Acidic ribosomal P proteins are phosphorylated in *Trypanosoma cruzi*

Eliana B. Gómez[a,b,1,2], Gabriel Medina[a,b,2], Juan P.G. Ballesta[c],
Mariano J. Levin[a,b], Maria T. Téllez-Iñón[a,b,*]

[a]*Instituto de Investigaciones en Ingeniería Genética y Biología Molecular (INGEBI), Universidad de Buenos Aires; Buenos Aires, Argentina*
[b]*Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires; Buenos Aires, Argentina*
[c]*Centro de Biología Molecular 'Severo Ochoa', Universidad Autónoma de Madrid, Canto Blanco 28049, Madrid, Spain*

Abstract

Trypanosoma cruzi ribosomes from epimastigote forms were purified as determined by electron microscopy and isoelectrofocusing was used to analyse this purified ribosome fraction. Silver stained gels revealed that acidic proteins are present in at least 10 different isoforms, in accord with previous cloning studies. To detect phosphorylation, in vitro phosphorylation assays using the recombinant protein TcP2β-mbp were carried out. The results showed that *T. cruzi* cytosolic fraction possesses protein kinase activity able to phosphorylate the recombinant protein. Purified ribosomes contain protein kinases that could also phosphorylate the recombinant protein TcP2β-mbp. Labelling parasites with [^{32}Pi] in a phosphate free medium demonstrated that ribosome proteins, recognised with a specific mouse antiserum against recombinant TcP2β proteins, are phosphorylated in vivo. All these results suggest that in vivo phosphorylation of ribosome TcP2β proteins are mediated by protein kinase(s) not yet identified. © 2001 Australian Society for Parasitology Inc. Published by Elsevier Science Ltd. All rights reserved.

Keywords: Trypanosoma; Ribosome; Protein phosphorylation; P proteins; Isoelectrofocusing

1. Introduction

One of the more studied characteristics of ribosomes is the highly flexible lateral protuberance (stalk) of the large subunit. The stalk consists of a pentameric complex which involves an rRNA binding protein, called L10 in eubacterias, and P0 in eukaryotes, and two homodimers of a set of strongly acidic proteins with low Mol. wt., L7/L12 or P1–P2, respectively. This complex is involved in the binding to the ribosome of soluble factors EF1 and EF2, the binding of aminoacid t-RNA and in the GTPase activity (Ballesta and Remacha, 1996; Liljas, 1991; Egebjerg et al., 1990; Lavergne et al., 1987; Bielka, 1982). It is also capable of regulating the pattern of protein expression (Remacha et al., 1995a).

The variety of P proteins is different in each organism, but they show a highly conserved amino acid sequence (Santos and Ballesta, 1995; Schijman et al., 1995, 1990; Shimmin et al., 1989). They are divided into two groups, P1 and P2,

according to their amino acid sequence identity to mammalian proteins (Wool et al., 1995). There is more than 80% amino acid identity in each group. One protein of each group is present in at least all the organisms studied. Only one type has been detected in human and rat cells (Rich and Steitz, 1987; Tsurugi et al., 1978), *Artemia salina* (Uchiumi et al., 1987; van Agthoven et al., 1978) and *Drosophila melanogaster* (Qian et al., 1987; Wigboldus, 1987), and two types in yeast (Remacha et al., 1995b; Beltrame and Bianchi, 1990; Mitsui and Tsurugi, 1988). In *Saccharomyces cerevisiae* the proteins are named YP1α and YP1β for the P1 group and YP2α and YP2β for the P2 group.

In eukaryotes these proteins are called P proteins due to their capacity to be phosphorylated. In *S. cerevisiae* the acidic proteins are found phosphorylated in vivo in a single position, the serine 96 (Zambrano et al., 1997). In mammalian cells multiple phosphorylation states of P1 and P2 proteins have been found (Lin et al., 1982). In some organisms phosphorylation is required for the reactivation and function of the ribosome in vitro (Rodriguez-Gabriel et al., 1998; Wool et al., 1995; Vidales et al., 1984), and restores the capability to bind GDP during the elongation step of translocation (Ballesta and Remacha, 1996).

In some eukaryotic cells a pool of cytoplasmic unpho-

sphorylated P proteins has been identified (Remacha et al., 1995a; Wigboldus, 1987). The amount of acidic proteins in ribosomes varies depending on the growth stage of the cell. These are the reasons why an exchange mechanism between the cytoplasm and ribosome which might be involved in the control of the particle activity, has been proposed (Ballesta and Remacha, 1996; Ballesta et al., 1993; Saenz-Robles et al., 1990).

