

# Substrate recognition by casein kinase-II: the role of histidine-160

Grazyna Dobrowolska<sup>a,\*\*</sup>, Flavio Meggio<sup>b</sup>, Oriano Marin<sup>b</sup>, Fred J. Lozeman<sup>a</sup>, Dongxia Li<sup>a</sup>,  
Lorenzo A. Pinna<sup>b</sup>, Edwin G. Krebs<sup>a,\*</sup>

<sup>a</sup>University of Washington, Department of Pharmacology, SL-15, Seattle, WA 98195, USA

<sup>b</sup>Università di Padova, Dipartimento di Chimica Biologica, CRIBI and Centro per lo Studio della Fisiologia Mitocondriale, via Trieste 75, 35121 Padova, Italy

Received 19 September 1994

**Abstract** Casein kinase-II (CK-II) belongs to the protein kinases recognizing serine/threonine in proximity to acidic residues in protein substrates. Crystallography and mutagenesis studies on the cAMP-dependent protein kinase (PKA) disclosed that glutamic acid-170 (E170), is important for interaction of substrates with the enzyme. At a position corresponding to E170 in PKA most Ser/Thr kinases have an aspartic or glutamic acid, while CK-II has a histidine residue (H160). In order to examine the relevance of this substitution for CK-II substrate specificity, a mutant of the catalytic  $\alpha$  subunit (H160D), in which H160 was changed to aspartic acid, was made. Our results show that H160 is not primarily involved in canonical substrate recognition, but does interact with an acidic residue located at position  $-2$  with respect to the target Ser/Thr.

**Key words:** Casein kinase-II;  $\alpha$  subunit; Specificity; Phosphorylation; Peptide

## 1. Introduction

Most of the Ser/Thr protein kinases can be divided into three groups according to the nature of the residues playing a major role in determining their substrate recognition sites [1]. The largest group includes the 'basophilic' enzymes that specifically recognize positively charged side chains, either arginine or lysine. Among these are cAMP-dependent protein kinase (PKA), protein kinase C (PKC) and essentially all other second messenger-dependent protein kinases. Then, an increasing number of Ser/Thr PKA, notably cyclin-dependent protein kinases (e.g. cdc2, cdk2) and mitogen-activated protein kinases (MAPKs) [2–5] have been shown to recognize sites specified by prolyl residue(s). Finally, six known Ser/Thr protein kinases are 'acidophilic' in that they recognize acidic determinants, namely the ubiquitous and pleiotropic casein kinases-I and -II (CK-I and CK-II), the Golgi mammary gland casein kinases (GEF-CKs; committed to the phosphorylation of newly synthesized casein), glycogen synthase kinase-3 (GSK-3), and the structurally related  $\beta$ ARK and rhodopsin kinases [6–8].

Among the acidophilic Ser/Thr protein kinases, CK-II, a pleiotropic enzyme playing an important role in cellular regulation [9–11], is the one for which the site specificity has been studied in greatest detail [9]. While its minimum consensus sequence (S/T-X-X-acidic) is determined by a negatively charged residue at the +3 position (D, E, (P)Y or (P)S), it has been shown that additional acidic residues play a favorable role at other positions between  $-2$  and  $+7$  [7,8] or even further downstream [14]. Three acidic residues, preferentially phosphorylated residues, at positions  $-2$ ,  $-1$  and  $+1$ , can create an alternative consensus sequence even if the canonical one (S/T-X-X-acidic) is lacking [15].

Elucidation of the crystal structure of the catalytic subunit of PKA [16], in conjunction with systematic mutational analysis [17], disclosed a number of acidic residues that are responsible

for interactions with the basic residues that specify the targeting of protein/peptide substrates by PKA. A prominent role is played by E170 [17–19], which binds one of the two crucial basic residues determining the consensus sequence of PKA, i.e. the arginine at position  $-2$  relative to serine. The acidic residue (D or E), corresponding to E170 in PKA, is conserved in most Ser/Thr protein kinases. It is replaced by a histidine (H160), however, in the catalytic subunits (either  $\alpha$  or  $\alpha'$  of CK-II). Such a substitution could, at least partially, account for the acidophilic properties of CK-II, in contrast to the basophilic properties of PKA and other related Ser/Thr protein kinases, assuming that the functional folding of all protein kinases is similar, as is suggested by their homology in primary structure. Such an assumption was confirmed by crystallography and modeling of the structures of such distantly related kinases as CDK2 [20], myosin light chain kinase [21], the EGF receptor [22] and MAP kinase [23].

