Generation of protein kinase Ck1 α mutants which discriminate between canonical and non-canonical substrates

Victor H. BUSTOS*, Oriano MARIN†‡, Flavio MEGGIO†‡, Luca CESARO†‡, Catherine C. ALLENDE*, Jorge E. ALLENDE* and Lorenzo A. PINNA†‡¹

*Programa de Biología Celular y Molecular, Instituto de Ciencias Biomédicas, Facultad de Medicina, Universidad de Chile, Casilla 70086, Santiago 7, Chile, †Dipartimento di Chimica Biologica, Università di Padova, viale G. Colombo 3, 35121 Padova, Italy, and ‡Istituto Veneto di Medicina Molecolare, via Orus 2, 35129 Padova, Italy

Protein kinase CK1 denotes a family of pleiotropic serine/threonine protein kinases implicated in a variety of cellular functions. Typically, CK1 acts as a ‘phosphate-directed’ kinase whose targeting is primed by a single phosphorylated side chain at position $n - 3$ or $n - 4$ relative to serine/threonine, but increasing evidence is accumulating that CK1 can also engage some of its substrates at sites that do not conform to this canonical consensus. In the present paper, we show that CK1 α phosphorylates with the same efficiency phosphopeptides primed by a phosphoserine residue at either $n - 3$ [pS(–3)] or $n - 4$ [pS(–4)] positions. The phosphorylation efficiency of the pS(–4) peptide, and to a lesser extent that of the pS(–3) peptide, is impaired by the triple mutation of the lysine residues in the K²²⁹KQK²³² stretch to alanine residues, promoting 40-fold and 6-fold increases of K_m respectively. In both cases, the individual mutation of Lys²³² is as detrimental as the triple mutation. A kinetic alanine-scan

analysis with a series of substituted peptide substrates in which the priming phosphoserine residue was effectively replaced by a cluster of four aspartate residues was also consistent with a crucial role of Lys²³² in the recognition of the acidic determinant at position $n - 4$. In sharp contrast, the phosphorylation of β -catenin and of a peptide including the non-canonical β -catenin site (Ser⁴⁵) lacking acidic/phosphorylated determinants upstream is not significantly affected by mutations in the KKQK stretch. These data provide a molecular insight into the structural features that underlie the site specificity of CK1 α and disclose the possibility of developing strategies for the preferential targeting of subsets of CK1 substrates.

Key words: casein kinase, consensus sequence, hierarchical phosphorylation, mutational analysis, phosphoacceptor site recognition, protein kinase CK1.

INTRODUCTION

The acronym CK1 denotes a family of ubiquitous pleiotropic serine/threonine protein kinases whose members, encoded by seven genes in the human genome (α , β , γ 1, γ 2, γ 3, δ and ϵ) [1] are variously implicated in a number of cellular functions [2–4], notably DNA repair [5–7], Wnt signalling [8,9], regulation of circadian rhythms [10–12], phosphorylation of G-protein-regulated receptors [13] and of NFAT (nuclear factor for activated T-cells) transcription factors [14,15]. Despite a growing list of proteins described as substrates of the different isoforms of CK1 and the general notion that CK1 shares with a tiny minority of serine/threonine protein kinases an ‘acidophilic’ character [16], accounting for its ability to phosphorylate casein and phosvitin *in vitro*, the structural features underlying the substrate specificity of CK1 isoforms are not fully understood and are, in some respects, controversial.

Early studies with artificial protein substrates [17,18] and synthetic peptides [19–21] disclosed a consensus sequence critically determined by a phosphorylated residue (either phosphoserine or phosphothreonine) at position $n - 3$ or, less efficiently, $n - 4$, relative to the target amino acid. This assigned CK1 to a small group of ‘primed’ or ‘phosphate-directed’ serine/threonine protein kinases, for which the term ‘hierarchical phosphorylation’ was coined [22] to indicate the fact that a primary phosphorylation event is a prerequisite for the generation of a consensus sequence leading to the subsequent phosphorylation of additional residue(s). CK1 shares with a few other kinases, notably CK2, G-

CK (Golgi casein kinase) and GSK3 (glycogen synthase kinase 3), the ability to propagate phosphorylation by such a hierarchical mechanism along a sequence of properly spaced serine/threonine residues.

It soon became clear, however, that phosphate-directed ‘hierarchical’ phosphorylation could not account for the targeting of certain proteins obviously devoid of phosphate(s) that were nevertheless affected by CK1. In particular, the phosphorylation of DARPP32 (dopamine- and cAMP-regulated phosphoprotein of 32 kDa) [23] and of protein phosphatase-1 inhibitor 2 [24] by CK1 disclosed situations where N-terminal clusters of numerous aspartate and/or glutamate residues could act as specificity determinants almost as efficiently as an individual phosphorylated residue at position $n - 3$ or $n - 4$ [25–27]. Advantage was also taken of this behaviour for the development of non-phosphorylated peptide substrates which are routinely used for the specific monitoring of CK1 activity [25,28].

