Anti-head and anti-tail antibodies against distinct epitopes in the catalytic subunit of protein kinase A

Use in the study of the kinase splitting membranal proteinase KSMP

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Received 5 February 1996

Abstract Protein kinases share a considerable sequence homology in their catalytic core (residues 40–300 in PKA). Each core is flanked by "head" and "tail" segments at its amino- and carboxy-termini, which are different in the various kinases. These end segments may play an important role in creating the preferential affinity of each kinase for its physiological substrates or regulatory ligands. Here we describe three anti-peptide antibodies (αP-1, αP-2, and αP-3) that specifically recognize the head and tail segments of the catalytic subunit (C) of PKA. (i) αP-1 (against ^{6}A-K^{23}) react with C when denatured but not when in its native structure; (ii) αP-2 (against ^{319}K-I^{335}), bind to the site in C cleaved by the kinase splitting membranal proteinase (KSMP) and inhibit this cleavage of C; (iii) αP-3 (against ^{338}S-F^{350}) react with C but not with the KSMP cleavage product C′, useful for detecting a KSMP-like activity in different tissues and subcellular loci. The combined use of the antibodies described here provides a strict definition of C, and thus a high degree of fidelity in its biorecognition.

Key words: Anti-peptide antibodies; Inhibitory antibodies; KSMP; Protein kinase A; Tails of protein kinases

1. Introduction

The catalytic subunit (C) of protein kinase A (PKA) [1,2] is regarded as a prototype for the large family of protein kinases [3,4], each having its own substrate specificity and a distinct mechanism of regulation. These kinases share a considerable sequence homology in a conserved catalytic core (residues 40–300 in PKA), which is flanked by a "head" and a "tail" segment found (respectively) at its amino- and at its carboxy-terminus (Fig. 1) [5–8]. Since the kinases differ significantly in the sequence of their head and tail segments, it seems reasonable to assume that these segments play an important structural and functional role, e.g., in forming the preferential affinity of each kinase for its physiological substrates or regulatory ligands.

We have previously identified a kinase splitting membranal proteinase (KSMP), which specifically cleaves C to yield a distinct clipped product C′ devoid of kinase activity [9–11]. The cleavage was shown to occur in the native structure of C, but not if C is pre-denatured [10,11], suggesting that KSMP recognizes the conformation of the kinase. More recently we have shown that the cluster of acidic amino acids at the carboxy-terminus tail of C (^{327}FDDYEEEEI^{335}) is the major biorecognition element for KSMP, since antiidiotypic monoclonal antibodies against a copolymer containing clusters of E and Y were found to inhibit KSMP [12]. A similar conformation-dependent cleavage by KSMP was shown to occur also in two other protein kinases which contain such clusters of acidic amino acids at their carboxy-terminus tail — the EGF receptor kinase, and the insulin receptor kinase [13,14], raising the possibility that the malleable tails at the carboxy terminus of these kinases may play an important role in their biorecognition and regulation [13–16]. This paper describes the preparation and characterization of anti-head and anti-tail antibodies against distinct epitopes in C, and their use in the specific identification of PKA and in the assay of KSMP by the determination of C and C′.

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Abbreviations: C, catalytic subunit of cAMP-dependent protein kinase; DAB, 3,3′-diaminobenzidine; KLH, keyhole limpet hemocyanin; KSMP, kinase-splitting membranal proteinase; C′, KSMP-cleaved C; PKA, protein kinase A (cAMP-dependent protein kinase).

2. Materials and methods

2.1. Preparation and assay of C

The C subunit of bovine PKA was purified as described by Reimann and Beham [17]. This purification involves chromatography on DE-52, affinity elution of C by cAMP, and chromatography on hydroxylapatite. To this procedure we added a purification step on a Mono S column (HR 5/5, Pharmacia, Sweden). The fractions with kinase activity obtained from the hydroxylapatite column were pooled, dialyzed against an equilibration buffer composed of 20 mM NaPi (pH 6.8), 1 mM EDTA and 1 mM DTT, and then applied on the Mono S column, which was pre-equilibrated with the same buffer. The loaded column was washed with the equilibration buffer, then eluted with a linear gradient of NaPi (20 mM to 200 mM, pH 6.8). This column yields two iso-forms of the C subunit: C_A and C_B [18,19]. The C_B isoform was used in this study, since it was previously shown that it constitutes a slightly better substrate for KSMP [20]. The enzyme was assayed as described previously [21] with the following modification: the phosphoprotein pellet formed by precipitation with trichloroacetic acid was washed on GF/C glass filters (Whatman, UK).

