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PKA from *Mucor circinelloides*: Model to study the role of linker I in the interaction between R and C subunits

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Abstract

Protein kinase A from the fungus *Mucor circinelloides* shows high affinity interaction between regulatory (R) and catalytic (C) subunits. Its R subunit shows a differential presence of several acidic residues in linker I region, in the amino terminus. Mutants R1, lacking the N-terminal region, and R2, lacking the acidic cluster, were used to analyze its effect on the interaction with the C subunit, assessed through inhibition of catalytic activity and cAMP activation of reconstituted holoenzyme. A similar decrease in the interaction was obtained when using R1 and R2 with the homologous C subunit; however when using heterologous bovine C, only R1 had a decreased interaction. The results show the general importance of linker I region in the R–C interaction in protein kinases A and point to the importance of the acidic cluster present in the N-terminus of *M. circinelloides* R subunit in the high affinity interaction between R and C in this holoenzyme.

Keywords: PKA; cAMP; Linker I; *Mucor circinelloides*; Interaction R–C

The cAMP-dependent protein kinase (PKA) holoenzyme in its inactive form is a tetramer (R_2C_2) with two copies of both regulatory (R) and catalytic (C) subunits. The C subunits are responsible for catalyzing the phosphoryl transfer reaction while the R subunits confer cAMP dependence and localize the holoenzyme [1] to discrete subcellular locations within the cell via interactions with anchoring proteins (AKAPs). R subunits have several functional domains: a dimerization domain (D/D), that is also the site for binding to AKAPs, is at the N-terminus while the C-terminus has the two tandem cAMP-binding domains. Between these domains is a variable linker region with dynamic properties that includes an inhibitor sequence (IS) that fills the active site cleft of the C and inhibits substrate binding and hence catalysis. The cAMP-binding domains are highly conserved among different species [2] while the linker regions show considerable variation [3]. The portion of the linker region preceding IS is named linker I and the portion located C-terminal to the IS is the linker II [4]. Type II R subunits have always a Ser at the consensus IS sequence which is autophosphorylated, while type I R subunits have a Gly or Ala at this position rendering a pseudo-phosphorylation site.

At low cAMP concentration, PKA is maintained as an inactive tetrameric holoenzyme R_2C_2. When the intracellular concentration of cAMP increases in response to specific cellular stimuli, two cAMP molecules bind to each R subunit thus releasing its inhibition of C [5]. Recent experiments suggest that cAMP does not fully dissociate the holoenzyme and that the substrate has a role in the process [6].

A crystal structure of a complex between the C subunit and a deletion mutant of RIα has been recently solved. This structure identifies a previously unidentified extended surface in C through which interaction with IS, linker II and the helical region of cAMP-binding domain A are being established [7]. In particular, linker II, disordered in free

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RIα, folds across the extended surface in C. At present there are no high-resolution structures of full-length R subunit homodimers or holoenzyme complexes, so the role of the linker I in the interaction R–C is not yet clear. However, it has been shown that the sequence immediately N-terminal to the IS (P-11, P-4) in RI and RII interacts with different sites at the proximal region of the active site cleft in the catalytic subunit [8]. Recent analysis of RIIα and RIIβ PKA isoforms through small-angle X-ray-scattering, has shown that linker regions are capable of organizing inter-domains and inter-subunit communication [4].

Mucor circinelloides and *Mucor rouxii* PKA are tetrameric holoenzymes present in a unique isoform [9–11]. They resemble higher eukaryotic counterparts as demonstrated by kinetic measurement and predicted by their aminoacidic sequence [12,13]. However, *M. rouxii* PKA has been shown to differ from PKAs in higher eukaryotes in the fact that it can not be dissociated by the sole addition of cAMP, but that NaCl or polycations have to be added together with cAMP, suggesting additional ionic interactions in the holoenzyme [10].

In this paper, we demonstrate that the PKA of *M. circinelloides* behaves like the *M. rouxii* PKA: it presents the same unique dissociation behavior by cAMP, indicating that the affinity of interaction between R and C subunits in this holoenzyme is higher than in higher eukaryotic holoenzymes. We demonstrate that a cluster of acidic residues in linker I has an important role in determining the high affinity of the *M. circinelloides* PKA holoenzyme, showing thus the importance of linker I region in the R–C interaction.

