Opposite and mutually incompatible structural requirements of type-2 casein kinase and cAMP-dependent protein kinase as visualized with synthetic peptide substrates

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The synthetic hexapeptide Ser-Glu-Glu-Glu-Val-Glu and its N-acetylated derivative are readily and specifically phosphorylated by rat liver casein kinase TS (type-2), while the derived heptapeptide with an additional N-terminal Arg is a very poor substrate. Conversely, the substitution of Glu for Val_5 in the synthetic peptide Arg-Arg-Ser-Thr-Val-Ala, which is a good substrate for cAMP-dependent protein kinase by virtue of the N-terminal arginyl residues, prevents its phosphorylation by this enzyme. These data indicate that the site specificities of these two classes of protein kinases, requiring acidic and basic residues on the C- and N-terminal sides of the target residue(s), respectively, are mutually incompatible.

Casein kinase type-2 *cAMP-dependent protein kinase* *Phosphorylation site* *Phoshorylatable peptide* *Protein phosphorylation* *Troponin-T*

1. INTRODUCTION

A property of many protein kinases is their capability of recognizing only a few phosphorylatable residues which are characterized by their inclusion within definite amino acid sequences (review [1,2]). Such a site specificity however is not always absolute since the structural requirements of distinct protein kinases can be simultaneously fulfilled around a single residue which is hence phosphorylatable by more than one protein kinase. Thus phosphorylation site-2 in skeletal muscle glycogen synthase is affected by several protein kinases including, besides cAMP-dependent protein kinase (A-kinase), phosphorylase kinase, glycogen synthase kinase-4 [3] and a calmodulin-dependent protein kinase distinct from phosphorylase kinase [4]. Similarly, the same residues affected by A-kinase in ribosomal protein S6 and in histone subfractions are also phosphorylated, though with different efficiencies, by H4-protein kinase [5] and cGMP-dependent protein kinase [6], respectively. Consequently, synthetic peptides have been prepared which can act as substrates for two different protein kinases (e.g., [7–9]).

While A-kinase requires two basic residues, at least one of which must be Arg, close to the N-terminal side of the target residue [1], type-2 casein kinases (Ck-TSs), a class of ubiquitous oligomeric cyclic nucleotide-independent protein kinases (review [10]) always affect, in either casein fractions or physiological substrates, seryl and threonyl residues having several acidic groups close to their C-terminal side [2,3,11–13]. Up to now, however, no direct evidence was available that such acidic residues actually represent the minimum structural requirement for this family of protein kinases. It was also conceivable that sites fulfilling the requirements of both A-kinase and Ck-TS could be affected by both enzymes.

Here we show with the aid of new synthetic peptides that the phosphorylation by rat liver Ck-TS

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actually requires a C-terminal acidic cluster but it is prevented by the N-terminal arginyl residue(s) which are required by A-kinase.

2. EXPERIMENTAL

Ck-TS, purified to greater than 95% homogeneity [14], and A-kinase [15] were prepared from rat liver as described.

The peptides Ser-Glu-Glu-Glu-Val-Glu, its acetylated derivative and Arg-Ser-Glu-Glu-Glu-Val-Glu were synthesized by the method in solution, following a strategy that will be detailed elsewhere. They migrated homogeneously upon thin-layer chromatography and paper electrophoresis and were over 95% pure according to amino acid analyses. The other hexapeptides were prepared as in [16] and the tetrapeptide Ser-Thr-Glu-Ala was obtained by trypsin digestion of Arg-Arg-Ser-Thr-Glu-Ala.

The activity of Ck-TS toward peptides was assayed as in [14], but replacing casein with variable amounts of peptide substrates (detailed in figures). A-kinase was assayed under the conditions described in [16]. The reaction was stopped with HCl, either 2 N or 6 N, and ^{32}P incorporated into the peptides was determined either by the isobutanol–benzene extraction procedure [17] after 15 min boiling, or by isolation of radiolabeled phosphoserine by high-voltage paper electrophoresis following 4 h hydrolysis at 105°C [18]. Comparable results were obtained by both procedures. Neither the direct paper electrophoresis method [16] nor the phosphocellulose paper method [19] was applicable to the acidic peptides.

