

Amino acid sequence of the β subunit of bovine lung casein kinase II

(cell-division-control protein/protein kinase/phosphorylation)

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ABSTRACT The amino acid sequence of the 209-residue β subunit of bovine lung casein kinase II has been determined. Excluding the amino-terminal blocking group, which was not identified, the molecular weight of the polypeptide chain is 24,239. A marked polarity of the β subunit is indicated by clusters of negative charges in the amino-terminal region and of positive charges in the carboxyl-terminal region. Whereas the β subunit shows no homology with any known protein, a segment of the sequence of the larger and microheterogeneous α subunit exhibits homology with the catalytic domains of other protein kinases, particularly with the yeast cell-division-control protein CDC28.

Casein kinase II is a ubiquitous protein kinase for which no clear cellular function or mode of control has yet been identified (reviewed in ref. 1). Originally named for its ability to phosphorylate casein, which is probably not a physiological substrate, the enzyme catalyzes the phosphorylation of a wide variety of other protein substrates including, for example, clathrin, acetyl-CoA carboxylase, inhibitor 2 of Mg^{2+} /ATP-dependent protein phosphatase, glycogen synthase, RNA polymerases I and II, spectrin, glycophorin, and several components involved in protein synthesis (2). The phosphorylation of some, but not all, of these substrates has been shown to occur in intact cells. The enzyme is unresponsive to Ca^{2+} /calmodulin and to cyclic nucleotides, but its activity is affected *in vitro* by agents that fall into two classes—cationic molecules (e.g., polyamines), which activate the enzyme under certain circumstances, and anionic molecules [e.g., 2,3-bis(phospho)glycerate or heparin], which inhibit enzyme activity (1).

Casein kinase II is isolated from mammalian sources as an $\alpha_2\beta_2$ or $\alpha\alpha'\beta_2$ tetramer (3–5) in which α and α' are closely related subunits of M_r 40,000 $\pm$ 4000 that possess catalytic activity (6). The function of the β subunit is unknown. No amino acid sequence data are available for the β subunit (M_r 25,000 $\pm$ 2000); however, a recent report (7) indicates that the α and α' subunits in yeast are the products of distinct but homologous genes.

In this report we describe the amino acid sequence of the β subunit of bovine lung casein kinase II and cite preliminary observations concerning microheterogeneity and homology of the α subunit from the same tissue.

MATERIALS AND METHODS

Casein kinase II was prepared from fresh bovine lung tissue. All procedures were carried out at 4°C. Five kilograms of bovine lung was homogenized in a Waring blender with 3 volumes of 40 mM Tris-HCl, pH 7.0/2 mM EDTA/1 mM dithiothreitol (TED buffer) containing 0.5 mM phenylmeth-

anesulfonyl fluoride and then was centrifuged for 30 min at 8000 $\times$ g. The supernatant was adjusted to a conductivity of 2 mS with distilled water and then applied to a column of DEAE-cellulose in TED buffer. Elution was with a linear gradient from 0 to 0.5 M NaCl in TED buffer. The active fractions from this step were pooled and applied to a phosphocellulose column in TED buffer and the column was developed with a linear gradient from 0 to 1.2 M NaCl in TED buffer. Active fractions were chromatographed on a column containing a 1:2 mixture of hydroxylapatite (Calbiochem) and Whatman C11 cellulose, with linear gradient elution from 15 to 300 mM potassium phosphate (pH 6.8). Active fractions were adjusted to 60% saturation with ammonium sulfate and the precipitate was resuspended in TED buffer containing 0.2 M NaCl and 25% (vol/vol) glycerol. This solution was fractionated by size on a Sephacryl S-300 column in the same buffer. Active fractions were applied to a heparin-agarose (Sigma) column in TED buffer, and bound proteins were eluted with a linear gradient from 0 to 1 M ammonium sulfate in TED buffer containing 25% glycerol. The active fractions were pooled and found to contain 0.1–0.4 mg of essentially homogeneous casein kinase II.

