SPECIFICITY OF A RAT LIVER PROTEIN PHOSPHATASE ACTIVE ON CASEINS PHOSOPHORYLATED BY TWO cAMP-INDEPENDENT PROTEIN KINASES

'Casein phosphatase' and the 'multifunctional' phosphoprotein phosphatase are different enzymes

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1. Introduction

An obvious postulate in the study of the frequently occurring phospho–dephosphorylation-mediated regulation of protein activity states the absolute specificity of such processes. In other words only one or few definite residues of the single target protein(s) should undergo the reversible modification thanks to their specific recognition by a protein kinase and a phosphoprotein phosphatase. Presumably at least three kinds of factors cooperate in determining such a goal:

(1) The compartmentation and, more in general, the 'morpho–functional' environment of both the protein substrate and the enzymes;
(2) The overall conformation of the protein substrate which has been shown to be important for masking residues otherwise available to the kinases [1,2] but which might possibly play an also positive role in the phosphorylation reaction (3);
(3) The local structural features around the target residue(s) determining what is frequently called the 'site specificity' of the enzyme.

This last factor has been thoroughly investigated with some protein kinases, both dependent and independent on cyclic nucleotides [4–11], showing that actually these enzymes are able to recognize residues characterized by definite primary structures which are quite different depending on the kinase.

However, much less information is available about the site specificity and the structural requirements of the phosphoprotein phosphatases, which are responsible for reversing the modifications induced by protein kinases. In most instances phosphoprotein phosphatases have been identified and studied by testing their activity on substrates pre-phosphorylated by cAMP-dependent protein kinase [including phosphorylase kinase, glycogen synthase, pyruvate kinase (type L), phosphohistones, phosphoprotamine, phosphocasein] with the important exception of phosphorylase *a* (e.g., see [12–14]). Apparently the phosphoprotein phosphatases of this kind from both muscle [12] and liver [14] display a rather broad specificity, being more or less active on all the above-mentioned substrates and even toward thiophosphorylated residues [15] and consequently are sometimes termed 'multifunctional' phosphatases [15]. The catalytic subunit of such enzymes is believed to be a polypeptide of ~35 000 M_r, dissociable from the holoenzymes by ethanol treatment [16] and also termed protein phosphatase C [17]. Such a protein phosphatase C consists of at least two different enzymes, exhibiting, however, an almost identical M_r and very similar physical properties, responsible for their co-purification [18].

In general, phosphohistones labeled by cAMP-dependent protein kinase can be regarded as suitable model substrates for the identification of such multifunctional phosphoprotein phosphatases, which are also active toward synthetic phosphopeptides pre-phosphorylated by the cAMP-dependent protein kinase [19]. There is no evidence however that the minimum structural requirement of such a kinase (i.e., the couples Arg–Arg or Lys–Arg close to the N-terminal side of the target residue [8]) is also critical for the site recognition by the phosphatase. On the contrary also phosphopeptides lacking any basic residue could be dephosphorylated [20].

However, a remarkable phosphoprotein phosphatase activity could be identified in rat liver cytosol also using as substrate casein labeled by 2 cAMP-independent protein kinases [21]. In contrast with the broad specificity of the multifunctional protein phosphatase, the protein phosphatase(s) responsible for such an activity (casein phosphatase) displays a noticeable specificity for only some of the phosphorylated residues of casein, while nothing was known about its activity toward phosphohistones and other proteins phosphorylated by cAMP-dependent kinase [21,22].

Since in the meantime the sites phosphorylated by the cAMP-independent casein kinases in casein fractions have been identified and shown to be quite different from the sites affected by the cAMP-dependent enzyme [9,11], the present study was designed to obtain more information about the substrate and site specificities of rat liver casein phosphatase and its possible relationship with the more widely known multifunctional phosphoprotein phosphatases.

2. Experimental

2.1. *Phosphorylated protein substrates*

^{32}P-Labeled whole casein and casein fractions were prepared by incubation with [γ-^{32}P]ATP (Radiochemical Centre, Amersham) and either rat liver casein kinase TS or S, under conditions in [23].

^{32}P-Labeled histones were prepared by incubating histones type IIA (Sigma) (final conc. 15 mg/ml) at 37°C for 60 min, with rat liver cAMP-dependent protein kinase (prepared as in [24]) in a medium containing: 25 μM [γ-^{32}P]ATP (spec. radioact. 0.40 μCi/nmol), 6 mM $MgCl_2$, 50 mM Tris–HCl buffer (pH 7.5) and 1 μM cAMP. The reaction was stopped by addition of formic acid (15% final conc.) and [γ-^{32}P]ATP removed from phosphohistones by Sephadex G-25 gel filtration in the same medium. The fractions eluted at the V_0, containing the labeled histones, were pooled, lyophilised and histones were dissolved in a small volume of 0.05 M HCl.

