Interaction Sites between Catalytic and Regulatory Subunits in Human Protein Kinase CK2 Holoenzymes as Indicated by Chemical Cross-Linking and Immunological Investigations†

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ABSTRACT: Protein kinase CK2, a heterotetramer composed of two catalytic subunits (α and/or α′) and two regulatory subunits (*β*), has been examined for intermolecular contact sites by methods that allow investigation of the native, unaltered proteins. Antibodies were raised against a series of 11 subunit peptides, affinity purified, and ensured for site specific binding by peptide competition. Chemical cross-linking of CK2 subunits with a hydrophilic carbodiimide and analysis of fused subunits and of CNBr-digested fusion products by immunoblotting with the sequence specific antibodies identified a tight interaction between positions *β*55−70 and α65−80 (α′66−81) of subunits *β* and α (α′), respectively. This was corroborated by cross-linking of subunits with peptides α65−80 and *β*55−70 by a peptide-based enzyme-linked immunosorbent assay in which peptides bound to wells via C-10 spacer arms are probed for complexing individual subunits and by immunoprecipitation with antibodies anti-α65−80 and anti-*β*55−70, resulting in precipitation but not coprecipitation of subunits. This α−*β* (α′−*β*) interaction site obviously is also of functional importance since subunits with attached antibodies cannot reconstitute to the fully active holoenzyme. Indeed, sites *β*55−70 and α65−80 (α′66−81) correspond to an acidic (*β*) and a basic (α or α′) domain involved in activity and stability control and in substrate and cosubstrate binding (kinase domain II/III), respectively. By contrast, a number of suspected contact sites were found to be rather loose and not essential for enzyme control as concluded from precipitation behavior of respective antibodies and the toleration of attached antibodies when active holoenzymes were being reconstituted. At subunit *β*, these include the terminal positions *β*2−14 and *β*204−213, the positions *β*97−105 and *β*140−156, and, surprisingly, also *β*171−186 which has been shown by deletion mutation and peptide replacement studies to represent a positively affecting interaction site. At subunits α and α′, these are the C-terminal positions α329−343 and α′336−350. Binding of antibodies to the positions α15−27 (α′16−28) and position α151−166 (α′152−167), on the other hand, inhibits activity.

Protein kinase CK2[1] (formerly casein kinase II) is a pleiotropic enzyme that phosphorylates proteins at serine and threonine residues. In the majority of proteins, the CK2 motif X-S/T-X-X-E/D belongs to a cluster of acidic residues. As a cosubstrate, CK2 accepts in addition to ATP other purine nucleoside triphosphates such as GTP or ITP. The kinase appears to act independently of any of the classical second messengers but becomes modulated by acidic and basic polyionic compounds such as heparin and polyamines. CK2 occurs ubiquitously and is vital for cells. It appears to be involved in the control of a variety of cellular processes, including DNA replication and transcription, RNA processing and translation, and cell metabolism and motility [for reviews, see Pinna (1990), Tuazon and Traugh (1991), Issinger (1993), Litchfield and Lüscher (1993), Pyerin (1994), Ahmed (1994), Ahmed et al. (1994), and Allende and Allende (1995)]. Of particular concern is the role which CK2 appears to play in mitogenic signal transduction from the cytoplasm into the nucleus and within the nucleus (Lorenz et al., 1993, 1994; Pepperkok et al., 1991, 1993, 1994), where it may phosphorylate nuclear proteins that control cell growth, including oncogene, proto-oncogene, and anti-oncogene products [for review, see Meek and Street (1992)]. When dysregulatedly expressed, CK2 may become oncogenic (Seldin & Leder, 1995). CK2 is oligomeric and shows an extremely high evolutionary conservation that is evident not only in the primary structures of subunits but also in CK2's quartenary structure and even in the structure of CK2 genes (Wirkner et al., 1992, 1994a,b).

Human CK2 is a tetramer composed of two catalytic (α and/or α′) and two regulatory subunits (*β*) with molecular masses of 43 kDa (α), 38 kDa (α′), and 28 kDa (*β*) when assessed by gel electrophoresis in the presence of SDS (Pyerin et al., 1987). As deduced from their cDNA sequences (Jakobi et al., 1989; Meisner et al., 1989; Lozeman et al., 1990), the subunits consist of 391 (α), 350 (α′), and

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[1] Abbreviations: BSA, bovine serum albumin; CK2, protein kinase CK2 (formerly casein kinase II); CNBr, cyanogen bromide; EDC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; SulfoSMCC, *N*-maleimidosulfosuccinimide methyl cyclohexane-1-carboxylate; Tris, tris(hydroxymethyl)aminomethane.

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215 (β) amino acid residues. Subunits α and α′ share an overall similarity of 85%. The 41 amino acid residues by which α′ is shorter than α are lacking at the C-terminus, and the 18 C-terminal residues of α′ have no sequence similarity whatsoever to the respective segment of α. An additional amino acid residue is present in α′ within the first six N-terminal residues.[2] The overall homology of the catalytic subunits to other protein kinases is modest; the homology of the nearest relatives, the cyclin dependent kinases (cdks), remains below 20% (Hanks & Quinn, 1991).

The CK2 tetramers may associate into higher ordered structures such as rings and filaments (Glover, 1986; Mamrack, 1989; Valero et al., 1995). However, the form purified from human tissues (Pyerin et al., 1987) and tissues of other mammals (Cochet & Chambaz, 1983; Filhol et al., 1994) or immunoprecipitated from extracts of human cells in culture (Chester et al., 1995) and obtained in reconstitutions with recombinant subunits both extracellularly and intracellularly (Bodenbach et al., 1994; Boldyreff et al., 1992, 1993) is the $\alpha_2\beta_2$, $\alpha'_2\beta_2$, and/or $\alpha\alpha'\beta_2$ tetramer. In vivo, the attachment of an additional protein (pp49) to the CK2 holoenzyme or to the α subunit alone was reported (Molina et al., 1991; Plana et al., 1994). The tetrameric holoenzymes are controlled in both their activity and selectivity by subunit β. While isoform specific differences in rates of assembly have been noted (Chester et al., 1995), no differences have been found yet in the interaction of subunits α and α′ with subunit β (Bodenbach et al., 1994). Very recently, Gietz et al. (1995) reported on the formation of $\alpha\beta$ and $\alpha'\beta$ heterodimers in a yeast two-hybrid system and the ability of subunit β to dimerize and thus bring the heterodimers into the CK2 tetramers. However, the question of which sites of the regulatory and the catalytic subunits contact each other directly remains to be answered.

On the basis of predominantly mutational analyses and peptide studies, a number of domains at both the regulatory and the catalytic subunits that appear to represent interaction sites have been identified. Subunit β contains a most noticeable conserved cluster of acidic residues in its N-terminal half (DLEPDEELED, position β55−64) that appears to exert control on the catalytic activity of α and α′ subunits because exchange of acidic residues or of Pro58 for Ala significantly increases their activation by β. Further, deletion mutations of various lengths indicate that the C-terminal part of subunit β contains contact sites responsible for both the interaction of regulatory and catalytic subunits and the tetramer formation. For interaction, the structural status of position β171−181 appears to be of particular importance (Boldyreff et al., 1993, 1994; Hinrichs et al., 1995). The N-terminus of subunit β constitutes a CK2 autophosphorylation element (MSSSEE, position β1−6) whose originally assumed importance for activity control was confirmed by neither replacement nor deletion mutations (Bodenbach et al., 1994; Hinrichs et al., 1993; Boldyreff et al., 1993). Both of the catalytic subunits contain as a striking feature a stretch of basic residues (KPVKKKKIKR, positions 71−80 and 72−81 in α and α′, respectively) located at the transition of protein kinase domains II and III (Hanks & Quinn, 1991). In protein kinases, this region is generally not basic but predominantly acidic and involved in substrate binding. Analogously, it could represent the site to which the (acidic) recognition consensus of CK2 substrates binds (Sarno et al., 1995) or also heparin and other polyanionic CK2 inhibitors (Hu & Rubin, 1990; Gatica et al., 1994). It is obvious to assume that this highly basic region interacts with the highly acidic region at subunit β and, consequently, was the first direct $\alpha-\beta$ contact to be suspected (Lozeman et al., 1990). However, experimental proof for such interaction is still lacking. Deletion and exchange of amino acid residues may allow identification of contact sites at the individual subunits but does not allow us to conclusively show which of the identified sites interact with each other. Further, mutations may result in alterations that affect parts of the molecules remote from those mutated [e.g., Jakobi and Traugh (1992)]. Peptide competitions, although a frequently employed tool when protein−protein interactions are being specified, are not necessarily conclusive; basic and acidic regions other than those mentioned are present in CK2 subunits and may interact, particularly at the usually employed enormous molar peptide excess.