Materials and methods

Materials. Kemptide and anti-rabbit IgG peroxidase-conjugated antibody, DEAE-cellulose, Sephadex G-25 were from Sigma Chemical Co. [γ^{32}P]ATP and [^{3}H]cAMP were from Perkin Elmer Life Sciences. Phosphocellulose paper was from Whatman. Recombinant *Bos taurus* C subunit was a generous gift from S. Taylor, University of California, San Diego. Nitrocellulose membrane was from MSI. 8-AHA-cAMP-agarose was from BioLog.

Mutagenesis of R subunit. The mutants of *M. racemosus* R subunit were obtained through PCR-based methods, using complementary mutagenic oligonucleotide primers and pYES-R (pYES plasmid with R subunit cDNA sequence) as template. R1 mutant lacks N-terminal residues (1–127) and R2 mutant lacks the acidic residues (110–125). For R1 mutant two primers were used B1:5′-CTAAGCTTTCAACTGAACCT TTGCCCTC-3′ and B2:5′-GTCGAATTCTTATGATTGCTGGTTAAT GACAG-3′. The 900 pb PCR product was cloned in HindIII–EcoRI pYES sites. For R2 mutant two primers were used for two pre-PCR single primer extension reactions of pYES-R. B3:5′-TCCCAACGACCAAGGT GCTCTTTCAT-3′ and B4:5′-GGGCAAAGGTTCAGTTGAAAATTT-3′. The two reactions were then mixed and used as template for PCR using B1 5′-CTAAGCTTATGATCACTGACGAACATCC-3′ and B6:5′-A CGGTAACCTTGTTTTGCCCAATAAAAC-3′. The 720 pb PCR product was cloned in HindIII–BstEII pYES-R sites. Constructions were verified by DNA sequencing.

Expression and purification of the R subunits. Rwt and mutants R1 and R2 were expressed and semipurified from *Saccharomyces cerevisiae* strain S13-3A containing pYES-R, pYES-R1, or pYES-R2 grown in YPGal. The strain S13-3A (Matα his3 leu2 ura3 trp1 ade8 tpk2::HIS3 tpk3::TRP1 bcy1::LEU2) harbors a deletion mutation that abolishes Bcy1 endogenous expression. Crude extracts were prepared using glass beads and buffer A (25 mM Tris–HCl pH 8, 5 mM EDTA, 3 mM EGTA, 10 mM 2-mercaptoethanol, complete protease inhibitor cocktail). They were loaded onto 4.5 ml 5–20% sucrose gradients in buffer A and centrifuged for 15 h at 35,000 rpm on a SW 55Ti rotor. The fractions with maximum cAMP-binding activity were pooled, freeze-dried and stored at −20 °C until use. These R preparations are not cAMP free.

Mucor circinelloides C subunit purification. Mucor circinelloides strain R7B (ATCC0680*)* (10^6 spores/ml) was cultivated in liquid YPG. Cultures were grown 6–7 h until the emission of germinative tube. PKA holoenzyme was prepared as described [12] using buffer A through DEAE-cellulose eluted with 0.35 M NaCl and further $(NH4)_2SO_4$ precipitation (60%). The C subunit was isolated by 8-AHA-cAMP-agarose purification as previously described [10]. The fractions with maximum phosphotransferase activity were pooled, freeze-dried and stored at −20 °C to use as enzyme source.

cAMP-binding assay. cAMP binding was measured by nitrocellulose filter assay. Purified R subunits were incubated for 30 min at 30 °C in a final volume of 70 μl with 0.3 μM [^{3}H]cAMP and 0.5 M NaCl in buffer A. An aliquot was spotted on nitrocellulose membrane filters under vaccum and washed with buffer Tris–HCl 20 mM pH 7.5.

Standard PKA assay. PKA C subunit activity was determined by assay of its phosphotransferase activity with kemptide as substrate. The assay was started by addition of either recombinant *B. taurus* catalytic subunit (C_B) or *M. circinelloides* C subunit (C_M), to a standard incubation mixture containing 15 mM $MgCl_2$, 0.1 mM [γ^{32}P]ATP (700 dpm/pmol), 300 μM kemptide (saturating for C_B or C_M enzymes) and 10 μM cAMP. After 15 min at 30 °C, aliquots were processed according to the phosphocellulose paper method [14]. PKA activity is expressed in units defined as pmol of phosphate incorporated into substrate/min, at 30 °C.

