Vol. 206, No. 1, 1995 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
January 5, 1995 Pages 171-179

MAPPING THE RESIDUES OF PROTEIN KINASE CK2 IMPLICATED IN SUBSTRATE RECOGNITION: mutagenesis of conserved basic residues in the α- subunit

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Received November 17, 1994

Six mutants of protein kinase CK2 α subunit in which basic residues have been mutated into alanines were assayed for their capability to phosphorylate the peptide RRRADDSDDDDD. Two mutants (R228A and R278K279R280A) behaved more or less as α wild type and one (H160,166A) was nearly inactive, hampering the calculation of kinetic parameters. In contrast 3 mutants (K74-77A, K79R80K83A and R191,195K198A) phosphorylated the peptide with reduced efficiency accounted for by increased K_m and decreased V_{max} values. By using derivatives of the RRRADDSDDDDD peptide in which individual aspartyl residues were variably replaced by alanine(s) and two peptide substrates derived from I-2 (KYRIREQESSGEEDSDL and RRKDLHDDEEDEEMSETADGE) it was shown that mutations in the 191-198, 74-77 and 79-83 regions were the least detrimental whenever the acidic determinants were lacking at positions +1, +4/+5 and +3, respectively. These data support the conclusion that the basic residues present in the p+1 loop of CK2α specifically recognize the acidic determinant adjacent to the C-terminal side of serine, while the specificity determinants located more downstream are variably recognized by different residues of the unique basic cluster spanning between Lys74 and Lys83.

Protein kinase CK2 is a ubiquitous Ser/Thr protein kinase implicated in a variety of cellular functions, with special reference to gene expression and cell proliferation [1-3]. It was generally known as casein kinase-2, but it has been re-

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ABBREVIATIONS:
CK2, casein kinase-2; CK1, casein kinase-1; CDK, cyclin dependent kinase; GSK-3, glycogen synthase kinase-3; I-2, inhibitor-2 of protein phosphatase-1; α^{wt}, α-subunit wild type, HBTU, 2-(1H-Benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate.

0006-291X/95 $5.00

cently proposed to abandon the explicit reference to casein in the name [4] since it leads to confusion about the physiological role of CK2, which is affecting a multitude of endogenous proteins [5] while casein is just an artificial substrate for it. Although CK2 belongs to the large superfamily of eukaryotic protein kinases, it displays a number of unusual features, among which its marked preference for sites specified by acidic residues, whereas most Ser/Thr protein kinases are basophilic, is especially notable. CK2 is normally composed by catalytic subunits (α and/or α') and non catalytic β-subunits to yield very stable heterotetramers of the $\alpha_2\beta_2$ type whose dissociation under physiological conditions was never demonstrated. Reconstitution experiments with wild type and variably mutated β-subunit [6-8] have outlined a number of functions of this non catalytic component, notably protection against denaturation and a dual effect on the catalytic activity, i. e. a positive one which is dependent on the integrity of the C-terminal domain of the β-subunit and a negative one mainly exerted by acidic residues clustered in its N-terminal moiety. It is not known which are the domain(s) of the α-subunit that interact with the β-subunit nor which are the residues responsible for the recognition of the acidic sites which are specifically phosphorylated by this enzyme. For both functions basic residues are reasonable candidates since they could account for down regulation by the acidic cluster of the β-subunit and for the binding of the multiple acidic residues that specify the sites affected by CK2. This prompted us to create a number of mutants of CK2α-subunit in which basic residues conserved in CK2 from various species but divergent from the homologous residues of other protein kinases have been replaced by alanines. These mutants will help to prove the role of the mutated residues in determining site specificity and mediating some of the effects of mutual interaction with the β-subunit. A first account of the results obtained is reported here.

MATERIALS AND METHODS

Synthetic peptides RRRADDSDDDDD and RRRAADSDDDDD were prepared as previously described [9]. The synthesis of the peptides RRRADASDDDDD, RRRADDSADDDD, RRRADDSDDADD and RRRADDSDDDAA was performed by Fmoc solid-phase chemistry with a manual multiple peptide procedure into polypropylene columns (5 ml) on a polystyrene support (Wang resin) preloaded with the first amino acid (0.7 mmol/g) The synthesis scale was 25 μmol/peptide. The chain elongation was accomplished via *in situ* activation (HBTU) using 5 molar excess for each Fmoc-amino acid. The crude peptides were purified by reverse phase semi-preparative HPLC and the purity (>95%) tested by aminoacid analysis and analytical HPLC. The I-2 derived synthetic peptides KYRIREQESSGEEDSDL and RRKDLHDDEEDEEMSETADGE have been prepared as previously described [10].

