Heterogeneity of rat liver cytosol casein kinase 2

Association between the α/α' -subunits of casein kinase 2 and the phosphorylatable protein pp49

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Casein kinase 2 activity could be resolved into three peaks by chromatography on DEAE-Sepharose. The peak eluted at high salt concentrations (casein kinase 2b) showed molecular and kinetic properties typical of the heterotetramer composed of α - (or α' -) and β -subunits. In contrast, the peak that was eluted at low salt concentrations (casein kinase 2a) contained no β -subunit but a phosphorylatable protein of 49 kDa (pp49), in addition to the α/α' -subunits. The presence of $\alpha/\alpha'/\alpha''$ -subunits in preparations of casein kinases 2a and 2b was confirmed by immunological assays. Casein kinase 2a had low specific activity and a very high apparent K_m for β -casein. The peak eluted at intermediate ionic strength contained the α/α' -subunits and variable amounts of β -subunit and pp49, and had kinetic properties intermediate between those of casein kinases 2a and 2b. Experiments based on heat inactivation, inhibition by low concentrations of heparin and ability to use GTP as substrate suggested that phosphorylation of pp49 was catalysed by the α/α' -subunits of casein kinase 2. No similarities were observed in the phosphopeptide maps of pp49 and β -subunit. These results show that the α/α' -subunits of rat liver cytosol casein kinase 2 can form complexes not only with the β -subunit but also with pp49, and that the complexes containing pp49 have a reduced affinity for the exogenous protein substrate β -casein.

INTRODUCTION

Casein kinases are cyclic nucleotide-independent and Ca^{2+} /calmodulin-insensitive protein kinases that show a remarkable preference to phosphorylate acidic substrates, such as casein or phosvitin, compared with basic substrates, such as histones. There are two types of casein kinases (casein kinase 1 and casein kinase 2), which differ in their chromatographic behaviour, molecular structure and kinetic properties [1].

Casein kinase 2 is a ubiquitous enzyme, which has been found in animal tissues, plants and yeasts. The possible importance of casein kinase 2 is indicated by the wide variety of its protein substrates, which include hormone receptors, regulatory enzymes and proteins involved in the control of gene expression and cell proliferation [2]. Casein kinase 2 is characterized *in vitro* by its ability to use GTP as well as ATP as a nucleotide substrate, and by inhibition by low concentrations of heparin [1]. However, the factors that control its activity *in vivo* remain obscure.

Casein kinase 2 isolated from most sources is an oligomeric enzyme with two dissimilar subunits, α (or α') and β , which are combined in $\alpha_2\beta_2$ or $\alpha\alpha'\beta_2$ complexes. The α/α' -subunits possess the catalytic site and are related polypeptides. The possibility that the α' -subunit is derived from the α -subunit by proteolysis or other chemical modifications has been suggested [3]. However, in yeast they are products of distinct but related genes [4], and there is also evidence that the α -subunit of human [5] and *Drosophila* [6] casein kinases 2 might each be codified by two genes.

The 24–28 kDa β -subunit, which is rapidly autophosphorylated *in vitro*, has an amino acid sequence that shows no similarity to that of any other known protein [4,7,8]. The β -subunit seems to be required for the enzyme to express maximal casein kinase activity [9] and affects the enzyme stability [10]. Furthermore, different types of experiments *in vitro* [11,12] as well as studies on the effects *in vivo* of epidermal growth factor indicate that autophosphorylation and/or transphosphorylation of the β -subunit affects the activity of casein kinase 2 [13].

In the present work evidence was obtained on the existence of

forms of casein kinase 2 as complexes between the α/α' -subunit and a pp49 protein, in addition to well-known complexes of α/α' - and β -subunits, and that the association of pp49 with the α/α' -subunits markedly reduces the affinity of the enzyme for the protein substrate.

EXPERIMENTAL

Materials

Staphylococcal V8 proteinase, ATP, GTP, β -casein, spermine, 3-dimethylaminobenzoic acid, 3-methyl-2-benzothiazolinone hydrazone and 3-amino-9-ethylcarbazole were from Sigma Chemical Co. Immobilon-P transfer membranes were from Millipore. Acrylamide was obtained from Fluka. Other reagents for electrophoresis and horseradish-peroxidase-conjugated goat anti-[rabbit IgG (H+L)] antibody were from Bio-Rad Laboratories. $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and $[\gamma\text{-}^{32}\text{P}]\text{GTP}$ were prepared from $[\text{}^{32}\text{P}]\text{orthophosphate}$ (New England Nuclear) as described in ref. [14]. The sources of other chemicals have been indicated previously [15].

