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In vivo phosphorylation of a synthetic peptide substrate of cyclic AMP-dependent protein kinase

(*Xenopus* oocytes/microinjection/protein phosphorylation/peptides)

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ABSTRACT A model synthetic peptide substrate of the cyclic AMP-dependent protein kinase (ATP:protein phosphotransferase; EC 2.7.1.37), Leu-Arg-Arg-Ala-Ser-Leu-Gly, closely resembling the local phosphorylation site sequence in porcine hepatic pyruvate kinase, was shown to be phosphorylated *in vivo* after microinjection into *Xenopus* oocytes. This result demonstrates that the microinjection technique, utilizing a synthetic peptide substrate, or possibly a synthetic substrate analog inhibitor [Kemp, B. E., Benjamini, E. & Krebs, E. G. (1976) *Proc. Natl. Acad. Sci. USA* 73, 1038–1042], can be used to study protein phosphorylation-dephosphorylation reactions in living oocytes. This follows, since it is clear that the injected peptide was accessible to the cellular compartment containing the protein kinase.

Since the discovery of cyclic AMP by Sutherland and Rall (1), considerable advances have been made in understanding the mechanism by which this important second messenger exerts its effects on intracellular processes. It was established that a cyclic AMP-dependent protein kinase (ATP:protein phosphotransferase; EC 2.7.1.37) is the primary target for control of glycogenolysis by cyclic AMP in skeletal muscle (2, 3) and the generality of this mechanism as applied to other cyclic AMP-stimulated systems was proposed (4). A considerable number of natural substrates of the cyclic AMP-dependent protein kinase have been identified, and their number and diversity of function illustrate the important role this protein kinase plays in coordinating various intracellular processes with the demands of the organism. It is apparent, therefore, that the protein substrate specificity of the protein kinase should have a crucial role in determining which processes are modulated in response to a given signal.

Recently, the molecular basis of the substrate specificity of the cyclic AMP-dependent protein kinase has been studied by using low molecular weight peptides (5–11). Studies with synthetic peptides both in this laboratory and elsewhere (11) indicate that the enzyme recognizes a rather restricted region of the primary structure around the phosphorylation site (6–11). The synthetic peptide substrates that have been most extensively studied correspond to the phosphorylation site sequence reported for pig liver and rat liver pyruvate kinase (10, 11). A heptapeptide studied in this laboratory, Leu-Arg-Arg-Ala-Ser-Leu-Gly, has an apparent K_m in the low micromolar range (10) and the V_{max} with this substrate is of the same order as that for physiological substrates.

Since the kinetic constants obtained with the heptapeptide as substrate were of the same order as those obtained with proteins as substrates, it seemed possible that this peptide would be capable of competing with natural substrates *in vivo* if it could cross the cell membrane. A suitable system for testing this is the living *Xenopus* oocyte into which volumes of up to 0.1 μl can be injected with a micropipet. The question of phosphorylation of the peptide is also of interest since we have found that meiosis in the oocyte, triggered by progesterone, is inhibited by microinjection of micromolar levels of the homogeneous catalytic subunit of the protein kinase and that it is induced without hormone by microinjection of protein kinase inhibitory proteins (12). These findings suggest that one of the substrates for the catalytic subunit is necessary and sufficient in its phosphorylated form to maintain the prophase block of the oocyte either directly or indirectly (12). If synthetic peptides at low concentrations *in vivo* could act as competitive inhibitors of the enzyme for normal substrates, as occurs *in vitro* (6), it might be possible to alter the progress of meiosis by microinjection of specific peptide substrates or suitable analogs. As a first step towards determining the feasibility of this approach, we show here that synthetic peptides introduced into oocytes of *Xenopus laevis* can be phosphorylated, thus demonstrating that such compounds, when injected, are accessible to the protein kinase.

MATERIALS AND METHODS

Cyclic AMP-Dependent Protein Kinase. Homogeneous beef skeletal muscle catalytic subunit was prepared by the method of Beavo *et al.* (13). The catalytic subunit of cyclic AMP-dependent protein kinase from *Xenopus* eggs was prepared as described (12). Protein kinase activity was measured by the method of Reimann *et al.* (14), with mixed histone type II-A as substrate. When synthetic peptide was used as substrate, aliquots of the reaction mixtures containing synthetic peptide were applied to discs of Whatman-P81 paper and washed with 30% acetic acid (four times for 20 min each). The paper discs were dried and radioactivity was determined in a Nuclear Chicago scintillation counter with a toluene-based scintillant. This ion-exchange filter paper procedure gives results quantitatively similar to those of the anion-exchange column procedure described previously (6). [γ-^{32}P]ATP was prepared according to the method of Glynn and Chappel (15).