We have therefore attempted to apply methods that allow examination of interactions of the native, unaltered subunit proteins. We have employed nonmutational methods, particularly chemical cross-linking and peptide-based immunomapping. Evidence is presented for a tight and essential contact between position 55−70 of subunit β and position 65−80 of subunit α (position 66−81 of α′), corresponding to results of mutational analyses. By contrast, others of the suspected contact sites were found to be rather loose and nonessential for enzyme control or facing outside holoenzyme(s).

EXPERIMENTAL PROCEDURES

Materials. Protein A−agarose was obtained from Boehringer, and streptavidin peroxidase and protein A/G−biotin were from Dianova. EAH-Sepharose was from Pharmacia, and the poly(vinyl difluoride) membrane was from Millipore. EDC from Pierce, and Maxisorp plates were from Nunc Intermed. All other biochemicals originated from Merck and Sigma.

Sequence Information. Data of predicted secondary structure (according to Chou-Fasman), hydrophobicity, and surface probability (according to EMINI) were calculated by the HUSAR program (Deutsches Krebsforschungszentrum, Heidelberg, Germany). According to these data, regions of interest for potential contact sites were selected and peptides representing 10−17 amino acid long stretches of the CK2 subunits were designed.

CK2 Subunit Peptides. Peptides were synthesized by Dr. R. Pipcorn (Deutsches Krebsforschungszentrum, Heidelberg) on a peptide synthesizer A431 (Applied Biosystems), purified, and controlled for fidelity by high-performance liquid chromatography (HPLC). Peptide designations were based on the nature of the respective CK2 subunit (α, α′, or β), followed by the positions of the amino acid residues in the respective sequences of human CK2 subunits. The peptides comprise α15−27 (VNTHRPREYWDYEc), α65−80 (cVVVKILKPVKKKKIKR), α151−166 (cGIMHRDVKPH-

[2] This additional amino acid residue causes a shift of position numbers, resulting in deviating designations of functionally equivalent parts of subunits α and α′. Throughout the paper, descriptions are based on the numbering of α, with the respective equivalent of α′ given in parentheses where appropriate.

NVMIDH), α329−343 (KDQARMGSSSMPGGSc), α′336−350 (CADNAVLSSGLTAAR), β2−14 (SSSEEVSWISWFC), β55−70 (DLEPDEELEDNPNQSDc), β97−105 (MLEKYQQGDc), β140−156 (CMDVYTPKSSRHHHTDG), β171−186 (HPEYRPKRPANQFVPRc), and β204−213 (cASNFKSPVKT). For experimental purposes, most of the peptides were synthesized with one additional cysteine residue at one of the termini unless a terminal cysteine residue was present in the original sequence. The added cysteine residues are indicated by a lowercase c.

Preparation of Polyclonal Antibodies. Rabbits were immunized by subcutaneous injections of the respective CK2 subunit peptides (see above) covalently linked via the thiol group of their terminal cysteine residues to amino groups of a carrier protein (keyhole limpet hemocyanine) following the procedure of Aldwin and Nitecki (1987) and emulsified in Freund's complete adjuvant (priming) or incomplete adjuvant (boostering). In the case of peptide β55−70 prepared without a terminal c, coupling to carrier protein was obtained by the aid of a carbodiimide procedure (Tamura et al., 1983). Injections were repeated in 4 week intervals followed by bleeding of the rabbits 10 days after each injection. Serum was prepared and affinity purified via peptide columns by application of a method devised by Chersi et al. (1989). Monospecificity of the antibodies was tested on blotted recombinant α, α′, and β proteins.

The affinity-purified antibodies were named according to the peptide they were raised against by addition of the prefix anti-. For instance, antibodies raised against peptide α329−343 were designated accordingly as anti-α329−343.

Preparation of Recombinant Subunits. Recombinant subunits (wild-type and mutants) were prepared as described in detail previously by Bodenbach et al. (1994).

Immunoprecipitation. One picomole of β subunit was incubated in 50 mM Tris-HCl (pH 7.4) and 0.1% BSA with 10 pmol of polyclonal antibody against a β sequence. In control reactions, the antibody was preincubated with the peptide against which it was raised (1 h at room temperature) before addition of the β subunit. An incubation overnight at 4 °C followed before 1 pmol of the α subunit was added to the solution. Subunits were given 30 min for reassociation at room temperature before the start of immunoprecipitation. The equivalent procedure was applied for respective precipitations by anti-α and anti-α′ antibodies and the respective catalytic subunits.

In immunoprecipitations performed for testing the accessibility of epitopes in potential contact sites, reassociation of the subunits was done overnight at 4 °C, followed by an antibody incubation for 2 h at room temperature. When the specificity of immunoreactions was probed by peptide competition, the peptides were added at an approximately 200-fold molar excess over antibodies.

Then in all cases described, protein A−agarose beads (preadsorbed with 1% BSA in PBS) in TNX buffer [50 mM triethanolamine (pH 7.4), 100 mM NaCl, and 0.5% Triton X100 [w/v]] were added (30 μL of sedimented material per sample) and the volume was adjusted to 500 μL with TNX. After 3 h at room temperature, immunoprecipitates were washed three times with 1 mL of TNX each (20 min at room temperature).

Protein A−agarose beads were sedimented at 6000 rpm in an Eppendorf centrifuge, the supernatant was removed, and SDS sample buffer [65 mM Tris-HCl (pH 6.8), 10% glycerol, 2% SDS, 5% 2-mercaptoethanol, and 0.001% bromophenol blue] was added to prepare samples for electrophoresis.

Polyacrylamide Gel Electrophoresis and Western Blotting. After electrophoresis on a 12% polyacrylamide gel in the presence of SDS (Lämmli, 1970) and transfer to a poly(vinyl fluoride) membrane (Immobilon P) by semidry electroblotting (Kyhse-Anderson, 1984), transferred protein and molecular weight markers were detected with 0.3% Ponceau S in 3% trichloroacetic acid (subsequent destaining in water). Blocking in poly(vinyl alcohol) (1 μg/mL PBS) for 10 s preceded a 2 h incubation with α or β specific polyclonal antibodies appropriately diluted in washing buffer [50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.1% BSA, and 0.05% Tween 20 [w/v]]. For the detection of intact antibodies bound onto the membrane (leaving the blotted antibodies of the immunoprecipitated material largely undetected), the blot was incubated for 45 min in protein A/G−biotin (0.05 μg/mL washing buffer) solution. Bound protein A/G−biotin was then specifically detected by streptavidin peroxidase (diluted 1/2000 in washing buffer). All previous incubations were conducted at room temperature, and washing steps (four times for 5 min) followed each incubation. A chemiluminescence reaction was used for detection using the peroxidase substrate Luminol (Schneppenheim & Rautenberg, 1987). After a washing step, signals were additionally detected with 4-chloro-1-naphthol as substrate directly on the blot (Kobayashi & Tashima, 1989).