Purification of casein kinase 2

Casein kinase 2 was purified from rat liver cytosol by the method of Itarte *et al.* [16], with the following modifications. Pooled fractions from the first phosphocellulose chromatography corresponding to maximum specific casein kinase activity were adjusted to 60% saturation with solid $(\text{NH}_4)_2\text{SO}_4$, and, after stirring for 30 min, the precipitate was collected by centrifuging at 20000 *g* for 30 min at 4 °C. The sediment was resuspended in 3 ml of 50 mM-Tris/HCl buffer, pH 7.5, containing 1 mM-dithiothreitol, 5% (v/v) glycerol, 0.1 mM-phenylmethanesulphonyl fluoride and benzamidine (60 $\mu\text{g}/\text{ml}$) (buffer A), dialysed extensively against buffer A containing 0.4 M-KCl and applied to a Bio-Gel A-0.5 m column (1 cm $\times$ 150 cm) equilibrated with the same buffer.

Fractions with casein kinase 2 activity were pooled, dialysed against buffer A and applied to a DEAE-Sepharose CL-6B column (1 cm $\times$ 2 cm) equilibrated with buffer A. After washing the column with 5 ml of buffer A and 25 ml of buffer A containing

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Fig. 1. Chromatography of casein kinase 2 on Bio-Gel A-0.5 m

(a) Samples from phosphocellulose were pooled and concentrated by $(\text{NH}_4)_2\text{SO}_4$ precipitation. After dialysis, the sample was applied to a Bio-Gel A-0.5 m column (1 cm $\times$ 150 cm) equilibrated with buffer A containing 0.4 M-KCl. Fractions (2.7 ml each) were collected and assayed for casein kinase activity with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (○) or $[\gamma\text{-}^{32}\text{P}]\text{GTP}$ (●) as phosphodonor. Protein concentration was determined by the absorbance at 280 nm (▲). The intensities of the bands corresponding to pp49 (▼) and to β -subunit (■), determined as indicated in (b), are represented in arbitrary units. (b) The indicated fractions were incubated with $[\gamma\text{-}^{32}\text{P}]\text{GTP}$ for 20 min as described in the Experimental section, electrophoresed in a 0.1 % (w/v) SDS/12.5 % polyacrylamide gel and autoradiographed.

0.1 M-KCl, a 10 ml/10 ml gradient of buffer A containing 0.1 M-KCl/buffer A containing 0.6 M-KCl was applied.

Samples with casein kinase 2 activity were pooled, dialysed against buffer A and applied to a 1 ml heparin-agarose column equilibrated with the same buffer. This column was washed with 2 ml of buffer A, then 4 ml of buffer A containing 0.2 M-KCl, and the enzyme was eluted with 8 ml of buffer A containing 0.5 M-KCl. Samples with casein kinase 2 activity were dialysed against buffer A and stored at -20°C until use.

Assays

Casein kinase activity was determined essentially as described previously [17]. One unit of casein kinase activity is defined as the amount that catalyses the transfer of 1 nmol of phosphate from $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ to casein (4 mg/ml) per min at 30°C . The apparent K_m values for casein kinase 2 were estimated from Hill plots as in ref. [17]. Cyclic AMP-dependent protein kinase activity was determined as indicated by Huang & Robinson [18]. Histone H1 kinase activity was determined as described by Quirin-Stricker & Schmitt [19].

Protein concentration was determined as described by Bradford [20], with BSA as standard.

Electrophoresis

(a) Denaturing gels. PAGE in the presence of 0.1 % (w/v) SDS was carried out by the method of Laemmli [21], with 12.5 % acrylamide in the separating gel and 5 % acrylamide in the stacking gel.

In some cases the samples were incubated previously with 25 μM - $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (30 $\mu\text{Ci}/\text{nmol}$) or 25 μM - $[\gamma\text{-}^{32}\text{P}]\text{GTP}$ (30 $\mu\text{Ci}/\text{nmol}$). The phosphorylation was stopped by addition of sample buffer. After electrophoresis the gels were dried and exposed to Kodak X-OMAT film. The intensity of the bands was quantified by densitometry.

