



## Characterization of the regulatory subunit of *Yarrowia lipolytica* cAMP-dependent protein kinase. Evidences of a monomeric protein

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### ABSTRACT

cAMP-dependent protein kinase (PKA) catalytic (C) and regulatory (R) subunits from *Yarrowia lipolytica* are encoded by single genes, *TPK1* and *RKA1*, respectively. Here we performed the heterologous expression, purification and characterization of the R subunit from *Y. lipolytica* yeast cells, and explored the main biochemical features of the PKA. The purified recombinant R, active and capable to interact with C subunit was used to prepare highly specific polyclonal antiserum. Sucrose-gradient centrifugation and gel filtration analysis of both recombinant and native R revealed the monomeric nature of this subunit. Hydrodynamic parameters of the holoenzyme indicated that *Y. lipolytica* PKA is a dimer of 90 kDa composed of an R subunit of 42 kDa and a C subunit of 39 kDa. The identification of the N-terminal sequence was carried out by mass spectrometry analysis of the purified native R subunit. The differences between N-terminal sequences of R subunits from *Y. lipolytica* and other organisms, particularly a short linker that spans the inhibitory site, were discussed as the possible cause of the lack of dimerization. R was identified as a type II subunit since our results indicated that it was phosphorylated *in vivo* by C at S124 identified by anti-phospho-PKA substrate (RRXS/T) antibody.

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### Introduction

*Yarrowia lipolytica* is a dimorphic fungus with promising biotechnological applications such as hydrocarbon and fatty acid degradations, heterologous protein expression and the industrial production of a series of organic acids and enzymes [1–4]. This microorganism is also a frequently used biological model in studies related to lipid metabolism, peroxisome biogenesis, general secretion pathways, and cell differentiation. *Y. lipolytica* has also become an appropriate alternative model for the study of dimorphic transitions since it is easily accessible to molecular biology and genetic manipulations; it has a well defined sexual cycle and different effectors reproducibly inducing preferential development of either yeast-like or mycelial morphologies have been described [1,5–8].

Fungal dimorphism is particularly important since genetic evidence indicates that in a number of fungi pathogenic in animals and plants, the morphogenetic transition is directly associated with virulence [9–13]. Furthermore, the induction conditions and the signal transduction pathways that regulate dimorphism in *Y. lipolytica* are similar to those of other fungi, including particularly *Candida albicans*, probably the most important human pathogenic fungus.

It is known that cAMP signaling mediates several physiological processes in fungi, including hyphal differentiation [14]. In *Y. lipolytica*, the addition of cAMP to the culture medium inhibits mycelium formation under inducing conditions [6]. The catalytic and regulatory subunits of PKA<sup>1</sup> from *Y. lipolytica* are known to be codified by single genes: *TPK1* and *RKA1*, respectively [15,16]. While *RKA1* appears to be essential in view of unsuccessful attempts to disrupt the gene [15], mutant strains deleted in *TPK1* are viable [16]. Merodiploid strains carrying several copies of the *RKA1* gene have been shown to be refractory to the negative effect exerted in a dose dependent manner by cAMP on the dimorphic transition, and it has also been shown that the *RKA1* gene is up-regulated at the transcriptional level under conditions that promote dimorphic transition [15]. In a previous work, we showed the necessity of an operative PKA pathway for normal yeast-like growth since a  $\Delta tpk1$  mutant grew constitutively in mycelial form under non-inducing conditions, and the inhibitory effect of cAMP during mycelial transition of wild-type strain was reverted by the specific PKA inhibitor Myr-PKI [16].

Typically, cAMP-dependent protein kinase (PKA) is a heterotetramer consisting of two regulatory (R) and two catalytic subunits

<sup>1</sup> Abbreviations used: PKA, cAMP-dependent protein kinase; IPTG, isopropyl  $\beta$ -D-thiogalactoside; MS, mass spectrometry; MALDI, matrix-assisted laser desorption ionization; TOF, time of flight; ORF, open reading frame; BLAST, basic local alignment search tool; PVDF, polyvinylidene difluoride.

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(C). Upon binding of cAMP, the R subunits dissociate from the complex, releasing active C subunits which phosphorylate many target proteins on serine or threonine residues (for a review see reference [17]). R subunits have a structure with conserved and well-defined domains [18]. These interact primarily with the C subunits at the inhibitory site and contain two tandem cAMP-binding domains at the carboxy terminus, designated A and B. In addition to its role as an inhibitor of the C subunit, the R subunit anchors the holoenzyme to specific intracellular locations, mainly through interaction of the N-terminal dimerization-docking domain with specific anchoring proteins [19].

In this study, we performed the heterologous expression, purification and characterization of *Y. lipolytica* recombinant R subunit and described the main biochemical characteristics of PKA from *Y. lipolytica* yeast cells. We present evidence for an unusual holoenzyme structure as a dimeric protein of the RC type. We also show that the native and the recombinant R subunits are monomeric proteins with a low apparent molecular mass. Our *in vivo* and *in vitro* experiments indicate that phosphorylation of the native R subunit by the C subunit occurs at the S124 residue located within the inhibitory site.

## Materials and methods

### Chemicals

Reagents were purchased as follows: kemptide (LRRASLG), PKA inhibitor (PKI) fragment (14–24), cAMP, anti-rabbit IgG (conjugated to alkaline phosphatase), Sigma Chemical Co.; phosphocellulose paper P-81 and DE-52 were from Whatman; [ $\gamma$ -<sup>32</sup>P]ATP, New England Nuclear; ‘Complete mini’ protease mix, Roche; pre-stained protein markers from Recomb; polyvinylidene difluoride (PVDF) membranes were from Immobilon-P; isopropyl  $\beta$ -D-thiogalactoside (IPTG), restriction endonucleases and pGEM-T easy vector were from Promega; His-Pur Cobalt Resin for purification of His-tagged proteins was from Thermo scientific. Anti-phospho-PKA substrate (RRXS/T) was from Cell Signalling. Phosphatase inhibitors cocktail set II, was from Calbiochem. All other chemicals were of the highest purity available.

### Organism and culture conditions

The *Y. lipolytica* strains PO1a (MatA, *ura 3–52*, *leu 2–270*) from INRA (Grignon, France) and AC11 (MatA, *ura 3–52*, *leu 2–270*,  $\Delta$ *tpk1*) previously described [16] were used throughout this study. Yeast cells cultures were maintained at 28 °C on YPD (1% yeast extract, 2% peptone, 2% glucose) solid (2% agar) medium. When indicated, they were transferred to liquid or solid YPD or YNB medium (0.67% YNB without amino acids/ammonium sulfate from Difco, 1% glucose, 0.5% ammonium sulfate, supplemented with 22.4 mg/L uracil or 262 mg/L leucine). *Escherichia coli* strains JM109 and BL21(DE3) were used for plasmid propagation and heterologous protein expression, respectively. *E. coli* was grown at 37 °C in Luria–Bertani medium (1% tryptone, 0.5% yeast extract and 0.5% sodium chloride) with ampicillin (100 mg/L) or kanamycin (50 mg/L) for plasmid selection.

### DNA manipulations

DNA purification was performed using Qiagen affinity columns, following the manufacturer's recommendations. Plasmid DNA isolation from *E. coli* was performed by the standard alkaline lysis method [20] or using the QIAprep Spin Miniprep Kit (Qiagen). Genomic DNA from *Y. lipolytica* was isolated according to Harju et al. [21]. DNA enzymatic reactions such as digestion, ligation

and vector dephosphorylation were performed as recommended by the manufacturers (Invitrogen, New England Biolabs, Promega).