The characterisation of *Trypanosoma cruzi* P proteins using biochemical techniques and their phosphorylation has not been studied. Here we describe the purification of ribosomes from *T. cruzi* epimastigote forms and the identification by electrofocusing of the P proteins. In addition, the phosphorylation of a recombinant TcP2β protein by parasitic extracts, and evidence of in vitro and in vivo TcP protein phosphorylation is shown.

2. Materials and methods

2.1. Parasites

Epimastigotes of *T. cruzi* (Tulahuen 2, strain) were cultured in axenic medium for 7 days at 28°C (Gómez et al., 1989). Cells were collected by centrifugation at $1000 \times g$ for 10 min, washed three times with 0.25 M sucrose, 5 mM KCl (SKS), and resuspended in the same buffer with the addition of 5 mM EGTA and a cocktail of protease inhibitors (final concentration: 0.5 mM TLCK, 0.1 mM PMSF, 25 μ/ml Trasylol, 10 μg/ml leupeptin). Parasites were broken after three cycles of freezing (-70°C) and thawing (4°C), and the rupture was controlled under optical microscopy.

2.2. Ribosome preparation

The parasite were disrupted as described above, with the addition of 0.25% w/v of deoxycholate to SKS buffer. The homogenate was centrifuged at $1000 \times g$ for 10 min and the supernatant was centrifuged at $12,000 \times g$ until no pellet was detected. The supernatant (S10) obtained was centrifuged at $105,000 \times g$ for 2 h, and the pellet P 100, enriched in ribosomes, was resuspended carefully in buffer II (20 mM Tris–HCl pH 7.7, 100 mM MgCl$_2$, 500 mM NH$_4$, Acetate, 5 mM β-mercaptoethanol). Afterwards the suspension was clarified by centrifugation (three–four times) at $12,000 \times g$ for 10 min. The supernatant was loaded on a sucrose discontinuous gradient in buffer II and centrifuged at $105,000 \times g$ for 18 h. The discontinuous gradient was formed with 40% v/v sucrose which corresponded to 44% of the final volume, and 20% v/v sucrose, which corresponded to 30% of the final volume. The translucent and gelatinous ribosome pellet was resuspended, with a glass rod, in a minimal volume of buffer I (10 mM Tris–HCl pH 7.5, 12.5 mM MgCl$_2$, 80 mM KCl, 5 mM βME). All buffers contained the protease inhibitors described before. All preparations were carried out at 4°C.

Saccharomyces cerevisiae pure ribosome fractions were prepared according to Sanchez-Madrid and Ballesta (1979) and used in control assays.

2.3. Electron microscopy

Ribosomes were fixed in glutaraldehyde (5% v/v final concentration) in cacodylate buffer (pH 7.2) and after fixation with 2% OsO$_4$ were prepared by conventional method to be analysed by EM. EM was performed in the Instituto Segrin-CRIBBAB, Conicet.

2.4. Electrophoresis

SDS-PAGE electrophoresis was done according to Laemmli, as was described by Ogueta et al. (1994). Samples were treated with dissociation buffer and heated 3 min at 100°C. After electrophoresis, gels were stained with 0.25% (w/v) Coomassie blue R250, or electrotransferred to nitrocelullose Hybond-C (Amersham) (Ogueta et al., 1994).

2.5. Western blot

Nitrocellulose sheets were blocked with 5% (w/v) low fat milk, 2% (w/v) glycine in buffer TBS (50 mM Tris–HCl pH 7.4, 0.15 M NaCl) for 1 h (Levin et al., 1989). The membranes were then incubated with antibodies diluted in blocking solution and revealed with the Vecta Stain reagent or Chemiluminiscence reagent from NEN laboratories.

2.6. Phosphorylation assays

Samples were incubated with exogenous or endogenous substrates in a mixture containing: 5 μM [γ^{32}P]ATP (10,000 cpm pmol^{-1}) (NEN laboratories), 20 mM Tris–HCl pH 7.5, 10 mM MgCl$_2$ and 10 mM β-glycerophosphate (βGP) during different times at 30°C. Reactions were stopped with dissociation buffer and analysed by SDS-PAGE and autoradiographed (Agfa Gevaert Curix films). As indicated some reactions were treated with a cocktail of phosphatase inhibitors: 50 mM FNa, 20 mM βGP, 10 mM Na pyrophosphate.

2.7. Phosphorylation by Casein Kinase II

Samples with TcP2β-mbp, TcP2β-mbp fragment or maltose binding protein were incubated 15 min at 30°C, with 0.14 μg α-subunit and 0.18 μg β-subunit of CKII, in a reaction mixture contained 50 μM [γ^{32}P]ATP (1000 cpm pmol^{-1}) and a cocktail of protease inhibitors. Reaction was stopped with dissociation buffer, analysed by SDS-PAGE and autoradiographed.