2.2. Preparation of the kinase splitting membranal proteinase (KSMP)

Brush-border membranes from rat kidney were purified by the procedure of Evers et al. [22], which is based on a precipitation of the membranes by Ca^{2+}-ions. This procedure yields a membrane preparation enriched with KSMP. This membrane preparation was solubilized with 1% octyl-β-D-glucopyranoside [10,11,23], the insoluble particles were removed by centrifugation at 100 000 × *g* for 30 min, and the resulting supernatant (protein concentration, 1 mg/ml) was used in the present study.

2.3. Assay of KSMP

KSMP was assayed as described previously [9,10] with the following modifications: the reaction mixtures used had a total volume of 20 μl and contained 1 μg of C, 100 ng of KSMP preparation in a buffer (pH 7.1) composed of Tris-HCl (20 mM), $MgCl_2$ (1.5 mM) and β-octyl-glucoside (0.2% w/v). The reaction was allowed to proceed at 23°C for the time indicated, and arrested by adding the sample buffer used for polyacrylamide gel electrophoresis in the presence of SDS, described

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by Laemmli [24], then boiling (5 min at 95°C). The gels used had usually a polyacrylamide gradient of 5 to 20%, and were stained with Coomassie blue (0.25%, w/v) in 50% (v/v) methanol and 7% (v/v) acetic acid.

2.4. Raising and purifying anti-peptide antibodies

The synthesized peptides were designated as follows: P-1, ^{6}AKKGSEQESVKEFLAKAK^{23}; P-2, ^{319}KGPGDTSNFDDYEEEEI^{335}; P-2′, ^{327}FDDYEEEEI^{335}, and P-3, ^{338}SINEKCGKEFSEF^{350}, where the numbers correspond to the positions of the amino acid residues in the C sequence. These peptides were synthesized on a solid-phase synthesizer, purified by reverse phase HPLC and analysed by amino acid composition or sequence before use. Three of these peptides (P-1, P-2 and P-3) were then cross-linked with KLH (Pierce, USA) as a carrier, and the resulting conjugates were used for immunization. The cross-linking and the protocol of injections were carried out as described by Harlow [25]. All the anti-peptide antibodies used in the present study were affinity purified as described below [25]. The immune sera obtained were fractionated by an ammonium sulfate precipitation (repeated twice), and the resulting IgG fractions were passed through a column with immobilized KLH to remove the anti-KLH specific antibodies. The resulting anti-KLH depleted IgG fractions were then affinity purified on the corresponding peptides immobilized on agarose beads. The elution from the KLH- and the peptide-columns was performed by Gly-HCl, pH 2.4, and by triethylamine, pH 11.5. The eluate from the KLH column was used as negative control.

2.5. Western blot analysis of C and C′

The transfer of proteins onto nitrocellulose paper and immunostaining were carried out as described in the literature [25]. Visualization of the C and C′ protein bands after SDS-PAGE was performed using horseradish peroxidase conjugated secondary antibodies (Sigma, USA), and either by the ECL detection system (Amersham, USA) or by the use of DAB (Sigma, USA) as a substrate. When the immunostaining was performed in the presence of different peptides, the peptides were preincubated with the first antibodies for 15–30 min at room temperature before applying on the nitrocellulose blots.

2.6. Preparation of C mutants

The wild type murine C_{α}-subunit gene was inserted into the pRSET-B vector (Invitrogen, USA) as described elsewhere (Yonemoto et al., submitted). It was a gift from Dr. Susan Taylor (University of California, San Diego). Site directed mutations were introduced by oligonucleotide-directed mutagenesis of a uracil-containing single-stranded Kunkel template [26]. The vectors harboring the mutated enzyme genes were used for the transformation of the *E. coli* BL21 (DE-3) strain. The conditions used for growing and for induction of the bacteria were described elsewhere [27,28]. After induction, the cells were collected, lysed in the SDS-PAGE loading buffer [24], and used for Western blot analysis.

