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Chemoattractant-elicited Increases in *Dictyostelium* Myosin Phosphorylation Are Due to Changes in Myosin Localization and Increases in Kinase Activity*

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We previously reported (Berlot, C. H., Spudich, J. A., and Devreotes, P. N. (1985) *Cell* 43, 307–314) that cAMP stimulation of chemotactically competent *Dictyostelium* amoebae causes transient increases in phosphorylation of the myosin heavy chain and 18,000-dalton light chain *in vivo* and *in vitro*. In this report we investigate the mechanisms involved in these changes in phosphorylation. In the case of heavy chain phosphorylation, the amount of substrate available for phosphorylation appears to be the major factor regulating the *in vitro* phosphorylation rate. Almost all heavy chain kinase activity is insoluble in Triton X-100, and the increase in the heavy chain phosphorylation rate *in vitro* parallels an increase in Triton insolubility of myosin. Changes in heavy chain phosphatase activity are not involved in the changes in the *in vitro* phosphorylation rate. In the case of light chain phosphorylation, increases in the *in vitro* phosphorylation rate occur under conditions where the amount of substrate available for phosphorylation is constant and phosphatase activity is undetectable, implicating light chain kinase activation as the means of regulation. The specificity of the myosin kinases operating *in vivo* and *in vitro* was explored using phosphoamino acid and chymotryptic phosphopeptide analysis. The light chain is phosphorylated on serine both *in vivo* and *in vitro*, and phosphopeptide maps of the light chain phosphorylated *in vivo* and *in vitro* are indistinguishable. In the case of the heavy chain, both serine and threonine are phosphorylated *in vivo* and *in vitro*, although the cAMP-stimulated increases in phosphorylation occur primarily on threonine. Phosphopeptide maps of the heavy chain show that the peptides phosphorylated *in vitro* represent a major subset of those phosphorylated *in vivo*. The kinetics of the transient increases in myosin phosphorylation rates observed *in vitro* can be predicted quantitatively from the *in vivo* myosin phosphorylation data assuming that there is a constant phosphatase activity.

Protein phosphorylation plays a major role in the regulation of many cellular processes such as cell motility, adaptation (or desensitization) of responses to external ligands, and cell proliferation (for review see Rosen and Krebs, 1981). In many systems increases in myosin light chain phosphorylation correlate with increased cell motility in the form of locomotion, shape changes, or organelle movement. Stimulation of polymorphonuclear leukocytes with the chemoattractant f-Met-Leu-Phe induces a series of shape changes which coincide with increases in myosin light chain phosphorylation (Fechheimer and Zigmond, 1983). Stimulation of platelets with ADP, which causes them to assume a spheroidal shape without aggregation or secretion, induces a light chain phosphorylation increase which slightly precedes the shape change (Daniel *et al.*, 1984). Addition of antibody reactive with cell-surface immunoglobulin to lymphocyte populations enriched for B cells stimulates locomotion of these cells and also increases *in vivo* phosphorylation levels of the 20,000-dalton light chain of myosin (Fechheimer and Cebra, 1982). Addition of colchicine to T-lymphoma cells induces capping of surface antigens concomitant with the intracellular accumulation of actin and myosin directly beneath the surface cap structure. It also increases the level of phosphorylation of the 20,000-dalton light chain of myosin by 5–8-fold (Bourguignon *et al.*, 1981).

We have shown (Berlot *et al.*, 1985) that cAMP stimulation of chemotactically competent *Dictyostelium discoideum* amoebae results in transient phosphorylation increases *in vivo* on both the heavy chain and the 18,000-dalton light chain of myosin. The time course of these *in vivo* phosphorylation changes is the same as that observed for changes in shape of the cells in response to a temporal jump in cAMP concentration (Fontana *et al.*, 1985; Chisholm *et al.*, 1985). The myosin heavy chain also displays a rapid transient decrease in phosphorylation *in vivo* which immediately precedes the increase and which correlates temporally with a "cringe" response (the cells cease all obvious movement) to cAMP seen immediately after stimulation (Futrelle *et al.*, 1982). We also observed increases in the rate of myosin phosphorylation *in vitro* when unlabeled amoebae were stimulated with cAMP and then lysed into a [γ-^{32}P]ATP-containing reaction mixture (Berlot *et al.*, 1985). These increases in amount and rate of myosin phosphorylation also occur in amoebae pretreated with caffeine (Berlot *et al.*, 1985), which was demonstrated by Brenner and Thoms (1984) to block cAMP-induced activation of adenylate cyclase.

A comparison of the kinetics of the cAMP-induced changes in amount of myosin phosphorylation *in vivo* and rate of myosin phosphorylation *in vitro* (Berlot *et al.*, 1985) shows that the *in vitro* kinetics are approximated by the derivative of the *in vivo* kinetics. In order to understand the cause(s) of the *in vivo* myosin phosphorylation changes, we have exam-

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ined the relationships between the transient increases in the rate of myosin phosphorylation measured *in vitro* and the *in vivo* changes in amount of phosphorylation. In this report we investigate the following questions: 1) Are the transient increases in myosin phosphorylation rates measured *in vitro* due to transient increases in myosin kinase activity, transient decreases in myosin phosphatase activity, or transient changes in the amount of myosin available as a substrate to kinases and/or phosphatases? 2) Are the specificities of the myosin kinases operating *in vivo* and *in vitro* consistent with the hypothesis that the *in vitro* phosphorylation reactions measure phosphorylation rate on the same site(s) which exhibit cAMP-stimulated increases *in vivo*? 3) Are the kinetics of the cAMP-stimulated changes in myosin phosphorylation observed *in vivo* and *in vitro* consistent with the hypothesis that the changes in phosphorylation rates measured *in vitro* reflect the rates of change of myosin phosphorylation *in vivo*?

EXPERIMENTAL PROCEDURES

Growth and development conditions and caffeine treatment of *D. discoideum* strain Ax-3, *in vivo* ^{32}P-labeling, immunoprecipitation and *in vitro* phosphorylation of myosin, polyacrylamide gel electrophoresis, and densitometry were performed as described (Berlot *et al.*, 1985). Developed amoebae are amoebae which have been shaken in MES[1] buffer (20 mM MES (pH 6.8), 0.2 mM $CaCl_2$, 2 mM $MgSO_4$) at 100 rpm for 3.5 h prior to being used for experiments.

Preparation and Phosphorylation of Triton-soluble and -insoluble Cell Fractions—Triton X-100 lysis of *Dictyostelium* amoebae can be used to isolate insoluble cytoskeletons which are enriched for myosin and actin (Giffard *et al.*, 1983). 100-μl aliquots of developed amoebae (2×10^7/ml) were lysed into 180 μl of an ice-cold mixture containing 0.2% Triton X-100, 2 mM $MgCl_2$, and 7.5 mM Tris (pH 7.5). The lysates were centrifuged in a microcentrifuge for 30 s. The supernatants were removed and phosphorylated by the addition of 20 μl of 200 μM [γ-^{32}P]ATP (10^4 Ci/mol, from Amersham Corp. or prepared according to the method of Walseth and Johnson, 1979). The pellets were suspended in 100 μl of MES buffer and then phosphorylated by the addition of 180 μl of the above ice-cold Triton mixture plus 20 μl of 200 μM [γ-^{32}P]ATP (10^4 Ci/mol). The phosphorylation reactions were incubated for 2 min on ice (during which time the reaction was linear) and then stopped by the addition of trichloroacetic acid to a final concentration of 2% or an equal volume of 2 × lysis buffer if myosin were to be immunoprecipitated. Samples were further processed for SDS-polyacrylamide gel electrophoresis as described (Berlot *et al.*, 1985).

