Reversible phosphorylation of a protein from *Trypanosoma equiperdum* that exhibits homology with the regulatory subunits of mammalian cAMP-dependent protein kinases

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Abstract

Homologous proteins of the cAMP-dependent protein kinase (PKA) regulatory and catalytic subunits have been identified in *Trypanosoma equiperdum* (TeqR-like and TeqC-like, respectively). Partially purified TeqR-like from parasites isolated in the presence of glucose migrated as an apparent 55 kDa/57 kDa polypeptide doublet when separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. However, a single polypeptide of 57 kDa was obtained when parasites were deprived of glucose, a condition that has been shown to activate a TeqC-like enzyme. As revealed by immunoblots using anti-phospothreonine antibodies, the 57 kDa band corresponded to a form of TeqR-like that was phosphorylated in threonine residues. TeqR-like phosphorylation was reversible since the level of phospho-TeqR-like decreased once glucose was readded to glucose starved-parasites. Dephospho- and phospho-TeqR-like proteins are monomers with native molecular masses of 54.93-57.41 kDa, Stokes radii of 3.42-3.37 nm, and slightly asymmetric shapes (frictional ratio *f/fo* = 1.36-1.32). A protein kinase of ~40 kDa was also partially purified from glucose deprived-trypanosomes, which corresponded to the TeqC-like enzyme by its ability to phosphorylate kemptide, its inhibition by the PKA-specific inhibitors PKI-α, and its immunorecognition by anti-PKA catalytic subunit antibodies. TeqR-like and TeqC-like did not coelute following anion-exchange chromatography, revealing that these proteins are not associated forming a complex in *T. equiperdum*. Yet, when TeqR-like was incubated *in vitro* with TeqC-like in the presence of Mg^{2+} and ATP, the 55 kDa dephospho form of the 55kDa/57 kDa polypeptide doublet of TeqR-like was converted in*to the* 57 kDa *phospho* form, demonstrating that TeqR-like is a substrate for TeqC-like.

Materials and Methods

Parasites and Glucose Deprivation: We employed the Venezuelan *T. equiperdum* TeAp-N/D1 strain and were inoculated intraperitoneally into adult albino rats (Sprague-Dawley). Parasites from infected blood were purified by chromatography using a fibrous diethylaminoethyl (DEAE)-cellulose column equilibrated with phosphate-buffered saline containing glucose (PBS-G). Purified parasites were immediately incubated in the absence of glucose with phosphate-buffered saline (PBS) for various times (0, 15, 30 and 60 min), at room temperature, under gentle and constant agitation. In both cases, parasites were centrifuged and pellets were stored at -80°C.

Partial Purification of TeqR-like Protein Two samples of purified TeAp-N/D1 *T. equiperdum* parasites were employed. One sample containing ~10^{10} trypanosomes was prepared in the presence of glucose using PBS-G buffer, and the other sample containing ~10^{9} parasites was deprived of glucose for 60 min as described above. Trypanosomes were lyzed on ice by sonication with protease inhibitors and then centrifuged (100,000 g for 60 min) The resulting supernatant was loaded onto a 50-ml DEAE-Sephacel column, which was previously equilibrated with 50 mM HEPES (pH 7.2) Proteins bound to the resin were eluted with a linear gradient from 0 mM NaCl to 500 mM NaCl in 50 mM HEPES (pH 7.2). Column chromatography was carried out at 4°C and fractions of 7 ml were collected. The eluting proteins were monitored at 280 nm and subsequently separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Fractions were subjected to western blot analyses using polyclonal antibodies raised in mice against the recombinant TeqR-like protein. Blots were also revealed with commercial rabbit polyclonal anti-phosphothreonine and anti-phosphoserine antibodies. In addition, fractions were assayed for kemptide kinase activity.