2.8. In vivo phosphorylation of T. cruzi cells

Epimastigotes cultured (80 ml, 5×10^7 cells/ml) as described above, were centrifuged 5 min at $1000 \times g$, resuspended and incubated 1 h in 20 ml depleted phosphate Krebs–Henseleit medium (25 mM NaHCO$_3$, 11 mM glucose, 118 mM NaCl, 4.7 mM KCl, 2 mM CaCl$_2$, 1.2 mM MgSO$_4$, 1.2 mM K$_2$HPO$_4$). Then, the culture was

centrifuged at $1000 \times g$ for 5 min and resuspended in 20 ml Krebs-Henseleit medium phosphate free with [^{32}Pi] (300 μCi/ml) for 4 h. After the sample was treated with 50 mM NaF and 0.25% (final concentration) deoxycholate. The suspension was frozen and thawed (three times) and centrifuged at $3000 \times g$ for 10 min at 4°C. The supernatant obtained was centrifuged at $16,000 \times g$ for 30 min a 4°C. The pellet, P16 and the supernatant S16, were subjected to 15% SDS-PAGE, transferred to nitrocellulose, autoradiographed and revealed by Western blot with a mouse specific antibody against P proteins.

2.9. Isoelectrofocusing (IEF) gels

Whole ribosomes prepared with protease and phosphatase inhibitors were directly analysed after treatment with RNAse (10 μg/ assay) for 40 min at room temperature. Aliquots of ribosomes were incubated with alkaline phosphatase, potato acid phosphatase or protein phosphatase 2A and stopped at 0°C. Samples were dehydrated in a Speed Vac and homogeneously resuspended with running buffer (20–30 μl). Running buffer IEF: BIEF: 9.5 M urea, 2% ampholytes (pH 2.5–5 range) and 0.1% (pH 5–7 range).

Gels were prepared according to the vertical IEF technique developed by Vidales et al. (1984). Gels with 5% polyacrylamide and 8 M urea were prepared with ampholytes 1.9% (range pH 2.5–5) and with the addition of 0.1% ampholytes from pH 5 to 7. Gradient was performed for 15 min at 50 V and 30 min each increasing the voltage from 100 to 600 V. Samples were loaded in buffer containing equal volume of 2 M urea to avoid modification of the proteins by the cathodic buffer (0.03 M NaOH). After running at 250 V for 18 h (maximum 0.6 mA), the voltage was raised to 600 V for 45 min.

Gels was silver stained according to Bravo and Ansorge (1982) or immunoanalysed. Protein concentration was determined by Bradford reagent using BSA as standard.

2.10. Ribosomes concentration

Purity of ribosomes was determined according to the absorbance ratio at 260/280 OD. The relation used was 13.75 OD 260 = 1 mg/ml ribosome, that was deduced considering 55% of ribosome is RNA, and 1 OD 260 = RNA = 40 μg/ml.

2.11. Materials

Recombinant casein kinase subunits was a gift of Dr Jorge Allende (Univ. Chile, Chile). The protein ribosomal P2β (10 kDa) of *T. cruzi* was cloned and expressed in an heterologous system coupled to maltose binding protein as a peptide of 46 kDa (TcP2β-mbp) and purified by an amylose resin (New England BioLabs Inc.). This recombinant protein was a gift of Dr P. López-Bergami (INGEBI). Protease and phosphatase inhibitors were from Sigma Chemical Co. Prestained protein markers were from

Gibco-BRL. Antibodies from Lupic P$^+$, Lupic P$^-$ patients, mouse antiserum against recombinant P proteins, mAB against C-terminal P acid proteins of *S. cerevisiae* (Vilella et al., 1991) were obtained from our lab.

3. Results

3.1. In vitro phosphorylation of the recombinant protein TcP2β-mbp

Protein fractions obtained by centrifugation from parasite extracts were used as source of protein kinase activity, and the recombinant protein P2β-mbp was used as substrate. Results shown in Fig. 1 indicate that the S10 (lane 2), P100 (lane 5) and S100 (lane 6) fractions have protein kinase activity able to phosphorylate the recombinant protein. Maltose binding protein was not phosphorylated by the *T. cruzi* fractions (lanes 3 and 8) and the TcP2β-mbp was not phosphorylated in the absence of protein extracts (data not shown). These results indicate that the parasite possesses protein kinase activity in the cytosolic fraction (S100) as well as in the P100 fraction, which includes unpurified ribosomes, that can phosphorylate the TcP2β recombinant protein.

