

## The phosphorylation sites of ribosomal P proteins from *Saccharomyces cerevisiae* cells by endogenous CK-2, PK60S and RAP protein kinases\*

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The phosphorylation sites of ribosomal acidic proteins (P proteins) from *Saccharomyces cerevisiae* were studied *in vivo* and *in vitro* by using CK-2, PK60S and RAP protein kinases. The three enzymes phosphorylate the last serine residues located in a highly conserved carboxyl end of the polypeptide chains. This was established by two-dimensional analysis of tryptic phosphopeptides from <sup>32</sup>P-labelled proteins YP1 $\alpha$ , YP1 $\beta$ , YP2 $\alpha$  and YP2 $\beta$ , and by kinetic studies of the protein kinases with synthetic peptides corresponding to the fragments of endogenous ribosomal acidic polypeptides.

In experiments with both endogenous P proteins and synthetic peptides as substrates protein kinase PK60S demonstrated unusual substrate specificity. In contrast to CK-2 and RAP protein kinases, PK60S phosphorylates predominantly two of the four P proteins, YP1 $\alpha$  and YP2 $\beta$ , with kinetic constants dependent on the primary structure of the N-terminal region of the polypeptide containing the target residue. The neutral amino acid, alanine, at position 3 in the peptide AAEESSDDD (polypeptide fragments of YP1 $\beta$  and YP2 $\alpha$ ) decreases the  $K_m$  value more than 10-fold by comparison with the basic lysine residue at the same position in the peptide AKEESSDDD (polypeptide fragments of YP1 $\alpha$  and YP2 $\beta$ ).

Ribosomes of all organisms contain a set of very acidic proteins (pI 3.5–4.5) named A proteins in prokaryotes and P proteins in eukaryotes. They are present in a few copies per ribosome and play an important role in translation. Bacterial (*Escherichia coli*) ribosomes have two A proteins encoded by the same gene. These proteins, called L7/L12, are responsible for binding enzymatic factors and aminoacyl-tRNA to the ribosome, as well

as for the factor GTP-dependent hydrolysis during protein synthesis (for reviews, see [1–3]).

In eukaryotic organisms, ribosomal P proteins play a similar functional role but differ from prokaryotic A proteins in some features. They can be divided into two subgroups named P1 and P2, which comprise a different number of proteins [4]. Eukaryotic ribosomal acidic proteins are phosphorylated both *in*

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**Abbreviations:** CK-2, protein kinase CK-2 (hitherto known as Casein Kinase II, EC 2.7.1.37); PK60S, protein kinase 60S ribosomal subunits; RAP kinase, ribosomal acidic proteins kinase.

*vitro* and *in vivo*, and for this reason they are called P proteins [5]. In the cells of *Saccharomyces cerevisiae*, four ribosomal acidic proteins are encoded by independent genes [6–8]. According to a uniform nomenclature they are called YP1 $\alpha$ , YP1 $\beta$ , YP2 $\alpha$ , YP2 $\beta$  [4]. Based on structural and functional homology to their mammalian counterparts, the acidic proteins from yeast ribosomes are classified into two pairs: YP1 $\alpha$ , YP1 $\beta$  as the P1 subgroup and YP2 $\alpha$ , YP2 $\beta$  as the P2 subgroup. All P proteins with molecular mass of about 13 kDa, are located on the surface of the 60S ribosomal subunit and can be removed from the particle by washing with NH<sub>4</sub>Cl/ethanol solution, yielding split proteins (SP fraction) [9]. It should be noted that YP1 $\beta$  can be found in the SP fraction as a truncated polypeptide, lacking eight amino-acid residues at the N-terminus. This form of YP1 $\beta$  is called YP1 $\beta$ ' [10]. The C-terminal domains of all four acidic proteins are identical. These polypeptide fragments have a serine residue as one of the potential phosphorylation sites. These and other features of those ribosomal acidic proteins are shown in Table 1. The primary structures of the P proteins have been derived from analyses of their genes and from amino-acid sequencing [6–8].