Peptide Synthesis and Purification. The synthetic peptide, Leu-Arg-Arg-Ala-Ser-Leu-Gly, was synthesized by the Merrifield solid phase synthesis technique and purified by ion-exchange chromatography on SP-Sephadex and gel chromatography on Sephadex G-25 (6). The amino acid composition of the synthetic peptide after acid hydrolysis (5.7 M HCl, 110°, 24 hr) determined on a Durrum D-500 amino acid analyzer was Ala (1.00), Arg (1.98), Gly (0.99), Leu (2.03), and Ser (1.00). The quantitative yield after total enzymic hydrolysis with amino-

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peptidase-M compared with acid hydrolysis was 101%, showing that the synthetic peptide was fully deprotected and had maintained its stereospecificity throughout the synthesis.

Microinjection of Oocytes. Ovaries were obtained from healthy *Xenopus laevis*, and the oocytes collected by collagenase treatment (16). Only unblemished large 1.3-mm-diameter (stage VI) oocytes were used. Microinjection procedures together with the construction and calibration of micropipets are described in detail elsewhere (17). Individual oocytes were injected with $^{32}P_i$ (2.5–3.5 μCi per oocyte) and subsequently with synthetic peptide (1.9–2.6 nmol) in a total volume of 50–70 nl and were then incubated in Wallace's medium OR2 (18) minus KCl (19) and NaH_2PO_4, for 10 min.

Isolation of Phosphorylated Peptide. Oocytes with $^{32}P_i$, with or without synthetic peptide, were fixed in 3 ml of 30% acetic acid for 12 hr at 0°. This procedure left the oocytes largely intact while allowing the synthetic peptide to leak out into the fluid above the oocytes. In control experiments with this technique, the recovery of phosphorylated peptide from oocytes injected with $^{32}P_i$-labeled phosphopeptide was greater than 80%. This procedure was used after it was found that extraction of the peptide from oocytes by oocytes by homogenization was complicated owing to the release of phosphorylated components of the oocyte which were not readily separated from the phosphorylated peptide. Phosphopeptide recovered from the oocytes was separated from residual $^{32}P_i$ and ^{32}P-labeled nucleotides by anion-exchange chromatography (AG 1X8 resin, Bio-Rad) in the presence of 30% acetic acid. For isolation of peptide phosphorylated *in vitro*, reaction mixtures (13) were diluted in 30% acetic acid and subjected to the same chromatographic procedure.

RESULTS

Phosphorylation of the synthetic peptide, Leu-Arg-Arg-Ala-Ser-Leu-Gly, *in vivo*

When oocytes were injected with carrier-free $^{32}P_i$ plus synthetic peptide and incubated for 10 min, approximately 109 × 10^3 cpm of $^{32}P_i$ per oocyte was recovered in the phosphopeptide fraction after anion-exchange chromatography. In contrast, only 4.4 × 10^3 cpm per oocyte was recovered in this fraction from control oocytes injected with $^{32}P_i$ without synthetic peptide.

The phosphorylated product isolated from the oocytes injected with peptide was characterized as follows. By high-voltage electrophoresis, it migrated toward the cathode in the same position as authentic phosphopeptide that had been prepared *in vitro* with the catalyted subunit of cyclic AMP-dependent protein kinase isolated from either beef skeletal muscle or *Xenopus* eggs (Fig. 1). As can be seen, several minor labeled components from the *in vivo* reaction were also present, which may represent partial proteolysis products derived from the peptide. In extracts of control oocytes that had been injected only with $^{32}P_i$, there was no detectable ^{32}P radioactive material migrating towards the cathode. The radioactivity associated with the peptide, phosphorylated either *in vitro* or in the intact oocyte, was alkali-labile (94% released in 15 min in 0.1 M NaOH at 100°) and stable to acid (100° remaining after 15 min in 0.1 M HCl at 100°). These properties are consistent with the presence of a serine phosphoester linkage in the phosphopeptide isolated from the oocyte. When the phosphorylated product was subjected to partial acid hydrolysis and high-voltage paper electrophoresis at pH 1.9, radioactivity in the same position as a phosphoserine marker was observed (Fig. 2).