In order to assess the actual role of H160 in determining the site specificity of CK-II, a mutant of CK-II  $\alpha$  subunit, in which H160 is replaced by an aspartic acid, was made. A number of peptide substrates varying with respect to the position of acidic residues were assayed for their ability to be phosphorylated either by the H160D mutant or by wild-type  $\alpha$  subunit.

## 2. Materials and methods

### 2.1. Cells

*Spodoptera frugiperda* (Sf9) cells were maintained in TNM-FH medium with 10% fetal calf serum.

### 2.2. Site-directed mutagenesis

Site-directed mutation was carried out using a polymerase chain reaction (PCR) method [24] consisting of 3 sequential PCRs. In two primary reactions two products were created, which overlapped in sequence, and contained the mutation (H160D) introduced as a part of the PCR primers. In each of them, template-Bluescript SK<sup>+</sup>, carrying the full-length human CK-II $\alpha$  cDNA [25] introduced into the *Bam*H1 site, was amplified over 20 cycles. The following primers were used: in the first reaction the 5' primer (sense) was ACCCGTGCCAAG-CAGGGCCAGAGT, corresponding to nucleotides 8–31 of the CK-II $\alpha$  coding sequence, and the antisense primer with an introduced mutation was ATGATCAATCATGACATTATCGGGCTTGACATCT-

\*Corresponding author. Fax: (1) (206) 543-0858.

\*\*Permanent address: Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Pawinskiego 5A str., 02-106 Warsaw, Poland.

CTGT; in the second reaction the sense primer was complementary to the mutated primer used in the first reaction, i.e. ACAGAGATGTC-AAGCCCGATAATGTCATGATTGATCAT, and the antisense 3' primer was ATCGCTTTCGAGAGTGTCTGCCCA, corresponding to the nucleotides 697–675 of CK-II $\alpha$  cDNA (nucleotides introducing the H160D mutation are underlined). The overlapping primary products that were obtained were purified with Gene Clean II Kit, denatured and allowed to reanneal producing a heteroduplex product that was amplified by the third PCR (20 cycles) using the same flanking 5' and 3' oligonucleotide primers that were used in the primary reactions. The final product was cut at unique restriction sites (*Asu*II and *Nco*I), and after purification with Gene Clean II Kit, a 573 bp DNA fragment carrying the introduced mutation was used to replace the analogous fragment in the wild-type CK-II $\alpha$  Bluescript SK<sup>+</sup> using standard procedures [26]. To verify that only the desired H160D mutation was introduced during PCR amplification, the subcloned part of the DNA was sequenced.

### 2.3. Cloning, expression and purification of human wild-type and H160D mutant CK-II $\alpha$ subunit

CK-II  $\alpha$  subunit wild-type and H160D mutant were expressed in Sf9 cells using the baculovirus expression system [27]. Standard techniques were used to generate the expression constructs [26]. Each of the clones (cDNA encoding wild-type and mutant H160D CK-II $\alpha$ ) was inserted into a modified viral polyhedrin locus contained in the transfer vector pVL941. The cDNAs were ligated into the *Bam*H1 site placed between the polyhedrin promoter and the polyadenylation signal. Each of the modified transfer vectors (carrying CK-II $\alpha$  or CK-II $\alpha$ H160D) and a *Bsu*I-digested Bac PAK 6 viral DNA (Clontech) were co-transfected into insect cells (Sf9 cells). Generation and purification of recombinant viruses was done according to the Clontech manufacturer protocol. Purified recombinant viruses were used to express enzymatic proteins.