Casein kinase II activity was determined by the incorporation of ^{32}P into the synthetic peptide Arg-Arg-Arg-Glu-Glu-Glu-Thr-Glu-Glu-Glu, as described by Kuenzel and Krebs (2).

Casein kinase II was reduced and S-carboxymethylated as described by Takio *et al.* (8). The S-carboxymethylated β subunit was specifically cleaved at methionine (2.4 nmol) or at arginine (1.3 nmol) as described by Takio *et al.* (9). Lysyl bonds were cleaved in 1.4 nmol of β subunit with *Achromobacter* protease I (generously provided by T. Masaki, Ibaraki, Japan) in 2 M urea/50 mM Tris-HCl (pH 9.0) at 37°C for 10 hr (10). The protein (1.6 nmol) was also digested with L-1-tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin (Worthington) in 0.1 M ammonium bicarbonate (pH 8) at 37°C for 2 hr. An amino-terminal N $^{\alpha}$ -blocked cyanogen bromide fragment (M1, 0.3 nmol) was subdigested with *Staphylococcus aureus* V8 protease (Miles) in 50 mM sodium phosphate (pH 7.8).

Peptides generated by cleavage at methionine, lysine, or arginine were separated by size exclusion on a TSK-G2000SW column in 6 M guanidine hydrochloride/10 mM sodium phosphate (pH 6.0), followed by reverse-phase HPLC using Beckman Ultrapore RPSC or Ultrasphere C₈ columns (4.6 $\times$ 75 mm) and an aqueous trifluoroacetic acid/acetonitrile system with a Varian model 5000 HPLC system. Tryptic and staphylococcal protease digests were separated by reverse-phase HPLC on a Brownlee Aquapore RP300 column (2.1 $\times$ 100 mm) with a Hewlett Packard model

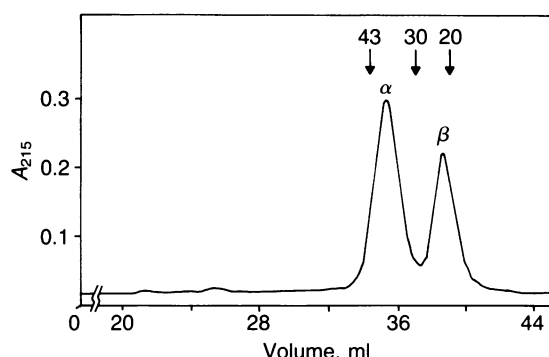


FIG. 1. Separation of the α and β subunits of S-carboxymethylated casein kinase II on two TSK-G4000SW columns in series (each 7.5×600 mm). Elution was with 6 M guanidine hydrochloride/10 mM sodium phosphate (pH 6.0) at 1.0 ml/min. Arrows indicate elution positions of standards (ovalbumin, M_r 43,000; carbonic anhydrase, M_r 30,000; soybean trypsin inhibitor, M_r 20,000).

1090M system and a Beckman Ultrapore C₈ column with the Varian system, respectively.

Amino acid compositions were determined with a Waters Picotag system (11). Amino-terminal amino acid sequence analysis by automated Edman degradation was done with a model 470A Applied Biosystems sequencer. Phenylthiohydantoin residues were identified on a Dupont Zorbax PTH column (12) and/or with an Applied Biosystems on-line HPLC system.

Homologous sequences were sought and aligned on a VAX 780 computer using the programs described by Dayhoff *et al.* (13) and the NBRF protein sequence database.¹¹

RESULTS

After reduction and S-carboxymethylation, the α and β subunits were separated on a tandem pair of TSK-G4000SW columns (Fig. 1). The S-carboxymethylated subunits α and β were eluted with apparent molecular weights of 38,000 and 23,000, respectively.

¹¹Protein Identification Resource (1986) Protein Sequence Database (Natl. Biomed. Res. Found., Washington, DC), Release 11.0.