Phosphorylation of acidic ribosomal proteins from yeast was initially reported twenty years ago [11, 12]. The role of this enzymatic process is not precisely recognized. It is known that the phosphorylated forms of the acidic proteins are always bound to the surface of the ribosomal particle. The transformation of non-phosphorylated acidic proteins to their phosphorylated forms associated with ribosomes, takes place during protein synthesis. Furthermore, phosphorylation of the acidic proteins is required for translational activity of ribosomes (for review, see [13, 14]). In yeast cells, three cAMP-independent protein kinases: multifunctional protein kinase CK-2 [12, 15], highly specific PK60S [16, 17] and the very recently discovered ribosomal acidic protein (RAP) kinase [18], are engaged in phosphorylation *in vitro* of P proteins. All three enzymes phosphorylate the 13-kDa P proteins, and additionally 38-kDa protein called P0 which belongs to the ribosomal core and is not

removable from ribosomes by washing with NH<sub>4</sub>Cl/ethanol [9, 19]. The P0 protein is not given further attention in this paper.

Ribosomal P proteins from yeast cells have been shown to be monophosphorylated [20], while acidic ribosomal proteins from rat liver are polyphosphorylated [5, 21]. Each of the individual P proteins from yeast has seven to eight serines as possible phosphorylation sites. From genetical and chemical analysis of ribosomal acidic phosphopeptides, Naranda *et al.* [22, 23] proposed serines 62, 73, 71 and/or 79 and 19 to be target residues in YP1 $\alpha$ , YP1 $\beta$ , YP2 $\alpha$  and YP2 $\beta$ , respectively, phosphorylated *in vivo* and *in vitro* by CK-2. The peptide sequences surrounding target residues are different (see Table 1) and do not accord with the documented peptide structures required for specific recognition by CK-2 [24]. To resolve this problem, we have analysed the tryptic phosphopeptides obtained from the individual P proteins phosphorylated *in vivo* and *in vitro* by endogenous CK-2, PK60S and RAP protein kinases. The experiments have been supplemented by kinetic studies using as substrates synthetic peptides corresponding to the fragments of the ribosomal acidic polypeptides containing target residues.

## MATERIALS AND METHODS

**Enzyme.** Protein kinases purified almost to homogeneity: CK-2 [25], PK60S [17] and RAP kinase [18] were obtained from *S. cerevisiae* cells. Enzymes activities were tested under standard conditions with either an endogenous protein substrate (80S ribosomes or extracted ribosomal P proteins) or synthetic peptides, as previously described [26]. One unit of protein kinase activity is defined as the amount of enzyme required for incorporation of 1 pmol of phosphate from [ $\gamma$ -<sup>32</sup>P]ATP into substrate per minute.

**Preparation of ribosomes and ribosomal acidic protein fraction (SP fraction).** 80S ribosomes devoid of endogenous protein kinase activity were isolated from yeast and purified as described earlier [17]. The acidic ribosomal proteins (P proteins) were extracted from purified ribosomes by

washing with 50% ethanol and 0.5 M ammonium chloride according to the procedure elaborated in Ballesta's laboratory [9].

***In vitro and in vivo phosphorylation of ribosomes.*** Highly purified 80S ribosomes (0.5 mg ribosomal proteins) were phosphorylated *in vitro* under standard conditions with 3–5 units of CK-2 [25], PK60S [17] or RAP [18] protein kinases and [ $\gamma$ - $^{32}$ P]ATP (Amersham) (500–2000 c.p.m./pmol).

*In vivo* labelling of ribosomes was performed in 40 ml cultures of yeast cells in a low-phosphate medium [27]. Briefly, after one hour of incubation at 30°C, 250  $\mu$ Ci of [ $^{32}$ P]H<sub>3</sub>PO<sub>4</sub> was added to the culture and incubation continued for 3–4 h to obtain the logarithmic growth phase. Cells were collected by centrifugation, washed with physiological fluid, and  $^{32}$ P-labelled ribosomes isolated and purified as described earlier [17].

***Analysis of acidic ribosomal phosphoproteins and phosphopeptide mapping.*** P proteins extracted from  $^{32}$ P-labelled 80S ribosomes were resolved by isoelectrofocusing on 5% polyacrylamide gel slabs containing 2% Pharmacia ampholytes, pH range 2.5–5.0, as described in detail earlier [28, 29]. Individual  $^{32}$ P-labelled acidic phosphoproteins were identified by autoradiography. Radioactive bands corresponding to phosphorylated YP1 $\beta$  and YP2 $\beta$  (YP1 $\beta$ p and YP2 $\beta$ p) were excised from the polyacrylamide gel and digested extensively with trypsin [30]. Analyses of tryptic peptides were performed by electrophoresis in the first dimension (3 h at 400V) and ascending chromatography in the second dimension, using Sigma TLC pre-coated plates type 100 cellulose [30].