In addition, evidence has accumulated that CK1 is implicated in the multiphosphorylation of proteins, notably NFAT4 [14], NFAT1 [15], β -catenin [8,9] and APC (adenomatous polyposis coli) [29], where the hierarchical propagation of phosphorylation is seemingly initiated by CK1 itself by phosphorylating residues which, however, do not display the canonical consensus for this kinase, either dictated by upstream phosphate or by acidic clusters. Based on the information obtained with a series of peptides with permutations of sequences surrounding the target serine residues phosphorylated by CK1 in these proteins, it has been proposed that there are common features recognized in these non-canonical

Abbreviations used: CK1, protein kinase CK1 (also known as casein kinase 1 or I); CK2, protein kinase CK2; Fmoc, fluorenylmethoxycarbonyl; GSK3, glycogen synthase kinase-3; NFAT, nuclear factor for activated T-cells; Ni-NTA, Ni²⁺-nitrilotriacetate; polyE,Y(4:1), poly(glutamate/tyrosine) (4:1); TFA, trifluoroacetic acid.

¹ To whom correspondence should be addressed (email lorenzo.pinna@unipd.it).

substrates. These common features include an S*LS (Ser-Leu-Ser) motif which involves the target serine (S*) and a cluster of acidic amino acids positioned several residues downstream [29–31]. In addition, it has also been postulated that some of these proteins, NFAT and Per, possess a motif FXXXX (Phe-Xaa-Xaa-Xaa-Phe) which signals their tight binding of CK1, presumably favouring their phosphorylation [15].

In order to shed light on the structural features of CK1 that are responsible for substrate recognition, we have started a mutational analysis of zebrafish (*Danio rerio*) CK1 α (almost identical, except for one amino acid, with its human homologue) aimed at identifying the elements responsible for canonical and non-canonical site recognition. A valuable clue to start with was provided by the three-dimensional structures of two CK1 isoforms, rat CK1 δ and *Schizosaccharomyces pombe* CK1 (related to the mammalian δ form) where two doubly charged anions, tungstate and sulphate respectively, were found co-crystallized [32,33]. These occupy an equivalent position in the two structures and are in close proximity to three residues, Arg¹⁷⁸, Gly²¹⁵ and Lys²²⁴ (numbering of human CK1 δ), suggesting that these anions might mimic the phosphate that acts as a specificity determinant in the canonical sequences recognized by CK1. These residues are also conserved in CK1 α isoforms (Arg¹⁸⁶, Gly²²³ and Lys²³² in the zebrafish CK1 α protein). In particular Lys²³² belongs to a basic cluster at the end of an exposed loop which has been shown to interact with several protein partners of CK1, either phosphorylatable or not, namely α -centaurin [34], axin [35], HMG1 (high-mobility group 1), importin, PP2Ac (protein phosphatase 2A catalytic domain), tubulin and actin [36]. In order to assess whether the basic residues in the conserved KKQK²³² stretch are implicated in substrate recognition, these were collectively or individually mutated to alanine residues. The mutants were then compared with wild-type CK1 α for their ability to recognize peptide substrates whose phosphorylation by CK1 is specified either by the non-canonical motif found in β -catenin or by the canonical consensus dictated by individual phosphoserine residues at position $n-3$ or $n-4$ or by a cluster of four aspartic acids spanning positions between $n-3$ and $n-6$. The present paper describes these results.

EXPERIMENTAL

Materials

Solvents, resin and coupling reagents for peptide synthesis were from Applied Biosystems (Foster City, CA, U.S.A.). All protected amino acids were purchased from Novabiochem (Laufelfingen, Switzerland). HPLC grade solvents were obtained from Merck (Darmstadt, Germany).

Expression and purification of proteins

The cDNA clone for the β -catenin of zebrafish was prepared as described previously [31]. The cDNA clone of CK1 α from zebrafish, with an N-terminal His₆ tag in a pT7-7 vector, was obtained as described previously [37]. The construction and purification of mutants CK1 α (K229A, K230A and K232A) was performed as described previously [35]. Mutants R186A and R186C were prepared using the overlapping PCR method [38]. For R186A, the forward and reverse primer oligonucleotides were 5'-CACAGGCACAGCTGCCTATGCCAGC-3' and 5'-GCTGGCATAGGCAGCTGTGCCTGTG-3' respectively. For R186C, the forward and reverse primer oligonucleotides were 5'-CACAGGCACAGCTTGCTATGCCAGC-3' and 5'-GCTGGCATAGGCAGCTGTGCCTGTG-3' respectively. Recombinant

Table 1 Structures and nomenclature of the peptide substrates used in the present study

Peptides 1–7 are derived, with substantial modifications, from inhibitor-2 of protein phosphatase-1 [26]. Peptide 8 reproduces the 38–64 sequence of β -catenin (β -cat) [31]. The phosphoacceptor residues are in bold and underlined. Substitutions relative to the D₄ peptide are in bold. **pS** denotes phosphoserine.