### Cloning, expression and purification of recombinant R subunits

As previously described by Cervantes-Chávez and Ruiz-Herrera [15], the *RKA1* gene has no introns, therefore the ORF was obtained by PCR using genomic DNA as template and specific oligonucleotides (5'-CGGCTAGCATGGCAAAGAGGTGATGGTCATT-3' and 5'-GGGGATCCCTACTTTGTGGGGTCATTTTCCTT-3'), and the PCR product was then ligated into the pGEM-T easy vector. The correctness of the sequence cloned in pGEM-T and inserted in pET28a-c (+) vector (Novagen) was verified by automated double strand DNA sequencing in a DNA automated sequencer (ABI PRISM) using universal primers (T7p and SP6). For *Y. lipolytica* R subunit expression as a His6-tagged fusion protein, the *RKA1* ORF was subcloned in the *NheI/BamHI* opened pET28a-c (+) vector, and used to transform BL21(DE3) cells according to standard procedures [20]. Similarly, *C. albicans* R subunit was expressed as a His6-tagged fusion protein using the same heterologous expression system as described previously [22].

Transformants were grown overnight in 3 mL of LB–kanamycin medium and these pre-cultures were used to inoculate 50 mL of fresh LB–kanamycin medium. Expression of *Y. lipolytica* and *C. albicans* R subunits was induced by the addition of 1 mM IPTG. After a 3 h incubation period, cells were harvested and used for protein extraction following Novagen's recommendations. The expressed fusion proteins were treated with 8 M urea, purified using His-Pur Cobalt Resin and renatured as described by Etchebere et al. [23] by dialysis against 10 mM Mops buffer, pH 7.2, 150 mM KCl, 0.5 mM dithiothreitol, with three changes of buffer over a 16 h period at 4 °C. Thrombin elimination of the His6-tag was performed with the Thrombin Cleavage Capture Kit (Novagen) according to the manufacturer's instructions.

### Preparation of antiserum against recombinant R subunit

The purified recombinant R subunit was subjected to 12% SDS-PAGE, and the band containing the protein stained with Coomassie brilliant blue was excised and used for immunization essentially following the procedure described by Dunbar [24]. Doses of 100  $\mu$ g of the purified protein were injected into two rabbits at days 1, 14 and 28. Blood was collected 7 days after the last booster. Pre-immune serum was kept as a control.

### Polyacrylamide gel electrophoresis and immunoblot analysis

Proteins were resolved by 12% SDS-PAGE according to Laemmli [25]. After electrophoresis, proteins were visualized by staining with Coomassie brilliant blue or transferred to PVDF membranes by semidry electroblotting for immunoblot analysis. The blots were blocked with 5% non-fat dried milk and incubated overnight with the specific antiserum. Immunological detection was performed using anti-rabbit IgG specific goat IgG conjugated to alkaline phosphatase.

### Preparation of soluble extracts

Yeast cells ( $1-2 \times 10^7$ ) were suspended in 500  $\mu$ L of 20 mM Tris-HCl buffer (pH 7.4) containing 1 mM EGTA, 1 mM EDTA, 10 mM  $\beta$ -mercaptoethanol and one tablet of ‘Complete mini’ protease mix per 10 mL (buffer A). All manipulations were thereafter performed at 4 °C. Cells were lysed by disruption with glass beads as described previously [16]. Crude extracts were centrifuged at 100,000g for 60 min and the supernatant obtained was referred as to S100.

### PKA activity and cAMP-binding assays

PKA activity was measured as previously described [26]. Briefly, the standard incubation mixture contained 20 mM Tris–HCl buffer pH 7.5, 10 mM MgCl<sub>2</sub>, 10 mM β-mercaptoethanol, 0.1 mM kemptide, 0.1 mM [ $\gamma$ -<sup>32</sup>P]ATP (0.1–0.5 Ci/mmol), and 10 μM cAMP when indicated, in a final volume of 60 μL. After incubation for 10 min at 30 °C, 50 μL aliquots were spotted on phosphocellulose paper squares and dropped into 75 mM phosphoric acid for washing. PKA specific activity was expressed as pmoles of [ $\gamma$ -<sup>32</sup>P] incorporated to kemptide/min/mg of protein. In all assays the amount of extract was adjusted in order to minimize endogenous kinase activity and the reactions were carried out under conditions of linearity respect to the amount of extract and the time of incubation. The measured activity was routinely checked to assure not less than 80% inhibition by PKI inhibitor.

cAMP-binding measurements were performed as previously described [26]. Samples were incubated at 28 °C for 30 min in a final volume of 50 μL containing 15 mM Tris–HCl buffer pH 7.5, 7 mM MgCl<sub>2</sub>, 1.4 mM β-mercaptoethanol, 600 mM NaCl and 210 nM [3H]cAMP (250,000 dpm). The reaction was stopped by filtering the samples through 0.45 μm nitrocellulose membranes. Non-specific binding was determined in the presence of 100 μM cAMP.

### Protein determination

Protein was quantified according to Bradford's method [27], using bovine serum albumin as standard.

### Preparation of partially purified holoenzyme

The S100 fraction (50 mg protein) obtained from 1 g cell (wet weight) was loaded onto a 5-mL DE-52 column equilibrated in buffer A. The column was washed with 10 volumes of buffer A and developed with a 50 mL linear gradient from 0 to 400 mM NaCl in buffer A. Fractions of 1 mL were collected and those exhibiting PKA and cAMP-binding activities were pooled and stored in aliquots at –20 °C.

### Preparation of partially purified c subunit

The C subunit was partially purified from *Y. lipolytica* DE-52 fraction as described above. The pooled DE-52 fractions were applied to 1-mL cAMP-agarose (Sigma A0144) column equilibrated in buffer A containing 150 mM NaCl. The column was washed with 10 mL of the same buffer and fractions of 0.5 mL were collected. Those containing kinase activity eluting behind the protein bulk, were pooled, concentrated and stored in aliquots at –20 °C. The kinase activity was tested to be independent of added cAMP.

### Purification of R subunit and MALDI–TOF mass spectrometry analysis

A DE-52 fraction (20 mg protein) was loaded onto 1-mL cAMP-agarose (Sigma A0144) column equilibrated in buffer A containing 150 mM NaCl. The cAMP-binding activity was retained, whereas the phosphotransferase activity passed through the column. The column was successively washed with 10 mL buffer A containing 2 M NaCl, 10 mL buffer A containing 10 mM AMP, and 10 mL buffer A containing 10 mM ATP. The R subunit was eluted by incubating the resin with 2 mL of 50 mM cAMP in buffer A for 180 min at 4 °C with gentle stirring. The suspension was centrifuged and the resin was incubated overnight in the same buffer. The eluates were pooled, concentrated to about 100 μL in a Centricon 10 and stored at –20 °C. Proteins from the sample were separated by 12% SDS–PAGE and visualized with the colloidal Coomassie brilliant blue reagent [28]. The band corresponding to the R subunit was excised

from the gel, digested in-gel with trypsin and submitted to matrix-assisted laser desorption ionization – time of flight (MALDI–TOF) mass spectrometry (MS) analysis (Unidad de Bioquímica Analítica, Instituto Pasteur de Montevideo, Uruguay). Selected peaks were subjected to tandem mass spectrometry (MS/MS) fragmentation in order to verify the protein sequence. All MS and MS/MS spectra were searched using the MASCOT search engine ([www.matrix-science.com](http://www.matrix-science.com)). The data was searched using trypsin or semi-trypsin as enzyme and N-terminal acetylation as a variable modification.