***Synthetic peptides.*** Five synthetic peptides, with amino-acid sequences corresponding to possible phosphorylation sites in ribosomal P proteins, were prepared. Each peptide had a triplet arginine residue additionally attached to the N-terminus of arginine to serve as a "sticky end", with which the phosphorylated peptide attached itself to phosphocellulose paper Whatman P81 during the preparation of samples for radioactivity counting [26]. The first three peptides, RRR•EITSDNL, RRR•EILSGFH and RRR•GAASGAA, reproduced the sequences 19–25, 55–61 and 70–76 of YP1 $\beta/\beta'$ . The

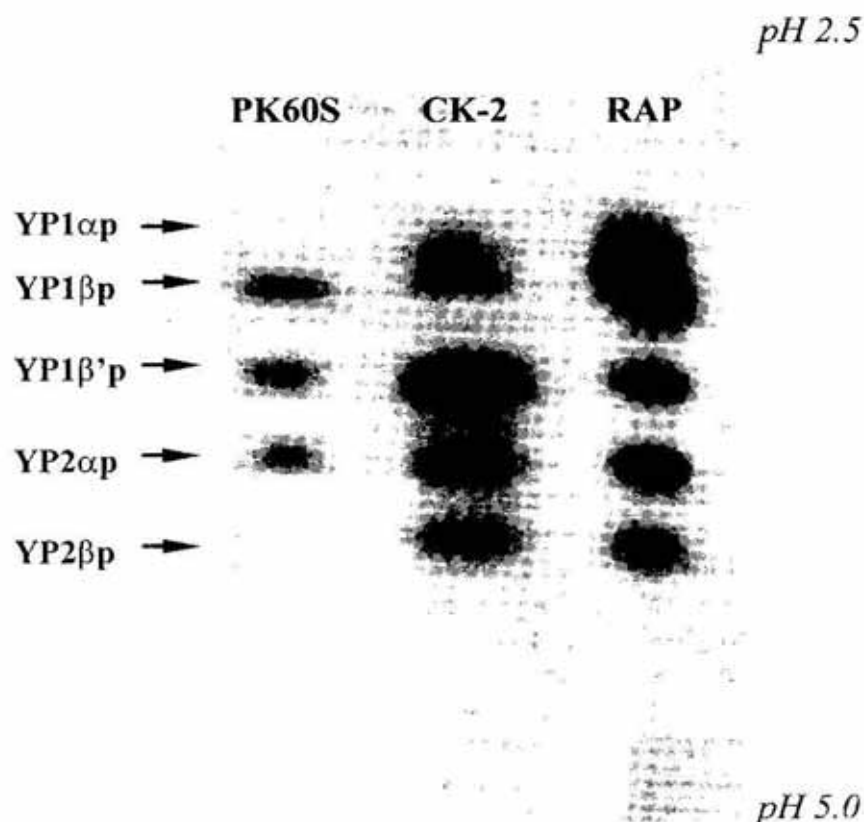
fourth synthetic peptide, RRR•AAEESDDD, reproduced the 92–99 fragments of the protein YP1 $\beta/\beta'$  and YP2 $\alpha$ . The fifth synthetic peptide, RRR•AKEESDDD, corresponds to two identical segments 92–99 and 96–102 of YP1 $\alpha$  and YP2 $\beta$ , respectively (see Table 1). All synthetic peptides were prepared on a peptide synthesizer Labortec AG model SP650 using the Fmoc-polyamide method on 4-(2',4'-dimethoxyphenyl-hydroxymethyl)-phenoxy resin (Tenta Gel S AC resin). Side-chain protections were as follows: 2,2,5,7,8-pentamethyl-chroman-6-sulphonyl (Pmc) for arginine, t-butyloxycarbonyl (Boc) for lysine, and t-butyl (t-But) for aspartic acid, glutamic acid, threonine and serine. After synthesis the peptides were cleaved from the resin with trifluoroacetic acid (TFA)/phenol/triisopropylsilane/water (88:5:2:5, by vol.). The crude peptides were purified on a preparative RP-HPLC Vydac C-18 column with a 5–30% linear gradient of acetonitrile. The purity of 95% was evaluated by amino-acid analysis on an analytical HPLC System Gold Beckman using a Spherisorb S 5X C-18 column.