Peptide	Conventional name	Amino acid sequence
1	D ₄	RRKHAAIGDDDDAYSITA
2	ADDD	RRKHAAIGADDDAYSITA
3	DADD	RRKHAAIGDADDDAYSITA
4	DDAD	RRKHAAIGDDADDDAYSITA
5	DDDA	RRKHAAIGDDDDAAYSITA
6	pS(–3)	RRKHAAIG pS AYSITA
7	pS(–4)	RRKHAAI pS AAYSITA
8	β -cat(38–64)	RRRGATTAP SL SGKGNPEDEDVDTNQVLYE

wild-type and mutant CK1 α from zebrafish with N-terminal His₆ tags were expressed in *Escherichia coli* BL21(DE3) cells and purified through Ni-NTA (Ni²⁺-nitrilotriacetate)–agarose columns. The various plasmids were used to transform *E. coli* BL21(DE3) cells. Cells were grown at 37°C to a D₆₀₀ of 0.4–0.6. At this point, protein expression was induced by adding IPTG (isopropyl β -D-thiogalactoside) to a final concentration of 100 μ M. In the case of CK1 α , induction was carried out overnight at 22°C, while, for β -catenin, the temperature was maintained at 37°C for 3 h. Afterwards the cells were pelleted at 3000 *g* for 20 min at 4°C, and cell pellets were resuspended in a buffer containing 50 mM Tris/HCl, pH 8.0, 500 mM NaCl, 1% Triton X-100, 1 mM PMSF and 2 μ g/ml each of leupeptin, aprotinin, pepstatin A and antipain. Lysozyme (1 mg/ml) was added, and cells were lysed for 30 min at room temperature (25°C). Lysates were sonicated and centrifuged at 10000 *g* for 30 min. In the case of His₆-tagged proteins, the recombinant proteins were purified using Ni-NTA–agarose columns which were washed and subsequently eluted with a buffer containing 50 mM Tris/HCl, pH 7.5, 0.2 M NaCl and 1% Triton X-100 with 250 mM imidazole. The protein was quantified by Western blotting, using an anti-His antibody and TATA-binding protein as standard. The specific activities of wild-type CK1 α and of the triple and individual mutants K229A, K230A and K232A were 132, 21, 42, 17 and 6 pmol of phosphate transferred per min respectively, using β -casein as substrate.

Peptides

The peptides used in the present study are listed in Table 1 together with their conventional names used in the text. The inhibitor-2 of protein phosphatase-1 derived peptides (peptides 1–5) were synthesized as described previously [39]. The β -catenin peptide (peptide 8 in Table 1) was synthesized as described by Marin et al. [31]. The phosphopeptides (peptides 6 and 7 in Table 1) were synthesized by the solid-phase peptide synthesis method using an automated peptide synthesizer (model 431-A; Applied Biosystems). The Fmoc (fluoren-9-ylmethoxycarbonyl) strategy was used throughout the peptide chain assembly [40]. Wang resin (loading of 0.96 mmol/g) was used as solid support to obtain 0.1 mmol of each peptide. The protected amino acids used were Fmoc-Ala, Fmoc-Thr(tert-butyl), Fmoc-Ile, Fmoc-Ser(tert-butyl), Fmoc-Tyr(tert-butyl), Fmoc-Ser[PO-(O-benzyl)OH], Fmoc-Ile, Fmoc-His(trityl), Fmoc-Lys(tert-butyloxycarbonyl) and Fmoc-Arg(2,2,4,6,7-pentamethylidihydrobenzofuran-5-sulphonyl). Coupling was performed on a Peptide Synthesizer Model 431A (Applied Biosystems) with

a single reaction for 45 min by a 0.45 M solution in DMF (*N,N*-dimethylformamide) of HBTU [2-(1*H*-benzotriazole-1-yl)-1,3,3,3-tetramethyluronium hexafluorophosphate] and HOBT (*N*-hydroxybenzotriazole) in the presence of DIEA (*N*-diisopropylethylamine) according to the manufacturer's protocol. Cleavage of the peptides was performed by reacting the peptidyl resins with TFA (trifluoroacetic acid)/water/thioanisole/ethane dithiol/phenol (10 ml/0.5 ml/0.5 ml/0.25 ml/750 mg) for 2.5 h. The peptides were precipitated with ice-cold diethyl ether and were isolated by centrifugation at 1500 *g* for 10 min. The pellets were washed several times with diethyl ether, dissolved in abundant water and freeze-dried. Crude peptides were purified by a preparative reverse-phase PrepNova-Pak HR C₁₈ HPLC column, 250 mm × 10 cm, 6 µm bead size (Waters–Micromass, Milford, MA, U.S.A.) at 12 ml/min using a linear gradient as described below. The molecular masses of the peptides were confirmed by MS with direct infusion on a Micromass ZMD-4000 mass spectrometer (Waters–Micromass). The purities of the peptides were approx. 95% as evaluated by analytical reverse-phase HPLC using a 5 µm, C₁₈ Symmetry300 column, 4.6 mm × 250 mm (Waters–Micromass) eluted with a linear gradient of 5–40% acetonitrile in 0.1% TFA at 1 ml/min.