Phosphoamino Acid Analysis—Immunoprecipitated myosin which had been phosphorylated *in vivo* or *in vitro* was subjected to electrophoresis on SDS-polyacrylamide gels. Myosin light chain and heavy chain bands were cut out of the polyacrylamide gels and washed in 100 ml of 25% isopropyl alcohol followed by 100 ml of 10% methanol at 37 °C with shaking. The myosin light chain bands contained 0.5–1.0 μg of protein, and the myosin heavy chain bands contained 1.0–2.0 μg of protein (*in vivo* phosphorylations) or approximately 7.5 μg of protein (*in vitro* phosphorylations). The gel slices were dried under a heat lamp, rehydrated in 1 ml of 50 mM NH_4HCO_3, 1 mM $CaCl_2$ containing 50 μg of trypsin (Sigma, Type XI, diphenyl carbamyl chloride-treated), and incubated at 37 °C overnight with rotation. The supernatant was then transferred to a screw-cap tube and evaporated under nitrogen. 0.25 ml of 6 N HCl was added, and the tubes were sealed under nitrogen. The samples were hydrolyzed for 2 h at 110 °C. The samples were then resuspended in 40 μl of acetic acid/pyridine/water, 10:1:189, v/v/v (pH 3.5). 5–10-μl aliquots of the samples (containing 20–2000 cpm) were spotted onto 20 × 20-cm cellulose-coated glass plates (MN 300-10, Macherey and Nagel). To compare samples taken from cells before and after cAMP stimulation, the amounts of radioactivity loaded onto the plates were proportional to the amounts of myosin phosphorylation before and after stimulation. Thin-layer electrophoresis was performed as described (Hunter and Sefton, 1980) at pH 3.5 followed by pH 1.9. The positions of the internal standards were determined by spraying the plates with ninhydrin. To quantitate radioactivity, the phosphoamino acids were scraped off the plates and counted for Cerenkov radiation.

Phosphopeptide Mapping—Phosphopeptide mapping was performed as described by Maruta *et al.* (1983). Myosin light chain and heavy chain bands were cut out of SDS-polyacrylamide gels, washed, and dried, as described above. They were then resuspended in 1 ml of 0.1 M NH_4HCO_3 (pH 8), 1 mM dithiothreitol containing 50 μg of chymotrypsin (Sigma, type VII, TLCK-treated) and incubated at 37 °C with rotation. After 12 h and again after 24 h, 50 μg of chymotrypsin were added. After a total incubation time of 36 h, the samples were evaporated under nitrogen, resuspended in acetic acid/pyridine/water, 10:1:89, v/v/v (pH 3.5), and spotted onto 20 × 20-cm cellulose-coated glass plates as described above. The samples were subjected to electrophoresis toward the cathode at 400 V for 1.5 h. In the second dimension, the phosphopeptides were chromatographed in acetic acid/pyridine/*n*-butyl alcohol/water, 10:33:50:40, v/v/v/v. The positions of the phosphopeptides were visualized by autoradiography.

Assay of Myosin Light Chain Phosphatase Activity—Substrate was prepared as follows. Amoebae developed at 4×10^7/ml were treated with 5 mM caffeine for 30 min and then incubated in [^{32}P]orthophosphate (0.5 mCi/ml) for 30 min before a 2×10^{-6} M cAMP stimulus was applied. 60 s after the stimulus, a portion of the suspension was quenched in an equal volume of 2 × lysis buffer (40 mM Tris-Cl (pH 7.5), 0.2% Nonidet P-40, 2 mM dithiothreitol, 10 mM EDTA, 2 mM phenylmethylsulfonyl fluoride, 2 mM Nα-*p*-tosyl-L-arginine methyl ester, 200 μM TPCK, 200 μM TLCK, 20 mM $NaHSO_3$, 100 μg/ml RNase A (Worthington), 50 mM sodium pyrophosphate, 200 mM NaF, 2 mM ATP, and 200 mM potassium phosphate (pH 7.5)). 500-μl aliquots of this lysate were then added to preincubated *Staphylococcus aureus* cell (Pansorbin)-myosin antibody complexes and immunoprecipitated as described (Berlot *et al.*, 1985).

Phosphatase assays were performed before and at timed intervals after cAMP stimulation of a developed cell suspension. For each assay, 50 μl of developed cell suspension (2×10^7/ml) were added to 200 μl of reaction mix (0.2% Triton X-100, 2 mM $MgCl_2$, 7.5 mM Tris-Cl (pH 7.5), 20 μM ATP, and ^{32}P-labeled myosin substrate), and incubated for 30 s at 22 °C. The reactions were quenched by the addition of 2 × lysis buffer and then processed as myosin immunoprecipitates (Berlot *et al.*, 1985).

RESULTS

Myosin Heavy Chain Phosphorylation

Increases in Phosphorylation Rate in Vitro Coincide with the Association of Myosin with the Triton-insoluble Cytoskeleton—As in previously reported experiments (Berlot *et al.*, 1985), the effects of cAMP stimulation of intact amoebae on myosin heavy chain phosphorylation were monitored both *in vivo* and *in vitro*. We previously demonstrated that the stimulus triggers immediate transient increases in myosin heavy chain phosphorylation (Berlot *et al.*, 1985). These measured increases were about 1.7-fold *in vivo* and 4–9-fold when assayed *in vitro*. We had also observed that the *in vitro* phosphorylation reaction mixtures, which represented a 300-fold dilution of the cells, could be further diluted by a factor of at least 7 without any effect on the rate of heavy chain phosphorylation (Berlot *et al.*, 1985), suggesting that myosin and the heavy chain kinase might be associated. In this report, we examined the solubilities of myosin and the heavy chain kinase in the Triton reaction mixture used for the *in vitro* phosphorylations. When a Triton-lysate of cells was fractionated into a soluble and an insoluble fraction by centrifugation before the addition of [γ-^{32}P]ATP, the majority of the heavy chain kinase activity was found in the insoluble fraction, both before (Fig. 1*C*, *lane 1*) and after (Fig. 1*C*, *lane 3*) cAMP stimulation. However, in unstimulated cells only ~7% of the myosin was Triton-insoluble (Fig. 1, *A* and *B*, *lanes 1* and *2*). After cAMP stimulation the amount of Triton-insoluble myosin transiently increased to ~50% of the total myosin (Fig. 1, *A* and *B*, *lanes 3* and *4*; Fig. 1*D*). The time course of the transient increase in myosin insolubility paralleled that

[1] The abbreviations used are: MES, 2-(*N*-morpholino)ethanesulfonic acid; TLCK, Nα-*p*-tosyl-L-lysine chloromethyl ketone; TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone; SDS, sodium dodecyl sulfate.

