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Studies on the Phosphorylation of a M_r 25,000 Protein, a Putative Protein Phosphatase 2A Modulator, by Casein Kinase I, and Analysis of Multiple Endogenous Phosphates[1]

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A M_r 25,000 protein, which was isolated from the cytosolic fraction of *Xenopus laevis* oocytes, is a newly identified substrate for casein kinase II and protein kinase C [Hashimoto *et al.* (1995) *J. Biochem.* 118, 453-460], and was recently shown to have the ability to modulate protein phosphatase 2A activity [Hashimoto *et al.* (1996) *J. Biochem.* 119, 626-632]. Acid phosphatase treatment of the protein shifted its electrophoretic mobility from 25 to 20 kDa on SDS-PAGE. The content of alkali-labile phosphate bound covalently to the protein was 53 mol per mol of M_r 25,000 protein. Amino acid composition analysis revealed that there are 50 serine residues and 6 threonine residues per mol of this protein. Therefore, this M_r 25,000 protein seems to be highly phosphorylated *in vivo*. The M_r 25,000 protein, once partially dephosphorylated by acid phosphatase, served as an efficient substrate for casein kinase I and casein kinase II. When entirely dephosphorylated, the M_r 25,000 protein was used as a substrate, the rate of phosphorylation with both casein kinases being decreased. This behavior of casein kinases toward the M_r 25,000 protein reflects the possible mechanism of multisite phosphorylation in which the introduction of a phosphate group facilitates sequential phosphorylation.

Key words: casein kinase I, endogenous phosphate, multisite phosphorylation, protein phosphorylation, *Xenopus laevis* oocyte.

Xenopus laevis oocytes are excellent cellular model for studying regulation of the cell cycle, because fully grown *Xenopus* oocytes are arrested at the G_2/M border of the first meiotic division and the arrest is relieved upon exposure to progesterone. Progesterone triggers the progression from the G_2 to the M phase of the cell cycle, marked by germinal vesicle breakdown (*1-3*). Among such events, protein phosphorylation and dephosphorylation reactions play important roles (*4-6*). From the cytosolic fraction of *X. laevis* oocytes, a common and effective phosphate acceptor for casein kinase II and protein kinase C was purified to near homogeneity (*7*). Its molecular mass was estimated to be approximately 25,000 by SDS-PAGE and gel filtration analyses. It is suggested that protein kinase C is one of the regulators for inducing the meiotic maturation of *X. laevis* oocytes (*8*), and casein kinase II may be involved in regulation of the cell cycle (*9, 10*).

Similar to casein kinase II, casein kinase I is independent of classical second messengers and acidotrophic, preferring acidic residues in its substrates. Casein kinase I is a ubiquitous Ser/Thr protein kinase that has been found in multiple subcellular compartments of eukaryotic cells (*11*). In vertebrates, four closely related casein kinase I isoforms (α, β, γ, and δ) have been reported (*12-14*), and casein kinase I isoforms of yeast were identified subsequently (*15-18*). The amino acid sequence of *X. laevis* casein kinase I is identical to that of the bovine casein kinase Iα subspecies (*19*). Casein kinase Iα shows cell cycle-dependent localization to mitotic spindles, implying a role of the enzyme in mitosis (*20*). It has been shown that the budding yeast HRR25 gene product is a casein kinase I isoform, and HRR25 mutants are defective in meiosis and show delays in G_2-M phase transition in the cell cycle (*16*). Taken together, in eukaryotes it seems likely that casein kinase I regulates, and is also regulated by, the cell cycle.

To learn more about the features of the M_r 25,000 protein and the possibility of its relationship with the cell cycle, we examined M_r 25,000 protein phosphorylation by casein kinase I. In this study, it became clear that this M_r 25,000 protein is a highly phosphorylated protein in *Xenopus* oocytes, and also is an effective phosphate acceptor for casein kinase I after partial dephosphorylation.

EXPERIMENTAL PROCEDURES

Materials and Chemicals—The following compounds were purchased from the indicated sources. α-Casein, BSA, and acid phosphatase from white potato were from Sigma. [γ-^{32}P]ATP was from Amersham. Okadaic acid and hydroxyapatite were from Wako. CKI-7, protein phosphatase 1, and protein phosphatase 2A were from Seikagaku Corporation. DEAE-Sephacel and Sephacryl S-200 were

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Abbreviations: CKI-7, *N*-(2-aminoethyl)-5-chloroisoquinoline-8-sulfonamide; PMSF, phenylmethylsulfonyl fluoride.

from Pharmacia. Phosphocellulose was from Whatman.

Casein kinase I was purified, in excess of 2,000-fold from rat liver as described previously (*12*) with slight modifications. All procedures were performed at 4°C. Rat liver was homogenized in buffer (5 mM HEPES, pH 7.4, and 250 mM sucrose) and then centrifuged at 15,400 × *g* for 20 min. The supernatant was recentrifuged at 100,000 × *g* for 60 min. Proteins insoluble in ammonium sulfate (60% saturation) were collected by centrifugation at 15,000 × *g* for 20 min, resuspended in buffer A (20 mM Tris-HCl, pH 7.8, 0.1 mM EDTA, 10 mM 2-mercaptoethanol, 0.1 mM PMSF, 5 mM $MgCl_2$, and 0.02% NaN_3), and then dialyzed against buffer A containing 0.04 M ammonium sulfate. The dialysate was adsorbed in a batch-wise manner on DEAE-Sephacel (270 ml). The protein not adsorbed was applied to a phosphocellulose column (2.8 × 16.2 cm), which was eluted with a linear gradient of 0.10-1.25 M NaCl in buffer A. The peak of casein kinase I activity was subjected to ammonium sulfate precipitation (60% saturation), and then dialyzed against buffer B (20 mM Tris-HCl, pH 7.8, 1 mM EDTA, 1 mM EGTA, 0.1 mM PMSF, 0.02% NaN_3, and 400 mM NaCl). The dialysate was loaded on a Sephacryl S-200 column (2.6 × 96.5 cm). The peak of casein kinase I activity was applied to a hydroxyapatite column (1.0 ml), and the pass-through was concentrated on a phosphocellulose column (1.4 ml) eluted with buffer C (50 mM Tris-HCl, pH 7.8, 10 mM 2-mercaptoethanol, 0.8 M NaCl, 0.5 mM $MgCl_2$, and 45% glycerol). The resulting casein kinase I was confirmed by the appearance of a silver-stained band at 30 kDa. Its specific activity was 15.7 units/mg, 1 unit being defined as the amount of enzyme transferring 1 nmol phosphate to α-casein per min. The final preparation was stable for several months when stored in 50% (v/v) glycerol at −20°C.

Casein kinase II was highly purified from rat brain as described previously with slight modifications (*7*).

M_r 25,000 protein (chromato-) was isolated from the cytosolic fraction of *X. laevis* oocytes by previously established procedures, including several column chromatographic steps (*7*). M_r 25,000 protein (heat) was prepared as follows: the cytosolic fraction of *X. laevis* oocytes was heat-treated initially at 100°C for 4 min, and then the soluble fraction was applied to a DEAE-Sephacel column and the M_r 25,000 protein was eluted with a linear gradient of 120-400 mM NaCl in buffer (20 mM Tris-HCl, pH 7.5, 10 mM 2-mercaptoethanol, and 0.5 mM EGTA). The procedures will be described in more detail elsewhere.

Dephosphorylation of the M_r 25,000 protein with acid phosphatase was performed in a 1 ml reaction mixture comprising 50 μg M_r 25,000 protein, 30 mM MES, pH 6.0, 3 mM EGTA, and 2.12 units/ml acid phosphatase from white potato for 30 min at 30°C (*21*). The reaction was blocked by boiling for 4 min in order to inactivate the phosphatase. A control sample was treated identically except that acid phosphatase was omitted. Other detailed reaction conditions are indicated for each experiment. The dephosphorylated samples were stored at −80°C and employed for phosphorylation experiments.