3. RESULTS AND DISCUSSION

While several sets of basic peptides have been prepared and utilized for studying the site specificity of A-kinase and other protein kinases with similar, though not identical, structural requirements (e.g., [7–9, 20–22]), up to now acidic peptide substrates have been synthesized only for tyrosine kinases [23,24]. However, very recently we synthesized acidic hexa- and heptapeptides reminiscent of the sites which are phosphorylated by type-2 casein kinase in glycogen synthase and troponin-T.

As shown in fig.1A, the hexapeptide Ser-Glu-Glu-Val-Glu and its *N*-acetylated derivative which is identical to the phosphorylation site of troponin-T except for the substitution of Glu_3 for Asp [11] are readily phosphorylated by Ck-TS. While *N*-acetylation does not substantially modify the phosphorylation efficiency, the addition of an Arg to the N-terminal side of the target serine is deleterious, giving rise to a heptapeptide which is almost inactive as a phosphate acceptor even after

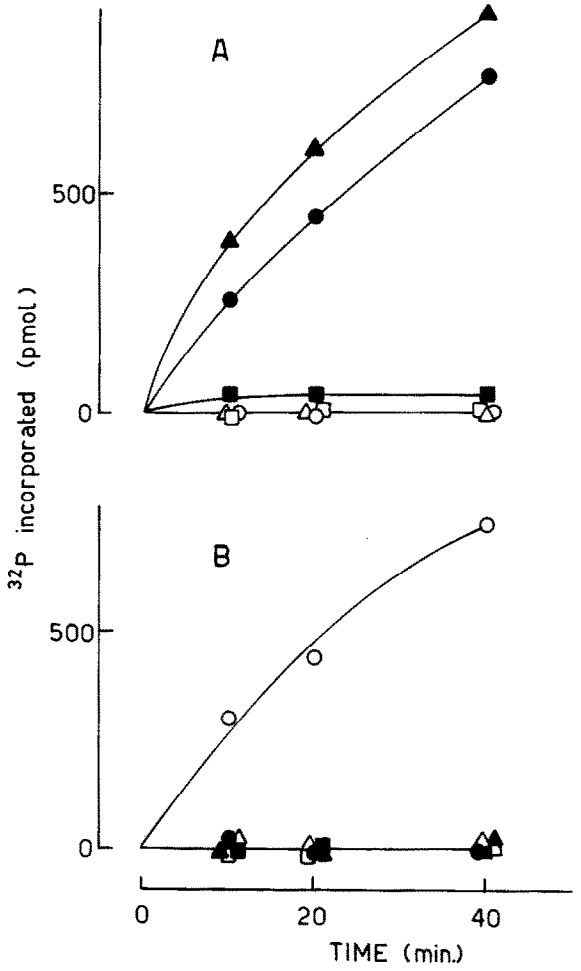

Fig.1. Time courses of phosphorylation of different synthetic peptides by either Ck-TS (A) or A-kinase (B). All experiments were performed with 50 μM [γ-^{32}P]ATP and 2 mM peptide substrates. Under these conditions ^{32}P incorporation into peptide Arg-Arg-Ser-Thr-Val-Ala by A-kinase occurred almost exclusively at its Ser_3 residue. Peptides were: Ser-Glu-Glu-Glu-Val-Glu (▲); AcSer-Glu-Glu-Glu-Val-Glu (●); Arg-Ser-Glu-Glu-Glu-Val-Glu (■); Arg-Arg-Ser-Thr-Val-Ala (○); Arg-Arg-Ser-Thr-Glu-Ala (△); Ser-Thr-Glu-Ala (□).

prolonged incubations with Ck-TS (fig.1A) and in a wide range of concentrations (fig.2). The kinetics in fig.2 also show that the K_m values for the peptide substrates are between 1 and 2 orders of magnitude greater than that for troponin-T [11]. The V_{max} values however are of the same order (unpublished). Fig.1A also shows that the basic peptides Arg-Arg-Ser-Thr-Val-Ala and Arg-Arg-Ser-Thr-Glu-Ala and the tetrapeptide Ser-Thr-Glu-Ala, lacking the extended acidic clusters C-terminal to the residues phosphorylated by type-2 casein kinase [3,11–13], are completely unaffected by Ck-TS.