***Protein*** concentration was measured by the Bradford procedure [31] with bovine serum albumin as a standard.

## RESULTS AND DISCUSSION

The three protein kinases PK60S, CK-2 and RAP kinases were used for phosphorylation of acidic ribosomal proteins from *S. cerevisiae* cells.  $^{32}$ P-labelled proteins were extracted from ribosomes and resolved by isoelectrofocusing in polyacrylamide gel. Identification of the individual P proteins was performed by autoradiography. Unlike CK-2 and RAP kinase, which intensively phosphorylated the whole set of P proteins, PK60S phosphorylated decidedly YP1 $\beta/\beta'$  and YP2 $\alpha$  (Fig. 1). Trace darkening of the film in the positions corresponding to YP1 $\alpha$ p and YP2 $\beta$ p, phosphorylated by PK60S, could be observed after a long exposure to autoradiography, but in this situation the other spots overlapped each other and rendered difficult identification of the P proteins. The results presented in Fig. 1 could suggest that target residue(s) in polypeptide chains of ribosomal acidic P





**Figure 1.** Autoradiogram of isoelectrofocusing gel of P proteins phosphorylated *in vitro*.

80S ribosomes, 1 mg, were labelled under standard conditions by [ $\gamma$ - $^{32}$ P]ATP (1000 c.p.m./pmol) with 3 units of PK60S, 5 units of CK-2 and RAP protein kinases. P proteins were extracted from  $^{32}$ P-labelled ribosomes with  $\text{NH}_4\text{Cl}$ /ethanol, precipitated with acetone and subjected to electrophoresis. Electrophoresis was run in a pH range from 2.5 (top) to 5.0 (bottom). The identified P proteins are marked by arrows.

proteins are either not the same for the three protein kinases studied, or that the kinases differ in affinity to a single phosphorylation site.

In order to resolve this problem, two-dimensional phosphopeptide mapping of YP1 $\beta$ p and YP2 $\beta$ p belonging to the P1 and P2 subgroups of P proteins, respectively, was performed. The amino-acid sequences of all P proteins presented in Table 1 show seven and eight serine residues in YP1 $\beta$  and YP2 $\beta$ , respectively. These residues can be possible targets for different protein kinases. Analyses of tryptic phosphopeptides obtained from YP1 $\beta$ p phosphorylated by CK-2, RAP kinase and PK60S showed that in each case a single  $^{32}$ P-labelled phosphopeptide migrated towards the anode (Fig. 2A, a–c). Similar results were obtained for YP2 $\beta$ p phosphorylated by CK-2 and RAP kinase (Fig. 2A, d–e). Since YP2 $\beta$  is only very weakly phosphorylated by PK60S, it was difficult to obtain an autoradiogram with a distinct spot on the film (Fig. 2A, f). When the mixed tryptic phosphopeptides from YP1 $\beta$ p and YP2 $\beta$ p, phosphorylated either by CK-2 or by RAP kinase, were analysed, two distinct spots shifted relative to each other were observed on the film (Fig.

2A, g–h). This indicates a similar but not identical, structure of the tryptic phosphopeptides obtained from the two P proteins. Based on the primary structures of P proteins presented in Table 1, only one fragment of each polypeptide chain can be taken into consideration, namely the C-terminal peptide formed after trypsin digestion. In the case of YP1 $\beta$ p and YP2 $\alpha$ p, the last cleavage of lysyl peptide bonds gives a peptide with eighteen amino-acid residues (EEEEAE-ESDDDMGFGLFD), which is also more acidic as compared with the peptide of thirteen amino-acid residues (EESDDDMGFGLFD) derived from YP1 $\alpha$  and YP2 $\beta$ . This is further confirmed by the results of tryptic phosphopeptide mapping of YP1 $\alpha$  phosphorylated by any of the three protein kinases. Only one spot was observed in the autoradiograms (Fig. 2B a–c). Similar phosphopeptide maps were obtained with YP2 $\beta$ p phosphorylated by CK-2 and RAP protein kinases (not shown).