Phosphorylation Reactions—Casein kinase I activity was assayed as the phosphorylation of α-casein (1.0 mg/ml) in a 50 μl reaction mixture comprising 20 mM HEPES, pH 7.4, 5 mM $Mg(Ac)_2$, 0.26 mg/ml BSA, 10 μM [γ-^{32}P]ATP (500-1,000 cpm/pmol), and the enzyme preparation.

Casein kinase II was assayed as described previously (*7*).

Control experiments were constantly performed, omitting either the substrate or casein kinase.

Phosphorylation of the M_r 25,000 protein or dephosphorylated M_r 25,000 protein by each protein kinase was performed under conditions essentially the same as those given above. The substrate concentrations were varied, as indicated in the figures and tables.

All phosphorylation reactions were initiated by the addition of [γ-^{32}P]ATP at 30°C, and terminated by the addition of 2 ml of 10% (w/v) trichloroacetic acid. After the addition of 7.5 μl of 0.2% BSA, heating for 10 min at 80°C, and standing on ice for 10 min, the acid-precipitated protein was collected on a Toyo membrane filter (0.45 μm pore size) and washed 3 times with 2 ml of 10% (w/v) trichloroacetic acid. The incorporated radioactivity was determined with a Beckman-LS 5801 liquid scintillation counter with Cerenkov radiation.

Phosphoproteins ^{32}P-labeled with casein kinase I were analyzed by SDS-PAGE. The phosphorylation reaction was terminated by boiling the reaction mixture for 3 min in the presence of a "stop solution" [final concentrations: 2% SDS (w/v), 62.5 mM Tris-HCl, pH 6.8, 10% glycerol (v/v), 5% 2-mercaptoethanol, and 0.001% bromphenol blue (w/v)]. Electrophoresis was performed, and the gel was stained for protein, dried, and then subjected to autoradiography.

Preparation and Dephosphorylation of the ^{32}P-Labeled M_r 25,000 Protein—The M_r 25,000 protein was converted to a ^{32}P-labeled M_r 25,000 protein with casein kinase I. The M_r 25,000 protein (35.4 μg/500 μl) was phosphorylated for 2 h at 30°C with casein kinase I (54 milliunits/500 μl) using 20 μM [γ-^{32}P]ATP in a buffer comprising 50 mM Tris-HCl, pH 7.4, 5 mM $Mg(Ac)_2$, 0.2 mg/ml leupeptin, 0.1 mM EGTA, 0.1% 2-mercaptoethanol (v/v), and 0.26 mg/ml BSA. The reaction was terminated by heating for 4 min at 100°C. The ^{32}P-labeled M_r 25,000 protein was separated from remaining [γ-^{32}P]ATP by gel filtration on a Sephadex G-25 column (1 × 7 cm) with a buffer comprising 50 mM Tris-HCl, pH 7.4, 0.1 mM EGTA, and 0.1% 2-mercaptoethanol (v/v). The BSA concentration of the preparation was adjusted to 0.26 mg/ml. It was kept at −80°C and then used as a substrate for phosphatase within 48 h.

Dephosphorylation of the ^{32}P-labeled M_r 25,000 protein (1.1 μM) with either protein phosphatase 1 or protein phosphatase 2A was carried out at 30°C for the times indicated in an incubation mixture comprising 50 mM Tris-HCl, pH 7.4, 0.1 mM EGTA, 1 mg/ml BSA, and 5.5 units/ml phosphatase 1 or phosphatase 2A. The remaining radioactive label was measured under conditions similar to those for the phosphorylation reaction. To determine the effects of okadaic acid on phosphatase 1 and phosphatase 2A, various concentrations of okadaic acid (10 nM, 100 nM, 1 μM, and 10 μM) were added.

Determination of Alkali-Labile Phosphate Bound Covalently to the M_r 25,000 Protein—The assay for protein-bound phosphate was combined with the hydrolysis of phosphoester bonds in the protein and the colorimetric determination of inorganic phosphate. Hydrolysis was carried out by incubation of the sample in 1 M sodium hydroxide at 60°C for 18 h. The inorganic phosphate released was determined using two different procedures, namely the method of Itaya and Ui (*22*), and that of Ames (*23*). In the case of the method of Itaya and Ui, after

hydrolysis, HCl and $HClO_4$ were added to stop hydrolysis and for deproteinization. After the addition of the carrier protein, BSA, the sample was left on ice for 20 min. The suspension was centrifuged and the supernatant was transferred quantitatively. A mixture of malachite green and acidified ammonium molybdate was added to the supernatant. Malachite green formed a complex with phosphomolybdate. The color developed was measured at 660 nm. When phosphate determination was carried out as described by Ames (*23*), trichloroacetic acid was used as the deproteinizing reagent. On the addition of a mixture of ascorbic acid and ammonium molybdate to the sample containing phosphate, the phosphomolybdate was reduced by ascorbic acid and the color of molybdenum blue was determined at 820 nm. Dried KH_2PO_4 was employed as a standard for inorganic phosphate. Control incubations were carried out in which 1 M sodium hydroxide was replaced by distilled water.

Amino Acid Analysis—Amino acids were analyzed with a Waters "PICO-TAG" system. The M_r 25,000 protein was hydrolyzed for 20 h at 110°C with 6 M HCl/1% phenol in the vapor phase *in vacuo*. The samples were dried, neutralized with ethanol/water/triethylamine (2 : 2 : 1), and then redried. Then these samples were derivatized with phenylisothiocyanate/ethanol/water/triethylamine (1 : 7 : 1 : 1). The phenylthiocarbamyl derivatives were then analyzed on a reverse-phase HPLC column. Correction of the Ser and Thr contents for destruction during hydrolysis was not performed.

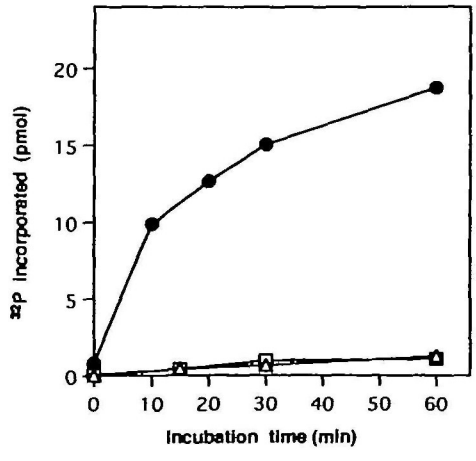
Other Procedures and Determinations—SDS-PAGE was performed with a 12.5% acrylamide running gel and a 4.5% stacking gel (*24*). Protein was determined with a commercial reagent from Bio-Rad by the method of Bradford, with BSA as a standard (*25*). The concentration of the M_r 25,000 protein was estimated by calculating the area of the stained band using a Toyo digital densitorol DMU-33C after SDS-PAGE. In this case, BSA was employed as the standard. Autoradiography was performed on a dried gel with X-ray film, Fuji Photo Film, for 4 h at −80°C.

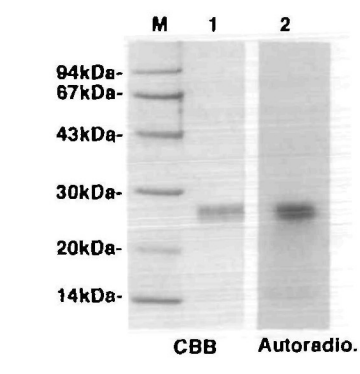
RESULTS

Phosphorylation of the M_r 25,000 Protein by Casein Kinase I—Phosphorylation of the M_r 25,000 protein was analyzed using purified rat liver casein kinase I and the M_r 25,000 protein preparation obtained on column chromatographic purification. When the M_r 25,000 protein was incubated with casein kinase I, time-dependent phosphorylation was observed, as shown in Fig. 1A. In the absence of either the substrate or the enzyme, the phosphorylation was essentially negligible. When the phosphorylated protein was analyzed by SDS-PAGE, the radioactive band was detected at the position of a M_r 25,000 protein on autoradiography, as indicated in Fig. 1B.