(b) Non-denaturing gels. Samples of casein kinases were phosphorylated with GTP as phosphodonor for 15 min at 30°C . After the incubation time, the reaction was stopped with non-denaturing sample buffer containing 0.25 M-Tris, 200 mM-NaF, 40 mM-EDTA, 10 % (v/v) glycerol and 0.01 % (w/v) Bromophenol Blue (final concentrations). Samples were run in 1 % (w/v) agarose gels with 50 mM-Tris/glycine buffer, pH 8.5, as elution buffer at 150 V for 45 min. After electrophoresis, the gel was dried and subjected to autoradiography. The bands were excised, treated with denaturing sample buffer and electrophoresed as indicated above.

Phosphopeptide maps

Samples of casein kinase 2 were autophosphorylated and then applied to a 0.1 % (w/v) SDS/12.5 % polyacrylamide gel (15 cm $\times$ 20 cm) in order to separate pp49 and the β -subunit by electrophoresis. The gel was dried and autoradiographed. The strips corresponding to pp49 and β -subunit were cut out, equilibrated with 125 mM-Tris/HCl buffer, pH 6.8, containing 0.1 % (w/v) SDS and then applied to a 0.1 % (w/v) SDS/10–20 % polyacrylamide gradient gel (15 cm $\times$ 20 cm) for electrophoresis. A 2 μg portion of staphylococcal V8 proteinase was added to each track. The digestion was done as described in ref. [22].

Immunological assays

The anti-(casein kinase 2) antibody used was from Dr. M. Pfaff. This antibody is an IgG fraction from a polyclonal antibody raised in rabbits against casein kinase 2 from mouse L210 cells [23].

(a) Immunoblotting. Samples of casein kinases 2a, 2b and 2a/2b were electrophoresed in 0.1 % (w/v) SDS/12.5 % polyacrylamide gels and the resolved bands were transferred on to Immobilon-P transfer membranes in 3 h at 150 V. The transfer buffer contained 39 mM-glycine, 48 mM-Tris, 0.0375 % (w/v) SDS and 20 % (v/v) methanol.

After the transfer, the membranes were treated with 3 % (w/v) BSA in phosphate-buffered saline (0.14 M-NaCl/12 mM-sodium phosphate buffer, pH 7.2) for 1 h at room temperature. Membranes were then incubated for 2 h at room temperature with the anti-(casein kinase 2) antibody diluted with 1 % (w/v) BSA/0.05 % (v/v) Tween 20 in phosphate-buffered saline. After being washed five times for 5 min with 0.1 % (v/v) Tween 20 in phosphate-buffered saline, membranes were treated for 2 h at room temperature with horseradish-peroxidase-conjugated goat anti-[rabbit IgG (H+L)] antibody diluted in 1 % (w/v) BSA/0.05 % (v/v) Tween 20 in phosphate-buffered saline (1:2000). Membranes were washed five times for 5 min with 0.1 % (v/v) Tween 20 in phosphate-buffered saline and then incubated with 3-amino-9-ethylcarbazole and H_2O_2 .

(b) E.I.s.a. Samples of casein kinases 2a, 2b and 2a/2b, diluted in 0.1 M-carbonate/bicarbonate buffer, pH 9.6, were incubated in micro-titre polystyrene plates overnight at room temperature in a humid atmosphere. The e.i.s.a. was developed under standard conditions with the use of 1 % (w/v) BSA/0.05 %

Fig. 2. Chromatography of casein kinase 2 on DEAE-Sepharose

(a) Fractions eluted from DEAE-Sepharose were assayed for casein kinase (CK) activity with [γ - 32 P]ATP as phosphodonor ($\circ$) and the protein concentration was determined by the absorbance at 280 nm ($\blacktriangle$). The intensities of the bands corresponding to β -subunit ($\blacksquare$) and pp49 ($\blacktriangledown$) determined as in (b) are represented in arbitrary units. (b) Samples of indicated fractions have been incubated with [γ - 32 P]ATP before electrophoresis in a 0.1% (w/v) SDS/12.5% polyacrylamide gel, and autoradiographed. (c) The indicated fractions were incubated with [γ - 32 P]GTP alone (panel 1) or in the presence of 1.13 units of casein kinase 2b/ml, before analysis as in (b).

