Regeneration of hybrid holoenzymes as a strategic approach for the purification of a cAMP dependent protein kinase catalytic subunit-like protein from *Trypanosoma equiperdum*

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Introduction

Trypanosoma equiperdum is a protozoan parasite of the order Trypanosomatida that is classified under the subgenus *Trypanozoon* together with *Trypanosoma brucei* and *Trypanosoma evansi*. *T. equiperdum* causes a contagious disease known as dourine in horses and other equids.

Phosphorylation by protein kinases (PKs) regulates almost every cellular process in eukaryotic cells, and as such, PKs play critical roles in the modulation of growth and cell signaling. The kinomes of all trypanosomatids which genome has been sequenced to date, including *T. equiperdum*, represent ~ 2% of their protein-coding capacity, indicating that PKs are key signaling molecules in these flagellate protozoa.

cAMP-dependent protein kinase (PKA) has served as a prototype of the family of PKs. The inactive PKA holoenzyme consists of a complex of two regulatory and two catalytic subunits. In the presence of cAMP, the holoenzyme dissociates into a regulatory subunit dimer that binds four cAMP molecules and two free and enzymatically active catalytic subunits. In mammals, there are two classes of regulatory subunits, R^{I} and R^{II}, which are subclassified into $R^{I}\alpha$, $R^{I}\beta$, $R^{II}\alpha$ and $R^{II}\beta$ subtypes. Similarly, there are three subtypes of mammalian catalytic subunits, which have been classified as Cα, Cβ, and Cγ. Interestingly, a gene homologous to those encoding for the mammalian PKA regulatory subunits and three genes that encode for PKs related to the catalytic subunits of mammalian PKAs (*PKAC1*, *PKAC2* and *PKAC3*) have been reported in the genomes of trypanosomatids. In *T. equiperdum*, we have described a PKA catalytic subunit-like protein, which enzymatic activity was prompted upon nutritional stress driven by glucose fasting [**1**]. Surprisingly, the kinase activity of the parasite PKA was independent of cAMP. In the present work, a method based on the formation of hybrid holoenzymes has been employed to purifiy a *T. equiperdum* PKA catalytic subunit-like protein from parasites that have been expanded in rats.

Materials and Methods

Production and purification of recombinant human PKA $H_6R^{I}\beta$ subunit and mouse PKA $H_6R^{II}\alpha$(R213K) subunit. *E. coli* BL21 (DE3) cells were transformed with either pRSETB-$H_6R^{I}\beta$ or pRSETB-$H_6R^{II}\alpha$(R213K). Both vectors were supplied by Dr. Susan S Taylor, UCSD, USA. Expression of $H_6R^{I}\beta$ and $H_6R^{II}\alpha$(R213K) was initiated with 1mM IPTG. After cell lysis and centrifugation, $H_6R^{I}\beta$ and $H_6R^{II}\alpha$(R213K) were purified by incubating the supernatant batch-wise with a nickel-chelating affinity resin. $H_6R^{I}\beta$ and $H_6R^{II}\alpha$(R213K) were eluted with 300mM imidazole.

Parasites. Cryopreserved *T. equiperdum* parasites from the Venezuelan TeAp-N/D1 strain were passaged using adult Sprague-Dawley albino rats. Trypanosomes were purified by ion exchange chromatography employing a DEAE-cellulose column.

Purification of the PKA catalytic subunit-like protein from *T. equiperdum*.
Purified $H_6R^{I}\beta$ and $H_6R^{II}\alpha$(R213K) were bound to a nickel-chelating resin with the purpose of generating $R^{I}\beta$ and $R^{II}\alpha$(R213K) affinity columns. In order to stimulate the *T. equiperdum* PKA catalytic subunit-like protein, freshly purified parasites were incubated in the absence of glucose for 1 h [**1**]. Cells were extracted by sonication. The homogenate was centrifuged, and the resulting supernatant was incubated batch-wise with either the $R^{I}\beta$ or the $R^{II}\alpha$(R213K) affinity columns. The parasite PKA catalytic subunit-like protein was eluted using 5 mM cAMP.