Sf9 cells ( $5 \times 10^7$  cells) were infected with either wild-type or mutant (H160D) CK-II $\alpha$  recombinant virus at a multiplicity of infection (MOI) of 10 and grown in 100 ml culture at 27°C. After 48 h the cells were centrifuged at  $2000 \times g$  for 10 min, washed 2 times with 10 ml of ice-cold PBS, and resuspended in 5 ml of homogenization buffer (50 mM Tris-HCl, pH 7.5, 0.25 M NaCl, 2 mM EDTA, 50 mM NaF, 0.25 M sucrose, 2 mM DTT, 1 mM PMSF, 10% glycerol, 0.05% Triton X-100, 10  $\mu$ g/ml leupeptin). Cells were sonicated 3 times for 20 s on ice, and the sonicates were centrifuged at  $12,000 \times g$  at 4°C for 30 min. The supernatants were diluted to a final concentration of 0.3 M NaCl and applied to 10 ml phosphocellulose columns previously equilibrated with buffer A (50 mM Tris-HCl, pH 7.5, 10% glycerol, 2 mM EDTA, 0.1 mM PMSF, 1  $\mu$ M leupeptin) containing 0.3 M NaCl. The columns were washed with 100 ml of equilibration buffer. The enzymes were step eluted by buffer A containing 1 M NaCl. Fractions with the highest CK-II activity were pooled, diluted to a concentration of 0.25 M NaCl and applied to 2 ml heparin-agarose columns preequilibrated with buffer B (buffer A containing 0.25 M NaCl). The columns were washed with 20 ml of buffer B and CK-II $\alpha$  and H160D were step eluted by 1 M NaCl in buffer A. Fractions containing CK-II activity were concentrated and applied to a Sephacryl S-200 (2.4  $\times$  92 cm) column equilibrated with buffer C (100 mM Tris-HCl, pH 7.5, 0.5 M NaCl, 10% glycerol, 2 mM EDTA, 0.1 mM PMSF, 1  $\mu$ M leupeptin). Gel filtration was performed in buffer C at a flow rate of 10 ml/h. CK-II $\alpha$  and H160D were eluted as single peaks of CK-II activity ( $M_r$  45,000). The specific activities of CK-II $\alpha$  and of H160D were 300 nmol/min/mg and 278 nmol/min/mg, respectively. The preparations were more than 95% pure as judged by SDS-PAGE followed by silver staining. The enzymes were concentrated, dialyzed against buffer A, aliquoted and stored at  $-70^\circ\text{C}$ .

### 2.4. Peptide synthesis

The peptides SEEEEE, SEEAEE and SAAEEE [12] were kindly provided by Prof. F. Marchiori (Padova, Italy). The phosphopeptides AcSpSpSSp and AcASpSSp [15] were provided by Dr. J.W. Perich (Melbourne, Australia). The peptides SEEKEE, AcDDSD, DDSD, DSD and RKMKDTDSEEEIR were synthesized by a manual synthesizer (Model Biolinx 4175, LKB) using the continuous flow variant of the Fmoc-polyamide method on Kieselguhr-supported polydimethylacrylamide resin functionalized with 4-hydroxymethylphenoxy-acetic acid (NovaSyn KA-Novabiochem AG) essentially following the procedure described in [28]. The synthesis of the peptides RRRAADSDDDD and RRRADDSDDDDDD has been reported in [29].

### 2.5. CK-II assay

CK-II activity was measured routinely as described earlier [13,30] except that 20 mM instead 150 mM NaCl was used in the reaction mixture. As a substrate for enzyme purification the peptide RRRDDSDDDD (100  $\mu$ M) was used. Incubation was for 10 min at 30°C.

### 2.6. Peptide phosphorylation

Reaction conditions for peptide phosphorylation experiments were as described earlier [15] except that 100  $\mu$ M [ $\gamma$ -<sup>32</sup>P]ATP instead of 25  $\mu$ M was used. In most instances isolation of radioactive amino acids after partial acid hydrolysis of phosphorylated peptide (6 N HCl, 4 h, 105°C) was performed as detailed in [15]. After high voltage electrophoresis and autoradiography the amount of incorporated <sup>32</sup>P was estimated by densitometric analysis and by counting radioactive (P)S and (P)T spots in a scintillation counter. Each experiment was carried out at least 3 times and the results averaged.

### 2.7. Protein determination

Protein concentration was determined by the Bradford method [31] using BSA as a standard.

## 3. Results

The effect of changing histidine-160 to aspartic acid was first examined using synthetic peptide substrates in which glutamic acid residues carboxy-terminal ('downstream') of the serine were altered. As shown in Table 1 the replacement of the critical glutamic acid at position +3 with either alanine or lysine in the peptide substrate SEEEEE was identically detrimental with either the wild-type CK-II $\alpha$  or its mutant H160D. Essentially the same result was obtained using the peptide substrate SAAEEE, which was phosphorylated similarly by either wild-type CK-II $\alpha$  or the H160D mutant (Table 1). These results were consistent with the finding that the pseudosubstrate peptide, YEEEE, displayed superimposable inhibition curves with either wild-type or mutated  $\alpha$  subunits (Fig. 1), showing that it interacted equally well with the two forms.