### Determination of sedimentation coefficients and Stokes' radii

Sedimentation coefficients were determined by sucrose gradient centrifugation according to Martin and Ames [29]. Aliquots of 200 μL from DE-52 fraction were loaded onto 4.5-mL linear sucrose gradient (from 5 to 20% w/v) prepared in buffer A. Gradients were run in a Beckman SW 55 Ti rotor at 38,000 rpm for 14 h at 4 °C and fractionated from the bottom in 0.65 mL fractions. Appropriate aliquots were assayed for [3H]cAMP-binding and phosphotransferase activity. Proteins used as markers were glucose oxidase (8.3 S), alkaline phosphatase (6.3 S), peroxidase (3.5 S) and cytochrome c (1.7 S).

The Stokes' radii were estimated according to Laurent and Killander [30] using a Sephacryl S-300 column (1.4 cm × 100 cm) equilibrated with buffer A containing 100 mM NaCl. Fractions of 1.6 mL were collected and aliquots were assayed for [3H]cAMP-binding and phosphotransferase activity. Proteins used for calibration of the column were glucose oxidase (4.18 nm), bovine serum albumin (3.48 nm), peroxidase (3.04 nm) and cytochrome c (1.80 nm). Molecular masses and frictional ratios were calculated according to Siegel and Monty [31].

### Phosphorylation of the R subunit

To assess *in vivo* phosphorylation of the R subunit all operations were performed in the presence of protein phosphatase inhibitors (inhibitor cocktail set II 150 mM). Cell extracts were prepared in buffer A as described above and R subunit was purified in a pull-down assay using a cAMP-agarose resin (Sigma A0144). The S100 fraction (2 mg protein) was incubated with 0.1-mL cAMP-agarose equilibrated in buffer A containing 150 mM NaCl for 60 min at 4 °C with gentle stirring. cAMP-agarose beads were successively washed with 2 mL buffer A containing 2 M NaCl, 10 mM AMP and 10 mM ATP. The proteins were eluted by boiling the resin 5 min in 1 volume of 2× Laemmli buffer.

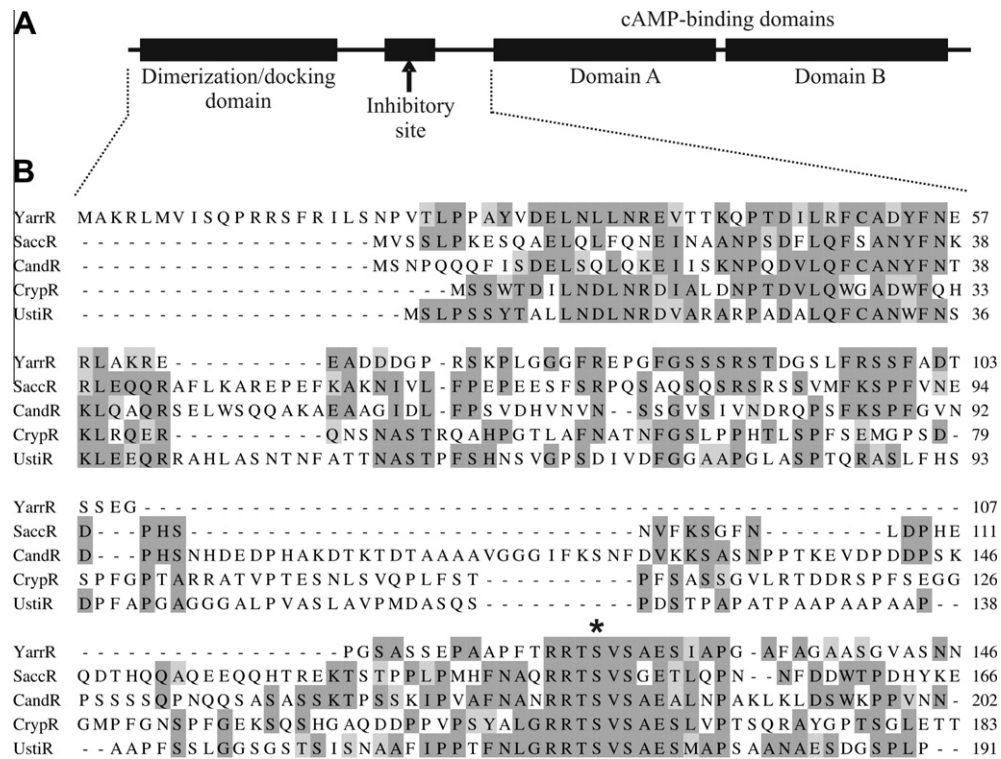
*In vitro* phosphorylation of the recombinant R subunit was performed at 30 °C using appropriate amounts of partially purified *Y. lipolytica* C subunit in a 40 μL assay mixture containing 20 mM Tris–HCl buffer pH 7.5, 10 mM MgCl<sub>2</sub>, 10 μM cAMP, 0.1 mM ATP. The reaction was stopped by adding 4× Laemmli buffer and boiling for 2 min.

The samples of *in vivo* and *in vitro* phosphorylation were submitted to 12% SDS–PAGE and phosphorylation of R subunit was determined by immunoblot analysis with anti-Rka1 and anti-phospho-PKA substrate (RRXS/T).

## Results

### Cloning *Y. lipolytica* R subunit

In order to perform the recombinant R subunit expression, the *RKA1* gene was cloned and sequenced as described in Materials and methods. The cloned sequence (HQ450396) resulted slightly different at the 5' region of the gene from the previously reported sequence (AJ579715.2), because of the reading of three additional nucleotides [15]. The ORF reported here matches perfectly with



**Fig. 1.** Schematic domain structure of a representative dimeric R subunit (A) and (B) multiple sequence alignment of the N-terminal sequence of R subunit from *Y. lipolytica* with those of other several organisms. Sequence conservation is shaded in boxes according to the percentage of identity and gaps introduced for alignment are indicated by dashes. The alignment was performed with Clustal W2 [48] and edited with Jalview 2.5 [49]. YarrR, *Yarrowia lipolytica*; SaccR, *Saccharomyces cerevisiae*; CandR, *Candida albicans*; CrypR, *Cryptococcus neoformans*; UstiR, *Ustilago maydis*. The asterisks indicates the position of autophosphorylation residue of *Y. lipolytica* Rka1p sequence.

the sequence located in the F chromosome of the *Y. lipolytica* genome (<http://cbi.labri.fr/Genolevures/>). It encodes a putative protein of 396 amino acids with a theoretical molecular mass of 42.8 kDa and a pI of 5.18. Analysis of the deduced amino acid sequence showed that the organization of the functional domains is similar to that of other fungi and mammalian homologues [18]. However, a high divergence was evident at the N-terminus, were the first 20 amino acids did not show any homology with N-terminal sequences of other R subunits (see Fig. 1). To assign a function to this tail sequence, a BLAST and a subcellular localization prediction analysis were conducted using different databases and algorithms; but neither functions nor signal peptide could be successfully ascribed to it.