Chemical Cross-Linking. Cross-linking of CK2 subunits was performed in MOPS buffer [10 mM 3-(*N*-morpholino)-propanesulfonic acid and 500 mM NaCl (pH 7.2)] with a concentration of 20 mM EDC and 284 μM of each CK2 subunit in a final volume of 500 μL. The chemical reaction lasted for 2 h at room temperature (Uy & Wold, 1977). Unless used for CNBr digest (see below), SDS sample buffer (5×) was added and samples as indicated in the legends to the figures were separated by 12% polyacrylamide gels in the presence of SDS.

Cyanogen Bromide Digest and Separation of Peptides. Cross-linked subunits were precipitated with 10% trichloroacetic acid for 1 min on ice, followed by centrifugation. Digest solution (500 μL; 70 mg of cyanogen bromide per milliliter of 70% formic acid) (Luo et al., 1991) was added to precipitates and digestion allowed to take place overnight at room temperature. Residual formic acid and CNBr were evaporated. For separation of peptides generated by the CNBr digestion, 17% polyacrylamide gels in the presence of SDS were run at 80 V on a BioRad minigel system and blotted for shortened periods (45−50 min). A Western blot was conducted as described above.

Kinase Assay. Protein kinase activity was assayed as described previously (Bodenbach et al., 1994) using phosvitin as a substrate. In all assays, 1 pmol of each CK2 subunit was applied. Reassociation of subunits was allowed to take place for 30 min at room temperature. When polyclonal antibodies were tested for their influence on reconstitution, the reassociation reaction was preceded by an overnight incubation at 4 °C of 1 pmol of the subunit in question with a 10-fold molar excess of specific antibody. Antibody preincubation and holoenzyme formation were performed in a final volume of 50 μL (50 mM Tris-HCl, 0.1% BSA, and 150 mM NaCl). Controls received an equivalent amount of (unspecific) rabbit IgG fraction.

Table 1: Precipitation and Coprecipitation of Human CK2 Subunits with Peptide Antibodies[a]

subunit	peptide region	antibody	precipitation	coprecipitation
α	α15−27	anti-α15−27	+	+
	α65−80	anti-α65−80	+	−
	α151−166	anti-α151−166	+	+
	α329−343	anti-α329−343	+	+
α′	α′336−350	anti-α′336−350	+	+
β	*β*2−14	anti-*β*2−14	+	+
	*β*55−70	anti-*β*55−70	+	−
	*β*97−105	anti-*β*97−105	+	+
	*β*140−156	anti-*β*140−156	+	−
	*β*171−186	anti-*β*171−186	+	+
	*β*204−213	anti-*β*204−213	+	+

[a] Peptides representing the given regions of CK2 subunits α, α′, or *β* were synthesized and employed as antigens to raise antibodies in rabbits. The affinity-purified antibodies were used to precipitate and to coprecipitate CK2 subunits as given in Figure 1. Positive and negative results are indicated by + and −, respectively.

ELISA. CK2 subunit peptides were covalently linked via their thiol groups to secondary amino groups attached to ELISA plates by a C-10 spacer (Covalink, NUNC). *N*-Maleimidosulfosuccinimide methylcyclohexane-1-carboxylate (SulfoSMCC) served as the coupling reagent (Sondergard-Andersen et al., 1990). After a 1.5 h preincubation with 1 mM SulfoSMCC at room temperature and a PBS washing step, the CK2 peptide to be investigated was added (10 mM in PBS) and left at 4 °C for coupling for 16 h. A repeated washing step with ELISA-washing buffer [50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.1% BSA, and 0.05% Tween 20] and a 2 h blocking with blocking solution [1% BSA, 10% milk powder, 0.9% NaCl, 1.0% Tween 20, and 10 mM Tris-HCl (pH 7.5)] were applied to avoid unspecific binding. Then, 2, 5, or 10 pmol of CK2 subunits whose binding capability was to be tested was added and incubated for 2 h at room temperature. Detection of binding was by means of a goat-anti-rabbit IgG peroxidase-coupled antibody and a peroxidase reaction with 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) as a substrate. Reactions were quantified by determination of the absorbance at 405 nm in an ELISA reader. Reaction volumes at each step of the ELISA were 100 *μ*L.

RESULTS

Examination of Subunit Regions for Their Possible Participation in CK2 Subunit Interaction. Antibodies were raised against a series of 11 synthetic peptides, 10−17 amino acid residues in length, that represent regions of subunits α, α′, and *β* of human CK2 (see Table 1) and that might participate in the formation of potential contact sites of subunits. Selection of regions was by a computer calculation program based on secondary structure prediction, hydrophobicity, and surface location probability (see Experimental Procedures). When the affinity-purified antibodies were incubated with the respective individual recombinant CK2 subunits, immunoprecipitates were obtained, with each of them indicating the epitopes formed under participation of the selected regions to be accessible and therefore obviously located at the surface of the native individual CK2 subunits.

When immunoprecipitations of subunits α or α′ were carried out in the presence of subunit *β* with one of the antibodies directed specifically against α or α′, the majority of them were able to coprecipitate subunit *β*. This is exemplified in Figure 1A for anti-α329−343. An example of an antibody with which coprecipitation was not achieved is provided in Figure 1B for anti-α65−80. Conversely, subunits α or α′ were coprecipitated by the majority of antibodies specifically directed against subunit *β*. An example for coprecipitation of α with *β* is shown in Figure 1C for anti-*β*2−14 and an example for a lack of coprecipitation in Figure 1D for anti-*β*55−70. The specificity of immunoreactions of each of the diverse antibodies was further ensured by peptide competition. The results obtained with the whole set of antibodies are summarized in Table 1.

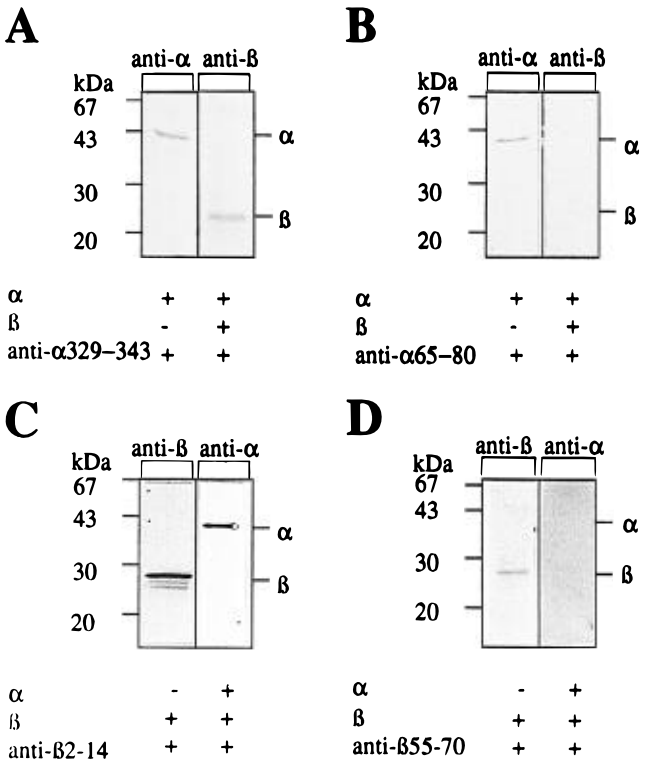

FIGURE 1: Immunoprecipitation and coprecipitation of CK2 subunits by antibodies directed to diverse regions. Antibodies raised against peptides representing the given regions of subunit α (A, anti-α329−343; B, anti-α65−80) or subunit *β* (C, anti-*β*2−14; D, anti-*β*55−70) were incubated with 1 pmol of individual subunits or subunit mixtures as indicated at bottom of each lane with + and − for the presence or absence of individual components, respectively. Precipitates were separated by polyacrylamide gel electrophoresis in the presence of SDS and immunoblotted using antibodies directed either to subunit α (anti-α) or to subunit *β* (anti-*β*) for detection as indicated at the top of each lane. Positions of molecular mass markers (left) and of CK2 subunits (right) are provided.

The results were comparable when, instead of a mixture of α and *β* subunits, the recombinant CK2 holoenzyme α*β*, expressed in *Escherichia coli* from a single mRNA containing in tandem the coding sequences of both *β* and α subunits (Bodenbach et al., 1994), was subjected to immunoprecipitation by the diverse CK2 antibodies.