The phosphorylated product obtained from the oocytes was further characterized by thin-layer chromatography on silica

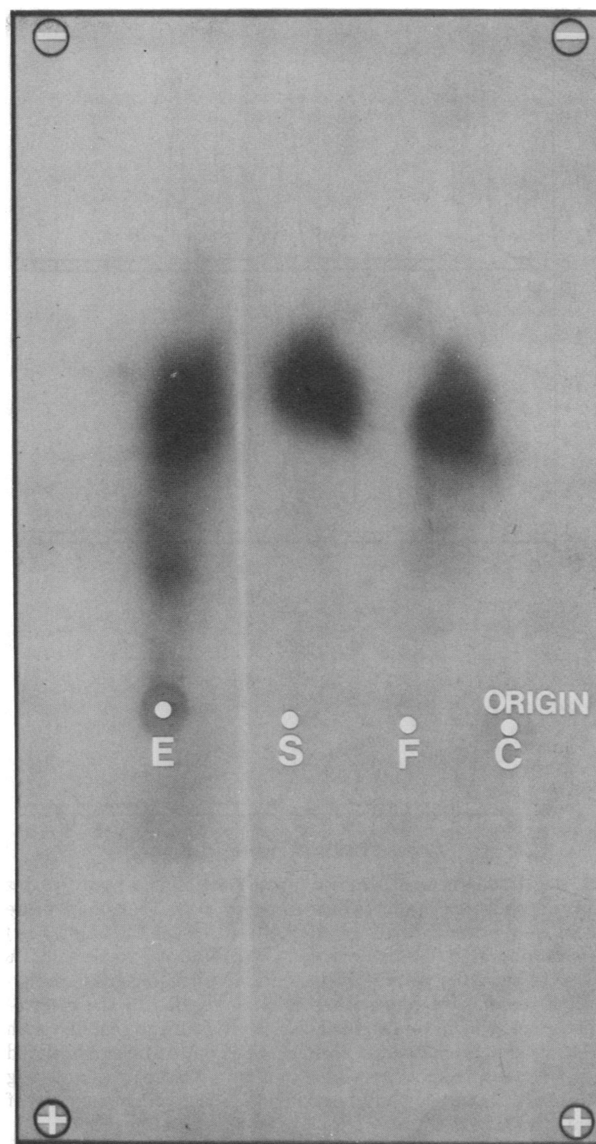

FIG. 1. Autoradiograph of an electrophoretogram of synthetic peptide phosphorylated *in vivo* and *in vitro*. The phosphopeptide fractions were concentrated by rotary evaporation and aliquots were electrophoresed for 2 hr at 1600 V (pH 4.7, 5% butanol/2.5% pyridine/2.5% acetic acid/water, by volume). Twenty-four-hour autoradiographs of the ninhydrin-stained electrophoretograms were prepared with Kodak x-ray film. E, synthetic peptide phosphorylated *in vivo* after microinjection into oocytes; S, synthetic peptide phosphorylated *in vitro* with catalytic subunit of beef skeletal muscle protein kinase; F, synthetic peptide phosphorylated *in vitro* with partially purified catalytic subunit of egg protein kinase; C, control oocytes microinjected with ^{32}P, but without synthetic peptide.

gel plates with four different solvent systems (Fig. 3). In each case, the phosphorylated product isolated from the oocytes migrated with the same R_F value as the phosphopeptide prepared *in vitro*. A certain amount of streaking occurred on the thin-layer plates, particularly in the case of phosphopeptide prepared *in vitro* using the egg catalytic subunit (see Fig. 3). This may have been due to slight contamination of the partially purified enzyme preparation with proteases.