Oligonucleotide directed mutagenesis was carried out as described in detail previously [11]. Expression and of mutated CK2 α-subunits was performed essentially as described by Grankowski et al. [12]. Soluble extracts were obtained as in

[12] in all of which a prominent band of apparent 44 kDa was detected by PAGE-SDS which reacted with monoclonal and specific polyclonal antibodies raised against CK2 α-subunit. These extracts were used for most of the experiments. Mutants R228A, R191,195K198A and K74-77A were purified by phosphocellulose [12] and used for kinetic experiments providing kinetic constants comparable to those obtained with the crude preparations. CK2 activity was assayed as previously described [9].

RESULTS

65 basic residues (26 arginines, 23 lysines and 16 histidines) are present in the α-subunit of human CK2 [3]. About 50 of them are >80% conserved in the α and α' subunits of various species [3, 13]. Thirty of these basic residues are totally or mostly divergent from the homologous residues of the other Ser/Thr protein kinases [14], hence they are reasonable candidates for mutations in order to explore the substrate specificity. Priority has been given among them to the following:

i) 6 lysines and one arginine which are clustered in subdomain III (K74, K75, K76, K77, K79, R80 and K83) giving rise to one of the most peculiar features of CK2. These residues have been mutated to yield two mutants, K74-77A and K79R80K83A.

ii) two histidines (H160, H166) belonging to a unique series of 4 histidines in subdomain VI with a regular 6 residues spacing between each other, the third of which (H160) is homologous to PKA E170, responsible for the binding of the basic residue at position -2 in the peptide substrate of this enzyme. H160 has been recently shown to contribute to the recognition of an acidic residue at position -2 whenever this is present in peptide substrates of CK2 [15].

iii) two arginines and one lysine (R191, R195 and K198) which are included in the so called p+1 loop [16] of subdomain VIII where their homologous residues in most protein kinases are hydrophobic and in PKA they have been shown to interact with the hydrophobic residue present at position +1 in most of its substrates.

iv) R228 which has been hypothetized to cooperate with His160 in recognizing the acidic determinant at position -2 in some CK2 substrates [15].

v) three consecutive basic residues (R278, K279, R280) present in the insert preceding subdomain XI of the CDK_s/CK2/GSK3 subfamily; these residues are mostly replaced by non basic ones in the other members of the CK2/cdc2/GSK3 subfamily.

The distribution of the mutated residues along the sequence of CK2α is outlined in Figure 1, where the 6 corresponding mutants obtained by variably replacing them with Ala are also shown.

The kinetic constants of α^{wt} and its 6 mutants for the peptide substrate RRRADDSDDDDD are reported in Table 1. This peptide is the best peptide substrate ever described for CK2 by virtue of its 7 acidic residues located at all positions where they are known to be strong specificity determinants, i.e. between -2 and +5 [9, 17].

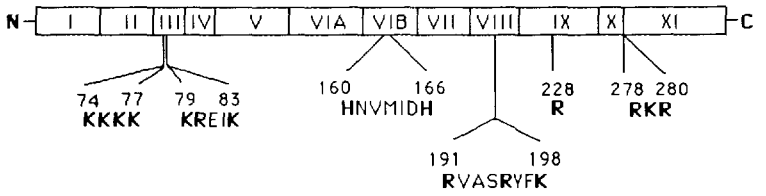

FIGURE 1.

Schematic representation of CK2 α-subunit outlining the mutations performed. The conserved I-XI subdomains are indicated. The aminoacid sequences in which basic residues (in bold) have been mutated to alanine are reported. The 7 basic residues included in the region 74-83 of the subdomain III were mutated to yield two mutants, K74-77A and K79R80K83A.

In order to optimize the phosphorylation efficiency, moreover, equimolar amounts of the β-subunit were added to α^{wt} and mutants in order to reconstitute fully active holoenzymes. As it can be seen, two mutants (R228A and R278K279R280A) behave more or less as α^{wt}; one (H160,166A) is totally inactive, thus hampering the calculation of reliable kinetic constants. The 3 remaining mutants phosphorylate the peptide substrate much less efficiently than α^{wt}, exhibiting either higher K_m values or, in the case of R191,195K198A, a much lower V_{max} value. In contrast the K_m values of these mutants for ATP were similar to that of the wild type enzyme (not shown). These data clearly indicate that basic residues located in at least two distinct regions of CK2α, notably the p+1

Table 1: **Kinetic constants for RRRADDSDDDDD phosphorylation by CK2 reconstituted with α^{wt} and mutated α-subunits**

