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Phosphotyrosine as a specificity determinant for casein kinase-2, a growth related Ser/Thr-specific protein kinase

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The motif Ser-Ser-Ser-Glu-Glu is readily phosphorylated by casein kinase-2 (CK-2), a growth-related protein kinase whose consensus sequence is Ser(Thr)-Xaa-Xaa-Glu(Asp) [(1990) Biochim. Biophys. Acta 1054, 267–283]. Here we show that phosphotyrosine can replace carboxylic acids as specificity determinant for CK-2 phosphorylation, the phosphotyrosyl peptide Ser-Ser-Ser-TyrP-TyrP actually being a substrate more efficient than Ser-Ser-Ser-Glu-Glu itself both in terms of K_m (0.69 vs 2.43 mM) and V_{max}. Prior dephosphorylation of phosphotyrosine entirely prevents the subsequent phosphorylation of serine by CK-2. While Ser-Ser-Ser-TyrP-TyrP is a better substrate than Ser-Ser-Ser-SerP-SerP, which in turn is better than Ser-Ser-Ser-Glu-Glu, Ser-Ser-Ser-ThrP-ThrP is a less efficient substrate than Ser-Ser-Ser-Glu-Glu. Thus the order of efficiency of phosphoamino acids as specificity determinants for CK-2 appears to be TyrP > SerP ≫ ThrP.

Protein kinase; Casein kinase-2; Phosphopeptide; Phosphotyrosine (as specificity determinant)

1. INTRODUCTION

Most, if not all, cellular functions are controlled by a network of reversible protein phosphorylation reactions whose co-ordinated occurrence is ensured by a variety of devices, including multi-site phosphorylation of individual targets [1]. In this connection, several examples of Ser/Thr-specific protein kinases whose phosphorylating activity toward either protein or model peptide substrates is potentiated by the previous phosphorylation of other seryl residue(s) in the proximity of the target amino acid have been reported [2–10]. Prior to this study it was unknown whether phosphotyrosine could also act as specificity determinant for serine phosphorylation. Such a circumstance, if proved, would disclose the possibility that the two major classes of protein kinases, Ser/Thr- and Tyr-specific, might cross-talk at substrate level. Here we show that the phosphopeptide Ser-Ser-Ser-TyrP-TyrP is indeed readily phosphorylated by casein kinase-2 (CK-2), a growth-related Ser/Thr-specific protein kinase [11,12], and that such a phosphorylation does not occur with prior dephosphorylation of the phosphotyrosine residues.

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Abbreviations: CK-1, casein kinase-1; GSK-3, glycogen synthase kinase-3; CK-2, casein kinase-2; Sp or SerP, phosphoserine; Tp or ThrP, phosphothreonine; Yp or TyrP, phosphotyrosine

2. MATERIALS AND METHODS

The peptides SSSEE-NHMe (**I**), SSSSpSp-NHMe (**II**) and SSSTpTp-NHMe (**III**) were prepared by Boc/solution-phase peptide synthesis using Boc-Ser(Bzl)-OH, Boc-Glu(OBzl)-OH, Boc-Ser(PO_3Ph_2)-OH or Boc-Thr(PO_3Ph_2)-OH for the synthesis of the protected peptides followed by their hydrogenolytic deprotection in 50% TFA/AcOH using either palladium/charcoal (for **I**) or platinum oxide (for **II** and **III**). The peptide SSSYpYp (**IV**) was prepared by Fmoc/solid-phase peptide synthesis using HMP polystyrene resin and Fmoc-Tyr(PO_3tBu_2)-OH for the synthesis of the protected peptide-resin followed by simultaneous resin cleavage and peptide deprotection by treatment with 95% TFA/anisole. All the peptides were >95% pure as judged from amino acid and phosphate analysis and HPLC profile. CK-2 was purified to nearly homogeneity from rat liver cytosol [13]. Phosphorylation of the peptides by CK-2 was performed in the presence of [γ-^{32}P]ATP and evaluated by determining [^{32}P]SerP released by acid hydrolysis, and isolated by high voltage paper electrophoresis [14].