Kemptide kinase activity using an electrophoretic gel-shift assay The heptapeptide kemptide (sequence = LRRASLG) was labeled with fluorescamine (fluram). Kinase reactions contained an aliquot (10 µl) of the chromatographic fractions, 0.14 mM fluram-kemptide, 25 mM Tris-HCl (pH 7.4), 12.5 mM $MgCl_2$ and 1.25 mM ATP, in a total final volumen of 20 µl. Samples were incubated for 30 min at room temperature. Following addition of 0.25% bromophenol blue and 10% glycerol, the mixtures were loaded into wells of a 1.2% agarose gel. Gels were revealed with UV light using a phototransilluminator (Fotodyne, Hartland, Wisconsin, USA). The PKA C subunit was purified from porcine heart and used as a positive control. The recombinant isoform α of the PKA heat stable inhibitor PKI from rabbit and a custom synthesized 20-amino acid inhibitor peptide that is derived from PKI-α, were also included to evaluate the inhibition of PKA-like enzymatic activities.

Gel Filtration Chromatography Fractions containing the TeqR-like protein were pooled, concentrated by lyophilization, and separated by size-exclusion chromatography using a Bio-Sil Sec-400-S high-performance liquid chromatography (HPLC) column using appropriate proteins as standards in order to establish the native molecular mass and Stokes radius of the parasite R-like protein.

