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PHOSPHOSERINE IN PEPTIDE SUBSTRATES CAN SPECIFY CASEIN KINASE II ACTION

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SUMMARY. Casein kinase II is a ubiquitous serine/threonine protein kinase which utilizes acidic amino acid residues as recognition determinants in its substrates, the motif -S/T-X-X-D/E- being particularly important. To test whether a phosphoserine residue can act as a substrate determinant, a peptide was synthesized, containing the sequence -S-X-X-S, which was not phosphorylated by casein kinase II. However, upon phosphorylation at the +3 position, the peptide became a substrate for casein kinase II. With another peptide, a positive influence of more distal phosphorylations was found. The results indicate the potential for casein kinase II to participate in hierarchal phosphorylation schemes.

Recent studies have established that phosphorylated amino acid residues in substrates may act as recognition factors for certain protein kinases (1-4). These protein kinases, glycogen synthase kinase-3 (GSK-3) and casein kinase I, are members of a small family of enzymes which in general identify their substrates by the presence of acidic amino acid residues near the target serine or threonine. GSK-3 and casein kinase I are implicated in hierarchal phosphorylation mechanisms in which an initial phosphorylation event determines subsequent phosphorylations.

Another protein kinase whose substrate sites have been defined in terms of acidic residues is casein kinase II (5-7), a ubiquitous enzyme in eukaryotic systems, which may be regulated by extracellular signalling (eg. 8,9). This protein kinase phosphorylates a large number of protein substrates (5-7) and there have been substantial efforts to define substrates for the enzyme based on the disposition of Glu and Asp residues in the vicinity of the phosphorylation site (10-15). An Asp or Glu in the +3 position has been deemed essential for casein kinase II action (13-15). A possible role for phosphate groups in casein

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kinase II substrates has also been inferred from recent studies (16-18). In the present work, we utilized enzymatic means to insert phosphate groups into the +3 position of a synthetic peptide which was not itself a substrate for casein kinase II. The enzymatic insertion of these phosphates created a substrate peptide for the protein kinase. The results showed additionally that more distal COOH-terminal phosphorylations could also influence casein kinase II action.

MATERIALS AND METHODS

Peptides. The peptide RRRDDDSDDD was the generous gift of Dr. Thomas Stephens, Eli Lilly and Company Research Laboratories. All other peptides utilized were synthesized on a phenylacetamidomethyl resin (0.61 mmol/g) using an Applied Biosystems Model 431A synthesizer run with the small scale (0.1 mmol) Boc program. Arginine side chains were protected by the Mts group. The completed peptides were cleaved from the resin with HF/10% anisole at 0° for 45 min, extracted into dilute acetic acid and lyophilized. Peptides were purified by reverse phase high pressure liquid chromatography (HPLC) using an acetonitrile gradient in 0.1% trifluoroacetic acid. Homogeneous fractions, as determined by analytical HPLC with the same system, were combined and lyophilized. The sequence of the peptides was confirmed using a Porton Instruments Model 1090 sequencer.

Enzymes. Casein kinase II and glycogen synthase kinase 3 were purified as previously described (19,3). Catalytic subunit of cAMP-dependent protein kinase was the generous gift of Dr. Edwin Krebs, University of Washington. The specific cAMP-dependent protein kinase inhibitor PKI-5-24-amide was purchased from Peninsula Laboratories (Belmont, CA).

Phosphorylation reactions. The peptides were typically assayed in 35 mM Tris-HCl pH 7.2, 1 mM EDTA, 0.22 mM EGTA, 0.4 mM [^{32}P]ATP (0.5-1.0 cpm/fmol), 6.0 mM magnesium acetate, 10 mM β-mercaptoethanol, and protein kinase in a total volume of 15μl. For determination of kinetic parameters, peptide substrates were varied in the range 5-200 μM. The reactions were terminated by the addition of EDTA and adenosine to final concentrations of 27.5 mM and 3.0 mM, respectively. Aliquots were spotted on 2.0 X 2.0 cm squares of Whatman P81 filter paper, washed with constant stirring four times with 75 mM phosphoric acid, and immediately washed once with 100% ethanol. Label incorporated into the peptides was determined in 0.5% diphenyloxazole in toluene, using a liquid scintillation counter.