Kinase assay

The assay has been described in detail previously [37]. Briefly, peptide substrates (0.2 mM unless otherwise indicated) were phosphorylated by incubation in a 30 µl volume containing 50 mM Tris/HCl buffer (pH 7.5), 10 mM MgCl₂, 100 mM NaCl and 100 µM [γ -³²P]ATP (specific radioactivity 500–1000 c.p.m./pmol). The reactions were incubated for 10 min at 37°C and stopped by cooling in ice and absorption on phosphocellulose paper. Papers were washed three times with 75 mM phosphoric acid, dried and counted in a scintillation counter. Initial rate data were fitted to the Michaelis–Menten equation to obtain *V*_{max} and *K*_m values. β -Casein (5 mg/ml) was phosphorylated under similar conditions, and the phosphate incorporated was quantified both by the phosphocellulose filter procedure and by SDS/PAGE and autoradiography. For β -catenin phosphorylation, 0.1 µM recombinant full-length β -catenin from zebrafish [31] was phosphorylated for 15 min at 37°C using the above standard conditions, except for the specific radioactivity of [γ -³²P]ATP which was 8000–10 000 c.p.m./pmol. The reaction was stopped by the addition of 5× concentrated Laemmli buffer and submitted to SDS/PAGE and autoradiography. PolyE,Y(4:1) [poly-(glutamate/tyrosine) (4:1)] (100 µM) was phosphorylated for 10 min at 37°C using the above standard conditions, except the specific radioactivity of [γ -³²P]ATP which was 50 000 c.p.m./pmol. The reaction was stopped by the addition of 5× concentrated Laemmli buffer and submitted to SDS/PAGE and autoradiography.

RESULTS

Generation of CK1 α mutants expected to be defective in substrate recognition

Based on the two crystal structures available [32,33], two basic residues were first-choice candidates for making positive interactions with the priming phosphate present in many canonical CK1 phosphoacceptor sites: an arginine residue located at the end of the activation plus p + 1 loop (whose homologue in CK1 α is Arg¹⁸⁶) and a lysine residue (Lys²³² in zebrafish CK1 α) just downstream from a basic element (the E-F loop) which has been shown to be implicated in the interaction with α -centaurin and

Figure 1 The triple mutant K229A,K230A,K232A is variably defective in substrate phosphorylation

Phosphorylation of various peptide and protein substrates by CK1 α , either wild-type (open bars) or triply mutated in the K²²⁹QKQ²³² cluster (closed bars), was performed and evaluated as detailed in the Experimental section. The activity of the mutant is normalized to that of wild-type (= 100%). Specific activities of wild-type CK1 α with the different substrates were 132, 280, 3, 14 and 45 pmol of phosphate transferred per min to β -casein, peptide D₄, polyE,Y(4:1), β -catenin and peptide β -cat(38–64) respectively. Similar results were obtained using the single mutant K232A.

other proteins [34,36]. These two basic residues embrace a doubly charged anion found in both structures (a sulphate in *S. pombe* CK1 [33], a tungstate in human CK1 δ [32]) suspected to mimic a phosphate. This prompted us to mutate Arg¹⁸⁶ and Lys²³², as well as two other lysine residues just preceding Lys²³² (Lys²²⁹ and Lys²³⁰).

The Arg¹⁸⁶ mutants are unstable and/or inactive

The two Arg¹⁸⁶ mutants generated (R186A and R186C) underwent rapid degradation even in the presence of protease inhibitors and they could not be purified. An assay of the R186A mutant in crude extract revealed no detectable casein kinase activity, suggesting that the mutant is inactive. However, the finding that some bacterial proteins were faintly phosphorylated in the crude extract where the CK1 α mutant was present (results not shown) would be also consistent with the concept that instead of being catalytically incompetent CK1 α R186A could be severely defective in casein recognition.

The triple CK1 α mutant K229A,K230A,K232A displays altered substrate specificity

Given the inactivity of the Arg¹⁸⁶ mutants, attention was focused on mutations that affected the E-F region and which gave rise to stable and catalytically active enzymes (see the Experimental section). To assess the possible implication of lysine residues within the basic motif K²²⁹QKQ²³² in the recognition of canonical sites, the triple mutant K229A,K230A,K232A was compared with wild-type for its ability to phosphorylate β -casein (whose phosphorylation by CK1 is typically primed by phosphoresidues at position *n* – 3/*n* – 4) and an inhibitor-2-derived peptide whose targeting is dictated by a DDDD cluster spanning positions *n* – 3 to *n* – 6 upstream from the serine residue (peptide D₄ in Table 1). As can be seen in Figure 1, the phosphorylation of both casein and of the inhibitor-2-derived peptide is severely impaired by the triple mutation. Likewise, the slow phosphorylation of tyrosine residues within the random polymer polyE,Y(4:1), symptomatic of the 'dual specificity' of CK1 [39,41], was also suppressed almost completely by this mutation. Conversely, β -catenin and a peptide substrate reproducing its non-canonical CK1 phosphoacceptor site (Ser⁴⁵), whose targeting is not specified by negatively charged side chains on the N-terminal side, are phosphorylated by wild-type CK1 α and by the triple mutant with comparable

Table 2 Alanine scan analysis of CK1 α mutants compared with substituted D₄ peptides

Kinetic constants were determined as described in the Experimental section. The values represent the means obtained from at least three independent experiments with S.E.M. never exceeding 15%. For peptide nomenclature see Table 1.