2.7. Cell-free translation of C and mutants

The coupled transcription and translation in the rabbit reticulocyte lysate system (TNT system, Promega, USA) was used for cell-free expression of the wild type or mutated C-subunit. pRSET expression vector (Invitrogen, USA), carrying the genes of the wild type or the mutated kinase, was used as a template. All the manipulations were done according to the Promega TNT system protocol. The translation products were labeled by [^{35}S]methionine (Amersham, UK) in the course of the reaction, then separated by SDS-PAGE and visualized by autoradiography.

2.8. Immunoprecipitation of in vitro translated C

The immunoprecipitation was performed as described by Harlow [25] with the following modifications: Affinity purified antipeptide or control antibodies (1 μg per assay) were preincubated with 5 μl of Protein A sepharose (Pharmacia, Sweden) in a final volume of 20 μl for 30 min at 4°C. The resin was washed three times with PBS with 0.05% Tween 20, and resuspended in 20 μl of the same buffer. To this resin 1 μl of TNT-translation mixture, containing [^{35}S]methionine-labelled C, was added and incubated for 1 h at room temperature with agitation. The resin was washed again three times with PBS with 0.05% Tween 20, two times with 20 mM Tris-HCl pH 7.1, and then resuspended in the SDS-PAGE loading buffer mentioned above [24].

3. Results and discussion

3.1. Choice of epitopes

The purpose of this study was (a) to obtain antibodies that would specifically recognize the C subunit of PKA, rather than other members of the protein kinase family, and refrain from cross-reacting with other protein kinases which share a considerable sequence homology with the catalytic core of C (residues 40–300) [3,4]), and (b) to obtain a set of antibodies with which it would be possible to detect both C and C′ and thus to detect and localize a KSMP-like activity in different cells and tissues, so as to assess the possible involvement of such an enzyme in the regulation of PKA. For this purpose we synthesized three peptides whose sequences were identical to three epitopes in C; one in the head of the enzyme and two in its tail (Fig. 1A).

The peptides had the following structures: P-1, ^{6}AKKGSEQESVKEFLAKAK^{23}; P-2, ^{319}KGPGDTSNFDDYEEEEI^{335}; and P-3, ^{338}SINEKCGKEFSEF^{350}. Each of them was purified by reverse phase HPLC, analysed, then cross-linked with KLH and used for immunization. The anti-peptide antibodies were then affinity purified as described below on a column containing the respective immobilized peptide. As seen in the example depicted in Fig. 1B, all three antibodies (αP-1, αP-2, and αP-3) reacted specifically with C on SDS-PAGE gels of total cell lysates.

3.2. Specificity of the αP-1, αP-2, and αP-3 antibodies

One of our major objects in the preparation of these antibodies was to be able to identify both C and its KSMP cleavage product C′. Since the substrate recognition region of KSMP was previously shown to reside mainly in the cluster of acidic amino acids (^{327}FDDYEEEEI^{335}) found at the carboxy-terminus tail of C [12], we expected that while αP-1 would recognize and stain both C and C′, αP-3 antibodies (which were raised against an epitope downstream from the cluster of acidic amino acids) would do so with C but not with C′. Indeed, this was found to be the case: when C was partially cleaved by KSMP to create a mixture of C and C′ and the components in the reaction mixture were stained with Coomassie blue (to stain both C and C′) or with αP-1 and αP-3 (to stain the head and the tail of C) it was found that αP-1 stains both C and C′ while αP-3 stains C but not C′ (Fig. 2A). Unexpectedly, the αP-2 antibodies, which were designed to recognize in C the binding site of KSMP [12], as well as the specific KSMP cleavage site (^{332}E-E^{333}) [29] stained also C′ (see Fig. 1A and 2A). To get an insight into this apparent discrepancy, we studied the effect of some of the peptides on the staining of C by αP-2. As seen in Fig. 2B, the peptide P-2, at concentrations $>10^{-7}$ M completely inhibited the binding of αP-2 to C, while the peptide P-2′ (^{327}FDDYEEEEI^{335}) failed to do so up to a concentration of 10^{-4} M. In that respect it was similar to the head peptide P-1 (see Fig. 1A and 2B) which also afforded C with no protection from staining by αP-2. This result suggests that the immuno-dominant moiety in the peptide resides in the stretch ^{319}KGPGDTSN^{326} rather than the stretch ^{327}FDDYEEEEI^{335}.

