Synthetic phosphopeptides are substrates for casein kinase II

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Casein kinase II is a protein serine/threonine kinase that exhibits a preference for acidic substrates. Previous studies have demonstrated that a glutamic acid 3 amino acids C-terminal (+3) to a serine or threonine is required for phosphorylation. To examine the ability of phosphoserine and phosphothreonine residues to serve as specificity determinants for casein kinase II, phosphopeptides containing either of these phosphoamino acids in the +3 position were synthesized and tested as substrates. Phosphopeptides containing phosphoserine in the +3 position were readily phosphorylated. In contrast, corresponding phosphothreonine-containing peptides were very poorly phosphorylated. These results imply that prior phosphorylation of substrate proteins on serine, but not threonine residues, may be important in regulating their phosphorylation by casein kinase II.

Casein kinase 2; Substrate specificity; Synthetic phosphopeptide; Peptide phosphorylation

1. INTRODUCTION

The majority of the protein kinases that have been characterized to date can be divided into two categories: the protein serine/threonine kinases [1] and the protein tyrosine kinases [2]. Within these categories, several of the protein kinases can be distinguished on the basis of their substrate specificity. The ability of particular protein kinases to phosphorylate a specific amino acid residue is at least in part determined by the primary amino acid sequence of the substrate protein. Considerable information about the primary sequence requirements for substrate phosphorylation has been obtained from studies using synthetic peptides. This approach has been successfully utilized for several protein serine/threonine kinases including casein kinase II [3–7].

Casein kinase II is a messenger-independent protein serine/threonine kinase found in the cytosol and nucleus of all eukaryotic cells [1,8,9]. A number of substrates have been identified as targets for this multifunctional enzyme, including a number of metabolic enzymes, some cytoskeletal and contractile proteins and a variety of proteins involved in the regulation of transcription and translation [8–12].

Previous studies using synthetic peptides have demonstrated that acidic amino acids C-terminal to the phosphorylatable serine/threonine are important specificity determinants [3–7]. Although multiple acidic residues are advantageous, a single acidic amino acid three residues C-terminal (+3 position) to the serine/threonine is required for phosphorylation. More recently, studies with partially dephosphorylated phosphoproteins or phosphopeptides have indicated that phosphoserine can also serve as a specificity determinant for casein kinase II [13,14]. The goal of the study presented here was to systematically examine the ability of phosphoserine or phosphothreonine in the +3 position to serve as a specificity determinant for casein kinase II. For this study, a series of eight novel peptides and phosphopeptides was synthesized and tested as substrates for purified bovine testis casein kinase II. To relate the results of this study to previous studies, the specific casein kinase II substrate peptide RRREEETEEE or the related peptide RRREEESEEE were used as parent peptides [5,7]. The data indicate that phosphoserine in the +3 position can serve as a specificity determinant for casein kinase II. However, a peptide with a phosphothreonine in the +3 position is not phosphorylated by casein kinase II when examined under the same conditions.

2. MATERIALS AND METHODS

2.1. Synthetic peptides

2.1.1. Solid phase synthesis and characterization

Peptides were synthesized from Boc-amino acids using an
2.1.2. HF cleavage of phosphopeptide from resin and removal of blocking groups

Synthetic peptides (0.15 mmol) were cleaved from the resin using anhydrous HF/anisole (1:1, v/v) for 45 min at 0°C. Reagents were evaporated and the crude peptides, still retaining their phenyl-phosphate blocking groups were extracted using 10% acetic acid (200 ml). Each phenyl-protected peptide was dissolved in 2 ml of 40% TFA/acetone, and 72 mg (1 equiv.) of anisomeric platinum (IV) oxide was added. Hydrogenolysis was performed for 24 h at room temperature under 4.05 bar (4 atm) of hydrogen pressure. The mixture was evaporated to dryness under vacuum, suspended in water, and the catalyst removed by filtration.

2.1.3. Peptide purification

Phosphorylated peptides were purified by preparative HPLC on a Whatman Partisil 10 ODS 3 column (22 × 250 mm) using a Beckman/Altex HPLC. Elution was performed with a linear gradient from 0.1% aqueous TFA to 30–40% acetonitrile-isopropanol (2:1) containing 0.1% TFA in 40 min at 14 ml/min with the eluent monitored at 220 nm, AUFS = 0.3. Phosphopeptides were separated from any unphosphorylated peptide or by-products by affinity chromatography on a Fe⁺⁺-Chelex column as described in detail elsewhere [15].

