Divalent cation hinder the solubilization of a tubulin kinase activity from *Trypanosoma cruzi* epimastigotes

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ABSTRACT

Trypanosoma cruzi epimastigotes were extracted under various conditions in order to examine the role of divalent cations in the solubilization of microtubule proteins. When epimastigotes were homogenized in the presence of 5 mM Mg^{+2} and 5 mM Ca^{+2} , a protein kinase responsible for phosphorylating tubulin, as well as the tubulin that became phosphorylated, remained tightly associated with the parasite particulate and detergent-resistant fractions. On the contrary, tubulin kinase and its substrate were predominantly released into the parasite cytosolic and detergent-soluble fractions, when epimastigotes were extracted in the presence of 5 mM EDTA and 5 mM EGTA. These evidences demonstrated a divalent cation-dependent solubilization of the enzyme responsible for the phosphorylation of tubulin in *T. cruzi* epimastigotes and suggested a tight association between tubulin and this kinase. Under all conditions tested, tubulin kinase activity in epimastigote extracts was lower than the addition of the corresponding value in the parasite cytosolic and membranous fractions, suggesting the presence of a kinase inhibitor or regulatory subunit which also seemed to be modulated by divalent cations. Additionally, inhibition experiments in the presence of heparin, 2,3-bisphosphoglycerate and GTP established that the parasite tubulin kinase corresponded to a protein kinase CK2.

Key terms: microtubules, post-translational modification of tubulin, protein kinase CK2, protein phosphorylation-dephosphorylation, signal transduction, *Trypanosoma cruzi*, tubulin.

INTRODUCTION

Chagas' disease is a major endemic in Latin America (WHO, 1996). *Trypanosoma cruzi*, its etiological agent, is a flagellate protozoan that undergoes complex morphological changes throughout its life cycle in both the insect vector and the vertebrate host (De Souza, 1984; Andrews et al., 1987). The differentiation process appears to be highly regulated, and includes significant changes in biochemical pathways (Urbina et al., 1993), which permits its adaptation to various environments (Burleigh & Andrews, 1995). Additionally, drastic changes in the cytoskeleton of the parasite are observed during its differentiation.

In most eukaryotic cells, the cytoskeleton consists of three major filament systems: microtubules, intermediate filaments, and actin microfilaments, which form interwoven scaffolding that permeates the cytoplasmic space of the cell. In contrast, the cytoskeleton of trypanosomes is composed almost entirely of microtubules and microtubule-associated proteins and is further distinguished by the conspicuous absence of transcellular filaments (Gull, 1999). A complex array of microtubules that includes the subpellicular network, the axoneme, the basal body, the paraflagellar rod, the flagellum attachment zone and the filaments responsible for parasite attachment conforms the trypanosome

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cytoskeleton (Robinson et al., 1991; Scherével, 1994).

Similar to other eukaryotes, trypanosome microtubules are composed of two main structural subunits, α and β tubulin, that interact with a number of less abundant microtubule-associated proteins. Trypanosome α and β tubulins have been well characterized and are similar in sequence to those of other eukaryotes (Kohl & Gull, 1998). Despite these similarities, trypanosome microtubules exhibit a number of unusual features that make them an attractive target for the development of anti-trypanosomal drugs (Hill et al., 2000): a highly-ordered and uniformly-polarized organization; extensive intermicrotubule and membrane-microtubule cross-links; and high stability at low temperatures.

Protein kinases CK1 and CK2, previously known as casein kinases, belong to the serine/threonine protein kinases superfamily and appear to be ubiquitous in eukaryotes. These enzymes have been postulated to regulate multiple pathways of cellular metabolism and gene expression (Guerra et al., 1999; Guerra & Issinger, 1999) and seem to play an important role in intracellular signaling during cell proliferation and differentiation (Carroll et al., 1988; Pinna & Meggio, 1997; Guerra & Issinger, 1999). Protein kinases CK1 are active monomers of 25-55 kDa (Tuazon & Traugh, 1991; Gross & Anderson, 1998). In contrast, protein kinases CK2 are $\alpha_2\beta_2$, $\alpha'\beta_2$, or $\alpha\alpha'\beta_2$ tetramers, where α and α' (35-44 kDa) correspond to their catalytic subunits and the β subunits (24-30 kDa) have regulatory properties (Tuazon & Traugh, 1991; Issinger, 1993; Pinna, 1994, 1997; Dobrowolska et al., 1999).

