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The consensus sequences for cdc2 kinase and for casein kinase-2 are mutually incompatible

A study with peptides derived from the β-subunit of casein kinase-2

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Two series of synthetic peptides that reproduce the amino- and carboxyl-terminal segments of the β-subunit of casein kinase-2, including the sites phosphorylated by CK2 and cdc2 kinase, respectively, have been used as model substrates for these enzymes. The N-terminal peptide β(1–9), MSSSEEVSW, is readily phosphorylated by CK2 but not at all by cdc2. The opposite is true of the C-terminal peptide β(206–215), NFKSPVKTIR, whose Ser-4 is a good target for cdc2 while being unaffected by CK2. The individual substitutions of Pro-5 and Lys-7 in the latter peptide with Gly and Ala (or Glu), respectively, prevent its phosphorylation by cdc2, whereas the substitution of Lys-3 with Ala is well tolerated and the substitution of the target Ser with Thr actually improves phosphorylation. Thus the consensus sequence for cdc2 is shown to be X-S-P-X-K. Such a requirement for a basic residue at position +3 is opposite to that of CK2 whose consensus sequence (S-X-X-E/D/Yp/Sp) includes an acidic residue at the same position. Moreover the motif Ser-Pro is detrimental for CK2, preventing the phosphorylation of otherwise suitable peptides. These observations would rule out the possibility that the site specificity of CK2 might overlap with that of cdc2 and possibly of other Pro-directed protein kinases.

Casein kinase-2; cdc2; Protein phosphorylation; Site specificity; Consensus sequence

1. INTRODUCTION

Casein kinase-2 (CK2) is a ubiquitous and pleiotropic protein kinase characterized by its heterotetrameric structure, independency of second messengers, predilection for acidic sites, and use of GTP besides ATP as phosphate donor (reviewed in [1,2]). Its catalytic domain belongs to the same branch of the protein kinase phylogenetic tree that also includes $p34^{cdc2}$ [3], i.e. the catalytic subunit of the cell division cycle protein kinase triggering the G2 to M phase transition [4,5]. Despite their relatively high sequence homology CK2 and cdc2 display different site specificities. CK2 is an acidophilic protein kinase whose consensus sequence is S-X-X-E/D/Sp/Yp [6–9]; its phosphorylation efficiency, moreover, is enhanced by multiple acidic residues, in addition to the crucial one at position +3, and can be altered by a number of other features [6,8,10]. No comparable systematic studies have been done on the specificity determinants of cdc2: it is firmly believed, however, that cdc2 is a 'proline-directed' protein kinase since the motif S/T-P is invariably found in all the sites affected by this enzyme [5,11]. A priori such structural requirements of CK2 and cdc2 are mutually compatible as they might co-exist within hypothetical sites suitable for both kinases. Here we show, however, that this is not the case since, on the one hand, the motif Ser-Pro is a strong negative determinant for CK2, preventing the phosphorylation of otherwise suitable peptide substrates; on the other the consensus sequence for cdc2 is shown to include, besides the prolyl residue, also a basic residue at position +3, where conversely CK2 requires an acidic residue.

Abbreviations: CK2, casein kinase-2; cdc2, cell division cycle protein kinase; Sp, phosphoserine; Tp, phosphothreonine; Yp, phosphotyrosine.

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2. MATERIALS AND METHODS

The cdc2 kinase, a complex by cyclin B and cdc2, from human HeLa cells arrested in mitosis with nocodazole, was purified according to [12]. Casein kinase-2 was purified to near homogeneity from rat liver cytosol as previously described [13] with a subsequent FPLC Mono Q chromatography.

The peptides were synthesized either by a manual synthesizer (Biolynx 4175-LKB) using the continous flow variant of the 'Fmoc-polyamide' method [14] on the Kieselguhr-supported polydimethylacrylamide resin functionalized with 4-hydroxy-methylphenoxy-acetic acid, or by an automated synthesizer from Applied Biosystems (Model 431-A), using FastMoc chemistry on hydroxymethylphenoxymethyl resin, according to the manufacturer's instructions [15]. The purity (95% or more) of the peptides, purified by ion-exchange chromatography, was checked by amino acid analyses and analytical HPLC.

