Cloning, expression and purification of the cAMP-dependent protein kinase catalytic subunit-like protein PKAC1 from *Trypanosoma equiperdum*

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Introduction

Dourine is a sexually transmitted infection caused by *Trypanosoma equiperdum*, a haemoparasite that belongs to the *Trypanozoon* subgenus of Salivarian trypanosomes. This disease is characterized by genital inflammation, skin plaques and neurological signs, and mainly affects horses and other equids.

The reversible phosphorylation of proteins is a ubiquitous mechanism of regulation that is vital for all living cells. In trypanosomatids, about 2% of their genome is composed of genes that encode for protein kinases (PKs), suggesting that protein phosphorylation plays a key role in the biology of these parasites.

The mammalian 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 the genomes of trypanosomatids, there are genes that encode for three AGC PKs related to the catalytic subunits of the mammalian PKA (*PKAC1*, *PKAC2* and *PKAC3*), and a gene homologous to the gene for the mammalian PKA regulatory subunits. Here, we have cloned and expressed the full gene sequence encoding for the PKAC1 protein from *T. equiperdum*.

Results

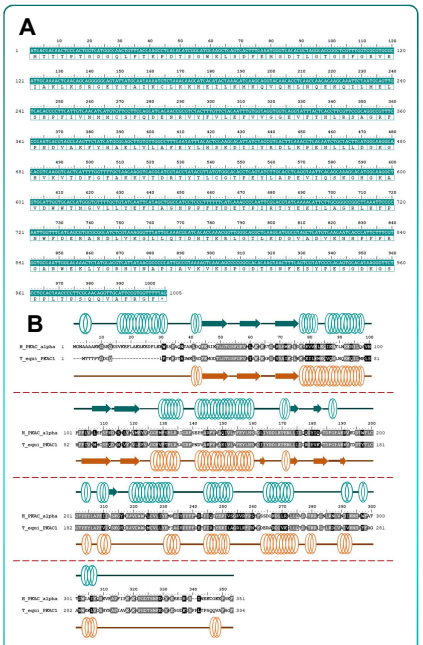

Figure 1. ***A*, Gene and amino acid sequence of PKAC1 from *T. equiperdum***. The *T. equiperdum* PKAC1 coding region was registered in the GenBank under the Accession No. MK754059. ***B*, Alignment of the amino acid sequences of the *T. equiperdum* PKAC1 and the human PKA catalytic subunit α**. Highlighted in grey and black are identical and similar amino acid residues, respectively. Shown in green and orange are the observed and predicted secondary structures of the human PKA catalytic subunit α (H_PKAC_alpha) and the parasite PKAC1 (T_equi_PKAC1), respectively.

Abstract

The full gene sequence encoding the cAMP-dependent protein kinase (PKA) catalytic subunit-like protein PKAC1 from *Trypanosoma equiperdum* was cloned, and a poly-His tagged construct was also generated. Both, the wild-type and poly-His tagged recombinant proteins were expressed in *Escherichia coli*. Most of the recombinant PKAC1 protein was highly insoluble and was probably packaged into inclusion bodies. When bacterial homogenates were separated into its soluble and particulate fractions, a polypeptide of about 38-40 kDa was predominantly seen in the insoluble fraction, whereas a band of approximately 45-50 kDa was primarily contained in the soluble fraction. In an attempt to solubilize the recombinant parasite protein, two non-ionic, non-denaturing surfactants, N-octyl-β-D-glucopyranoside (OG, 19 mM) and N-octanoyl-N-methylglucamine (Mega 8, 79 mM), were initially incorporated into the lysis buffer, either alone or in combination; however, these detergents were not appropriate for solubilizing the PKAC1 protein. We also tried the anionic detergent N-laurylsarcosine (*aka* sarkosyl), and the treatment with 3% sarkosyl enhanced the solubilization of the expressed trypanosome protein although some insoluble material still remained. Incorporation of the non-ionic surfactant Triton X-100 (4%) into de sarkosyl-containing lysis buffer yielded a slight increase in the solubilization of the protein. On the basis of these findings, we used a mixture of 3% sarkosyl, 4% Triton X-100 and Mg-ATP (15 mM $MgCl_2$ and 5mM ATP) to solubilize the PKAC1 protein. We observed that the addition of Mg-ATP during bacterial homogenization improved the solubilization of the recombinant PKAC1. Moreover, the soluble PKAC1 mainly corresponded to the form of the protein that migrated as a 45-50 kDa polypeptide, indicating that this higher band was probably produced by covalent phosphorylation. The kinase enzymatic activity of the expressed 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 recombinant protein was expressed in a functional form given that it was capable of phosphorylating kemptide. The poly-His tagged PKAC1 was purified by affinity chromatography using a Ni^{2+}-chelating resin. In addition, a recombinant purification strategy based on the formation of hybrid holoenzymes between the recombinant *T. equiperdum* wild type PKAC1 and mammalian PKA regulatory subunits was employed for the purification of the parasite PKAC1 protein. By immobilizing the hybrid holoenzyme complexes on a Ni^{2+}-chelating affinity resin, the *T. equiperdum* PKAC1 protein was specifically purified using high concentrations of cAMP.