Recently, Casas et al. (2002) have demonstrated that tubulin was the predominant phosphoprotein in homogenates of three different differentiation stages of *T. cruzi*: epimastigotes, trypomastigotes and spheromastigotes. The phosphorylation of tubulin was inhibited with heparin, 2,3-bisphosphoglycerate (2,3-BPG), and GTP in a dose dependent manner, indicating that tubulin kinase corresponded to a protein kinase CK2-like activity (Casas et al.,

2002). Interestingly, CK1-like and CK2-like activities have been previously shown in *T. cruzi* epimastigotes (Calabokis et al., 2002). Since divalent cations have been reported to strongly influence the distribution of protein kinases and their substrates in other systems (Ikeda et al., 1996; Nikula et al., 1987; Clement-Chomienne & Walsh, 1996), we have studied here the effect of Ca^{2+} and Mg^{2+} on the solubilization of the CK2-like activity responsible for the phosphorylation of tubulin in *T. cruzi*.

MATERIALS AND METHODS

Materials

Reagents and materials were purchased from the following sources: [γ - ^{32}P] ATP (3000 Ci/mmol), New England Nuclear; P81 phosphocellulose chromatography paper, Whatman; OptiPhase Hisafe II (scintillation liquid), LKB; polyvinylidene difluoride (PVDF) microporous membranes, Millipore; dephosphorylated casein, leupeptin, benzamidine, phenyl methyl sulfonyl fluoride (PMSF), L-trans-epoxysuccinyl-leucylamido(4-guanidino) butane (E-64), monoclonal anti- α tubulin (clone DM 1A), monoclonal anti- β tubulin (clone TUB 2.1), heparin, 2,3-BPG, cAMP-dependent protein kinase inhibitor PKI (6-22), 1-[N,O-bis(5-isoquinolinesulfonyl)-N-methyl-L-tyrosyl]-4-phenylpiperazine (KN-62), GTP, cAMP, bestatin, Sigma; N-(2-aminoethyl)-5-chloroisoquinoline-8-sulfonamide (CKI-7), Seikagaku America. Dr. Susan S. Taylor (University of California, San Diego, U.S.A.) generously donated synthetic Pep1 (RRKDLHDDEEDEAMSITA) and Pep2 (RRRADDSDDDDD) peptides. All other chemicals were of the highest-quality grade available.

Parasite culture

Trypanosoma cruzi epimastigote forms (EP strain) were grown at 28°C, in LIT-medium (liver infusion tryptose), as described (Rangel-Aldao et al., 1983). Epimastigotes

at stationary phase were collected by centrifugation at 3500x g, at 4 °C for 15 min, and washed three times with phosphate buffered saline (PBS) solution (0.1 M, pH 7.4) containing 10 mM NaCl and 42 mM sucrose. During the final wash, an aliquot of the resuspended epimastigotes was used to count the number of parasites using a Neubauer chamber. The final cell pellet was kept frozen at -80 °C until further use.

Homogenization of parasites and preparation of soluble, particulate, detergent-soluble and detergent-resistant fractions

All steps were performed at 4 °C. *T. cruzi* epimastigotes (~ 5 x 10⁹) were suspended in 10 ml of various buffers containing the following cocktail of protease inhibitors: 50 µM PMSF, 10 µM leupeptin, 10 µM E-64 and 1 mM benzamidine.

Initially, parasite homogenates were prepared using three different buffer conditions: 1) 50 mM Pipes (pH 6.9), 2 mM EGTA, 1 mM MgSO₄, 0.1 mM EDTA, 100 µM GTP, 5% glycerol (Buffer A); 2) 50 mM Tris-HCl (pH 8.0), 1mM MgCl₂, 1 mM CaCl₂ (Buffer B); and 3) 50 mM Tris-HCl (pH 8.0), 2 mM EDTA, 5% glycerol (Buffer C). The various parasite suspensions were homogenized on ice by sonication (35 W), using four cycles of 30 seconds each, separated by 2 min resting periods. The homogenates were then centrifuged at 100000x g for 45 min producing supernatants and sedimented fractions. For each buffer condition, the supernatant or soluble fraction (S1) was conserved, and the resulting pellet (P1) was resuspended, sonicated and centrifuged twice more, as described above, obtaining the corresponding fractions S2, P2, S3 and P3. Then, the P3 fraction was resuspended in the corresponding buffer containing 2% Triton X-100, and three consecutive extractions were carried out. The resulting supernatants and pellets were successively numbered. Resuspended pellet volumes were maintained throughout the experiment in order to keep the relative protein

concentration with respect to the corresponding homogenate in each step.