Phosphopeptides. Different phosphorylated forms of the peptides EEESEAEPAPSRRGSARR and EEESEASPAPSRRGSARR were prepared as follows. Large scale phosphorylation reactions (1 mg peptide) were run, incubating with cAMP-dependent kinase first. [^{32}P]ATP was removed by passage over Dowex 1 X 8 equilibrated in 30% acetic acid. The phosphorylated forms of the peptides were separated from the mixture by affinity chromatography using iminodiacetic acid-epoxy activated Sepharose 6B (Sigma, St. Louis) charged with ferric chloride (20). After drying, portions of each monophosphopeptide were phosphorylated with multiple additions of GSK-3 and incubation at 30°C overnight. The diphosphorylated form of EEESEAEPAPSRRGSARR and the triphosphorylated form of

EEESEASPAPSRRGSARR (as identified using isoelectric focussing) were separated from these reaction mixtures using reverse phase HPLC and two different solvent systems. Separation was first accomplished using a 0-70% acetonitrile gradient in 25 mM MES pH 6.0, then using a 0-70% acetonitrile gradient in 0.1% trifluoroacetic acid. The yields and concentrations of the phosphorylated forms of the peptides were determined using Cherenkov counting.

Other procedures. Isoelectric focusing was performed using previously described procedures (1). The kinetic constants for the various peptides were determined from Lineweaver-Burk plots of the kinetic data for each peptide. Protein concentrations were determined by the method of Bradford (21). Other chemical reagents were obtained in the highest quality available from commercial vendors.

RESULTS

The rationale behind the design of synthetic peptides for this study was to create a phosphoserine at the +3 position with respect to a potential casein kinase II site. Since chemical synthesis of phosphoserine containing peptides is not yet routine, our strategy was to build a protein kinase site at the +3 position. A successful design was based on the sequence of a phosphorylated region of the G-component of protein phosphatase-1 (Fig. 1). The synthetic peptide with this sequence was an excellent substrate for GSK-3 after prior phosphorylation by cAMP-dependent protein kinase (2). We synthesized a variant of this peptide with an NH_2-terminal extension containing a potential casein kinase II and with a GSK-3 site at the +3 amino acid (Fig. 1). Basic residues were added at the carboxyl terminus to enable the peptide to bind to P81 paper (see "Materials and Methods"). A control peptide was synthesized containing glutamate at the +3 amino acid position from the casein kinase II site. As a reference, we also analyzed phosphorylation of the peptide

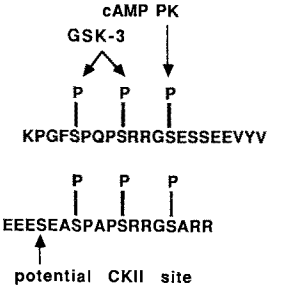

Figure 1. Peptides based on the sequence of the G-component of protein phosphatase 1. The upper peptide is identical in sequence to the G-component protein and contains phosphorylation sites for the indicated protein kinases. The lower peptide is based on the same sequence, but additionally contains a potential site for phosphorylation by casein kinase II (CKII) after prior phosphorylation by cAMP-dependent protein kinase (cAMP PK) and glycogen synthase kinase-3 (GSK-3).

Table I. Kinetic Parameters for the Phosphorylation of Synthetic Peptides by Casein Kinase II

Peptide	V_{max} (nmoles/min)	K_m (mM)	V_{max}/K_m
RRRDDDSDDD	97.2	58.2	1.67
EEESEAEPAPSRRGSARR	29.8	122.3	0.244
EEESEAEPAPSRRGS(P)ARR	9.7	20.9	0.464
EEESEAEPAPS(P)RRGS(P)ARR	38.4	49.3	0.780
EEESEASPAPSRRGSARR	<0.1	---	---
EEESEASPAPSRRGS(P)ARR	<0.1	---	---
EEESEAS(P)PAPS(P)RRGS(P)ARR	2.04	38.6	0.0528

RRRDDDSDDD, which is an excellent synthetic substrate for casein kinase II (14).

The peptides EEESEAEPAPSRRGSARR and EEESEASPAPSRRGSARR were phosphorylated as described under "Materials and Methods" with cAMP-dependent kinase, GSK-3, or a sequential combination of those kinases. When cAMP-dependent protein kinase was utilized, the 5-24-amide inhibitor peptide was added to a final concentration of 5.0 µg/ml before addition of other kinases (22). As expected from work on the related G-component peptide (2), both peptides were excellent substrates for cAMP-dependent kinase. The peptides were not substrates for GSK-3 until after they had been phosphorylated by cAMP-dependent kinase. Analysis by isoelectric focussing indicated that phosphorylation of the peptide EEESEAEPAPSRRGS(P)ARR by GSK-3 generated a single new phosphorylated species corresponding to the introduction of a single phosphate group while phosphorylation of the peptide

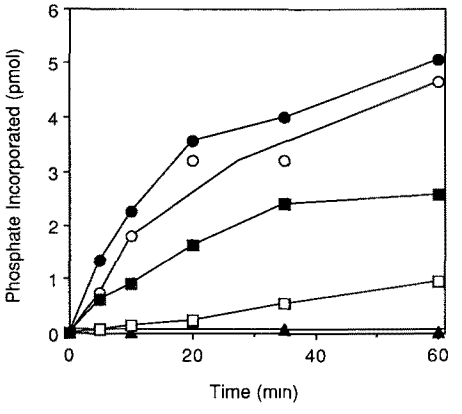

Figure 2. Time course of the phosphorylation of the various phosphorylated forms of the synthetic peptides by casein kinase II. The incorporation of phosphate into the diphosphorylated (●), monophosphorylated (○), and unphosphorylated (■) forms of the peptide EEESEAEPAPSRRGSARR and into the triphosphorylated (□) and monophosphorylated (▲) forms of the peptide EEESEASPAPSRRGSARR were monitored as a function of time. The concentration of the peptides was 20.0 µM.

