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Research paper

Biochemical characterization of the cAMP-dependent protein kinase regulatory subunit-like protein from *Trypanosoma equiperdum*, detection of its inhibitory activity, and identification of potential interacting proteins

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ABSTRACT

An enriched fraction of an inhibitor of both the catalytic subunit of the cAMP-dependent protein kinase (PKA) from pig heart and a *Trypanosoma equiperdum* PKA catalytic subunit-like protein (TeqC-like) was obtained from the soluble fraction of *T. equiperdum* parasites after three consecutive purification steps: sedimentation through a linear 5–20% sucrose gradient, diethylaminoethyl-Sepharose anion-exchange chromatography, and Bio-Sil Sec-400-S size-exclusion high-performance liquid chromatography. The inhibitor was identified as the *T. equiperdum* PKA regulatory subunit-like protein (TeqR-like) on the basis of Western blot and mass spectrometry analyses, and behaved as an uncompetitive or anti-competitive inhibitor of the parasite TeqC-like protein, with respect to a fluorescently labeled substrate (kemptide, sequence: LRRASLG), showing a K_i of 1.17 μM. The isolated protein possesses a molecular mass of 57.54 kDa, a Stokes radius of 3.64 nm, and a slightly asymmetric shape with a frictional ratio $f/fo = 1.43$. As revealed during the purification steps and by immunoprecipitation experiments, the TeqR-like and TeqC-like proteins were not associated forming a heterooligomeric complex, differing from traditional PKA subunits. Co-immunoprecipitation results followed by mass spectrometry sequencing identified two isoforms of the parasite heat-shock protein 70, α-tubulin, and β-tubulin as candidates that interact with the TeqR-like protein in *T. equiperdum*.

1. Introduction

Trypanosoma equiperdum is the etiological agent of dourine, a neglected venereal sickness that mainly affects equines [1]. Unlike other trypanosomal infections, this disease is not transmitted by an insect vector but almost exclusively by sexual intercourse. The trypanosomes, which are present in the semen and exudates of the genital mucosa of the infected donor animal, are transmitted to the recipient during coitus [1]. Thus, *T. equiperdum* is mainly a tissue trypanosome that is scarcely detected in the blood. However, during chronic conditions of the disease, a few parasites may appear in the host peripheral blood, which give the chance for bloodsucking vectors to mechanically transmit this trypanosome [2]. *T. equiperdum* is a long and slender monomorphic parasite that belongs to the subgenus Trypanozoon of Salivarian trypanosomes, and its reproduction in the host occurs through a process of longitudinal binary division [2,3].

The genome of the other two members of the Trypanozoon subgenus, *Trypanosoma brucei* and *Trypanosoma evansi*, have been

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Abbreviations

PKA	cAMP-dependent protein kinase
PKA_R	PKA regulatory subunit
PKA_C	PKA catalytic subunit
TeqR-like	*Trypanosoma equiperdum* PKA regulatory subunit-like protein
TeqC-like	*T. equiperdum* PKA catalytic subunit-like protein
D/D	dimerization/docking
IS	inhibitory site
CNB	cyclic nucleotide binding
PBC	phosphate binding cassette
LRR	Leu-rich repeats
AKAP	A-kinase anchoring protein
HSP70	heat-shock protein 70
DEAE	diethylaminoethyl
PBS	phosphate-buffered saline
PBS-G	PBS containing glucose
PMSF	phenyl methyl sulfonyl fluoride
E-64	L-trans-epoxysuccinyl-leucylamido (4-guanidino) butane
poly(ethylene glycol) compound	2,2'-[(methyl-ethylidene)-bis (4,1-phenyleneoxymethylene)]-bis-oxirane polymer with α-hydro-ω-hydroxypoly-(oxy-1,2-ethanediyl]
SDS	sodium dodecyl sulfate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
HPLC	high-performance liquid chromatography
MALDI	matrix-assisted laser desorption/ionization
TOF	time-of-flight
K_{av}	gel filtration chromatography partition coefficient
f/f_0	frictional coefficient

sequenced (http://tritrypdb.org), and drafts of the *T. equiperdum* genome have also been reported [4,5]. Since these three parasites belong to the same subgenus, they appear to share most of their genes [6,7]. Interestingly, about 2% of the *T. brucei* and *T. evansi* genomes are made up of genes that encode for protein kinases, demonstrating that protein phosphorylation plays an important physiological function in these parasites. More specifically, there are three genes that encode for AGC Ser/Thr protein kinases that are related to the catalytic subunits of the mammalian cAMP-dependent protein kinase (protein kinase A or PKA), and a gene homologous to those encoding for the mammalian PKA regulatory subunits (http://tritrypdb.org). Recently, we reported a *T. equiperdum* PKA catalytic subunit-like protein (TeqC-like), which enzymatic activity was triggered upon nutritional stress driven by glucose starvation [8], and a *T. equiperdum* PKA regulatory subunit-like protein (TeqR-like), which is monomeric and is not capable of binding cyclic nucleotides [9,10]. Interestingly, the TeqR-like protein appeared to be highly immunogenic and possessed the capability to be used as a new diagnostic tool for animal trypanosomosis [11]. In addition, the *T. equiperdum* PKA kinase activity was not dependent on cAMP [8], which is consistent with a recent report of a cAMP-independent PKA from *T. brucei* that was activated by nucleoside analogues [12].

In higher eukaryotes, the PKA regulatory subunits (PKA_R) are modular and multifunctional proteins that, in the absence of cAMP, bind with high affinity to the PKA catalytic subunits (PKA_C) and inhibit their kinase activity [13]. Canonical PKA_R subunits show a similar domain organization consisting of: i) an amino-terminal region that includes a dimerization/docking (D/D) domain responsible of maintaining their dimeric structure and of interacting with scaffolding proteins (A-kinase anchoring proteins or AKAPs), ii) a linker region containing the inhibitory site (IS) that docks to the active site of the PKA_C subunits, and iii) a carboxyl-terminal region bearing two tandem cyclic nucleotide binding (CNB) domains responsible of ligating cAMP. In contrast, the TeqR-like protein from *T. equiperdum* possesses an amino-terminal region that is curiously long when compared to higher eukaryotic PKA_R subunits, and lacks the D/D domain [9,10]. In addition, the N-terminal portion of the parasite protein shows high homology with proteins that contained Leu-rich repeats (LRR), and the role of this N-terminal LRR-containing domain in the trypanosome protein remains unknown [10,14]. However, the TeqR-like protein seems to contain two putative CNB domains located at its carboxy-terminal region. Both CNB domains apparently fold as the corresponding sites of all known PKA regulatory subunits by predictive analyses, but differences in the immediate environment of the nucleotide binding pockets are evident [10,14]. Particularly, a conserved Arg known to maintain a salt bridge interaction with the nucleotide cyclic phosphate in regular CNB domains is replaced by residues that are not capable of supporting the necessary ionic balance [9]. Consistently, the parasite TeqR-like protein is not able of binding neither cAMP nor cGMP, as demonstrated by direct binding assays and molecular docking approximations [10]. Co-crystallization of the PKA regulatory subunit-like protein from *Trypanosoma cruzi* with 7-cyano-7-deazainosine revealed how the replacement of key residues in both CNB domains of the protein, together with its unique C-terminal αD hélix, account for this ligand swap between orthodox cAMP-dependent PKAs and trypanosome cAMP-independent PKAs [12]. Although the TeqR-like protein is not a dimer and is not capable of binding cyclic nucleotides, it contains the inhibitory or pseudosubstrate site, IS, that in other eukaryotes is involved in the inactivation of the PKA_C subunits, and holds a Thr in its consensus recognition sequence that is located at the right position to be potentially phosphorylated [9,10]. In the present work, we partially purified a parasite inhibitor that was capable of inactivating both a mammalian PKA_C subunit and a TeqC-like protein from *T. equiperdum*. On the basis of Western blot and mass spectrometry analyses, the parasite native inhibitor was identified as the TeqR-like protein. Moreover, co-immunoprecipitation experiments showed that two variants of the parasite heat-shock protein 70 (HSP70), α-tubulin and β-tubulin appeared to interact with the TeqR-like protein in *T. equiperdum*.

2. Materials and methods

2.1. Parasites

Cryopreserved *T. equiperdum* parasites from the Venezuelan TeAp-N/D1 isolate were passaged using Sprague-Dawley adult albino rats. When more than 100 trypanosomes per microscopic field were observed in blood smears, the infected anesthetized rats were bled via heart puncture using 5% EDTA (pH 8.0) as anticoagulant. Trypanosomes were purified chromatographycally by using a coarse anion-exchange diethylaminoethyl (DEAE)-cellulose column equilibrated with phosphate-buffered saline containing glucose (PBS-G) [57 mM Na_2HPO_4, 3 mM NaH_2PO_4, 43.8 mM NaCl, and 1% glucose (pH 8.0)] [15]. Parasites eluting from the column were collected by centrifugation at 1475*g*, for 20 min, and rinsed 3

times with PBS-G buffer. The number of parasites was determined using a Neubauer chamber, and the final cell pellet was kept frozen at - 70 °C until further use.

2.2. Purification of the mammalian PKA_C subunit

The PKA_C subunit was purified from porcine heart as described by Nelson and Taylor [16].

2.3. Kemptide kinase activity using an electrophoretic gel-shift assay

Kemptide kinase activity was measured using the commercial Pep-Tag® non-radioactive kit (Promega) containing the fluorescently labeled kemptide (PepTag® A1 Peptide-LRRASLG) as substrate. Assays were performed following the instructions of the company, and the reaction mixtures were loaded on a 1.2% agarose gel and separated by electrophoresis. To obtain qualitative results, gels were revealed with UV light using a phototransilluminator (Fotodyne).

A calibration curve of the amount of phosphorylated fluorescent kemptide versus the intensity of its corresponding fluorescent band was built to quantify the enzymatic activity. In order to estimate the moles of phosphate incorporated into the fluorescent kemptide, the fluorescence intensity of the phosphorylated and dephosphorylated substrate was determined at 532 nm on a Typhoon Fla 9500 (General Electric) laser scanner [17]. One unit of activity represents one μmole of phosphate incorporated into the fluorescently labeled peptide substrate per minute.