The efficiency of M_r 25,000 protein phosphorylation catalyzed by casein kinase I was examined, relative to α-casein, at varying Mg^{2+} concentrations. α-Casein had been used as a common substrate for assaying the activity of casein kinases *in vitro*. As shown in Fig. 2A, casein kinase I exhibited an absolute requirement for Mg^{2+}, when both the M_r 25,000 protein and α-casein were used as substrates. When the M_r 25,000 protein was used as a substrate, the enzyme activity was strongly stimulated by Mg^{2+} concentrations up to 5 mM, but higher concentrations inhibited the activity. Accordingly, the Mg^{2+} concentration of 5 mM was used for all kinetic assessments of casein kinase I activity using the M_r 25,000 protein as a phosphate acceptor. The saturated Mg^{2+} concentration for α-casein was 15 mM, and higher concentrations had no inhibitory effect on the activity. The effects of NaCl on the phosphorylation of the M_r 25,000 protein and α-casein by casein kinase I were examined (Fig. 2B). NaCl had little effect on either M_r 25,000 protein or α-casein phosphorylation at low concentrations. However, higher concentrations inhibited the phosphorylation of the M_r 25,000 protein, but increased the phosphorylation of α-casein.

The phosphorylation of the M_r 25,000 protein by casein kinase I exhibited a broad pH optimum (Fig. 3A), whereas





lane M, molecular mass markers
lane 1, *Mr* 25,000 protein
lane 2, phosphorylated *Mr* 25,000 protein

Fig. 1. **Phosphorylation of the M_r 25,000 protein by casein kinase I.** (A) Time course of phosphorylation. The M_r 25,000 protein (1.3 μg) was phosphorylated with rat liver casein kinase I (2.7 milliunits) under the conditions given under "EXPERIMENTAL PROCEDURES." Blank reactions lacking the M_r 25,000 protein or casein kinase I were run. At the times indicated, an aliquot (50 μl) of the reaction mixture was removed and acid-precipitable radioactivity was determined. ●, complete system; □, minus M_r 25,000 protein; and △, minus casein kinase I. (B) SDS-PAGE analysis of the phosphorylated protein. SDS-PAGE analysis of the phosphorylated protein and autoradiography were performed under the conditions given under "EXPERIMENTAL PROCEDURES." Lane M, molecular mass markers; lane 1, Coomassie Brilliant Blue-stained M_r 25,000 protein; lane 2, autoradiography of the ^{32}P-labeled M_r 25,000 protein phosphorylated by casein kinase I. An aliquot of the reaction mixture with the complete system (Fig. 1A) was subjected to SDS-PAGE and autoradiography.

maximal incorporation of phosphate into α-casein was observed at pH 7.4, as shown in Fig. 3B. In this study the reaction was routinely conducted at pH 7.4, in HEPES.

Chijiwa *et al.* (*26*) synthesized and characterized a specific inhibitor of casein kinase I called CKI-7. Therefore, we examined whether or not CKI-7 inhibited the phosphorylation by casein kinase I (Fig. 4A). Indeed, CKI-7 potently inhibited the activity toward both M_r 25,000 protein and α-casein.

Heparin is widely considered to be a specific inhibitor for casein kinase II, and have no effect on other protein kinases including casein kinase I at concentrations below 1 μg/ml (*27*). However, heparin has been reported to be a potent inhibitor not only of casein kinase II but also of casein kinase I, and inhibition of casein kinase I is dependent on the substrate used (*28, 29*). The results, shown in Fig. 4B, indicated that the α-casein phosphorylation by casein kinase I was not inhibited by heparin at all concentrations

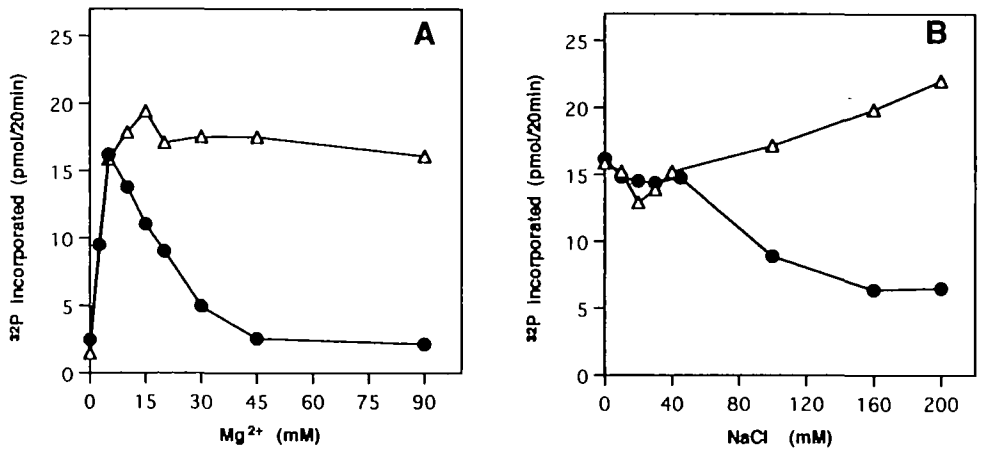


Fig. 2. **Effects of Mg^{2+} (A) and NaCl (B) on casein kinase I activity.** The M_r 25,000 protein (0.7 μg) or α-casein (50 μg) was phosphorylated with rat liver casein kinase I (2.7 milliunits for M_r 25,000 protein, 1.8 milliunits for α-casein) for 20 min under the conditions given under "EXPERIMENTAL PROCEDURES," except that Mg^{2+} and NaCl were varied as indicated. ●, M_r 25,000 protein; and △, α-casein.

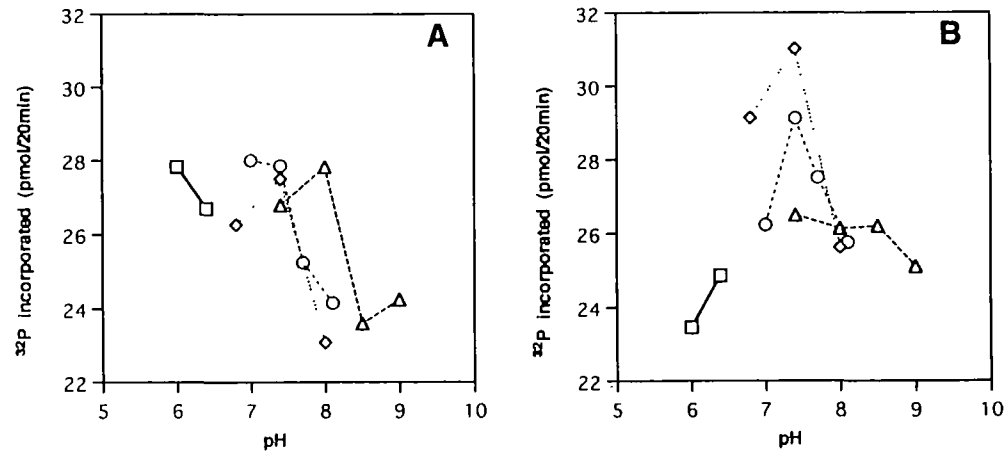


Fig. 3. **Effect of pH on casein kinase I activity.** The M_r 25,000 protein (0.7 μg) (A) or α-casein (50 μg) (B) was phosphorylated with rat liver casein kinase I (2.7 milliunits for M_r 25,000 protein, 1.8 milliunits for α-casein) for 20 min under the conditions given under "EXPERIMENTAL PROCEDURES," except that the following 20 mM buffers were employed. □, MES (pH 6.0-6.4); ◇, TES (pH 6.8-8.0); ○, HEPES (pH 7.0-8.1); and △, Tris-HCl (pH 7.4-9.0).