3.3. Deletion mutants of C confirm the specificity of αP-2

For further analysis of the specificity of the αP-2 antibodies we constructed three deletion mutants of C lacking different fragments of the designed epitope of the αP-2 antibodies.

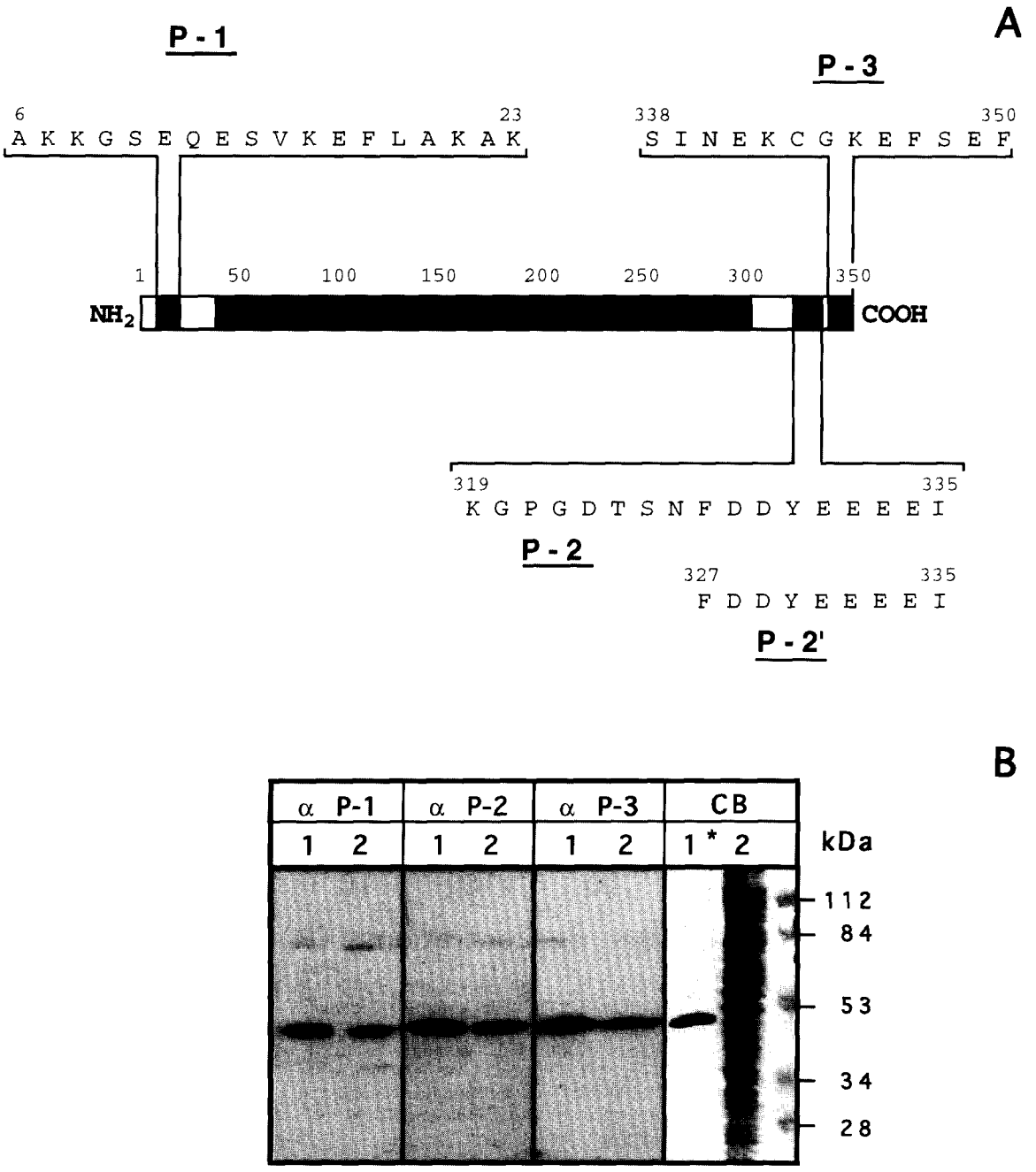

Fig. 1. Design of synthetic peptides and the specificity of the raised anti-peptide antibodies. (A) Sequence of the peptides used for the raising of epitope-specific antibodies against the C subunit of PKA. The gray part represents the conserved core of protein kinases (residues 40–300). The sequence and location in C of the head and tail peptide segments used for the preparation of the anti-peptide antibodies are indicated and designated P-1, P-2, P-3. A fragment peptide of P-2 with the indicated sequence (used in inhibition studies) is designated P-2′. (B) Specificity of the anti-peptide antibodies. A Western blot analysis was carried out with samples of purified bovine C (20 ng protein per lane) without (lanes marked 1), or with (lanes marked 2) 20 μg of a protein mixture obtained from a crude lysate of 293 cells. The panel marked CB was stained with Coomassie blue, and the other panels were stained each with the indicated antibody. In the CB panel lane 1* contained 0.5 μg (rather than 20 ng) of purified bovine C to allow visualization with CB.

These mutants of C had deletions of the following segments: ^{319}K-F^{327} (Δ9), ^{319}K-D^{323} (Δ5), and ^{324}T-F^{327} (Δ4) (Fig. 3A) These mutant C subunits were expressed in *E. coli* as described in section 2 [27,30], then subjected to a Western blot analysis staining with either αP-2 or αP-3. As seen in Fig. 3B, all of these deletion mutants interacted with αP-3, but none of them interacted with αP-2. Since these deletions in C were found to prevent the recognition of both C and C′ by αP-2, and since these findings are in agreement with the results described above regarding the inhibition of the interaction between αP-2 and C by peptides, it seems reasonable to conclude that the segment ^{319}K-F^{327} in C is indeed the immunodominant determinant of the αP-2 antibodies.