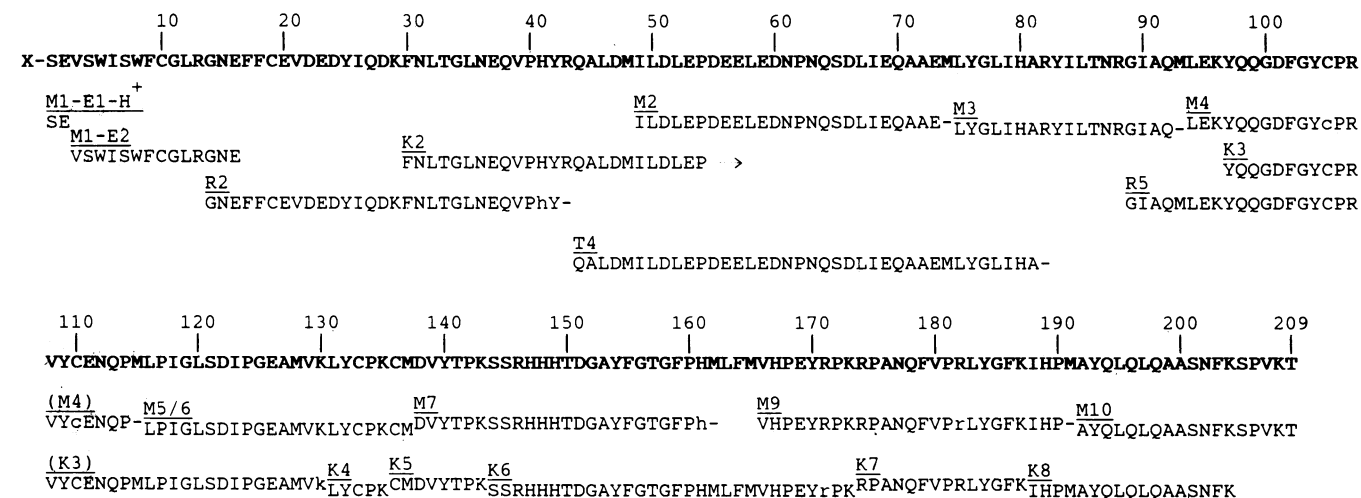


FIG. 2. Summary of the proof of sequence of the β subunit of casein kinase II. Sequences of specific peptides (names are underlined) appear in one-letter code below the summary sequence (bold type). Prefixes K, M, R, and T denote peptides generated by specific cleavage at lysine, methionine, or arginine and by trypsin cleavage, respectively. Peptides from subdigests (M1-E1-H⁺ and M1-E2) are identified in the text. Dashes represent single unidentified residues; arrows, longer unidentified sequences; and X, the unidentified blocking group at the amino terminus. Lowercase letters indicate tentative identifications.

The isolated S-carboxymethylated β subunit was blocked at the amino terminus, preventing direct Edman degradation of the whole protein and of peptides derived from the amino terminus. Most of the sequence was provided by analyses of two sets of peptides derived by cleavage at methionine (peptides M1-M10) and at lysine (peptides K2-K8). These two sets overlapped sufficiently to provide the sequence of residues 94-209 (Fig. 2). These peptides were isolated by HPLC of fractions pooled from sizing columns (Fig. 3). Results of their individual sequence analyses are summarized in Fig. 2, and one set of amino acid compositions is listed in Table 1.

In the amino-terminal half of the subunit, sequence analysis of K2 overlapped six residues of M2, but not M3 or M4. However, the fragment R5, derived by tryptic cleavage at arginine, linked M3 to M4; another peptide (R2) from the same digest overlapped and extended the sequence of K2 to Gly-14 (Fig. 2). K2, M2, and M3 were all overlapped by a tryptic peptide (T4), completing the sequence from residue 14 to the carboxyl terminus, Thr-209.