Alignment of Human R isoforms sequences and *T. equiperdum* R-like sequence

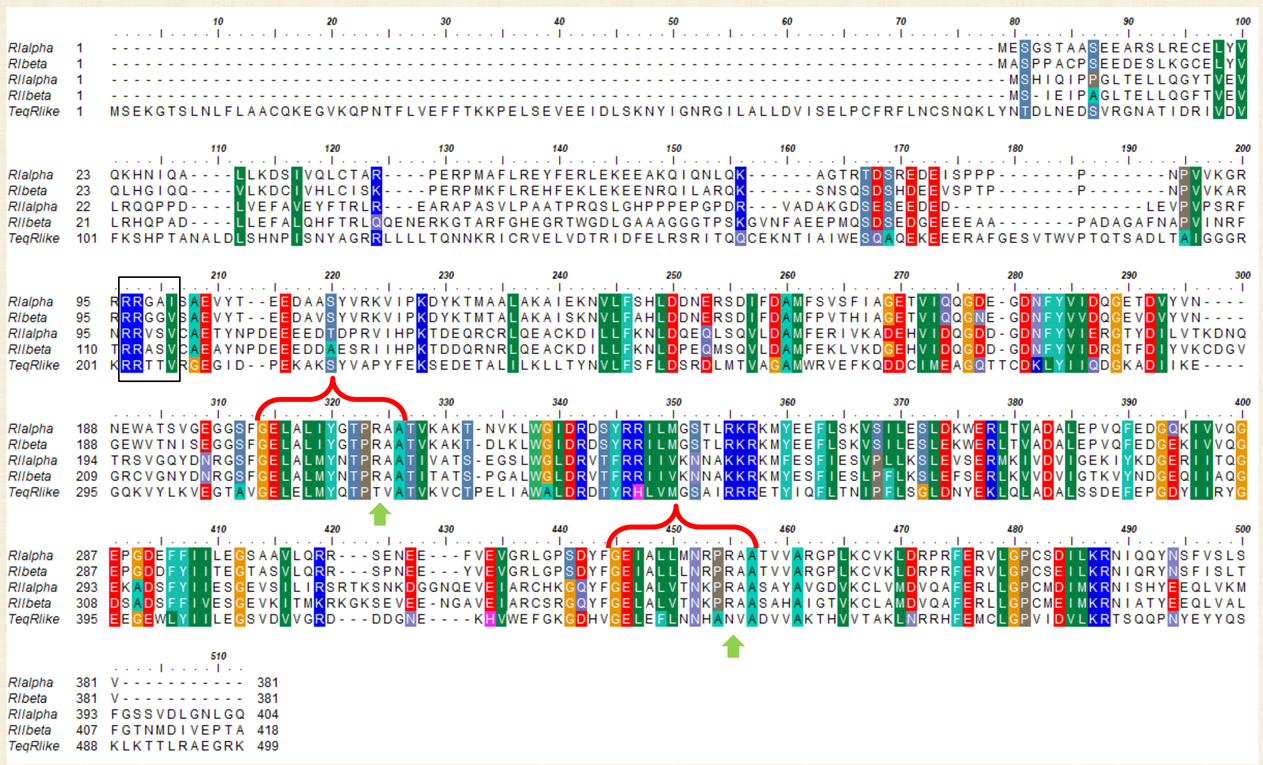

Fig. 1. Comparison of the primary structure of the four human PKA Regulatory subunit isoforms and the *T. equiperdum* PKA R-like protein Multiple sequence alignment was accomplished by using the Clustal Omega program (https://www.ebi.ac.uk/Tools/msa/clustalo/). The single letter amino acid code is used and gaps are indicated by dashes. Highlighted in color are identical and similar amino acid residues. The PBC domains are shown in red brackets, and conserved arginines that are essential for the interaction with the phosphate group of cAMP in both PBC domains of the human PKA R-subunits are shown by green arrows. The *T. equiperdum* R-like protein contains the consensus inhibitor or pseudosubstrate site that in other eukaryotes is involved in binding and inactivation of the PKA C subunits. In the parasite R-like protein, this motif corresponds to residues 202-206 (sequence = RRTTV) in which Thr^{205} is at the correct position to be phosphorylated by a trypanosome PKA-like enzyme. The inhibitor or pseudosubstrate sequence is framed inside a black box.