CKII protein kinase is able to phosphorylate yeast ribosomal acidic proteins (Szyszka et al., 1995; Hasler et al., 1991). To test if the *T. cruzi* kinase activity could be CKII-like, experiments using a CKII recombinant protein kinase from *Xenopus* were performed, employing the TcP2β as a substrate. This kinase was not able to phosphorylate the fusion protein TcP2β nor maltose binding protein. The CKII was an active enzyme since it could autophosphorylate the α and β subunit (data not shown).
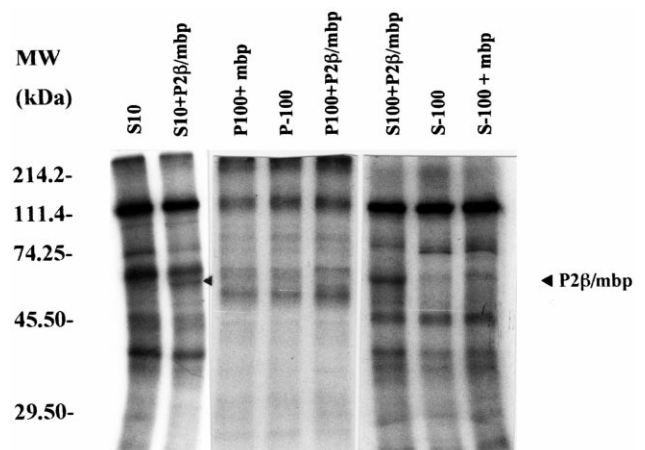

Fig. 1. In vitro phosphorylation of the recombinant protein TcP2β-mbp. Subcellular fractions from *Trypanosoma cruzi* extracts were incubated in a protein kinase reaction with [γ^{32}P] ATP alone or with the addition of the recombinant protein TcP2β or maltose binding protein. Samples resolved by 15% SDS-PAGE were visualised by autoradiography. (lane 1) S10 fraction; (lane 2) S10 fraction plus TcP2β-mbp; (lane 3) P100 fraction plus maltose binding protein; (lane 4) P100 fraction; (lane 5) P100 fraction plus TcP2β; (lane 6) S100 plus TcP2β; (lane 7) S100 fraction; (lane 8) S100 plus maltose binding protein. Molecular weight markers are indicated.

3.2. Purification of T. cruzi ribosomes

The pellet of purified ribosomes was visualised under EM (Fig. 2). All micrographs analysed showed an unique type of structure corresponding to ribosomes without cellular membranes or other organelle fragment (Stöffler and Stöffler-Meilicke, 1986). The ribosome major longitude was of 250 Å, which is the expected size for fixed ribosomes visualised by EM.

3.3. Immunodetection of P proteins from purified ribosomes

To detect the presence of acidic proteins in ribosomes, Western blot analysis using Lupus patients P+ serum (Fig. 3, lane 1) and mouse antibodies against the TcP2β-mbp (Fig. 3, lane 2), were carried out. As a positive control the recombinant protein P2β-mbp was employed and a Lupus P⁻ serum was used as a negative control (data not shown). The Lupus P⁺ antisera recognised a triplet between 14 and 16 kDa. (Fig. 3, lane 1). Some Lupus P⁺ serum also recognised a protein of approximately 18 kDa which has not previously been identified (Levin et al., 1993; Levitus and Levin, 1998).

Neither the Lupus P⁺ nor the mouse antibodies against TcP2β identified acidic proteins in the cytosolic fraction (S100) suggesting that there are no cytosolic P proteins
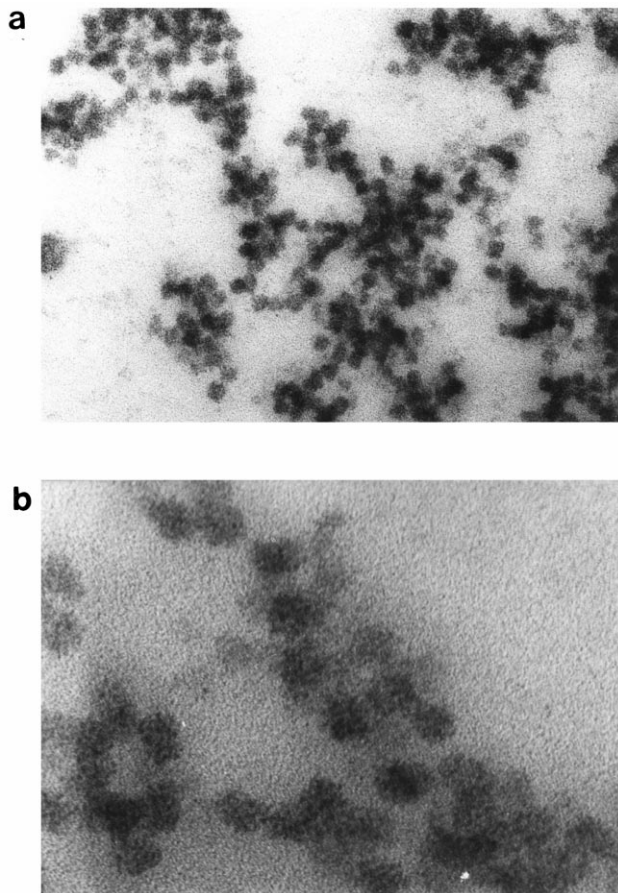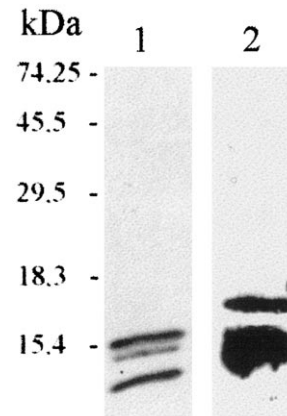
Fig. 3. Immunodetection of acidic P proteins from purified ribosomes. Purified ribosomes (0.5 mg) were resolved by 15% SDS-PAGE and transferred to nitrocellulose membranes. Western blot analysis were carried out with sera from Lupus patients P+ (lane 1) and with mouse antibodies raised against the TcP2β (lane 2).