As mentioned earlier (see above), it is known that acidic amino acids amino-terminal to the serine ('upstream') also play a positive role in substrate recognition by CK-II [15]. The positive role of an acidic residue at position -2 is illustrated by comparing the phosphorylation rates of the peptides, RRRADDSDDDD and RRRAADSDDDD, by wild-type CK-II $\alpha$ , the former being preferred over the latter (see

Table 1  
Phosphorylation of synthetic peptide substrates by wild-type and mutated  $\alpha$  subunit

Peptide (1 mM)	Relative phosphorylation rate (%) with	
	$\alpha^{\text{wt}}$	$\alpha^{\text{H160D}}$
SEEEEE	100	100
SEEAEE	6	6
SEEKEE	3	4
SAAEEE	31	46
AcD D S D	100	100
D D S D	18	50
A D S D	8	33
K D S D	0.7	7.5
R R R A D D S D D D D D	100	100
R R R A A D S D D D D D	24	43
Ac Sp Sp S Sp	100	100
AcA Sp S Sp	41	63

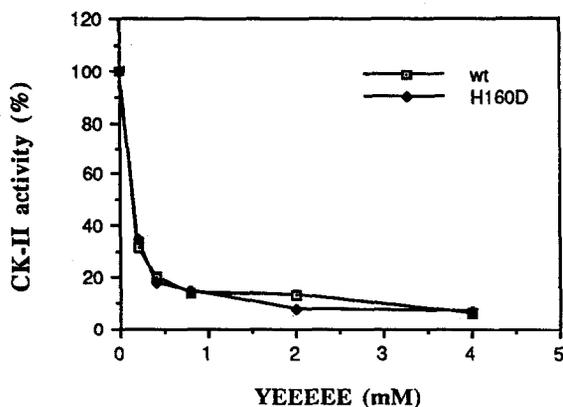


Fig. 1. Inhibitory effect of YEEEE peptide on CK-II $\alpha$  wild-type and mutant H160D. CK-II activity of both wild-type and mutated  $\alpha$  subunit was assayed as described in section 2 using the peptide DSD (100  $\mu$ M) as the phosphorylatable substrate

Table 1). In previous work it was determined that the favourable influence of an acidic residue at position -2 is even more evident with those peptide substrates of CK-II that are atypical in that they lack the critical acidic determinant at position +3. The phosphorylation of these substrates by wild-type CK-II $\alpha$  appears to depend on the presence of three acidic residues flanking serine, at positions -2, -1 and +1 [15], and the importance of an acidic residue at position -2 was emphasized in experiments that compared the phosphorylation rates of AcSpSpSSp vs. AcASpSSp and AcDDSD vs. DDSD, respectively. As shown in Table 1, in each instance the peptide bearing a net negative charge on the residue at position -2 is clearly preferred over the one lacking this feature. It should be noted, in fact, that the peptide DDSD bears both a negative and positive charge on its N-terminal aspartyl residue, i.e. on the  $\gamma$  and  $\alpha$  carboxyl and amino groups, respectively, while only the negative charge is present in its N-acetylated derivative. The relevance of the charge residing on the residue on position -2 is further outlined by phosphorylation rates of ADSD (slower than that of DDSD) and of KDSD (even slower than that of ADSD) (Table 1).

Whenever the peptides differing in the charge residing on

their residue at position -2 are phosphorylated by mutated CK-II $\alpha$  (Table 1, right column) it turns out that the presence of a negative charge on the residue at position -2 is invariably perceived as a less-essential feature than it is by wild-type CK-II $\alpha$ . Such a different behaviour appears to be mostly accounted for by the  $K_{cat}$ , as shown in Table 2 for the peptides RRRAADSDDDD and RRRADDSDDDD. The additional acidic residue at position -2 increases the  $K_{cat}$  values 4- and 2-fold for wild-type CK-II $\alpha$  and mutant H160D, respectively.