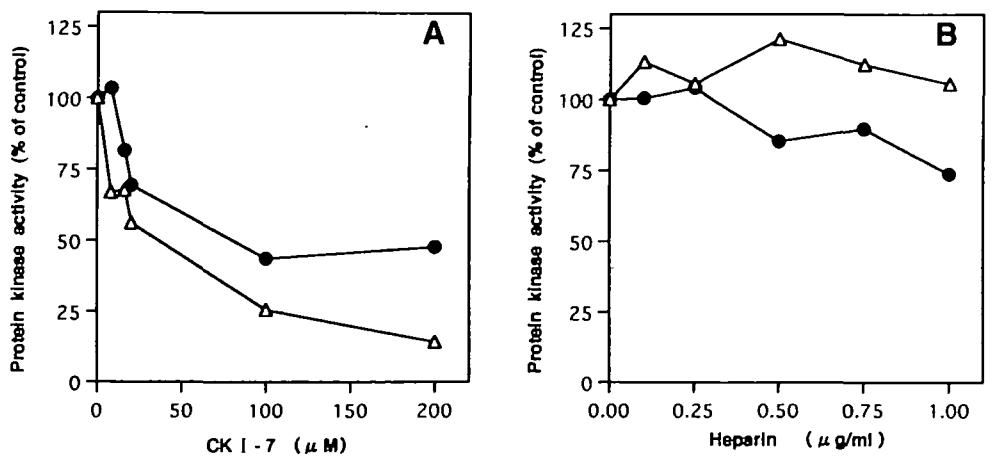


Fig. 4. **Effects of CKI-7 (A) and heparin (B) on phosphorylation by casein kinase I.** The M_r 25,000 protein (0.7 μg) or α-casein (50 μg) was phosphorylated with rat liver casein kinase I (2.7 milliunits for M_r 25,000 protein, 1.8 milliunits for α-casein) for 20 min under the conditions given under "EXPERIMENTAL PROCEDURES," except that CKI-7 or heparin was added at the concentrations indicated. ●, M_r 25,000 protein; and △, α-casein.

tested. But the M_r 25,000 protein phosphorylation was inhibited slightly by heparin.

The purification of the M_r 25,000 protein from *X. laevis* oocytes was performed employing established procedures, including several column chromatographic steps. The preparation by this method is termed M_r 25,000 protein (chromato-) (7). When the cytosolic fraction of oocytes was heat-treated initially, followed by DEAE-Sephacel column chromatography, the M_r 25,000 protein was purified with a higher yield and became nearly homogenous, as judged on SDS-PAGE. This preparation is termed M_r 25,000 protein (heat). Into these two different M_r 25,000 protein preparations, heat-treated and chromatographic, the stoichiometries of phosphate incorporation by casein kinase I were examined. As shown in Fig. 5, by 120 min incubation, phosphate incorporation into M_r 25,000 protein (heat) by casein kinase I was saturated at 0.44 mol per mol of this protein. Under similar conditions, approximately 1.73 mol of phosphate was incorporated per mol of M_r 25,000 protein (chromato-). Kinetic parameters were determined by regression analysis of double-reciprocal plots constructed from initial rate measurements (Table I). For M_r 25,000 protein (heat) and M_r 25,000 protein (chromato-), the K_m values were calculated to be 1.12 and 0.55 μM, and the apparent V_{max} values to be 8.0 nmol/min/mg and 15.1 nmol/min/mg, respectively. The V_{max}/K_m for M_r 25,000 protein (chromato-) was 4.2-fold greater than that for M_r 25,000 protein (heat). These results led to the hypothesis that casein kinase I sites were already partially occupied in the heat-treated preparation.

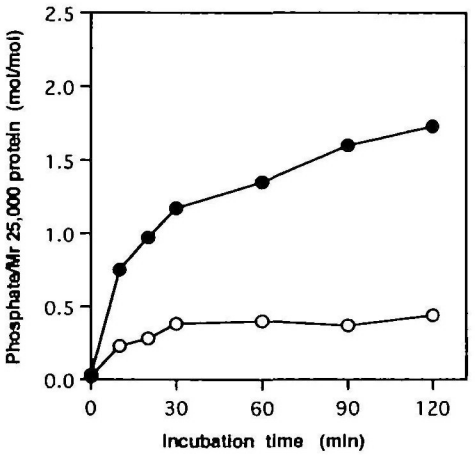


Fig. 5. **Stoichiometry of M_r 25,000 protein phosphorylation by casein kinase I.** M_r 25,000 protein (chromato-) (0.44 μg) and M_r 25,000 protein (heat) (0.43 μg) were phosphorylated with rat liver casein kinase I (2.7 milliunits) under the conditions given under "EXPERIMENTAL PROCEDURES." At the times indicated, an aliquot (25 μl) of the reaction mixture was removed and the acid-precipitable radioactivity was determined. ●, M_r 25,000 protein (chromato-); and ´ , M_r 25,000 protein (heat).

The Influence of Dephosphorylation of the M_r 25,000 Protein on Its Electrophoretic Mobility—Phosphorylation has been shown to alter the electrophoretic mobility of substrates on SDS-PAGE. Therefore, the M_r 25,000 protein was treated with acid phosphatase before SDS-PAGE and the influence of dephosphorylation of the M_r 25,000 protein on electrophoretic mobility was examined. Figure 6A shows the gradual mobility shift accompanying M_r 25,000 protein dephosphorylation. The more highly dephosphorylated the M_r 25,000 protein became, the more rapidly it migrated on SDS-PAGE. The mobility shift became maximal at the position of 20 kDa, and then did not increase further. One control experiment showed that phosphatase inhibitors (50 mM sodium fluoride and 10 mM sodium pyrophosphate) blocked the effect of acid phosphatase on the electrophoretic mobility (Fig. 6B, lane 3). Another control experiment (Fig. 6B, lane 4) with protease

TABLE I. **Kinetic parameters of phosphorylation of the M_r 25,000 protein by casein kinase I.** M_r 25,000 protein (heat) (0.43–8.57 μM) and M_r 25,000 protein (chromato-) (0.08–1.74 μM) were phosphorylated with rat liver casein kinase I (2.7 milliunits) for 10 min under the conditions given under "EXPERIMENTAL PROCEDURES." The results were estimated from a double-reciprocal plot.

Substrate	K_m(μM)	V_{max} (nmol/min/mg)
M_r 25,000 protein (heat)	1.12±0.16	8.01±2.55
M_r 25,000 protein (chromato-)	0.55±0.04	15.14±0.22

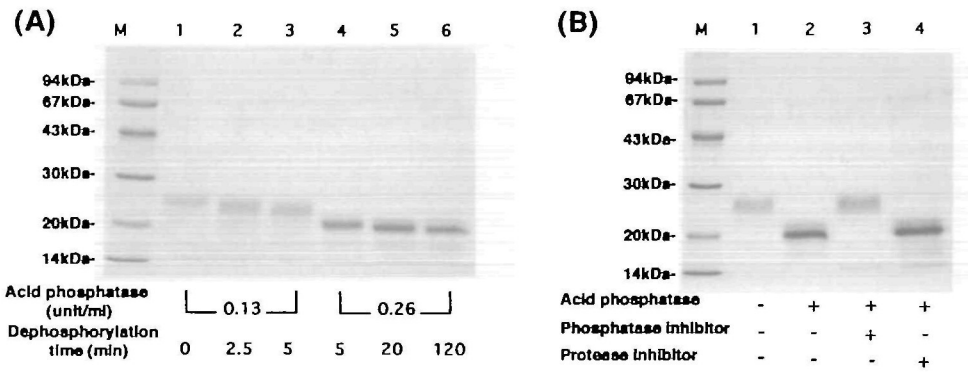


Fig. 6. **Influence of dephosphorylation by acid phosphatase on the electrophoretic mobility of the M_r 25,000 protein on SDS-PAGE.** (A) Effect of dephosphorylation by acid phosphatase on the mobility of the M_r 25,000 protein. Partially dephosphorylated M_r 25,000 protein samples were prepared by varying the time of dephosphorylation and the amount of acid phosphatase. Lane M, molecular mass markers; lanes 1-6, Coomassie Brilliant Blue-stained native and dephosphorylated M_r 25,000 protein (1.0 μg protein was loaded on each lane). (B) Effects of acid phophatase inhibitors or protease inhibitors on the mobility shift of the M_r 25,000 protein. The M_r 25,000 protein was incubated for 20 min at 30 C in the absence (lane 1) or presence (lane 2) of acid phosphatase (2.12 units/ml). Control reactions were performed with incubation of acid phosphatase (2.12 units/ml) plus phosphatase inhibitors (50 mM sodium fluoride and 10 mM sodium pyrophosphate) (lane 3) or protease inhibitors (2 mM PMSF, 0.4 mM leupeptin, 10 mM EDTA, 2 μg/ml aprotinin, and 0.2 mg/ml trypsin inhibitor) (lane 4). An aliquot of each of these reaction mixtures was analyzed by SDS-PAGE as described under "EXPERIMENTAL PROCEDURES." Lane M, molecular mass markers; lanes 1-4, Coomassie Brilliant Blue-stained M_r 25,000 protein previously treated under the various conditions described above (1.25 μg protein was loaded on each lane).

inhibitors (2 mM PMSF, 0.4 mM leupeptin, 10 mM EDTA, 2 μg/ml aprotinin, and 0.2 mg/ml trypsin inhibitor) showed no effect on the mobility shift by acid phosphatase. These findings constituted evidence that the mobility shift was not caused by proteolytic degradation, but by dephosphorylation. These results were consistent with the idea that the M_r 25,000 protein is a phosphorylated protein.