	V_{max} (nmol/min/mg)	K_m (μM)	V_{max}/K_m
α^{wt}	288	27	10.6
R228A	344	32	10.7
R191,193K198A	15	50	0.3
R278K279R280A	302	22	13.7
H160,166A	n. d.	n.d.	-
K74-77A	192	416	0.46
K79,R80,K83A	115	250	0.46

n. d.: not determined because of very low phosphorylation rate, hampering the calculation of kinetic parameters.

loop and the basic stretch of subdomain III, are primarily implicated in the recognition of the phosphoacceptor substrate. In order to get a better insight into the individual acidic residues of the substrate that are recognized by the basic residues replaced by alanine in mutants K74-77A, K79R80K83A and R191,195K198A advantage has been taken of 5 derivatives of the peptide RRRADDSDDDDD in which aspartyl residues had been replaced by alanine. The phosphorylation rates of each peptide, normalized to that of the same peptide by α^{wt} are shown in Table 2 and they allow to identify those mutations which are less detrimental with (a) given substituted peptide(s) than with the parent peptide, for having affected residues that interact with the acidic determinants that have been substituted in these peptides. From this analysis it can be inferred that one or more of the 4 lysines clustered in the 74-77 sequence are interacting with the acidic determinants at position +4 and/or +5 and, to a lesser extent, with that at position +3. One or more of the residues K79, R80 and K83 primarily recognize the acidic determinant at position +3 and, to a lesser extent, those at position +4 and/or +5. The three basic residues of the p+1 loop (R191, R195 and K198), on the other hand, specifically recognize the acidic determinant at position +1. In contrast none of the 10 basic residues located either in the 74-83 sequence or in the p+1 loop appear to play any prominent role in the interactions with the acidic determinants at positions -1 and/or -2 since neither of the two peptides with these substitutions displays a relative phosphorylation rate higher than that of the reference peptide by the 3 mutants bearing substitutions in these regions (see Table 2).

The above conclusions are strengthened by the kinetic constants for the phosphorylation of two peptides derived from the protein phosphatase-1 inhibitor-2 [10] and differing for the distribution of acidic residues around the target serines (see Table 3). The 120/121 site derived peptide in fact is lacking the acidic deter-

Table 2: **Effect of variably substituting aspartyl residues with alanine in the peptide substrate on the relative phosphorylation rate by CK2α mutants**

	Relative phosphorylation rate[a]					
	RRRADDSDDDDD	RRRAASDDDDD	RRRADASDDDDD	RRRADDSADDDD	RRRADDSDDADD	RRRADDSDDDAA
α^{wt}	100	100	100	100	100	100
K74-77A	21	3	4	27	47	**72***
K79R80K83A	5	2	5	11	**45***	29
R191,195K198A	4	3	5	**24***	6	4

All experiments were performed in the presence of equimolar amounts of the β-subunit in order to optimize the catalytic efficiency. The concentration of all peptides was 100 μM. Underlining denotes the Ala residues replaced for Asp in the peptides. Bold typing and an asterisk denote the highest value of relative phosphorylation rate observed with each mutant.

[a] Expressed as per cent of the phosphorylation rate by α^{wt}.

Table 3: **Kinetic constants for I-2 derived peptides of CK2 reconstituted with α^{wt} and mutated α-subunits**

	Peptide substrate			
	KYRIREQE<u>SS</u>GEEDSDL (site 120/121)		RRKDLHDDEEDEEM<u>S</u>ETADGE (site 174)	
	Vmax (nmol/min/mg)	Km (μM)	Vmax (nmol/min/mg)	Km (μM)
α^{wt}	143	50.0	143	20.8
R228A	162	43.1	162	32.2
R191,195K198A	51	50.0	14	181.1
R278K279R280A	189	36.3	189	29.4
H160,166A	n. d.	n. d.	n. d.	n. d.
K74-77A	180	1,250.0	180	47.6
K79R80K83A	39	1,538.4	39	31.2

Underlining denotes the seryl residue(s) that are phosphorylated within the peptides.

minant at position +1, while bearing the ones at positions +3, +4 and +5. In contrast the site 174 derived peptide is an atypical substrate of CK2 for lacking the important acidic determinant at position +3 [18, 19] which is present in 92 out of 105 sites examined [17]. As shown in Table 3 the phosphorylation efficiency of the 120/121 site peptide is dramatically compromised by the two mutations in the 74-83 basic cluster, with a more than 20-fold increase of K_m values, while it is only slightly affected by the mutations in the p+1 loop (same K_m and just 3-fold decreased V_{max}). By contrast the mutation affecting the p+1 loop is very detrimental for the phosphorylation of the 174 site peptide (10-fold lower V_{max} and 9-fold higher K_m values), whereas the phosphorylation efficiencies of this peptide by the other two mutants, with special reference to mutant K74-77A, are comparable to that observed with CK2 α^{wt}.