CK1 α	Kinetic value for peptide														
	D ₄			DDDA			DDAD			DADD			ADDD		
	<i>K_m</i> (μ M)	<i>V_{max}</i> (c.p.m.)	<i>V_{max}</i> / <i>K_m</i>	<i>K_m</i> (μ M)	<i>V_{max}</i> (c.p.m.)	<i>V_{max}</i> / <i>K_m</i>	<i>K_m</i> (μ M)	<i>V_{max}</i> (c.p.m.)	<i>V_{max}</i> / <i>K_m</i>	<i>K_m</i> (μ M)	<i>V_{max}</i> (c.p.m.)	<i>V_{max}</i> / <i>K_m</i>	<i>K_m</i> (μ M)	<i>V_{max}</i> (c.p.m.)	<i>V_{max}</i> / <i>K_m</i>
Wild-type	318	2250	4.62	330	465	1.41	146	565	3.87	190	1060	5.58	168	1199	7.14
K229A,K230A,K232A	2697	1070	0.39	753	50	0.066	281	1649	5.87	3782	1135	0.3	3033	1225	0.40
K229A	1127	1730	0.55	1245	78	0.063	386	1107	2.87	2307	547	0.24	1622	587	0.36
K230A	967	290	0.72	4240	208	0.049	169	651	3.85	3552	941	0.26	1382	677	0.49
K232A	4500	1280	0.33	3629	160	0.044	656	1797	2.74	5235	1199	0.23	3005	970	0.32

rates. These results are consistent with the view that defective phosphorylation of canonical substrates by the triple mutant is because of impaired substrate recognition rather than to a decrease in catalytic efficiency, which would affect all substrates equally. The concept that the triple mutation does not impinge on the catalytic mechanism is also supported by the finding that wild-type CK1 α and the triple mutant display identical *K_m* values for ATP (10 μ M; results not shown). In sharp contrast, the impaired phosphorylation of the D₄ peptide by the triple mutant is mainly accounted for by a large increase in the *K_m* value (see Table 2), consistent with the concept that the mutation is detrimental to substrate binding affinity.

Alanine scan analysis of point-mutated CK1 α compared with substituted DDDD peptides highlights the role of Lys²³² in recognizing the acidic determinant at position *n* – 4

A powerful tool for identifying interactions between individual specificity determinants and the residues committed to their recognition is provided by the so-called ‘charged residues to alanine scan approach’, by which the polar residues suspected to be implicated in kinase–substrate interactions are mutated individually to alanine both in the kinase and in the peptide substrate; each mutant is assayed kinetically with each substituted peptide, and their kinetic constants are compared with those calculated for the wild-type kinase–parent peptide reaction. Such an approach proved to be quite successful in a number of cases, e.g. to disclose the interactions of PKA (protein kinase A) [42] and CK2 [43] with their peptide substrates.

In order to apply the alanine scan approach to CK1, three new CK1 α mutants were generated in which Lys²²⁹, Lys²³⁰ or Lys²³² was replaced separately by alanine. On the other side of this matrix, derivatives of the parent D₄ peptide, each having one of the four aspartate residues replaced by alanine were synthesized (see Table 1 for peptide structures and nomenclature).

The kinetic constants of each mutant for each peptide substrate were calculated experimentally. They are shown in Table 2, where the data for the triple mutant K229A,K230A,K232A are also included.

Focusing first on wild-type CK1 α , it should be noted that, as pointed out previously [27], the replacement of individual acidic determinants in the peptide substrate does not promote any increase in *K_m* values, which rather tend to slightly decrease, but exerts its detrimental effect by causing a decrease in *V_{max}* which, in the case of the *n* – 3 and *n* – 4 substitutions, is dramatic. This can be explained by the ‘compensation’ theory [16], which implies that, whenever a specificity determinant is lacking at the

canonical position, but is present at adjacent positions, it tends nevertheless to bind to the anchoring residue(s), giving rise to a structural alteration which makes the peptide less prone to be readily phosphorylated. This may reflect in decreased *V_{max}* rather than in increased *K_m*, even though it was generated by altered binding. Based on these premises, the analysis of the kinetic data was conducted, taking into account alterations in both *K_m* and *V_{max}*, integrated into the ‘efficiency’ parameter, expressing the *V_{max}*/*K_m* ratio (also termed pseudo-first-order rate constant).

The rationale underlying the alanine scan approach is that a mutation in the kinase will be less detrimental if the kinase activity is measured with a peptide already lacking the determinant that would be recognized by the residue(s) mutated in the enzyme. The analysis of the efficiencies of substituted peptides (normalized to that of the parent peptide) will therefore bring to light the peptide(s) lacking the determinant which would have been recognized by the mutated residue(s) by displaying efficiencies higher than that of the non-substituted peptide. Vice versa, efficiencies lower than that of the parent peptide will denote substituted peptides which have lost a residue that is able to interact with side chain(s) still present in the mutated kinase.