#### Heterologous expression, purification and biochemical properties of recombinant R subunit

The *RKA1* gene was overexpressed as a His6-tagged fusion protein in *E. coli*. It was found that the majority of the recombinant protein appeared in inclusion bodies; therefore, purification of the recombinant protein required previous solubilization of protein aggregates with 8 M urea, before enrichment through an affinity column chromatography step and subsequent refolding with a renaturation buffer as described in Materials and methods. Fig. 2A shows SDS-PAGE and western blot analysis with anti-His-tag antibody and anti-Rka1p antiserum (see below) of protein extracts from non-induced and induced *E. coli*.

In order to estimate the functionality of the recombinant R subunit, the cAMP-binding capacity and the ability to inhibit kemptide phosphorylation by the C subunit were evaluated. The purified His6-tagged protein showed a  $[3H]cAMP$  binding capacity of  $2980 \pm 80$  pmol/mg, indicating that cAMP-binding sites are active in the recombinant protein. With the purpose of analyzing the interaction of the C subunit and the recombinant R subunit, partially purified C subunit was preincubated with increasing concen-

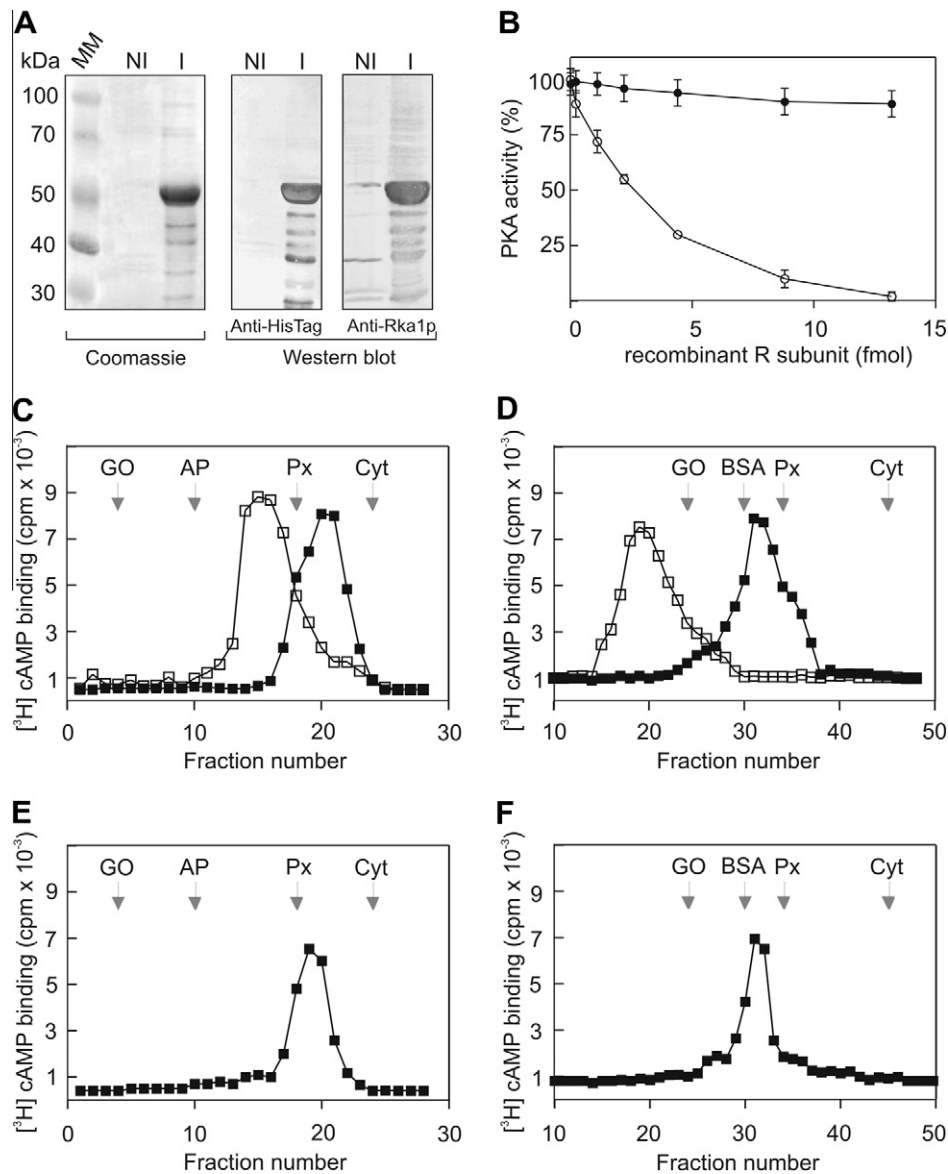
trations of purified His6-tagged protein and their kinase activities were measured using kemptide as substrate in the absence or presence of 10  $\mu M$  cAMP (Fig. 2B). The addition of increasing amounts of recombinant R subunit resulted in complete inhibition of the phosphotransferase activity and full original activity was restored by the addition of cAMP. Both results indicated that the recombinant protein was active and exhibited the typical biochemical characteristics of native R subunit.

Analysis of the His6-tagged recombinant protein by sucrose gradient and gel filtration was performed with the purpose of exploring its polymeric nature. The results shown in Fig. 2C and D indicate that the *Y. lipolytica* recombinant R subunit was isolated as a unique peak of cAMP-binding activity with an apparent sedimentation coefficient of 3.0 and a Stokes radius of 3.3. According to Siegel and Monty [31], the molecular mass was estimated at 48 kDa, a value compatible with a monomeric protein. *C. albicans* recombinant R subunit was included as a control since we had previously shown that it is a dimeric protein [26]. As can be seen in Fig. 2C and D, *C. albicans* subunit resulted in a single peak with a sedimentation coefficient of 4.6 and the Stokes radius of 5.1, both values expected for a dimeric structure.

It is worthwhile mentioning that all results obtained with the *Y. lipolytica* recombinant protein were identical whether the protein carried the His6-tag or not (Fig. 2E and F).

#### Preparation, reactivity and specificity of *Y. lipolytica* R subunit antiserum

To identify native R subunit in a complex protein mixture, an antiserum against purified recombinant R subunit was prepared as described in Materials and methods and used to identify the corresponding protein in crude extracts and in cAMP-agarose eluates. The immunoblotting experiments in Fig. 3 showed that the Rka1p antiserum recognized a mayor band in the crude extract (lane 1)



**Fig. 2.** Heterologous expression and biochemical analysis of recombinant R subunit. (A) Protein extracts from non-induced (NI) and induced (I) *E. coli* were subjected to 12% SDS-PAGE and proteins were stained with Coomassie brilliant blue (left panel); or transferred to PVDF membranes and analyzed by Western blot with monoclonal anti-His-Tag antibody (middle panel) or anti-Rka1p subunit (right panel). Molecular mass markers and their values are shown, respectively in lane MM and on the left. (B) Inhibition of the partially purified catalytic subunit by recombinant R subunit. Catalytic subunit was preincubated for 15 min at 30 °C with increasing concentrations of purified R subunit and residual phosphotransferase activity was measured in the presence (●) or absence (○) of 10  $\mu\text{M}$  cAMP as described in Materials and methods. (C) Sucrose gradient centrifugation and (D) Sephacryl S-300 column chromatography analysis of His6-tagged recombinant *Y. lipolytica* (■) and *C. albicans* (□) R subunit. (E) Sucrose gradient centrifugation and (F) Sephacryl S-300 column chromatography analysis of recombinant *Y. lipolytica* R subunit without His6-tag. The arrows in C, D, E and F indicate the position of marker proteins: GO, glucose oxidase; AP, alkaline phosphatase; BSA, bovine serum albumin; Px, peroxidase; Cyt, cytochrome c.