2.4. Partial purification of the PKA_C subunit inhibitor from T. equiperdum

T. equiperdum parasites (2×10^{11} trypanosomes) were homogenized with 5 ml of lysis buffer [50 mM Tris-HCl (pH 7.0), 10 mM EDTA, 10 mM EGTA, 50 μM phenyl methyl sulfonyl fluoride (PMSF), 1 mM benzamidine, 50 μM leupeptin and 10 μM L-trans-epoxysuccinyl-leucylamido (4-guanidino) butane (E−64)] by sonication (4 rounds of 30 s each, with pausing recesses of 2 min per round). The cell extract was centrifuged at 100,000 *g* for 1.5 h, at 5 °C, in order to obtain the parasite soluble fraction, which was concentrated by dialysis at 5 °C against solid poly (ethylene glycol) compound (2,2'-[(methyl-ethylidene)-bis (4,1-phenyleneoxymethylene)]-bis-oxirane polymer with α-hydro-ω-hydroxypoly-(oxy-1,2-ethanediyl]). Various back-to-back purification steps were performed (*see below*), and the fractions containing the parasite PKA_C subunit inhibitor were identified by determining the inactivation of the phosphotransfer enzymatic activity using the fluorescent kemptide assay.

The inhibitory activity was assessed by measuring the remaining kinase activity of either the pig heart PKA_C subunit or the trypanosome TeqC-like protein in the presence of the fractions generated throughout the various purification steps. The decrease of phosphotransfer activity was calculated following subtraction of the amount of phosphate incorporated in the absence of the inhibitor minus the amount of phosphate incorporated in the presence of the inhibitor. A unit of inhibitor is defined as the amount that inhibits one unit of the enzyme.

Additionally, the resulting fractions from the purification steps were analyzed by absorbance at 280 nm, sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting using mice polyclonal antibodies raised against the recombinant TeqR-like protein.

The following purification steps were carried out in a consecutive manner:

i) The *T. equiperdum* soluble fraction (2 ml) was loaded on a linear 5–20% sucrose gradient (24 ml) made in 50 mM Tris-HCl (pH 7.0), 0.1 mM EDTA, 0.15 M NH_4Cl, and 0.2 mM dithiothreitol. The sample was centrifuged at 100,000 *g*, for 20 h, at 5 °C, without brake, using a SW 32 Ti swinging-bucket rotor (Beckman Coulter). Fractions of 500 μl were carefully taken from the top to the bottom of the centrifuge tube, and an aliquot of each fraction was assayed for inhibition of the kemptide kinase activity of the pig heart PKA_C subunit. Fractions containing the inhibitor were pooled and concentrated by lyophilization.

ii) The concentrated inhibitor sample was applied to a DEAE-Sepharose anion-exchange column, and the column was eluted using a NaCl gradient (0.01–2.0 M), at 5 °C. Fractions of 1 ml were collected, and an aliquot of each fraction was assayed for inhibition of the kinase activity of the mammalian PKA_C subunit. Fractions from the DEAE-Sepharose chromatography having the inhibitor were pooled and concentrated by lyophilization.

iii) The inhibitor-containing mixture obtained from the ion-exchange column was separated by size-exclusion chromatography following injection of the pooled sample into a Bio-Sil Sec-400-S high-performance liquid chromatography (HPLC) column (300 × 7.8 mm, Bio-Rad). The separation was carried out at 5 °C, with a flow rate of 1 ml/min, and using a buffer containing Tris-HCl 50 mM, NaCl 100 mM (pH 7.0). Fractions of 200 μl were collected, and an aliquot of each fraction was assayed for inhibition of the kinase activity of the porcine heart PKA_C subunit. In order to establish the native molecular mass and Stokes radius of the parasite PKA_C subunit inhibitor, the column was calibrated by employing the following protein standards: β-amylase (200 kDa, 51 Å), alcohol dehydrogenase (150 kDa, 45 Å), bovine serum albumin (67 kDa, 35.5 Å), carbonic anhydrase (32 kDa, 21.4 Å), chymotrypsinogen A (25 kDa, 20.9 Å), and ribonuclease A (13.7 kDa, 16.4 Å). The excluded and included volumes were measured by chromatographing blue dextran and potassium dichromate, respectively.

2.5. Isolation of an enriched fraction of the TeqC-like protein

Since glucose removal triggers a TeqC-like protein from *T. equiperdum* [8], freshly purified parasites (3×10^{10} trypanosomes) were incubated in the absence of glucose with phosphate-buffered saline (PBS) [57 mM Na_2HPO_4, 3 mM NaH_2PO_4, and 43.8 mM NaCl (pH 8.0)], for 1 h, at room temperature, under mild but continuous agitation. Then, the trypanosome TeqC-like protein was partially purified from the soluble fraction of glucose-starved parasites, using the two initial steps of the procedure described by Guevara et al. [8], which consisted of a sedimentation step throughout a linear 5–20% sucrose gradient, followed by DEAE-Sepharose column chromatography.

2.6. Determination of the K_i for the PKA_C subunit inhibitor from T. equiperdum

The inhibitory effect on the TeqC-like protein of the parasite inhibitor, which co-eluted with the TeqR-like protein, was assayed using various concentrations of the PepTag® A1 Peptide-LRRASLG (5.3, 9.1 and 12.9 μM) and two concentrations of the partially purified TeqR-like protein (0.18 and 0.37 μM). As the TeqR-like protein was not completely pure, the concentration of the protein was roughly estimated by comparing the signal of the stained polypeptide band, with known amounts of bovine serum albumin.

Parallel experiments were carried out in the absence of the enriched fraction of the TeqR-like protein. Products of the reactions were separated by electrophoresis on agarose gels, and the fluorescence intensity of the phosphorylated and dephosphorylated substrate was quantified at 532 nm [17]. Following evaluation of the initial rate (V_o) of the reactions, the type of inhibition was determined by regression analysis of double reciprocal plots [18], and the K_i value was measured by plotting the intercept on the $1/V_0$ axis (y-intercept) against the concentration of the TeqR-like inhibitory protein.

2.7. Immunoprecipitation experiments

In order to reduce non-specific binding, a sample of the *T. equiperdum* soluble fraction (1 ml) containing 159 μg of total protein, was pre-incubated with 20 μl of 10% of either protein A-Sepharose or protein G-agarose, depending whether the antibodies were raised in rabbits or mice, respectively. The pre-incubation was carried out for 1 h, on ice, and centrifuged at 14,000 rpm (Eppendorf microcentrifuge 5412) for 10 min, at 5 °C. This procedure was repeated three times. Then, the resulting supernatant was incubated for 1 h, at 5 °C, under constant agitation with either commercial rabbit polyclonal antibodies against the α isoform of the human PKA_C subunit (Santa Cruz Biotechnology, Inc.) or mice polyclonal antibodies raised against the recombinant TeqR-like protein. Immune complexes were precipitated by using 20 μl of either the protein A-Sepharose or protein G-agarose 10% suspension for 1 h, on ice, and centrifuged at 14,000 rpm, for 10 min, at 5 °C. The supernatant was discarded, and the sedimented pellet was washed four times with a buffer containing 60 mM NaH_2PO_4/Na_2HPO_4 (pH 8.0) and 150 mM NaCl. Finally, the pellet was resuspended in SDS-PAGE sample buffer [19] and separated by gel electrophoresis. Immunoprecipitated proteins were analyzed by western blotting employing either commercial rabbit polyclonal antibodies against the α isoform of the human PKA_C subunit or mice polyclonal antibodies against the recombinant TeqR-like protein. Immunoprecipitation experiments were also performed using a mixture of commercial mice anti-chicken embryo brain α-tubulin monoclonal antibodies (Clone DM1A, Sigma) and anti-rat brain β-tubulin monoclonal antibodies (Clone TUB 2.1, Sigma). The precipitated proteins were evaluated by immunoblotting using either the same mixture of mice anti-α-tubulin and anti-β-tubulin antibodies or the mice polyclonal antibodies raised against the recombinant TeqR-like protein. A parallel assay was carried out by immunoprecipitating the parasite soluble fraction with the anti-TeqR-like protein antibodies, and the precipitated proteins were evaluated by western blotting using either the same antibodies raised against the TeqR-like protein or the mixture of commercial anti-α-tubulin and anti-β-tubulin antibodies.

2.8. Identification of immunoprecipitated proteins using matrix-assisted laser desorption/ionization- time-of-flight/time-of-flight (MALDI-TOF/TOF) tandem mass spectrometry

Immunoprecipitated proteins were separated by SDS-PAGE on a 17-cm long 8–12% gradient slab gel, which was colored with Coomasie brilliant Blue R-250. The stained-bands were excised, and incubated with a solution containing 30% acetonitrile and 250 mM ammonium bicarbonate. Once destained, the gel slices were washed with water, cut in small cube pieces of about 1 mm^3, dehydrated with 90% acetonitrile, and dried using a SpeedVac vacuum concentrator. For in-gel digestion, the gel powder was incubated for 16 h, at 37 °C, with 50 mM sodium bicarbonate (pH 8.3) containing 12.5 ng/μl of sequencing grade trypsin. The resulting peptides were adsorbed onto C18-ZipTip® (Millipore) tips. Following removal of salts and contaminants, tryptic peptides were eluted with 1% formic acid in 60% acetonitrile. Eluted peptides were co-crystallized with a saturated solution of α-cyano-4-hydroxycinnamic acid prepared in 70% acetonitrile containing 0.1% trifluoroacetic acid, using a 1:1 ratio, and analyzed with a Bruker MALDI-TOF/TOF Autoflex III Smartbeam mass spectrometer system. Protein fingerprintings were determined by measuring the *m/z* values of the resulting tryptic peptides. Interpretable fragmentation spectra were also obtained by post-source decay analysis, given that the instrument is coupled with a sequence extension algorithm.