Coprecipitation implies that the respective epitope of a given subunit which is seen by an antibody remains accessible despite complexation of that subunit to another subunit, and consequently, lack of coprecipitation should indicate masking of the epitope due to subunit interaction. It was therefore concluded that, in CK2 holoenzyme complexes, the epitopes formed under participation of the regions α65−80 (α′66−81), *β*55−70, and *β*140−156 are not facing the

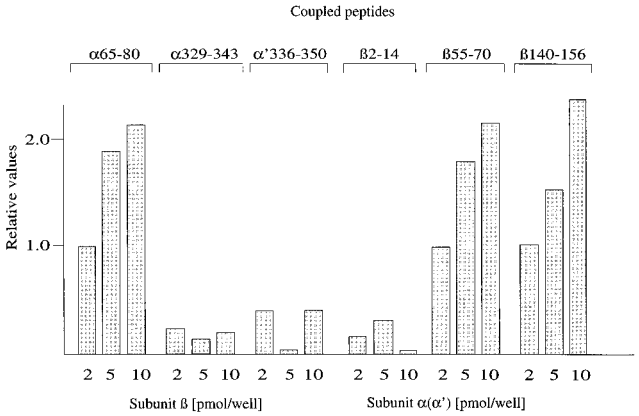

FIGURE 2: Peptide-based ELISA assay. Synthetic peptides representing regions of subunits α or α′ (α65−80, α329−343, or α′336−350) or subunit β (β2−14, β55−70, or β140−156) were covalently linked to wells of ELISA plates by reaction of cysteine residues to secondary amino groups of C-10 spacers using SulfoSMCC (1 nmol of peptide/well). Wells received the indicated amounts of subunits β (β) or α or α′ (α or α′). The peptide coupled to wells and the subunit assayed for binding are indicated at the top and bottom, respectively. Binding of subunits was detected by subunit specific antibodies raised in rabbits and peroxidase-coupled goat-anti-rabbit IgG. Peroxidase reactions are given in relative values, with the maximum reading of each experimental series at 2 pmol of β or α/well set to 1.0.

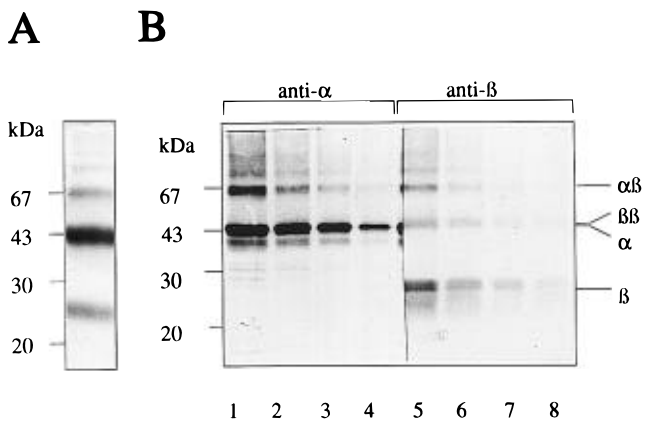

FIGURE 3: Cross-linking of subunits in the CK2αβ holoenzyme complex. Recombinant α and β subunits were reconstituted to active CK2αβ holoenzyme and then reacted with EDC (1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide). The products were separated by polyacrylamide gel electrophoresis in the presence of SDS and either stained with Coomassie blue (A) or immunoblotted (B) and detected with antibodies specific for subunit α or β as indicated at the top of the lanes. The immunoblot shows reaction products obtained with varying amounts (lanes 1 and 5, 14 pmol; lanes 2 and 6, 7 pmol; lanes 3 and 7, 2.8 pmol; and lanes 4 and 8, 1.4 pmol) of holoenzyme. Positions of molecular mass markers are given on the left side and positions of individual subunits (α or β) and cross-linked subunits ($\beta\beta$ or αβ) on the right side. Note that, due to the filter being cut vertically near the edge but within lane 5, immunostaining of this lane occurred for both α and β. In this lane, the band marked αβ (and bands above) provides α and β signals at exactly the same position. By contrast, the band marked $\beta\beta$ migrates slightly above α and is recognized by anti-β but not anti-α antibodies.

surface of holoenzymes and perhaps are directed toward another subunit.

More direct information for this location came from a peptide-based ELISA. Synthetic peptides representing sequences of subunits α, α′, and β (see Table 1) were covalently linked to ELISA plates. Linking was achieved with the aid of a cysteine residue at either the C- or N-terminus of the individual peptides that were introduced synthetically or were already naturally available there. To allow for free accessibility in wells, the peptides were linked via a C-10 spacer arm. The plates were then detected for binding of individual native CK2 subunits. Wells containing peptide α65−80 showed strong binding signals when subunit β was added, the binding obeying a saturation characteristic (Figure 2). By contrast, signals in wells containing peptides α329−343 or α′336−350 remained at the level of the controls (wells not coated by a peptide) and therefore did obviously not bind subunit β. Wells coupled with peptide β55−70 or peptide β140−156 and incubated with subunit α also provided strong binding signals and saturation characteristics, whereas peptide β2−14 was nonbinding. Thus, a peptide-based ELISA confirmed the immunoprecipitation data of an interaction site for subunit β on subunit α (α′) at position α65−80 (α′66−81) and two interaction sites for subunit α (α′) on subunit β at positions β55−70 and β140−156. It should be noted that the ELISA procedure includes remarkably harsh washing steps. As a result, tight interactions are determined while loose interactions are lost.

Cross-Linking Indicates Tight Interaction in CK2 Holoenzyme Complexes of Positions α65−80 (α′66−81) and β55−70. EDC is a hydrophilic cross-linking agent that covalently links basic and acidic amino acid residues situated in extremely close proximity. It is a "zero-length" cross-linker that does not introduce a spacer between reaction partners but rather fuses residues directly (Uy & Wold, 1977). When the CK2αβ holoenzyme complex was allowed to react with EDC, a ladder of bands was obtained upon gel electrophoresis in the presence of SDS (Figure 3A). Immunoblot analysis identified aside the unaltered individual subunits α and β, cross-linked homopolymers and heteropolymers (Figure 3B). The presence of a $\beta-\beta$ homodimer was indicated by its reaction with anti-β but not with anti-α antibodies. Formation of a $\beta-\beta$ homodimer is in line with the results obtained very recently by the two-hybrid system (Gietz et al., 1995; O.-G. Issinger, personal communication). It, however, was not the subject of the present study and was therefore not further investigated. The presence of an $\alpha-\beta$ heterodimer was indicated by its recognition with both anti-α and anti-β antibodies.

In order to answer the question of whether two of the sequence elements that had been identified above as potential contact sites at the individual α and β subunits had been located closely enough in the holoenzyme as to react with each other in the presence of EDC, cross-linking of subunits was competed by the respective peptides. When peptide α65−80 was present in reaction assays, the $\alpha-\beta$ heterodimer band was significantly weaker (Figure 4A). At the same time, an upshift of the β band occurred. The upshifted band corresponded in size to a heterodimer composed of subunit β and peptide α65−80, βα65−80. When the cross-linking was carried out in the presence of peptide β55−70, the $\alpha-\beta$ heterodimer was decreased in favor of a band with a size estimated for a heterodimer composed of subunit α and peptide β55−70, αβ55−70 (Figure 4B). Peptides such as α329−343 or β140−156 did not give this effect (data not shown). Thus, in CK2 holoenzyme complexes, subunits α (α′) and β appeared to contact each other tightly through their regions α65−80 (α′66−81) and β55−70. This was investigated further by comparative CNBr digestion.