(v/v) Tween 20 in phosphate-buffered saline to block the unspecific binding. The anti-(casein kinase 2) antibody was diluted with 1% (w/v) BSA/0.05% (v/v) Tween 20 in phosphate-buffered saline (1:7000). Horseradish-peroxidase-conjugated goat anti-[rabbit IgG (H+L)] antibody was diluted with 1% (w/v) BSA/0.05% (v/v) Tween 20 in phosphate-buffered saline (1:2000), and 3-dimethylaminobenzoic acid, 3-methyl-2-benzothiazolinone hydrazide and H_2O_2 were added to detect the antigen-antibody complexes.

RESULTS

Co-purification of casein kinase 2 and a 49 kDa phosphoprotein

During the purification of casein kinase 2 it was observed that the enzyme was eluted from phosphocellulose together with several phosphorylatable proteins (results not shown). When this

enzyme preparation was subjected to chromatography on Bio-Gel A-0.5 m a clear overlapping was observed between the casein kinase activity, determined with either ATP or GTP, and autophosphorylation of the 28 kDa β -subunit of casein kinase 2. In addition, an overlapping of a 49 kDa phosphoprotein (designated pp49) and the casein kinase 2 activity eluted in the retarded fractions was also detected in this chromatography (Fig. 1).

The fractions containing casein kinase 2 activity were pooled and subjected to DEAE-Sepharose chromatography. This step resolved casein kinase 2 activity into two major peaks (Fig. 2). The first one (casein kinase 2a/2b) contained both β -subunit and pp49 protein, whereas the second peak (casein kinase 2b) was enriched in β -subunit. A small peak of casein kinase activity eluted in the first fractions of the gradient was also consistently observed. This peak (casein kinase 2a) contained pp49 and very low amounts of β -subunit. Addition of exogenous casein kinase 2b to the initial fractions of the gradient lacking casein kinase

Fig. 3. Analysis of casein kinases 2a, 2a/2b and 2b obtained after heparin-agarose chromatography

(a) Samples of casein kinase 2a (track 1), casein kinase 2b (track 2) or intermediate casein kinase (track 3), purified by heparin-agarose chromatography, were incubated with [γ - 32 P]ATP, electrophoresed in a 0.1% (w/v) SDS/12.5% polyacrylamide gel and then stained with Coomassie Brilliant Blue. (b) Autoradiography of the gel shown in (a). On the right hand positions of molecular-mass markers are indicated in kDa. (c) Samples of casein kinase 2b (track 1) and casein kinase 2a/2b (track 2) were phosphorylated in the presence of [γ - 32 P]GTP, then subjected to non-denaturing electrophoresis in 1% (w/v) agarose gel as indicated in the Experimental section, and the bands were detected by autoradiography. (d) The phosphorylated bands from the non-denaturing gel were excised and electrophoresed in 0.1% (w/v) SDS/12.5% polyacrylamide gel. On the right hand positions of molecular-mass markers are indicated in kDa.

Fig. 4. Immunoblot analysis of casein kinases 2b, 2a/2b and 2a

Casein kinase 2b (track 1), casein kinase 2a/2b (track 2) and casein kinase 2a (track 3), purified by heparin-agarose chromatography (1 μ g each), were electrophoresed in a 0.1% (w/v) SDS/12.5% polyacrylamide gel, electrotransferred to Immobilon-P, as described in the Experimental section, and treated with anti-(casein kinase 2) antibodies that were diluted 600-fold in (a) and 300-fold in (b).

activity, before the incubation with [γ - 32 P]ATP, did not reveal the presence of phosphorylated pp49 (Fig. 2c). A better resolution between pp49 and β -subunit was achieved when only the trailing fractions from Bio-Gel A-0.5 m chromatography were applied to

the DEAE-Sepharose column and a shallow gradient was used (results not shown).

The fractions corresponding to each of the peaks showing casein kinase activity were pooled and chromatographed on heparin-agarose. This step did not achieve any further resolution between the casein kinase activity, pp49 and/or β -subunit in samples corresponding to the different peaks of casein kinase 2 (results not shown).

Fractions from heparin-agarose were pooled, dialysed against buffer A and stored at -20°C until use.

The contribution of casein kinase 2a was only 2% of the total casein kinase 2 activity recovered. The peaks corresponding to casein kinase 2b and casein kinase 2a/2b accounted for the remaining activity in similar amounts.