Peptides were analyzed by using the Mascot software tool that identifies proteins by interpreting mass spectrometry data [20]. Peptides were assigned by searching into the annotated NCBI (https://www.ncbi.nlm.nih.gov/protein) and SwissProt [21] protein sequence databases. The Mascot program computes a score based on the probability that the peptides from a sample match those in the selected databases. Another statistical parameter is the peptide-spectrum match (PSM), which is a scoring function that provides a numerical score quantifying the matching quality of alignment between the observed fragment ions of the experimental tandem mass spectra and the predicted theoretical spectra containing all the expected ions theorized to be generated by a given peptide [22–24]. In this manner, the probability that the observed match is a random event is also taken into account. Correspondingly, Mascot scores and PSMs were calculated and reported for the sequenced peptides.

2.9. Other procedures

Protein concentration was measured using bovine serum albumin as protein standard [25]. Regular separations by SDS-PAGE were performed on 1.5-mm thick mini-slab gels containing 12% polyacrylamide [19]. For Western blot analyses, proteins separated by SDS-PAGE were electrically transferred from the gels to nitrocellulose membranes [26]. cAMP binding was evaluated by filtration throughout nitrocellulose membrane disks following ammonium sulfate salting-out precipitation [27]. A list of some ortholog gene products in related species was built by employing the primary structures of the identified *T. equiperdum* proteins as imputs for the BLAST tool [28] of the TriTrypDB kinetoplastid genomics resource (https://tritrypdb.org/tritrypdb/). TriTrypDB accession codes and percentages of sequence identity of the orthologs were reported.

3. Results

3.1. Identification of the TeqR-like protein in T. equiperdum homogenates, soluble and particulate fractions

The gene sequence encoding for the TeAp-N/D1 *T. equiperdum* ortholog of the PKA_R subunits was cloned (GenBank Accession No. KJ636459, NCBI ID: AID53025.1, https://www.ncbi.nlm.nih.gov/genbank/), and the recombinant protein was expressed in *Escherichia coli*, purified to homogeneity, and used to raise polyclonal antibodies in mice [10,11]. As shown in a, western blotting revealed that these antibodies recognized a polypeptide band with an apparent molecular mass of about 58 kDa in *T. equiperdum* whole cell lysates, and in both the parasite soluble and particulate fractions. On the basis of the intensity of the band, the parasite protein appeared to be mainly soluble (Fig. 1a).

Interestingly, the parasite soluble fraction was capable of inhibiting the enzymatic activity of the PKA_C subunit from pig heart, in an analogous manner as the recombinant PKI-α from rabbit, which is a 77-amino acid protein that is a PKA-specific heat-

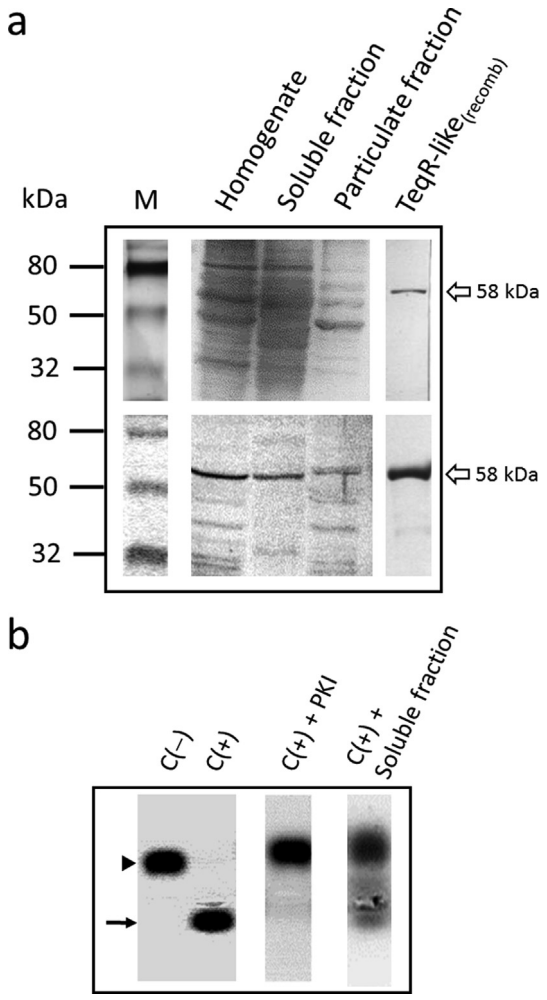

Fig. 1. Identification of an inhibitor of the PKA_C from porcine heart in *T. equiperdum*. **a**, Gel separation stained with silver (top), and Western blot using polyclonal antibodies against the recombinant TeqR-like [TeqR-$like_{(recomb)}$] (bottom) of the proteins in the homogenate and the soluble and particulate fractions of *T. equiperdum* parasites. Purified TeqR-$like_{(recomb)}$ (1 μg) was included as a positive control. The migration of the trypanosome TeqR-like is indicated by white arrows. M = molecular weight protein markers. **b**, The *T. equiperdum* soluble fraction contained an inhibitor of the PKA_C from pig heart. Kemptide kinase activity was determined by means of the Promega Pep-Tag® non-radioactive assay. The reaction mixture without any added enzyme was employed as a negative control (C-), and the purified PKA_C from pig heart was used as a positive control (C+). The recombinant PKA-specific heat-stable inhibitor PKI-α was included as a control for inhibition of the enzymatic activity. The arrowhead and arrow designate the non-phosphorylated and phosphorylated fluorescently labeled peptide, respectively.

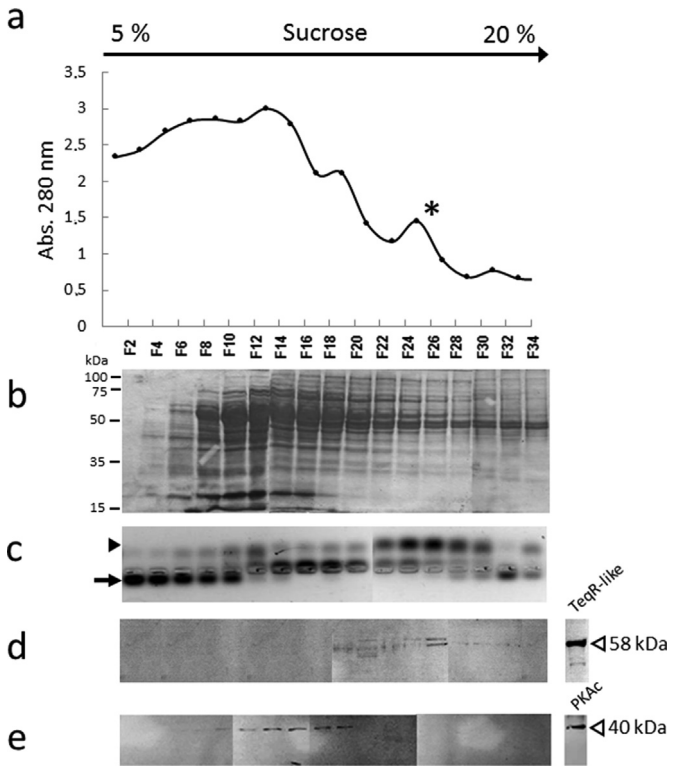

Fig. 2. Sedimentation of the *T. equiperdum* soluble fraction by ultracentrifugation on a sucrose gradient. **a**, The trypanosome soluble fraction was loaded on a 5–20% sucrose gradient and fractionated by ultracentrifugation. Proteins were determined by measuring the absorbance of the fractions at 280 nm **b**, SDS-PAGE separation showing the polypeptide profile of the resulting fractions following sedimentation. Proteins were revealed by silver staining. The migration of the molecular weight standards is indicated. **c**, Inhibition of the kemptide kinase activity of the mammalian PKA_C by fractions 22–30 (F22–F30). The arrowhead and arrow show the non-phosphorylated and phosphorylated fluorescently labeled kemptide, respectively. In panel a, the peak of kemptide kinase inhibitory activity is highlighted with an asterisk (*). **d**, Immunoblot analysis using antibodies against the recombinant TeqR-like protein. **e**, Western blot analysis using antibodies against the α isoform of the human PKA_C. Purified TeqR-like (recombinant) and PKA_C from pig heart were included as controls. White arrowheads indicate the migration and apparent molecular masses of TeqR-like and PKA_C.

stable inhibitor (Fig. 1b), and mammalian type I and II PKA_R subunits (data not shown). In an attempt to identify the parasite inhibitor, and explore whether it corresponded to the parasite TeqR-like protein, we purified an enriched fraction of this PKA inhibitor from the *T. equiperdum* soluble fraction.

3.2. Partial purification of the PKA_C subunit inhibitor from T. equiperdum

Parasites were homogenized by sonication, and centrifuged to separate the soluble cytosolic fraction from the particulate fraction. Then, the soluble fraction was loaded on a 5–20% sucrose gradient and fractionated by ultracentrifugation (Fig. 2a). Fig. 2b illustrates the SDS-PAGE separation showing the polypeptide profile of the resulting fractions following sedimentation. Since the fractions were taken from the top to the bottom of the tube, polypeptides with lower apparent molecular weights were observed in the former fractions, whereas bands with higher apparent molecular weights were contained in the latter fractions (Fig. 2b). Fractions 22–30 inhibited the kinase activity of the mammalian PKA_C subunit (Fig. 2c), with a major inhibitory peak between fractions 24 and 26 at a concentration of approximately 15% sucrose (highlighted with an asterisk in Fig. 2a). Western blot analysis showed that fractions 18–32 contained either a band of ~58 kDa or a doublet band of ~58–59 kDa that was recognized by the mice polyclonal antibodies raised against the recombinant parasite TeqR-like protein (Fig. 2d). The highest staining intensity was observed at fraction 26 (Fig. 2d). Interestingly, immunoblots using polyclonal antibodies against the α isoform of the human PKA_C subunit revealed that fractions 8–18 contained the peak of the trypanosome TeqC-like protein (Fig. 2e), demonstrating that the TeqC-like and TeqR-like proteins are dissociated. Fractions 22–30 containing the kinase activity native inhibitor were pooled and the sample was identified as SG22-30.