The results of *in vitro* experiments were confirmed by *in vivo* studies (Fig. 3). Phosphopeptide mapping analysis of YP1 $\beta$ p and YP2 $\beta$ p  $^{32}$ P-labelled *in vivo* shows, in either case, a single spot on the film (Fig. 3, a–b).

Table 1. Amino-acid sequences of acidic ribosomal P proteins from *Saccharomyces cerevisiae*

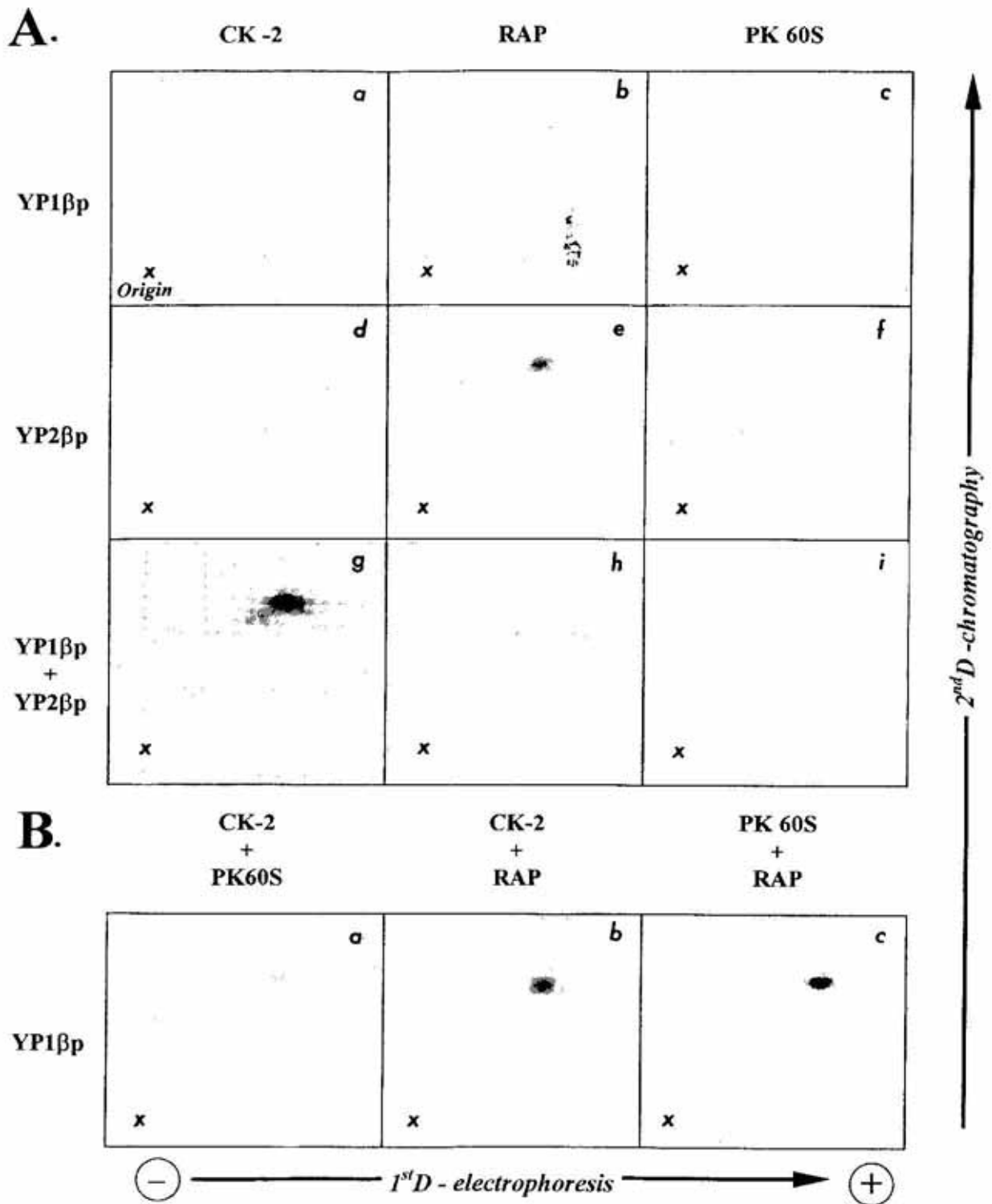
Protein	Amino-acid sequence
YP1 $\alpha$	<p style="text-align: center;">10                    20                    30                    40</p> MSTESALSYAALILADSEIEISSEKLLTLTNAANVPVENTWADIFAKA <p style="text-align: center;">50                    60                    70                    80                    90</p> LDKQNLKDLLVNF $\overline{S}$ AGAAAAPAGVAGGVAGGEAGEAEAEKEEEEEAK <p style="text-align: center;">100</p> EESDDDMGFGLFD
YP1 $\beta/\beta'$	<p style="text-align: center;">10                    20                    30                    40</p> MSDSIIS $\overline{F}$ AAFILADAGLEITSDNLLTITKAAGANVDNVWADVAKA <p style="text-align: center;">50                    60                    70                    80                    90</p> LEGKDLKEILSGFHNAGPVAGAGAA $\overline{S}$ GAAAAGGDAAAEEEEKEEEAA <p style="text-align: center;">100</p> EESDDDMGFGLFD
YP2 $\alpha$	<p style="text-align: center;">10                    20                    30                    40</p> MKYLAAYLLLNAAGNTPDATKIKAILESVGIIEDEKVVSSVLSALEGK <p style="text-align: center;">50                    60                    70                    80                    90</p> SVDELITEGNEKLAAVPAAGPA $\overline{S}$ AGGAAAA $\overline{S}$ GDAAEEEEKEEEAA <p style="text-align: center;">100</p> EESDDDMGFGLFD
YP2 $\beta$	<p style="text-align: center;">10                    20                    30                    40</p> MKYLAAYLLLVQGGNAAP $\overline{S}$ AADIKAVVESVGAEVDEARINELLSLEGK <p style="text-align: center;">50                    60                    70                    80                    90</p> GSLEEILAEQKKFATVPTGGASSAAAGAAGAAAGGDAEEEEKEEEAK <p style="text-align: center;">100                    110</p> EESDDDMGFGLFD