Additional evidence that His-160 of CK-II $\alpha$  plays a relevant role in substrate recognition came from experiments with the tridecapeptide substrate RKMKDTDSEEEIR, based on the main calmodulin site affected by CK-II. The threonyl (T79) and the seryl (S81) residues are phosphorylated by CK-II in calmodulin [32] as well as in the derived peptide [33]. Both T79 and S81 display the minimum structural requirement for being phosphorylated, by virtue of an acidic residue at position +3. They differ, however, in a number of additional features, and notably for the presence of a positively charged lysine two residues upstream from T79, which can be expected to hinder the phosphorylation of this residue more effectively than that of S81. As shown in Fig. 2A, if the calmodulin peptide is phosphorylated by wild-type CK-II $\alpha$ , its seryl residue is substantially preferred over the threonyl residue. The opposite is observed if the phosphorylation is performed with H160D (Fig. 2B). With the wild-type enzyme S81 displayed a two-fold higher  $V_{max}$  and a lower  $K_m$  values as compared to T79. Such differences disappear with the mutant, which exhibited similar  $V_{max}$  toward either T79 or S81, and in this case a lower  $K_m$  toward T79 (Fig. 3).

All the observed differences between CK-II $\alpha$  wild-type and H160D are consistent with the concept that H160 is implicated in the recognition of an acidic residue located at position -2 relative to serine; however, the replacement of H160 with aspartic acid does not confer any increased catalytic efficiency toward substrates bearing a basic residue at position -2. KDSD was a worse substrate than DDSD also for H160D (see Table 1). One possible explanation could be that the peptide's overall charge was drastically changed by the introduced lysine, and this could negatively influence the other, more critical, substrate determinants (especially at position +1). The above find-

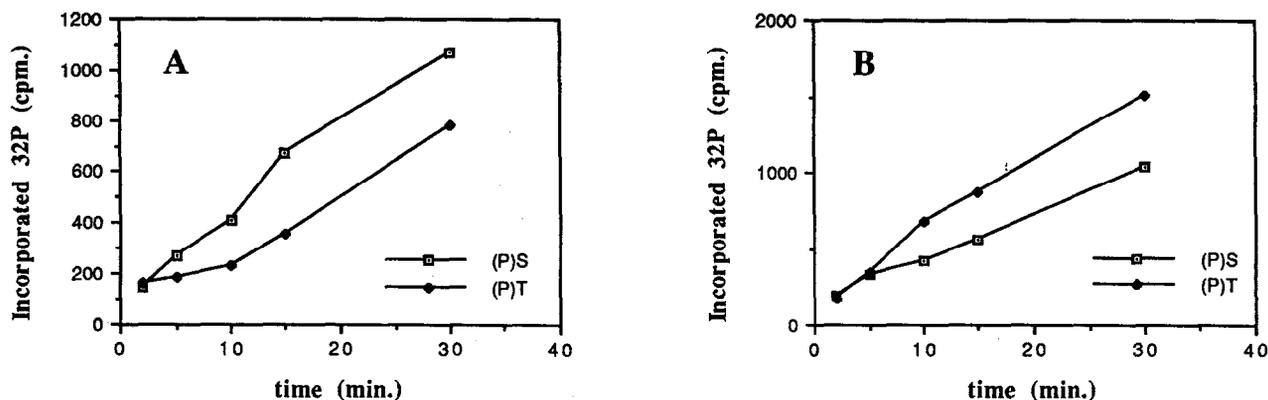


Fig. 2. Time-courses of serine and threonine phosphorylation in the peptide RKMKDTDSEEEIR by wild-type (A) and mutated H160D (B)  $\alpha$  subunit. Peptide (1 mM) phosphorylation and phosphoamino acid analysis were performed as described in section 2.

ing also supports the concept that some other residues, besides H160, recognize the amino acid at position -2 in the CK-II substrate.

#### 4. Discussion

As mentioned earlier, the tertiary structure of the PKA catalytic subunit showed that E170 cooperates in the binding of the arginyl residue at position -2 [19] in the consensus sequence of PKA, R-R-X-S/T [34]. Assuming a high degree of conservation throughout the catalytic domains of all protein kinases it was conceivable that H160 (homologous to PKA E170) might participate in CK-II substrate specificity. It should be recalled in this respect that the targeting by CK-II is primarily determined by multiple acidic residues located downstream from serine/threonine, the residue at position +3 playing an especially critical role by generating the canonical consensus sequence S/T-X-X-Acidic; acidic residues have, nevertheless, been shown to display a favourable effect also at positions -1 and -2 [12,13,15].