Characteristics of purified casein kinases

As shown in Fig. 3, the final preparations of casein kinases 2a, 2a/2b and 2b contained the α - and α' -subunits but were distinct in that casein kinase 2a contained pp49 but no detectable β -subunit, whereas casein kinase 2b was free of pp49 and contained the β -subunit, as determined by either Coomassie Blue staining or autoradiography. Both pp49 and β -subunit were present in samples of casein kinase 2a/2b.

The existence of a rather strong association between pp49 and casein kinase 2 was also suggested by the co-migration of this

Table 1. Summary of the structural and kinetic properties of casein kinases 2a and 2b

Property	Casein kinase 2a	Casein kinase 2b
Molecular structure:		
Components	α_T -Subunit + pp49	α_T -Subunit + β -subunit
Molar ratio	1:0.90 $\pm$ 0.26 (8)	1:0.79 $\pm$ 0.06 (8)
Phosphorylatable component detected	pp49	β -Subunit
Kinetic properties:		
Specific activity (units/mg of protein)	11.9 $\pm$ 3.4 (8)	200.3 $\pm$ 15.1 (7)
GTP/ATP activity ratio	0.77 $\pm$ 0.08 (8)	0.71 $\pm$ 0.06 (7)
Apparent K_m value for:		
ATP (μ M)	6.2 $\pm$ 1.3 (5)	8.6 $\pm$ 0.5 (5)
β -Casein (mg/ml)	> 5	0.29 $\pm$ 0.03 (6)

Fig. 5. Effect of heat treatment on casein kinase 2a

A sample of casein kinase 2a with a specific activity of 16.5 units/mg was incubated at 80 °C (a) or at 50 °C (b and c). At indicated times samples were removed and assayed for endogenous phosphorylation with $[\gamma\text{-}^{32}\text{P}]\text{GTP}$ (a and b) or for casein kinase 2 activity with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (○) or with $[\gamma\text{-}^{32}\text{P}]\text{GTP}$ (●) (c). In tracks 10+ and 14+ of (a) and tracks 30+ and 60+ of (b), casein kinase 2b was added (2 units/ml in the assay). Tracks C correspond to casein kinase 2b alone.

band and the β -subunit in non-denaturing gel electrophoresis (Figs. 3c and 3d).

Densitometric analysis of the stained gels allowed estimation of the molar proportions of the different components. With each type of casein kinase the molar ratios of α' -subunit to α -subunit varied with the different preparations in the range 0–2:1. These changes were not reflected in significant differences in the specific activity of the enzyme. Thus all those subunits were considered together (α_T), as indicated in previous studies by other groups [1].

Both the α -subunit and the α' -subunit as well as a third α'' -subunit present in samples of casein kinases 2a, 2b and 2a/2b were recognized by the anti-(casein kinase 2) antibody (Fig. 4). The presence of an α'' -subunit has also been reported previously in samples of casein kinase 2 from different sources [24,25]. Increasing the amount of antibody used in the assays led to detection of the β -subunit in the samples of casein kinases 2b and 2a/2b but no band was observed in casein kinase 2a fractions.

As indicated in Table 1, the molar ratio of β -subunit to α_T -

subunits detected by densitometry of Coomassie Blue-stained gels was 0.79:1 for casein kinase 2b. This indicated that the enzyme corresponded to the typical $\alpha_2\beta_2$ form of casein kinase 2. Densitometric analysis of different samples of casein kinase 2a indicated that the enzyme existed as a 1:1-molar-ratio complex of α_T -subunits and pp49.

The catalytic properties of casein kinases 2a and 2b were then studied. No cyclic AMP-dependent protein kinase activity or histone H1 kinase activity was detected in either enzyme preparation. Both casein kinases had a similar apparent K_m value for ATP and were also able to use GTP as phosphodonor. Casein kinase activities of fractions 2a, 2b and 2a/2b were stimulated 1.3–1.8-fold by 2.5 mM-spermine. However, they were clearly different in their apparent K_m values for β -casein. In fact, it was not possible to determine the value of this parameter for casein kinase 2a since the activity of this enzyme still increased considerably within the highest range of β -casein concentrations used in the assays (up to 10 mg/ml). In contrast, casein kinase 2b

Fig. 6. Inhibition of pp49 phosphorylation by heparin

Autophosphorylation of casein kinase 2a/2b, with a specific activity of 172 units/mg, was assayed with [γ - 32 P]GTP as a substrate, in the presence of the indicated amounts of heparin.

showed a much lower apparent K_m value. The extremely high K_m value of casein kinase 2a could be the reason for its low specific activity as compared with that of casein kinase 2b determined with 4 mg of β -casein/ml.