Phosphate Content and Amino Acid Composition of the M_r 25,000 Protein—Since the dephosphorylated M_r 25,000 protein exhibited various forms with faster mobilities on SDS-PAGE (Fig. 6A), it was likely that the protein was phosphorylated at multiple sites. Additional evidence showing multisite phosphorylation was obtained by determining the alkali-labile phosphate bound covalently to M_r 25,000 protein preparations. A summary of the values is given in Table II. The alkali-labile phosphate content of M_r 25,000 protein preparations was analyzed using the sodium hydroxide procedure for hydrolysis of protein-bound phosphate. Inorganic phosphate released was determined by either the malachite green method of Itaya and Ui (*22*) or the method of Ames (*23*). Quite similar results were obtained with both methods (Table II), suggesting that these data seem to be reliable. The phosphate content was found to be 53 mol per mol of M_r 25,000 protein (heat), and 45 mol per mol of M_r 25,000 protein (chromato-). The difference between them might be due to the dephosphorylation by endogenous phosphatase during the column chromatographic purification. When both M_r 25,000 protein (heat) and M_r 25,000 protein (chromato-) were completely dephosphorylated, almost all of the phosphates were released. The entirely dephosphorylated M_r 25,000 protein migrated to the position of 20 kDa on SDS-PAGE (Fig. 6A, lane 6). Since the presence of a high content of phosphate

TABLE II. **Alkali-labile phosphate content of the M_r 25,000 protein.** Phosphate was analyzed as described under "EXPERIMENTAL PROCEDURES." Dephospho M_r 25,000 protein refers to the M_r 25,000 protein treated with 2.21 units/ml acid phosphatase at 30°C for 30 min. Values are means±SD with the numbers of independent determinations in brackets.

	Phosphate content (mol/mol M_r 25,000 protein)	
	Itaya and Ui method	Ames method
M_r 25,000 protein (heat)	53.08±2.02 (4)	—
M_r 25,000 protein (chromato-)	45.10±2.41 (9)	47.14±2.74 (2)
Dephospho M_r 25,000 protein (heat)	2.49 (1)	—
Dephospho M_r 25,000 protein (chromato-)	1.11±0.83 (6)	2.02 (1)

TABLE III. **Amino acid composition of the M_r 25,000 protein.** The M_r 25,000 protein was analyzed for amino acids as described under "EXPERIMENTAL PROCEDURES." The data are presented as assumed numbers of residues per molecule. The molecular weight of the M_r 25,000 protein is assumed to be 20,000, as described in the text.

Amino acid	Residues per mol
Asp/Asn	8.1
Glu/Gln	40.4
Ser	50.0
Gly	11.1
His	11.9
Arg	19.3
Thr	6.0
Ala	7.4
Pro	2.4
Tyr	0.0
Val	2.6
Met	0.0
Ile	0.0
Leu	0.0
Phe	0.0
Lys	19.7

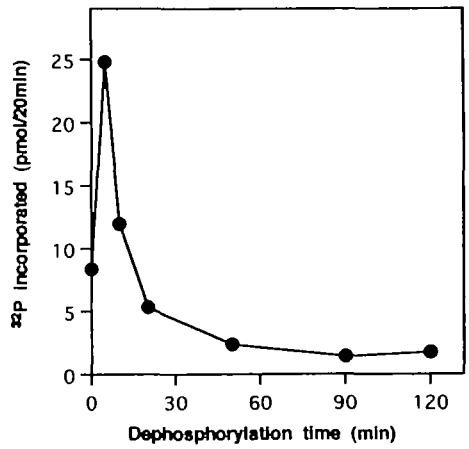


Fig. 7. **Effect of previous dephosphorylation of the M_r 25,000 protein by acid phosphatase on casein kinase I activity.** The details of the preparation of variably dephosphorylated M_r 25,000 protein (1.77 μg) and the conditions for phosphorylation in the presence of casein kinase I (2.7 milliunits) were given under "EXPERIMENTAL PROCEDURES," except that the amount of acid phosphatase was 0.265 unit/ml and the dephosphorylation time was varied as indicated.

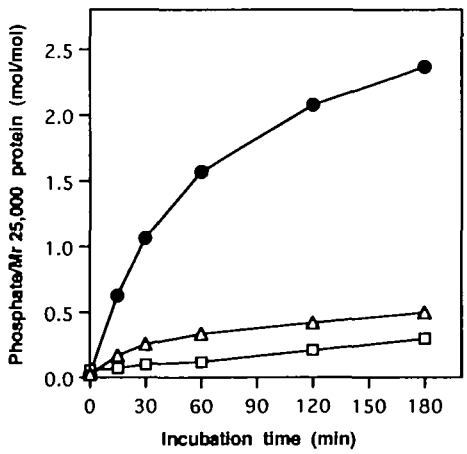


Fig. 8. **Time-dependent phosphorylation of the native and previously dephosphorylated M_r 25,000 protein by casein kinase I.** The M_r 25,000 protein was phosphorylated with rat liver casein kinase I (1.35 milliunits) as described under "EXPERIMENTAL PROCEDURES." At the times indicated, an aliquot (25 μl) of the reaction mixture was removed and acid-precipitable radioactivity was determined. △, native M_r 25,000 protein (0.89 μg); ●, M_r 25,000 protein partially dephosphorylated with 0.13 unit/ml acid phosphatase for 7.5 min (0.89 μg); and □, M_r 25,000 protein entirely dephosphorylated with 2.12 units/ml acid phosphatase for 30 min (0.85 μg).

groups in the protein did not reflect the true molecular weight on SDS-PAGE, we reasoned that their removal would result in a normal protein with respect to its mobility on SDS-PAGE. Therefore, the apparent molecular weight of the M_r 25,000 protein was calculated to be 20,000 in this study.

Determination of the amino acid composition of the M_r 25,000 protein would allow more complete interpretation of the physicochemical properties of the protein. Table III shows that there are 50 Ser residues and 6 Thr residues per mol of M_r 25,000 protein. The content of hydroxyamino acid residues was extraordinary high, accounting for about 30% of all residues, while those of acidic and basic residues were high, and that of hydrophobic residues was low. The phosphate content of the M_r 25,000 protein was determined by alkali hydrolysis, and phosphoserine and phosphothreonine were sensitive to hydrolysis by sodium hydroxide. Therefore, the Ser and Thr residues of the M_r 25,000 protein were almost in the phosphorylated state.

Phosphorylation of Previously Dephosphorylated M_r 25,000 Proteins by Casein Kinase I and Casein Kinase II—In order to clarify the relationship between endogenous phosphates and additional phosphorylation by casein kinase I or casein kinase II, the M_r 25,000 protein was dephosphorylated with acid phosphatase. The native, fully phosphorylated M_r 25,000 protein and its variably dephosphorylated forms were subjected to enzymatic phosphorylation with casein kinase I in the presence of [γ-^{32}P]ATP. As shown in Fig. 7, partial dephosphorylation led to much greater incorporation, but further dephosphorylation impaired subsequent enzymatic phosphorylation. A similar experiment was performed with casein kinase II. A suitable substrate for casein kinase II required more progressive dephosphorylation than one for casein kinase I (not shown).