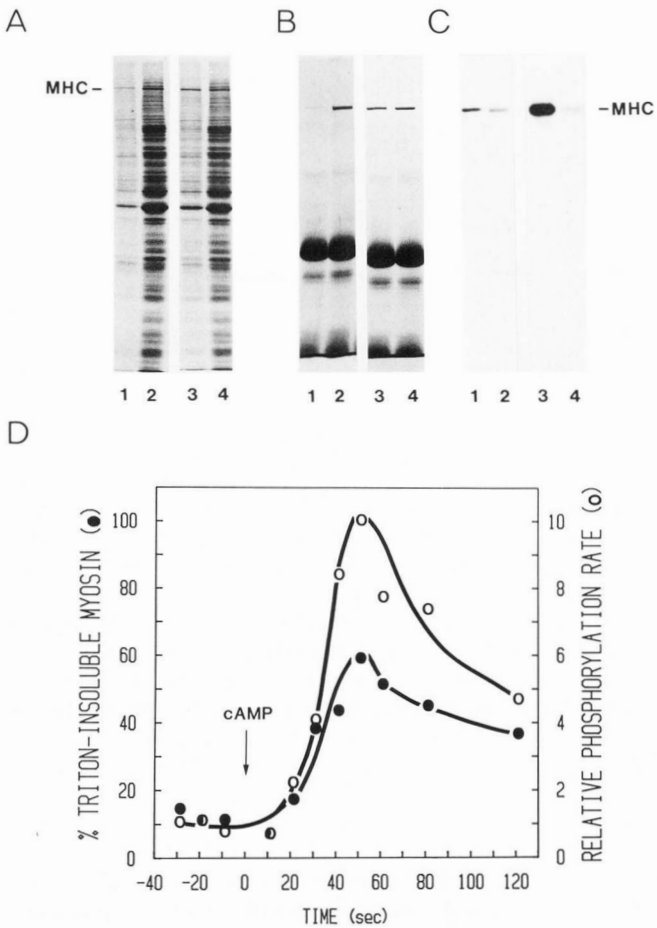

FIG. 1. **cAMP-induced increases in Triton insolubility of myosin coincide with increases in heavy chain phosphorylation rate.** In *A–C*, aliquots of a developed and caffeine-treated cell suspension were removed before and after stimulation with 2×10^{-6} M cAMP, added to ice-cold Triton mixtures, and centrifuged as described under "Experimental Procedures." Gel *lanes* in *A–C*: *1*, pellet from unstimulated cells; *2*, supernatant from unstimulated cells; *3*, pellet from cells, 50 s after stimulation; *4*, supernatant from cells, 50 s after stimulation. *A*, Coomassie-stained SDS-polyacrylamide gel (10%) of total lysate. Samples were trichloroacetic acid-precipitated as described under "Experimental Procedures." *B*, Coomassie-stained SDS-polyacrylamide gel (7.5%) of myosin immunoprecipitates. Samples were phosphorylated with [γ-^{32}P]ATP as described under "Experimental Procedures," and myosin was immunoprecipitated as described (Berlot *et al.*, 1985). The myosin heavy chain (*MHC*) migrates faster here than in *A* due to the lower percentage of acrylamide. *C*, autoradiograph of *B*. In *D*, aliquots of a developed and caffeine-treated cell suspension were added to ice-cold Triton mixtures and centrifuged at the indicated times. The pellets and supernatants were phosphorylated as described under "Experimental Procedures," and myosin was then immunoprecipitated. Percent Triton-insoluble myosin (*closed circles*) was determined by quantitating the amount of myosin in the pellets and the supernatants using densitometry as described (Berlot *et al.*, 1985) and taking the ratio of the amount of myosin in the pellet to the total amount of myosin for each point. Relative phosphorylation rate (*open circles*) is the ratio of the myosin phosphorylation rate measured *in vitro* at a given time after cAMP stimulation (2×10^{-6} M) to the average of the myosin phosphorylation rates measured *in vitro* before stimulation. Phosphorylation rate was determined by measuring the amount of phosphate incorporated into myosin during a linear 2-min reaction on ice and normalizing for variability in aliquot size by dividing the values obtained by the total amount of myosin in each assay. The amount of myosin was determined by densitometry and the amount of phosphorylation was determined by measuring Cerenkov radiation of the myosin bands cut out of the polyacrylamide gels.

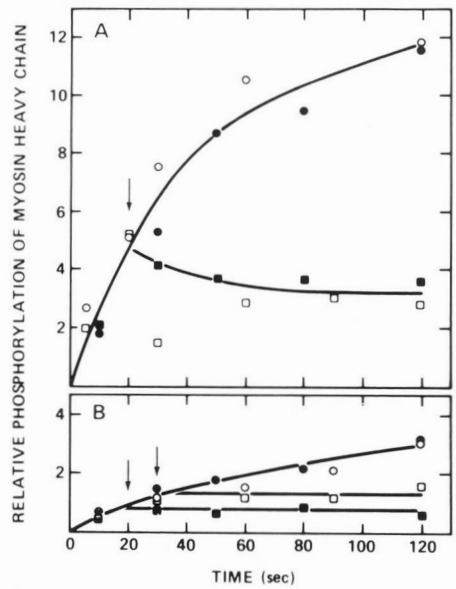

FIG. 2. ***In vitro* assay of myosin heavy chain phosphatase activity before and after cAMP stimulation.** *A*, caffeine-treated developed cells at a density of 2×10^7/ml (1 volume) that had been stimulated with 2×10^{-6} M cAMP for 50 s were lysed into 2 volumes of a reaction mix containing 0.2% Triton X-100, 2 mM $MgCl_2$, 7.5 mM Tris-Cl (pH 7.5), 1 mM phenylmethylsulfonyl fluoride, 100 μM TPCK, 100 μM TLCK, and 20 μM [γ-^{32}P]ATP (10^4 Ci/mol). The lysate was split in half. One half (*circles*) was incubated at 22 °C for the times shown, and phosphorylation of the myosin heavy chain was measured. The other half (*squares*) received a cold chase of unlabeled ATP at 20 s (*arrow*) and was similarly incubated. The *open symbols* represent time points taken in an experiment in which a cold chase of 2 mM ATP was added. The *closed symbols* represent time points taken in an experiment in which a cold chase of 100 mM ATP was added. At the indicated times 300-μl aliquots were removed from the reactions and brought to a final concentration of 2% trichloroacetic acid. Samples were processed for SDS-polyacrylamide gel electrophoresis as described (Berlot *et al.*, 1985). Relative phosphorylation was quantitated using densitometry. In *B*, the same experiment described for *A* was carried out with cells before stimulation. The 2 mM ATP cold chase in this experiment was applied after 30 s of incubation (*arrow*), whereas the 100 mM ATP cold chase was applied after 20 s of incubation (*arrow*). The cAMP-elicited increases in heavy chain phosphorylation rate are of the same magnitude regardless of whether phosphorylation rates are measured in the presence of 20 μM or 2 mM ATP.

of the transient increase in heavy chain phosphorylation rate *in vitro* (Fig. 1*D*). This result suggests that the observed changes in rate of heavy chain phosphorylation are due to changes in the availability of the heavy chain to its kinase rather than changes in kinase activity.

Increases in Heavy Chain Phosphorylation Rate in Vitro Do Not Involve Phosphatase Inhibition—To determine whether heavy chain phosphatase activity plays a role in the transient cAMP-induced heavy chain phosphorylation rate increases measured *in vitro*, cells were taken before (Fig. 2*B*) or after (Fig. 2*A*) cAMP stimulation and lysed into a reaction mix containing [γ-^{32}P]ATP. After 20 or 30 s of incubation, while the phosphorylation levels were still increasing linearly, a cold chase of ATP was added, and myosin dephosphorylation was measured as a function of time. For both basal and stimulated cells, addition of [^{32}P]phosphate to the myosin heavy chain by *in vitro* kinase activity ceased upon addition of the cold chase. Phosphorylation levels did not subsequently decrease significantly (Fig. 2). This result demonstrates that, under the *in vitro* conditions in which increases in myosin heavy chain phosphorylation rate are observed after cAMP stimulation of intact cells, there is minimal myosin phosphatase

activity in either stimulated or unstimulated cell extracts. Inhibition of phosphatase, therefore, does not play a role in these transient *in vitro* phosphorylation rate increases. It is important to emphasize that the absence of significant levels of phosphatase activity in the *in vitro* assays does not imply that there is no phosphatase *in vivo*. A phosphatase is clearly essential *in vivo* to bring the heavy chain phosphorylation levels back to base line after the transient increase in phosphorylation.