Protein kinase activity assays. Kinase activity was determined using an electrophoretic gel-shift non-radioactive assay [**1**,**2**]. A synthetic heptapeptide known as kemptide (sequence: LRRASLG) was fluorescently labeled with fluorescamine and employed as substrate. Reaction mixtures were separated by electrophoresis on a 1.0% agarose gel, and the gels were revealed in a phototransilluminator using UV light. Known PKA substrates such as histone type II-AS and a custom synthesized 20-residue peptide, SP20 (sequence: TTYADFIASGRTGRRNSIHD), were also modified with fluorescamine and used as substrates. The inhibitory effect of the PKA heat-stable inhibitor PKI-α was measured by using 0.9, 9 and 36 units of the PKA-specific inhibitor. The synthetic peptide IP20 (sequence: TTYADFIASGRTGRRNAIHD) was also evaluated as an inhibitor peptide.

Abstract

cAMP-dependent protein kinase (PKA) has served as a prototype of the family of protein kinases. Three genes that encode for protein kinases related to the catalytic subunits of mammalian PKAs, namely *PKAC1*, *PKAC2* and *PKAC3*, have been identified in the genome of various trypanosomatids (http://tritrypdb.org). In agreement with these findings, we are reporting a PKA catalytic subunit-like protein from *Trypanosoma equiperdum*, which enzymatic activity was prompted upon nutritional stress driven by glucose fasting. The kinase enzymatic activity of the parasite protein was measured with an electrophoretic gel-shift assay that employs a fluorescently modified synthetic heptapeptide known as kemptide (sequence: LRRASLG), which is a specific substrate for PKA and PKA-like enzymes. The parasite enzyme was capable of phosphorylating histone type II-AS, the synthetic peptide SP20 (sequence: TTYADFIASGRTGRRNSIHD), and the α isoform of the PKA type II regulatory subunit (RIIα-subunit), which are known to function as substrates for the PKA catalytic subunit. Additionally, the ATP:phosphotransferase activity of the *T. equiperdum* protein kinase was inhibited by both the PKA-specific heat-stable peptide inhibitor PKI-α and the synthetic peptide IP20 (sequence: TTYADFIASGRTGRRNAIHD), which is derived from PKI-α. Surprisingly, the kinase enzymatic activity of the *T. equiperdum* PKA was independent of cAMP. In the present work, a methodology based on the formation of hybrid holoenzymes has been employed to purify the *T. equiperdum* PKA catalytic subunit-like protein from parasites that have been expanded in rats. By using polyhistidine-tagged mammalian PKA regulatory subunits that have been immobilized on a Ni^{2+}-chelating affinity resin, stable heterologous holoenzyme complexes can be regenerated between the mammalian PKA regulatory subunits and the trypanosome protein kinase enzyme. Increasing the concentration of cAMP in the elution buffer resulted in the release and subsequent purification of the *T. equiperdum* PKA catalytic subunit-like protein.

Results

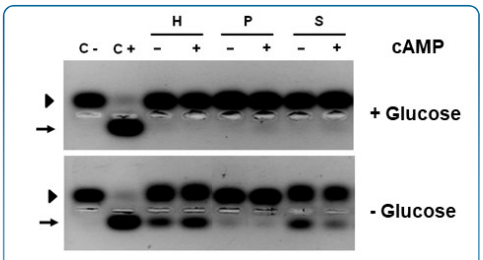

Figure 1. Effect of glucose deprivation on the parasite kemptide kinase activities. *T. equiperdum* parasites were purified through a DEAE-cellulose column using PBS-G (PBS containing 1% glucose) and either immediately used (+ Glucose, Top) or incubated with PBS lacking glucose for 1 h (- Glucose, Bottom). Enzymatic activities were estimated on the homogenates (H), particulate (P) and soluble fractions (S). Measurements were made in the absence (-) or presence of 5 μM cAMP (+). The arrowhead and arrow indicate the non-phosphorylated and phosphorylated peptide, respectively. Pig heart PKA catalytic subunit (C +). No addition (C -). No kinase activities were detected when trypanosomes were purified in the presence of glucose; however, glucose starvation activates kemptide kinase activities from *T. equiperdum*. Interestingly, the parasite kinase activities were independent of cAMP.