Analytical HPLC was performed on a reverse phase Whatman Partisil 5 ODS-3 column (4.6 × 250 mm) using a linear gradient of aqueous 0.1% H₂PO₄ to 0.1% H₂PO₄ in acetonitrile over 40 min at 1 ml/min with the eluent monitored at 210 nm with AUFS = 0.25 and on Waters cation exchange cartridge (Partisil SCX 8 mm × 10 cm) with a gradient from 10% acetonitrile in 0.01 M potassium phosphate, pH 3.5 to 10% acetonitrile in 0.4 M sodium phosphate, pH 2.5 over 40 min.

2.1.4. Other peptides

The peptide RRREEETEEE was obtained from Peninsula Laboratories. The synthesis of RRREEESAAA and RRREEESEEE 
were performed at the 0.3 mmol scale in a 24 ml reaction vessel using PAM resin and the DIC/HOBt coupling strategy as described [15]. Automated monitoring assured a minimum efficiency of 99.5% at each step. Identity of the peptides was verified by amino acid analysis and fast atom bombardment mass spectroscopy [15].

3. RESULTS

Synthetic peptides and phosphopeptides that are variants of RRREEETEEE and RRREEESEEE [5,7] have been used to systematically examine the ability of phosphoserine or phosphothreonine in the +3 position to serve as specificity determinants for casein kinase II. As a basis for comparison, peptides containing either glutamic acid, alanine or the unphosphorylated hydroxy-amino acids serine and threonine in the +3 position were examined (table 1). The kinetic parameters (Kₘ and kcat) for the phosphorylation of each of the peptides by casein kinase II are reported in table 2. A ratio of the phosphorylation rate and substrate concentration dependence (kcat/Kₘ) serves as a comparative index for the various peptides as substrates.

As expected from previous studies, replacement of an alanine with a glutamic acid in the +3 position results in a dramatic improvement in peptide phosphorylation (RRREEESAAA vs RRREEESEEE) [6,7]. This is evidenced by large increases in relative phosphorylation (table 1) and in the kcat/Kₘ ratio (table 2). A large increase (10-fold) in relative phosphorylation rate (table 1) is also observed when a serine is replaced with a phosphoserine (RRREEESAAA vs RRREEEESAS(P)A) and RRREEEETEEE vs

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Relative phosphorylation rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>RRREEETEEE</td>
<td>100</td>
</tr>
<tr>
<td>RRREEESAAA</td>
<td>4</td>
</tr>
<tr>
<td>RRREEESEEE</td>
<td>190</td>
</tr>
<tr>
<td>RRREEEESASA</td>
<td>6</td>
</tr>
<tr>
<td>RRREEEESAS(P)A</td>
<td>64</td>
</tr>
<tr>
<td>RRREEEETAAA</td>
<td>1</td>
</tr>
<tr>
<td>RRREEEETAS(P)A</td>
<td>11</td>
</tr>
<tr>
<td>RRREEEETATA</td>
<td>&lt;1</td>
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<tr>
<td>RRREEEETAT(P)A</td>
<td>5</td>
</tr>
<tr>
<td>RRREEEETATA</td>
<td>1</td>
</tr>
<tr>
<td>RRREEEETAT(P)A</td>
<td>&lt;1</td>
</tr>
</tbody>
</table>

Assays were conducted as described in section 2 using each peptide at a concentration of 2 mM. Data were compiled from six separate experiments. From these experiments, the actual specific activity of casein kinase II towards RRREEETEEE was 0.85 ± 0.07 γmol phosphate transferred/min/mg (average ± SE).
Table 2

Kinetic constants for casein kinase II phosphorylation of synthetic peptides

<table>
<thead>
<tr>
<th>Peptide</th>
<th>$k_{cat}/K_m$ (mM)</th>
<th>$K_m$ (mM)</th>
<th>$k_{cat}/K_m$</th>
<th>Relative $k_{cat}/K_m$</th>
</tr>
</thead>
<tbody>
<tr>
<td>RRREEEETEEE</td>
<td>1.55</td>
<td>0.56</td>
<td>2.77</td>
<td>100</td>
</tr>
<tr>
<td>RRREEEESAAA</td>
<td>0.13</td>
<td>4.88</td>
<td>0.03</td>
<td>1</td>
</tr>
<tr>
<td>RRREEEESAE</td>
<td>2.81</td>
<td>0.96</td>
<td>2.93</td>
<td>106</td>
</tr>
<tr>
<td>RRREEEESAAS</td>
<td>0.35</td>
<td>7.67</td>
<td>0.05</td>
<td>2</td>
</tr>
<tr>
<td>RRREEEESAAS(P)A</td>
<td>1.20</td>
<td>0.57</td>
<td>2.11</td>
<td>76</td>
</tr>
<tr>
<td>RRREEETAASA</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>&lt;1</td>
</tr>
<tr>
<td>RRREEETAS(A)P</td>
<td>0.24</td>
<td>0.85</td>
<td>0.28</td>
<td>10</td>
</tr>
<tr>
<td>RRREEFSAAAT</td>
<td>0.03</td>
<td>3.33</td>
<td>0.01</td>
<td>&lt;1</td>
</tr>
<tr>
<td>RRREEETASAT(P)A</td>
<td>0.16</td>
<td>4.16</td>
<td>0.04</td>
<td>1</td>
</tr>
<tr>
<td>RRREEETAT</td>
<td>0.02</td>
<td>1.93</td>
<td>0.01</td>
<td>&lt;1</td>
</tr>
<tr>
<td>RRREEETAT(P)A</td>
<td>0.03</td>
<td>10.49</td>
<td>&lt;0.01</td>
<td>&lt;1</td>
</tr>
</tbody>
</table>