DISCUSSION

The data presented in this paper provide clear-cut evidence that a number of basic residues responsible for substrate recognition by CK2 are located in at least two distinct regions of the α-subunit, notably the so called p+1 loop [16] (R191, R195 and K198) and the basic stretch spanning from Lys 74 to Lys 83. The p+1 loop is a segment of subdomain VIII which in PKA has been shown to interact with the

hydrophobic residue adjacent to the C-terminal side of most PKA substrates [16]. The residues homologous to CK2 R191, R195 and K198 are in PKA L198, P202 and L205, respectively. These residues are hydrophobic in nature in most protein kinases with the notable excxeption of CK2. The finding that the replacement of R191, R195 and K198 with alanines dramatically worsen the phosphorylation efficiency of the peptides RRRADDSDDDDD and RRKDLHDDEEDEEMSETADGE but not that of the peptide KYRIREQESSGEEDSDL in which neither of the two phosphorylatable serines (underlined) is adjacent to the N-terminal side of an acidic residue strongly support the view that also in CK2 the "p+1 loop" is committed with the binding of the residue located at position +1 in the phosphoacceptor substrate This conclusion is corroborated by the observation that the mutation of residues R191, R195 and K198 reduces much more severely the phosphorylation of all the derivatives of the peptide RRRADDSDDDDD bearing an aspartic acid at position +1 (which is a strong specificity determinant) than that of the derivative lacking this determinant (RRRADDSADDDD). Apparently the basic residues of the p+1 loop in CK2α are not only structurally homologous but also functionally analogous to the hydrophobic residues of the PKA p+1 loop.

The basic residues aligned in the 74-83 stretch of subdomain III are also clearly implicated in substrate recognition, they apparently recognize, however, acidic residues which are more remote than the one at position +1 from the target residue. This conclusion is supported by the experiments with the Ala substituted derivatives of peptide RRRADDSDDDDD (see Table 2) and is also consistent with the observation that the mutation of the basic residues of subdomain III is much more detrimental with an I-2 derived peptide whose specificity determinants are located at positions +2, +3 and +4 than with another peptide derived from a site crucially specified by an acidic residue at position +1. It should be also mentioned in this connection that the mutation of K74 and K75 to glutamic acid has been shown to impair heparin inhibition but not the affinity for the substrate [20] while the double mutation of K75 and K76 to glutamic acid had a modest effect on the K_m value [21]. Our finding that the quadruple mutation of K74, K75, K76 and K77 to alanine dramatically impairs the affinity for KYRIREQEESSGEEDSDL but only slightly that of RRKDLHDDEEDEEMSETADGE would be consistent with the concept that K77 is especially important for substrate recognition, and that its counterpart in the substrate would be one of the acidic residues at positions +2, +3 or +5 lacking in the latter peptide. Moreover, its implication in the recognition of acidic residues at positions +4 and/or +5 is supported by the finding that the mutant K74-77A phosphorylates the peptide RRRADDSDDDAA (but not RRRADDSDDDDD) almost as readily as CK2 wild type, as expected assuming that the residues important in the recognition of the acidic determinants +1 to +3 have not been altered in it (see Table 2). On the other hand the 3 basic residues downstream from K77 in the 74-83 segment appear to be more specifically involved in the recognition of acidic determinant at position +3,

since the peptide lacking this determinant (RRRADDSDDADD) is the one whose relative phosphorylation rate is the least reduced by the mutations affecting these residues (45% residual phosphorylation as compared of 5, 2, 5, 11 and 29% observed with the other peptides of the series (see Table 2)).

A priori also the 3 consecutive basic residues (R278, K279 and R280) present in the large insert of domain X were candidates for determining substrate recognition since they are unique to CK2 while being variably replaced in the members of the CK2/CDK$_s$/GSK3 subfamily exhibiting site specificity different from that of CK2. Our data would rule out however any major involvement of these residues in substrate recognition since the corresponding mutant exhibits kinetic constants comparable to those of α^{wt}, with all substrates tested. It is quite possible on the other hand that R278, K279 and R280 as well as K79, R80 and K83 are implicated in some kind of interactions with the β-subunit since the corresponding mutants undergo a higher and lower than normal stimulation by the β-subunit, respectively (unpublished data). Reconstitution experiments of CK2 holoenzyme using these mutants in combination with mutants and synthetic fragments of the β-subunit are in progress to shed light on this point.

ACKNOWLEDGMENTS

This work was supported by grants to F.M. from MURST and CNR (grant 94.02450.CT04), to L.A.P. from CNR (grant 93.02055.CT14 and ACRO) and from AIRC and to O.G.I. (grant SFB246/B3).

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