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

(Xenopus oocytes/microinjection/protein phosphorylation/peptides)

JAMES L. MALLER*, BRUCE E. KEMP†, AND EDWIN G. KREBS*

Department of Biological Chemistry, University of California, Davis, California 95616

Contributed by Edwin G. Krebs, November 4, 1977

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 Kₐ in the low micromolar range (10) and the Vₘₐₓ 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 vitro if it could cross the cell membrane. A suitable system for testing this idea 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 (15). 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 90% 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-32P]ATP was prepared according to the method of Glynn and Chappell (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 (2.08), and Ser (1.00). The quantitative yield after total enzymic hydrolysis with amino-
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 

\[ ^{32}P \] 1 (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 NaH₂PO₄ for 10 min.

Isolation of Phosphorylated Peptide. Oocytes with 

\[ ^{32}P \] 1, 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 

\[ ^{32}P \] 1-labeled phosphopeptide was greater than 80%. This procedure was used after it was found that extraction of the peptide from 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 

\[ ^{32}P \] 1 and 

\[ ^{32}P \] 3 labeled nucleotides by anion-exchange chromatography (AG 1×8 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 

\[ ^{32}P \] 1 plus synthetic peptide and incubated for 10 min, approximately 109 × 10^6 cpm of 

\[ ^{32}P \] 3 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 

\[ ^{32}P \] 1 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 catalyzed 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 

\[ ^{32}P \] 1, 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 gel plates with four different solvent systems (Fig. 3). In each case, the phosphorylated product isolated from the oocytes migrated with the same RF 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
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 oocyte's ability to 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 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).

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 kinases might have 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 is not paralleled by 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 to proteolytic degradation as a function of the region of the oocyte into which they are introduced by microinjection. This kind of approach might prove useful in characterizing the reported involvement of proteases in meiotic maturation of Xenopus oocytes (30).

Purified protein kinase catalytic subunit was generously supplied by Dr. Peter J. Bechtel, and Ms. Edwina Beckman kindly undertook the amino acid analysis. We are grateful to Dr. T. M. Lincoln of Vanderbilt University and Dr. David Glass of this laboratory for a gift of homogeneous cyclic GMP-dependent protein kinase. We thank Prof. J. Hedrick for providing healthy Xenopus laevis. E.G.K. is a recipient of an Australian National Heart Foundation Overseas Fellowship. J.M. is the recipient of a postdoctoral fellowship from the Muscular Dystrophy Association of America. E.G.K. is an Investigator of the Howard Hughes Medical Institute. This work was supported by grants from the National Institutes of Health (AM 12842 and AM 16716).


* J. L. Maller, unpublished results.