$\overline{MSDSIISF}$ , truncated sequence in polypeptide YP1 $\beta$ ;  $\overline{S}$ , proposed phosphorylation sites [22, 23].

Mixed tryptic phosphopeptides from the two assayed P proteins gave two shifted radioactive phosphopeptides (Fig. 3, c) similar to those obtained in the *in vitro* experiments. The data presented in Figs. 1, 2 and 3 indicate: firstly, the acidic character of the labelled tryptic phosphopeptides; secondly, different primary structure of tryptic phosphopeptides from the assayed P proteins proved by their electrophoretic and chromatographic mobilities (mixed phosphopeptides); thirdly, a possibility that only one serine residue in polypeptide chains of YP1 $\beta$  and YP2 $\beta$  is modified by all three assayed protein kinases.

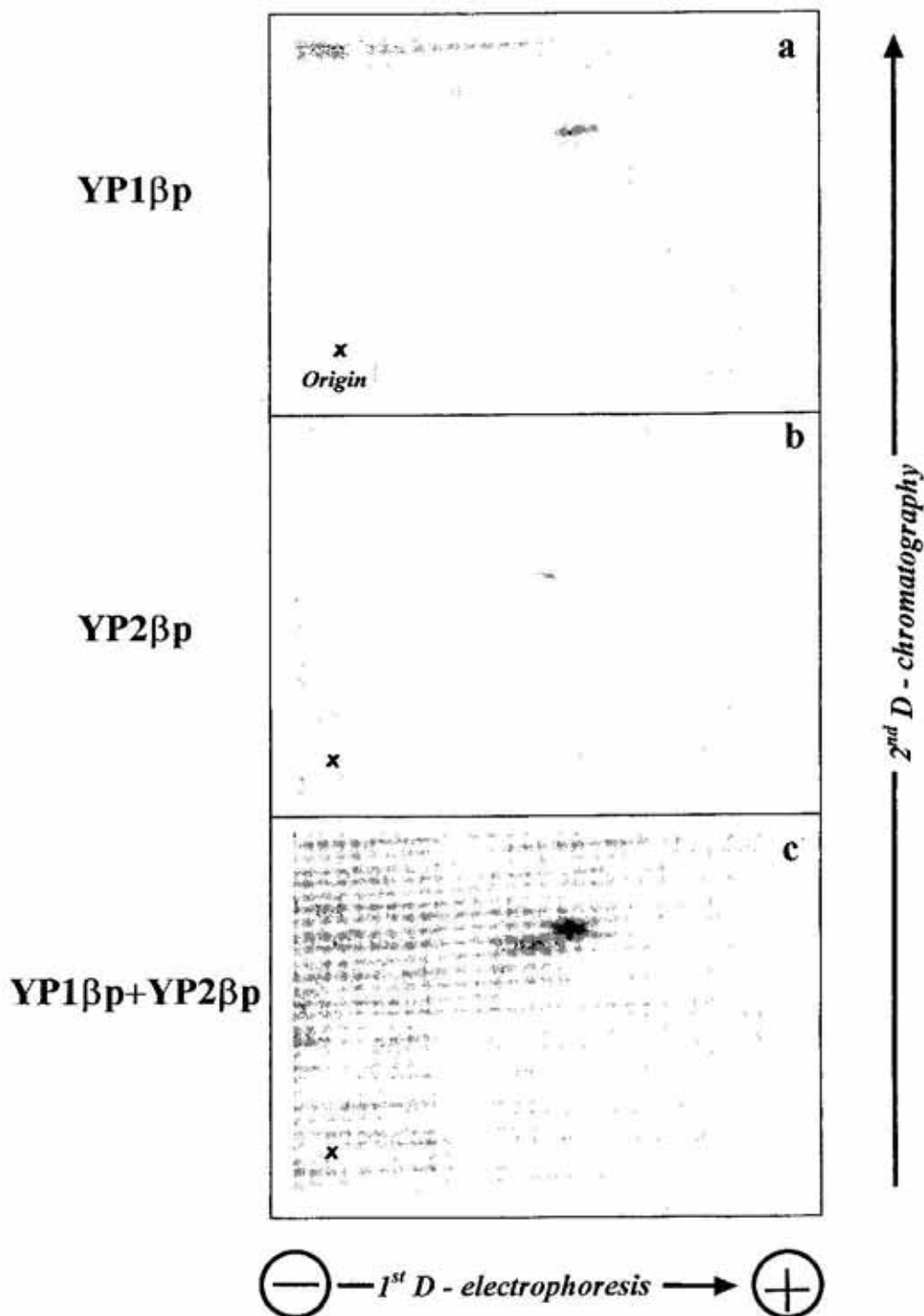
The starting point for our kinetic studies with synthetic peptides as substrates was the primary structure of the P proteins and the distribution of serine residues along the pep-

ptide chains (see Table 1). YP1 $\beta'$ , which is shorter by eight amino acids, was phosphorylated as well as untruncated YP1 $\beta$ . This suggested that none of the three serine residues (Ser-2, Ser-4 and Ser-7) located in the missing N-terminal fragment was phosphorylated by the assayed enzymes. Taking this into consideration, we prepared five synthetic peptides whose amino-acid sequences imitated the structure of the chosen polypeptide fragments. Peptides