CK2 and cdc2 activities were assayed as in [16] and [12], respectively, by a 5 min incubation using 1 mM peptide as phosphoacceptor substrate, unless differently indicated. The specific activity of [^{32}P]ATP was 900 cpm/pmol and its concentration was 25 μM and 100 μM with CK2 and cdc2, respectively. ^{32}P incorporated into peptides with 2 or more basic residues (peptide β(206–215) and its derivatives) and histone H1 was evaluated by the phosphocellulose paper procedure [17]. The procedure consisting of phosphoamino acid isolation and quantitation after partial acid hydrolysis [18] was applied to the other peptides. Kinetic constants of peptide substrates were determined by regression analysis of double-reciprocal plots constructed from initial velocity data relative to 5 substrate concentrations in the 10–250 μM range (in triplicate) fitted to the Michaelis–Menten equation.

3. RESULTS

The non-catalytic β-subunit of CK2 can be phosphorylated either by CK2, through an autophosphorylation mechanism [1,2], or by cdc2 kinase [19,20], at distinct sites located in the amino- and carboxyl-terminal segments, respectively. Ser-2, included into the sequence MSSSEE, and thus fulfilling the consensus sequence for CK2, is the main autophosphorylation site [1]; the target for cdc2 in the β-subunit of A431 cells CK2 is Ser-209, displaying the typical Ser-Pro motif [20]. The same residue is also phosphorylated by cdc2 in the recombinant human β-subunit incubated with cdc2 (unpublished data in collaboration with B. Boldyreff and O.-G. Issinger).

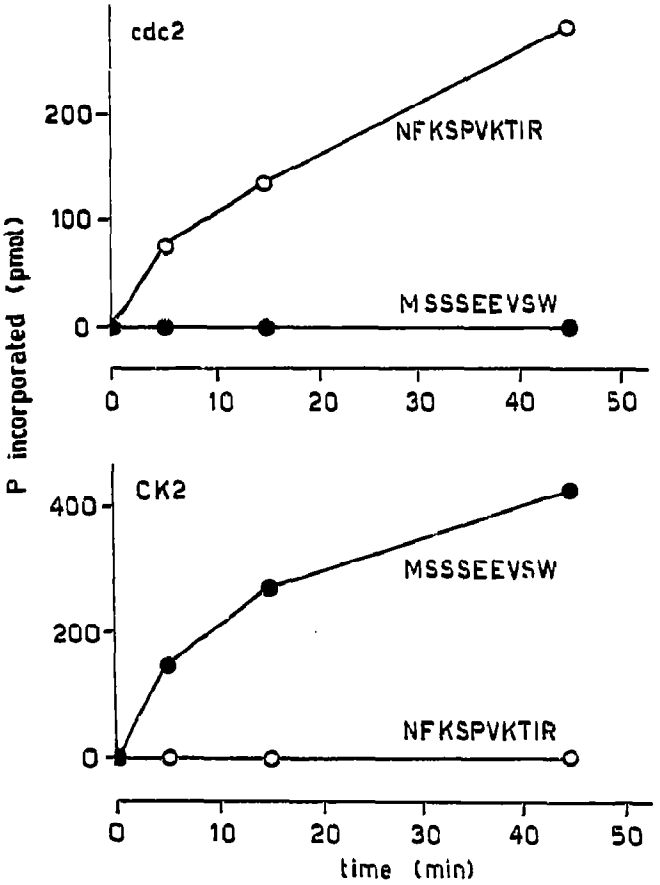

Fig. 1. Time-courses of phosphorylation of synthetic peptides derived from the β-subunit of CK2 by CK2 and cdc2. Phosphorylation by cdc2 (upper figure) and by CK2 (lower figure) of synthetic peptides NFKSPVKTIR (○) and MSSSEEVSW (●), corresponding to the carboxy- and amino-terminal ends of CK2 β-subunits, was performed and evaluated as described in Materials and Methods.