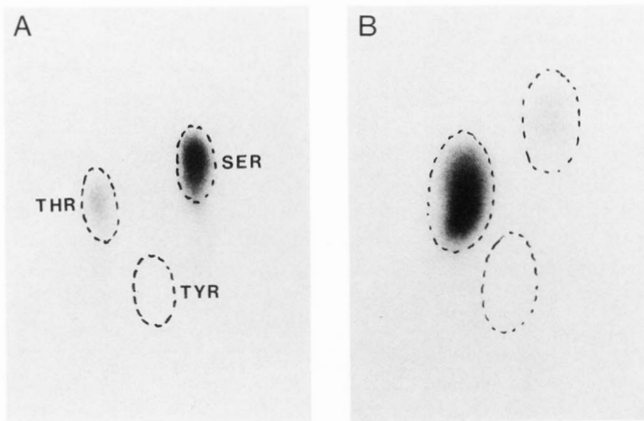

FIG. 3. **Phosphoamino acid analysis of the myosin heavy chain phosphorylated *in vivo* and *in vitro*.** Myosin heavy chain from unstimulated developed cells was phosphorylated *in vivo* (*A*) or *in vitro* (*B*). For *A*, the developed cell suspension (2×10^7 cells/ml) was labeled with [^{32}P]orthophosphate (5 mCi/ml) for 20 min before the sample was taken. For *B*, 0.5 ml of a developed cell suspension (2×10^7 cells/ml) was added to 1 ml of reaction mix containing 0.2% Triton X-100, 2 mM $MgCl_2$, 7.5 mM Tris-Cl (pH 7.5), and 20 μM [γ-^{32}P]ATP (1.25×10^5 Ci/mol) and reacted for 30 s at 22 °C. The reaction was stopped and myosin was immunoprecipitated as described (Berlot *et al.*, 1985). In the phosphoamino acid maps shown, the vertical dimension is the result of electrophoresis toward the anode at pH 3.5, whereas the horizontal dimension is the result of electrophoresis toward the anode at pH 1.9. The positions of the ^{32}P-labeled phosphoamino acids were visualized by autoradiography. The positions of the ninhydrin-stained internal standards are marked by *dotted lines*. *THR*, threonine; *SER*, serine; *TYR*, tyrosine.

Analysis of Phosphoamino Acids and Chymotryptic Phosphopeptides Phosphorylated in Vivo and in Vitro—We compared the specificity of the myosin heavy chain kinase(s) operating *in vivo* and *in vitro* using phosphoamino acid and chymotryptic phosphopeptide analysis. Prior to cAMP stimulation, the myosin heavy chain was phosphorylated on both serine and threonine residues (Fig. 3). There was relatively more phosphoserine than phosphothreonine in the *in vivo* labeling conditions (~80% phosphoserine; Fig. 3*A*), whereas the opposite was observed in the *in vitro* phosphorylations (~75% phosphothreonine; Fig. 3*B*). After cAMP stimulation, threonine phosphorylation accounted for most of the phosphorylation increases both *in vivo* and *in vitro*. Threonine phosphorylation increased by a factor of 2.0 (SD = 0.5, n = 3) *in vivo* and a factor of 3.8 (SD = 0.9, n = 2) *in vitro*, whereas serine phosphorylation increased by a factor of only 1.3 *in vivo* (SD = 0.15, n = 3) and *in vitro* (SD = 0.14, n = 2).

A comparison of the chymotryptic phosphopeptide maps of the myosin heavy chain phosphorylated *in vivo* and *in vitro* revealed that five out of the seven peptides phosphorylated *in vivo* (*1, 2, 3, 5*, and *6* in Fig. 4*A*) were also phosphorylated *in vitro* (Fig. 4*B*). All of the peptides phosphorylated *in vitro* were phosphorylated *in vivo*. Phosphopeptide maps prepared from mixtures of *in vivo* and *in vitro* labeled peptides confirmed that the *in vitro* labeled peptides co-migrated with *in vivo* labeled peptides (Fig. 4*C*). We conclude that the *in vitro* phosphopeptides, which contain predominantly threonine phosphorylation, are a major subset of the *in vivo* phosphopeptides. The phosphopeptide maps of myosin heavy chain

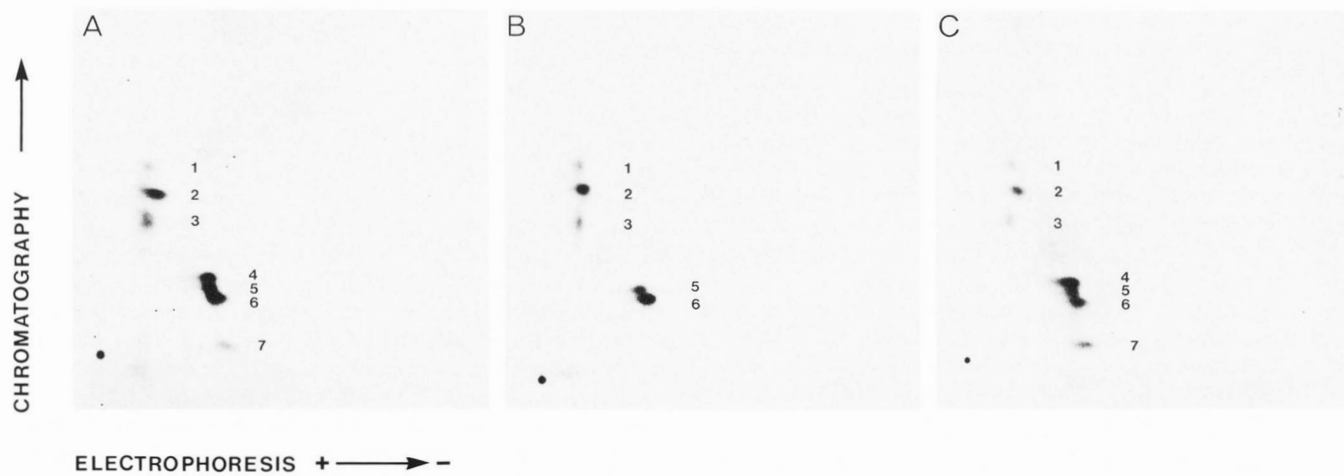

FIG. 4. **Chymotryptic phosphopeptide maps of the myosin heavy chain phosphorylated *in vivo* and *in vitro*.** *A*, phosphopeptide map of the myosin heavy chain phosphorylated *in vivo* 50 s after cAMP stimulation (2×10^{-6} M). Developed cells at a density of 10^8/ml were treated with caffeine for 30 min and then were labeled with [^{32}P]orthophosphate (3 mCi/ml) for 30 min. *B*, phosphopeptide map of the myosin heavy chain from caffeine-treated unstimulated cells phosphorylated *in vitro*. A developed cell suspension (0.5 ml; 2×10^7 cells/ml) was added to 1 ml of reaction mix containing 0.2% Triton X-100, 2 mM $MgCl_2$, 7.5 mM Tris-Cl (pH 7.5), and 20 μM [γ-^{32}P] ATP (1.7×10^4 Ci/mol), reacted for 30 s at 22 °C, and processed as described under "Experimental Procedures" for phosphopeptide mapping. *C*, phosphopeptide map of simultaneously loaded samples (equal amounts of radioactivity) of *in vivo* and *in vitro* labeled myosin heavy chain, both from developed, caffeine-treated, unstimulated cells. Samples were prepared as described for *A* and *B*. In the phosphopeptide maps shown, the samples were applied at the *lower left corner*. The first dimension (*horizontal*) is the result of electrophoresis toward the cathode at pH 3.5, whereas the second dimension (*vertical*) is the result of chromatography. The positions of the phosphopeptides were visualized by autoradiography. The quantities of radioactivity loaded onto the TLC plates and the amounts of time that the autoradiographs were exposed were determined so as to maximally visualize all of the peptides. Therefore, the plates were not loaded relative to the amounts of phosphorylation on myosin before and after cAMP stimulation, and the amounts of phosphorylation cannot be directly compared between plates.