3.4. αP-2 antibodies inhibit the KSMP cleavage of C

In view of the specificity established above for the αP-2

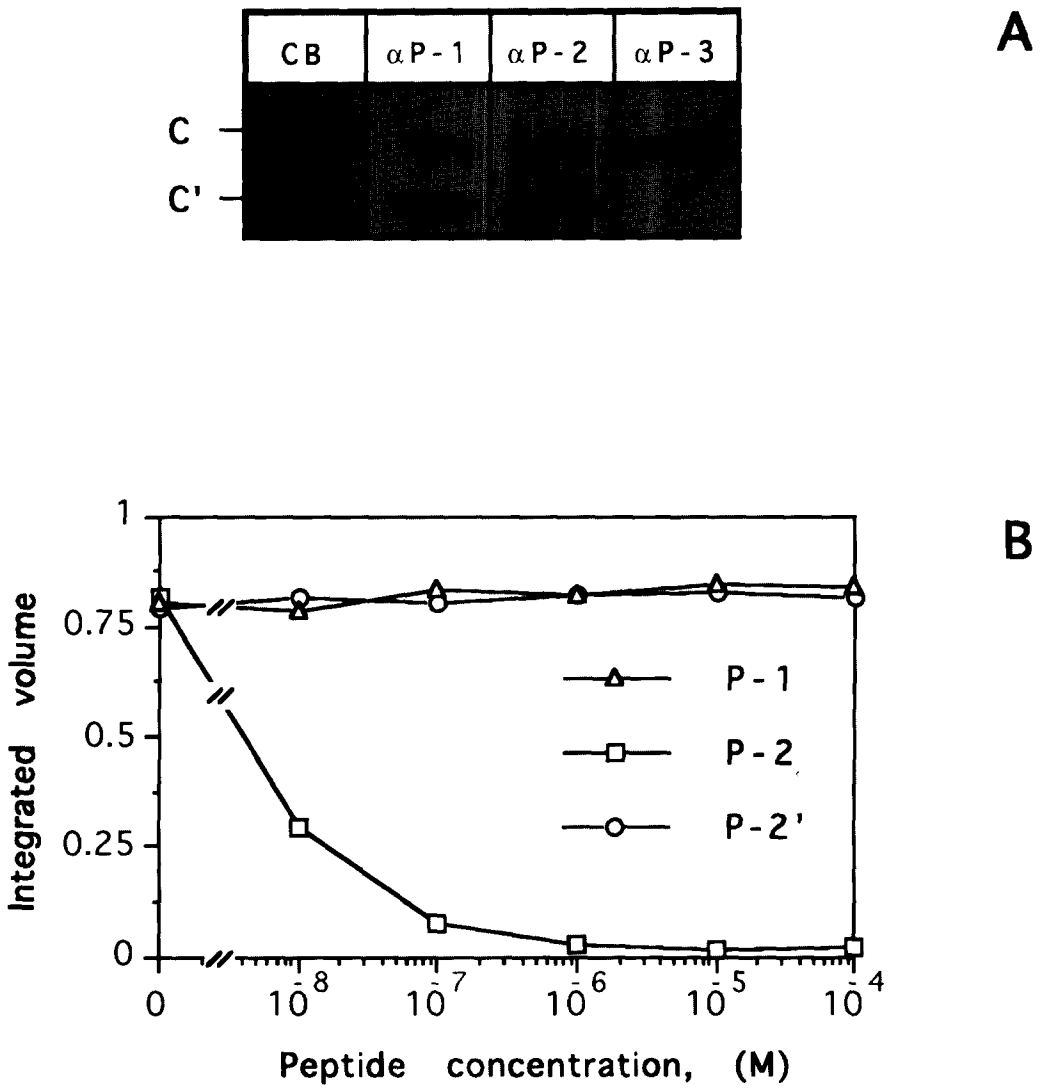

Fig. 2. Specificity of the anti-peptide antibodies. (A) Western blot of C and C′ after SDS-PAGE, developed with the indicated antibodies. A preparation of pure bovine C was subjected to proteolysis by KSMP under conditions yielding a ~50% degradation of the kinase. Aliquots from the resulting reaction mixture (50 ng protein for immunodetection, and 0.5 μg protein for staining with Coomassie blue (CB)) were subjected to SDS-PAGE, blotted onto nitrocellulose, and stained with the indicated antibodies (αP-1, αP-2, and αP-3). (B) Quantitative assessment of the αP-2 inhibition of the staining by the synthetic peptides indicated. A Western blot of the C preparation (bovine, 50 ng per lane) was stained by αP-2 antibodies that were preincubated (22°C, 30 min) with the indicated concentrations of P-1, P-2, and P-2′. The blot was developed by an ECL detection system, and the relative cross-reactivity with C was assessed by the integrated volume of the stained bands determined by computer densitometry scanning of the film.

antibodies, it was of interest to find out whether they could be used as inhibitors of the KSMP cleavage of C since they could sterically hinder the access of KSMP to the site of cleavage. Indeed, we found that while αP-1 did not affect the cleavage at all (the rate was identical to that observed with the αKLH