Inhibition of C kinase activity by R subunits. Interaction between C and R subunits was assayed by measuring the ability of the R subunits to inhibit kemptide phosphorylation by C. Purified C subunits were preincubated for 30 min at 30 °C with various concentrations of R subunits (0.02–1.5 nM) in 20 mM Tris–HCl buffer (pH 7.5), 1 mM 2-mercaptoethanol, 10 mM $MgCl_2$ and 0.1 mM ATP in a final volume of 70 μl. The amounts of C subunit used in the assays were 0.02 pmol of C_B (5 U) and 2 U of C_M.

The phosphorylation reactions were started by the addition of 10 μl of a mixture containing kemptide and [γ^{32}P] ATP so as to give final concentrations of 300 μM kemptide and 0.1 mM ATP (1000–1500 cpm/pmol). Incubations were performed at 30 °C for 10 min. Aliquots of 50 μl were processed as described above.

Reconstitution and dissociation of the holoenzyme. PKA holoenzymes were reconstituted by combining C_M or C_B subunits with wild-type and mutant R subunits in 20 mM Tris–HCl buffer (pH 7.5), 1 mM 2-mercaptoethanol, in the presence of 10 mM $MgCl_2$ and 0.1 mM ATP and preincubated for 30 min at 30 °C. The reconstituted holoenzymes were used for measurement of kinase activity at various cAMP concentrations as described above. The amounts of subunits used for the reconstitution of the holoenzymes were the following: (1) 0.7 U of C_M with 0.005 pmol of R subunits; (2) 0.03 pmol of C_B (7.5 U)with 0.05 pmol of R subunits. The different amounts of C_B and C_M used in the inhibition and activation experiments do not affect the conclusions of the work.

Results and discussion

PKA holoenzyme from Mucor circinelloides displays high affinity R–C interaction

In order to analyze whether *M. circinelloides* PKA holoenzyme (PKAMc) resembles its *M. rouxii* counterpart in the high affinity interaction of its subunits we studied its biochemical behavior in response to cAMP. We have already shown that partial activation of *M. rouxii* PKA

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by cAMP, using kemptide as substrate, could be turned to complete activation by addition to the assay of 0.5 M NaCl or polycations together with cAMP [15]. We therefore assayed the activity of increasing concentrations of PKAMc in the presence of 10 μM cAMP ± 0.5 M NaCl. The results (Supplementary Fig. 1A) indicate that PKAMc activity measured in the presence of cAMP was higher and the linearity was maintained, when NaCl was added to the incubation mixture. The results indicate that the lack of proportionality, in the absence of NaCl, was due to incomplete activation.

Another property that distinguishes *M. rouxii* PKA from other holoenzymes is the behavior on cAMP affinity chromatography. While most of the PKAs dissociate into its subunits upon contact with the cAMP-agarose ([16] and Supplementary Fig. 1C), the *M. rouxii* PKA needs the addtion of NaCl to promote dissociation [10]. We therefore assayed the behavior of PKAMc on this affinity chromatography. Neither catalytic nor cAMP-binding activities were detected in the flow through indicating that the holoenzyme was bound to the resin and the dissociation had not occurred *in situ* by the effect of cAMP. The C subunit was eluted only when 0.3 M NaCl was added to the elution buffer (Supplementary Fig. 1B).

These observations, taken as a whole, indicate that PKAMc shares with the PKA from *M. rouxii* the property of a higher affinity between R and C in which ionic interactions seem to be involved.

A comparison of the sequences from higher eukaryotic RI and RII subunits and from different fungi shows that the hinge region, outside the IS, comprising linker I and linker II presents almost no conservation among the species (Fig. 1A). In linker I region we observe the presence of acidic residues with different distribution and number among the different sequences. In particular, R subunits from both *Mucor* fungi and from *Blastocladiella emersonii* have a cluster of around 12 acidic residues in a row. This fact, together with the necessity of polycations to achieve complete activation of *Mucor* holoenzymes by cAMP, suggested us that this acidic cluster participates in the R–C interaction.

Inhibition of C subunit kinase activity by R subunits

In order to evaluate the impact of the acidic cluster in the R–C interaction in the PKAMc, we generated mutant R proteins, R1 and R2, to study whether the N-terminal sequence has an effect on the affinity of interaction with the C subunit. R1 mutant has a deletion of the N-terminus from the first methionine residue up to twenty residues before the autophosphorylation site (1–127). R2 lacks only the sixteen aminoacids corresponding to the acidic cluster (110–125) (Fig. 1B).