A different set of experiments was performed in order to confirm that divalent ions affected the solubilization of tubulin and its kinase. Briefly, epimastigote homogenates were prepared using the following buffers: 1) 50 mM Tris-HCl pH (8.0), 5 mM MgCl₂, 5 mM CaCl₂ (Buffer D), and 2) 50 mM Tris-HCl (pH 8.0), 5 mM EGTA, 5 mM EDTA (Buffer E). In each case, homogenates were sonicated and centrifuged as previously described. Following three extraction cycles with buffer alone, three additional extractions with the corresponding buffer containing 2% Triton X-100 were also conducted.

Protein kinase assay

Protein kinase assays were performed according to Calabokis et al. (2002). The reaction mixtures were carried out in a final volume of 70 µl and contained 50 mM Tris-HCl (pH 8.0), 12 mM MgCl₂, 20 mM KF, 100 µM [γ -³²P] ATP (specific activity ≈ 4500 cpm/pmol), and 50 µl of the parasite fraction. The reactions were incubated for 20 min at room temperature, and an aliquot (50 µl) was spotted on P81 phosphocellulose filter papers (2 cm x 2 cm). The papers were washed with 50 mM phosphoric acid three times (15 min per wash), dried, and analyzed for radioactivity by liquid-scintillation counting. One unit of activity was defined as the amount of enzyme that catalyzed the incorporation of 1 pmol of inorganic phosphate (Pi) from [γ -³²P] ATP per min. Since the volumes were kept constant throughout the extraction experiments, relative enzymatic activities were reported as total pmoles [γ -³²P] Pi incorporated/min.

The remainder of the samples (20 µl) was boiled at 100°C, for 5 min, in the presence of sample buffer for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970) and completely loaded on 12% polyacrylamide slab gels. The resulting [³²P]-labeled phosphopeptides were separated by electrophoresis (Laemmli,

1970) and electrotransferred to PVDF membranes (Towbin et al., 1979). Then, the membranes were exposed to Kodak X-Omat X-ray film at -80 °C using intensifying screens, and the phosphorylated bands were qualitatively analyzed by autoradiography. For protein staining, the radioactively-labeled membranes were colored with Coomassie blue R-250.

Casein kinase activity was measured by including dephosphorylated casein (1 mg/ml) in the reaction mixtures. A similar procedure was employed when the synthetic peptides Pep1 (200 µM) and Pep2 (40 µM) were used as exogenous substrates, except that 20 µM of the peptidase inhibitor bestatin was added to the reaction mixture. Specific casein, Pep1 and Pep2 kinase activities were assessed by subtracting the incorporation of [³²P] ATP in the absence of substrates. To determine the specific tubulin kinase activity, [³²P]-phosphorylated tubulin was excised from the Coomassie blue stained-PVDF membranes, transferred into scintillation vials, and counted.

Phosphorylation assays in the presence of well-known inhibitors for mammalian protein kinases

The fractions obtained from epimastigotes homogenized with Buffer D and E were assayed for kinase activity in the presence of increasing concentrations of heparin (0-133 µg/ml), 2,3-BPG (0-1.33 mM), GTP (0-133 µM), CKI-7 (0-1 µM), PKI (6-22) (0-1 µM), or KN-62 (0-2 µM). The assay was performed with and without cAMP (50 µM) when the effect of PKI (6-22) was evaluated. Parallel experiments were also carried out using 1 mg/ml of dephosphorylated casein as an exogenous substrate.

RESULTS

Epimastigote samples were homogenized in the presence of various buffers (A, B, or C) in order to examine the role of divalent cations in the extraction of parasite

microtubule proteins. Following centrifugation, the supernatants (S) and pellets (P) were separated, yielding the solubilized and remaining particulate fractions for each condition. Re-extractions with the various buffers containing 2% Triton X-100 were also carried out, generating their corresponding detergent-soluble and detergent-resistant components. All the fractions were consecutively numbered and were assayed for their kinase activity in the absence or presence of casein. S1-S3 and P1-P3 corresponded to the fractions resulting from the extractions with the buffers, while S4-S6 and P4-P6 were subsequently produced by the buffers containing 2% Triton X-100. Figure 1 summarizes the Coomassie blue stained-polypeptide profile (I), the endogenous protein kinase (II) and casein kinase (III) activities obtained under the various buffers conditions, following separation of the resulting fractions by SDS-PAGE. A predominant 55-kDa-polypeptide band was detected by protein staining (Fig. 1, I), which was strongly phosphorylated in the parasite extracts (p55, Fig. 1, II). This band was recognized by anti-α and anti-β tubulin monoclonal antibodies following Western blot analysis (data not shown). Additionally, anti-α and anti-β tubulin monoclonal antibodies immunoprecipitated the phosphorylated 55-kDa polypeptide from epimastigote homogenates (data not included), verifying that p55 corresponded to the parasite tubulin.