SG22-30 was loaded on a DEAE-Sepharose anion-exchange column and the column was eluted using a sodium chloride gradient (Fig. 3a). Fig. 3b shows the polypeptide content of the

eluting fractions as the ionic strength was increased. As illustrated in Fig. 3c, fractions 21–35 clearly contained an inhibitor of the kinase activity of the pig heart enzyme. The major inhibitory peak eluted at fraction 27 (NaCl concentration of ~250 mM). Moreover, fractions 21–35 contained a polypeptide band of ~58 kDa that was recognized by the mice anti-recombinant parasite TeqR-like protein antibodies following Western blot analysis (Fig. 3d), indicating that the TeqR-like protein might be responsible of the inhibition that was seen. Interestingly, the slowing migrating band of the doublet seen in Fig. 2d was not seen here, suggesting that the higher band of about 59 kDa was probably caused by covalent posttranslational modification of the protein, perhaps phosphorylation. The additional chemical groups seemed to be hydrolyzed and lost during storage and further chromatography of the SG22-30 fraction. Also, two peaks with cAMP binding activity were observed before the elution of the parasite TeqR-like protein, at a NaCl concentration lower than 200 mM (Fig. 3a), confirming that the TeqR-like protein is not capable of binding cAMP, as previously reported using both the native protein [9] and the recombinant protein [10]. In order to continue with the next purification step, fractions 23–33 were collected because they contained the strongest inhibitory activity, and the pooled sample was identified as DEAE23-33.

DEAE23-33 was injected into a Bio-Sil Sec-400-S HPLC column, which separates compounds by size exclusion chromatography (Fig. 4). The resulting chromatogram and the polypeptide content of the eluting fractions are shown in Fig. 4a and b, respectively. As illustrated in Fig. 4a and c, fractions containing the inhibitory activity of the pig heart PKA_C subunit (Fractions 40–46) eluted at a retention time of 9.5–11 min. Interestingly, these fractions also contained the band recognized by the mice anti-recombinant parasite TeqR-like protein antibodies (Fig. 4d). Fractions 40–46 were pooled and the sample was identified as SEC40-46.

By combining these three consecutive purification steps, an enriched fraction of a *T. equiperdum* inhibitor of the ATP:-phosphotransferase activity of the PKA_C subunit from pig heart was achieved. As shown in Table SI, this parasite inhibitor was purified more than 100 fold; however, it was not purified to homogeneity (Fig. S1). The peak of the PKA_C subunit inhibitor always coincided

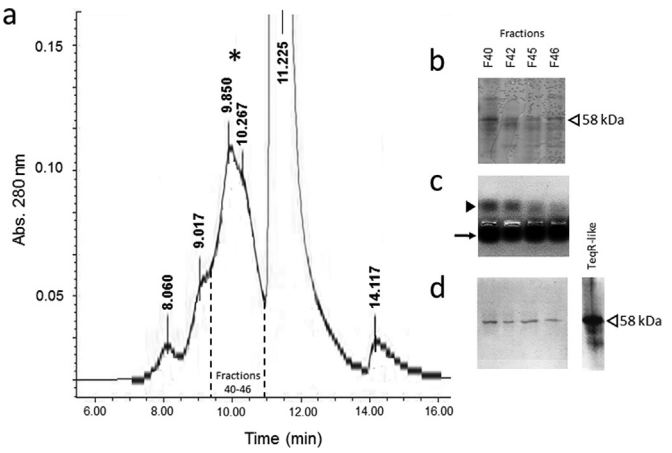

Fig. 4. Separation of DEAE23–33 by gel-filtration chromatography. **a**, Fractions 23–33 from the DEAE-Sepharose column (Fig. 3) were pooled and loaded on a size exclusion Bio-Sil Sec-400-S HPLC column. Proteins were determined by measuring the absorbance at 280 nm **b**, SDS-PAGE separation showing the polypeptide content of fractions 40, 42, 45 and 46 (F40, F42, F45 and F46). Proteins were revealed by silver staining. **c**, Inhibition of the kemptide kinase activity of the porcine heart PKA_C by F40, F42, F45 and F46. The arrowhead and arrow point out to the non-phosphorylated and phosphorylated fluorescently labeled kemptide, respectively. In panel a, the peak of kemptide kinase inhibitory activity is highlighted with an asterisk (*). **d**, Western blot analysis using antibodies against the recombinant TeqR-like. Purified recombinant TeqR-like was included as a control. White arrowheads indicate the migration and apparent molecular mass of TeqR-like.

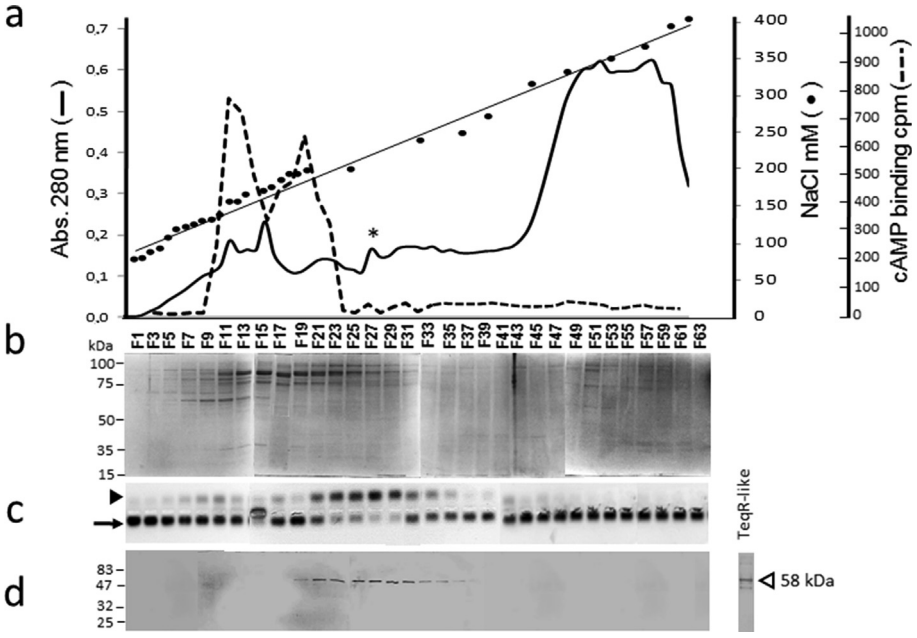

Fig. 3. Separation of SG22–30 by anion-exchange chromatography. **a**, Fractions 22–30 from the sucrose gradient (Fig. 2) were pooled and loaded on a DEAE-Sepharose anion-exchange column, which was eluted by increasing the ionic strength employing a NaCl gradient (•). Proteins were determined by measuring the absorbance at 280 nm (full line). Counts per minute (cpm) of [^{3}H] cAMP binding was measured by Millipore filtration following ammonium sulfate precipitation (dashed line). **b**, Separation by SDS-PAGE showing the polypeptide content of the eluting fractions. Proteins were revealed by silver staining. **c**, Inhibition of the kemptide kinase activity of the mammalian PKA_C by fractions 21–35 (F21–F35). The arrowhead and arrow point out to the non-phosphorylated and phosphorylated fluorescently labeled kemptide, respectively. In panel a, the peak of kemptide kinase inhibitory activity is highlighted with an asterisk (*). **d**, Western blot analysis using antibodies against the recombinant TeqR-like. Purified recombinant TeqR-like was included as a control. The white arrowhead indicates the migration and apparent molecular mass of the TeqR-like protein.

with the peak of the TeqR-like protein, demonstrating that both activities are linked to the same trypanosome protein (Figs. 2–4).

3.3. Determination of the molecular weight and Stokes radius of the PKA_C subunit inhibitor from T. equiperdum

The trypanosome enriched fraction containing both the PKA_C subunit inhibitor and the TeqR-like protein was applied to an HPLC size-exclusion column that was calibrated using markers of known molecular masses. By plotting the partition coefficient (K_{av}) against the logarithm of the molecular weight of each standard [29], a molecular mass of 57.54 kDa was calculated for the parasite inhibitor of the PKA_C subunit, which coincided with the size calculated for the trypanosome TeqR-like protein (Fig. 5a). As shown in Fig. 5b, a linear relationship was also obtained when the value of $(-\log K_{av})^{1/2}$ was plotted versus the Stokes radius of each protein marker [30], and a Stokes radius of 3.64 nm was acquired for the parasite protein. By using the established molecular weight and Stokes radius, a frictional coefficient f/f_o of 1.43 was determined for the *T. equiperdum* protein [31]. This result indicated that the parasite PKA_C subunit inhibitor/TeqR-like protein is slightly asymmetric, bearing a conformation that lies in the boundary between a globular and a moderately elongated protein [32]. Since an apparent molecular mass of ~58 kDa was obtained for the parasite TeqR-like protein by electrophoresis under denaturing conditions, the native *T. equiperdum* protein appears to be a monomer as previously shown for the recombinant protein [10]. Therefore, the parasite TeqR-like protein differed from mammalian PKA_R subunits that are known to be dimeric proteins [33].