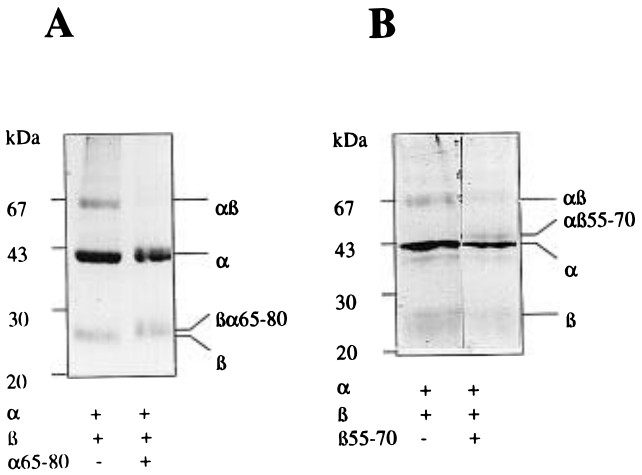

FIGURE 4: Cross-linking of subunits in the CK2αβ holoenzyme complex in the presence of CK2 peptides α65−80 or β55−70. Cross-linking reaction as in Figure 3 but in the presence of peptide α65−80 (A) or β55−70 (B). The products were separated by polyacrylamide gel electrophoresis in the presence of SDS and stained with Coomassie blue. Positions of molecular mass markers are given on left side and positions of individual subunits (α or β) and heterodimers (αβ, βα65−80, or αβ55−70) on the right side.

Following EDC cross-linking, CK2αβ or CK2α′β holoenzyme was digested by CNBr and the digests were analyzed by gel electrophoresis in the presence of SDS and immunoblotting. As shown in Figure 5, the majority of the bands that were detected were present in digests of both the cross-linked subunits and the individual subunits (investigated in parallel as a control) regardless of the antibody used for detection of fragmented subunits. However, there was one important exception; a band in the 20 kDa range was exclusively observed in digests of cross-linked subunits (αβdig and α′βdig). This band was recognized by antibodies in a strict regiospecific manner. It was recognized by anti-α15−27 and anti-α′16−28 in digests of αβ and α′β holoenzyme, respectively, but not by anti-α151−166, anti-α329−343, or anti-α′336−350 (Figure 5A,B). It was also seen by anti-β55−70 but not by anti-β140−156. Thus, the digestion products obviously consisted of fragments of both α (α′) and β subunits. But none of the fragments seemed to contain a C-terminal part of any of the individual subunits. They appeared to rather consist of N-terminal subunit fragments. Because CNBr specifically attacks methionine residues, it should, according to computer calculation (HUSAR program, Deutsches Krebsforschungszentrum, Heidelberg), generate a large N-terminal fragment of subunit α (α′) of roughly 16 kDa that includes position α65−80 (α′66−81), and subunit β should be cleaved in a way as to produce, among others, a fragment of roughly 3 kDa of the N-terminal part that contains position β55−70 (Figure 5C). When cross-linked, these two fragments would generate a product that had a size indeed matching those of αβdig and α′βdig.

Together, the cross-linking results seem to indicate that sequences α65−80 (α′66−81) and β55−70 in all likelihood directly and tightly contact each other in CK2 holoenzyme complexes. By contrast, site β140−156, located in the C-terminal half of subunit β and facing also toward another subunit in holoenzyme complexes, may rather be excluded from contacting to site α65−80 (α′66−81).

The α65−80 (α′66−81) to β55−70 Contact is Involved in Control of CK2 Kinase Activity. Peptide antibodies are expected to regiospecifically bind to subunits and, in representing bulky molecules with a molecular mass of approximately 160 kDa, to impose spatial obstacles for subunit interaction at antibody binding sites and adjacent to these. Interaction sites of importance for proper control of CK2 should therefore be disclosed by an affected enzyme activity. As shown in Table 2, the presence of anti-α65−80 but not of IgG fractions (controls) significantly inhibited the stimulation of α activity by β. Also, the presence of anti-β55−70 was inhibitory for the stimulation of α activity by β. By contrast, anti-β140−156 that shares with anti-α65−80 and anti-β55−70 the property of being unable to coprecipitate CK2 subunits (see Table 1) had little effect on activity when preincubated with β in reassociation assays. All the other antibodies directed against the different peptides of subunit β were also without effect, including antibodies directed against the N-terminus (anti-β2−14), the C-terminus (anti-β204−213), the presumed positive interaction site to subunit α (anti-β171−186), and a central region (anti-β97−105). Other antibodies directed against peptides of subunits α and α′ differed in their effects. Anti-α151−166 had a particularly strong inhibitory effect (the strongest of all antibodies tested). Also inhibitory was anti-α15−27 directed against an N-terminal region, whereas antibodies directed against the C-termini of subunits α and α′, anti-α329−343 and anti-α′336−350, had no effect on kinase activity.

DISCUSSION

Examining the native, unaltered catalytic, and regulatory subunits of CK2 for interaction sites, we find evidence for a tight and functionally important contact between amino acid side chains of position 65−80 of subunit α (position 66−81 of α′) and position 55−70 of subunit β. The evidence is based on the following observations. (i) The affinity-purified peptide antibodies anti-α65−80 (anti-α′66−81) and anti-β55−70 precipitate the subunits α (α′) and β, respectively, in their isolated states but not when complexed to each other, indicating that the antibody binding sites are located at surface of the individual subunits but not of CK2 holoenzyme complexes. Regiospecificity of antibodies was ensured by competition with the subunit peptides α65−80 (α′66−81) and β55−70, respectively. The data are in line with early observations by Litchfield et al. (1990), who employed an antibody against β51−64. (ii) When covalently linked via a C-10 spacer arm to ELISA plates, peptide α65−80 (α′66−81) complexes subunit β, and peptide β55−70 complexes subunit α (α′) with high affinity. Peptides representing other parts of subunits, except β140−156, were unable to do so. (iii) The chemical cross-linker EDC covalently links subunits α (α′) and β under conditions that favor the heterotetrameric holoenzyme structure. Because EDC linking needs the reacting side chains to be located closely enough to each other for allowing the van der Waals radii of reacting atomic groups to touch each other, tight α−β (α′−β) contacts are obviously established in holoenzymes. These are shown by CNBr digestion of cross-linked α−β (α′−β) followed by antibody mapping of the digest to be located in the N-terminal parts of subunits, the parts where α65−80 (α′66−81) and β55−70 are located. (iv) EDC also cross-links subunit α and peptide β55−70 as well as subunit