Two peptides reproducing such sites have been synthesized and assayed for their capability to undergo phosphorylation by either CK2 or cdc2. As shown in Fig. 1 the N-terminal β(1–9) peptide was readily phosphorylated by CK2 byt not at all by cdc2; the C-terminal β(206–215) peptide was conversely a good substrate for cdc2 alone, while it was not affected by CK2.

The effect of substituting individual residues of the β(206–215) peptide on its susceptibility to phosphorylation by cdc2 is shown in Table I. It can be seen that threonine was phosphorylated more efficiently than serine, while tyrosine was not affected to any detectable extent. The replacement of Lys-3 with Ala was quite well tolerated, both in terms of K_m and V_{max} (see Table II). In contrast the replacements of either Pro-5 (with Gly) or Lys-7 (with either Ala or Glu) were deleterious. In particular the derivative lacking the prolyl residue was not a substrate, even if tested at high concentration (up to 2 mM). The Lys-7 → Ala (Glu) derivatives still

Table I

Effect of structural modifications on the phosphorylation of peptide substrates by either cdc2 or CK2

Peptides (1 mM)	Phosphorylation rate by:	
	cdc2	CK2
NFKSPVKTIR [β(206–215)]	100	<1
NFASPVKTIR	92	<1
NFKSGVKTIR	<1	<1
NFKSPVATIR	10	<1
NFKSPVETIR	12	<1
NFKTPVKTIR	151	<1
NFKYPVKTIR	<1	<1
MSSSEEVSW [β(1–9)]	<1	100
SSSEE	<1	13
SGGEE	<1	17
SPGEE	<1	<1
SGPEE	<1	10
GSSSEE	<1	36
PSSSEE	<1	40
SAEEEE	<1	104
SPEEEE	<1	<1

The phosphorylation rates are expressed relative to those of the parent peptides, β(206–215) and β(1–9), reproducing the carboxy- and amino-terminal ends of CK2 β-subunit, for cdc2 and CK2, respectively. The specific activity of cdc2 and CK2 determined with peptide substrates β(206–215) and β(1–9) was 200 and 121 nmol P · min^{-1} · mg^{-1}, respectively. Substitutions relative to the parent peptides are underlined. Prolyl residues are bold typed. The lack of any detectable phosphorylation of tyrosine in the peptide NFKYPVKTIR has been assessed also by phosphoamino acid analysis after 2 h hydrolysis with 6 N HCl.

underwent detectable phosphorylation; they were, however, substrates too poor for performing accurate determinations of their kinetic constants.

The above results clearly indicate that the consensus sequence for peptide phosphorylation by cdc2 must be X-S-P-X-K. The requirement for a basic residue at position +3 relative to the target residue is opposite to that of CK2 which needs an acidic residue at the same position [6]. Basic residues in the vicinity of serine are invariably negative determinants for CK2 [8].

The irreconciliability between the specificity determinants of CK2 and cdc2 was reinforced by the additional finding that the motif Ser-Pro, which is a conditio sine qua non for phosphorylation by cdc2, actually prevented the phosphorylation of otherwise suitable peptide substrates by CK2. This can be seen in Table I where the phosphorylation rate of the pentapeptide SSSEE, reproducing the phosphorylated motif of the β(1–9) peptide, is compared with those of a number of derivatives. While SGGEE proved a substrate as good as the parent pentapeptide, its prolyl derivative SPGEE was not phosphorylated to any detectable extent, although it still fulfilled the consensus sequence of CK2. Likewise the replacement of Pro for Ala in the hexapeptide substrate SAEEEE fully compromised its phosphoacceptor activity. The motif Ser-Pro seemingly accounts also for the failure of CK2 to phosphorylate the β(206–215) derivative NFKSPVETIR despite the presence in it of the Ser-X-X-Glu consensus sequence. The crucial position where proline acts as a negative determinant is the one adjacent to the C-terminal side of serine, since peptides bearing prolyl residues at different positions were fairly good substrates (compare SGPEE and PSSSEE with SGGEE and GSSSEE, respectively).