This kind of analysis applied to the data of Table 2 is shown in the form of histograms in Figure 2 and leave no doubt that the mutations of CK1 α within the basic cluster K²²⁹QKQ²³² primarily hamper the recognition of the acidic determinant at position *n* – 4, since the peptide bearing an alanine residue at this position (DDAD) exhibits much higher efficiencies than the parent peptide (D₄) with the triple CK1 α mutant, as well as with the individual CK1 α mutants, in particular with K232A, but not with wild-type. In contrast, the substituted peptide with alanine at position *n* – 3 (DDDA) displays reduced relative efficiencies not only with wild-type, but also with all the mutants, consistent with the concept that the aspartic acid at position *n* – 3 can interact efficiently with residue(s) still present in the mutants.

The observation that the relative efficiency of the peptide DDAD is increased by the mutation of any of the three basic residues of the 229–232 CK1 α cluster can be explained in two ways: either all of the three residues, and, in particular, Lys²²⁹ and Lys²³², contribute to the interaction with the aspartic acid at position *n* – 4 (which could account for the fact that the triple mutation increases its relative efficiency more than any of the individual mutations) or, more likely, the aspartate residue at *n* – 4 interacts with only one lysine of the cluster, whose proper orientation for binding, however, is altered by the substitution of any of the other two (see the Discussion). In this connection, the special relevance of Lys²³² is demonstrated by the observation that this individual mutation is more detrimental than all the others

Figure 2 Relative efficiencies for the phosphorylation of singly substituted peptides by CK1 α mutants

The histograms have been constructed with data taken from Table 2. Efficiencies are normalized to that of the parent peptide D₄ (=100%).

Table 3 Kinetic constants for the phosphorylation of primed peptides bearing the priming phosphate at either the $n-3$ or $n-4$ position, by wild-type CK1 α and mutants

Kinetic constants were determined as described in the Experimental section. The values represent the means obtained from at least three independent experiments with S.E.M. never exceeding 15%. For peptide nomenclature see Table 1.

Kinase	pS(-3)		pS(-4)	
	K_m (μ M)	V_{max} (c.p.m.)	K_m (μ M)	V_{max} (c.p.m.)
Wild-type	30	446	32	428
K229A,K230A,K232A	150	463	1350	483
K229A	57	403	104	409
K230A	74	360	140	391
K232A	180	454	1218	717

(the triple mutant included) on the K_m for the parent peptide (see Table 2).

Role of CK1 α K232 in recognition of primed phosphopeptide substrates

To assess whether mutations within the K²²⁹KQK²³² cluster are also detrimental to the phosphorylation of primed peptides, i.e. of peptide substrates whose canonical consensus sequence is specified by phosphorylated residues at positions $n-3$ or $n-4$, two phosphopeptides were synthesized reminiscent of the inhibitor-2-derived peptides used above, in which an individual phosphoserine was placed either at position $n-3$ or at $n-4$ instead of the DDDD cluster. These two phosphopeptides were analysed kinetically as substrates for wild-type CK1 and CK1 mutants. The results are shown in Table 3. A number of points in these results deserve attention.

First, the two peptides primed with pS(-3) and pS(-4) are equally well phosphorylated by wild-type CK1 α , in terms of both

K_m and V_{max} . This came as a surprise, since previous studies with native CK1 preparations indicated a marked preference for peptides that bore the priming phosphoserine at position $n-3$ over those whose priming phosphoserine was at position $n-4$ [19,20].

Secondly, the K_m values of primed peptides, which is much lower than those of canonical non-primed peptides (see above and Table 2), were increased by mutations that affected phosphorylation efficiency, while V_{max} remained almost unchanged. This behaviour is clearly symptomatic of alterations in affinity, as expected upon mutating anchoring residues without substantial alteration in the catalytic event.

Thirdly, mutations within the KKQK basic stretch cause a much more dramatic decrease in the phosphorylation efficiency of the pS(-4) peptide as compared with the pS(-3) peptide.

Fourthly, the data with primed phosphopeptides show very neatly that the crucial residue for substrate recognition within the K²²⁹KQK²³² cluster is Lys²³², since its individual replacement with alanine has a far more marked effect than those of Lys²²⁹ and Lys²³⁰, being almost as detrimental as the triple mutation with both the pS(-3) and pS(-4) peptides.

Taken together, the kinetic analyses with the primed phosphopeptides, in conjunction with the information drawn from the DDDD substituted peptides, provide the unambiguous confirmation that the KKQK basic cluster is implicated in the recognition of canonical substrates by CK1 α and they demonstrate that a crucial interaction is the one occurring between Lys²³² and the negatively charged determinant at position $n-4$. On the other hand, the recognition of the determinant at position $n-3$ appears to be less strictly dependent on Lys²³² as the mutation of this residue causes a 6-fold increase in K_m (reflected in a comparable fall in phosphorylation efficiency) compared with the almost 40-fold alteration observed with the peptide primed at position $n-4$. Also, in the case of the determinant at $n-3$, the crucial residue appears to be Lys²³², with Lys²²⁹ and Lys²³⁰ playing a role even less pronounced than in the case of the determinant at $n-4$.