As shown in Figure 1, although the fraction of tubulin that became phosphorylated (fP-Tub) and the tubulin kinase activity (TubK) seemed to remain primarily associated with the pellet fractions, both proteins were present in S1 and S2 when divalent metal chelating agents were employed in the buffers (Fig. 1, II-A and II-C). Interestingly, no difference was obtained when 1 mM Mg⁺² was added together with 2 mM EGTA and 0.1 mM EDTA (Fig. 1, II-A). In contrast, TubK and fP-Tub were only obtained in S1 when 1 mM Mg⁺² and 1 mM Ca⁺² were used (Fig. 1, II-B), remaining tightly bound to the particulate fractions. Similar results were obtained when Triton X-100

Figure 1. SDS-polyacrylamide gel electrophoresis separation of the cytosolic, particulate, detergent-soluble and detergent-resistant fractions obtained from epimastigotes extracted with Buffer A (A), Buffer B (B) or Buffer C (C). I, polypeptide profile; II, endogenously phosphorylated bands; III, casein kinase activity. H = homogenate. S1-S3 and P1-P3 = soluble and particulate fractions, respectively. S4-S6 and P4-P6 = detergent-soluble and detergent-resistant fractions, respectively. Cas = casein

was included in the buffers. When the phosphopolypeptide profile of S4 was compared with S3, a slight increase in the solubilization of fP-Tub and TubK by the detergent was observed when EGTA and EDTA were used during the re-extraction (Fig. 1, II-A and II-C). However, further extractions with Triton X-100 did not enhance the solubilization of fP-Tub and TubK (Fig. 1, II-A and II-C, lanes S5 and S6). On the other hand, fP-Tub and TubK were not obtained in the detergent-soluble fractions (S4-S6) and completely remained in the detergent-resistant fractions (P4-P6) when divalent metal ions were included in the buffers (Fig. 1, II-B). All these results clearly indicated that fP-Tub and TubK were strongly associated to the parasite particulate fraction in the presence of Mg^{+2} and Ca^{+2} , and their solubilization was slightly elevated when divalent metal chelating agents were employed in the buffers.

Since Casas et al. (2002) have shown that TubK is a protein kinase CK2-like activity in *T. cruzi*, we also examined the kinase activity of the fractions obtained under the various conditions, using casein as an

exogenous substrate. Calabokis et al. (2002) have reported soluble CK1-like and CK2-like activities in *T. cruzi* epimastigotes. Thus, levels of casein kinase activity in the soluble and detergent-soluble fractions (S1-S6), as well as in the particulate and detergent-resistant components (P1-P6), represent the sum of all protein kinases CK1 and CK2 present in those fractions, including the tubulin kinase activity under study here. Therefore, the intensity of the phosphorylated casein band cannot be interpreted as being related exclusively to this tubulin kinase activity, which only corresponds to a portion of the total parasite CK2 activity. Accordingly, major differences were seen between the endogenous phosphorylation of tubulin and the phosphorylation of casein (Fig. 1, III). With the exception of S1, which showed an important casein kinase activity, the casein phosphorylating activity remained tightly bound to the particulate and detergent-resistant fractions (P1-P6) when 1 mM Mg^{+2} and 1 mM Ca^{+2} was used (Fig. 1, III-B). Hence, the phosphorylation of casein closely paralleled the endogenous phosphorylation of tubulin when Buffer B