Synthetic peptides, prepared and kindly provided by Dr F. Marchiori, were phosphorylated by the cAMP-dependent kinase under conditions similar to those applied to histones except that [γ-^{32}P]ATP was 50 μM. [γ-^{32}P]ATP was removed by high-voltage paper electrophoresis at pH 1.9 (formic acid/acetic acid/water, 2.5:7.8:89.7). The radioactive areas corresponding to the [^{32}P]peptides were eluted from the paper with 30% acetic acid, lyophilised and dissolved with the minimum volume of 0.5 M imidazole–acetate buffer (pH 6).

2.2. *Phosphoprotein phosphatase determinations*

Phosphoprotein phosphatase activities were routinely assayed by incubating 5–15 μg enzyme preparations at 37°C for 30 min in 0.2 ml medium containing 0.5 M imidazole–acetate (pH 6.0) and the chosen ^{32}P-labeled substrate at 7 mg/ml final conc. In the assays of phosphohistone phosphatase the pH of the medium was 7 instead of 6. Cysteine and other sulfhydryl reagents were found to be ineffective and were omitted. For measurements of the rate of dephosphorylation the incubation time was shortened to either 5 or 10 min in order to prevent dephosphorylation exceeding 20% of the total ^{32}P incorporated. The reaction was stopped by addition of trichloroacetic acid (5% final conc.) and the $^{32}P_i$ liberated was converted into its phospho–molybdic complex, extracted with isobuthanol–benzene and evaluated as in [22].

2.3. *Isolation of phosphopeptides and phosphoamino-acids*

The macropeptide 27–141 (CN5) was obtained by CNBr digestion of RCm-α_{s2}-casein according to [25]. Tryptic [^{32}P]peptides from α_{s1}-, α_{s2}- and β-caseins were obtained and isolated as in [9,23]. [^{32}P]Ser and [^{32}P]Thr were isolated from the phosphorylated substrates both before and after incubation with phosphatase by hydrolysis for 4 h in 6 N HCl at 110°C followed by high-voltage paper electrophoresis at pH 1.9, and were determined as in [10].

2.4. *Partial purification of rat liver cytosol casein phosphatase*

The procedure followed was a modification of that in [22] and it is preferable since it gives preparations that are fairly stable for $\geq$1 month at −18°C. For a typical preparation the 105 000 × *g* supernatant of 6 Wistar rat livers, homogenised in 0.25% sucrose containing 50 μM phenylmethylsulphonyl fluoride (PMSF) was dialysed overnight against 50 mM Tris-acetate (pH 6.0) containing 10% glycerol and 50 μM PMSF (buffer A), and chromatographed through a DEAE-cellulose column (2.5 × 12 cm) equilibrated and initially eluted with $\geq$350 ml same buffer. Subsequently a 0–0.4 M linear NaCl gradient was started. Fractions of 7.3 ml were collected and analysed for their absorbance at 280 nm and for their protein

phosphatase activity toward both whole [^{32}P]casein (labeled with casein kinase TS) and [^{32}P]histones (labeled with the cAMP-dependent protein kinase). The first of the 3 peaks of casein phosphatase activity, eluted by ~0.08 M NaCl and almost free of any phosphohistone phosphatase activity was collected as indicated in fig.1A, concentrated by Diaflo UM10 ultrafiltration and submitted to gel filtration through a Sepharose 6B column (1.8 X 121 cm) equilibrated with buffer A. A single peak of casein phosphatase activity was constantly obtained (fig.1B). This fraction was kept at −18°C and utilised for most of the experiments described.