The remaining sequence in the amino-terminal region was established by subdigestion of the N-blocked cyanogen bromide fragment, M1, with *S. aureus* V8 protease. An arginine-containing peptide (M1-E2) overlapped R2 by three residues, extending the sequence to Val-3. The breakthrough fraction from the Ultrasphere C₈ column separation of the same digest contained nearly equal quantities of serine and glutamic acid/glutamine by amino acid composition and very small amounts of aspartic acid/asparagine and valine. Sequence analysis of an aliquot of this pooled fraction yielded only Val-Asp-Glu (residues 21-23). The remainder was treated with 12 M HCl at 25°C for 15 hr to promote a N $\rightarrow$ O acyl migration and then subjected to Edman degradation. A major sequence, Ser-Glu, was revealed (M1-E1-H⁺ in Fig. 2). It remained only to determine whether any peptide intervened between Ser-Glu and Val-Ser-Trp-. The N-blocked tryptic peptide R1 (residues 1-13) was subjected to digestion with the V8 protease. When the digest was analyzed without fractionation it yielded the single sequence Val-Ser-Trp-Ile-Ser-Trp-Phe-Cys, indicating that the protease cleaved only one glutamyl bond within R1 to give the blocked peptide X-Ser-Glu (M1-E1-H⁺) and the Val-Ser-Trp- (M1-E2) sequence.

With the small quantity of protein available, it was not possible to identify the amino-terminal blocking group. It may be an acetyl group, which is common to many proteins.

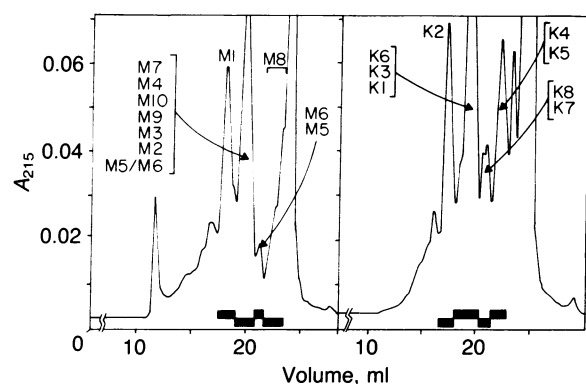


FIG. 3. Products of cleavage of 1.4 nmol of S-carboxymethylated β subunit at methionine (Left) or lysine (Right), separated on a TSK-G2000SW column (7.5×600 mm) by elution as in Fig. 1 but at 0.4 ml/min. Symbols M1–M10 and K1–K8 identify the locations of fragments. Their listed order (from the top) indicates their order of elution on subsequent reverse-phase HPLC of fractions represented by black boxes. Peptide M5/M6 overlapped M5 and M6.

The only evidence supporting this contention is that the blocked peptide X-Ser-Glu was not retained by the reverse-phase Ultrasphere C₈ column. If a longer acyl group had been present, such as the myristoyl group found, for example, in cAMP-dependent protein kinase (14), the peptide would have been eluted much later (15).

Evidence that Thr-209 is carboxyl-terminal rests only on the results of Edman degradation of M10, where no phenylthiohydantoin was observed after that threonine. The

amino acid composition of M10 is indicative of another glycine and/or proline, but compositions determined at this level (15–30 pmol) are susceptible to variation from trace contaminants and we find the Edman degradation data more convincing.

The complete 209-residue amino acid sequence (Fig. 2) is in accord with the amino acid composition of the whole protein (Table 1). The calculated molecular weight (24,239, plus the unidentified blocking group) is within 10% of 27,000 estimated by polyacrylamide gel electrophoresis (16) and of 23,000 estimated by size-exclusion HPLC (Fig. 1). There was no indication of microheterogeneity or polymorphism in the β subunit during the analysis.

Potential secondary structure in the β subunit was sought by the method of Chou and Fasman (17). The hydrophobic profile (18) was also plotted (Fig. 4). Other than a predicted tendency toward α -helical structure in the amino-terminal half of the β subunit, there was little in the way of unusual secondary structure patterns.