Figure 2. SDS-polyacrylamide gel electrophoresis separation of the soluble and particulate fractions obtained from epimastigotes homogenized with Buffer D (A) or Buffer E (B). I, polypeptide profile; II, endogenously phosphorylated bands; III, casein kinase activity. H = homogenate. S1-S3 and P1-P3 = soluble and particulate fractions, respectively. Cas = casein

was used during the parasite extraction (Fig. 1, II-B and III-B). However, some casein kinase activity was also obtained in the rest of the soluble and detergent soluble fractions (S2-S6) (Fig. 1, III-B). On the other hand, some differences were observed when Figure 1 III-A, III-B and III-C were compared. The phosphorylation of casein was higher in the solubilized fractions (S1-S6) in the presence of 2 mM EGTA, 0.1 mM EDTA and 1 mM Mg^{+2} (Fig. 1, III-A), in contrast with the casein phosphorylation observed in the same fractions when only 2 mM EDTA was included in the buffer (Fig. 1, III-C). Under this condition (Fig. 1, III-C), the casein kinase and TubK activities remained primarily confined to the

particulate and detergent-resistant fraction, following the two first extractions (S1 and S2).

An aliquot of each reaction mixture was spotted onto P81 phosphocellulose papers to quantitatively determine the endogenous protein kinase and casein kinase activities of whole-cell homogenates, supernatants and pellets yielded by the various buffer conditions. The TubK activity was also measured by excising and counting the [^{32}P]-phosphorylated tubulin bands. Initially, the kinetics of [^{32}P] incorporation into endogenous substrates and exogenous casein by the various *T. cruzi* fractions was examined at room temperature and showed a linear kinase activity during the first 40

min (data not shown). Upon longer incubation, the activity reached saturation levels and started to plateau. Consequently, all subsequent enzymatic assays were performed during the linear phase of the curve, using a total incubation time of 20 min. As shown in Table I, the relative values determined were consistent and agreed with the results qualitatively achieved by autoradiography in Figure 1. Interestingly, the TubK, casein kinase and endogenous protein kinase activity values obtained in the original extract were lower than the sum of the corresponding activities measured in the S1 and P1 fractions, under all conditions. Since these assays were performed in a linear mode, our results strongly indicated the presence of an enzyme inhibitor in the parasite homogenate.

Following our initial observations (Fig. 1), two additional parasite extraction conditions were also examined: one containing elevated amounts of divalent cations (Buffer D) and another containing higher concentrations of EDTA and EGTA (Buffer E). The results obtained in Figure 1B were corroborated when epimastigotes were homogenized in the presence of 5 mM

Mg²⁺ and 5 mM Ca²⁺ (Fig. 2, A-II). TubK and fP-Tub remained tightly associated with the pellet, and only a minor amount of TubK was detected in the first solubilized fraction (S1). In contrast, a prominent increase in the solubilization of fP-Tub and TubK was achieved when epimastigotes were extracted in the presence of 5 mM EDTA and 5 mM EGTA (Fig. 2, B-II, S1). Moreover, fP-Tub and TubK were obtained in all fractions when divalent ion chelating agents were included in the buffer (Fig. 2, B-II). When the phosphopolypeptide profile of S4 was investigated, a slight increase in the solubilization of fP-Tub and TubK in comparison to S3 was evidenced as a result of re-extracting P3 with Buffer E containing 2% Triton X-100 (data not included). Under these conditions, S5 and S6 also contained fP-Tub and TubK although in lesser amount than S4 (data not shown). In contrast, fP-Tub and TubK were not obtained in the detergent-soluble fractions (S4-S6) and remained in the detergent-resistant fractions (P4-P6) when Buffer D in the presence of 2% Triton X-100 was employed during the re-extractions (data not included). The phosphorylation of exogenously-added casein was also evaluated for comparison

Table I
Endogenous protein kinase, tubulin kinase and casein kinase activities in fractions obtained from epimastigotes of *T. cruzi* extracted with Buffer A, Buffer B or Buffer C.

BUFFER A										
	PK	TubK	CK		PK	TubK	CK		PK	TubK
H	21500	20000	38889	S1	23120	21200	31872	P1	18040	17500
				S2	5060	4800	10474	P2	12880	11960
				S3	1320	1100	4977	P3	12320	11880
				S4	1505	1234	7935	P4	11340	10920
				S5	1052	945	7042	P5	10000	9800
				S6	907	760	3772	P6	7800	7030
BUFFER B										
	PK	TubK	CK		PK	TubK	CK		PK	TubK
H	21352	19500	40500	S1	10560	9800	29211	P1	21120	19680
				S2	2760	1840	3278	P2	16100	13900
				S3	980	440	3571	P3	15840	13420
				S4	420	84	2275	P4	14280	13020
				S5	389	165	1920	P5	12000	11200
				S6	342	152	1898	P6	9500	8360
BUFFER C										
	PK	TubK	CK		PK	TubK	CK		PK	TubK
H	24000	22500	37850	S1	15360	13920	30137	P1	17760	16800
				S2	4600	3220	39086	P2	8280	6900
				S3	1320	880	17798	P3	8360	7040
				S4	1680	1260	17942	P4	5880	4200
				S5	1210	900	15103	P5	6400	4800
				S6	760	380	10400	P6	3800	3520