Figure 3 The inhibitory efficacy of polyE,Y(4:1) is abrogated by the K²²⁹KQK²³² triple mutation

Phosphorylation of β -casein (5 mg/ml) by wild-type CK1 α (●) and triple mutant K229A,K230A,K232A (■) was determined in the presence of increasing polyE,Y(4:1) concentrations, under conditions detailed in the Experimental section. The activities of both wild-type and mutated CK1 α were normalized to those observed in the absence of polyE,Y(4:1) which were 161 and 25 pmol/min per μ g respectively.

PolyE,Y(4:1) inhibits CK1 α by interacting with the KKQK motif

Various isoforms of CK1 (the α form included) have been reported to display dual specificity for being able to phosphorylate the random polymer polyE,Y(4:1) [39,41], a typical broad-specificity substrate of protein tyrosine kinases. As shown above (see Figure 1), such a phosphorylation is abolished by the triple mutation of lysine residues within the K²²⁹KQK²³² cluster, suggesting that its recognition follows the same rules as the canonical substrates. It should be noted in this respect that polyE,Y(4:1) is a fairly good substrate only in terms of K_m , while its k_{cat} is extremely low when compared with those of serine/threonine substrates [39,41], as also confirmed in the present study where the phosphorylation rate of polyE,Y(4:1) was 3 pmol $\cdot$ min⁻¹ as compared with 132 pmol $\cdot$ min⁻¹ calculated for casein phosphorylation under identical conditions (see legend of Figure 1). This confers to polyE,Y(4:1) the potential of a competitive inhibitor with respect to serine/threonine substrates sharing the same binding site(s). In fact, polyE,Y(4:1) inhibits in a dose-dependent manner the phosphorylation of casein by wild-type CK1 α (Figure 3). Its inhibitory activity, however, is lost almost completely with triply mutated CK1 α K229AK230AK232A, as also shown in Figure 3, consistent with the view that this basic element is also responsible for polyE,Y(4:1) binding (see above and Figure 1).

DISCUSSION

The data reported in the present paper provide the first biochemical and functional insight into the structural features governing the peculiar substrate specificity of protein kinase CK1. They also show that, while the recognition of canonical sites, defined either by a phosphorylated residue or by an acidic cluster upstream of the target amino acid is determined by identical structural elements, the targeting of non-canonical sites lacking these features is not grounded on the same mechanism.

In the case of numerous protein kinases, the molecular features underlining their site specificity have been disclosed by solving their structure in complex with phosphoacceptor substrates and/or by mutational analysis of residues suspected of being implicated in substrate recognition. Neither of these approaches had been

applied before to CK1. The most valuable hint was provided by divalent anions, sulphate and tungstate, which were found in the crystal structure of *S. pombe* CK1 and mammalian CK1 δ respectively [33,32] embedded in a basic cavity formed by an arginine residue at the end of the p+1 loop and a lysine one just at the beginning of helix F, which in CK1 is preceded by a basic loop implicated in interaction with a number of protein partners [34,36]. Both of these residues are conserved throughout the different CK1 isoforms. This circumstance and the analogy between the anions found in the crystals and a phosphate group suggested that the same basic cavity might also be responsible for interaction with the phosphorylated side chain generating the consensus of primed CK1 substrates.

To shed light on this point, these two basic residues, as well as a few others clustered in the proximity of the lysine in zebrafish CK1 α , have been mutated to alanine in order to check how these mutations might affect substrate recognition. The data obtained provide the clear-cut demonstration that Lys²³² is definitely implicated in the recognition of canonical substrates, whose consensus is specified by either a phosphorylated serine or by multiple carboxylic side chains. In both cases, the K232A mutant is severely defective in substrate recognition. In sharp contrast, this individual mutation and the triple mutation of Lys²²⁹, Lys²³⁰ and Lys²³² only marginally affect the capacity of CK1 α to phosphorylate the non-canonical substrate β -catenin and a β -catenin-derived peptide. The observation that Lys²³² belongs to a basic region, which has been shown to be also implicated in the binding of non-substrate proteins [34,36], discloses the possibility that these two mutants might down-regulate in a competitive manner the phosphorylation of canonical substrates. The effect of mutating the other basic residue suspected of being implicated in substrate recognition (Arg¹⁸⁶) could not be evaluated, since both the mutants generated (R186A and R186C) were extremely unstable and seemingly inactive.