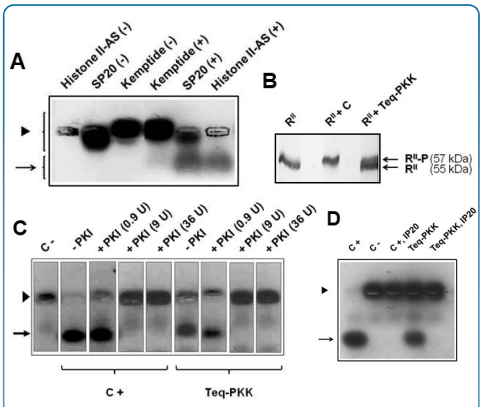

Figure 2. Evaluation of PKA catalytic subunit substrates and inhibitors on the *T. equiperdum* protein kemptide kinase (TeqPKK). ***A***, The parasite enzyme was capable of phosphorylating kemptide, histone type II-AS, and SP20. Kinase reactions were performed in the absence (-) or presence (+) of Teq-PKK. The arrowhead and arrow indicate the migration of the non-phosphorylated and phosphorylated substrates, respectively. ***B***, Purified pig heart PKA $R^{II}\alpha$ subunit alone (R^{II}) or in the presence of either the pig heart PKA catalytic subunit (R^{II} + C) or Teq-PKK (R^{II} + Teq-PKK) was incubated with 1 mM cAMP, 10 mM $MgCl_2$, and 1 mM ATP. The reaction mixtures were separated by SDS-PAGE and electrotransferred to nitrocellulose. The blot was revealed by using anti-PKA $R^{II}\alpha$ subunit antibodies. Arrows indicate the migration and apparent molecular masses of the dephosphorylated (R^{II}) and phosphorylated (R^{II}-P) forms of the monomeric $R^{II}\alpha$ subunit. ***C***, Teq-PKK was incubated with various units (U) of PKI-α (0.9, 9 and 36 units). As seen, PKI-α inhibits Teq-PKK. ***D***, Teq-PKK was incubated in the absence or presence of 0.25 mg/ml IP20. As shown, IP20 inhibits Teq-PKK. In ***C*** and ***D***, the arrowhead and arrow indicate the non-phosphorylated and phosphorylated peptide, respectively. Porcine heart PKA catalytic subunit (C +). No addition (C -).

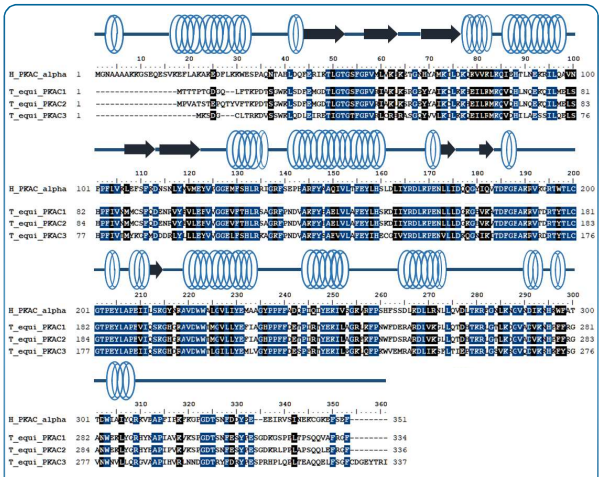

Fig. 3. Multiple alignment of the amino acid sequences of the three *T. equiperdum* PKA catalytic subunit-like proteins (PKAC1, PKAC2 and PKAC3) and the human PKA catalytic subunit α. The single letter amino acid code is used and gaps are indicated by dashes. Highlighted in blue and black are identical and similar amino acid residues, respectively. Also shown is the secondary structure of the human PKA catalytic subunit α based on the solved X-ray crystal structure of the mammalian protein. The parasite proteins were identified as RHW70011.1 (T_equi_PKAC1), SCU68910.1 (T_equi_PKAC2), and SCU64246.1 or RHW69208.1 (T_equi_PKAC3). The human protein was identified as P17612.2 (H_PKAC_alpha).