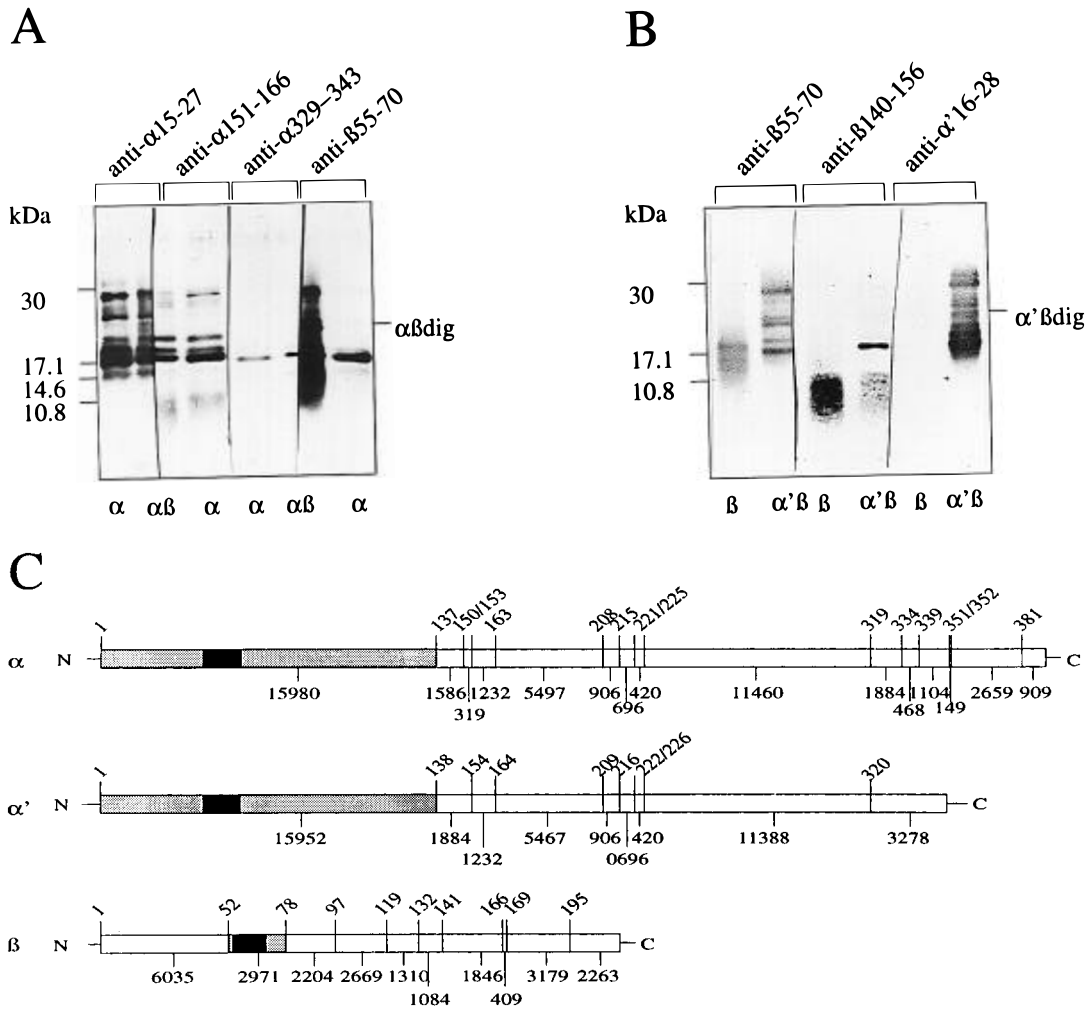

FIGURE 5: CNBr digests of cross-linked CK2 subunits analyzed by regiospecific CK2 antibodies. Subunits α or α′ were cross-linked to subunit β by EDC. The cross-linked products (αβ or α′β) and, as a control, individual nonlinked subunits (α or β) were digested with CNBr and the digests separated by polyacrylamide gel electrophoresis in the presence of SDS followed by immunoblotting using various regiospecific antibodies. (A) Immunoblots of digestion products of cross-linked α−β and of individual α using antibodies against subunit α (anti-α15−27, anti-α151−166, and anti-α329−343) or subunit β (anti-β55−70). (B) Immunoblot of digestion products of cross-linked α′−β and of individual β using antibodies against subunit β (anti-β55−70 and anti-β140−156) or subunit α′ (anti-α′16−28). Note that, because the filter was cut vertically within lanes αβ in panel A, each of the two lanes was stained by two different antibodies, allowing for direct comparisons: left side, positions of molecular mass markers; right side, position of cross-linked digestion products composed of α and β fragments (αβdig) or α′ and β fragments (α′βdig) as indicated by the CNBr digestion maps below. (C) CNBr digestion maps of subunits α, α′, and β. Subunits are drawn to scale as bars. Shaded areas represent suspected fragments contained in αβdig and α′βdig, respectively; black boxes indicate cross-linking positions (α65−80, α′66−81, and β55−70). Numbers above each subunit provide positions of methionine residues in each of the subunits (CNBr digestion sites), and numbers below the bars indicate calculated sizes of digestion fragments.

β and peptide α65−80 (α′66−81), and the peptides compete for cross-linking with subunits. (v) The regiospecific antibodies anti-β55−70 and anti-α65−80 were both inhibitory for the stimulation of kinase activity of subunit α and α′ by subunit β.

The demonstration of a tight interaction between α (α′) and β subunits via positions α65−80 (or α′66−81) and β55−70 is the experimental confirmation of an idea suggested already when the coding sequences of CK2 subunits had become available (Lozeman et al., 1990). These regions are strongly basic (KPVKKKKIKR, position α71−80) and strongly acidic (DLEPDEELED, position β55−64), respectively. It is obvious that these two regions, upon being approached, would strongly electrostatically attract each other. Both of the two regions appear to be intimately involved in CK2 function. Position α65−80 (α′66−81) harbors parts of protein kinase subdomains II and III and is important for substrate and cosubstrate binding (Hanks & Quinn, 1991; Sarno et al., 1995). Position β55−70 is involved in enzyme control and stabilization of catalytic subunits. The substitution of acidic residues or of Pro58 for Ala results in hyperactive CK2 holoenzymes (Boldyreff et al., 1994; Hinrichs et al., 1995), and peptide β55−71 increases stabilization against inactivation by heat (Meggio et al., 1992). Also, peptide replacement studies indicate the importance of these two regions for CK2 control (Meggio et al., 1992; Sarno et al., 1993). Their successful covalent linking by EDC indicates that the amino acid side chains that had reacted are located so close to each other in α−β complexes that their van der Waals radii can touch each other and that these side chains presumably are the carboxylic acid group of an aspartic or a glutamic acid and the primary amino group of a lysine side chain (Uy & Wold, 1977). It remains to be determined which of the seven aspartic and glutamic acid residues present at β and of the six lysine residues present at α are fusing.

Table 2: Effect of Sequence Specific Antibodies on CK2 Activity[a]

subunit	peptide region	antibody	inhibition of kinase activity (% of control)
α	α15−27	anti-α15−27	27 ± 3
	α65−80	anti-α65−80	50 ± 15
	α151−166	anti-α151−166	85 ± 8
	α329−343	anti-α329−343	−
α′	α′336−350	anti-α′336−350	−
β	β2−14	anti-β2−14	−
	β55−70	anti-β55−70	25 ± 2
	β97−105	anti-β97−105	−
	β140−156	anti-β140−156	−
	β171−186	anti-β171−186	−
	β204−213	anti-β204−213	−

[a] Affinity-purified antibodies raised against peptides representing the given regions of CK2 subunits α, α′, and β were investigated for their inhibitory effect on kinase activity when assayed with phosvitin as a substrate. Antibodies were preincubated with the respective subunits prior to reassociation to holoenzymes. − indicates no inhibition. To rule out unspecific antibody effects, an IgG fraction (rabbit) was added to controls run in parallel. Note that reconstitution inhibition may in some cases result in free catalytic subunit contributing some activity to the values provided.

The third region shown by our immunoprecipitation series to represent a potential contact site is region β140−156. This is a rather basic sequence fragment containing a prominent His cluster (HHH, position β151−153) adjacent to an Arg (position 150) and near a Lys (position 147). The fragment also contains a potential phosphorylation site for cdks at Thr145. Although cdks definitely interact with CK2 and phosphorylate further downstream at Ser209 (Litchfield et al., 1992), phosphorylation at Thr145 has not been reported. This might result from a hindered access for cdks and therefore might support the idea that region β140−156 becomes masked or structurally altered when interacting with α (α′) in holoenzymes. The ability of β140−156 to interact with site(s) at α (α′) subunits is particularly indicated by the results obtained with the peptide-linked ELISA; coupled to ELISA plates by a C-10 linker arm, peptide β140−156 complexes subunit α (α′). Because site β140−156 is located in the C-terminal part of subunit β, it is obviously not involved in the α−β (α′−β) EDC cross-linking. We have been noting that the presence of anti-β140−156 does not hinder subunit β from stimulating subunit α. This appears to indicate proper α−β interaction despite hindrance of site β140−156 to participation due to an antibody bound to it. Site β140−156 may therefore be classified a loose contact site to α (α′) and play a minor role in enzyme control. This is in line with results by Hinrichs et al. (1995) who mutated the His cluster of the *Xenopus* β subunit into an Ala cluster and found little effect on catalytic activity of reconstituted holoenzyme.

