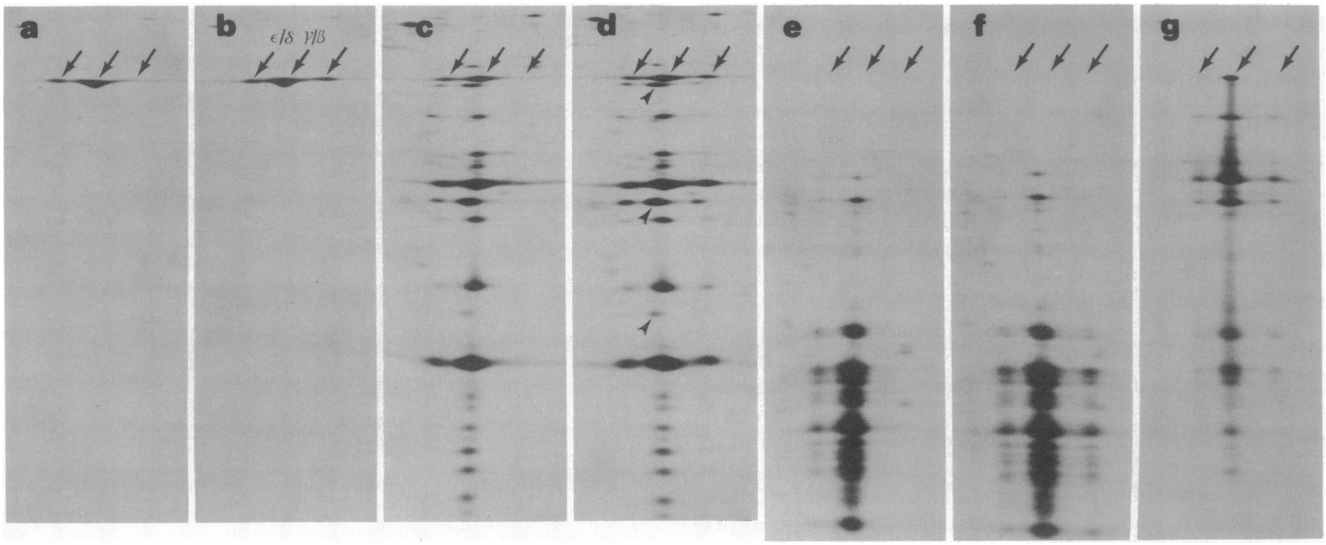

FIG. 3. Peptide mapping of S49 cell actins by partial proteolysis. Cells were labeled for 1.5 hr with [^{35}S]methionine (150 μCi/ml) in the absence (*a*, *c*, and *e*) or presence (*b*, *d*, *f*, and *g*) of But$_2$cAMP, extracts were prepared, and actins were purified on DNase I-agarose. Samples from these preparations containing 2×10^5 cpm were subjected to the modified two-dimensional gel procedure; the first-dimension gels were overlayered with agarose containing no protease (*a* and *b*), 3.3 μg of staphylococcal protease per ml (*c* and *d*), 67 ng of papain per ml (*e* and *f*), or 10 ng of papain per ml (*g*). Arrows indicate, from left to right, the positions of the metabolically labile δ/ϵ actins, the Coomassie blue staining β/γ actins, and protein O; arrowheads in panel d indicate major off-line peptides discussed in the text. Autoradiographic exposures were for 1 (*a* and *b*), 9 (*c*–*f*), or 10 (*g*) days.