from unstimulated cells contain the same peptides, in roughly the same proportion, as those of myosin heavy chain from stimulated cells (for example, compare Fig. 4, *A* and *C*). Thus the increases in myosin heavy chain phosphorylation after cAMP stimulation are on the same chymotryptic peptides as those phosphorylated before stimulation. These results support the hypothesis that the *in vitro* phosphorylation reactions measure phosphorylation rate on the same site(s) which exhibit cAMP-stimulated increases *in vivo*.

It should be emphasized that although SDS-denatured myosin heavy chain was digested with an excess of chymotrypsin (20–150-fold (w/w)) for 36 h, the presence of multiple spots in the peptide maps does not necessarily indicate that there are multiple sites of phosphorylation. Myosin heavy chain was also digested with an excess of trypsin (20-fold (w/w)) as well as with a combination of trypsin plus chymotrypsin (20-fold excess of each (w/w)), and multiple phosphopeptides were also obtained (data not shown). It is possible that the peptides overlap (Maruta *et al.*, 1983) or represent different oxidation states of the same peptide. In this regard, the maximum amount of myosin heavy chain phosphorylation we have measured in these experiments is less than 1 mol of phosphate per mol of heavy chain or light chain (Berlot *et al.*, 1985). The level of myosin phosphorylation was determined (Berlot *et al.*, 1985) by comparing the incorporation of [^{32}P] phosphate into myosin with the specific radioactivity of the [^{32}P]ATP. When unstimulated developed cells are incubated with [^{32}P]orthophosphate, myosin incorporates 0.05 mol of phosphate per mol of heavy chain *in vivo*. When developed cells are treated with caffeine before ^{32}P-labeling, myosin from unstimulated cells incorporates 0.03 mol of phosphate per mol of heavy chain *in vivo*. In the *in vitro* phosphorylation reactions myosin from stimulated cells incorporates 0.03 mol of phosphate per mol of heavy chain during a 30-s incubation at 22 °C (Berlot *et al.*, 1985).

Myosin Light Chain Phosphorylation

Increases in Light Chain Phosphorylation Rate in Vitro Are Due to Kinase Activation—We previously demonstrated that cAMP stimulation of intact amoebae triggers immediate transient increases in light chain phosphorylation (Berlot *et al.*, 1985). These increases are 3–10-fold *in vivo* and 20-fold when assayed *in vitro*. Since phosphorylation of the myosin light chain is assayed *in vitro* using a constant, exogenous source of myosin as a substrate (Berlot *et al.*, 1985), the observed increases in rate of light chain phosphorylation in lysates from cAMP-stimulated cells must be due to some alteration in kinase or phosphatase activity rather than in availability of the myosin substrate.

To determine whether changes in myosin light chain phosphatase were involved in the increase in the light chain phosphorylation rate *in vitro* after cAMP stimulation, excess ^{32}P-labeled myosin substrate was incubated with cell lysates prepared before and after cAMP stimulation. Under these conditions there was no detectable light chain phosphatase activity. Since the conditions of this phosphatase assay were identical to those in which an increase in light chain phosphorylation rate was observed (Berlot *et al.*, 1985), changes in phosphatase activity cannot be the cause of these cAMP receptor-mediated changes in the light chain phosphorylation rate *in vitro*. Therefore, the transient increases in light chain phosphorylation rate *in vitro* must be due to the transient activation of a light chain kinase. As in the case of the heavy chain, the absence of measurable phosphatase activity in the *in vitro* assay does not imply that there is no phosphatase activity *in vivo*. A light chain phosphatase must act *in vivo* to bring the light chain phosphorylation levels back to base line after the transient activation of light chain kinase.

Analysis of Phosphoamino Acids and Phosphopeptides Phosphorylated in Vivo and in Vitro—We compared the specificity of the myosin light chain kinase(s) operating *in vivo* and *in vitro* using phosphoamino acid and chymotryptic phosphopeptide analysis. The myosin light chain was phosphorylated on serine both *in vivo* and *in vitro* before and after cAMP stimulation of developed amoebae (Fig. 5). The same result was obtained in the presence or absence of caffeine (Fig. 5, *A–D*). The *in vivo* and *in vitro* chymotryptic phosphopeptide maps of the light chain were identical (Fig. 6). These phosphopeptide maps revealed a discrete class of peptides which were present in the same proportion both before and after cAMP stimulation of intact amoebae (data not shown). Thus the increases in light chain phosphorylation after cAMP stimulation occur within the same chymotryptic peptides as those phosphorylated before stimulation. These results support the hypothesis that the light chain phosphorylation reactions *in vitro* measure phosphorylation rate on the same sites which exhibit cAMP-stimulated phosphorylation increases *in vivo*.

As in the case of heavy chain phosphorylation, the presence of multiple spots in the light chain phosphopeptide maps does not necessarily indicate that there are multiple phosphorylation sites. When unstimulated developed cells are incubated with [^{32}P]orthophosphate, myosin incorporates 0.03 mol of phosphate per mol of light chain *in vivo* when labeling occurs in the absence of caffeine. When the developed cells are treated with caffeine before ^{32}P-labeling, myosin incorporates 0.007 mol of phosphate per mol of light chain *in vivo* (Berlot *et al.*, 1985).

Kinetic Analysis of the in Vivo and in Vitro Increases in Myosin Phosphorylation

The previously reported kinetics of the *in vitro* changes in rate of myosin phosphorylation approximate the derivative of the *in vivo* changes in amount of myosin phosphorylation (Berlot *et al.*, 1985). Our *in vitro* phosphorylation assays suggest that the transient increase in heavy chain phosphorylation rate is due to a change in the availability of the heavy chain to its kinase, whereas the increase in light chain phosphorylation rate is due to kinase activation. The conditions of these *in vitro* phosphorylation assays resemble the *in vivo* myosin phosphorylation conditions in that cAMP stimulation results in transient increases in myosin phosphorylation in both cases. Although the *in vitro* phosphorylation assays do not measure myosin phosphatase, myosin phosphatase must be present *in vivo* because myosin phosphorylation levels return to prestimulus levels after the transient cAMP-induced increases. In order to compare the kinetics of the transient increases in myosin phosphorylation *in vivo* and *in vitro*, we assumed that, *in vivo*, there is a phosphatase with an unchanging rate constant equal to k_p, which dephosphorylates myosin with first order kinetics. We modeled the relationship between the *in vitro* phosphorylation rates and the *in vivo* phosphorylation amounts as follows:

$$\alpha \frac{d[\mathrm{M} \sim \mathrm{P}]}{dt} = v(t) - k_p[\mathrm{M} \sim \mathrm{P}] \quad (1)$$

where [M ~ P] is the concentration of phosphorylated myosin, $v(t)$ is the time-dependent myosin phosphorylation rate, k_p is the time-independent phosphatase rate constant, and α is simply a proportionality factor relating the measured quantities *in vivo* and *in vitro*. The term $v(t)$ can be expressed as k_k[M] where either or both k_k, the rate constant for the kinase