4. DISCUSSION

Althought it is commonly held that the motif Ser/Thr-Pro represents an absolute requisite for phosphorylation by cdc2, such a concept is drawn from the observation that all the sites affected by this enzyme display this feature, rather than from a systematic analysis with substituted substrates, which is still needed. The data presented here partially obviate this gap by providing the unambiguous evidence that a prolyl residue at position +1 and a basic residue at position +3 are indeed both stringently required for attaining appreciable phosphorylation of peptide substrates by cdc2. Basic residues on the C-terminal side appear, conversely, not to be stringently required for substrate recognition. It is hard to reconcile the crucial relevance of the basic residue at position +3 with the observation that several sites reported to be affected by cdc2 actually lack this feature [5,21]. Further work is needed in order to assess whether such a discrepancy is accounted for by different species of cdc2, or cdc2-related kinases, with variable specificities, or whether it might reflect the capability of the tertiary structure of the protein substrate to overcome the primary structure requirements disclosed with short peptide substrates. It should be noted anyway that short synthetic peptides reproducing phosphoacceptor sites for cdc2 can behave as substrates as good as intact protein targets since the decapeptide β(206–215) and its threonyl derivative display kinetic constants quite comparable to those of histone H1.

Table II

Kinetic constants of CK2 β-subunit derived peptides for cdc2

Peptides	K_m (μM)	V_{max} (nmol·min^{-1}·mg^{-1})
NFKSPVKTIR	43	209
NFASPVKTIR	58	134
NFKSPVATIR	n.d.	≈27[a]
NFKSGVATIR	n.d.	<2
NFKTPVKTIR	40	313
Histone H1	35	203

Substitutions relative to the parent peptide are underlined. n.d., not determined due to too low a phosphorylation rate.
[a] This value is not a real V_{max} but the phosphorylation rate measured with 1,500 μM peptide.

An intriguing outcome of our study is that the specificity determinants of cdc2 act as powerful negative determinants for CK2 and vice versa. Firstly, in fact, the latter enzyme crucially needs an acidic residue at position +3 where a basic residue is conversely required for cdc2 targeting; secondly, the prolyl residue at position +1, which is stringently required by cdc2, completely prevents the phosphorylation of otherwise suitable peptide substrates by CK2. This latter finding accounts for the observation that Ser-46 of DARPP inhibitor, displaying the Ser-Pro motif within the sequence SSPEEE is not affected by CK2 despite the fact that it fulfills the consensus sequence for CK2 phosphorylation, while the adjacent residue Ser-45 is extensively phosphorylated [22]. Likewise CK2 phosphorylates Ser-120 and Ser-121 of protein phosphatase inhibitor-2, but not Ser-129 [23], which also fulfills the consensus sequence for CK2, being adjacent, however, to the N-terminal side of a prolyl residue. These findings support the view that the Ser-Pro motif exerts its negative effect also on the phosphorylation of intact protein substrates by CK2. Another remarkable difference between CK2 and cdc2 is that while the former by far prefers seryl over threonyl residues [6] the latter actually phosphorylates threonyl peptides more readily than the seryl counterparts.

It can be concluded therefore that the site specificities of CK2 and cdc2 are mutually incompatible and it seems very unlikely that these enzymes might share any common phosphorylation sites, as it conversely happens with several other protein kinases. It should be recalled in this connection that the number of 'Pro-directed' protein kinases [24], sharing with cdc2 the ca-

pability to recognize the Ser-Pro doublet, is continuously growing, including among others, MAP2 and ERT kinases [25,26]. Such a motif, therefore, may represent a primary consensus recognized by a variety of growth-related protein kinases. The capability of this motif to adversely effect the phosphorylation of the same sites by CK2 (which also can be considered a growth-related kinase) may reflect different, if not opposite, physiological commitments of CK2 and Pro-directed protein kinases.

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