The complete amino acid sequence of the larger subunit, α , has not yet been determined. Cleavage of the S-carboxymethylated α subunit (2.3 nmol) with cyanogen bromide yielded a mixture of fragments that were separated on a TSK-G2000SW column followed by reverse-phase HPLC on a Brownlee Aquapore RP300 column. The amino-terminal sequences of two of these fragments, M-X and M-Y, are illustrated in Fig. 5, aligned with segments of other protein kinases. In accord with a report (6) that the α subunit is the catalytic subunit of this protein kinase, homology is observed, particularly with myosin light chain kinase and with the yeast protein CDC28. Analysis of the α subunit has been hindered by its limited availability (≈ 7.5 nmol) and by

Table 1. Amino acid compositions of the β subunit of S-carboxymethylated casein kinase II (CKII β) and its cyanogen bromide peptides (M1–M10)

Amino acid*	M1 (1–48)	M2 (49–74)	M3 (75–93)	M4 (94–115)	M5 (116–128)	M6 (129–137)	M7 (138–162)	M8 (163–165)	M9 (166–191)	M10 (192–209)	CKII β (1–209)
<i>Residues per molecule[†]</i>											
Asx (D/N)	4.4 (7)	4.9 (6)	1.1 (1)	1.4 (2)	0.7 (1)		1.5 (2)		1.0 (1)	1.2 (1)	18.9 (21)
Glx (E/Q)	6.1 (8)	7.2 (8)	1.3 (1)	4.4 (5)	0.9 (1)		1.4	0.5	2.3 (2)	3.5 (3)	30.0 (28)
Cys (C)	(2)			0.3 (2)		0.9 (2)					2.2 (6)
Ser (S)	3.3 (3)	0.9 (1)	0.5		1.1 (1)		2.1 (2)		1.0	2.2 (2)	9.5 (9)
Gly (G)	2.8 (3)		2.3 (2)	2.5 (2)	2.3 (2)		3.3 (3)	0.8	2.4 (1)	0.9	14.2 (13)
His (H)	0.7 (1)		1.2 (1)				2.9 (4)		1.9 (2)		7.4 (8)
Arg (R)	1.5 (2)		1.8 (2)	0.9 (1)			0.8 (1)		2.7 (3)		9.7 (9)
Thr (T)	1.2 (1)		0.9 (1)				1.8 (3)			0.9 (1)	5.5 (6)
Ala (A)	2.2 (1)	2.0 (2)	2.0 (2)		1.0 (1)		1.0 (1)		1.4 (1)	3.0 (3)	9.7 (11)
Pro (P)	1.7 (1)	2.5 (2)	0.8	2.1 (2)	2.2 (2)	1.2 (1)	1.9 (2)		5.0 (5)	1.6 (1)	16.2 (16)
Tyr (Y)	0.8 (2)		1.3 (2)	2.4 (3)		1.0 (1)	0.8 (2)		0.9 (2)	0.7 (1)	11.6 (13)
Val (V)	1.4 (3)			1.0 (1)		1.0 (1)	0.9 (1)		1.7 (2)	1.1 (1)	10.1 (9)
Met (M)	+	0.7 (1)	0.8 (1)	0.6 (1)	0.7 (1)	0.8 (1)	0.6 (1)	0.7 (1)	0.8 (1)		7.7 (9)
Ile (I)	2.6 (2)	1.8 (2)	2.5 (3)		1.8 (2)				1.1 (1)		10.1 (10)
Leu (L)	4.0 (4)	4.0 (4)	3.0 (3)	1.0 (1)	2.0 (2)	1.0 (1)		1.0 (1)	1.4 (1)	2.0 (2)	19.0 (19)
Phe (F)	1.6 (4)			0.9 (1)			1.5 (2)	0.9 (1)	1.8 (2)	1.1 (1)	11.0 (11)
Lys (K)	1.0 (1)			1.2 (1)		1.9 (2)	1.1 (1)		2.1 (2)	2.4 (2)	9.6 (9)
Trp (W)	ND (2)										ND (2)
Total	(48)	(26)	(19)	(22)	(13)	(9)	(25)	(3)	(26)	(18)	(209)
<i>Amount analyzed,[‡] pmol</i>											
	3.9	67.7	26.9	36.5	20.3	23.1	22.8	92.2	16.9	28.3	8.7
<i>Yield,[§] %</i>											
	16	30	44	51	25	26	40	50	30	15	

Names of peptides and (in parentheses) the sequence positions they span are given in column heads.