antibodies, results not shown), the αP-3 antibodies slowed the rate of the KSMP cleavage by about 50%. However, the αP-2 antibodies were found to block the KSMP cleavage (Fig. 4), a finding which will be useful in assessing the consequences of the KSMP cleavage of C and thus its physiological significance.

3.5. *Accessibility of the P-1, P-2 and P-3 epitopes to the corresponding antibodies when C is in its native structure*

As seen in Fig. 2A, all three epitopes chosen in this study (P-1, P-2 and P-3) are accessible to interaction with the corresponding antibodies (αP-1, αP-2, and αP-3) when C is in its unfolded (denatured) state, i.e., when C is stained by the antibodies after SDS-PAGE (Fig. 2A) However, when C is in its folded and catalytically active state, two of the antibodies react with C (αP-2, and αP-3) and one (αP-1) does not. As seen in Fig. 5A, when the cDNA of C was translated in a rabbit reticulocyte lysate system in the presence of [^{35}S]methionine and the ^{35}S-labeled enzyme was subjected to immunoprecipitation with αP-1, αP-2, αP-3, or αKLH, an immunoprecipitate was obtained with either αP-2 or αP-3 but not with αP-1 (the ^{35}S-labeled precipitate obtained in this case was identical to that obtained with the antibodies against the carrier protein, KLH). The failure of the αP-1 antibodies to precipitate C in its native structure, contrasted with the ability to stain C if it is pre-denatured, may be due to the fact that the P-1 epitope in the native structure of the