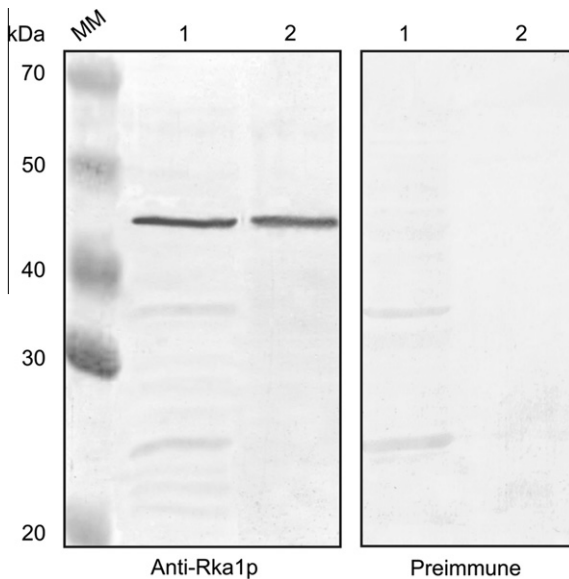
and a single band in the cAMP-agarose eluate (lane 2), with an estimated molecular mass of 46 kDa. This band was not detected with the pre-immune serum. In the crude extracts, the minor lower molecular mass bands very probably represent non-specific proteins since they were also detected with the pre-immune serum. These results indicate that the molecular mass of the R subunit from crude extracts was identical to that of cAMP-agarose purified protein suggesting that the 46 kDa protein represents the native form of the R subunit.

#### Hydrodynamic parameters of the partially purified holoenzyme and dissociated subunits

To further characterize the PKA components, the native holoenzyme was partially purified from the S100 fraction of wild-type *Y. lipolytica* strain by chromatography on DE-52 column. Phospho-

transferase activity eluted as a single peak at 100 mM NaCl and showed almost absolute dependence on the addition of cAMP (data not shown). Neither cAMP-binding activity nor kemptide kinase activity was detected in the flow-through. Column fractions containing kemptide kinase activity eluting at 0.1 M NaCl were pooled, concentrated in a Centricon 10, and subjected to sucrose gradient centrifugation and gel filtration analysis (see below).

In order to confirm the monomeric structure of R subunit, the hydrodynamic parameters of native PKA and its subunits were determined by sucrose-gradient centrifugation and gel filtration. Upon sucrose-gradient centrifugation of the partially purified holoenzyme (Fig. 4A) the protein kinase activity sedimented in two peaks where only the heaviest minor peak of 4.9 S was activated by cAMP, indicating that it corresponds to the PKA holoenzyme. Our results also showed that the peaks with either only cAMP-binding or only phosphotransferase activity that appeared with



**Fig. 3.** Western-blot analysis of native R subunit from *Y. lipolytica*. Lane MM, molecular mass markers; lane 1, crude extract; lane 2, purified R subunit. Proteins were subjected to 12% SDS-PAGE, blotted to a PVDF membrane, and revealed with an antiserum anti-Rka1p or pre-immune serum as described in Materials and methods. Values of the molecular mass markers are indicated on the left.

lower sedimentation coefficients (2.6 and 3.3 S, respectively), very probably represented free subunits due to partial dissociation of the holoenzyme during sucrose-gradient centrifugation. The fact that the minor peak with cAMP-binding activity co-sedimented with the peak of cAMP-dependent phosphotransferase activity, and was absent in sucrose-gradients from the  $\Delta tpk1$  mutant, reinforces the idea that this small peak corresponds to the R subunit associated with the C subunit in the holoenzyme.

Analysis of the partially purified holoenzyme by gel filtration showed that the bulk of the protein kinase activity eluted after the glucose oxidase (Fig. 5). This peak represented protein kinase holoenzyme, since its activity was highly dependent on cAMP. The minor peak of phosphotransferase activity eluting between peroxidase and cytochrome *c* very probably corresponded to free catalytic subunit since its activity was independent of cAMP. A significant proportion of cAMP-binding activity co-eluting with peroxidase was very likely the free R subunit.

Comparison of the molecular masses of the holoenzyme and the dissociated catalytic and regulatory subunits, estimated according to Siegel and Monty [31], clearly suggests that the PKA from *Y. lipolytica* is a dimer of 90 kDa composed of one R and one C subunit with molecular masses of 42 kDa and 39 kDa, respectively. Table 1 summarizes the structural parameters and molecular masses estimated for *Y. lipolytica* PKA and its subunits.

In order to preclude the possibility that the low molecular mass of the isolated R subunit was due to proteolytic degradation of the native protein, comparison of the molecular mass of the purified R subunit with the value derived from less purified preparations was performed by western blot analysis with the antiserum raised against the recombinant R subunit. As can be seen in Fig. 6, the molecular mass (46 kDa) of purified protein was identical to that of R subunits from whole cell lysate, crude extract and cAMP-agarose eluate as well as those of R peaks from sucrose gradient and gel filtration.

#### Studies of the amino acid sequence of the native R subunit

The results presented above indicate that the dimeric RC structure of the holoenzyme of *Y. lipolytica* is a consequence of the

monomeric nature of the R subunit. In order to assess the N-terminal sequence of the native R subunit and further rule out the possibility of proteolysis occurring on the putative dimerization domain, which would prevent the homodimer formation, sequences of tryptic peptides from the purified protein were determined by MALDI-TOF MS. The protein was digested with trypsin as described in Materials and methods and some of the resulting peptides were sequenced by tandem mass spectrometry. Fig. 7 shows the mass spectrum and the peptides that were identified. As can be seen in panel C, two peptides align with sequences localized at the N-terminal domain of the deduced amino acid sequence confirming that the native R subunit did not undergo extensive proteolytic cleavage since the predicted sequence was conserved from T23 downwards, spanning the putative dimerization domain. The first 22 amino acids could not be identified, perhaps as a consequence of a protein maturation event. Further support for the idea that the first peptide corresponds to the N-terminus of the native protein came from the result of its MS/MS spectrum which revealed it was acetylated (Fig. 7B), one of the most common *in vivo* N-terminal covalent modifications of eukaryotic proteins (for a review see reference [32]).