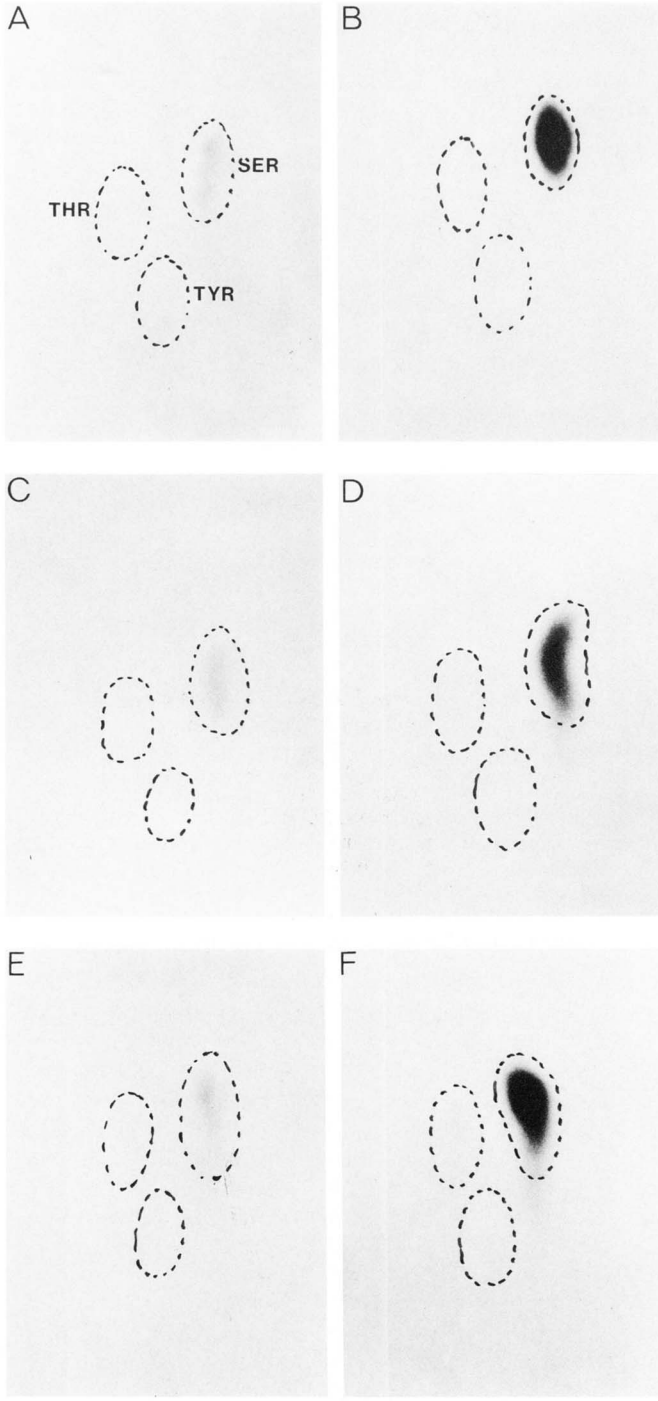

FIG. 5. **Phosphoamino acid analysis of the myosin light chain phosphorylated *in vivo* and *in vitro*.** Myosin light chain was phosphorylated *in vivo* (*A–D*) or *in vitro* (*E* and *F*). Phosphoamino acids of the light chain phosphorylated *in vivo* (*A*) before cAMP stimulation (2×10^{-6} M) and (*B*) 30 s after stimulation. The developed cell suspension (2×10^7 cells/ml) was labeled with [^{32}P]orthophosphate (5 mCi/ml) for 20 min before the points were taken. In the presence of caffeine, phosphoamino acids of the light chain phosphorylated *in vivo* (*C*) before cAMP stimulation (2×10^{-6} M) and (*D*) 60 s after stimulation and *in vitro* (*E*) before cAMP stimulation (2×10^{-6} M) and (*F*) 30 s after stimulation. For *C* and *D*, the developed cell suspension (2×10^7 cells/ml) was labeled with [^{32}P]orthophosphate (10 mCi/ml) for 30 min before the points were taken. For *E* and *F*, 50 μl of developed cell suspension (2×10^7 cells/ml) were added to 330 μl of reaction mix containing 0.2% Triton X-100, 2 mM $MgCl_2$, 7.5 mM Tris-Cl (pH 7.5), 20 μM [γ-^{32}P]ATP (4.5×10^5 Ci/mol), and immunoprecipitated myosin. The reactions proceeded for 30 s at 22 °C and were then processed as described (Berlot *et al.*, 1985). In the phosphoamino acid maps shown, the *vertical dimension* is the result of electrophoresis toward the anode at pH 3.5, whereas the *horizontal dimension* is the result of electrophoresis toward the anode at pH 1.9. The positions of the ^{32}P-labeled phosphoamino acids were visualized by autoradiography. The positions of the ninhydrin-stained internal standards are marked by *dotted lines*. The quantities of radioactivity loaded onto each TLC plate were in proportion to the -fold increases in light chain phosphorylation. Therefore, for each condition of labeling, the amounts of radioactivity in the maps from stimulated *versus* unstimulated cells can be compared directly.

reaction, and [M], the concentration of phosphorylatable myosin, may be time-dependent variables. In the case of the heavy chain phosphorylation response it appears to be [M] which increases, whereas during the light chain phosphorylation response, k_k appears to transiently increase. Rearranging Equation 1, we obtain

$$v(t) = \alpha \frac{d[\mathrm{M} \sim \mathrm{P}]}{dt} + k_p[\mathrm{M} \sim \mathrm{P}] \quad (2)$$

Thus the kinetic relationship between [M ~ P] (as determined *in vivo*) and $v(t)$ (as determined *in vitro*) is defined up to two constants, α and k_p. The phosphorylation rate is a linear combination of [M ~ P] and its first derivative.

The *in vivo* phosphorylation data from a 15-min ^{32}P-labeling was used to provide [M ~ P] during a response to 2×10^{-6} M cAMP. Data obtained for the heavy chain (Fig. 7*A*) and the light chain (Fig. 7*B*) were used to predict the time course of $v(t)$, the rate of myosin phosphorylation, using Equation 2. There is good agreement between the predicted phosphorylation rates and the rates measured *in vitro* for both myosin chains. Thus, the kinetics of the cAMP-induced changes in myosin phosphorylation rates measured *in vitro* appear to reflect the kinetics of the changes in myosin phosphorylation amounts *in vivo* after a cAMP stimulus. The best-fit value for k_p, the phosphatase rate constant, was the same for both chains and equal to 0.05 s^{-1}, corresponding to a $t_{1/2}$ of 14 s for the dephosphorylation reaction.