I–III are copies of the fragments 19–25, 55–61 and 70–76 of the polypeptide chain of YP1 $\beta/\beta'$ . Peptide IV reproduced the 92–99 C-terminal fragments of YP1 $\beta/\beta'$  and YP2 $\alpha$ . Peptide V comprised the sequences 92–99 and 96–102 of YP1 $\alpha$  and YP2 $\beta$ , respectively. The potential targets for the protein kinases appear to be the last serine residues located close to the C-termini



**Figure 2. Phosphopeptide maps of YP1 $\beta$ p and YP2 $\beta$ p phosphorylated *in vitro*.**

Phosphorylation of 80S ribosomes was carried out under standard conditions using [ $\gamma$ - $^{32}$ P]ATP (2000 c.p.m./pmol) and 5 units of each protein kinase. P proteins after extraction from ribosomes were resolved by isoelectrofocusing on polyacrylamide gel, digested extensively with trypsin and the phosphopeptides mapped as described in Materials and Methods. A, phosphorylation by individual kinases; B, phosphorylation by combined kinases.



**Figure 3. Phosphopeptide maps of YP1βp and YP2βp phosphorylated *in vivo*.**

Ribosomes phosphorylated *in vivo* were isolated, purified and P proteins extracted before being resolved by isoelectrofocusing. Other details are as in previous Figures and Materials and Methods.

of all ribosomal acidic polypeptides. These serine residues are surrounded by acidic clusters of aspartic and glutamic acids which are necessary for recognition of the target residue by CK-2 [24].