*One-letter symbols are given in parentheses.

[†]Determined by amino acid analysis or (in parentheses) from the sequence. +, Present but not quantified; ND, not determined. Cysteine was determined as S-carboxymethylcysteine. Methionine was recovered as homoserine in cyanogen bromide peptides. Values in *italics* were set as integers.

[‡]For composition.

[§]From cyanogen bromide digest.

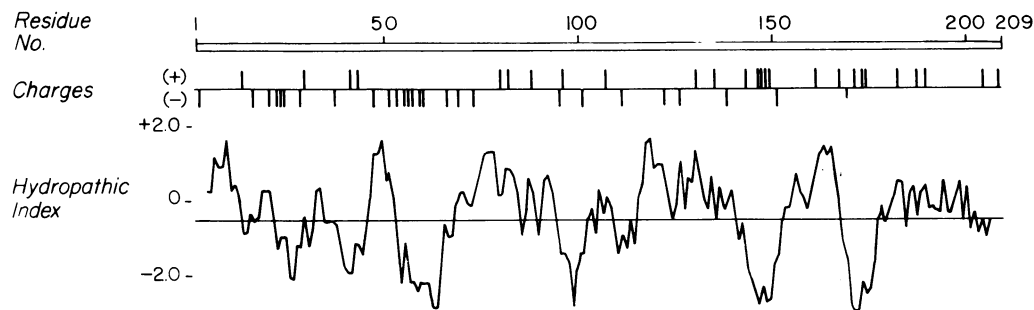


FIG. 4. (Upper) Diagrammatic summary of the location of positive and negative charges along the β subunit. (Lower) The hydropathic profile was calculated by the procedure of Kyte and Doolittle (18), and the global average hydropathic index is indicated by the horizontal line.

evidence that the preparation contains at least three copurified homologous species, as demonstrated by microheterogeneity at at least 31 positions (unpublished data).

DISCUSSION

The present data provide the complete 209-residue amino acid sequence of the β subunit of bovine lung casein kinase II. All identifications were verified in a complementary HPLC system except for residues 41–43, which rely only on the system of Glajch *et al.* (12). Moreover, 195 residue identifications were confirmed in more than one peptide. The amino-terminal blocking group was not identified, but if it is an acetyl group as in most other blocked proteins, this would give a calculated molecular weight of 24,281 for the β subunit.

Difficulties associated with the determination of the amino acid sequence were related primarily to the limited availability of the protein. A total of 170 μ g (7 nmol) of S-carboxymethylated β subunit was used in the four digests. This approaches the lower limit for conventional isolation procedures by HPLC. Some uncertainty in the identification of the carboxyl terminus is also related to the small amounts of peptide available for confirmation of composition and structure. Toward the end of this study, and in the examination of

the α subunit, it was necessary to work with even smaller quantities. Greater sensitivity has been achieved by using minibore HPLC columns (2-mm internal diameter), slower flow rates (0.5 ml/min), and a diode-array detector.

The protein sequence data base was searched for homologs of the β subunit and of two fragments of the α subunit. Although none were found for the β subunit, segment M-X of the α subunit was clearly homologous with the catalytic domains of certain other protein kinases (Fig. 5). This segment is highly conserved among other protein kinases, regardless of their mode of regulation, and it is generally accepted that it represents a central part of each catalytic domain (9). Segment M-Y bore a greater resemblance to the yeast protein CDC28, which is involved in control of cell division, than to the corresponding segments of the catalytic domains of the other protein kinases. Since casein kinase II has been detected in nuclei, and inasmuch as many of the substrates for casein kinase II are important in cell growth and/or control of gene expression (1), its relationship to CDC28 may be of particular interest.