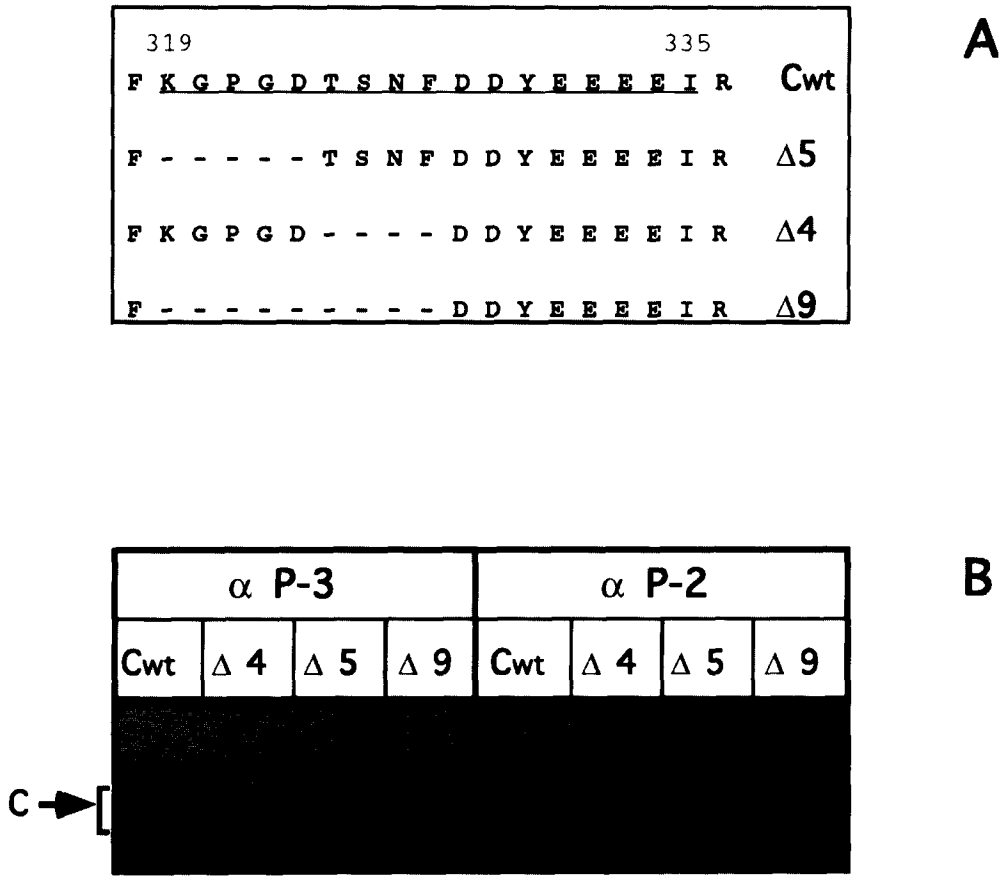

Fig. 3. Elucidation of the specificity of the αP-2 antibodies by immunostaining of deletion mutants of C. (A) Structure of the C deletion mutants used. Dashes indicate deleted amino acid residues; Cwt represents the wild type C structure; Δ4, Δ5, and Δ9 represent the structure of deletion mutants of C lacking (respectively) the 4, 5, or 9 amino acid residues indicated. The underlined region in the Cwt structure corresponds to the sequence of the P-2 peptide that was used for the raising of the αP-2 antibodies. (B) Western blot analysis of the deletion mutants mentioned in the above panel. The mutants were expressed in *E. coli*, and the whole bacterial lysate (5 μg) was subjected to SDS-PAGE and blotted onto nitrocellulose. The immunodetection was performed with the indicated antibodies (αP-3 and αP-2). The arrow (marked "C") indicates the position of Cwt, or of the indicated C mutants.

enzyme (residues 6–23) may be inaccessible to interaction with the antibodies due to steric restrictions. In fact, this epitope is part of the A-helix which, in the mammalian enzyme structure, is firmly anchored to the core of the enzyme, contributing V15, F18 and L19 to the formation of the hydrophobic pocket accommodating the myristyl chain and anchoring it tightly to the enzyme [8].

3.6. Concluding remarks

The αP-1 antibodies react equally well with C and with KSMP-cleaved C (C') when in the denatured state (e.g. on SDS-PAGE gels). However, they fail to react with C when it is in its native structure. Since the P-1 epitope (in helix A) has, at least in the myristylated mammalian enzyme, an important structural role in creating the hydrophobic pocket which accommodates the myristyl chain [8], and since the conformation of this site may be altered if the myristyl chain is to be pulled out to allow the kinase to anchor itself to a hydrophobic membrane, these antibodies may become useful for monitoring such conformational changes. In this context it should be mentioned that, in the non-myristylated recombinant enzyme, the first 14 amino acid residues are not visible in the crystals [5,6], probably because of a loosening in the structure.