Time-dependent phosphorylation with casein kinase I showed that 0.5, 2.4, and 0.3 mol of phosphate were incorporated in 180 min into 1 mol of fully phosphorylated (heat), partially dephosphorylated (containing 24.2 mol of phosphate), and entirely dephosphorylated M_r 25,000 protein, respectively (Fig. 8). These observations on phosphorylation by casein kinase II suggested that 0.7, 3.7, and 0.7 mol of phosphate were incorporated in 180 min into 1 mol of fully phosphorylated (heat), partially dephosphorylated (containing 6.7 mol of phosphate), and entirely dephosphorylated M_r 25,000 protein, respectively (Fig. 9). The partially dephosphorylated M_r 25,000 protein was phosphorylated 8.0 and 5.3 times more rapidly than the entirely dephosphorylated counterpart by casein kinase I and casein kinase II, respectively. The present findings constitute evidence that the partially dephosphorylated M_r 25,000 protein contained recognition sites which were created by Ser/Thr(P) for casein kinase I and casein kinase II, whereas the entirely dephosphorylated M_r 25,000 protein had no such recognition sites, and some of the endogenous phosphates of the fully phosphorylated M_r 25,000 protein were localized to casein kinase I- and casein kinase II-phosphorylatable sites. These results supported the idea that the presence of phosphoryl residues facilitates further phosphorylation, as has been described in other instances (*30-32*).

Dephosphorylation of the ^{32}P-Labeled M_r 25,000 Protein with Protein Phosphatase—The M_r 25,000 protein was converted to a ^{32}P-labeled M_r 25,000 protein by phosphorylation with casein kinase I. The ^{32}P-labeled M_r 25,000 protein was separated from remaining [γ-^{32}P]ATP by gel filtration, using Sephadex G-25. The ^{32}P-labeled M_r 25,000 protein was used as a substrate for protein phosphatases.

Protein phosphatase 1 and protein phosphatase 2A were examined as to their ability to dephosphorylate the M_r 25,000 protein ^{32}P-labeled with casein kinase I. Incubation

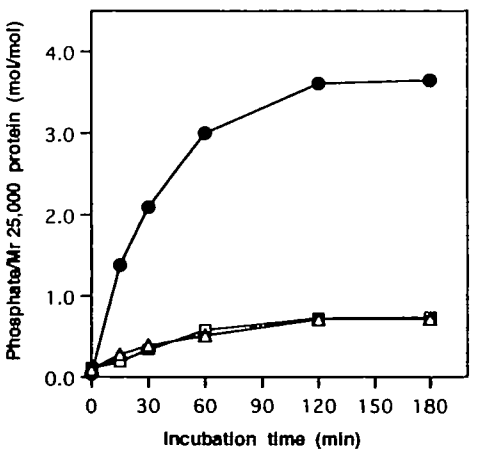


Fig. 9. **Time-dependent phosphorylation of the native and previously dephosphorylated M_r 25,000 protein by casein kinase II.** The M_r 25,000 protein was phosphorylated with rat brain casein kinase II (1.41 milliunits/ml) under the conditions given under "EXPERIMENTAL PROCEDURES." At the times indicated, an aliquot (25 μl) of the reaction mixture was removed and acid-precipitable radioactivity was determined. △, native M_r 25,000 protein (0.37 μg); ●, M_r 25,000 protein partially dephosphorylated with 0.53 unit/ml acid phosphatase for 7.5 min (0.21 μg); and □, M_r 25,000 protein entirely dephosphorylated with 2.12 units/ml acid phosphatase for 30 min (0.35 μg).

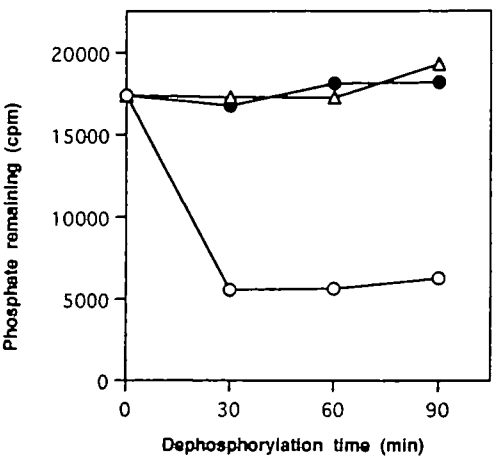


Fig. 10. **Dephosphorylation of the ^{32}P-labeled M_r 25,000 protein by protein phosphatase 1 and protein phosphatase 2A.** The M_r 25,000 protein (35.4 μg/500 μl) was phosphorylated for 2 h at 30°C with casein kinase I (54 milliunits/500 μl) using 20 μM [γ-^{32}P]ATP. Dephosphorylation of the ^{32}P-labeled M_r 25,000 protein (1.1 μM) with either 5.5 units/ml phosphatase 1 (△) or 5.5 units/ml phophatase 2A (□) was carried out at 30°C as described under "EXPERIMENTAL PROCEDURES." At the times indicated, an aliquot (20 μl) of the reaction mixture was removed and the radioactivity remaining in the M_r 25,000 protein was measured. A control was treated identically except that the phosphatase was omitted (●).

of the ^{32}P-labeled M_r 25,000 protein with phosphatase 2A led to rapid release of ^{32}P from the substrate. This indicates that the phosphorylation of the M_r 25,000 protein by casein kinase I could be reversed by phosphatase 2A. However, no release of ^{32}P was observed in the presence of phosphatase 1 (Fig. 10).

Dephosphorylation (76.6–88.8%) of the ^{32}P-labeled M_r 25,000 protein was inhibited by 10 nM–10 μM okadaic acid (not shown).

DISCUSSION

The experiments reported here suggest that the M_r 25,000 protein was already substantially phosphorylated at multiple sites *in vivo*. From the results in Figs. 8 and 9, it was clarified that some endogenous phosphates of the protein were localized to casein kinase I- and casein kinase II-phosphorylatable sites. The results obtained on phosphate determination, summarized in Table II, show that M_r 25,000 protein (heat) contained 53 mol of alkali-labile phosphate per mol of protein. Considering the amino acid composition (Table III), nearly all Ser and Thr residues of the M_r 25,000 protein might be phosphorylated. The yolk protein, phosvitin, is known as one of the most highly phosphorylated proteins in nature. Of the 239 amino acid residues of this protein, 93 are Ser residues, and almost all of them are phosphorylated (*33*). Taborsky has described the amino acid compositional features of phosvitin, as follows: the extraordinarily high Ser content, including the extensive if not complete phosphorylation of these residues; the relatively dominant presence of Lys and Arg with respect to all residues but Ser; and the complete or nearly complete absence of a number of amino acids, especially those with nonpolar or sulfur-containing side chains (*34*). This description is true of the amino acid composition of the M_r 25,000 protein (Table III). Therefore, we are interested in examining the relationship between phosvitin and the M_r 25,000 protein. All yolk proteins are derived from the circulating phosphoprotein, vitellogenin. In *Xenopus*, as in all oviparous vertebrates, vitellogenin is synthesized by and secreted from the liver in response to estrogenic stimulation (*34*). After uptake into developing oocytes, vitellogenin is cleaved to produce phosvitin, phosvette I, phosvette II, lipovitellin I, and lipovitellin II (*33*). Phosvitin, phosvette I, and phosvette II contain clustered Ser residues, almost all of which are phosphorylated, and the estimated molecular weights for them are 33, 19, and 13.5 kDa, respectively. It has been shown that there are at least four distinct but related genes (A1, A2, B1, and B2) for vitellogenin in *Xenopus* (*35*). One of these four genes, gene A2, was sequenced completely and its amino acid sequence was predicted (*36*). We compared the amino acid sequence of the amino-terminal region of the M_r 25,000 protein (*7*) with that around the clustered Ser residues of the vitellogenin A2 gene product. There was no sequence similarity. The amino acid composition of the M_r 25,000 protein (Table III) does not agree with those of phosvitin, phosvette I and phosvette II of *X. laevis* (*36*), in particular, the Glx content of the M_r 25,000 protein is extraordinarily higher than theirs. As shown by Hashimoto *et al.*, an amino acid sequence closely matching that of the amino-terminal region of the M_r 25,000 protein was not found on database analysis performed at the Peptide Institute, Osaka (*7*).