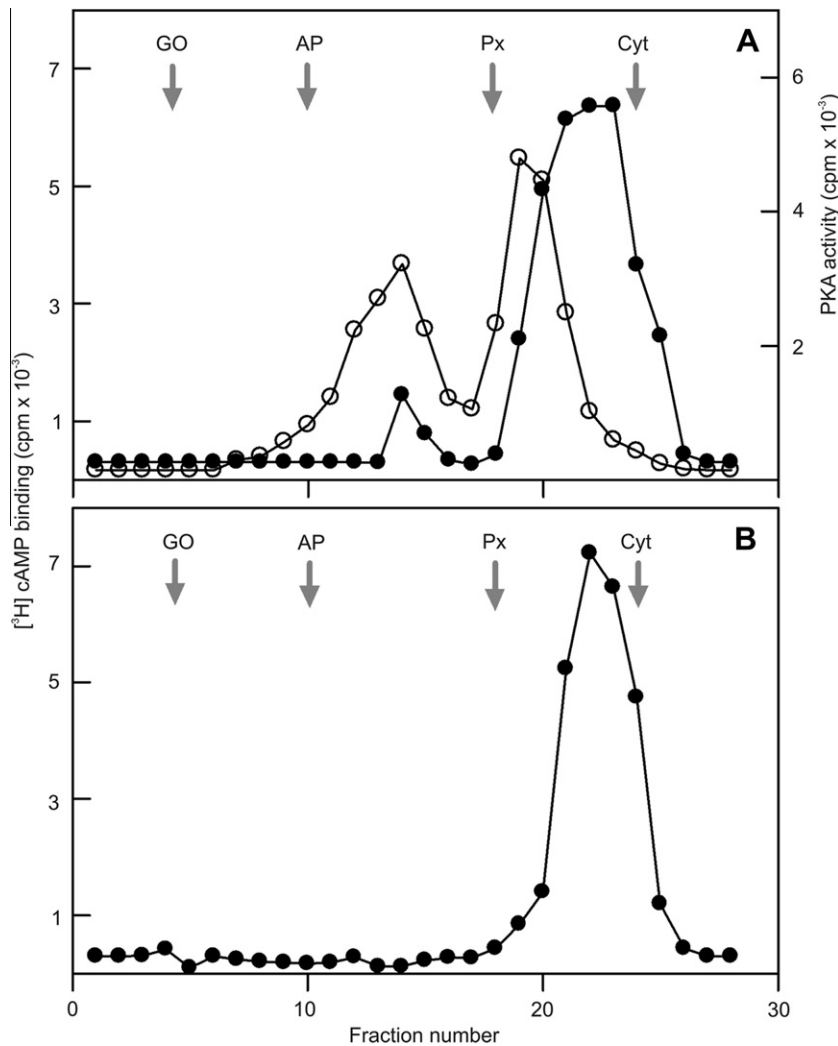
#### Phosphorylation of R subunit by *c* subunit

PKA regulatory subunits have typically been classified according to their physicochemical properties, namely, overall charge and molecular mass, and the presence or absence of a serine residue susceptible to autophosphorylation at the inhibitory site [18]. *In silico* analysis of *Y. lipolytica* amino acid sequence pointed at S124 as the unique autophosphorylation site (see Fig. 1). Therefore, susceptibility to undergo autophosphorylation of the R subunit at the inhibitory site through the endogenous C kinase activity in the holoenzyme was analyzed *in vivo* as described in Materials and methods. The R subunit from the wild-type strain was enriched from the S100 extract by cAMP-agarose column in the presence of a phosphatase inhibitor cocktail and phosphorylation of Ser124 was assessed by recognition with anti-phospho-PKA substrate (RRXS/T) antibody. Purified R subunit from  $\Delta tpk1$  mutant strain was included as a control. Fig. 8A shows that only R subunit from wild-type strain was phosphorylated under these conditions (lanes 1 and 2) Fig. 8B also shows *in vitro* phosphorylation of recombinant R subunit by the partially purified C subunit (lanes 1 and 2). These results indicate that *Y. lipolytica* R subunit belongs to the type-II subclass given that only R from the wild-type strain was detected by the anti-phospho-PKA substrate (RRXS/T) antibody, and that the recombinant R could be phosphorylated *in vitro* by partially purified C.

#### Discussion

This paper describes the biochemical and structural characterization of the partially purified PKA from *Y. lipolytica* and its constituent catalytic and regulatory subunits. We also present data on the expression, purification and characterization of the recombinant R subunit.

The *RKA1* gene was accurately overexpressed as a His6-tagged fusion protein in *E. coli* as shown by the correctness of the DNA sequence of the cloned ORF and the expected molecular mass of the recombinant protein observed in extracts from induced cells analyzed by western blot with anti-His-Tag antibody (Fig. 2). Despite the fact that the bulk of the recombinant R subunit appeared in inclusion bodies, the heterologous protein was correctly refolded after solubilization of protein aggregates with 8 M urea, affinity chromatography purification and *in vitro* folding, as revealed by its cAMP-binding activity and its capacity to interact with partially

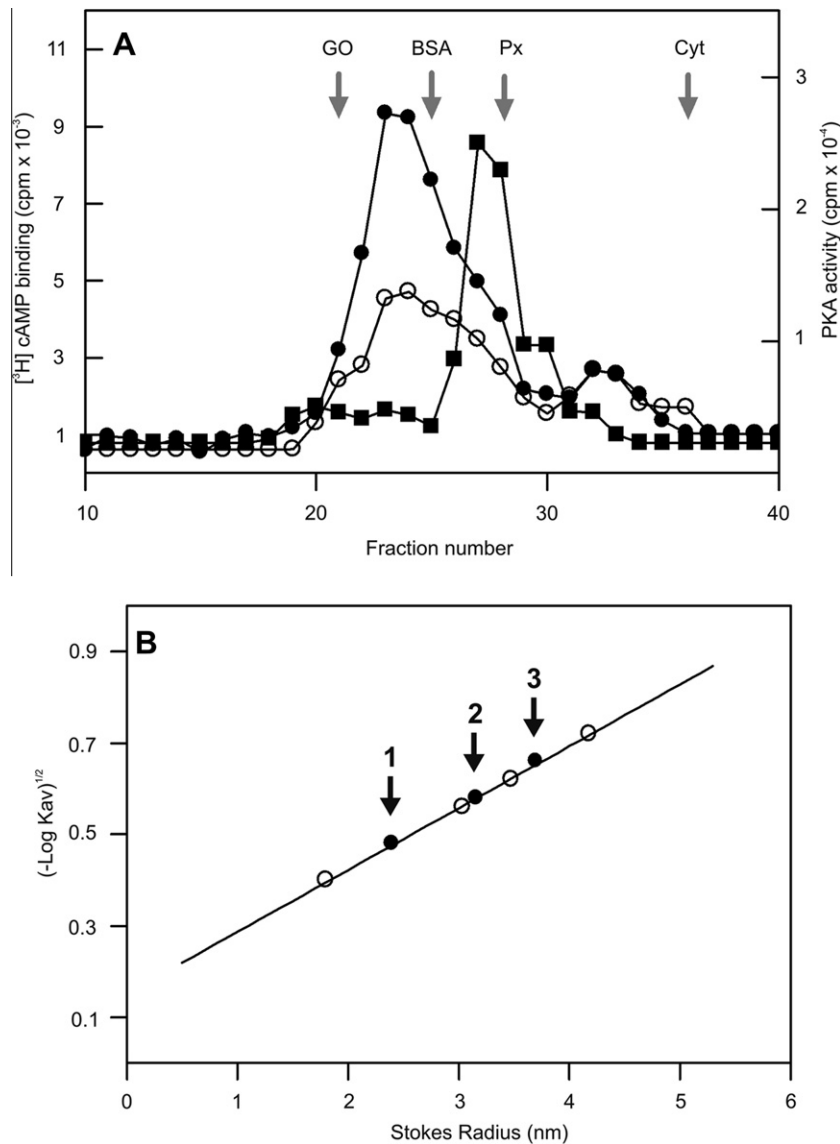


**Fig. 4.** Sucrose gradient centrifugation analysis of partially purified native PKA from wild-type and  $\Delta$ atp1 *Y. lipolytica* strains. Sucrose gradient centrifugation was performed as described in Materials and methods. Fractions of 0.65 ml were collected from the bottom of the tubes and appropriate aliquots were assayed for kemptide phosphotransferase activity in the presence of 10  $\mu$ M cAMP ( $\circ$ ) and cAMP binding capacity ( $\bullet$ ). (A) Partially purified PKA from wild-type and (B) partially purified R subunit from  $\Delta$ atp1 strain. The arrows indicate the position of marker proteins: GO, glucose oxidase; AP, alkaline phosphatase; Px, peroxidase; Cyt, cytochrome c.