Casein kinase 2a/2b preparations that contained different amounts of pp49 and β -subunit differed in their specific activity and apparent K_m for β -casein. As an example, a preparation of casein kinase 2a/2b with a β -subunit/pp49 molar ratio of 0.75:1 had an specific activity of 112 units/mg of protein and an apparent K_m value for β -casein of 1.71 ± 0.29 mg/ml. Thus we consider the preparations of casein kinase 2a/2b to be intermediate forms or a mixture of casein kinases 2a and 2b.

The amount of α -subunit(s) present in each type of casein kinase 2 preparation was then quantified by e.l.i.s.a. as described in the Experimental section. This allowed estimation of the activity of each type of casein kinase 2 expressed in terms of units of enzyme activity/mg of α -subunit. The value of this parameter was found to be 50 ± 11 for casein kinase 2a and 530 ± 46 for casein kinase 2b.

Phosphorylation of pp49

It is well established that the catalytic activity of casein kinase 2 resides in its α/α' -subunits. In order to determine whether pp49

had protein kinase activity able to phosphorylate itself or whether its phosphorylation was due to the casein kinase activity of the α/α' -subunits, experiments with heat-inactivated preparations were carried out.

Casein kinase 2a was incubated at either 50 °C or 80 °C. At different times samples were removed and assayed for casein kinase activity, autophosphorylation and phosphorylation in the presence of exogenous casein kinase 2b.

As shown in Fig. 5(a), autophosphorylation of pp49 is negligible after 2 min of incubation at 80 °C, and decreased dramatically during the incubation at 50 °C (Fig. 5b). Phosphorylation of β -casein by casein kinase 2a, determined with either ATP or GTP, also decreased at a similar rate when the sample was incubated at 50 °C (Fig. 5c).

Casein kinase 2a was not able to phosphorylate the β -subunit of heat-inactivated casein kinase 2b (results not shown), whereas when purified casein kinase 2b was added to the inactivated sample of casein kinase 2a the 49 kDa protein was phosphorylated (Figs. 5a and 5b). Phosphorylation of the β -subunit to a similar extent to that observed with casein kinase 2b alone was also observed. The rate of phosphorylation of pp49 by exogenous casein kinase 2b also depended on the concentration of pp49, with an apparent K_m value of 12 μ g/ml.

The effect of heparin on pp49 phosphorylation was then assayed with a preparation of casein kinase 2a/2b that contained both β -subunit and pp49 in a molar ratio of 1.8:1. This preparation had a specific activity of 172 units/mg of protein and an apparent K_m value for β -casein of 0.71 ± 0.09 mg/ml. As shown in Fig. 6, phosphorylation of both β -subunit and pp49 was strongly inhibited by low concentrations of heparin. These results indicate that the casein kinase activity of the α/α' -subunits was responsible for the phosphorylation of pp49.

Phosphorylation of pp49 by casein kinase 2a was already noticeable at very short times (5 s), increased linearly in the first 5 min and then proceeded at a lower rate, reaching values of 0.62 ± 0.06 mol of phosphate incorporated/mol of pp49 after 2 h of incubation (Fig. 7). The stoichiometry of this reaction is similar to that observed for β -subunit phosphorylation by casein kinase 2b (results not shown).

Fig. 7. Time course of pp49 phosphorylation by casein kinase 2a

pp49 phosphorylation of casein kinase 2a (3.25 units/ml and 0.187 mg/ml in the assay) was carried out in the presence of 80 μ M-[γ - 32 P]GTP. The reaction was stopped at the indicated times by addition of sample buffer. Samples were boiled for 4 min and electrophoresed in a 0.1 % (w/v) SDS/12.5 % polyacrylamide gel followed by autoradiography. 32 P incorporation into pp49 was quantified by excising the bands and counting their Čerenkov radiation.