Materials and Methods

Cloning of the *T. equiperdum PKAC1* gene. *T. equiperdum* genomic DNA was prepared from isolated parasites from the Venezuelan TeAp-N/D1 strain. PCR primers were designed based on the *Trypanosoma brucei* strain TREU297 *PKAC1* gene (Accession No. XM822292.1). The construct sequence was verified by DNA sequencing and the coding region sequence was registered in the GenBank (Accession No. MK754059).

Construction of a site-directed mutant of the *T. equiperdum* PKAC1 protein tagged with 8 His residues at its COOH-terminal [TeqPKAC1(His)$_8$]. A poly-His tagged construct of the parasite PKAC1 protein was built by modifying its stop codon to a Trp codon, and incorporating two additional amino acids followed by 8 His at its COOH-terminus.

Heterologous overproduction of the wild-type TeqPKAC1 and mutant TeqPKAC1(His)$_8$ proteins. Broth cultures containing 100 mg/ml ampicillin and 50 mg/ml kanamycin were inoculated with single colonies of *E. coli* M15[pREP4] containing the gene sequences for either TeqPKAC1 or TeqPKAC1(His)$_8$ inserted into the pQE-TriSystem vector. Expression of the proteins was initiated by the addition of 0.5mM IPTG.

Purification of the TeqPKAC1(His)$_8$ protein. After cell lysis and centrifugation, TeqPKAC1(His)$_8$ was purified by incubating the supernatant batch-wise with a nickel-chelating affinity resin. TeqPKAC1(His)$_8$ was eluted with 300mM imidazole.

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) subunits were eluted with 300mM imidazole.

Purification of the wild-type TeqPKAC1 protein. Purified PKA $H_6R^I\beta$ and $H_6R^{II}\alpha$(R213K) subunits were bound to a nickel-chelating resin in order to generate $R^I\beta$ and $R^{II}\alpha$(R213K) affinity columns. *E. coli* cells expressing TeqPKAC1 were extracted by sonication. The total 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. TeqPKAC1 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.

Conclusions

Both TeqPKAC1 and TeqPKAC1(His)$_8$ were expressed in a functional manner given that they were capable of phosphorylating kemptide. Although most of the recombinant proteins were highly insoluble, both proteins were solubilized by using a mixture of sarkosyl and Mg-ATP. By using poly-His tagged mammalian PKA regulatory subunits that have been immobilized on a nickel-chelating affinity resin, stable hybrid holoenzymes were regenerated between the mammalian PKA regulatory subunits and the wild-type TeqPKAC1. Increasing the concentration of cAMP resulted in the release and subsequent purification of TeqPKAC1. TeqPKAC1(His)$_8$ was purified by affinity chromatography using a nickel-chelating resin. Freshly purified TeqPKAC1 and TeqPKAC1(His)$_8$ were active; however, they were labile and/or unstable since their kinase activity was inactivated after 1-week storage at – 20 °C.

Results

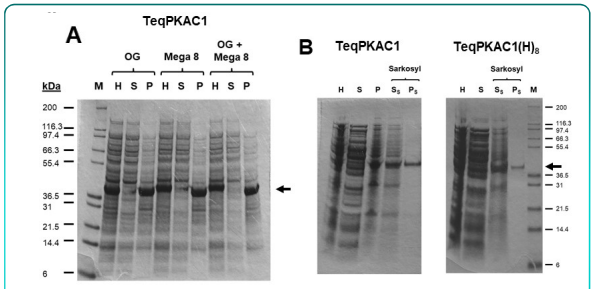

Figure 3. **Effect of detergents on the solubility of recombinant TeqPKAC1 and TeqPKAC1(H)$_8$.** ***A***, TeqPKAC1 remained insoluble when 19 mM N-octyl-β-D-glucopyranoside (OG) and 79 mM N-octanoyl-N-methylglucamine (Mega 8), were incorporated into the lysis buffer, either alone or in combination. ***B***, Treatment with 3% N-laurylsarcosine or sarkosyl enhanced the solubilization of TeqPKAC1 (Left) and TeqPKAC1(H)$_8$ (Right), although some insoluble material still remained. M = markers, H = total lysates, S and P = soluble and particulate fractions, S_S and P_S = fraction solubilized and final pellet after sarkosyl treatment.