Kinase activities are expressed as total pmoles of [γ -³²P] Pi incorporated/min. PK = endogenous protein kinase, TubK = tubulin kinase, CK = casein kinase, H = whole-cell homogenate, S1-S3 = soluble fractions, S4-S6 = detergent-soluble fractions, P1-P3 = particulate fractions, and P4-P6 = detergent-resistant fractions.

(Fig. 2, A-III and B-III). As previously described, levels of casein kinase activity in the different fractions may result from the various *T. cruzi* CK1 and CK2 isoforms and cannot be inferred to be solely associated to the parasite TubK.

A phosphopolypeptide band of approximately 79 kDa (p79) was also noticed in Figure 2, which probably corresponded to an autophosphorylated, unrelated protein kinase or to a parasite-endogenous substrate for another kinase. Interestingly, p79 showed a different behavior than fP-Tub and TubK and remained primarily associated with the parasite particulate fraction under both extraction conditions (Buffers D and E, Fig. 2). These results unequivocally indicated that the differential solubilization of TubK and fP-Tub seen in the absence or presence of divalent cations did not correspond to an experimental artifact.

Figure 3 quantifies the relative casein kinase, TubK and endogenous protein kinase activities determined in the soluble and particulate fractions obtained following homogenization of the parasites with Buffers D and E. The TubK and endogenous protein kinase activities were

predominantly found in the particulate fractions (Fig. 3, P1-P3), when Buffer D was used. In contrast, these kinase activities were primarily evidenced in the soluble fractions (Fig. 3, S1-S3), when Buffer E was employed. Although the parasite casein kinase activity was also primarily attained in the particulate fractions when Buffer D was used (Fig. 3A), casein kinase activities were evidenced in all soluble and particulate components obtained employing either Buffer D or E (Fig. 3A and B). These results were expected since the casein kinase activity measured in each parasite fraction must represent the total activity of all isozymes present in that individual component (Fig. 3). As previously shown in Table I, the sum of the kinase activities obtained for S1 and P1 was always higher than the corresponding activities obtained in the original homogenate, under both conditions (data not included), suggesting again the presence of a protein kinase modulator in the whole-cell extract.

CK2 inhibitors were employed to demonstrate that the TubK was the CK2-like activity previously reported to be responsible for phosphorylating tubulin in *T. cruzi* epimastigotes (Casas et al., 2002).

Figure 3. Endogenous protein kinase (PK), tubulin kinase (TubK) and casein kinase (CK) activities in the soluble (SF) and particulate (PF) fractions obtained from epimastigotes homogenized with Buffer D (A) or Buffer E (B). SF panels: filled, open and vertically-striped bars correspond to the S1, S2 and S3 fractions, respectively. PF panels: filled, open and vertically-striped bars correspond to the P1, P2 and P3 fractions, respectively.

Since divalent cations influenced the compartmentalization of the parasite TubK and fP-Tub, three specific CK2 inhibitors, heparin, 2,3-BPG and GTP (Tuazon & Traugh, 1991) were tested on the TubK-enriched fractions originated when the parasites were extracted with Buffer D or E, *i.e.*, the corresponding P1 or S1 fractions, respectively. Compared to the control assay, a significant decrease in the phosphorylation of tubulin was observed when the P1 fraction produced by the extraction with Buffer D was incubated with increasing concentrations of heparin (Fig. 4A, top). Similarly, a strong inhibition in the phosphorylation of tubulin was also observed in the presence of heparin, for the S1 fraction that was released by homogenization with Buffer E (Fig. 4B, top). Additionally, 2,3-BPG (Fig. 4A and B, bottom) and GTP (data not shown) inhibited the TubK present in both fractions in a dose-dependent manner. TubK was also assayed in the presence of increasing concentrations of well-known inhibitors for

other mammalian protein kinases: KN-62 for the Ca^{2+} /calmodulin-dependent protein kinase, CKI-7 for the protein kinase CK1, and PKI (6-22) for the cAMP-dependent protein kinase. In particular, the effect of PKI (6-22) was evaluated in the absence and presence of cAMP. However, no inhibition on the TubK activity was observed with any of these compounds (data not shown). These results confirmed that the TubK under investigation here represented a CK2-like enzyme, and argued against the possible contribution of other protein kinases in the divalent metal dependent-redistribution of TubK and fP-Tub. When casein was added to the reaction mixtures, a concentration-dependent inhibition of the phosphorylation of both tubulin and the exogenous substrate casein was also evidenced in the presence of heparin, GTP and 2,3-BPG (data not included).