Fig. 8. Phosphopeptide maps of β -subunit and pp49

Preparations of casein kinases 2a and 2b were autophosphorylated in the presence of [γ - 32 P]GTP and electrophoresed in a 0.1% (w/v) SDS/12.5% polyacrylamide gel. Bands corresponding to pp49 (track 1) and to β -subunit (track 2) were excised from the gel, digested with staphylococcal V8 proteinase (2 μ g/track) and the phosphopeptides analysed by electrophoresis as described in the Experimental section. Positions of molecular-mass markers are indicated on the right in kDa.

of considering pp49 as a precursor of the β -subunit. On the other hand, the fact that pp49 serves as a substrate of the α/α' -subunits does not preclude that it should be considered as a mere tightly bound substrate, since it can form complexes with the α/α' -subunits in which no β -subunit was detected. In addition, our data indicate that pp49 can also interact with the α -subunits present as $\alpha_2\beta_2$ complexes in preparations of casein kinases 2b and 2a/2b. What is clear is that the presence of pp49 in complexes of casein kinase 2 renders the enzyme poorly active towards the exogenous β -casein, as detected not only by the increase in the apparent K_m for this protein but also by the decrease in the specific activity when expressed as units of enzyme activity/mg of α -subunit. Thus, in any case, pp49 may modulate the activity of the catalytic subunits.

The existence in eukaryotic cells of enzymes with kinetic properties similar to those of casein kinase 2 but with distinct molecular structure has already been reported. In fact, the relative amounts of the α - and β -subunits were described to vary among different preparations of casein kinase 2 obtained from either rat liver nuclei [27] or from cytosol [28]. This led to the suggestion that the 25 kDa component could represent a tightly bound substrate [29], although it was later confirmed as a subunit of the enzyme [11]. Heterogeneity in the molecular structure of enzymes behaving kinetically as casein kinase 2 has been observed in maize seedlings [29]. An illustrative example of this heterogeneity is provided by the different reports on yeast, where enzymes containing β -subunits of either 24 kDa [30] or 41 kDa [4], as well as homotetrameric forms [10] or even monomeric structures [31], share kinetic properties similar to those of casein kinase 2.

Despite this diversity, and with very few exceptions, the enzymes reported so far as kinetically similar to casein kinase 2 share in common the presence of α - and/or α' -subunits, which contain the catalytic site [11]. Thus it is attractive to consider the possibility that these catalytic subunits could interact with diverse polypeptides, giving rise to a variety of forms showing different molecular and catalytic or regulatory properties, one of which would be the well-characterized $\alpha_2\beta_2$ heterotetramer. This possibility is reminiscent of what occurs with protein phosphatases, in particular with the type 1 enzymes [32]. Changes in the relative amounts of α/α' -subunit-interacting proteins, such as pp49, may condition the type of form and the kinetic properties of casein kinase 2 present in eukaryotic tissues, and may serve as a regulatory device for controlling the activity of this protein kinase.

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Effect of pp49 on β -casein phosphorylation by casein kinase 2b

When the effect of pp49, present in heat-inactivated samples of casein kinase 2a, on the apparent K_m of casein kinase 2b for β -casein was studied, it was observed that the value of this parameter increased from 0.3 mg/ml (in the absence of pp49) to 0.4, 0.5, 1.6 or 2.6 mg/ml in the presence of 1.25, 2.5, 13.75 or 23.25 μ g of pp49/ml in the assay. On the contrary, the apparent K_m of casein kinase 2b for ATP was $8.4 \pm 1.3 \mu$ M in the absence of pp49 and $7.0 \pm 0.3 \mu$ M when pp49 was present.

Comparison of pp49 and the β -subunit

The possible existence of precursor forms of the β -subunit has been reported for the enzyme from rat liver nuclei [26]. This led us to study the possible relationship between pp49 and the β -subunit.

As shown in Fig. 8, clear differences were observed when the phosphopeptide maps derived from the β -subunit and pp49 were compared. This fact suggests that β -subunit and pp49 are unrelated polypeptides.

DISCUSSION

The heterotetrameric $\alpha_2\beta_2$ form of casein kinase 2 is the one most frequently observed in enzyme preparations purified from different types of eukaryotic cells [1]. The data obtained in the present work agree with the existence of rat liver casein kinase 2 as this complex, but they also demonstrate that this enzyme can form complexes with another phosphorylatable protein, designated pp49.

The role played by pp49 in the samples showing casein kinase 2 activity is difficult to ascertain. The peptide maps of the V8-proteinase-digests of phosphorylated β -subunit and pp49 suggest that they are not related proteins, which is against the possibility

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