The data presented here show that H160 is not primarily involved in recognition of the acidic residues of the substrate, situated on the C-terminal side of the target serine, but is capable of interacting with the residue located at position -2, thus accounting at least partially, for the positive role exerted by an acidic amino acid in this position. While in fact the mutation of H160 to an aspartic acid does not affect the specificity of CK-II $\alpha$  towards peptide substrates having C-terminal modifications, it significantly alters the responsiveness to changes occurring at position -2 in the peptide substrate. The observed alterations are quite consistent with a positive role for H160 in binding an acidic residue at position -2 since the suppression of a negative charge and/or the inclusion of a positive charge at position -2, which are invariably perceived as very negative determinants by the wild-type enzyme, are less detrimental to the CK-II $\alpha$  mutant in which H160 has been replaced by an aspartic acid.

It should be noted, on the other hand, that a lysyl residue replaced for an acidic one at position -2 fails to improve the phosphorylation efficiency by the H160D mutant, but is less detrimental than it is with the wild-type enzyme. This finding strongly suggests that additional residue(s) might cooperate with H160 in binding position -2 of the peptide substrate. This would also be consistent with the observation that in PKA other acidic residue(s) besides E170, namely E230, and, under certain circumstances, E203, are implicated in the binding of the basic residue at position -2 of the peptide substrate [19]. It is quite conceivable therefore that additional residue(s) are also involved in the recognition of the same position by CK-II as well.

The homologue residue to E230 in human CK-II $\alpha$  would be

Table 2  
Kinetic constants of peptide substrates for wild-type and mutated  $\alpha$  subunit

Peptide	$K_{cat}$ ( $\text{min}^{-1}$ )		$K_m$ (mM)	
	wt	H160D	wt	H160D
R R R A A D S D D D D D	4.6	5.4	0.050	0.036
R R R A D D S D D D D D	19.2	10.8	0.047	0.031

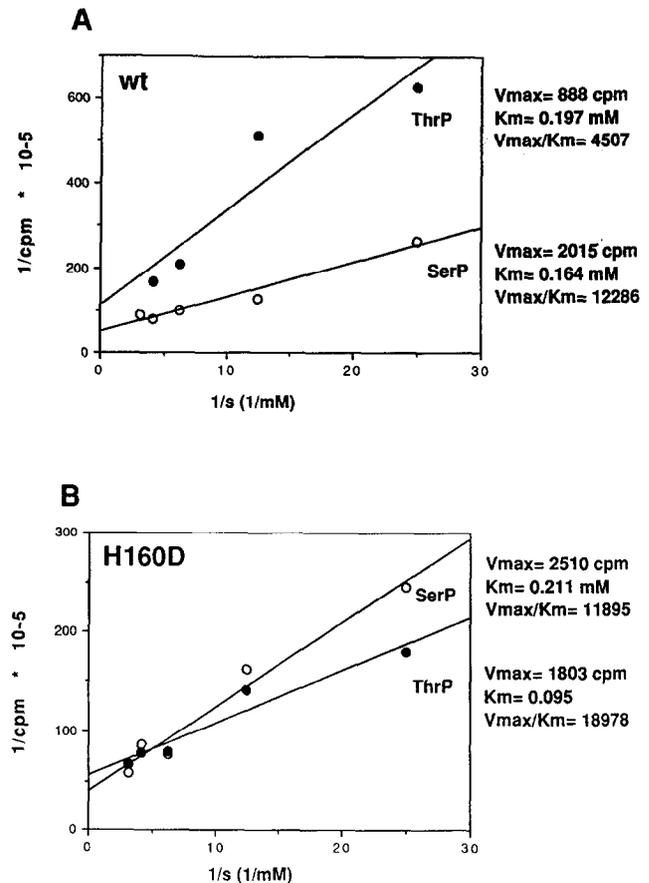


Fig. 3. Double-reciprocal plots for serine and threonine phosphorylation by CK-II $\alpha$  wild-type (A) and mutant H160D (B) in the peptide RKMKDTDSEEEIR. Phosphorylation conditions and phosphoamino acid analysis are described in section 2. Peptide concentration ranged from 0.02 to 0.5 mM.