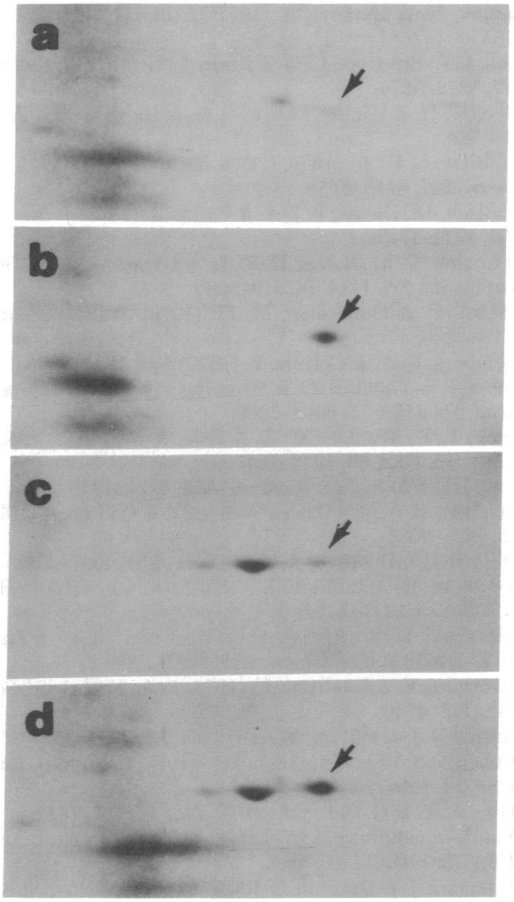

FIG. 4. Protein O is phosphorylated actin. Cells were labeled for 6 hr with $^{32}PO_4$ in the presence or absence of But_2cAMP, extracts were prepared, and actins were purified on DNase I-agarose. (*a* and *b*) Portions of gel patterns of the $^{32}PO_4$-labeled preparations from cells labeled in the absence (*a*) and presence (*b*) of But_2cAMP. (*c*) The corresponding pattern from cells labeled with [^{35}S]methionine in the presence of But_2cAMP (from the preparation used in the experiment of Fig. 3). (*d*) The pattern from a mixture of the samples for *b* and *c*. The position of protein O is indicated by arrows. The load was 3×10^5 cpm of ^{32}P or ^{35}S per gel. Autoradiograms were exposed for 1 day at −78°C with intensifying screens.