purified C subunit (see Figs. 2 and 8). It is worthwhile to mention that other research groups using the same heterologous expression system for obtaining recombinant R subunit from several sources [22,33–35] including *Saccharomyces cerevisiae* and *C. albicans*, always reported soluble protein production. The fact that *Y. lipolytica* recombinant R subunit was found in inclusion bodies may be the consequence of the peculiar features of its N-terminal sequence. As can be seen in Fig. 1, the linker sequence from *Y. lipolytica* R subunit connecting the putative dimerization docking domain with the inhibitory site is smaller than the other R subunits, similarly Vigil et al. (2006) have reported a mutant of bovine RII $\beta$  subunit lacking 12 amino acid residues within the linker region ( $\Delta$ 68–79) which yielded an insoluble heterologous protein, possibly due to the hydrophobic amino acid residues preceding the deleted sequence [33].

Regarding the structure of the holoenzyme, we here present evidence compatible with a dimeric protein of type RC with an estimated molecular mass of 90 KDa. No higher molecular mass species than this were observed during our study. Native and recombinant R subunits were isolated as monomeric proteins with a low apparent molecular mass. Dimeric species of the PKA holoenzyme are not rare, and have been isolated and described in several systems [36–42]; some of them have been obtained by endogenous proteol-

ysis or trypsin treatment of the tetrameric holoenzyme [38,41], while a few others like those from *Saccobolus platensis*, *Dictyostelium discoideum*, *Paramecium tetraurelia*, *Neurospora crassa* and *Myxococcus xanthus* resulted dimeric as a consequence of a monomeric native form of the regulatory subunits [36,37,39,40,42,43]. We propose that the dimeric structure of the *Y. lipolytica* holoenzyme is a consequence of the inability of the R subunit to form a homodimer, due to differences in the amino acid sequence from N-terminus to the beginning of the cAMP-binding domains. Most R subunits have a well-defined domain structure with a stable dimerization/docking domain at the N-terminus joined to cAMP-binding domains by an extended highly disordered linker that spans the inhibitory site. Within these domains, the *Y. lipolytica* R subunit has a number of particularities in the amino acid sequence that may be responsible for the dimerization incapacity (Fig. 1). Since the first 22 amino acids are present in the recombinant R subunit and they were not detected by MS/MS analysis in the native R subunit, and both proteins appeared as monomeric, these first 22 amino acids seem not to be responsible for the lack of dimerization. A careful comparison of the putative dimerization/docking domain of the *Y. lipolytica* R deduced amino acid sequence with that of well characterized dimeric R subunits revealed the absence of several highly-conserved residues especially near the end of the domain. These resi-



**Fig. 5.** Sephacryl S-300 gel filtration of PKA from *Y. lipolytica*. (A) Elution profile for partially purified PKA from wild-type strain. Gel filtration was performed as described in Materials and methods and aliquots of the fractions were assayed for protein kinase activity in the absence (○) or in the presence (●) of 10 μM cAMP, and cAMP binding (■). The arrows indicate the positions of marker proteins: GO, glucose oxidase; BSA, bovine serum albumin; Px, peroxidase; Cyt, cytochrome c. (B) Determination of Stokes radii of partially purified PKA. The data were plotted according to Siegel and Monty [31]. Empty circles indicate the position protein markers and filled circles the position of (1) C subunit, (2) R subunit and (3) holoenzyme.

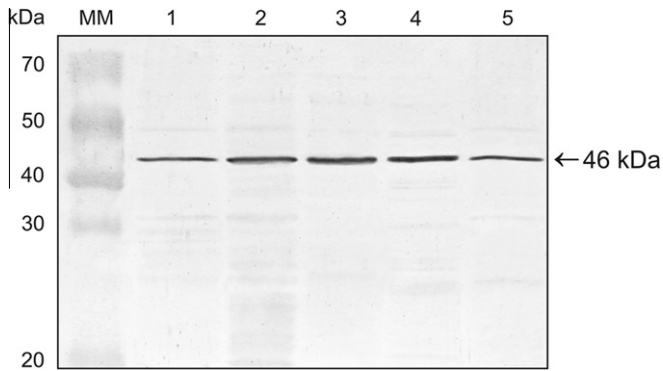
**Table 1**  
Hydrodynamic parameters of *Y. lipolytica* PKA.

PKA	Sedimentation coefficient (S)	Stokes radius (nm)	Molecular mass (kDa)	Frictional ratio $f/f_0$
Holoenzyme	4.9	3.8	90	1.28
C subunit	3.3	2.4	39	1.07
R subunit	2.6	3.2	42	1.41

dues can alter the electrostatic environment of the exposed faces of the structure which conforms this domain and consequently alter its interaction [44]. It is also possible that the linker sequence between the putative dimerization/docking domain and the inhibitory sequence, shortened in *Y. lipolytica* than that of the R subunit from other fungi, may contribute to destabilize the structures that promote dimerization. Supporting this idea, there are several studies that recognize the importance of the linker region in the overall architecture of the free R subunits and of their corresponding holo-

enzymes [33,45]. In addition, a computerized analysis of hydrophobic profiles from the N-terminal domain of the *Y. lipolytica*, *S. cerevisiae* and human RIIβ R subunits sequences obtained with the ProtScale application [46], revealed a noticeable difference in the electrostatic profile that is potentially relevant for inter-subunit interaction. It can not be ruled out that the *Y. lipolytica* N-terminal domain may fulfill physiological functions other than dimerization, including interactions with A kinase anchoring proteins and subcellular localization of PKA.