DISCUSSION

Our previous observations (Berlot *et al.*, 1985) that cAMP stimulation of amoebae results in increases both in amount of myosin phosphorylation *in vivo* and in rate of myosin phosphorylation as measured *in vitro* suggested that the *in vitro* phosphorylation assays could be exploited to investigate mechanisms underlying the phosphorylation responses. Using these assays, we observed that most of the myosin heavy chain phosphorylated *in vitro* is Triton-insoluble, as is nearly all of the heavy chain kinase activity measured under these conditions. Furthermore, the cAMP-stimulated increase in heavy chain phosphorylation rate *in vitro* correlates temporally with an increase in Triton insolubility of myosin. Thus, the major factor responsible for the transient heavy chain phosphorylation rate increase observed *in vitro* appears to be the amount of myosin heavy chain available for phosphorylation. On the other hand, the major factor responsible for the light chain phosphorylation rate increase measured *in vitro* appears to be activation of light chain kinase, since the cAMP-stimulated increase in phosphorylation rate occurs under conditions where the amount of light chain available for phosphorylation is constant and light chain phosphatase activity is undetectable. Our analysis of the specificity of the myosin kinase(s) operating *in vivo* and *in vitro* is consistent with the hypothesis that the site(s) phosphorylated *in vitro* are the same site(s) which exhibit cAMP-stimulated increases *in vivo*. Furthermore, our analysis of the kinetics of change of *in vivo* amounts of phosphorylation and *in vitro* rates of phosphorylation supports the idea that the changes in rate

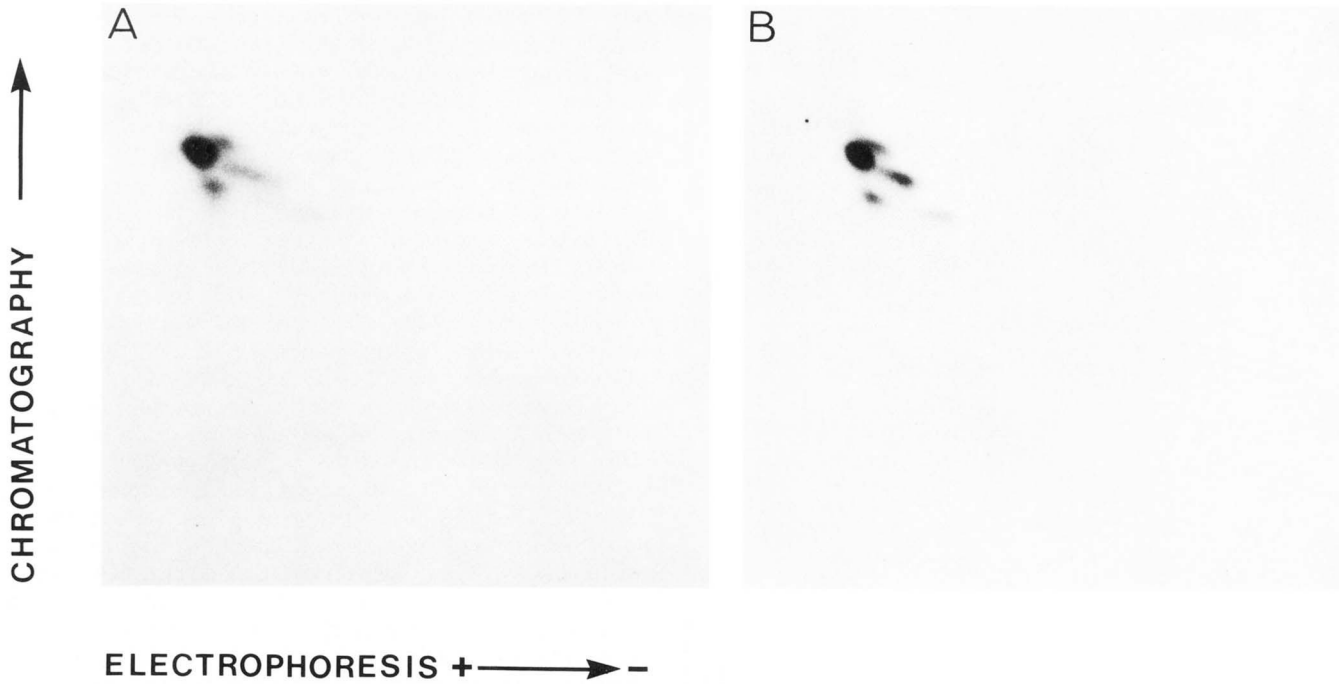

FIG. 6. **Chymotryptic phosphopeptide maps of the myosin light chain phosphorylated *in vivo* and *in vitro*.** *A*, phosphopeptide map of the myosin light chain phosphorylated *in vivo* 50 s after cAMP stimulation (2×10^{-6} M). Developed cells at a density of 10^8/ml were treated with caffeine for 30 min and then were labeled with [^{32}P]orthophosphate (3 mCi/ml) for 30 min. *B*, phosphopeptide map of the myosin light chain phosphorylated *in vitro* 25 s after cAMP stimulation. 50 μl of a developed cell suspension (2×10^7 cells/ml) which had been treated with caffeine for 30 min were added to 200 μl of reaction mix containing 0.2% Triton X-100, 2 mM $MgCl_2$, 7.5 mM Tris-Cl (pH 7.5), 20 μM [γ-^{32}P]ATP (2.5×10^5 Ci/mol), and immunoprecipitated myosin. The reaction proceeded for 30 s at 22 °C. The sample was then processed as described (Berlot *et al.*, 1985). In the phosphopeptide maps shown, the samples were applied at the *lower left corner*. The first dimension (*horizontal*) is the result of electrophoresis toward the cathode at pH 3.5, whereas the second dimension (*vertical*) is the result of chromatography. The positions of the phosphopeptides were visualized by autoradiography.

observed *in vitro* reflect the actions of the kinases operating *in vivo*.

We have not yet rigorously localized the serine and threonine sites of myosin heavy chain phosphorylation. However, a 58,000-dalton fragment of the heavy chain, which represents approximately the carboxyl-terminal half of the tail, has been cloned and expressed in *Escherichia coli*, and incubation of this fragment in the *in vitro* reaction mixture results in its phosphorylation on both serine and threonine.[2] Therefore, the sites of phosphorylation are probably in the terminal half of the tail. Also, the serine and threonine phosphorylation sites appear to be close together. This conclusion is based on two observations: 1) five out of the seven *in vivo* labeled phosphopeptides are also phosphorylated *in vitro* and 2) the *in vivo* phosphorylation is predominantly on serine, whereas the *in vitro* phosphorylation is predominantly on threonine. Therefore, those phosphopeptides common to both maps must contain both serine and threonine.

We previously observed that the cAMP-stimulated heavy chain phosphorylation increases are greater *in vitro* (4–9-fold) than *in vivo* (1.7-fold) (Berlot *et al.*, 1985). We have now determined that these phosphorylation increases occur predominantly on threonine. The increase in threonine phosphorylation *in vivo* is occurring over a high background of ^{32}P-labeled serine which is not changing much upon stimulation. In the *in vitro* labeling experiments, the phosphorylated serine is only minimally ^{32}P-labeled, and the total increase in ^{32}P-labeling of the myosin heavy chain *in vitro* therefore appears higher than that *in vivo*.