Region β171−186 had been extensively analyzed for a role in α−β interaction by mutational analyses. Comparative reconstitution experiments with mutants carrying deletions of different lengths downstream of position 150 of subunit β resulted in the assumption that region β171−181 may be involved in proper α−β interaction, because no reconstitution was obtained with mutant βΔ171−215 but some interaction was obtained with mutant βΔ181−215. The latter was suspected to form heterodimers, and the interaction was found sufficient to provide almost full protection of α against heat inactivation and to activate α in phosphorylation of a model CK2 substrate. Because mutants in which charged residues between position 171 and 181 had been substituted for Ala behaved like wild type β in properties such as activating subunit α, forming heterooligomers, and protecting against thermal inactivation, it has been concluded that the overall structure of β171−181is responsible rather than the charge of individual residues (Boldyreff et al., 1993, 1994). This view was strengthened by the suggestion that Pro179 of β may contribute to subunit interaction as concluded from differences in affinity to α of mutants βΔ179−215 and βΔ181−215 (Hinrichs et al., 1995) and particularly by peptide replacement studies ascribing the positive effect of subunit β on subunit α to the C-terminal region (β170−215) (Marin et al., 1995). We therefore had expected that site β171−181 would face subunit α (α′) and that binding of antibody anti-β171−186 would affect reconstitution and holoenzyme activity. To our surprise, the results obtained are inconsistent with this expectation. Anti-β171−186 not only immunoprecipitates isolated β subunit but also coprecipitates α or α′ subunits, and preincubation of subunit β with anti-β171−186 does not affect generation of holoenzyme as active as the controls, indicating accessibility of site β171−186 for the antibody in holoenzyme complexes and therefore its rather surface-oriented location and arguing rather against its importance as an α−β interaction site. How can the contradictory observations be explained? Three possibilities might be mentioned. First, a structure of CK2 might establish under the conditions used other than the canonical heterotetramer that would allow the mutually exclusive α−β interaction and anti-β171−186 binding to simultaneously occur. One might speculate that the ability of β to dimerize (Gietz et al., 1995) might tolerate the formation of structures such as heterotrimers $\alpha\beta_2$ ($\alpha'\beta_2$). This would result in an activating α−β interaction of one of the β subunits while exposing site 171−186 at the second β for antibody interaction. Such a structure was compatible with the results of mutational analyses; the "intermediate" behavior of βΔ181−215 in reconstitution was suspected to stem from a heterodimer formation leading to an activated catalytic state similar to that obtained with wild type β (Boldyreff et al., 1993), and the promotion of more effective catalytic activities with α and mutants of β was generally obtained by shifting the molar ratios toward β mutants (Boldyreff et al., 1993; Hinrichs et al., 1995). Second, the deletion mutations of β may not affect the considered parts alone but in addition other parts of β remote from the sites deleted. Third, site β171−186 may possess a structural feature with a surface-oriented part that would allow binding of antibody, leaving the other part(s) unaltered that are involved in subunit interaction allowing for normal functioning.

Common to all other sequences calculated by a computer for surface location and treated in the present paper (α15−27, α151−166, α329−343, α′336−350, β2−14, β97−105, and β204−213) is the fact that the antibodies specifically directed against them precipitate the respective subunit and coprecipitate the other subunit interacting to form a holoenzyme. All of the sequences may therefore be concluded to face outside holoenzyme or are located in an antibody-accessible manner. The catalytic subunit sites α15−27, α329−343, and α′336−350 represent amino or carboxy terminal regions beyond defined protein kinase domains. This location appears easily compatible with the mentioned prerequisites for antibody binding. The lack of an influence of antibody binding on kinase activity together with the lack

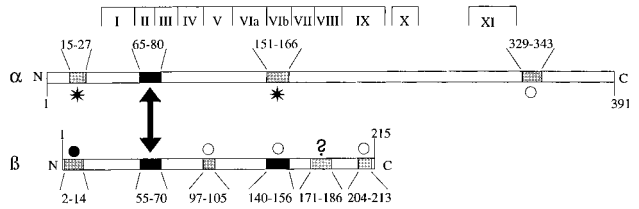

FIGURE 6: Schematic representation of how the investigated sequences of catalytic and regulatory CK2 subunits may interact with each other. Subunits α and β are drawn to scale as bars, I−XI indicating positions of kinase domains in the catalytic subunit. Boxes indicate the location of investigated sequences and numbers of the respective amino acid positions: shaded boxes, sequences accessible to antibody binding both in individual subunits and in the holoenzyme (surface-located sites); black boxes, sequences accessible to antibody binding in individual subunits but not in the holoenzyme (not surface-located sites). The double arrow between the α and β subunits indicates tight interaction of sites α65−80 and β55−70. ○, sites without significance for proper holoenzyme formation and minor role in enzyme control (loose contacts); ●, contact site at β affecting active center at α; ?, assumed contact site for proper α−β interaction by mutation analyses and peptide replacement studies that has not been confirmed by the investigations presented in this paper; *, sites at α affecting or involved in the formation of the catalytic center.

of binding of subunit β in peptide-based ELISAs is compatible with the idea that these regions are involved in only a loose interaction with subunit β or in no interaction at all. However, anti-α15−27 does affect kinase activity of CK2 holoenzyme. Because the antibody also affects isolated α (α′) (data not shown), it appears to reach an area of α (α′) that is functionally important for this subunit. According to the three-dimensional structure model of cyclic AMP dependent protein kinase, this might be the equivalent of two β sheets (positions 171−175 and 178−183) linking to the active center (Knighton et al., 1991; Bossemeyer et al., 1993).

Site α151−166 belongs to protein kinase domain VIb. It contains the invariant His160 involved in substrate binding, the counterpart to Glu170 of cyclic AMP dependent protein kinase (Taylor et al., 1993; Bossemeyer et al., 1993). Further, it is part of a unique series of six basic amino acid residues equidistant from each other spaced by five residues, respectively ($KX_5HX_5HX_5HX_5HX_5R$, position 142−172 in α and 143−173 in α′), and exchange of His160 and His166 for Ala totally inactivates α (Sarno et al., 1995). It has therefore been concluded that the site is involved in providing a molecular basis for the specific recognition of CK2 substrate proteins. Indeed, anti-α151−166 strongly affects kinase activity of reconstituted holoenzyme and also of isolated subunit α (α′) (data not shown). The ability of this antibody to coprecipitate and inhibit activity is compatible with a substrate binding cleft that is located, at least in part, at the surface of the holoenzyme to allow for free access of proteins and peptides.

The regulatory subunit sites β2−14 and β204−213 are also expected for their locations at the amino and carboxy terminal parts, respectively, to be rather accessible to antibodies in holoenzyme complexes. In fact, coprecipitation has been obtained with both of the respective antibodies. Site β2−14 contains the autophosphorylation sites (Bodenbach et al., 1994; Boldyreff et al., 1993; Hinrichs et al., 1993) and therefore can principally interact with the active center of α (α′). However, this contact is obviously not sufficiently strong to keep α (α′) complexed during peptide-based ELISA. Site β204−213 contains at Ser209 the cdk phosphorylation site which is phosphorylated in CK2 holoenzyme complexes (Litchfield et al., 1992) and thus is obviously accessible for cdks, supporting the surface location which has been concluded from the presented antibody precipitation and coprecipitation studies. Finally, site β97−105 obviously is also a surface-located sequence whose particular functions, if any, are not known yet.

Figure 6 provides a schematic summary of subunit sequences dealt with and their interactions, if any, when subunits complex to CK2 holoenzyme. For the sake of clarity, only subunits α and β are shown.