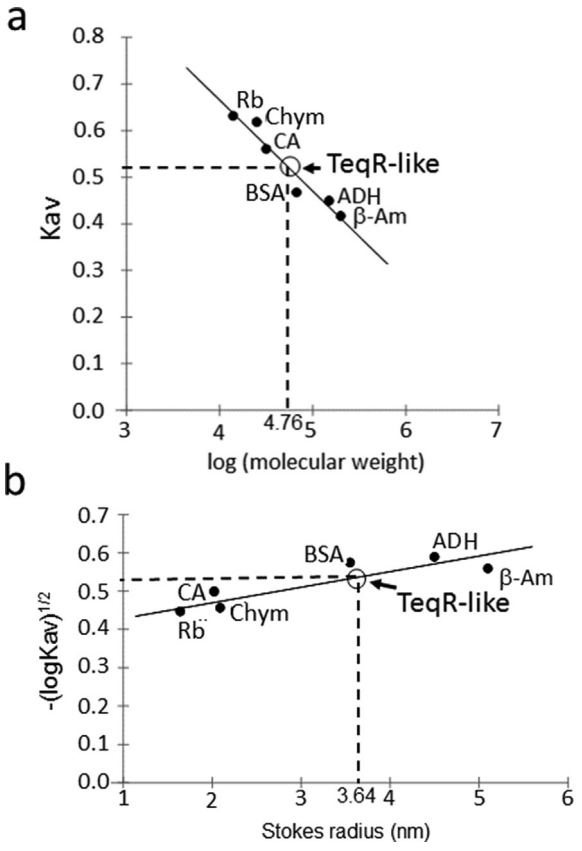

Fig. 5. Molecular weight and Stokes radius of the PKA_C inhibitor from *T. equiperdum*. The partially purified *T. equiperdum* PKA catalytic subunit inhibitor, which co-eluted with the parasite TeqR-like protein, was applied to an HPLC size-exclusion column. Protein standards were used to calibrate the column and were chromatographed together with the trypanosome protein. **a**, Plot of the partition coefficient Kav value of each standard versus the logarithm of its molecular weight. **b**, Plot of the $(-\log Kav)^{1/2}$ value of each marker against its Stokes radius. The calibration curve for the column was generated with β-amylase (β-Am, 200 kDa, 51 Å), alcohol dehydrogenase (ADH, 150 kDa, 45 Å), bovine serum albumin (BSA, 67 kDa, 35.5 Å), carbonic anhydrase (CA, 32 kDa, 21.4 Å), chymotrypsinogen A (Chyn, 25 kDa, 20.9 Å), and ribonuclease A (Rb, 13.7 kDa, 16.4 Å). The elution position of the *T. equiperdum* PKA_C inhibitor was determined by assaying for kinase activity using the fluorescently labeled kemptide as substrate, and coincided with the elution position of the parasite TeqR-like that was followed by Western blot.

3.4. Inhibition of the parasite TeqC-like protein by the partially purified TeqR-like protein

The trypanosome TeqC-like protein was partially purified from the soluble fraction of glucose-starved *T. equiperdum* parasites, using the two initial purification steps reported by Guevara et al. [8]. As illustrated in Fig. 6, the parasite TeqR-like protein was able of inhibiting the kinase activity of the TeqC-like protein. As also shown in Fig. 6, the kinase activity of the parasite enzyme was not recovered when cAMP was added to the reaction mixture. These results confirmed that the parasite TeqC-like protein is a cAMP-independent enzyme as previously shown [8].

3.5. Determination of the K_i of the TeqR-like protein on the enzymatic activity of the parasite TeqC-like protein

As shown in Fig. 7a, the kinetics of inhibition of the kinase activity of the TeqC-like protein by the parasite TeqR-like protein was followed by using increasing concentrations of the fluorescently labeled kemptide in the absence of the TeqR-like protein, or with two fixed concentrations of this inhibitor (0.18 and 0.37 μM). The fluorescence intensity of the phosphorylated and dephosphorylated substrate was quantified using a laser scanner. The slope of the curve of V_o versus the substrate concentration decreased as the concentration of the parasite TeqR-like increased (data not shown), demonstrating that the TeqR-like protein behaved as a reversible inhibitor. Fig. 7b shows the Lineweaver-Burk plots that were obtained using different doses of the fluorescently labeled kemptide, in the absence or presence of each of the two concentrations of the inhibitor. Since the relationship of 1/Vo with 1/kemptide shows parallel straight lines (Fig. 7b), the trypanosome TeqR-like protein appears to behave as an uncompetitive or anti-competitive

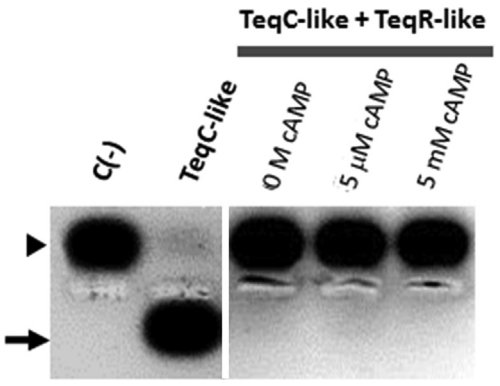

Fig. 6. Inactivation of the TeqC-like protein by the partially purified TeqR-like. TeqC-like was partially purified from the soluble fraction of glucose-starved *T. equiperdum* parasites, and its kemptide kinase activity was determined by using the electrophoretic gel-shift assay in the absence or presence of TeqR-like. Determinations were made in the absence (0 M) or presence of either 5 μM or 5 mM cAMP. The reaction mixture without any added enzyme was employed as a negative control (C-). The arrowhead and arrow point out to the non-phosphorylated and phosphorylated fluorescently labeled kemptide, respectively.

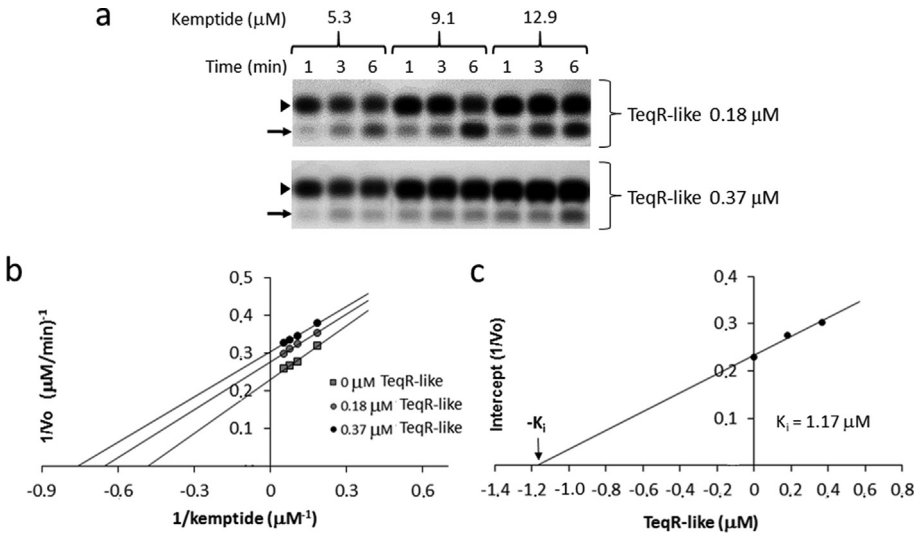

Fig. 7. K_i determination. **a**, Kinetic analysis of the inhibition of the kemptide kinase activity of the TeqC-like protein by the TeqR-like protein using the electrophoretic gel-shift assay. Measurements were made using increasing concentrations of the fluorescently labeled kemptide (5.3, 9.1 and 12.9 μM), in the absence (0 μM) or presence of two concentrations of TeqR-like (0.18 and 0.37 μM). The arrowhead and arrow indicate the dephosphorylated and phosphorylated fluorescently labeled substrate, respectively, and the intensity of the unmodified and modified peptide was quantified using a laser scanner. **b**, Lineweaver-Burk plots for the partially purified TeqC-like protein, in the absence or presence of two different concentrations of the inhibitor. Vo = initial rate. **c**, Plot of the intercept of the 1/Vo value from panel b versus the concentration of the TeqR-like protein.

inhibitor with respect to the fluorescently labeled kemptide, with a K_i of 1.17 μM (Fig. 7c).

3.6. *Immunoprecipitation of the T. equiperdum soluble fraction using antibodies directed either against the isoform α of the human PKA_C subunit or against the recombinant TeqR-like protein*

The parasite soluble fraction was immunoprecipitated with commercial antibodies raised against the isoform α of the mammalian PKA_C subunit, and the precipitate was analyzed by immunoblotting using either the same antibodies or anti-recombinant TeqR-like protein antibodies. The parasite TeqC-like protein immunoprecipitated with the antibodies against the isoform α of the mammalian PKA_C subunit (Fig. 8a, Panel I); but, no co-immunoprecipitation of the parasite TeqR-like was observed (Fig. 8a, Panel II). Similarly, the trypanosome TeqR-like protein was immunoprecipitated by the anti-recombinant TeqR-like protein antibodies (Fig. 8b, Panel I); however, the anti-PKA_C subunit antibodies did not recognize any band by Western blot following immunoprecipitation with the anti-recombinant TeqR-like protein antibodies (Fig. 8b, Panel II). These results suggest that the TeqC-like and TeqR-like proteins are not capable of associating to form a stable holoenzyme type of complex, at least under the conditions that were employed in this experiment.

3.7. *Immunoprecipitation using anti-recombinant TeqR-like protein polyclonal antibodies and identification of prospective interacting proteins by mass spectrometry*

The parasite soluble fraction was immunoprecipitated with anti-recombinant TeqR-like protein polyclonal antibodies prepared in mice, and the resulting immunoprecipitate was separated by SDS-PAGE revealing the presence of four polypeptide bands (Fig. 9), with apparent molecular masses of approximately 70 kDa (Band 1), 58 kDa (Band 2) and 55 kDa (Bands 3 and 4).

All four bands were analyzed by mass spectrometry. MALDI-TOF mass spectra of the in-gel trypsin digests revealed the tryptic peptide mass fingerprints of the four polypeptide bands (Fig. 10a and S2a-S4a). In order to find out the identity of the immunoprecipitated proteins, further fragmentation of several of their tryptic peptides was performed using MALDI-TOF/TOF (Fig. 10b–f, S2b-d, S3b-c and S4b-d). For example, five tryptic peptides with molecular masses of 1615.707, 2060.829, 2251.937, 2347.988, and 2421.018 Da, respectively, were selected to identify the polypeptide corresponding to Band 2 (Fig. 10). As shown in Table 1, the obtained sequences for the five peptides perfectly matched with tryptic peptides of the TeqR-like protein. As expected, Band 2 was recognized by immunoblotting using the same anti-TeqR-like polyclonal antibodies (Fig. 9). In a similar way, the rest of the immunoprecipitated polypeptide bands were analyzed by MALDI-TOF/TOF mass spectrometry (Figs. S2–S4, Table 1), and the following proteins were identified: two different isoforms of the parasite HSP70, a putative *T. equiperdum* HSP70 mitochondrial precursor and another HSP70 variant that probably corresponds to the putative *T. equiperdum* glucose-regulated protein 78 (Band 1), α-tubulin (Band 3), and β-tubulin (Band 4). Although Fig. S2 only illustrates the spectra of the tryptic peptides that were sequenced from the trypanosome mitochondrial HSP70 variant, the peptides from the other parasite HSP70 isoform were analyzed and sequenced as well (Table 1). Mascot scores and PSMs for the reported peptides were also calculated and are included in Table 1. On the basis of these co-immunoprecipitation results, the two isoforms of HSP70, α-tubulin and β-tubulin appear to maintain protein-protein interactions with the TeqR-like protein in *T. equiperdum*. As shown in Table IIS, all five identified proteins from *T. equiperdum* showed very high homology with ortholog gene products from related organisms.