pool in *T. cruzi* epimastigotes or they are present in low concentrations, not detectable with these antiserum. The serum used was unable to immunoprecipitate the P proteins from the parasite.

3.4. Isoelectrofocusing assay of ribosomal proteins

To determine if endogenous *T. cruzi* P proteins are phosphorylated in vivo, purified ribosome fractions, treated or not with phosphatases were analysed by isoelectrofocusing (IEF) assays. The IEF method allows the separation of proteins according to their isoelectric point so, the removal of the phosphate group from a protein raises its isoelectric point changing its relative position in a gel. Purified ribosomes treated or not with different phosphatases were resolved by IEF and the gel was silver stained (Fig. 4A) or transferred to nitrocellulose membranes for Western blot detection (Fig. 4B). As a control for the phosphatase treatment, *S. cerevisiae* purified ribosomes were used (Fig. 4C). Western blot analysis of the IEF *T. cruzi* ribosome proteins showed that the Lupus P⁺ serum detected at least six types of acidic P proteins (Fig. 4B). The silver stained counterpart shows a series of double bands which are differentially recognised by the Lupus P⁺ serum (Fig. 4A). After alkaline phosphatase treatments yeast protein displays a mobility shifts as was indicated in the work of Szyszka et al. (1995), and confirmed in experiments shown in Fig. 4C. No shift was obtained when *T. cruzi* purified ribosomes were treated with different phosphatases (alkaline, PP2A or potato acidic phosphatase) under different conditions of time and temperature (Fig. 4A and B, lane 2). Alkaline phosphatase treatment carried out with ribosomes preincubated in the presence of phosphatase inhibitors showed dissimilar results (data not shown) suggesting that endogenous ribosomal phosphatase activity might interfere with the experiment and phosphorylation assays with purified ribosomes.

Fig. 2. Purification of *Trypanosoma cruzi* ribosomes: (a) at × 140,000, (b) at × 370,000.
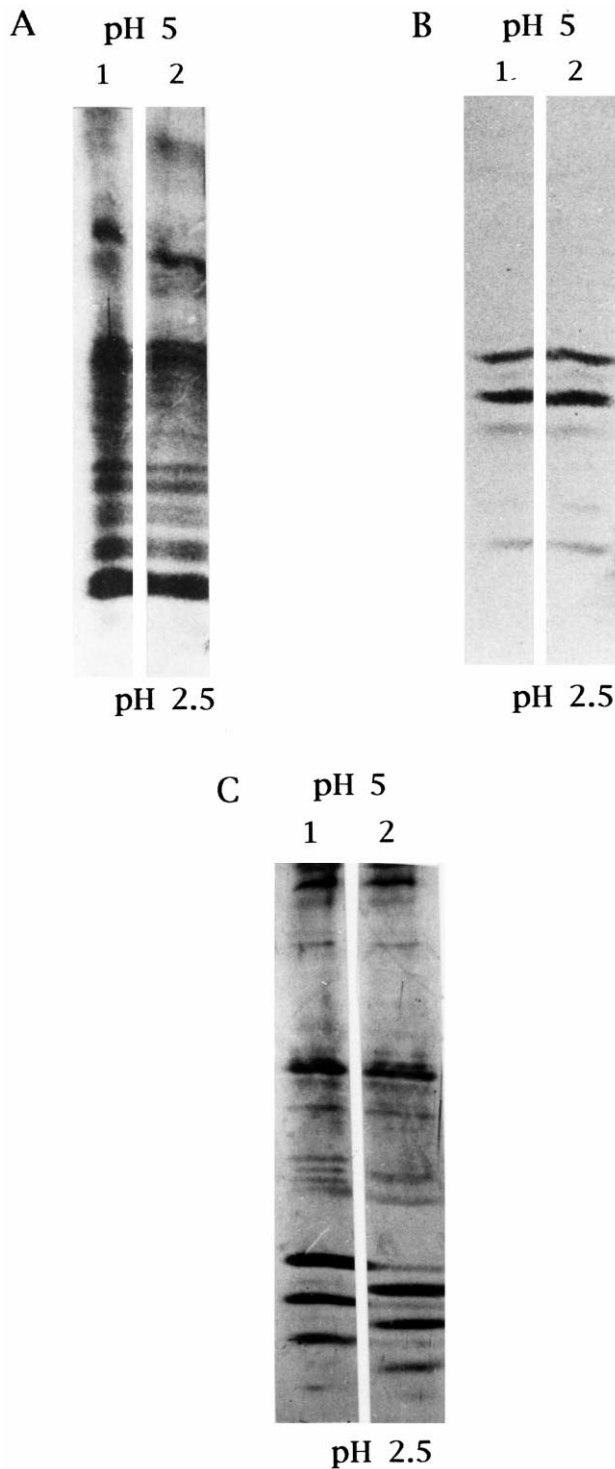
Fig. 4. Isoelectrofocusing assay of ribosomal proteins. Purified ribosomes (0.5 mg) from *Trypanosoma cruzi* were incubated in the absence (panel A and B, lane 1) or presence of alkaline phosphatase (1 unit/sample) (panel A and B, lane 2) and resolved by isoelectrofocusing (IEF) assays in a 2.5–5.0 pH range. The gel was silver stained (panel A) or transferred to nitrocellulose membranes for Western blot detection with Lupus P$^+$ serum (panel B). Control *S. cerevisiae* purified ribosomes treated with phosphatase (panel C, lane 1) or not (panel C, lane 2) were used as a control of phosphatase treatment and silver stained.