E224 which, however, is not conserved in CK-II $\alpha$  from yeast (CK2A) [35], *Dictyostelium discoideum* [36] and *Zea mays* [37]. Therefore it is possible that amino acid(s) with cationic side chain(s), which are not exactly homologous to PKA E230, might cooperate with H160 in the binding of an acidic residue at position -2 in the peptide substrates of CK-II. A reasonable candidate could be R228, quite close to S224. Arginine-228, in fact, lacks an homologous counterpart in PKA as well as in most other protein kinases, while it is conserved or conservatively replaced by lysine in all known CK-II $\alpha$  and - $\alpha'$  subunits, with the one exception being the  $\alpha$  subunit from the amoeba *Dictyostelium discoideum* [36]. Quite intriguingly CK-II $\alpha$  from *Dictyostelium discoideum* displays the unique feature of having a seryl residue substituted for H160, which is otherwise conserved in higher animals and plants, in one of the yeast CK-II $\alpha$  subunits and even in *T. parva*, a protozoan much more recently developed than *Dictyostelium* as a parasite of mammals.

It is conceivable, therefore, that the ability to recognize an acidic residue at position -2 as a positive determinant of specificity may have appeared throughout evolution in parallel with the necessity for phosphorylating protein targets, exhibiting such a feature, more efficiently. It is worth noting in this respect that only a relatively small number of known CK-II targets possess acidic residues upstream of the phosphorylatable

amino acid, as opposed to acidic residues downstream (with special reference to position +3) which are a recurrent feature of all known CK-II sites.

In a compilation of 27 sites affected by CK-II [9], only 5 of them include an acidic residue at position -2, namely acetyl-coA carboxylase, inhibitor-2 of protein phosphatase-1 (only one site), hsp90 (two sites), and DARPP-32. It is tempting to speculate that the functional targeting of these proteins by CK-II evolved in parallel with the capability of CK-II to recognize an acidic residue at position -2 as a positive specificity determinant. Regardless of the validity of this hypothesis, our data unambiguously show that H160 plays a role in the recognition of this structural element, which is rather infrequent among CK-II targets.

*Acknowledgements:* We thank Lynda Munar for her excellent assistance in cell culture. Dr J.W. Perich and Prof. F. Marchiori are gratefully acknowledged for providing samples of synthetic peptides. The work was supported by grants from National Institutes of Health (DK 42528 to E.G.K.) and from AIRC, Italian MURST and CNR (Target Project on Biotechnology and Bioinstrumentation and ACRO) to L.A.P. and F.M.