Table 1 contains those peptides with both higher Mascot scores and positive values of PSMs. However, other peptides with lower Mascot scores and negative values of PSMs, but matching 100% with the identified protein were also obtained. For example, for the polypeptide band that corresponded to the parasite TeqR-like protein (Band 2), three additional tryptic peptides with molecular masses of 2234.918, 1250.502, and 1266.492 Da were also acquired. These peptides revealed the following sequences: i) SHPTANALDLSHNPISNYAGR for the peptide of 2234.918 Da, ii) DLMTVAGAMWR for the peptide of 1250.502 Da, and iii) DLMTVAGAMWR for the peptide of 1266.492 (where M represents the oxidation of

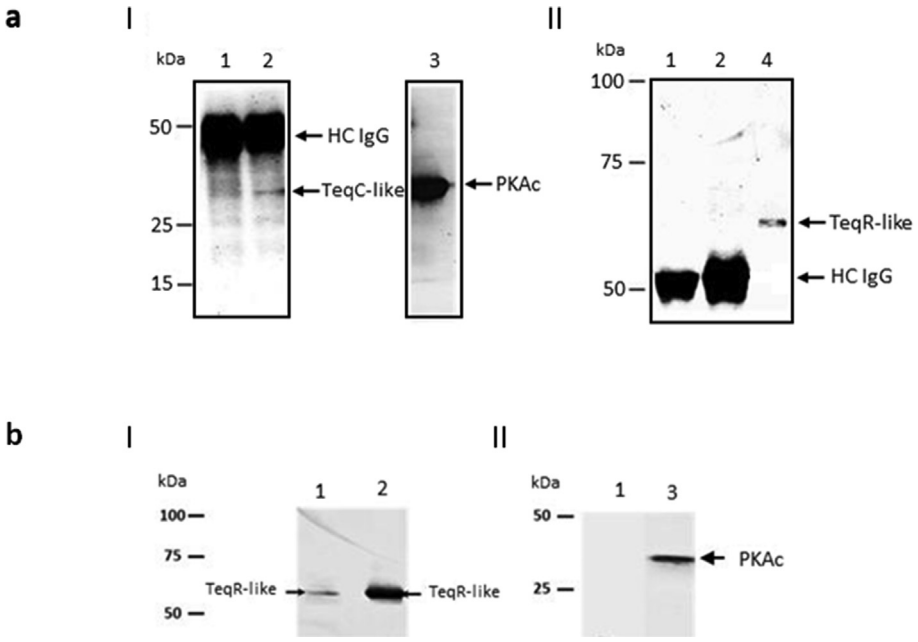

Fig. 8. Immunoprecipitation of the *T. equiperdum* cytosolic fraction. **a**, The trypanosome soluble fraction was immunoprecipitated with rabbit anti-human PKA_C antibodies, and the precipitate was revealed by Western blot using either the same antibodies (Panel I) or mice anti-recombinant TeqR-like antibodies (Panel II). **1**, Control sample of just the antibodies bound to protein A-Sepharose; **2**, the trypanosome soluble fraction was incubated with the antibodies and then precipìtated with protein A-Sepharose; **3** and **4**, purified PKA_C from pig heart and recombinant TeqR-like, respectively. HC IgG = Immunoglobulin heavy chain. Cross-reactivity between the anti-mouse secondary antibody and the heavy chain of the rabbit primary antibody was seen (Panel II). **b**, The parasite soluble fraction was immunoprecipitated with anti-recombinant TeqR-like antibodies raised in mice (lane **1**), and the precipitate was revealed by immunoblot using either the same antibodies (Panel I) or anti-human PKA_C antibodies (Panel II). In this case, protein G-agarose was employed to immunoprecipìtate the antibodies. Lanes **2** and **3**, purified TeqR-like and PKA_C, respectively. Blots were cut either below (Panel I) or above (Panel II) the 50 kDa protein marker in order to circumvent detection of the HC IgG.

the methionine residue to methionine sulfoxide). These sequences were 100% identical to residues 103–123 (^{103}SHPTANALDLSHNPISNYAGR^{123}) and 251–261 (^{251}DLMTVAGAMWR^{261}) of the identified trypanosome TeqR-like protein; yet, the acquired Mascot scores were ≤16.8 (scores greater than 24–29 and 45–46 indicate homology and identity, respectively, according to the NCBInr database). Similarly, the PSMs for these peptides were negative. Regardless of the outcomes of the Mascot scores and PSMs, obtaining these additional peptides demonstrated that Band 2 contained the parasite TeqR-like protein. Likewise, for the polypeptide band that corresponded to the parasite α-tubulin (Band 3), three extra tryptic peptides with molecular masses of 1341.516, 1631.679, and 1227.500 Da with the sequences INVYFDEATGGR, SVLIDLEPGTMDSVR, and VGEQFTLMFR, respectively, were obtained. These sequences were 100% identical to residues 47–58 (^{47}INVYFDEATGGR^{58}), 63–77 (^{63}SVLIDLEPGTMDSVR^{77}), and 381–390 (^{381}VGEQFTLMFR^{390}) of the identified trypanosome β-tubulin. Since both α-tubulin and β-tubulin mígrate with apparent molecular masses of ~55 kDa following separation by SDS-PAGE, some contamination of Band 3 with Band 4 may easily occur. Therefore, acquiring these extra peptides confirmed that the polypeptide bands 3 and 4 contained α-tubulin and β-tubulin, respectively. However, these peptides show low Mascot scores and negative values of PSMs (Data not shown).

In order to demonstrate the interaction between the parasite TeqR-like protein and α/β-tubulin, an additional experiment was carried out in which the *T. equiperdum* soluble fraction was immunoprecipitated using a mixture of commercial anti-α/β-tubulin monoclonal antibodies. The precipitated proteins were evaluated by immunoblotting using the anti-TeqR-like polyclonal antibodies. As seen in Fig. S5, the parasite TeqR-like protein was co-immunoprecipitated with the mixture of anti-α/β-tubulin antibodies. As expected, the parasite α-tubulin and β-tubulin were also immunoprecipitated with the mixture of anti-α/β-tubulin antibodies (Data not shown). Similarly, the antibodies against the recombinant TeqR-like protein co-immunoprecipitated the trypanosome α/β-tubulin subunits from the *T. equiperdum* soluble fraction (Fig. S5). Additionally, the parasite TeqR-like was also immunoprecipitated by the anti-TeqR-like antibodies (Data not shown).

4. Discussion

We obtained an enriched fraction of a *T. equiperdum* inhibitor of the TeqC-like enzyme, which was identified here as the parasite TeqR-like protein. During its partial purification, we observed that the TeqR-like protein was not capable of binding cAMP (Fig. 3), although it possesses two presumptive CNB domains with sequences that are similar to phosphate binding cassettes (PBCs) [9,10]. In typical PKA_R subunits, the PBC motif is responsible of docking the cAMP phosphate. However, sequence diversity has been reported within this motif in several organisms [34,35], implying that the CNB domain has evolved as a platform for binding not only cAMP, but also a variety of other ligands. In *T. equiperdum*, both CNB sites of the TeqR-like protein possess non-conventional PBC sequences. Therefore, these structural motifs in the parasite protein might be used to link a different type of ligand. Consistently, a cAMP-independent PKA that was activated by nucleoside analogues was found in *T. brucei* [12]. It appears that in trypanosomes, PKA has developed to respond to an endogenous nucleoside related signaling molecule that has not been identified to date but is clearly different than cAMP and cGMP [10,12].

We also showed here that the *T. equiperdum* PKA subunit-like proteins were not associated forming a heterooligomeric holoenzyme complex. First, both PKA subunit-like proteins sedimented at different sucrose concentrations. Moreover, no co-