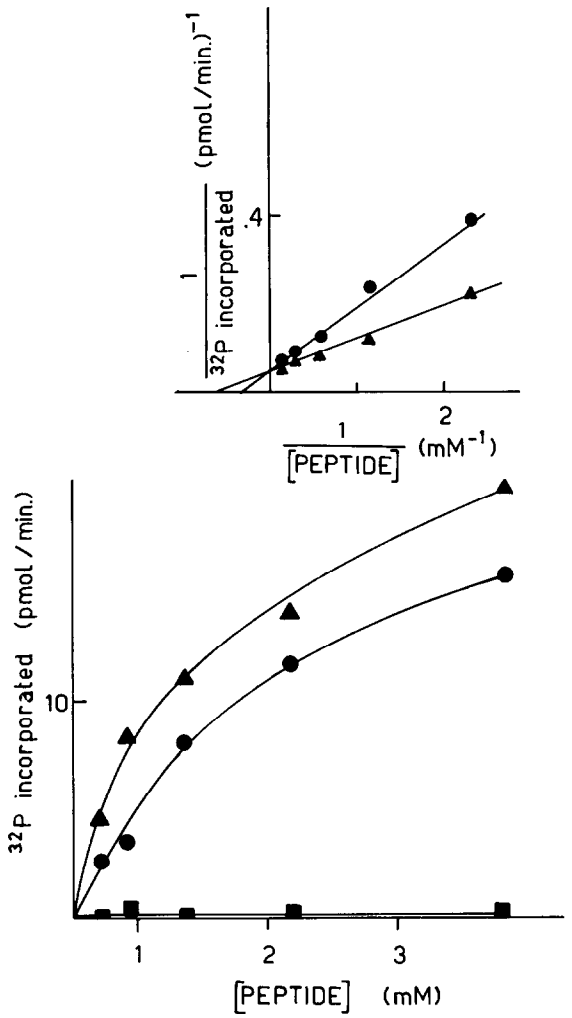

Fig.2. Kinetics of phosphorylation of peptides Ser-Glu-Glu-Glu-Val-Glu (▲), its *N*-acetylated derivative (●), and Arg-Ser-Glu-Glu-Glu-Val-Glu (■) by Ck-TS. The experimental conditions are either described or quoted in section 2 and in the legend to fig.1. Incubation time was 5 min. Double-reciprocal plots are shown in the upper panel. The resulting K_m values were 1.6 and 3.6 mM for Ser-Glu-Glu-Glu-Val-Glu and its *N*-acetylated derivative, respectively.

Collectively, the above results support the conclusion that an acidic cluster C-terminal to the target residue actually represents a necessary condition for phosphorylation by Ck-TS, yet it is not always a sufficient condition, as a basic residue on the opposite side of the phosphorylatable serine can suppress its favorable effect.

As expected from its known structural requirements, the behavior of A-kinase, toward the same set of peptides, is opposite to that of Ck-TS. As shown in fig.1B, in fact, A-kinase is inactive toward all the acidic peptides and readily phosphorylates only the basic peptide Arg-Arg-Ser-Thr-Val-Ala mainly at its Ser_3 residue. Interestingly, however, such phosphorylation is fully prevented if a glutamic acid is substituted for Val_5.

In conclusion, the opposite effects of local structural features on the phosphorylation efficiencies by Ck-TS and A-kinase (schematically summarized in table 1) would indicate that the requirements of the former prevent phosphorylation by the latter and vice versa. Considering that several proteins, including glycogen synthase and R_{II}, are substrates of both A-kinase and Ck-TS, the strictly distinct site specificities of these two enzymes may represent a device for preventing mutual interference.

Table 1

Positive and negative primary structure determinants for phosphorylation by Ck-TS and A-kinase

Peptide substrate	Phosphorylation by	
	Ck-TS	A-Kinase
Ser-Glu-Glu-Glu-Val-Glu	100	<1
[Arg]-Ser-Glu-Glu-Glu-Val-Glu	5	<1
Arg-Arg-Ser-Thr-Val-Ala	<1	100
Arg-Arg-Ser-Thr-[Glu]-Ala	<1	<1

The residues supposed to act as positive determinants are underlined, the ones hindering phosphorylation are shown in a box. Phosphorylation is expressed as a % of the best substrate (100%)

This would also suggest that A-kinase and casein kinase-2 play different, or maybe opposite, roles, in contrast with the well documented additive effects mediated by other protein kinases exhibiting partially overlapping site specificities (e.g., [25]).

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