As presented in Table 2, only peptide IV (RRR•AAEESDDD) and peptide V (RRR•AKEESDDD), which reproduced the C-terminal fragments of both groups: P1 and P2, of acidic ribosomal phosphoproteins,

**Table 2. Phosphorylation of synthetic peptides reproducing selected fragments of ribosomal acidic polypeptides from *Saccharomyces cerevisiae***

No	Peptide		Phosphorylation (pmol/sample)		
	structure	descent	CK-2	RAP	PK60S
I	RRR•EITSDNL				
	Ser-22	YP1 $\beta$ / $\beta'$	0.26	0.11	0.06
II	RRR•EILSGFH				
	Ser-58	YP1 $\beta$ / $\beta'$	0.13	0.09	0.08
III	RRR•GAASGAA				
	Ser-73	YP1 $\beta$ / $\beta'$	0.25	0.12	0.09
IV	RRR•AAEESDDD				
	Ser-96	YP1 $\beta$ / $\beta'$ , YP2 $\alpha$	30.81	8.62	9.05
V	RRR•AKEESDDD				
	Ser-96	YP1 $\alpha$	10.06	6.64	2.39
	Ser-100	YP2 $\beta$			

Phosphorylation of 2 nmoles synthetic peptides was carried out for 15 min under standard conditions, using [ $\gamma$ - $^{32}$ P]ATP (500 c.p.m./pmol) and 5 units of each enzyme. The samples for counting were prepared as described earlier [30]. The triple arginine residues (RRR) at the N-terminus of each peptide are required for the phosphocellulose paper assay as described in Materials and Methods.

were in fact phosphorylated by the three kinases. Special attention was given to the specific PK60S, which phosphorylated peptides IV and V with differing efficiency. PK60S displayed over a tenfold lower  $K_m$  value for peptide IV (16.1  $\mu$ M) than for peptide V (170.0  $\mu$ M) (Table 3). The difference between these two peptides concerns only one amino-acid residue at position 3. Peptide IV had a neutral alanine, whereas peptide V had a basic lysine residue. This single amino-acid replacement significantly altered the phosphorylation of YP1 $\beta$ / $\beta'$  and YP2 $\alpha$  in comparison to YP1 $\alpha$  and YP2 $\beta$  by PK60S protein kinase. It also partially explains the difficulty of obtaining a phosphopeptide map of YP2 $\beta$  phosphorylated by PK60S (see Fig. 2A, f).

Serine residues surrounded by clusters of acidic amino acids are always found at similar positions in polypeptide chains of all known ribosomal P proteins [2]. These residues are phosphorylated both in lower [32] and higher [33] eukaryotic organisms. Similar polypeptide structures form a domain typical for CK-2 phosphorylation sites [24]. Nevertheless, the last serines located within the highly conserved and acidic C-terminal domain of P proteins from *S. cerevisiae* were initially excluded as target residues for protein kinases [22, 23]. It is, consequently, of interest to note that the same research group subsequently retracted this proposal in a review paper [13].

The physiological role of P protein phosphorylation is still inadequately elucidated, and

**Table 3. Kinetic constants of CK-2, RAP and PK60S kinases with the best synthetic substrates (peptides IV and V)**

Peptide	CK-2			RAP			PK60S		
	$K_m$	$V_{max}$	$V_{max}/K_m$	$K_m$	$V_{max}$	$V_{max}/K_m$	$K_m$	$V_{max}$	$V_{max}/K_m$
(IV) RRR•AAEESDDD	16.7	56.61	3.39	15.2	17.55	1.15	16.1	20.67	1.28
(V) RRR•AKEESDDD	20.0	23.18	1.16	17.9	16.88	0.94	170.0	19.18	0.11

Enzyme reactions were assayed for 3 min under standard conditions with synthetic peptides in the concentration range of 5–160  $\mu$ M.  $K_m$  and  $V_{max}$  are expressed as  $\mu$ M and pmol phosphate  $\times$  min $^{-1}$  per mg peptide, respectively. The data presented are the means of at least three separate determinations.



forms the subject of ongoing studies in our laboratory.

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