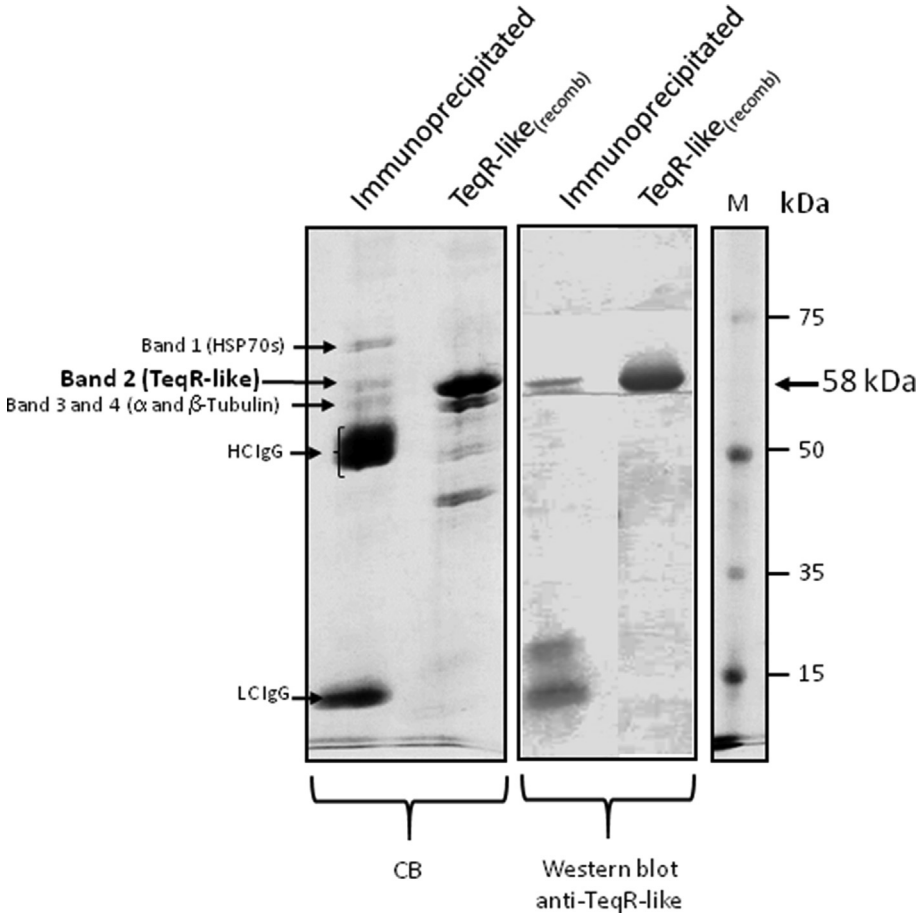

Fig. 9. Identification of prospective TeqR-like-binding partners by co-immunoprecipitation. The parasite soluble fraction was immunoprecipitated with anti-recombinant TeqR-like antibodies (anti-TeqR-like), and the resulting immunoprecipitate was separated by SDS-PAGE. **Left**, Coomassie blue R-250 staining (CB). **Middle**, immunoblot using the same antibodies. **Right**, protein markers (M). Shown are the polypeptide bands that were identified by MALDI-TOF/TOF mass spectrometry as HSP70s, α-tubulin and β-tubulin. TeqR-like$_{(recomb)}$ = purified recombinant TeqR-like. HC IgG = Immunoglobulin heavy chain. LC IgG = Immunoglobulin light chain. Based on the migration of the protein markers, the piece of the blot containing the parasite TeqR-like was cut and revealed in order to avoid detection of the HC IgG, which may mask the signal.

immunoprecipitation of the parasite TeqR-like protein was observed following immunoprecipitation with antibodies raised against the isoform α of the human PKA$_C$ subunit. Similarly, the parasite TeqC-like protein did not co-immunoprecipitate using anti-*T. equiperdum* TeqR-like protein polyclonal antibodies. These findings suggest that in trypanosomes these proteins differed from traditional PKA subunits. Paradoxically, Bachmaier et al. [12] demonstrated that the PKA subunit genes in *T. brucei* do encode protein products able to form heterodimeric holoenzyme complexes of regulatory and catalytic subunits. Specifically, pull down experiments using *T. brucei* cell clones expressing epitope-tagged PKA catalytic subunit-like or regulatory subunit-like proteins showed protein-protein interactions between the regulatory and each of the three catalytic subunits (PKAC1, PKAC2, and PKAC3) from *T. brucei* [12]. These contrasting results may be a consequence of Bachmaier et al. [12] employing *T. brucei* cell lines that were genetically manipulated to overexpress the proteins of interest, whereas we used native proteins from *T. equiperdum* parasites that were expanded in rats. Obviously, the level of total PKA subunit-like proteins must differ in each case. It is plausible that an increase in the concentration of the unknown nucleoside signaling molecule that stimulates this atypical cAMP-independent PKA occurs under the conditions used in the present work throughout parasite isolation and manipulation, producing in turn the dissociation of the PKA subunit-like proteins. If that is the case, some degradation of the free trypanosome TeqR-like and TeqC-like proteins may also occur following holoenzyme dissociation, decreasing in turn the amount of these proteins in the parasite soluble fraction. Analogous results have been reported in mammalian cells [36]. Since Guevara et al. [8] showed that glucose deprivation activates a cAMP-independent protein kinase from *T. equiperdum*, the rise in the level of the nucleoside molecule responsible of activating the parasite cAMP-independent PKA might probably occur as a sequel of some nutritional stress process. Moreover, since the TeqC-like enzyme appears to sense the nutritional status of the trypanosome, it would be interesting to link the pool of this novel nucleoside signaling molecule to the adenylate energy charge in these microorganisms.

On the basis of the pattern of the Lineweaver-Burk inhibition plots that yielded parallel rather than intersecting lines, the TeqR-like protein seems to work as an uncompetitive inhibitor with respect to kemptide. This is uncommon when compared to canonical PKAs, in which PKA$_R$ subunits are thought to behave as competitive inhibitors with respect to known PKA$_C$ subunit substrates. For example, Geahlen and Krebs [37] found that the regulatory subunit of the type I PKA was a competitive inhibitor of the cGMP-dependent protein kinase, which is a different but related AGC Ser/Thr protein kinase. Then, finding that the parasite TeqR-like protein is an uncompetitive inhibitor is unexpected. The trypanosome protein possesses features that are typical of

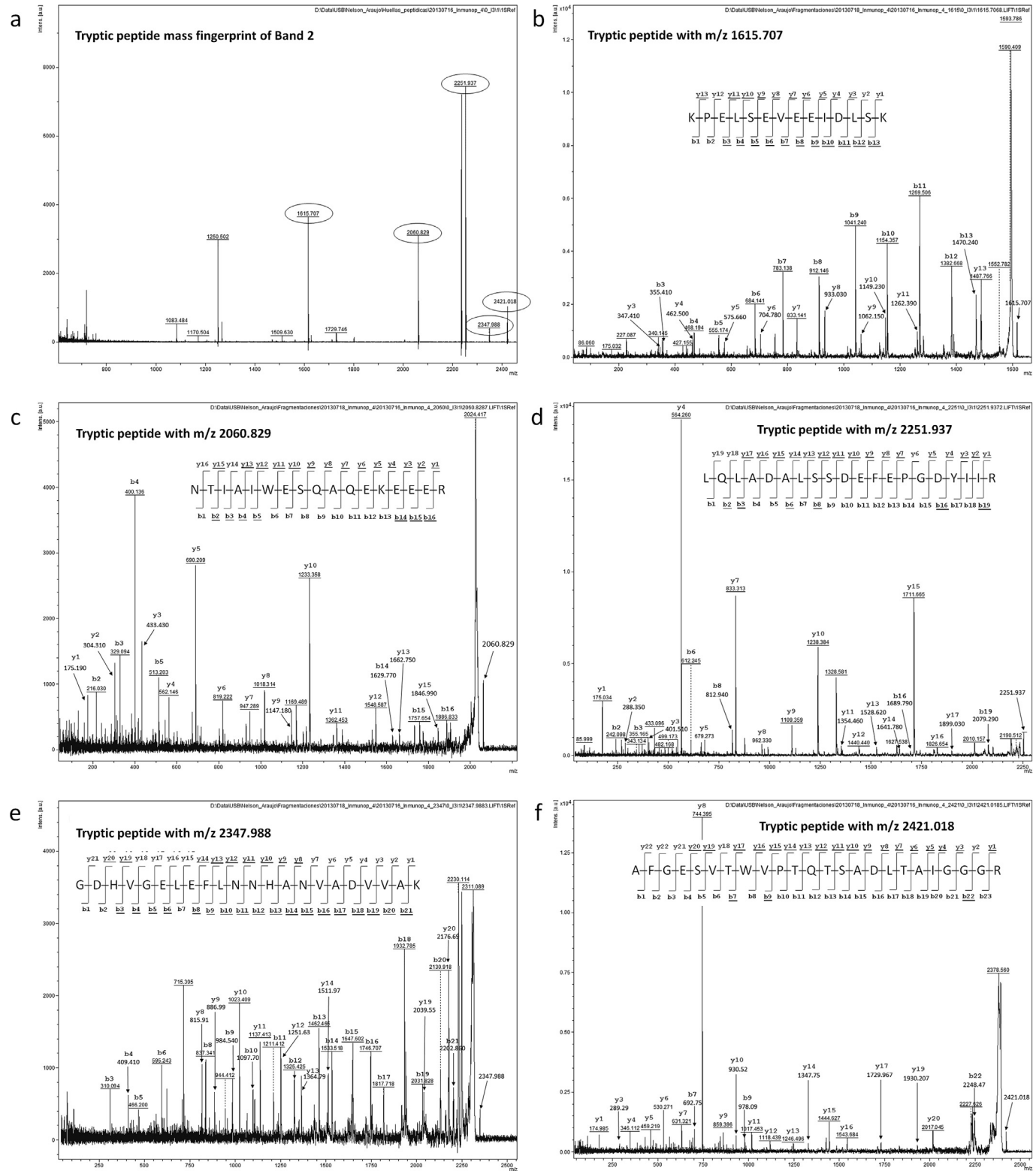

Fig. 10. Mass spectrometry analyses of Band 2. **a**, MALDI-TOF mass spectrum of the in-gel trypsin digest of Band 2 (Fig. 9). Peaks surrounded by ovals correspond to the five tryptic peptides that were sequenced in panels **b**–**f**. Sequencing by MALDI-TOF/TOF of tryptic peptides from Band 2 with *m/z* values of 1615.707 (**b**), 2060.829 (**c**), 2251.937 (**d**), 2347.988 (**e**), and 2421.018 (**f**). Identified fragments from the series b and y were underlined.

competitive inhibitors, such as a linker region containing the IS sequence, which similar to mammalian PKA_R subunits should be capable of binding to the active site of the parasite TeqC-like proteins. The IS region in the parasite protein also contains a Thr residue located at the right position in the consensus sequence, which allegedly can be recognized and phosphorylated by the parasite PKA-like enzymes. Therefore, the TeqR-like protein should behave as a phosphate acceptor substrate similar to kemptide;

Table 1
Immunoprecipitated proteins from the parasite soluble fraction using anti-recombinant TeqR-like polyclonal antibodies.