Wild-type R subunit and mutants, R1 and R2, were expressed and purified from *S. cerevisiae*. There was no significant difference in the expression level and in the cAMP-binding affinity for the three proteins indicating that the deletion mutations present in the N-terminus of the R subunit did not have an effect neither on protein stability nor on cAMP-binding affinity (data not shown).

The first approach to evaluate R–C interaction was to measure the ability of Rwt, R1, and R2 subunits to inhibit kemptide phosphorylation by C_M. Fig. 2A shows that the PKA activity was inhibited by nM concentrations of the three subunits. However, the ability of R1 and R2 to associate with C_M subunit was several-fold lower than that of Rwt. The IC_{50} for R inhibition is dependent on the concentration of C used in the assay (data not shown). In the particular assay represented in Fig. 2A the IC_{50} for Rwt was 0.075 nM, while concentrations higher than 0.5 nM of R1 and R2 proteins were needed to inhibit around 50% of phosphotransferase activity. The deletion of only the acidic cluster in R2 produced the same decrease in the interaction as the deletion of the complete N-terminus of R subunit, indicating that the acidic aminoacids have a role in the high affinity R–C interaction in PKAMc.

To analyze whether the effect observed upon deletion of the cluster of acidic residues was also observed in an heterologous interaction, the same assay of Fig. 2A was performed using purified recombinant *B. taurus* C subunit (C_B). The results, shown in Fig. 2B, indicate that the difference in inhibition of R1 and R2, when compared to Rwt, is not so significant as the effect observed in Fig. 2A when using homologous interaction. These results suggest a C subunit component in the effect the deletion of the acidic residues has on C_M. No conclusions can be drawn on the relative affinities of Rwt-C_M and Rwt-C_B, since as stated before; the IC_{50} is dependent on C concentration used in the assays. Although we can estimate the concentration of the purified recombinant C_B, no estimate of the semipurified C_M can be attempted. Besides, the comparison of enzymatic units used in both cases is also not valid as it has not been yet demonstrated whether different C subunits have the same catalytic efficiency (k_{cat}) toward kemptide.

Dissociation of reconstituted holoenzymes by cAMP

A second approach was used to assay the interaction between R and C subunits, and this is the reconstitution and further activation by cAMP of wt or mutated holoenzymes. The rationale in this assay is that the lower the affinity between R and C, the lower the cAMP concentration needed to promote activation. Once again, heterologous holoenzyme reconstitution using C_B was included, not to compare the absolute parameters with those of the homologous holoezyme, but to analyze whether the observed effect is only due to the mutated R subunit or whether there is a C subunit component. Reconstituted holoenzymes with R1, R2 and Rwt and C_M or C_B were prepared by mixing C subunit with equal concentrations of R1, R2, or Rwt, so as to obtain 60–90% inhibition. The phosphotransferase activity of the reconstituted holoenzymes was then measured in the presence of variable cAMP concentration.