## References

- [1] Pinna, L.A. (1991) in: *Cellular Regulation by Protein Phosphorylation*, ASI series vol. 56 (Heilmeyer Jr., L.M.G. ed.) pp. 179–193. Springer-Verlag, Berlin.
- [2] Joseph, C.K., Byun, H.S., Bittmann, R. and Kolesnick, R.N. (1993) *J. Biol. Chem.* 268, 20002–20006.
- [3] Alvarez, E., Northwood, I.C., Gonzalez, F.A., Latour, D.A., Seth, A., Abate, C., Curran, T. and Davis, R.J. (1991) *J. Biol. Chem.* 266, 15277–15285.
- [4] Hall, F.L., Braun, R.K., Mihara, K., Fung, Y.T., Berndt, N., Carbonaro-Hall, D. and Vulliet, P.R. (1991) *J. Biol. Chem.* 266, 17430–17440.
- [5] Northwood, I.C., Gonzalez, F.A., Wartmann, M., Raden, D.L. and Davis, R.J. (1991) *J. Biol. Chem.* 266, 15266–15276.
- [6] Pinna, L.A., Meggio, F. and Marchiori, F. (1990) in: *Peptides and Protein Phosphorylation* (Kemp, B.E. ed.) pp. 145–169, CRC Press, Boca Raton, FL.
- [7] Kennelly, P.J. and Krebs, E.G. (1991) *J. Biol. Chem.* 266, 15555–15558.
- [8] Onorato, J.J., Palczewski, K., Regan, J.W., Caron, M.G., Lefkowitz, R.J. and Benovic, J.L. (1991) *Biochemistry* 30, 5118–5125.
- [9] Pinna, L.A. (1990) *Biochim. Biophys. Acta* 1054, 267–284.
- [10] Tuazon, P.T. and Traugh, J.A. (1991) *Adv. Second Messenger Phosphoprotein Res.* 23, 123–164.
- [11] Issinger, O.-G. (1993) *Pharmac. Ther.* 59, 1–30.
- [12] Marin, O., Meggio, F., Marchiori, F., Borin, G. and Pinna, L.A. (1986) *Eur. J. Biochem.* 160, 239–244.
- [13] Kuenzel, E.A., Mulligan, J.A., Sommercorn, J. and Krebs, E.G. (1987) *J. Biol. Chem.* 262, 9136–9140.
- [14] Hrubey, T.W. and Roach, P.J. (1990) *Biochem. Biophys. Res. Commun.* 172, 190–196.
- [15] Perich, J.W., Meggio, F., Reynolds, E.C., Marin, O. and Pinna, L.A. (1992) *Biochemistry* 31, 5893–5897.
- [16] Knighton, D., Zheng, J., Ten Eyck, L.F., Ashford, V.A., Xuong, N.H., Taylor, S.S. and Sowadski, J.M. (1991) *Science* 253, 407–414.
- [17] Gibbs, C.S. and Zoller, M.J. (1991) *J. Biol. Chem.* 266, 8923–8931.
- [18] Tomoda, T., Murata, T., Arai, K. and Muramatsu, M. (1993) *Biochim. Biophys. Acta* 1175, 333–342.
- [19] Knighton, D.R., Zheng, J., Ten Eyck, L.F., Xuong, N.H., Taylor, S.S. and Sowadski, J.M. (1991) *Science* 253, 414–420.
- [20] De Bond, H.L., Rosenblatt, J., Jancarik, J., Jones, H.D., Morgan, D.O. and Kim, S.-H. (1993) *Nature* 363, 595–602.
- [21] Knighton, D., Pearson, R.B., Sowadski, J.M., Means, A.R., Ten Eyck, L.F., Taylor, S.S. and Kemp, B.E. (1992) *Science* 258, 130.
- [22] Knighton, D.R., Cadena, D.L., Zheng, J., Ten Eyck, L.F., Taylor, S.S., Sowadski, J.M. and Gill, G.N. (1993) *Proc. Natl. Acad. Sci. USA* 90, 50001–5005.
- [23] Zhang, F., Strand, A., Robbins, D., Cobb, M.H. and Goldsmith, E.J. (1994) *Nature* 367, 704–710.
- [24] Higuchi, R. (1990) in: *PCR Protocols* (Innis, M.A., Gelfand, D.H., Sninsky, J.J. and White, T.J. eds) pp. 177–183, Academic Press, NY.
- [25] Lozeman, F.J., Litchfield, D.W., Piening, C., Takio, K., Walsh, K.A. and Krebs, E.G. (1990) *Biochemistry* 29, 8436–8447.
- [26] Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
- [27] Summers, M.D. and Smith, G.E. (1988) *A Manual of Methods for Baculovirus Vectors and Insect Cell Culture Procedures*. Texas Agricultural Experiment Station Bulletin No. 1555.
- [28] Marin, O., Donella-Deana, A., Brunati, A.M., Fischer, S. and Pinna, L.A. (1991) *J. Biol. Chem.* 266, 17798–17803.
- [29] Marin, O., Meggio, F. and Pinna, L.A. (1994) *Biochem. Biophys. Res. Commun.* (in press).
- [30] Litchfield, D.W., Lozeman, F.J., Piening, C., Sommercorn, J., Takio, K., Walsh, K.A. and Krebs, E.G. (1990) *J. Biol. Chem.* 265, 7638–7644.
- [31] Bradford, M.M. (1976) *Anal. Biochem.* 72, 248–254.
- [32] Meggio, F., Brunati, A.M. and Pinna, L.A. (1987) *FEBS Lett.* 215, 241–246.
- [33] Meggio, F., Boldyreff, B., Marin, O., Marchiori, F., Perich, J.W., Issinger, O.-G. and Pinna, L.A. (1992) *Eur. J. Biochem.* 205, 939–945.
- [34] Kemp, B.E., Bylund, D.B., Huang, T. and Krebs, E.G. (1975) *Proc. Natl. Acad. Sci. USA* 72, 3448–3452.
- [35] Padmanabha, R., Chen-Wu, J.L.-P., Hanna, D.E. and Glover, C.V.C. (1990) *Mol. Cell. Biol.* 10, 4086–4099.
- [36] Kikkawa, U., Mann, S.K.O., Firtel, R.A. and Hunter, T. (1992) *Molec. Cell. Biol.* 12, 5711–5723.
- [37] Dobrowolska, G., Boldyreff, B. and Issinger, O.-G. (1991) *Biochim. Biophys. Acta* 1129, 139–140.