SDS-PAGE band	Tryptic peptide *m/z*	Sequenced tryptic peptide by MALDI-TOF/TOF[a]	Corresponding sequence in the identified protein[a,b]	Identity (%)	Mascot score	PSMs[c]	Identified protein	NCBI GenBank ID No.
Band 1	1389.665	EISEVVLVGGMTR	^{353}EISEVVLVGGMTR^{365}	100	108.3[d]	74.5	HSP70 (mitochondrial variant)	RHW71876.1
	1879.936	GVNPDEAVALGAATLGGVLR	^{384}GVNPDEAVALGAATLGGVLR^{403}	100	159.5[e]	126.7		
	2404.002	EMAADNQMMGQFDLVGIPPAPR	^{466}EMASDNQMMGQFDLVGIPPAPR^{487}	95.5	111.0[e]	78.8		
	2638.121	SQIFSTYSDNQPGVHIQVFEGER	^{429}SQIFSTYSDNQPGVHIQVFEGER^{450}	100	60.1[f]	28.2	HSP70 (another variant)	RHW68443.1 or
	2003.794	WLNDNQEASLDEYNHR	^{583}WLNDNQEASLDEYNHR^{598}	100	54.7[e]	22.2		RHW67553.1
Band 2	1615.707	KPELSEVEEIDLSK	^{34}KPELSEVEEIDLSK^{47}	100	91.3[g]	58.2	PKA regulatory subunit-like protein (TeqR-like)	RHW67040.1
	2060.829	NTIAIWESQAQEKEEER	^{160}NTIAIWESQAQEKEEER^{176}	100	121.0[g]	88.2		
	2421.018	AFGESVTWVPTQTSADLTAIGGGR	^{177}AFGESVTWVPTQTSADLTAIGGGR^{200}	100	127.3[g]	95.2		
	2251.937	LQLADALSSDEFEPGDYIIR	^{373}LQLADALSSDEFEPGDYIIR^{392}	100	125.7[g]	93.3		
	2347.988	GDHVGELEFLNNHANVADVVAK	^{428}GDHVGELEFLNNHANVADVVAK^{449}	100	173.3[g]	141.1		
Band 3	1701.742	AVFLDLEPTVVDEVR	^{65}AVFLDLEPTVVDEVR^{79}	100	47.9[h]	15.1	α-Tubulin	RHW74511.1
	2329.809	AFVHWYVGEGMEEGEFSEAR	^{403}AFVHWYVGEGMEEGEFSEAR^{422}	100	46.7[i]	13.6		
Band 4	1341.571	INVYFDEATGGR	^{47}INVYFDEATGGR^{58}	100	67.0[h]	33.9	β-Tubulin	RHW74472.1
	1631.756	SVLIDLEPGTMDSVR	^{63}SVLIDLEPGTMDSVR^{77}	100	21.1[j]	12.1		
	1227.548	VGEQFTLMFR	^{381}VGEQFTLMFR^{390}	100	35.6[k]	2.8		

[a] Peptide sequences are reported using amino acid single-letter abbreviations.
[b] Residues are numbered according to the primary structure of the translated gene products stored in the NCBI GenBank database (https://www.ncbi.nlm.nih.gov/). Organism: *T. equiperdum* IVM-t1 strain [5].
[c] PSMs = peptide-spectrum matches.
[d] Score greater than 46 and 23 indicates identity according to the NCBInr and SwissProt databases, respectively.
[e] Score greater than 45 and 21 indicates identity according to the NCBInr and SwissProt databases, respectively.
[f] Score greater than 44 and 20 indicates identity according to the NCBInr and SwissProt databases, respectively.
[g] Score greater than 45 indicates identity according to the NCBInr database.
[h] Score greater than 45 and 22 indicates identity according to the NCBInr and SwissProt databases, respectively.
[i] Score greater than 46 and 22 indicates identity according to the NCBInr and SwissProt databases, respectively.
[j] Score greater than 21 indicates identity according to the SwissProt database.
[k] Score greater than 23 indicates identity according to the SwissProt database.

however, the protein works as an uncompetitive inhibitor.

Uncompetitive inhibition does not affect the affinity of the substrate to the enzyme. The combination of an uncompetitive inhibitor to an enzyme seems to distort indirectly the active site of the enzyme affecting its catalytic function and rendering a catalytically inactive enzyme. In general, this type of inhibition mechanism takes place when an inhibitor binds to the complex formed between the enzyme and its substrate but not to the free enzyme. Our results clearly reflect that ligation of the kemptide substrate to the trypanosome TeqC-like protein precedes binding of the TeqR-like protein to the parasite enzyme. Consistently, immunoprecipitation experiments have shown that there is no formation of a holoenzyme type of complex between the TeqR-like and the TeqC-like proteins. Although uncompetitive inhibition is a rare mechanism in monosubstrate enzymatic reactions, it is more frequent in the bisubstrate enzymatic reactions that are characteristic of protein kinase enzymes [38–40]. Evidently, an understanding of the molecular basis of the inhibition of the trypanosome TeqC-like protein by the uncompetitive TeqR-like protein will require an analysis of the three-dimensional crystal structure of the enzyme-substrate-inhibitor complex.

Our kinetic data revealed a reversibility in the inhibition profile of the parasite TeqR-like protein. Since TeqR-like behaved as an uncompetitive inhibitor of the TeqC-like enzyme, the inhibition by TeqR-like and the affinity of the kemptide substrate for TeqC-like are not correlated. In other words, the binding of the inhibitor should not affect the affinity of the substrate to the enzyme. However, we do not know if there is a correlation between the reversibility in the inhibition profile by the TeqR-like protein and dephosphorylation events. A conjecture would be to postulate that the TeqR-like protein might be capable of interacting and/or activating parasite protein phosphatases. If so, the inhibition by the parasite TeqR-like protein might be correlated with reversible phosphorylation/dephosphorylation events. This would be a very compelling hypothesis; yet, it would be just speculation given that there are no experimental evidences to support that assumption.

Searching for interacting proteins, an association between the trypanosome TeqR-like protein and two HSP70 variants was found here. Proteins that belong to the HSP70 family are celular chaperones that play prime household management functions assisting in protein synthesis, folding, assembly, translocation, and degradation [41,42]. Therefore, trypanosome HSP70s are probably required to interact with the TeqR-like protein during synthesis and folding. The trypanosome TeqR-like protein possesses a particular N-terminal domain of unknown function that is characterized for having LRR sequences [10,14]. The TeqR-like protein LRR domains might be probably involved in the interaction with trypanosome HSP70s given that the chaperone consensus binding motif has been shown to contain sequences enriched in non-polar amino acids, such as Leu, Ile, Val, Phe and Tyr [43]. Interestingly, the LRR motif is a structural pattern that has been reported to be especially suitable for protein-protein interactions. Moreover, various studies have shown that mammalian HSP70 can interact and be phosphorylated by PKA_C subunits [44–46], and phosphoproteome analysis of PKA downstream targets has revealed that HSP70 can also be phosphorylated in *T. brucei* [12]. Based on all these results, the interaction between the *T. equiperdum* TeqR-like protein and HSP70 variants can be easily accounted.

We also found an association between the parasite TeqR-like protein and the *T. equiperdum* α/β-tubulin. Interestingly, analysis of the three-dimensional structure of the ternary complex of human α-tubulin with TBCE and TBCB, two chaperones known as tubulin-binding cofactors, revealed that α-tubulin-β-tubulin heterodimer dissociation occurs through a disruption of the interface between both tubulin subunits that is produced by a steric connection between β-tubulin and the TBCE cytoskeleton-

associated protein Gly-rich and LRR domains [47]. Moreover, the structure of the α-tubulin-TBCE-TBCB complex showed that the LRR domain and the linker site of TBCE interact with the H12 helix of α-tubulin [47]. Therefore, it is appealing to hypothesize that the LRR domain of the TeqR-like protein might be involved in the association with the parasite α/β-tubulin in a similar manner as these tubulin-binding cofactors, and may participate in the regulation of tubulin heterodimer association/dissociation processes. In addition, given that no typical AKAPs have been reported in trypanosomes, it is also tempting to postulate that the parasite TeqR-like protein might work in an equivalent manner as an AKAP under certain conditions, tethering and directing the trypanosome TeqC-like proteins to their downstream substrate targets. Interestingly, a PKA-mediated phosphorylation of α-tubulin has been reported in other biological systems [48]. Alternatively, the parasite α/β-tubulin might behave as an AKAP for the PKA subunit-like proteins, as reported for type I PKA in *Aplysia* and mouse neurons [49].

Although the TeqR-like protein is monomeric and is not capable of binding cyclic nucleotides, the parasite protein possesses sequence homology with the four isoforms of the mammalian PKA_R subunits, RIα, RIβ, RIIα, and RIIβ. Surprisingly, most of the similarities with its mammalian counterparts lie at its carboxy-terminal region containing the CNB domains [10]. In contrast, the corresponding amino-terminal regions of the mammalian PKA_R subunits hold almost no homology with the trypanosome protein [10]. These structural dissimilitudes, together with the functional differences shown here, and the fact that RNAi-mediated repression of the PKA regulatory subunit-like protein in *T. brucei* has been reported to cause growth inhibition [12], strongly indicate that this parasite protein represents an appealing target that may lead to the discovery of new anti-trypanosome chemotherapeutic drugs.

5. Conclusions

An inhibitor of a TeqC-like protein that corresponded to the TeqR-like protein was partially purified and biochemically characterized here. The inhibitor inactivated the TeqC-like protein in an uncompetitive manner, when fluorescently labeled kemptide was used as the substrate. Interestingly, the parasite TeqR-like and TeqC-like proteins were not associated suggesting that they are not capable of forming a heterooligomeric holoenzymatic type of complex. However, other trypanosome proteins that appeared to interact with the TeqR-like protein were recognized by co-immunoprecipitation followed by mass spectrometry analyses, such as two HSP70 variants, α-tubulin and β-tubulin.

Authors' contributions

N.A.A., M.C., and J.B. designed research. N.A.A. and J.B wrote the paper. N.A.A., and M.R. performed research. N.A.A., M.R., E.V., M.C., and J.B. analyzed data. All authors read and approved the final manuscript.

Author agreement form

The material submitted is original, and all authors are in agreement to have the article published.

Declaration of competing interest

The authors declare that there is no conflict of interests regarding the publication of this article.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biochi.2019.10.020.

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