EEESEASPAPSRRGS(P)ARR resulted also in a more acidic species correlating with modification at two sites (not shown). Again, these results are consistent with earlier studies (1,2). By varying the protein kinases used, we were thus able to generate a set of four different phosphopeptides (Table I), which were purified as described under "Materials and Methods".

The peptides and phosphopeptides were then tested as substrates for casein kinase II. A representative time course of phosphorylation of the peptides is shown in Fig. 2. Table I compares the kinetic constants for casein kinase II phosphorylation of the same peptides. The unphosphorylated peptide EEESEAEPAPSRRGSARR was a substrate for casein kinase II. The monophosphorylated form of the peptide, generated after phosphorylation by cAMP-dependent protein kinase, was a better substrate than the unphosphorylated form, in terms of Km or V_{max}/K_m (Table I). The diphosphorylated form of the peptide, formed by phosphorylation with both cAMP-dependent protein kinase and glycogen synthase kinase-3, had a higher K_m than the monophosphorylated form, but had the highest V_{max} of any of the three forms of this peptide, making it overall the best substrate of the three in terms of V_{max}/K_m values.

The peptide EEESEASPAPSRRGSARR, with Ser in the +3 position, was not an effective substrate for casein kinase II nor was the same peptide after phosphorylation by cAMP-dependent protein kinase alone. However, phosphorylation of the peptide by both cAMP-dependent protein kinase and GSK-3 did create a substrate for casein II. The K_m value of 39 μM was excellent but the V_{max} was low. Comparing V_{max}/K_m values, the triphosphorylated form of the peptide EEESEASPAPSRRGSARR was about an order of magnitude less effective than any of the forms of the peptide with Glu at the +3 position.

DISCUSSION

The results of this investigation demonstrate that phosphoserine residues can act as specificity determinants for casein kinase II. Attachment of a phosphate group to a Ser in the +3 position of a peptide not recognized by casein kinase II generated a peptide substrate with respectable kinetics of phosphorylation and a very favorable K_m of 39 μM. The control peptide with the -S-X-X-E- motif actually had a significantly higher K_m but its improved V_{max} made it a substantially better substrate based on V_{max}/K_m. The K_m for one of the best known substrates, RRRDDDSDDD, is around 60 μM (14; Table I). The work of Pinna and colleagues (16,17) had provided the first evidence for a role of phosphate groups in casein kinase II action by showing that partial dephosphorylation of certain multiply phosphorylated substrates increased the rate of phosphorylation by

casein kinase II. Lichfield et al. (18) showed that chemically synthesized RRREEESAAS(P)A was phosphorylated by casein kinase II with a K_m of 0.57 mM, 10-fold greater than for our peptide with the same -S-X-X-S(P)- motif. Rationalizing this difference is hard since the peptides differ in other respects. It is significant though that phosphoserine is capable of specifying casein kinase II action with low K_m.

The effect of placing a phosphoserine at the +3 position is consistent with the previous work suggesting that an acidic residue at this location is essential and demonstrates that phosphoserine can partly substitute Glu or Asp in this role. The nature of other residues surrounding the phosphorylation site is also known to influence casein kinase II action (10-15). In our studies, the control peptide was improved as a substrate after phosphorylations quite distal to the casein kinase II site, at the +7 or at the +7 and +11 positions. These locations are more remote than residues usually implicated in casein kinase II recognition and may not be involved directly in the enzyme-substrate interactions. One possibility is that these phosphates may "shield" the positive charges on the Arg residues at +8, +9, +13 and +14 which could conceivably act as negative determinants.

The ability of phosphoserine to direct casein kinase II action suggests that some physiological substrates of the enzyme might require prior phosphorylation. In our work we have shown that the presence of a GSK-3 site at the +3 position is compatible with casein kinase II action. The same may be true of sites for other protein kinases although sites for enzymes requiring NH_2-terminal basic residues would likely introduce strong negative determinants for casein kinase II. Of course, phosphorylation at other positions could also influence casein kinase II. Casein kinase II joins GSK-3 and casein kinase I in being able to act as secondary protein kinases in hierarchal phosphorylation schemes, mechanisms whereby initial phosphorylations provoke subsequent ones. Casein kinase II would thus be a rare example of protein kinase able to serve as either a primary or a secondary kinase.

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