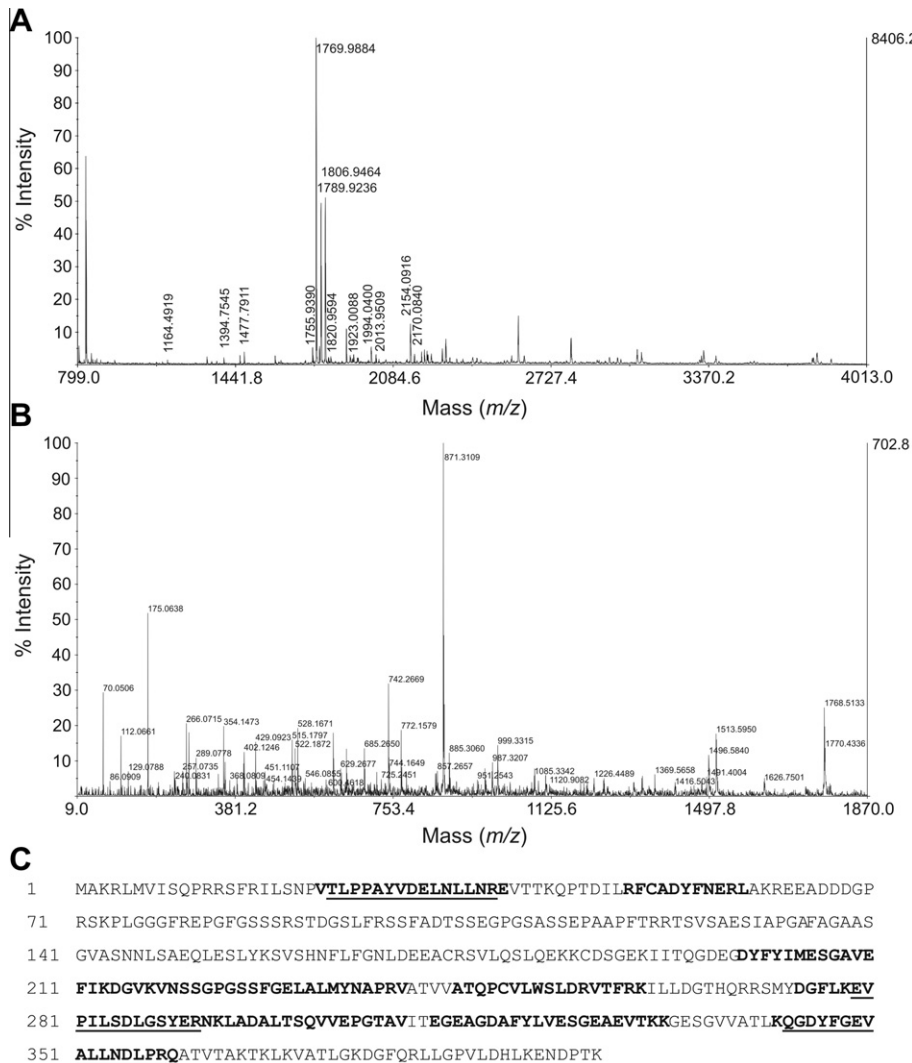
The possibility that limited proteolysis is responsible for the monomeric structure of the regulatory subunit can be ruled out since the recombinant R subunit having a His6-tag in its N-terminus behaved as the monomeric protein (Fig. 2C and D). Also the deduced molecular mass of R native protein purified by chromatography on DE-52 column and cAMP-agarose was identical to those obtained for R subunits from whole cells lysates, crude extracts, and the peaks of sucrose gradient centrifugation and Sephacryl gel filtration (Fig. 6). In addition, MS/MS analysis of the



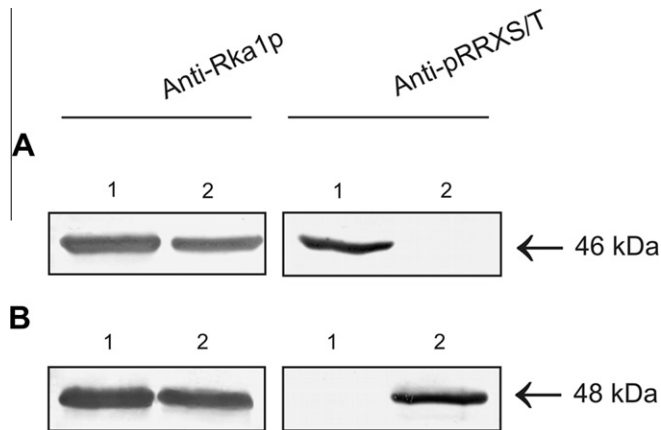
**Fig. 6.** Western-blot analysis of *Y. lipolytica* R subunit along the purification steps and from peaks from sucrose gradient and Sephacryl S-300 gel filtration. Lane MM, molecular mass markers; lane 1, total cell lysate; lane 2, crude extract; lane 3, cAMP-agarose purified R subunit; lane 4, fraction 22 of sucrose gradient; lane 5, fraction 27 gel filtration. Proteins were subjected to 12% SDS-PAGE, blotted to a PVDF membrane, and revealed with an anti-Rka1p antiserum as describe in Materials and methods. Values of the molecular mass markers are indicated on the left.

tryptic fragments of the purified R subunit allowed the identification of an N-terminal sequence which spans the putative dimerization/docking domain, since the predicted sequence was conserved from T23 downwards.

In eukaryotic PKAs, R subunits are classified into two major types, RI and RII subunits which contain the inhibitory sequences RRX[A/G] and RRXS, respectively [18]. In the absence of cAMP, only the serine residue in the inhibitory sequence of the RII subunit can be phosphorylated by the C subunit in the holoenzyme complex. Here, we demonstrated that *Y. lipolytica* R subunit belongs to Type II since it was *in vivo* phosphorylated by C. We can point at S124 as the autophosphorylation site since analysis of the amino acid sequence showed this residue located within the inhibitory site, as the only serine residue contained in the consensus RRTS sequence recognized by the anti-phospho-PKA substrate (RRXS/T) antibody. A decrease in the affinity between R and C subunits upon phosphorylation of R subunits has been reported in *C. albicans* and *S. cerevisiae* [22,47]. This decreased affinity points to the important role a phosphate group may play in hindering the proper assembly of distal domains involved in holoenzyme formation. Further work



**Fig. 7.** Mass spectrometry analysis of R subunit. (A) MS spectra of the tryptic-treated R subunit purified from *Y. lipolytica* as described in Materials and methods. Peptides labeled in the figure were identified as part of the R subunit using MASCOT search engine setting trypsin or semi-trypsin as enzyme and N-terminal acetylation as variable modification. (B) The MS/MS spectrum of the tryptic peptide ion with  $m/z$   $[M+H]^+$  value 1769.9884, corresponding to the putative N-terminal peptide. (C) Amino acid sequence of *Y. lipolytica* R subunit showing tryptic and semi-tryptic peptide fragments detected by MALDI-TOF MS. Boldface residues recovered from the tryptic digestion supernatant. Boldface underlined residues were confirmed by de novo peptide sequencing performed by ms/ms spectrometry analysis. The sequence coverage for tryptic digestion is of 41%.



**Fig. 8.** *In vivo* and *in vitro* phosphorylation of R subunit. (a) *In vivo* phosphorylation was assessed by purification of R subunit in the presence of phosphatase inhibitors from wild-type (lane 1) or  $\delta tpk1$  strain (lane 2); and (b) *in vitro* phosphorylation was carried out by incubation of purified recombinant R subunit in the absence (lane 1) or in the presence (lane 2) of partially purified c as described in Materials and methods. The samples were submitted to 12% SDS-PAGE, blotted to a PVDF membrane, and phosphorylation was determined by immunoblot analysis with specific anti-Rka1p and anti-phospho-PKA substrate (rrxs/t) antibodies.

is needed to evaluate the effect of *Y. lipolytica* autophosphorylation of the R subunit in its interaction with the C subunit. If, as in the above mentioned fungi, the effect is similar, then the existence of the autophosphorylated PKA holoenzyme would allow cells to respond more easily to small changes in cAMP levels due to the weaker affinity between the R and C subunits. This study is now under progress in our laboratory.

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We thank Dr. Eduardo Passeron for critical reading of the manuscript. This work was supported by Grants from the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) and the Agencia Nacional de Promoción Científicas y Técnicas (ANPCyT).

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