3. RESULTS

The peptide SSSEE has been chosen as a reference because of its identity with a motif recurrent at several sites affected by CK-2 in casein and soybean antiprotease fractions [15], clathrin light chain-b [16] and the β-subunit of CK-2 itself [12]. For both β-casein [17] and synthetic peptides [18] this motif is mainly phosphorylated by CK-2 at its first serine in virtue of the penultimate glutamic acid fulfilling the minimum structural requirement of an acidic residue at position +3. In Fig. 1 the phosphorylations of SSSEE and of its three derivatives in which the two C-terminal glutamic acids have been replaced by either phosphotyrosine, phosphoserine or phosphothreonine are compared. The phos-

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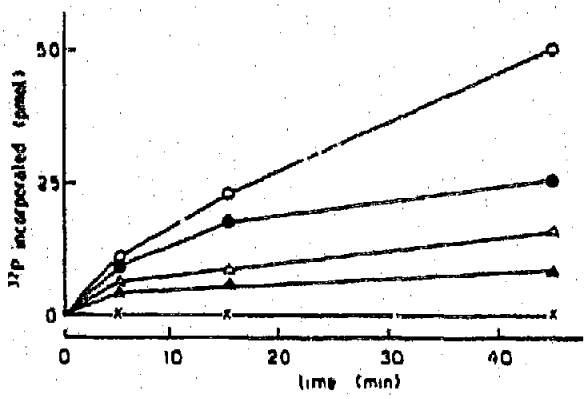

Fig. 1. Phosphorylation of the peptides Ser-Ser-Ser-TyrP-TyrP (•), Ser-Ser-Ser-SerP-SerP (•), Ser-Ser-Ser-ThrP-ThrP (▲), Ser-Ser-Ser-Glu-Glu (▲) and Ser-Ser-Ser-Tyr-Tyr (×) by CK-2.

photyrosyl peptide was found, by far, to be the best substrate of this peptide series with the order of phosphorylation efficiency being TyrP > SerP > Glu > ThrP.

The crucial relevance of the phosphate group of TyrP for determining the substrate targeting by CK-2 was proved by the deleterious effect of prior enzymatic dephosphorylation which prevents the subsequent phosphorylation of the peptide by CK-2 (Fig. 2). The same detrimental effect of dephosphorylation was also observed with the phosphoseryl and phosphothreonyl peptides (not shown). This observation supports the concept that phosphothreonines also play a favourable phosphorylation-directing role, although their efficacy

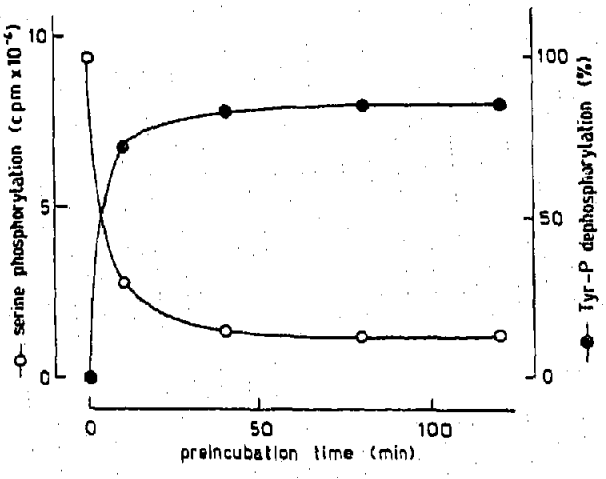

Fig. 2. Inverse relationship between prior phosphotyrosine dephosphorylation (•) and subsequent serine phosphorylation (○) by CK-2. The phosphopeptide Ser-Ser-Ser-TyrP-TyrP was preincubated with potato acid phosphatase for the indicated times and the extent of dephosphorylation evaluated from the inorganic phosphate released [25]. Boiled aliquots of the variably dephosphorylated peptide were subjected to phosphorylation by 10 min incubation with CK-2 in the presence of [^{32}P]ATP, and radiolabeled phosphoserine was estimated as indicated in the experimental section.