The observation that increases in myosin heavy chain phosphorylation rate are regulated by changes in the availability of myosin to its kinase rather than by changes in kinase or phosphatase activity places myosin in a class with a growing number of proteins whose phosphorylation has been shown to be substrate-regulated. Increases in β-tubulin phosphorylation during differentiation of a mouse neuroblastoma cell line were demonstrated to correlate with increases in the amount of microtubule polymer (Gard and Kirschner, 1985). In that case the mechanism appears to be that a phosphatase preferentially dephosphorylates β-tubulin in the monomer form. Both rhodopsin (Kuhn and Dreyer, 1972; Bownds *et al.*, 1972; Frank *et al.*, 1973) and the β-adrenergic receptor (Stadel *et al.*, 1983; Sibley *et al.*, 1985) are preferentially phosphorylated after interaction with their respective ligands. Rhodopsin kinase phosphorylates only the light-bleached form of rhodopsin (Kuhn and Dreyer, 1972; Bownds *et al.*, 1972; Shichi and Somers, 1978), whereas β-adrenergic receptor kinase phosphorylates only the agonist-occupied β-adrenergic receptor (Benovic *et al.*, 1986). In these cases phosphorylation appears to play a role in adaptation of the responses associated with the receptors (Shichi *et al.*, 1984; Stadel *et al.*, 1983; Sibley *et al.*, 1985).

A further understanding of how cAMP stimulation brings about myosin phosphorylation and intracellular localization changes will involve determining how the various myosin responses influence each other. A key question is whether heavy chain and/or light chain phosphorylation causes asso-

[2] A. De Lozanne, C. H. Berlot, R. Chasan, L. Leinwand, and J. A. Spudich, manuscript in preparation.

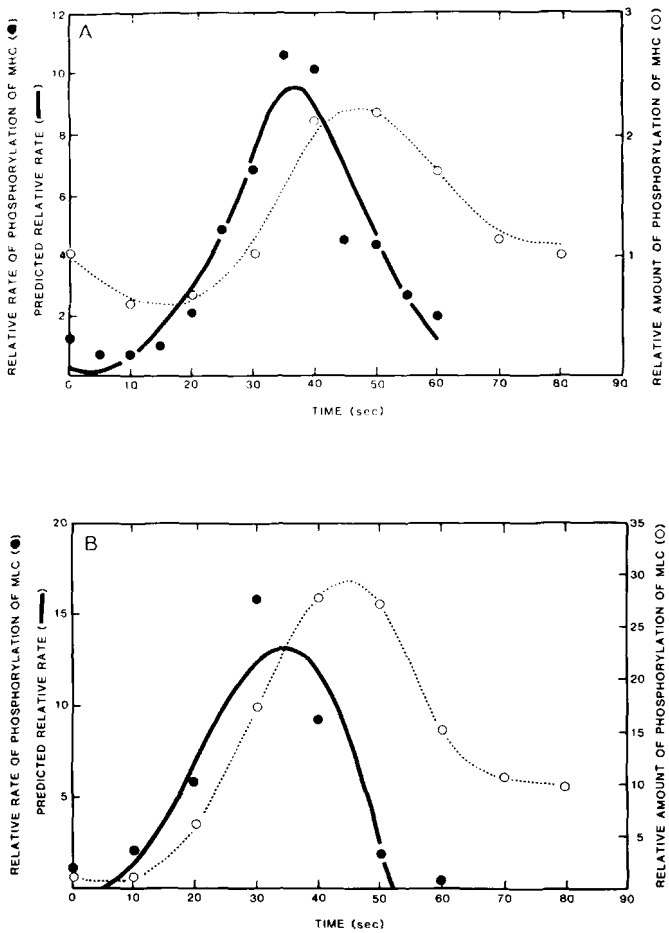

FIG. 7. **Predicted and observed rates of myosin phosphorylation during responses to cAMP.** *A*, the predicted rate of myosin heavy chain (*MHC*) phosphorylation ($v(t)$, shown as a *solid line*) was derived from the *in vivo* amounts of phosphorylation (*open circles, dotted line*) using Equation 2 (see text) and compared to actual measurements taken *in vitro* (*solid circles*). Rates were measured relative to a base line determined from additional points taken from $t = -30$ s to $t = 0$ s (data not shown). The constants α and k_p were fit using a linear least-squares procedure that minimized χ^2 between the data points (*closed circles*) and the function. Best-fit values were: $\alpha = 45$, $k_p = 0.05$ s^{-1}. *B*, the predicted rate of myosin light chain (*MLC*) phosphorylation ($v(t)$, shown as a *solid line*) was derived from the *in vivo* amounts of phosphorylation (*open circles, dotted line*) using Equation 2 (see text) and compared to actual measurements taken *in vitro* (*solid circles*). Rates were measured relative to a base line determined from additional points taken from $t = -25$ s to $t = 0$ s (data not shown). The fitting procedure was identical to *A*. Best-fit values were: $\alpha = 6.0$, $k_p = 0.05$ s^{-1}. Myosin from caffeine-treated developed cells was phosphorylated *in vivo* or *in vitro* as described (Berlot *et al.*, 1985). Relative amounts of myosin phosphorylation *in vivo* and rates of myosin phosphorylation *in vitro* were quantitated by densitometry. Relative amount of myosin phosphorylation is the ratio of the amount of myosin phosphorylation at a given time after cAMP stimulation (2×10^{-6} M) to the amount measured before stimulation. Relative rate of myosin phosphorylation is the ratio of the rate of myosin phosphorylation at a given time after cAMP stimulation (2×10^{-6} M) to the rate measured before stimulation. Data for myosin phosphorylation levels over time (open circles in *A* and *B*) were fit by a cubic spline fit/smoothing algorithm (Reinsch, 1971) that generated a set of arrays suitable for interpolation. The smoothed spline fits to the phosphorylation data are shown by the *dotted lines*. As a result of the cubic spline method, these functions are continuous in both first and second derivative. Both the spline-fit function and its first derivative were evaluated at the time points to be fit (*solid circles*). A set of normal equations for α and k_p was derived from Equation 2 and used together with the data to yield best-fit values. These values, in turn, were used to generate the predicted kinetics (shown by the *solid lines*).

ciation of myosin with the Triton-insoluble cytoskeleton or whether association must first occur to permit phosphorylation. The kinetics of the increase in the light chain phosphorylation rate *in vitro* slightly precede those of the increase in the heavy chain phosphorylation rate *in vitro* and the movement of myosin into a Triton-insoluble phase. Perhaps light chain phosphorylation, which has been shown (Griffith *et al.*, 1987) to increase actin-activated ATPase and motility in the *Nitella*-based movement assay of Sheetz and Spudich (1983), plays a causative role in the movement of myosin into the Triton-insoluble cytoskeleton.

Yumura and Fukui (1985), using fluorescently labeled myosin antibodies and fluorescence microscopy, demonstrated a transient cAMP-stimulated movement of myosin from the endoplasm to the cortex. How does this relate to the transient increase in myosin insolubility? Comparison of the kinetics of these two responses suggests that the movements observed by immunofluorescence may be more rapid than the Triton insolubility response described here. In fact, the kinetics observed by Yumura and Fukui (1985) may even precede the light chain phosphorylation increase. If so, the sequence of events following cAMP stimulation may be that some early signal triggers a rapid movement of myosin to the cortex where it is subsequently phosphorylated on the light chain and then more firmly attached to the Triton-insoluble cytoskeleton. This then results in phosphorylation of the heavy chain. Previous work demonstrated that heavy chain phosphorylation inhibits assembly of thick filaments (Kuczmarski and Spudich, 1980). Further work will determine whether heavy chain phosphorylation plays a role in terminating the transient increase in myosin insolubility. It should be noted that these kinetic comparisons are tentative, and a direct comparison of the immunofluorescence changes and those described here under the same conditions could yield new insights.

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