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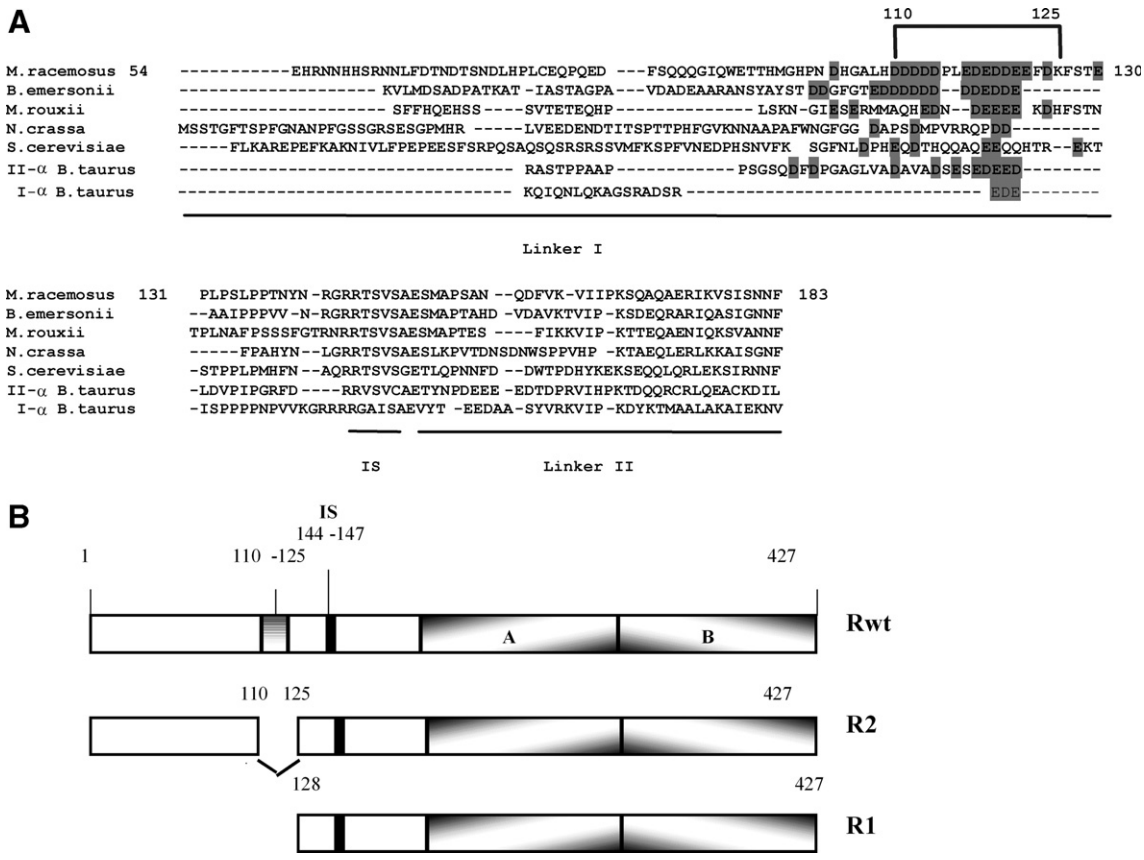

Fig. 1. Sequence alignment and schematic representation of the N-terminus of R subunits. (A) Aminoacid sequence alignment of the N-terminus region. The inhibitor consensus site is indicated as IS. The acidic cluster is shaded, and the residues deleted in R2 are over lined. (B) Schematic representation of *M. circinelloides* Rwt and mutants R1 and R2. (A and B) cAMP-binding domains A and B, respectively. The acidic cluster includes residues 110–125.

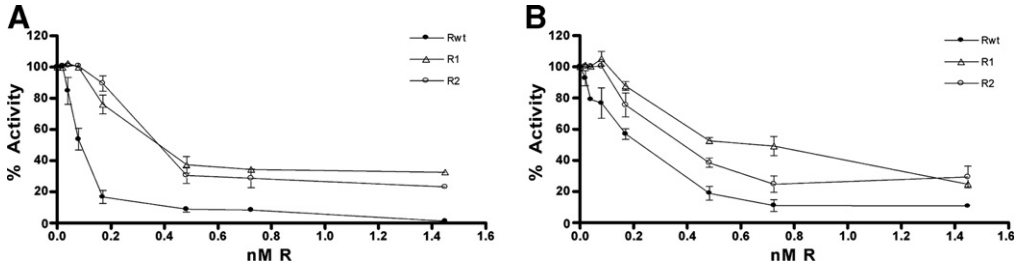

Fig. 2. Inhibition of C subunit kinase activity by Rwt , R1, and R2 subunits. Purified C_M (2.1 U) (A) or C_B subunit (0.02 pmol) (B) were preincubated for 30 min at 30 °C with various concentrations of Rwt, R1, or R2 subunits (0.02–1.5 nM) previous to kinase assay. Each value represents the means ± SD for three independent experiments.

Fig. 3A shows the cAMP activation of homologous holoenzymes reconstituted with Rwt, R1, and R2 with C_M. Activation of R1-C_M and R2-C_M holoenzymes were attained at similar cAMP concentration, while the $A_{0.5}$ for cAMP for the Rwt-C_M holoenzyme activation is one order higher. These results agree with those of Fig. 2A in the sense that the deletion of the acidic cluster or the deletion of the whole amino terminus of R subunit has as a consequence the same decrease in the affinity with the homologous C subunit.