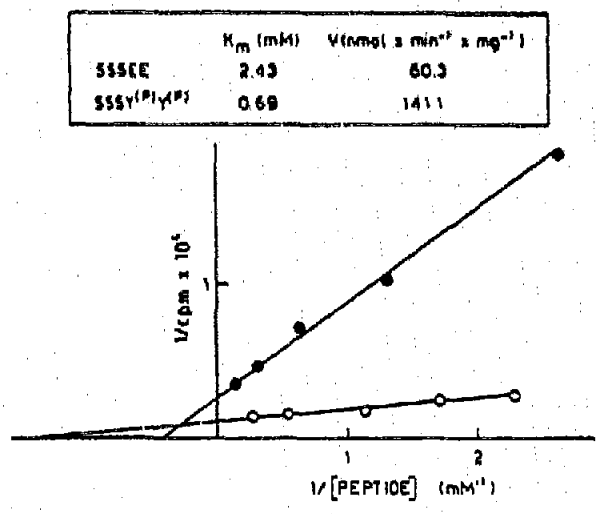

Fig. 3. Determination of the kinetic constants of the peptides Ser-Ser-Ser-TyrP-TyrP (○) and Ser-Ser-Ser-Glu-Glu (•) for CK-2. Kinetic parameters were calculated by double-reciprocal plots constructed from initial rate measurements fitted to Michaelis-Menten equation.

is quite modest in comparison to those of phosphotyrosine and phosphoserine.

As shown in Fig. 3, the suitability of the phosphotyrosyl peptide as a target for CK-2 is quite remarkable both in terms of K_m, which is more than 4-fold lower than that of the reference peptide SSSEE, and of V_{max}, which is significantly higher.

4. DISCUSSION

The data presented provide the first evidence that phosphorylated tyrosine can act as a canonical specificity determinant for a Ser/Thr-specific protein kinase, thus disclosing the possibility that the targeting by some members of this class of protein kinases may be triggered by tyrosine protein kinases through a substrate level phosphorylation mechanism. Such an intriguing hypothesis is of special interest in the case of CK-2, given the involvement of this enzyme in cell proliferation and its response to mitogens, like insulin and EGF (reviewed in [12]), whose signals are transmitted into the cell by receptorial tyrosine protein kinases. In addition, it will also be interesting to examine whether phosphotyrosine could generate a consensus sequence for two other protein kinases, GSK-3 and CK-1, both of these kinases being known to recognize phosphoserine as specificity determinant [5,8].

Although tyrosine residues are not present in the sequence sites which have, to date, been elucidated for some CK-2 phosphorylated substrates (reviewed in [12]) the sequence sites of many other substrates which are affected by this enzyme have yet to be examined. In this connection it is particularly interesting that the amino acid sequence of the autophosphorylation site of the insulin receptor protein kinase (IYDTDYYR) includes

three tyrosyl residues all of which undergo phosphorylation [19]. Once triply phosphorylated, this peptide sequence should be a good phosphorylation target for CK-2, by virtue of the threonine residue which will fulfill the consensus sequence: -Thr-Asp-TyrP-TyrP-. This may account for the reported phosphorylation of insulin receptor by CK-2 [20]. The same applies to the IGF-II receptor which is also a good target for CK-2 [21] and whose segment homologue to the insulin receptor is entirely conserved [22].

In addition the phosphorylation of a tyrosyl residue may also play a favourable role for the subsequent phosphorylation of the serine residue of the C-terminal flanking peptide of rat progastrin [23]. While this peptide sequence has been shown to be a good target for CK-2, a downstream tyrosine residue at the +7 position is also readily phosphorylated by two tyrosine protein kinases from spleen (unpublished observation in collaboration with J.G. Dockray and A. Varro). Although the tyrosine residue is located at position +7 relative to serine (SAEEEDQY), it may nevertheless influence serine phosphorylation since it has been shown previously that a glutamic acid at that position is still recognized as a favourable feature by CK-2 [24]. In the case of native progastrin such a downstream tyrosine is extensively sulphated (J.G. Dockray, personal communication) and it would be of interest to examine the possibility that besides phosphotyrosine, sulphotyrosine may also act as a specificity determinant for CK-2.

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