Results

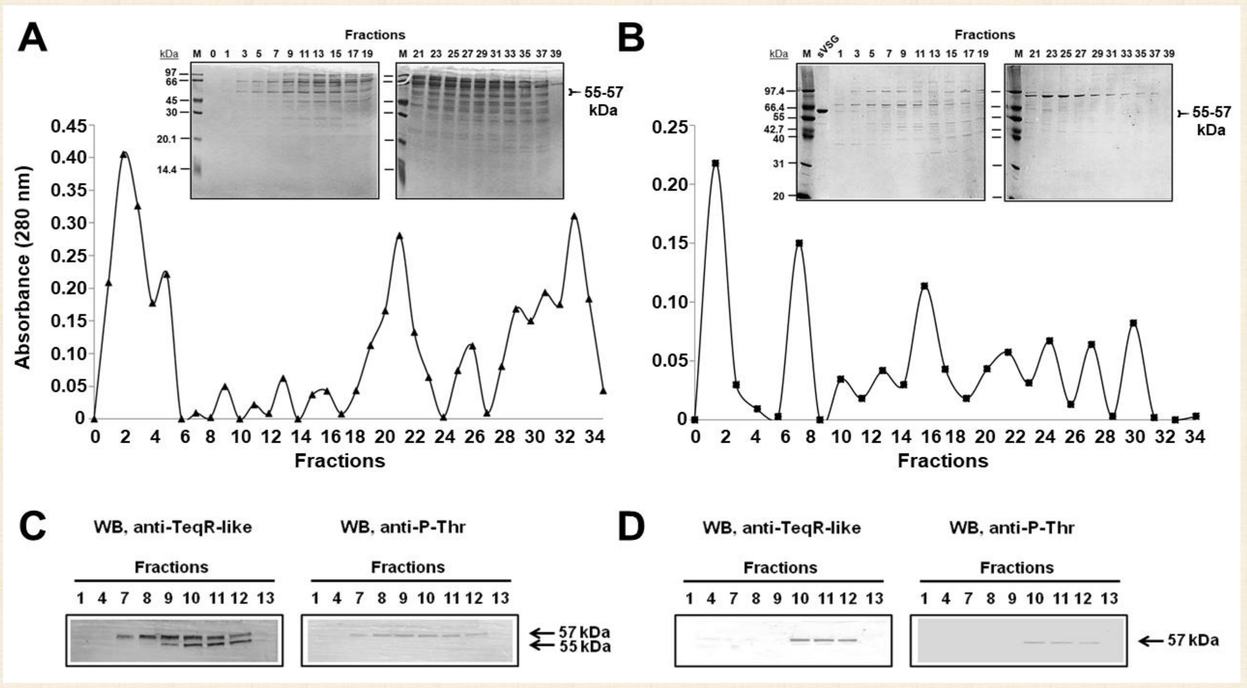

Figure 2. Partial purification of TeqR-like, and identification of its dephosphorylated and phosphorylated forms. The soluble fractions from glucose-enriched and glucose-starved *T. equiperdum* parasites were subjected to separation throughout a DEAE-Sephacel anion-exchange chromatography column. **A** and **C**, Parasites purified in the presence of glucose. **B** and **D**, Glucose-deprived parasites. **A** and **B**, Chromatograms showing the protein profiles (Absorbance at 280 nm). **Insets**, Aliquots (50 µl) of the chromatography fractions were evaluated by SDS-PAGE. Polypeptides were detected after staining with Coomassie blue R-250. M = molecular weight markers. sVSG = purified soluble form of VSG from the *T. equiperdum* TeAp-N/D1 strain (apparent molecular mass of 64 kDa). The small parenthesis designates the migration of the 55-57 kDa polypeptide bands. **C** and **D**, Aliquots (50 µl) of the resulting fractions were analyzed by western blot (WB) using either mouse polyclonal antibodies against the recombinant TeqR-like protein (anti-TeqR-like, **left**, dilution 1:10,000) or 5 µg/ml anti-phosphothreonine polyclonal antibodies raised in rabbits (anti-P-Thr, **right**). Then, membranes were incubated with the appropriate dilution of alkaline phosphatase conjugated secondary antibodies against mouse or rabbit IgG depending on the case, following the instructions of the supplier (dilution 1:15,000). Arrows indicate the apparent molecular masses of the immunorecognized polypeptide bands.

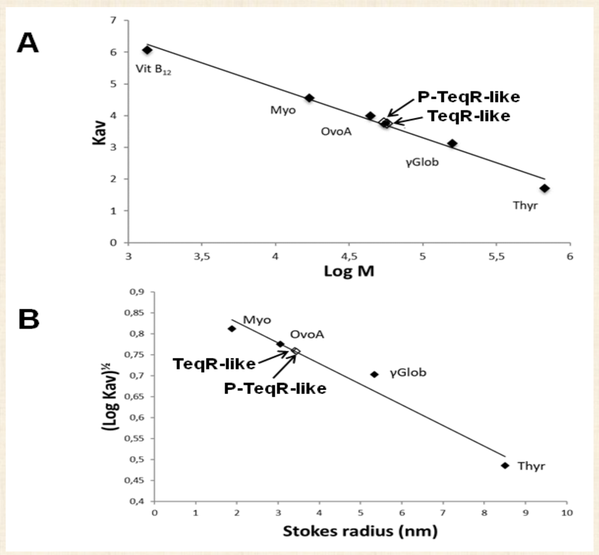

Figure 2. Molecular weight and Stokes radius of TeqR-like proteins that were partially purified from glucose-enriched and glucose-starved parasites. Partially purified TeqR-like proteins from either glucose-enriched or glucose-deprived parasites were applied to an HPLC size-exclusion column. Protein 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 (M). **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 thryroglobulin (Thyr), gamma globulin (γGlob), ovalbumin (OvoA), myoglobin (Myo) and vitamin B_{12} (Vit B_{12}).