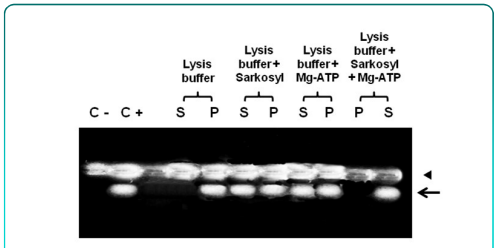

Figure 4. **Effect of sarkosyl, Mg-ATP and the combination of sarkosyl and Mg-ATP on the solubility of the recombinant PKAC1 protein**. Kinase enzymatic activity was measured with an electrophoretic gel-shift assay that uses fluorescently labeled kemptide. The protein was expressed in a functional form since it was able of phosphorylating the substrate. As shown, the PKAC1 protein completely solubilized by the combination of sarkosyl and Mg-ATP. The arrowhead and arrow indicate the non-phosphorylated and phosphorylated peptide, respectively. Pig heart PKA catalytic subunit (C +). No addition (C -).

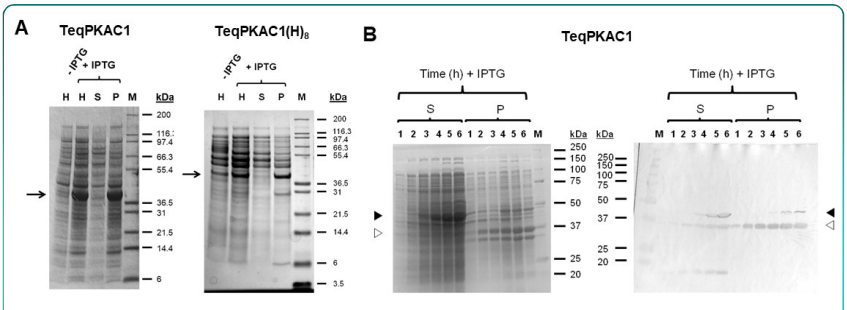

Figure 2. **Bacterial expression of the *PKAC1* gene from *T. equiperdum***. In addition to the wild-type PKAC1 (TeqPKAC1), a poly-His tagged construct of the PKAC1 protein was generated [TeqPKAC1(H)$_8$]. ***A***, Expression of TeqPKAC1 (Left) and TeqPKAC1(H)$_8$ (Right) in total extracts (H), soluble (S) and particulate fractions (P) after addition of IPTG (+ IPTG). A polypeptide of 38-40 kDa was seen (arrow), which remained in the pellet. ***B***, Time course of expression of wild-type TeqPKAC1. Samples were removed at the indicated times, centrifuged to separate the soluble (S) from the particulate fractions (P), and analyzed by SDS-PAGE. Left, Coomassie blue staining (Cb). Right, Analysis by western blot using anti-human PKA catalytic subunit antibodies (WB). A band of 38-40 kDa was mainly seen in the insoluble fractions (▷), whereas a band of 45-50 kDa was primarily contained in the soluble fractions (▶). M = molecular weight markers.

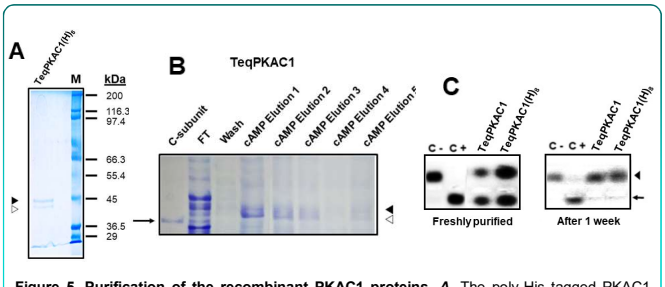

Figure 5. **Purification of the recombinant PKAC1 proteins**. ***A***, The poly-His tagged PKAC1, TeqPKAC1(H)$_8$, **Figure 5**. **Purification of PKAC1 proteins**. ***A***, TeqPKAC1(H)$_8$ was purified using a Ni^{2+}-chelating affinity resin. ***B***, TeqPKAC1 was purified by regenerating hybrid holoenzymes with poly-His tagged mammalian PKA regulatory subunits. By immobilizing the hybrid holoenzymes on a Ni^{2+}-chelating resin, TeqPKAC1 was eluted using cAMP. The arrow indicates the migration of the pig heart PKA catalytic subunit. In ***A*** and ***B***, two bands are seen, a polypeptide of 38-40 kDa (▷,◁) and a polypeptide of ~ 45 kDa (▶,◀). M = markers, FT = flow throughout. ***C***, TeqPKAC1 and TeqPKAC1(H)$_8$ were assayed for kemptide kinase activity. The arrowhead and arrow indicate the non-phosphorylated and phosphorylated peptide, respectively. Freshly purified proteins possessed enzymatic activity (Left), but the kinase activity was inactivated after 7-day storage at – 20 °C (Right). Pig PKA catalytic subunit (C +). No addition (C -).

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.