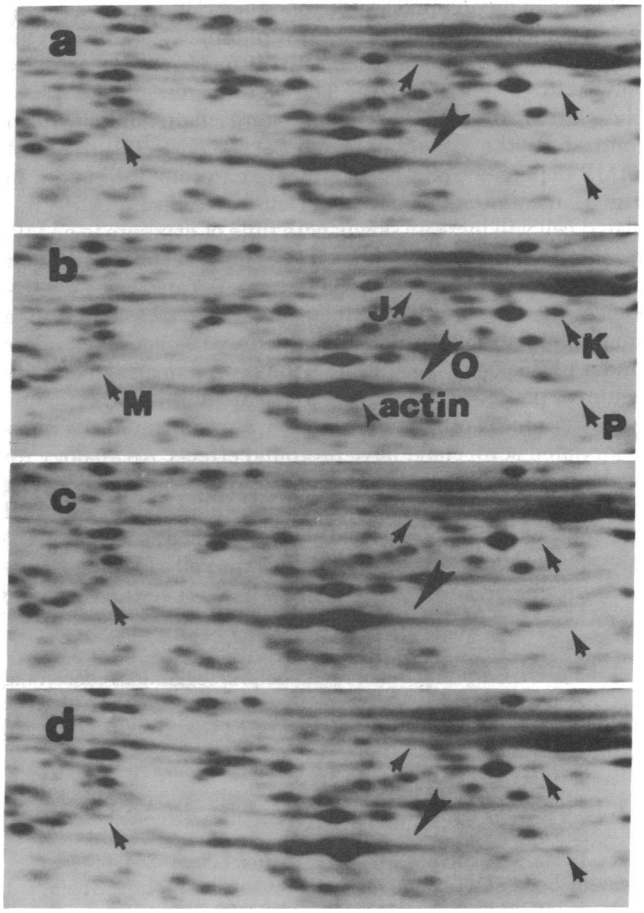

FIG. 5. cAMP-dependent appearance of protein O requires concomitant protein synthesis. [^{35}S]Methionine (500 μCi) was added to 1 ml of cells preincubated in low-methionine medium. After 5-min incubation at 37°C, 0.25-ml samples were removed and incubated an additional 5 min without (*a*) or with (*b*) 10 μM D,L-isoproterenol; then they were harvested and extracted. After 8 min 50 sec of labeling, emetine was added to the remainder of the culture to a concentration of 100 μM; 10 sec later, samples were again removed and incubated 5 min without (*c*) or with (*d*) isoproterenol as above. (This concentration of emetine was sufficient to inhibit incorporation of [^{35}S]-methionine by more than 90%.) From each sample, 2×10^6 acid-precipitable cpm was subjected to two-dimensional gel electrophoresis and autoradiograms were prepared. Autoradiographic exposures were for 5 days. Large arrowheads, position of protein O; smaller arrows, positions of several other spots shown previously to appear reproducibly in gel patterns from S49 cells labeled with [^{35}S]methionine in the presence of analogs or inducers of cAMP (12); letter designations in *b* are from ref. 12. The position of the major Coomasie blue-staining form of actin is also indicated in *b*. Portions of gel patterns shown include proteins with molecular weights from about 40,000 to 60,000 and isoelectric points in the range of about 5.6 to 6.7.

cised from the gel of Fig. 4*b*, hydrolyzed with hydrochloric acid and subjected to high-voltage electrophoresis. The only ^{32}P-labeled material detected in the hydrolyzate comigrated with phosphoserine.

Fig. 5 shows that the appearance of protein O in gel patterns of [^{35}S]methionine-labeled proteins requires increased cAMP during the period of labeling. Protein O is seen when isoproterenol is present during the latter half of an 11-min labeling period (Fig. 5*b*), but it is not seen when cells are treated with isoproterenol after labeling has been terminated with emetine, a potent inhibitor of protein chain elongation (Fig. 5*d*). Fig. 5 *a* and *c* show patterns from control cultures labeled in parallel with the cultures for *b* and *d*. Proteins M and P, whose appearances are cAMP-dependent and independent of new protein synthesis, provide a positive control for this experiment. The cAMP-dependent proteins J and K, which failed to appear in an earlier label–chase experiment (12), also failed to appear in the emetine-treated cells. The previous label–chase experiment, using centrifugation to remove label and But_2cAMP to activate endogenous protein kinase (12), left a margin of about 20–30 min for the period during which protein O might appear in the absence of new protein synthesis. The experiment of Fig. 5 reduces this margin to less than 2–3 min by using emetine to inhibit protein synthesis within seconds and isoproterenol to increase intracellular cAMP and activate endogenous kinase within 1 min (unpublished results). In view of the evidence above that protein O is phosphorylated actin, the results of Fig. 5 suggest that phosphorylation occurs concomitantly with actin synthesis. This implies that a site accessible for cAMP-dependent phosphorylation in actin nascent chains becomes inaccessible in the completed protein.

The results presented in this report demonstrate that S49 cell actin is subject to cAMP-dependent phosphorylation *in vivo* during or for a few minutes after its synthesis. Only a small fraction (about 10%) of the newly synthesized actin is phosphorylated and, from the earlier study (12), the phosphorylated actin is stable in the continued presence of cAMP. Both β and

γ actins can be phosphorylated, phosphorylation is on a serine residue, and only a single residue is phosphorylated per actin chain [because phosphorylated actin differs from β/γ actin by only a single charge (18)]. The significance of this phosphorylation, if any, to actin function remains unknown. In contrast to the situation for more orthodox substrates of cAMP-dependent phosphorylation (e.g., proteins M and P in Fig. 5), the amount of phosphorylated actin in a cAMP-stimulated cell will increase slowly with time of exposure to cAMP, reaching a maximum of about 10% of the level of total cellular actin only after several cell generations. Because most physiological increases of intracellular cAMP are transient, only a small amount of phosphorylated actin will ever accumulate under physiological conditions. However, if one or a few molecules of phosphorylated actin were capable of modifying the behavior of an entire actin filament, a small amount of phosphorylated actin could have profound effects. An analogy for such an effect is provided by the substoichiometric poisoning of microtubule assembly by colchicine–tubulin dimers (26). If the actin phosphate were stable in the absence as well as in the presence of cAMP, it could also serve the cell as a memory device for delayed (and, perhaps, cell cycle-specific) responses to cAMP increases.

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