The conclusion that the M_r 25,000 protein is a heavily phosphorylated protein has raised the questions of where and how this multisite phosphorylation occurs. Roach has reviewed the mechanisms of multisite and hierarchal phosphorylation (*37, 38*). A mechanism unique to multiply phosphorylated protein is that one phosphate introduced by a primary kinase generates a new recognition site for a secondary kinase. The candidates that can act as secondary protein kinases in the above sense are casein kinase I, casein kinase II, and GSK-3 (glycogen synthase kinase-3), which recognize motifs S(P)-X_{1-3}-*S*, *S*-X_{0-2}-S(P)/E/D_{1-3}, and *S*-X_3-S(P), respectively. S(P) denotes a phosphoserine, the phosphoacceptor residue is *underlined*, X is any amino acid and Thr can substitute for Ser (*37–39*). Therefore, casein kinase II can act as either a primary or secondary protein kinase. It can be speculated that the introduction of phosphates into the clustered Ser (Thr) residues by these kinases yields new targets for themselves, thus phosphorylation occurs sequentially. When appropriate phosphate groups were present, the M_r 25,000 protein became a better substrate for casein kinase I and casein kinase II (Figs. 7, 8, and 9). When the entirely dephosphorylated M_r 25,000 protein was used as a substrate, dramatic decreases in the rate of phosphorylation with both casein kinase I and casein kinase II were observed (Figs. 8 and 9). These results support the idea of the mechanism of multisite phosphorylation described above. Casein kinase I has been reported to participate in the hierarchal phosphorylation of simian virus 40 large T antigen (*30*), glycogen synthase (*31*), and the transcriptional regulator, cAMP-response elements modulator (CREM) (*32*). Casein kinase I phosphorylation alters the activities of these substrates. Both casein kinase I and casein kinase II are constitutively active when purified and second messenger-independent. But casein kinase I and casein kinase II activity could be regulated and recruited into second messenger-dependent phosphorylation cascades, if primary phosphorylation could be controlled. As shown in Figs. 8 and 9, phosphates incorporated by casein kinase I and casein kinase II into the M_r 25,000 protein were less than expected. For *in vitro* experiments, using compulsively dephosphorylated substrates, it is difficult to reproduce hierarchal and ordered multisite phosphorylation occurring *in vivo*. As several kinases might participate in multisite phosphorylation of the M_r 25,000 protein, the possibility of involvement of casein kinase I and casein kinase II cannot be excluded.

Incidentally, it has been reported that the lumen of the Golgi apparatus or endoplasmic reticulum is a likely site for the phosphorylation of vitellogenin (*40*). But the understanding of the mechanisms of vitellogenin post translational modification is still incomplete.

Recently, the crystal structure of a truncated variant of casein kinase I from *Schizosaccharomyces pombe*, which corresponds to the casein kinase I catalytic core, was determined in a complex with MgATP (*41*). It resembles the "closed," ligand-bound conformations of both cyclin-dependent kinase 2 (cdk2) and cAMP-dependent protein kinase (cAPK), with differences in surface loops. The crystal structure analysis of casein kinase I suggested the structural basis of the substrate selectivity, and the putative regulatory mechanisms through which casein kinase I may be controlled by the cell cycle.

Phosphorylation has been shown to alter electrophoretic

mobility in several instances. Phosphorylation of ATP-Mg dependent phosphatase by F_A/GSK-3 (*42*) and of PHAS-I (phosphorylated heat- and acid-stable protein regulated by insulin) by MAP (mitogen-activated protein) kinase (*43*) reduces the electrophoretic mobilities of these two substrates on SDS-PAGE, whereas phosphorylation of DARPP 32 (a dopamine- and cAMP-regulated phosphoprotein) by casein kinase I (*44*) and of cdc2 by MO15 gene product (*45*) induces more rapid migration. The effect of acid phosphatase treatment on the mobility of the M_r 25,000 protein shown in Fig. 6A suggests that the gradually increasing mobility is a reflection of the degree of dephosphorylation, and the presence of phosphates probably accounts for the lower electrophoretic mobility. By utilizing this property, it is possible to estimate the relative amounts of endogenous phosphates of the M_r 25,000 protein to a certain extent.

The physiological function and structure of the M_r 25,000 protein, and the role of the phosphorylation of the M_r 25,000 protein remain to be resolved. Recently, the possible role of the M_r 25,000 protein was suggested to be as a modulator for protein phosphatase 2A in our laboratory (*46*). This modulatory effect was abolished on treatment of the M_r 25,000 protein with acid phosphatase (Kobayashi, N. and Hashimoto, E., manuscript in preparation). This suggests that the multiple phosphates play an important role in the function of this protein. However, it is not clear at this time how many phosphates were introduced into this M_r 25,000 protein by casein kinase I in the *in vivo* reaction. So, the role of phosphorylation by casein kinase I in the function of this putative phosphatase modulator remains to be determined. We are currently engaged in cloning of the cDNA that encodes the M_r 25,000 protein in order to determine the amino acid sequence of the protein. It may then be possible, by comparing the M_r 25,000 protein to proteins of known structure and function, to elucidate its biochemical properties and functions.