DISCUSSION

The results reported here demonstrate that a model synthetic peptide substrate of the cyclic AMP-dependent protein kinase

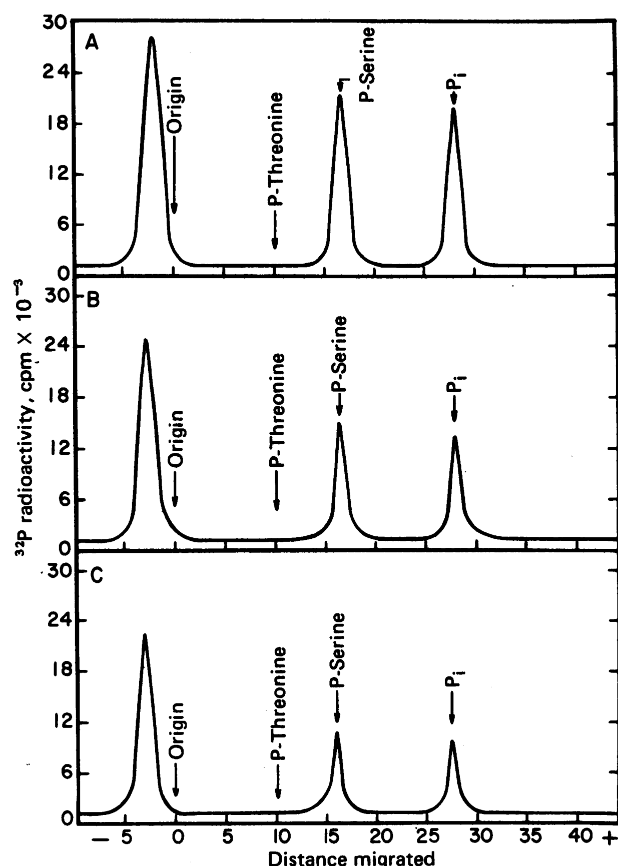

FIG. 2. Radiochromatogram scans of partial acid hydrolysates of peptide phosphorylated *in vitro* and *in vivo*. Phosphopeptide fractions were partially hydrolyzed in HCl (5.7 M at 110° for 1.5 hr) and electrophoresed for 60 min at 3500 V (pH 1.9, 4% acetic acid/1% formic acid/water by volume). Authentic *O*-phosphoserine and *O*-phosphothreonine reference markers were applied to the electrophoretograms, which were stained with ninhydrin and scanned with a Packard radiochromatogram scanner. (*A*) Peptide phosphorylated *in vivo;* (*B*) peptide phosphorylated *in vitro* with the exogenous egg enzyme, and (*C*) peptide phosphorylated *in vitro* with exogenous beef skeletal muscle enzyme.

is phosphorylated *in vivo* when microinjected into *Xenopus* oocytes. This finding supports the concept that microinjection of specific peptide substrates or peptide analogues may prove useful as a tool for studying protein phosphorylation reactions in intact oocytes.

Previous characterization of protein phosphorylation reactions in the oocyte has focused on the period just prior to nuclear membrane breakdown, when a large burst of protein phosphorylation occurs involving exclusively cytoplasmic substrates and enzymes (20). The site of phosphorylation inside the intact oocyte of the synthetic peptide used in the present study is not known; however, it is clear that the peptide substrate became accessible to a protein kinase capable of phosphorylating it. In the earlier experiments, i.e., the microinjection of homogeneous catalytic subunit of cyclic AMP-dependent protein kinase, the specific biological effect that resulted (12) implies that the injected enzyme is also able to find access to a substrate at an appropriate intracellular site. In the latter experiments (12) and in the ones reported here, the substance injected was introduced into the cytoplasm of the animal hemisphere of the oocyte approximately halfway between the plasma membrane and the germinal vesicle. In the light of previous work demonstrating the amphibian oocytes actively redistribute injected proteins according to their nuclear or cytoplasmic origin (21, 22), it might be expected that both catalytic subunit and the synthetic peptide would remain in the cytoplasm since the enzyme was derived from cytosol and since the synthetic peptide represents the phosphorylation site of a cytoplasmic enzyme. On the other hand, at least some of of the nuclear proteins in oocytes are known to be synthesized in the cytoplasm and transported to the nucleus (23, 24). In addition, for protein kinase, translocation from the cytoplasm to the nucleus or the microsomes has been reported for several cell types as a consequence of hormone administration (25–27). These considerations make uncertain the assumption that the injected substrate remained in the cytoplasm for the phosphorylation event. However, the amphibian oocyte seems particularly suited for approaching the question of intracellular protein migration since one can microinject into either the cytoplasm or the germinal vesicle (28). In addition, one can manually enucleate individual oocytes and recover the nucleus within 30 sec (24).