The αP-2 antibodies, which are shown here to be specific to the ^{319}K-F^{327} segment in C (very close to the KSMP cleavage site of the kinase, ^{332}E-E^{333} [29]), act as an effective inhibitor of the cleavage. Thus they are useful for monitoring the con-

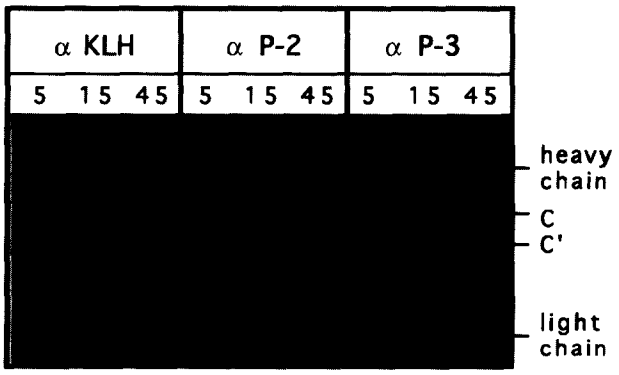

Fig. 4. Inhibition of the KSMP cleavage of C by αP-2 and αP-3. A preparation of bovine C (0.5 μg) was preincubated (22°C, 30 min) with the indicated antibodies (2 μg), and then a KSMP cleavage was carried out (22°C, pH 7.1) for the indicated times (5, 15 and 45 min). αKLH antibodies were used as a control for non-specific interaction. "Heavy chain" and "light chain" indicate (respectively) the position of the IgG heavy and light chains.

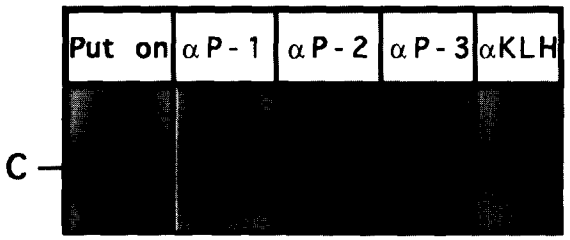

Fig. 5. Biorecognition of the native conformation of C by the anti-peptide antibodies described in this study. The immunoprecipitation of native [^{35}S]methionine-labeled C, translated in a rabbit reticulocyte translation mixture, was carried out using the indicated antibodies (αP-1, αP-2, αP-3, and αKLH). Each antibody (1 μg) was bound to 5 μl of protein A sepharose, then 1 μl of the rabbit reticulocyte translation mixture was added to the immunosorbent. "Put on" indicates the starting material (1 μl of the standard translation mixture) from which the C was immunoprecipitated. "C" indicates the position of intact native C.

sequences of blocking the cleavage and for gaining an insight into the possible physiological role of KSMP.

The αP-3 antibodies, which are shown above to react with C but not with C′, provide us with the means of identifying the cleavage product of C by KSMP (C′). This inactive cleavage product would stain with αP-1 but not with αP-3 and could be used to indicate the possible presence of KSMP in a certain tissue or at a given cellular locus.

As a group, the anti-head and anti-tail antibodies described here provide us with a useful tool for the study of PKA itself, its conformational changes, and its possible regulation by a KSMP-like proteinase. Being epitope-specific antibodies based on distinct sequences outside the core of C (which shares a high homology with all members of the protein kinase family [3]), these antibodies are bound to be specific to C. Furthermore, since they are directed against two distal segments of the molecule (the N-terminus and the C-terminus), their combined use provides a more restricted definition of C and thus a greater degree of fidelity in biorecognition.

Acknowledgements: This work was supported by the Ministry of Science (Israel) with the GSF Nürenberg (Germany); by the Minerva Foundation, Munich (Germany); and by the Ministry of Health (Israel). Partial support was also obtained from the Ebner Family Biomedical Research Foundation, the Henri Gutwirth Fund, and the Simone Mallah Fund at the WIS. S.Sh. is the incumbent of the Kleeman Professorial Chair in Biochemistry at the WIS.

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