Two synthetic peptides, Pep1 (RRKDLHDDEEDEAMSITA), which is specific for CK1 (Marin et al., 1994), and

Figure 4. Inhibition of the endogenous phosphorylation of tubulin by heparin and 2,3-BPG in the P1 fraction originated from epimastigotes extracted with Buffer D (A) and in the S1 fraction obtained when the parasites were homogenized with Buffer E (B). The arrow indicates the migration of phosphorylated tubulin (p55).

Pep2 (RRRADDSDDDD), which is specifically recognized by CK2 (Marin et al., 1994) were also examined as substrates for the TubK-enriched fractions originated when the parasites were extracted with Buffer D or E. The P1 fraction produced by the extraction with Buffer D phosphorylated Pep2 but not Pep1 (Fig. 5A), indicating that P1 contained exclusively protein kinases of the CK2 type. This result is consistent with the observation that TubK, which is a CK2, was predominantly associated with the parasite particulate fraction under this condition. As shown in Figure 5B, the S1 fraction that was released by homogenization with Buffer E was capable of phosphorylating Pep1 and Pep2. Consequently, both classes of isozymes, protein kinases CK1 and CK2, were present in S1. However, Pep1 was a better substrate for this fraction than Pep2. Since a soluble CK1-like activity has been previously reported (Calabokis et al., 2002), S1 probably contains this enzymatic activity besides the TubK activity that was released by extraction with Buffer E.

DISCUSSION

Casas et al. (2002) recently reported the co-purification of tubulin and a CK2-like

enzyme responsible for its phosphorylation from *T. cruzi* epimastigotes using DEAE-Sephacel chromatography. In an attempt to find conditions under which tubulin can be separated and purified from its kinase, we examined the effect of various homogenization buffers on the solubilization of both proteins. Our results clearly indicated a strong association between tubulin and its kinase, since no separation of tubulin kinase and the fraction of tubulin that became phosphorylated was obtained under the various conditions tested, even when detergents were included in the extraction buffers. Moreover, an analogous solubilization pattern was demonstrated for both proteins fP-Tub and TubK. The presence of Mg^{2+} and Ca^{2+} in the extraction buffer produced the association of a predominant amount of TubK and fP-Tub with the parasite particulate fraction. In contrast, a high proportion of fP-Tub and its kinase was solubilized when both chelating agents EDTA and EGTA were included in the homogenization step. Co-immunoprecipitation and *in vitro* binding studies have evidenced that CK2 is strongly associated to tubulin in mammalian cell extracts (Faust et al., 1999), which argues in favor of our results with *T. cruzi* epimastigotes.

Other authors have demonstrated the influence of divalent cations on the compartmentalization of several protein kinases and their substrates. Similar effects caused by Ca^{2+} have been reported for PKC in a number of tissues. PKC was also solubilized in the presence of chelating agents and remained associated with the particulate fraction in the presence of Ca^{2+} (Nikula et al., 1987; Ikeda et al., 1996). In *T. cruzi* epimastigotes, Ogueta et al. (1994, 1996) have reported a Ca^{2+} /calmodulin (CaM)-dependent protein kinase (TcCaMK) the cellular distribution of which relied primarily on its phosphorylation state. Based on their results, they established a model in which the Ca^{2+} /CaM-dependent autophosphorylation of TcCaMK promotes its activation and release into the parasite cytosol, while the dephosphorylated form of TcCaMK is inactive and remains attached to the parasite cytoskeletal elements

Figure 5. The P1 fraction obtained when epimastigotes were homogenized with Buffer D (A) and the S1 fraction originated from parasites extracted with Buffer E (B) were assayed for kinase activity using the synthetic peptides Pep1 and Pep2.