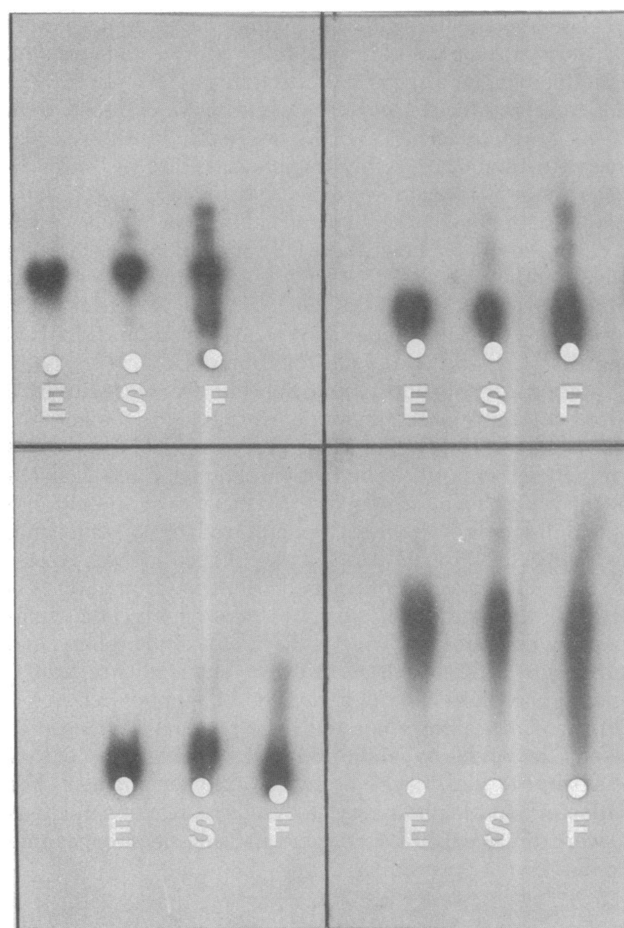

FIG. 3. Autoradiographs of thin-layer chromatograms of synthetic peptide phosphorylated *in vitro* and *in vivo*. Samples (1 μl) were applied to thin-layer silica gel-coated glass plates (50 X 65 nm). The plates were developed in glass jars with the following solvents (by volume). (*Upper Left*) Acetic acid/ethylacetate/pyridine/water (1:5:5:3); (*Upper Right*) acetic acid/1-butanol/pyridine/water (3:15:10:12); (*Lower Left*) acetic acid/pyridine/water (30:50:15); (*Lower Right*) acetic acid/1-butanol/water (1:4:1). The phosphopeptide fractions E, S, and F refer to peptide phosphorylated *in vivo* and *in vitro* as described in the legend to Fig. 1. The R_F values for the phosphopeptide fractions in the four solvents were (*Upper Left*) 0.12; (*Upper Right*) 0.33; (*Lower Left*) 0.56; and (*Lower Right*) 0.80.

The synthetic peptide used in this study was chosen because it contains the specificity determinants for the well-characterized cyclic AMP-dependent protein kinase from muscle and

because it is phosphorylated *in vitro* by the catalytic subunit of the presumably analogous enzyme from eggs. It is possible, of course, that after microinjection other protein kinases could also phosphorylate the peptide substrate to yield an electrophoretically identical product. Hence, it cannot be concluded with absolute certainty what enzyme was acting in this case. For example, cyclic GMP-dependent protein kinase has a substrate specificity similar to that of cyclic AMP-dependent protein kinase and can phosphorylate *in vitro* the peptide used in this study (29). Interestingly, the biological activity inside the oocyte of cyclic GMP-dependent protein kinase kinase also parallels the cyclic AMP-dependent enzyme, since the homogeneous cyclic GMP-dependent enzyme also inhibits meiosis when microinjected, half-maximal inhibition occurring at an internal concentration of approximately 50 nM.[‡]

The most important specificity determinant for substrates of cyclic AMP-dependent protein kinase appears to be the presence of arginine residues near the phosphorylatable serine. These particular residues also serve as determinants for trypsin-like proteases, and thus it is not surprising that the peptide substrate used in this study was susceptible to partial degradation after microinjection (Fig. 1). This finding demonstrates that protein kinases and proteases share or overlap the same intracellular compartment and suggests experiments to compare the susceptibility of nuclear and cytoplasmic proteins or peptides or proteolytic degradation as a function of the region of the oocyte into which they are introduced by micropipet. This kind of approach might prove useful in characterizing the reported involvement of proteases in meiotic maturation of *Xenopus* oocytes (30).

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[‡] J. L. Maller, unpublished results.