3.5. In vitro T. cruzi ribosome phosphorylation

Due to the results obtained in the IEF experiments, we decided to test endogenous phosphatases in the ribosomal preparations. The purified *T. cruzi* ribosomes were used in different phosphorylation assays. Ribosomes were incubated with labelled ATP (Fig. 5A, lane 3), labelled ATP plus TcP2β-mbp (Fig. 5A, lane 1) and labelled ATP plus phosphatase inhibitors (lane 2). After SDS-PAGE the gel was blotted on nitrocellulose membranes, which were autoradiographed and used in Western blots experiments employing the mouse anti TcP2β serum.

The recombinant P protein TcP2β-mbp was not phosphorylated by the purified ribosomal fraction (Fig. 5A, lane 1). A band of about 15 kDa was phosphorylated in all treatments (Fig. 5A, lanes 1–4), and was recognised by the serum (Fig. 5B, lanes 1–4). A second band of about 14 kDa was also phosphorylated in the assays shown in lanes 1 and 3 of Fig. 5A. The Western blot assay showed that the antiserum recognised a protein which migrated in the same
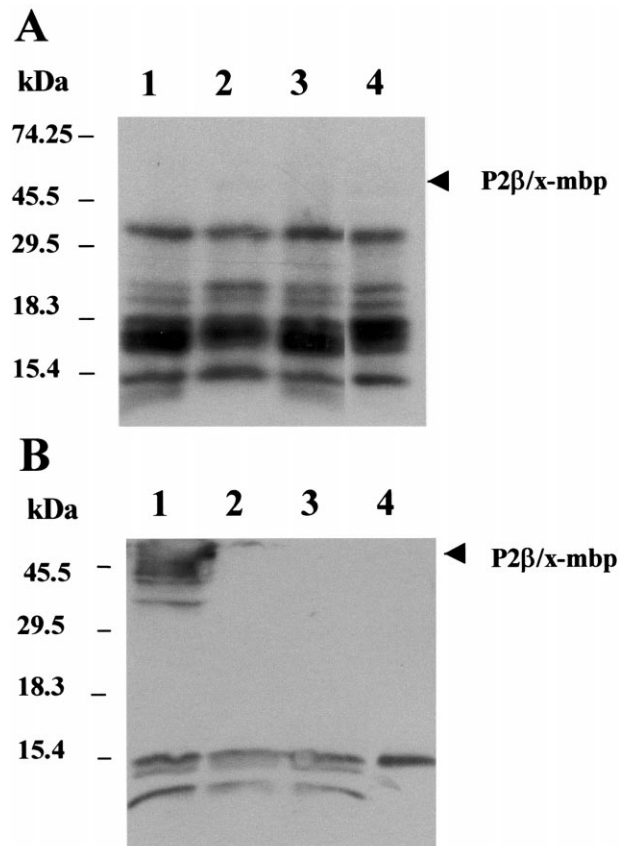