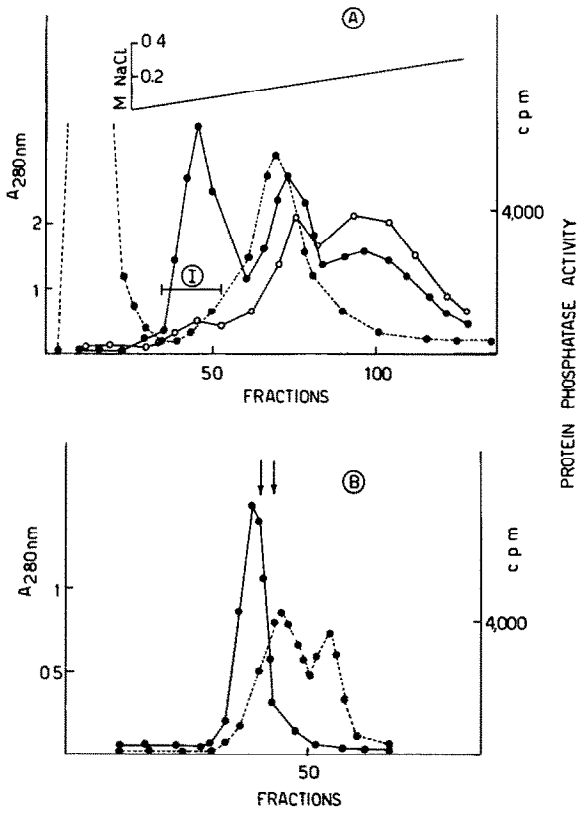

Fig.1. Partial purification of rat liver cytosol casein phosphatase, and its separation from phosphohistone phosphatase by subsequent DEAE-cellulose (A) and Sepharose 6B (B) column chromatography. Conditions are in section 2. The first casein phosphatase peak eluted from DEAE-cellulose was collected as indicated, concentrated by Diaflo UM10 ultrafiltration and submitted to Sepharose 6B gel filtration. Arrows indicate the elution position of marker proteins serum albumin and ovoalbumin (M_r 67 000 and 45 000, respectively): (●——●) casein phosphatase; (○——○) phosphohistone phosphatase; (●– – –●) A_{280}.

2.5. *PAGE of partially purified casein phosphatase*

Polyacrylamide (5%) gel electrophoreses were run at pH 7.5 [26]. Before applying the enzyme preparation, persulfate (present in the gels as catalyzer of polymerization) was removed by a 20 min pre-run. At the end of the run the gels were sliced into 2–4 mm segments which were extracted overnight with 0.4 ml 0.5 M imidazole–acetate (pH 6.0). The phosphatase activity of the extracts was assayed toward [^{32}P]casein (labeled by either casein kinase TS or S) and phosphohistones phosphorylated by the cAMP-dependent protein kinase. Parallel gels were stained with Coomassie brilliant blue.

3. Results

Rat liver cytosol was submitted to DEAE-cellulose gradient chromatography and the phosphoprotein

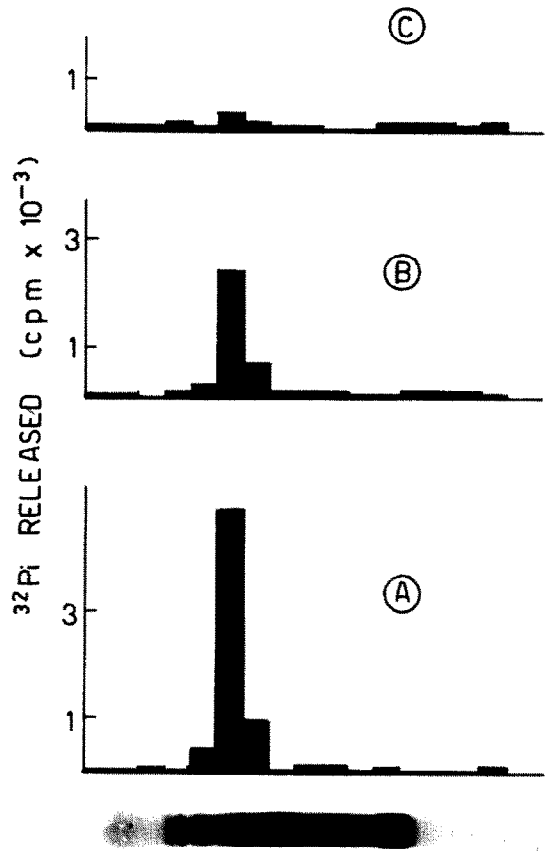

Fig.2. PAGE pattern of partially purified casein phosphatase, tested on [^{32}P]casein labeled with either casein kinase TS (A) or S (B) and on phosphohistones (C). General conditions are in section 2.

phosphatase activities identified by using either [^{32}P]-casein labeled with casein kinase TS or [^{32}P]histones labeled by the cAMP-dependent kinase as substrates. The two profiles, reported in fig.1A are only partially overlapping, indicating that, at least in part, the casein phosphatase activity is not accounted for by the general 'phosphohistone phosphatase(s)'. In particular the first peak of casein phosphatase was almost completely devoid of any phosphohistone phosphatase and was further purified by Sepharose 6B gel filtration (fig.1B) displaying an elution volume consistent with M_r slightly >70 000. No appreciable variation of the elution volume was observed when the crude cytosol was used instead of the purified fraction from DEAE-cellulose, either before or after ethanol treatment, a widely employed procedure for dissociating the multifunctional protein phosphatase into its 35 000 M_r catalytic subunit [13,17].