Fig. 3B shows that cAMP activation of the reconstituted R1-C_B holoenzyme was very different from the behavior of either R2-C_B or Rwt-C_B. The R subunit with a complete deletion of the amino terminus has a clear decrease in the interaction with the heterologous C_B, In fact, while at the concentrations of cAMP used in this assay, activation of Rwt-C_B and R2-C_B holoenzymes was just beginning, the R1-C_B holoenzyme was already 100% active. This approach makes more evident the different behavior of R1 in its interaction with C_B than the inhibition assay used in Fig. 2B.

These results confirm that the acidic cluster in linker I region in the R subunit from *M. circinelloides* is decisive in the interaction between R and C in PKAMc. From these

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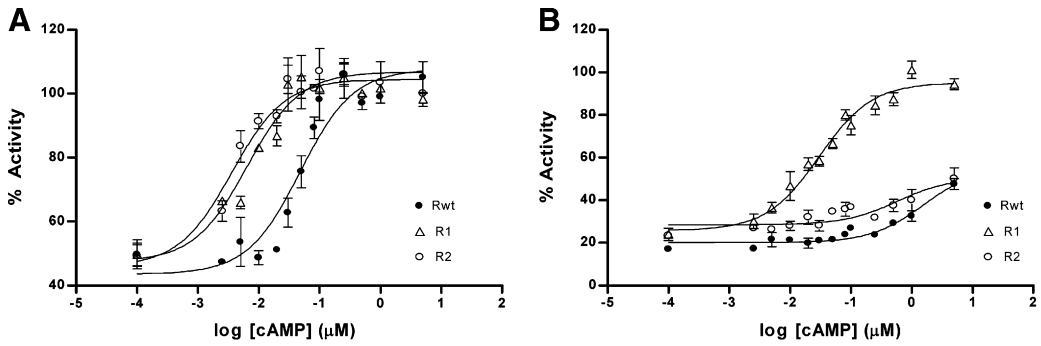

Fig. 3. Dissociation of reconstituted PKA holoenzymes by cAMP. C_M or C_B were preincubated with wt or mutant R subunits to reconstitute PKA holoenzymes. Measurement of kinase activity was performed at various cAMP concentrations (0.001–10 μM). (A) The reconstituted holoenzymes were prepared with 0.7 U C_M and 0.005 pmol of Rwt, R1, or R2; (B) the holoenzymes were prepared using 0.03 pmol C_B and 0.05 pmol of Rwt, R1, or R2. Each value represents the means ± SD for three independent experiments.

results we can also suggest that determinants present in the surface of C subunit are also important since the behavior of the deletion mutant R1 was different for C_B or C_M.

Conclusion

In higher eukaryotes, a mulitvalent mode of interaction between R and C subunits of PKA has been shown [17]. The active cleft of C subunit docks to the pseudosustrate site (IS) of R [18] as well as to a second non-contiguous site which is C-terminal to the IS [19]. Regarding the importance of linker I in the R–C interaction these models, until now only the region P-11 to P-4 of this domain has been shown to be important in RI and RII interaction with C as well as in isoform-specific differences [9].

In this paper, we point to the non-conserved sequences in linker I as a domain that could explain the interspecies difference in R and C subunit affinities. Particularly, we were interested in the cluster of acidic residues, which appeared in linker I region of some fungal R subunits, such as *M. rouxii* and *M. circinelloides*. From previous studies [11,20,21] and this work we know that the interaction between R and C in the PKA from these two fungi is higher than in PKA from other models, with an ionic component in this extra-strength. We now demonstrate that the removal of the cluster of acidic residues in the R subunit of *M. circinelloides* produces a decrease in affinity toward its homologous C subunit equivalent to the decrease obtained by deletion of the complete amino terminus of R. This result is in accordance with the need of ionic strength or polycations together with cAMP to promote dissociation or complete activation. At the same time, we have observed that the deletion of the acidic residues has a small impact on the heterologous interaction of the R subunit from *M. circinelloides* with bovine C subunit, indicating the need of a complementary surface in the catalytic subunit to interact with these residues. However, the deletion of the complete N-terminus in the fungal R subunit decreased its interaction with bovine C, pointing to a general importance of the linker I region in R–C interaction in PKAs.

Summarizing, we have demonstrated not only that the linker I region is important in the interaction R–C in general, but also that its aminoacid sequence has a role in determining a species-specific characteristic in these subunits.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2007.08.052.

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