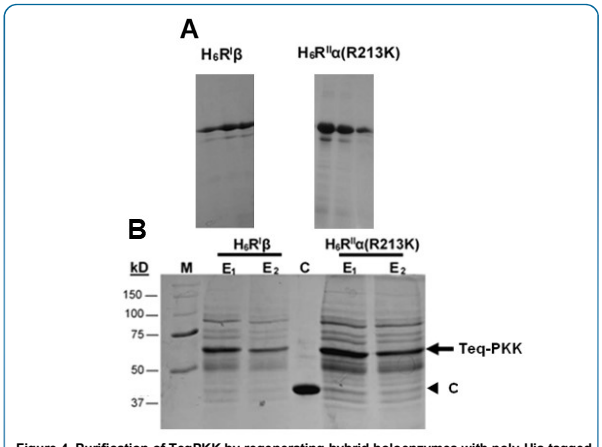

Figure 4. Purification of TeqPKK by regenerating hybrid holoenzymes with poly-His tagged mammalian PKA regulatory (R) subunits. ***A***, **Purification of poly- His tagged mammalian PKA regulatory subunits**. Both $H_6R^{I}\beta$ and $H_6R^{II}\alpha$(R213K) were purified to homogeneity by using a Ni^{2+}-chelating resin. ***B***, **Purification of TeqPKK**. $R^{I}\beta$ and $R^{II}\alpha$(R213K) affinity columns were prepared by rebinding the purified $H_6R^{I}\beta$ and $H_6R^{II}\alpha$(R213K) to new batches of nickel-chelating resins. In order to reconstitute the hybrid holoenzyme complexes, the clarified fraction of glucose-starved *T. equiperdum* parasites was incubated with the $R^{I}\beta$ and $R^{II}\alpha$(R213K) affinity columns. Heterologous holoenzymes were generated as demonstrated by the predominant elution of a protein by using cAMP. M = markers. E_1 and E_2 = first and second cAMP elutions. C = pig heart PKA catalytic subunit.

Conclusions

By using poly-His tagged mammalian PKA regulatory subunits that have been immobilized on a nickel-chelating affinity resin, stable heterologous holoenzyme complexes were regenerated between the mammalian PKA regulatory subunits and the trypanosome protein kinase enzyme. Increasing the concentration of cAMP in the elution buffer resulted in the release and subsequent purification of the *T. equiperdum* PKA catalytic subunit-like protein. The freshly purified parasite protein was active; however, it was labile and/or unstable since its kinase activity was inactivated after 1-week storage at – 20 °C. The purified trypanosome PKA catalytic subunit-like protein may correspond to either of the gene products of the *T. equiperdum* *PKAC1*, or *PKAC2* or *PKAC3* genes.

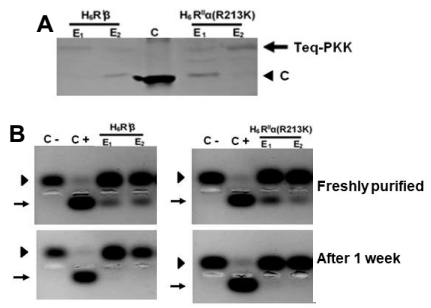

Figure 5. Purified TeqPKK was recognized by antibodies directed against the PKA catalytic subunit and possessed enzymatic activity. ***A***, Samples of the first and second cAMP elutions (E_1 and E_2) from the $R^{I}\beta$ and $R^{II}\alpha$(R213K) affinity columns were separated by SDS-PAGE and electrotransferred to nitrocellulose. The blot was revealed using anti-human PKA catalytic subunit antibodies. C = pig heart PKA catalytic subunit. ***B***, Samples of E_1 and E_2 were assayed for kemptide kinase activity. The arrowhead and arrow indicate the non-phosphorylated and phosphorylated peptide, respectively. Porcine heart PKA catalytic subunit (C +). No addition (C -). Freshly purified TeqPKK possessed enzymatic activity (Top); however, the enzyme was inactivated after 7-day storage at – 20 °C (Bottom).

References

1. Guevara, A., Lugo, C., Montilla, A.J., Araujo, N.A., Calabokis, M., Bubis, J. (2019) Glucose deprivation activates a cAMP-independent protein kinase from Trypanosoma equiperdum. Parasitology 146, 643-652. doi: 10.1017/S0031182018001920.

2. Araujo, N.A., Guevara, A., Lorenzo, M.A., Calabokis, M. and Bubis, J. (2016). Fluram-kemptide-Lys8 non-radioactive assay for protein kinase A. The Protein Journal 35, 247-255. doi: 10.1007/s10930-016-9667-9.