Phosphorylation was monitored and kinetic constants determined as described in section 2; n.d., not determined.

RRREEETAS(A)P. The $k_{cat}/K_m$ ratios for these peptides (table 2) demonstrate that this effect is even greater than indicated by the relative phosphorylation rates.

A peptide containing a phosphorylatable serine is more readily phosphorylated than the corresponding threonine-containing peptide (RRREEFSASAS(P)A vs RRREEETAS(A)P). This preference for serine over threonine by casein kinase II has been noted in previous studies [6,7].

The data in table 2 clearly demonstrate that glutamic acid or phosphoserine, but not phosphothreonine, in the +3 position can serve as a specificity determinant for casein kinase II. Although the $k_{cat}$ for RRREEESAAE is significantly greater than that for RRREEEESAAS(P)A, the former peptide also has a higher $K_m$. Therefore, the $k_{cat}/K_m$ ratio of RRREEEESAAS(P)A is only moderately lower than that of RRREEESAAE. This suggests that phosphoserine and glutamic acid have a similar capacity to serve as specificity determinants for casein kinase II, at least in the +3 position. By comparison, even the best of the phosphothreonine-containing peptides (RRREEESAAT(P)A) had a 10-fold lower phosphorylation rate than the corresponding phosphoserine-containing peptide (RRREEEESAAS(P)A). The kinetic constants for the two peptides also illustrate this point since the $k_{cat}/K_m$ ratio for the phosphothreonine-containing peptide was more than 50-fold lower than that of its phosphoserine-containing counterpart.

4. DISCUSSION

The observations presented here extend those of two previous reports that demonstrated that casein kinase II can use partially dephosphorylated phosvitin [13] or phosphotripeptide [14] as substrates. In the previous reports, the position of the phosphoserine residue with respect to the phosphorylatable residue was not defined. Furthermore, the $K_m$ of the partially dephosphorylated tripeptide was approximately 5-fold higher than that observed in this study with RRREEEESAAS(P)A.

The inability of phosphothreonine to function as a specificity determinant may provide valuable insight into mechanisms of substrate recognition by casein kinase II. Clearly, a model stating that a negatively charged residue in the +3 position will have a positive effect on phosphorylation is not entirely correct. The methyl group on the $\beta$ carbon of threonine could impair interactions between the phosphate group on the substrate and casein kinase II. Based on the results of this study, it will clearly be of interest to examine the ability of phosphotyrosine or even the N-phosphorylated amino acids (phosphohistidine, phospholysine, phosphoarginine) as specificity determinants for casein kinase II.

The demonstration that at least one type of phosphoamino acid can have a positive influence on phosphorylation by casein kinase II has some interesting implications. Casein kinase II joins casein kinase I [18] and glycogen synthase kinase 3 [19-23] as protein serine/threonine kinases that can utilize phosphotriesters as specificity determinants. The ability to utilize phosphoserine in such a manner suggests the potential for synergistic phosphorylations where prior phosphorylation of a substrate by one protein kinase permits phosphorylation by a second enzyme. Indeed, casein kinase II is known to participate in the synergistic phosphorylation of glycogen synthase [19-21], the RI1 regulatory subunit of cAMP-dependent protein kinase [22], and inhibitor 2 of protein phosphatase 1 [23]. Prior phosphorylation of either protein by casein kinase II is obligatory for subsequent phosphorylation by glycogen synthase kinase 3. On the basis of the results presented here and elsewhere, it is probable that casein kinase II will also phosphorylate some proteins only after phosphorylation by another protein kinase.

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REFERENCES