After the gel filtration step the purification factor of casein phosphatase approaches 120, a rather low

Table 1
Dephosphorylation rates of different sites of casein fractions phosphorylated by either casein kinase TS or S

Labeled substrate[a]	Labeling kinase	Dephosphorylation rate (cpm/min as $^{32}P_i$)[b]	Amino acid sequence around the phosphorylated residues[c]
β-Casein	TS	810	Gln–Gln–Gln–Thr(^{32}P)–Glu–Asp–Glu
β-Casein trypsin digested[d]	TS	354	
β-Casein	S	270	(Ser-P)$_3$–Glu–Glu–Ser(^{32}P)–Ile–Thr–Arg
Macropeptide CN5 from α_{s2}-casein	S	1410	Glu–Glu–Asn–Ser(^{32}P)–Lys–Lys–Thr
50% Dephospho-α_{s1}-casein	TS	n.d.	Ser-P–Ile–(Ser(^{32}P)-P)$_3$–Glu–Glu–Ile
Macropeptide CN5 from 30% dephospho-α_{s2}-casein	TS (site 1)[e]	n.d.	Ser–Ile–Gly–(Ser(^{32}P)-P)$_3$–Glu–Glu–Ser-P
Macropeptide CN5 from 30% dephospho-α_{s2}-casein	TS (site 2)[e]	570	Gln–Leu–Ser-P–Thr(^{32}P)–Ser-P–Glu–Glu–Asn
α_{s1}-casein	TS (site 1)[e]	n.d.	Ser-P–Ile–(Ser(^{32}P)-P)$_3$–Glu–Glu–Ile
α_{s1}-casein	TS (site 2)[e]	540	Glu–Ser-P–Thr(^{32}P)–Glu–Asp–Gln–

[a] The total radioactivity incorporated in all the substrates was equivalent to 45 000 (± 2000) cpm. In cases of two-site substrates, the ^{32}P was approximately equally distributed between the two sites

[b] Incubation time, 5 min

[c] The identification of the phosphorylated residues has been described in [9–11,23]

[d] β-[^{32}P]Casein was digested with trypsin (1%) in 0.1 M NH_4HCO_3 for 3 h at 37°C. Trypsin was inactivated by 3 min boiling. Controls with β-[^{32}P]casein undergoing the same procedure in absence of trypsin behaved as 'native' β-[^{32}P]casein

[e] The entity of dephosphorylation occurring at the distinct Ser and Thr sites was checked by determining the decrease of [^{32}P]Ser and [^{32}P]Thr induced by the phosphatase after 5 and 30 min incubation. The decrease of [^{32}P]Ser in α_{s2}-casein and in 30% dephospho-α_{s2}-casein was neglegible even after prolonged incubations, inducing the almost complete disappearance of [^{32}P]Thr. The dephosphorylation of unlabeled Ser-P residues at site 2 of α_{s2}-casein was also neglegible

n.d., not detectable

value largely accounted for by the great loss of activity that occurred during the purification procedure. It is important to underline however that the PAGE profiles of the partially purified preparations, though still showing a number of contaminating proteins, clearly shows a single faint protein band accounting for both the whole phosphatase activity toward casein, labeled by either casein kinase TS or S, and the low residual phosphohistone phosphatase activity (fig.2). Hence a single enzyme is apparently responsible for the different protein phosphatase activities exhibited by our partially purified preparation of casein phosphatase.

The dephosphorylation rates by casein phosphatase of several sites phosphorylated in casein fractions and derivatives by either casein kinase TS or S are reported in table 1. It can be seen that the 3 [^{32}P]Thr residues labeled by casein kinase TS in α_{s1}-, α_{s2}- and β-caseins are all fairly good targets for the phosphatase. On the other hand the [^{32}P]Ser residues labeled by the same kinase and included in the typical $(Ser\text{-}P)_3$ clusters of casein fractions are completely unaffected by the phosphatase. However the 2 [^{32}P]Ser residues phosphorylated by casein kinase S undergo dephosphorylation, though at quite different rates, the [^{32}P]-Ser_{135} of α_{s2}-casein behaving as the best substrate among all the tested residues.