Fig. 5. In vitro *Trypanosoma cruzi* ribosome phosphorylation. Purified *T. cruzi* ribosomes (50 μg proteins) were used in different phosphorylation assays, and after resolved in 15% SDS-PAGE, the gel was blotted onto nitrocellulose membranes, which were autoradiographed (A) and used in Western blots experiments employing the mouse anti- TcP2β serum (B). Ribosomes were incubated with [γ^{32}P]ATP and TcP2β (lane 1), with TcP2β plus phosphatase inhibitors (lane 2) or without the recombinant protein (lane 3). (lane 4) Another preparation of ribosomes incubated without the recombinant protein. Molecular weight markers are indicated.

position as the 14 kDa protein phosphorylated in the kinase assays (Fig. 5B, lanes 1–4). Ribosomes incubated with phosphatase inhibitors did not phosphorylate the 14 kDa protein (Fig. 5A, lane 2), but the Western blot analysis showed that the protein was present (Fig. 5B, lane 2). When using another ribosomal preparation (Fig. 5A, lane 4) which lacks the 14 kDa protein, as determined by Western blot (Fig. 5B, lane 4), the 14 kDa phosphorylated band was also absent (Fig. 5A, lane 4). A third band of about 12 kDa which was not phosphoryalated, was also recognised by the serum (Fig. 5A, lanes 1–3). In the other ribosome preparation the 12 kDa protein was not present (Fig. 5B, lane 4), and phosphorylation of proteins smaller than 15 kDa was not observed (Fig. 5A, lane 4).

Treatment with polylysine, an activator, and heparin, an inhibitor of CKII, showed the same phosphorylation pattern as the control (data not shown).

3.6. In vivo phosphorylation of T. cruzi parasites

Trypanosoma cruzi epimastigote forms were labelled with [^{32}Pi] orthophosphate in a free phosphate medium. Different parasite subcellular fractions were resolved by SDS-PAGE and the proteins were blotted to a nitrocellulose membrane that was autoradiographed and analysed by Western Blot with the mouse anti- TcP2β-mbp serum (Fig. 6). Phosphorylation of some peptides was observed in the range of low Mol. wt. (Fig. 6A, lanes 1–3). The antibodies recognised one protein of about 15 kDa in the S16 fraction, which includes ribosomes (Fig. 6B, lanes 1–3). A protein with the same mobility is phosphorylated in the S16 fraction suggesting that some of the P protein variants could be phosphorylated in vivo. The serum did not recognise any protein in the fractions without ribosomes.

4. Discussion

The *T. cruzi* P proteins were first characterised for the antigenic activity present in patients with Chagas disease. Actually, a complex P protein family has been identified in this parasite (Levin et al., 1993). Several ribosomal acidic P proteins genes, P1, TcP1 (109 aminoacids), two P2, named TcP2α (112 aminoacids) and TcP2β (107 aminoacids) according to their similarity with the *S. cerevisiae* analogous proteins, and one P0, TcP0 (321 aminoacids) (Levin et al., 1993; Schijman et al., 1990) have been cloned. Multiple independent genes encode some of these proteins. Nucleotide sequence analyses identified four variants for TcP2β, two for TcP2α, one for TcP1 and two for TcP0 (Schijman et al., 1995).

The small acidic P proteins from *T. cruzi* present a conserved common structure consisting of a rigid amino terminal region, a hinge region rich in proline and alanine, and a globular region in the carboxy terminal, with a well conserved consensus sequence of 13 acidic aminoacids EE**E**DDDMGFGCFD. This sequence differs from the common one (EE**S**DDDMGFGCFD), in the last serine, which is known to be phosphorylated in almost all eukaryotic organisms (Shimmin et al., 1989). The protozoan *Tetrahymena* lacks the last serine, and experiments suggest that they are not phosphorylated (Hansen et al., 1991; Sandermann et al., 1979).

This conserved sequence is an epitope recognised by sera obtained from an appreciable number of patients with systemic lupus erythematosus (Elkon et al., 1986), and also by sera obtained from patients with Chagas disease with cardiac failure (Kaplan et al., 1997; Levitus et al., 1991; Mesri et al., 1990; Levin et al., 1989). This was also reported against laminin, spectrin and tubulin proteins (Unterkircher et al., 1993), adrenergics receptor (Borda et al., 1984) and cytoskeletal proteins (Levitus and Levin, 1998).