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REFERENCES

Ahmed, K. (1994) *Cell. Mol. Biol. Res. 40*, 1−11.
Ahmed, K., Issinger, O.-G., Marshak, D. R., & Pyerin, W., Eds. (1994) *Cell. Mol. Biol. Res. 40* (5/6).
Aldwin, L., & Nitecki, D. E. (1987) *Anal. Biochem. 164*, 494−501.
Allende, J. E., & Allende, C. C. (1995) *FASEB J. 9*, 313−323.
Bodenbach, L., Fauss, J., Robitzki, A., Krehan, A., Lorenz, P., Lozeman, F. J., & Pyerin, W. (1994) *Eur. J. Biochem. 220*, 263−274.
Boldyreff, B., Meggio, F., Pinna, L. A., & Issinger, O.-G. (1992) *Biochem. Biophys. Res. Commun. 188*, 228−234.
Boldyreff, B., Meggio, F., Pinna, L. A., & Issinger, O.-G. (1993) *Biochemistry 32*, 12672−12677.
Boldyreff, B., Meggio, F., Pinna, L. A., & Issinger, O.-G. (1994) *Cell. Mol. Biol. Res. 40*, 391−399.
Bossemeyer, D., Engh, R. A., Kinzel, V., Ponstingl, H., & Huber, R. (1993) *EMBO J. 12*, 849−859.
Chersi, A., Ruocco, E., & Murati, E. (1989) *J. Immunol. Methods 122*, 285−289.
Chester, M., Yu, I. J., & Marshak, D. R. (1995) *J. Biol. Chem. 270*, 7501−7514.
Cochet, C., & Chambaz, E. M. (1983) *J. Biol. Chem. 258*, 1403−1406.
Filhol, O., Cochet, C., Loue-Mackenbach, P., & Chambaz, E. M. (1994) *Biochem. Biophys. Res. Commun. 198*, 660−665.
Gatica, M., Jedlicki, A., Allende, C. C., & Allende, J. E. (1994) *FEBS Lett. 339*, 93−96.
Gietz, R. D., Graham, K. C., & Litchfield, D. W. (1995) *J. Biol. Chem. 270*, 13017−13021.
Glover, C. V. C (1986) *J. Biol. Chem. 261*, 14349−14354.
Hanks, S. K., & Quinn, A. M. (1991) *Methods Enzymol. 200*, 38−62.
Hinrichs, M. V., Jedlicki, A., Tellez, R., Pongor, S., Gatica, M., Allende, C., & Allende, J. E. (1993) *Biochemitsry 32*, 7310−7316.
Hinrichs, M. V., Gatica, M., Allende, C. C., & Allende, J. E. (1995) *FEBS Lett. 368*, 211−214.
Hu, E., & Rubin, C. S. (1990) *J. Biol. Chem. 265*, 20609−20615.
Issinger, O.-G. (1993) *Pharmacol. Ther. 59*, 1−30.
Jakobi, R., & Traugh, J. A. (1992) *J. Biol. Chem. 267*, 23894−23902.
Jakobi, R., Voss, H., & Pyerin, W. (1989) *Eur. J. Biochem. 183*, 227−233.
Knighton, D. R., Zheng, J., Ten Eyck, L. F., Ashford, V. A., Yuong, N.-H., Taylor, S. S., & Sowadski, J. M. (1991) *Science 235*, 407−414.
Kobayashi, R., & Tashima, Y. (1989) *Anal. Biochem. 183*, 9−12.
Kyhse-Andersen, J. (1984) *J. Biochem. Biophys. Methods 10*, 203−209.
Lämmli, U. K. (1970) *Nature (London) 227*, 680−685.
Litchfield, D. W., & Lüscher, B. (1993) *Mol. Cell. Biochem. 127/128*, 187−199.

Litchfield, D. W., Lozeman, F. J., Piening, C. H., Sommercorn, J., Takio, K., Walsh, K. A., & Krebs, E. G. (1990) *J. Biol. Chem. 265*, 7638−7644.
Litchfield, D. W., Lüscher, B., Lozeman, F. J., Eisenman, R. N., & Krebs, E. G. (1992) *J. Biol. Chem. 267*, 14943−14951.
Lorenz, P., Pepperkok, R., Ansorge, W., & Pyerin, W. (1993) *J. Biol. Chem. 268*, 2733−2739.
Lorenz, P., Pepperkok, R., & Pyerin, W. (1994) *Cell. Mol. Biol. Res. 40*, 519−527.
Lozeman, F. J., Litchfield, D. W., Piening, C., Takio, K., Walsh, K. A., & Krebs, E. G. (1990) *Biochemistry 29*, 8436−8447.
Luo, K., Hurley, T. R., & Sefton, B. M. (1991) *Methods Enzymol. 210*, 149−152.
Mamrack, M. D. (1989) *Mol. Cell. Biochem. 85*, 147−157.
Marin, O., Meggio, F., Boldyreff, B., Marin, O., Issinger, O.-G., & Pinna, L. A. (1995) *FEBS Lett. 363*, 111−114.
Meek, D. W., & Street, A. J. (1992) *Biochem. J. 287*, 1−15.
Meggio, F., Boldyreff, B., Marin, O., Issinger, O.-G., & Pinna, L. A. (1992) *Eur. J. Biochem. 204*, 293−297.
Meisner, H., Heller-Harrison, R., Buxton, J., & Czech, M. P. (1989) *Biochemistry 28*, 4072−4076.
Molina, E., Plana, M., & Itarte, E. (1991) *Biochem. J. 277*, 811−818.
Pepperkok, R., Lorenz, P., Jakobi, J., Ansorge, W., & Pyerin, W. (1991) *Exp. Cell Res. 197*, 245−253.
Pepperkok, R., Herr, S., Lorenz, P., Pyerin, W., & Ansorge, W. (1993) *Exp. Cell Res. 204*, 278−285.
Pepperkok, R., Lorenz, P., Ansorge, W., & Pyerin, W. (1994) *J. Biol. Chem. 269*, 6986−6991.
Pinna, L. A. (1990) *Biochim. Biophys. Acta 1054*, 267−284.
Plana, M., Gil, C., Molina, E., & Itarte, E. (1994) *Cell. Mol. Biol. Res. 40*, 455−462.
Pyerin, W. (1994) *Adv. Enzyme Regul. 34*, 225−246.
Pyerin, W., Burow, E., Michaely, K., Kübler, D., & Kinzel, V. (1987) *Biol. Chem. Hoppe-Seyler 368*, 215−227.
Sarno, S., Marin, O., Meggio, F., & Pinna, L. A. (1993) *Biochem. Biophys. Res. Commun. 194*, 83−90.
Sarno, S., Boldyreff, B., Marin, O., Guerra, B., Meggio, F., Issinger, O.-G., & Pinna, L. A. (1995) *Biochem. Biophys. Res. Commun. 206*, 171−179.
Schneppenheim, R., & Rautenberg, P. (1987) *Eur. J. Clin. Microbiol. 6*, 49−51.
Seldin, D. C., & Leder, P. (1995) *Science 267*, 894−897.
Sondergard-Andersen, J., Lauritzen, E., Lind, K., & Holm, A. (1990) *J. Immunol. Methods 131*, 99−104.
Tamura, T., Bauer, H., Birr, C., & Pipkorn, R. (1983) *Cell 34*, 587−596.
Taylor, S. S., Knighton, D. R., Zheng, J., Ten Eyck, L. F., Sowadski, J. M., Gibbs, C. S., & Zoller, M. J. (1993) *TIBS 18*, 84−89
Tuazon, P. T., & Traugh, J. A. (1991) *Adv. Second Messenger Phosphoprotein Res. 23*, 123−164.
Uy, R., & Wold, F. (1977) *Adv. Exp. Med. Biol. 86A*, 169−186.
Valero, E., DeBonis, S., Filhol, O., Wade, R. H., Langowski, J., Chambaz, E. M., & Cochet, C. (1995) *J. Biol. Chem. 270*, 8345−8352.
Wirkner, U., Voss, H., Lichter, P., Weitz, S., Ansorge, A., & Pyerin, W. (1992) *Biochim. Biophys. Acta 1131*, 220−222.
Wirkner, U., Voss, H., Lichter, P., & Pyerin, W. (1994a) *Cell. Mol. Biol. Res. 40*, 489−499.
Wirkner, U., Voss, H., Lichter, P., Ansorge, A., & Pyerin, W. (1994b) *Genomics 19*, 257−265.

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