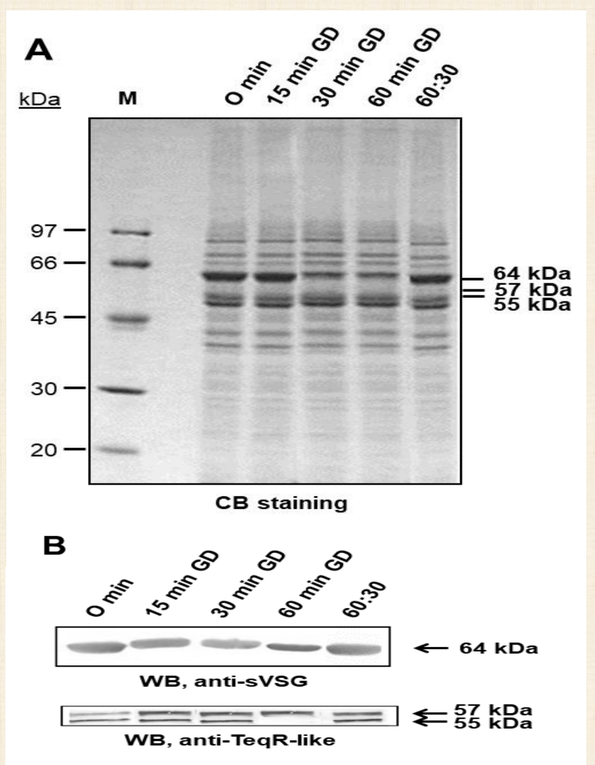

Figure 3. Reversible phosphorylation of TeqR-like. *T. equiperdum* parasites were purified through a DEAE-cellulose column using PBS containing 1% glucose (PBS-G), and the purified trypanosomes were immediately used (0 min). Also, freshly purified parasites were incubated with PBS lacking glucose for 15, 30 and 60 min (GD = glucose deprivation). In addition, a sample of the parasites deprived of glucose for 60 min was reincubated with PBS-G for 30 min (60:30).In all cases, trypanosomes were extracted and the corresponding soluble fractions were evaluated by SDS-PAGE on a 12% polyacrylamide slab gel, and directly stained with Coomassie blue R-250 (CB staining, A) or analyzed by western blot (WB, B) employing either mouse polyclonal antibodies against the soluble form of the variant surface glycoprotein from the same T. equiperdum isolate (anti-sVSG, top, dilution 1:10,000) or mouse anti-recombinant TeqR-like polyclonal antibodies (anti-TeqR-like, bottom, dilution 1:10,000). Membranes were revealed by using alkaline phosphatase conjugated secondary antibodies against mouse IgG (dilution 1:15,000). M = molecular weight protein markers. Arrows indicate the apparent molecular masses of the immunorecognized polypeptide bands.

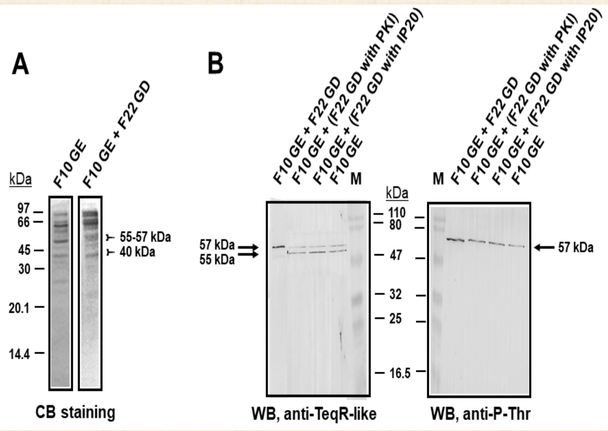

Figure 6. TeqR-like was phosphorylated in vitro by TeqC-like. A sample (30 µg of total protein) of the fraction containing the TeqR-like protein partially purified from glucose-enriched (GE) trypanosomes (Fraction 10 from Fig. 1A, F10GE) was incubated with 30 µg (total protein) of the fraction containing the TeqC-like protein partially purified from glucose-deprived (GD) parasites (Fraction 22 from Fig. 1B, F22GD) previously evidenced by kemtpide kinase assay (data not shown). A, Separation by SDS-PAGE of F10GE and the mixture of F10GE and F22GD (F10GE + F22GD) on 12% polyacrylamide slab gels. Polypeptides were detected after staining with Coomassie blue R-250 (CB staining). Small parentheses designate the migration of the 55/57 kDa and 40 kDa polypeptide bands corresponding to TeqR-like and TeqC-like, respectively. B, Phosphorylation of TeqR-like was followed by western blot (WB) using anti-recombinant TeqR-like antibodies raised in mice (anti-TeqR-like, left, dilution 1:10,000) and anti-phosphothreonine antibodies raised in rabbits (anti-P-Thr, right, 5 µg/ml). As controls, samples of F22GD that were preincubated with either 9 units of PKI-α (F22GD with PKI) or 0.25 mg/ml IP20 (F22GD with IP20) before combining it with F10GE in the reaction mixture were included. Likewise, a sample of F10GE with no addition was also incorporated. Then, nitrocellulose membranes were incubated with the appropriate dilution of alkaline phosphatase conjugated secondary antibodies against mouse or rabbit IgG depending on the case, following the instructions of the supplier (dilution 1:15,000). M = molecular weight protein markers. Arrows indicate the apparent molecular mass of the immunorecognized polypeptide bands.

Highlights

- ✓ Native TeqR-like exists as monomeric dephosphorylated and phosphorylated variants
- ✓ The phosphorylation of TeqR-like is reversible and prompted by parasite starvation
- ✓ Phospho-TeqR-like is phosphorylated in threonine residues
- ✓ TeqR-like is a substrate of TeqC-like even though both proteins are not associated