In a recent report (7) two distinct α subunits of casein kinase were found to be encoded by separate genes in yeast and to be distinct from yeast CDC28. Both of their amino-

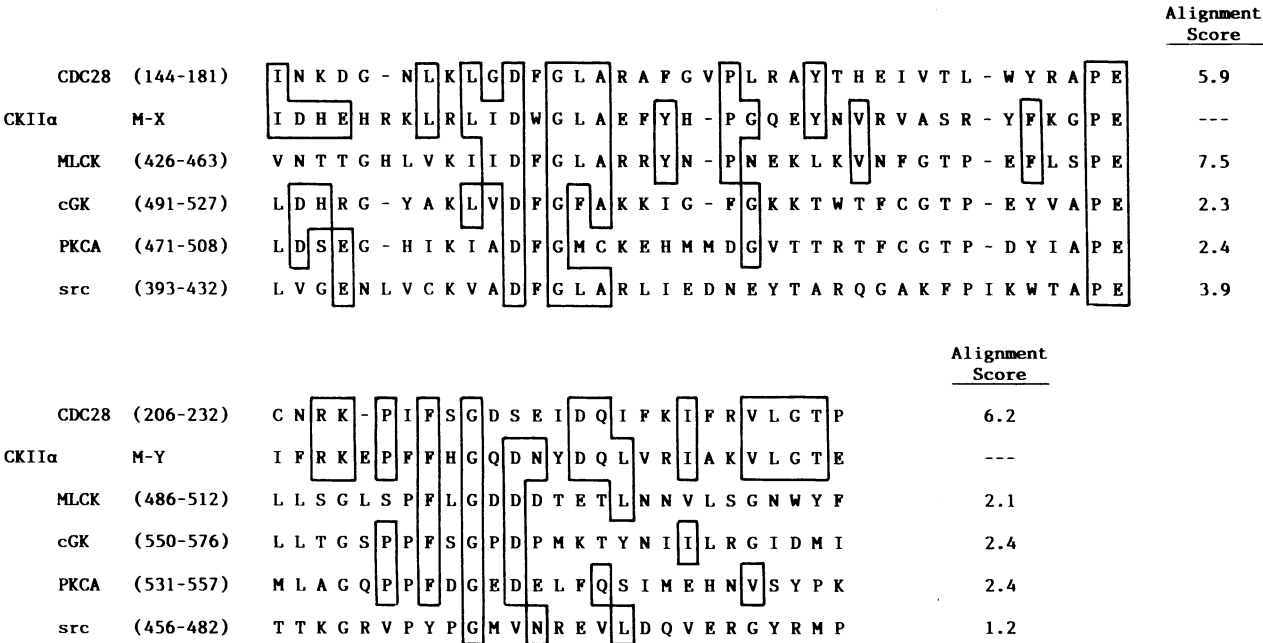


FIG. 5. Homology of segments M-X and M-Y of the major component of casein kinase II (CKII α) with segments of a yeast cell-division-control protein (CDC28) (19), myosin light chain kinase (MLCK) (9), cGMP-dependent protein kinase (cGK) (20), protein kinase C (PKCA) (21), and the tyrosine protein kinase product of the *src* gene (*src*) (22). Residues identical with CKII α residues are enclosed in boxes. Hyphens indicate gaps introduced to optimize the alignments. Alignment score was calculated as in ref. 13.

terminal sequences were homologous with a segment of the bovine lung α subunit (unpublished data). The microheterogeneity we observe in the bovine lung α subunit is another indication that the difference between α and α' subunits reflects different gene products rather than posttranslational processing.

Although the β subunit does not appear to be homologous with any known protein, it has unusual polarity in its clustering of acidic and basic residues. If the β subunit is considered in three regions, the amino-terminal 75 residues carry 19 negative charges and the carboxyl-terminal 69 residues carry 15 positive charges (Fig. 4). At this time one can only speculate whether these observations are related to the highly charged characteristics of the two classes of agents that are known to affect the activity of casein kinase, polyanionic molecules, such as heparin, that inhibit the enzyme (23–25) and certain polycationic molecules (polyamines) that activate it (24, 25). Alternatively, the β subunit may be involved in substrate selectivity or in a yet-unidentified regulatory response.

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