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REFERENCES

1. Maller, J.L. (1985) Regulation of amphibian oocyte maturation. *Cell Differ.* **16**, 211-221
2. Dennis Smith, L. (1989) The induction of oocyte maturation: Transmembrane signaling events and regulation of the cell cycle. *Development* **107**, 685-699
3. Maller, J.L. and Krebs, E.G. (1977) Progesterone-stimulated meiotic cell division in *Xenopus* oocytes. *J. Biol. Chem.* **252**, 1712-1718
4. Gu, Y., Rosenblatt, J., and Morgan, D.O. (1992) Cell cycle regulation of CDK2 activity by phosphorylation of Thr 160 and Tyr 15. *EMBO J.* **11**, 3995-4005
5. Nurse, P. (1990) Universal control mechanism regulating onset of M-phase. *Nature* **344**, 503-508
6. Matten, W., Daar, I., and Vande Woude, G.F. (1994) Protein kinase A acts at multiple points to inhibit *Xenopus* oocyte maturation. *Mol. Cell Biol.* **14**, 4419-4426
7. Hashimoto, E., Takeuchi, F., Tanaka, Y., and Yamamura, H. (1995) Purification and phosphorylation of a M_r 25,000 protein, an effective phosphate acceptor for casein kinase II and protein kinase C, detected in the cytosolic fraction of *Xenopus laevis* oocytes. *J. Biochem.* **118**, 453-460
8. Stith, B.J. and Maller, J.L. (1987) Induction of meiotic maturation in *Xenopus* oocytes by 12-*O*-tetradecanoylphorbol 13-acetate. *Exp. Cell Res.* **169**, 514-523
9. Litchfield, D.W., Lüscher, B., Lozeman, F.J., Eisenman, R.N., and Krebs, E.G. (1992) Phosphorylation of casein kinase II by $p34^{cdc2}$ *in vitro* and at mitosis. *J. Biol. Chem.* **267**, 13943-13951
10. Russo, G.L., Vandenberg, M.T., Yu, I.J., Bae, Y.-S., Franza, B.R., Jr., and Marshak, D.R. (1992) Casein kinase II phosphorylates $p34^{cdc2}$ kinase in G1 phase of the HeLa cell division cycle. *J. Biol. Chem.* **267**, 20317-20325
11. Tuazon, P.T. and Traugh, J.A. (1991) Casein kinase I and II-multipotential serine protein kinases: Structure, function and regulation in *Advances in Second Messenger and Phosphoprotein Research* (Greengard, P. and Robison, G.A., eds.) Vol. 23, pp. 123-164, Raven Press, New York
12. Rowles, J., Slaughter, C., Moomaw, C., Hsu, J., and Cobb, M.H. (1991) Purification of casein kinase I and isolation of cDNAs encoding multiple casein kinase I-like enzymes. *Proc. Natl. Acad. Sci. USA* **88**, 9548-9552
13. Graves, P.R., Haas, D.W., Hagedorn, C.H., DePaoli-Roach, A.A., and Roach, P.J. (1993) Molecular cloning, expression, and characterization of a 49-kilodalton casein kinase I isoform from rat testis. *J. Biol. Chem.* **268**, 6394-6401
14. Zhai, L., Graves, P.R., Longenecker, K.L., DePaoli-Roach, A.A., and Roach, P.J. (1992) Recombinant rabbit muscle casein kinase Iα is inhibited by heparin and activated by polylysine. *Biochem. Biophys. Res. Commun.* **189**, 944-949
15. Robinson, L.C., Jane Albert Hubbard, E., Graves, P.R., DePaoli-Roach, A.A., Roach, P.J., Kung, C., Haas, D.W., Hagedorn, C.H., Goebl, M., Culbertson, M.R., and Carlson, M. (1992) Yeast casein kinase I homologues: An essential gene pair. *Proc. Natl. Acad. Sci. USA* **89**, 28-32
16. Demaggio, A.J., Lindberg, R.A., Hunter, T., and Hoekstra, M.F. (1992) The budding yeast *HRR25* gene product is a casein kinase I isoform. *Proc. Natl. Acad. Sci. USA* **89**, 7008-7012
17. Wang, P.-C., Vancura, A., Desai, A., Carmel, G., and Kuret, J. (1994) Cytoplasmic forms of fission yeast casein kinase-1 associate primarily with the particulate fraction of the cell. *J. Biol. Chem.* **269**, 12014-12023
18. Kearney, P.H., Ebert, M., and Kuret, J. (1994) Molecular cloning and sequence analysis of two novel fission yeast casein kinase-1 isoforms. *Biochem. Biophys. Res. Commun.* **203**, 231-236
19. Tapia, C., Featherstone, T., Gómez, C., Taillon-Miller, P., Allende, C.C., and Allende, J.E. (1994) Cloning and chromosomal localization of the gene coding for human protein kinase CK 1. *FEBS Lett.* **349**, 307-312
20. Brockman, J.L., Gross, S.D., Sussman, M.R., and Anderson, R.A. (1992) Cell cycle-dependent localization of casein kinase I to mitotic spindles. *Proc. Natl. Acad. Sci. USA* **89**, 9454-9458
21. Cala, S.E. and Jones, L.R. (1991) Phosphorylation of cardiac and skeletal muscle calsequestrin isoforms by casein kinase II. *J. Biol. Chem.* **266**, 391-398
22. Itaya, K. and Ui, M. (1966) A new micromethod for the colorimetric determination of inorganic phosphate. *Clin. Chim. Acta* **14**, 361-366
23. Ames, B.N. (1966) Assay of inorganic phosphate, total phosphate and phosphatases in *Methods in Enzymology* (Neufeld, E.F. and Ginsburg, V., eds.) Vol. 8, pp. 115-118, Academic Press, New York and London
24. Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophase T4. *Nature* **227**, 680-685
25. Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248-254
26. Chijiwa, T., Hagiwara, M., and Hidaka, H. (1989) A newly synthesized selective casein kinase I inhibitor, *N*-(2-aminoethyl)-5-chloroisoquinoline-8-sulfonamide, and affinity purification of casein kinase I from bovine testis. *J. Biol. Chem.* **264**, 4924-4927
27. Hathaway, G.M., Lubben, T.H., and Traugh, J.A. (1980) Inhibition of casein kinase II by heparin. *J. Biol. Chem.* **255**, 8038-8041

28. Singh, T.J. (1988) Inhibition of glycogen synthase (casein) kinase-1 by heparin. *Arch. Biochem. Biophys.* **260**, 661-666
29. Ahmad, Z., Camici, M., DePaoli-Roach, A.A., and Roach, P.J. (1984) Glycogen synthase kinases. *J. Biol. Chem.* **259**, 3420-3428
30. Umphress, J.L., Tuazon, P.T., Chen, C.-J., and Traugh, J.A. (1992) Determinants on simian virus 40 large T antigen are important for recognition and phosphorylation by casein kinase I. *Eur. J. Biochem.* **203**, 239-243
31. Flotow, H., Graves, P.R., Wang, A., Fiol, C.J., Roeske, R.W., and Roach, P.J. (1990) Phosphate groups as substrate determinants for casein kinase I action. *J. Biol. Chem.* **265**, 14264-14269
32. Groot, R.P., Hertog, J.D., Vandenheede, J.R., Goris, J., and Sassone-Corsi, P. (1993) Multiple and cooperative phosphorylation events regulate the CREM activator function. *EMBO J.* **12**, 3903-3911
33. Wiley, H.S. and Wallace, R.A. (1981) The structure of vitellogenin. *J. Biol. Chem.* **256**, 8626-8634
34. Taborsky, G. (1983) Phosvitin. *Adv. Inorg. Biochem.* **5**, 235-279
35. Wahli, W., Dawid, I.B., Ryffel, G.U., and Weber, R. (1981) Vitellogenesis and the vitellogenin gene family. *Science* **212**, 298-304
36. Gerber-Huber, S., Nardelli, D., Haefliger, J.-A., Cooper, D.N., Givel, F., Germond, J.-E., Engel, J., Green, N.M., and Wahli, W. (1987) Precursor-product relationship between vitellogenin and the yolk proteins as derived from the complete sequence of a *Xenopus* vitellogenin gene. *Nucleic Acids Res.* **15**, 4737-4760
37. Roach, P.J. (1991) Multisite and hierarchal protein phosphorylation. *J. Biol. Chem.* **266**, 14139-14142
38. Roach, P.J. (1990) Control of glycogen synthase by hierarchal protein phosphorylation. *FASEB J.* **4**, 2961-2968
39. Kennelly, P.J. and Krebs, E.G. (1991) Consensus sequences as substrate specificity determinants for protein kinases and protein phosphatases. *J. Biol. Chem.* **266**, 15555-15558
40. Capasso, J.M., Keenan, T.W., Abeijon, C., and Hirschberg, C.B. (1989) Mechanism of phosphorylation in the lumen of the Golgi apparatus. *J. Biol. Chem.* **264**, 5233-5240
41. Xu, R.-M., Carmel, G., Sweet, R.M., Kuret, J., and Cheng, X.-D. (1995) Crystal structure of casein kinase-1, a phosphate-directed protein kinase. *EMBO J.* **14**, 1015-1023
42. DePaoli-Roach, A.A. (1984) Synergistic phosphorylation and activation of ATP-Mg-dependent phosphoprotein phosphatase by F_A/GSK-3 and casein kinase II (PC 0.7). *J. Biol. Chem.* **259**, 12144-12152
43. Haystead, T.A.J., Haystead, C.M.M., Hu, C., Lin, T.-A., and Lawrence, J.C., Jr. (1994) Phosphorylation of PHAS-I by mitogen-activated protein (MAP) kinase. *J. Biol. Chem.* **269**, 23185-23191
44. Desdouits, F., Cohen, D., Nairn, A.C., Greengard, P., and Girault, J.-A. (1995) Phosphorylation of DARPP-32, a dopamine- and cAMP-regulated phosphoprotein, by casein kinase I *in vitro* and *in vivo*. *J. Biol. Chem.* **270**, 8772-8778
45. Fesquet, D., Labbé, J.-C., Derancourt, J., Capony, J.-P., Galas, S., Girard, F., Lorca, T., Shuttleworth, J., Dorée, M., and Cavadore, J.-C. (1993) The MO15 gene encodes the catalytic subunit of a protein kinase that activates cdc2 and other cyclin-dependent kinases (CDKs) through phosphorylation of Thr161 and its homologues. *EMBO J.* **12**, 3111-3121
46. Hashimoto, E., Kobayashi, N., Kubota, N., Tanaka, Y., and Yamamura, H. (1996) Phosphorylated sites of M_r 25,000 protein, a putative protein phosphatase 2A modulator, and phosphorylation of the synthetic peptide containing these sites by protein kinase C. *J. Biochem.* **119**, 626-632