(Ogueta et al., 1996). Interestingly, a differential solubilization of a protein kinase CK2 and its endogenous substrate tubulin, depending on the presence of divalent cations, have never been previously reported in any other tissue or organism. Here, we clearly showed that Ca^{2+} and Mg^{2+} hindered the solubilization of this enzymatic activity from *T. cruzi* epimastigotes, suggesting a regulatory mechanism modulating the subcellular translocation of both proteins in the parasite.

Calabokis et al. (2002) have recently reported the identification of two casein kinase activities in the clarified cytosolic fraction of *T. cruzi* epimastigotes. One of these, Q-I, belongs to the CK1 family, whereas the other, Q-II, appears to contain either a mixture of CK1 and CK2 or a protein kinase with both activities. Evidently, *T. cruzi* possesses several casein kinases activities, and TubK must correspond to one of the parasite CK2 isozymes. Previous studies have described CK2-like enzymes in other trypanosomes. Aboagye-Kwarteng et al. (1991) identified a protein kinase CK2-like activity in *T. brucei brucei* and found that the phosphorylation of two polypeptide substrates, p37 and p42, increased as the parasites differentiate from long, slender, rapidly dividing to short, stumpy forms. Additionally, the genes encoding the α subunits of CK2 from both *Leishmania chagasi* and *T. brucei* have been cloned (Bhatia et al., 1998; Park et al., 2002). Interestingly, two genes encoding for CK2 catalytic subunits, CK2 α and CK2 α' , were identified in *T. brucei* (Park et al., 2002). Although the alpha isoform is preferentially nucleolar, it also localizes with the nucleus and in a lower proportion with the cytoplasm (Park et al., 2002).

The mammalian CK2 holoenzyme is composed of two catalytic subunits ($\alpha\alpha$, $\alpha'\alpha'$ or $\alpha\alpha'$), which are essential for cell viability, and a dimer of two non-catalytic β subunits. In humans, Shi et al. (2001) have reported a third catalytic subunit isoform, α'' . Although both the isolated catalytic subunits and the holoenzyme are constitutively active, the β subunits

profoundly affect many properties of CK2, whose precise function is still poorly understood (Pinna, 2002). Briefly, the CK2 β subunit has been shown to have trifunctional properties: it enhances the stability, the activity and the specificity of the CK2 catalytic subunits (Guerra & Issinger, 1999). Moreover, a dual role has also been reported for the CK2 β subunit, being an activator or an inhibitor depending on the substrate employed (Benitez et al., 2001). The phosphorylation of calmodulin by the CK2 catalytic subunits is inhibited when the β subunit is present. In contrast, the phosphorylation of casein is enhanced when both CK2 components are included in the assay. Additionally, given the structure of the holoenzyme, the β subunits could undergo reversible dissociation under physiological conditions and play a role as anchoring elements and/or docking platforms for protein substrates and effectors (Pinna, 2002). Recently, Park et al (2002) reported the molecular cloning of the *T. brucei* CK2 β subunit, but its function has not yet been elucidated.

By homology with *T. brucei*, CK2 β subunits may also exist in *T. cruzi*. Here, we evidenced the presence of a negative regulator of the CK2-like enzyme that phosphorylates tubulin in *T. cruzi*. Interestingly, this TubK regulator also seemed to be modulated by divalent cations. Then, if *T. cruzi* possesses CK2 β subunits, they could contribute with the modulation of the parasite CK2 catalytic subunits responsible for phosphorylating tubulin. However, other molecules playing inhibitory functions cannot be discarded, since it has been suggested that CK2 β subunits might not exist in some organisms (Boldyreff et al., 1993; Dobrowolska et al., 1991; Kikkawa et al., 1992; Ospina et al., 1992).

Various studies have indicated that calcium plays an important role in *T. cruzi* host cell invasion. Pretreatment of trypomastigotes with chelating agents decreased the association of parasites to myoblasts by approximately 50% (Moreno et al., 1994). On the contrary, pretreatment of trypomastigotes with ionomycin, which elevated the level of Ca^{2+} in the parasites,

significantly enhanced their infectivity capability (Yakubu et al., 1994). In addition, *T. cruzi* macrophage infection was blocked by incubating the parasites with inhibitors of protein kinases (Vieira et al., 1994). The physiological role that divalent cations play in the interaction between tubulin and its kinase calls for further study. However, since the parasite tubulin kinase is a protein kinase CK2-like activity that is involved in the modulation of processes such as movement, proliferation and differentiation in other organisms, it is plausible that CK2 might also play an important role in regulating these processes in *T. cruzi*, in a calcium-dependent manner.

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