In the case of primed peptide substrates, considering the juxtaposition of Lys²³² and Arg¹⁸⁶ at the right distance to accommodate a phosphate group (see the Supplementary Figure at <http://www.BiochemJ.org/bj/391/bj3910417add.htm>), it is difficult to conceive that a phosphate group interacting with Lys²³² would not also be in contact with Arg¹⁸⁶, unless profound structural alterations occur upon substrate binding. In this respect, the observation that the mutations affecting Lys²³² are more detrimental if the primed peptide bears the phosphorylated side chain at position $n-4$ instead of at $n-3$ is intriguing. Since two instead of one phosphate groups at positions $n-3$ and $n-4$ do not improve the binding efficiency significantly [44], it is likely that the priming phosphate binds to the same anchoring site if it is located at either position $n-3$ or position $n-4$. This is also compatible with the modelling performed with either the pS(-3) or pS(-4) peptides, showing that both peptides can correctly bind in order to undergo phosphorylation with the priming phosphate embedded between Arg¹⁸⁶ and Lys²³² and the phosphorylatable serine reaching the γ phosphate of ATP (see the Supplementary Figure at <http://www.BiochemJ.org/bj/391/bj3910417add.htm>). In contrast, a hypothetical pS(-2) peptide anchored in the same position would be too short to reach the catalytic site with its phosphorylatable serine (results not shown). Interestingly, modelling also discloses a different orientation of the phosphate group, depending on its belonging to the pS(-3) or pS(-4) peptides. This could account for the fact that mutation of Lys²³² is variably detrimental in the two cases. We therefore propose that the basic cavity formed by Lys²³² and Arg¹⁸⁶ is responsible for the binding of the phosphodeterminant at either position, having to assume, however, that the relative importance of Lys²³² is greater and its mutation has a more dramatic effect if the phosphate is

bound to the side chain at position $n - 4$ than it would be if the phosphate were at position $n - 3$.

It is possible, on the other hand, that, whenever the individual phosphate is replaced by numerous acidic side chains which play a synergistic role in substrate binding [25,27], the accommodation of this cluster of acidic residues alters the conformation of the binding site, making it more 'shallow' in order to allow separate interactions between each aspartate residue and distinct basic residues nearby. This may also explain why the mutation of Lys²²⁹ and Lys²³⁰ has almost no effect on the kinetics with the primed peptides (Table 3) while substantially affecting the kinetic parameters with the DDDD peptide (Table 2). Clearly, the solution of the crystal structure of complexes between CK1 α and canonical peptide substrates used for the present study will be required to prove the exactness of our hypothesis.

Two collateral outcomes of the present work may also deserve a comment. One is the observation that in our experiments a priming phosphate at position $n - 4$ is as powerful as one at position $n - 3$ as a specificity determinant. This contradicts previous reports showing that priming at position $n - 3$ was much more effective [19,20]. At present, the reasons for this discrepancy are not clear. In principle, it is possible that it is accounted for either by different CK1 isoforms used in this investigation (recombinant CK1 α) and in those early experiments (run with native CK1 preparations composed of a mixture of various isoforms) or by the structure of the phosphopeptides used. The former possibility, however, seems unlikely in the light of the fact that the pS(-3) and pS(-4) peptides used in the present study are also phosphorylated with similar efficiencies by recombinant CK1 δ from zebrafish and by native CK1 partially purified from rat liver, which is a mixture of several isoforms (results not shown). It seems more likely therefore that the preferential phosphorylation of the pSXXS compared with the pSXXXS motif is dictated by structural features of the peptide substrates, whose variable sequence, especially between the priming and the primed residues, may influence their mode of binding. A practical consequence of our present observation is to expand the repertoire of potential CK1 targets in order to include not only the pS/pTXXS/T consensus, but also the pS/pTXXXS/T one. Consequently, any sequence in which two phosphorylated residues are separated not only by three residues can be generated by GSK3 (consensus S/TXXXpS/pT), but also by CK1 (consensus pS/pTXXXS/T), depending on which residue is phosphorylated first. In a recent yeast phosphoproteome analysis, 145 doubly phosphorylated peptides were identified [45], 40 of which were classified as potential CK1 sites for displaying the pS/TXXpS/T motif [46] while 25, conforming to the pS/TXXXpS/T motif, were assigned to GSK3 assuming that downstream phosphorylation occurred first. It is now possible that at least some of these latter hierarchical phosphorylations are performed by CK1 instead of GSK3, especially if the upstream serine, but not the downstream one, displays a clear-cut consensus for being phosphorylated by a specific kinase which would in turn generate a canonical consensus for CK1.

Another observation worthy of note concerns the dual specificity of CK1, reflected in its ability to phosphorylate, albeit very slowly, the random polymer polyE,Y(4:1) [39,41]. Our data demonstrate that recognition of polyE,Y(4:1) is grounded on the same mechanism as those of canonical serine/threonine substrates, since the triple mutant K229A,K230A,K232A is also defective in polyE,Y(4:1) phosphorylation and is no longer susceptible to inhibition by polyE,Y(4:1) with casein as substrate. This discloses the possibility of using inhibition by polyE,Y(4:1) as a cursory criterion to discriminate between protein substrates which are phosphorylated at canonical sites [susceptible to com-

petitive inhibition by polyE,Y(4:1)] and those which are phosphorylated at non-canonical sites [unaffected by polyE,Y(4:1)].

This work was supported by FONDECYT (Fondo Nacional de Desarrollo Científico y Tecnológico)-Chile Project 1030462 and by the Wellcome Trust to J.E.A. and by grants from Italian MIUR (Ministero dell'Istruzione, dell'Università e della Ricerca Scientifica) (PRIN 2003 and FIRB 2001) and European Commission (PRO-KINASERESEARCH 503467) to L.A.P.

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Received 3 May 2005/20 June 2005; accepted 24 June 2005

Published as BJ Immediate Publication 24 June 2005, doi:10.1042/BJ20050717