These data strongly suggest that while phosphothreonyl residues are the preferred targets of the phosphatase, the dephosphorylation of phosphoseryl residues is conditioned by the fulfillment of more specific structural requirements. Actually phosphothreonyl residues are readily affected even if included within sequences preventing the dephosphorylation of phosphoserine: this is confirmed by the fast dephosphorylation of the labeled [^{32}P]Thr residue included within the cluster Ser-P–[^{32}P]Thr–Ser-P–Glu–Glu of α_{s2}-casein and shown in table 1, in contrast with the resistance of the adjacent unlabeled Ser-P residues. The dephosphorylation rates of some synthetic peptides phosphorylated by cAMP-dependent protein kinase are reported in table 2. It should be noted that the dephosphorylation of a [^{32}P]Ser residue included in a hexapeptide lacking any acidic residue is nevertheless neglegible in comparison with those of [^{32}P]-Thr included in the same or in very similar peptides. On the contrary a [^{32}P]Ser residue included in a very basic dodecapeptide is readily split.

Table 2
Amino acid sequences of synthetic peptides phosphorylated by the cAMP-dependent kinase and used as substrates of casein phosphatase

Synthetic phosphopeptides	Susceptibility to casein phosphatase: ($^{32}P_i$ released/10 min (cpm))
Arg–Arg–[^{32}P]Ser–Thr–Val–Ala	36
Arg–Arg–[^{32}P]Ser–[^{32}P]Thr–Val–Ala	250
Arg–Arg–Ala–[^{32}P]Thr–Val–Ala	340
Pro–$(Arg)_5$–[^{32}P]$(Ser)_2$–Arg–Pro–Val–Arg	402

General conditions are as in section 2 except for the concentration of the phosphopeptides (free of non-phosphorylated congeners) which was only 0.12 nmol/ml (spec. radioact. 70 000 cpm/nmol). Incubation time, 10 min. The dodecapeptide was phosphorylated at only 1 of its 2 Ser residues

4. Conclusions

(1) Some of the 'casein phosphatase' activity of rat liver cytosol is accounted for by an enzyme distinguishable from the 'multifunctional' protein phosphatases in virtue of its almost neglegible activity toward phosphohistones phosphorylated by the cAMP-dependent protein kinase in contrast to its fast dephosphorylation of ^{32}P-labeled casein fractions phosphorylated by two cAMP-independent 'casein kinases'. Its insensitivity to Mn^{2+} [22] and to ethanol treatment are in agreement with such a conclusion.

(2) The structural determinants of the site specificity of casein phosphatase are obviously not the same responsible for the site recognition by any of the 3 protein kinases employed for preparing the labeled substrates, since the activity of casein phosphatase is equally well directed toward residues phosphorylated by the casein kinases TS and S and by the cAMP-dependent kinase, which are enzymes displaying quite different site specificities.

(3) The most remarkable feature of casein phosphatase is perhaps its preferential cleavage of the phosphothreonyl residues, independently of their inclusion in more or less acidic or definitely basic

fragments. Few cases are known of physiologically meaningful reversible phosphorylations of Thr, rather than Ser, residues [27–29] and it is possible that casein phosphatase-like protein phosphatases might be responsible for the dephosphorylation of such residues. It should be recalled that free phosphothreonine is not affected by our enzyme at all [22] and, moreover, a remarkable fall of activity is induced by the previous tryptic digestion of the protein substrate including the target [^{32}P]Thr (see table 1). It is evident therefore that structural factors more complicated than the local amino acid sequence is, may be required for the dephosphorylation of phosphothreonine residues.

(4) The dephosphorylation of phosphoseryl residues appears to be under the control of structural requirements more stringent than those allowing the dephosphorylation of phosphothreonyl residues. In particular the Ser-P residues included within acidic clusters are insensitive to the phosphatase while Thr-P is not. On the other hand the extremely fast dephosphorylation of α_{s2}-casein [^{32}P]Ser$_{135}$, included within the sequence Asn–Ser-P–Lys–Lys, and the slower but appreciable dephosphorylation of β-casein [^{32}P]Ser$_{22}$ and of the Ser-P residue of the dodecapeptide of table 2, both having an Arg residue near to their C termini, compared with the neglegible dephosphorylation of the phosphopeptide Arg–Arg–Ser-P–Thr–Val–Ala, might suggest the requirement of C terminal basic residues for the Ser-P cleavage by casein phosphatase.

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