Here we report biochemical studies on pure *T. cruzi* ribosomes which allows the study of structural and biochemical features of these particles. The results show the existence of at least 10 different *T. cruzi* P protein variants as determined
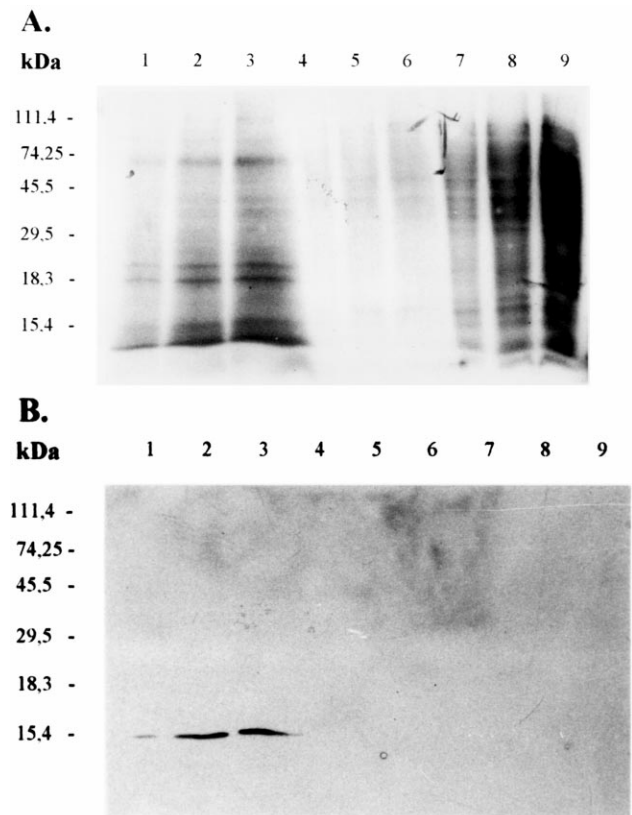

Fig. 6. In vivo phosphorylation of *Trypansoma cruzi* parasites. *Trypanosoma cruzi* epimastigotes were labelled with [^{32}Pi] ortophosphate in a free phosphate medium. Different fractions obtained by centrifugation were resolved by SDS-PAGE and the proteins were blotted to a nitrocellulose membrane that was autoradiographed (A) and analysed by Western Blot using the mouse anti TcP2β-mbp serum (B). (lane 1) S16 fraction (25 μg protein); (lane 2) S16 fraction (50 μg protein), (lane 3) S16 fraction (100 μg protein); (lane 4) P16 fraction (6 μg protein); (lane 5) P16 fraction (12 μg protein); (lane 6) P16 fraction (25 μg protein); (lane 7) P3 fraction (45 μg protein); (lane 8) P3 fraction (90 μg protein); (lane 9) P3 fraction (180 μg protein).

by IEF. We present evidence that suggests that *T. cruzi* P proteins can be phosphorylated in vivo, and demonstrate that the recombinant TcP2β-mbp can be phosphorylated by cytosolic kinase(s). The fact that this protein could not be phosphorylated by a purified ribosomal fraction indicates that the protein(s) responsible for its phosphorylation is/are not in the ribosome preparation or, if present, they are inactive. On the other hand, although the treatment of purified ribosomes with phosphatases and IEF analysis suggest that the P proteins are not phosphorylated, the experiments with phosphatase inhibitors indicate that endogenous phosphatases are present and could be affecting the IEF results. The inhibition of the 14 kDa protein phosphorylation by phophatase inhibitors (Fig. 5A) can be explained by at least two models: (1) in order to phosphorylate the protein, it must be first dephosphorylated so, in the presence of phosphatase inhibitors the phosphate group is not removed and as a consequence the radiolabelled phosphate can not be incorporated; (2) the responsible kinase(s) must be activated by a dephosphorylation process, so the phosphatase inhibitors prevent phosphatase(s) to act on the kinase(s).

These experiments suggest that associated with the *T. cruzi* ribosomes there is at least one protein kinase able to phosphorylate certain variants of P proteins. This conclusion is supported by the fact that the pattern of ribosome proteins phosphorylation is modified by phophatase inhibitors, which specifically inhibit the phosphorylation of the 14 kDa variant. This result also indicates that phosphatase(s) co-purified with the ribosomes. The experiments carried out with the other ribosome preparation further sustain that some P proteins are able to be phosphorylated, since, when the 14 kDa P protein variant is not present, no phosphorylation of a 14 kDa protein can be observed.

In addition, the in vitro phosphorylation studies using purified ribosomes show the existence of kinases and phosphatases in this particles which have different substrate specificity. These enzymes could control the phosphorylation and activity of *T. cruzi* ribosomal P proteins, as was reported for *S. cerevisiae* (Naranda et al., 1993). In this organism the acidic proteins are found phosphorylated in vivo in a single position, the serine 96 (Zambrano et al., 1997), and various types of protein kinases have been identified from pure ribosomes which had distinct specificity for the phosphorylation of the P proteins variants (Szyszka et al., 1995, 1996). The phosphorylation state of these proteins could affect ribosome function (Szyszka et al., 1995; Vard et al., 1997), and it was shown that the composition of the stalk selectively modifies the efficiency of yeast ribosomes in translating certain mRNAs affecting the pattern of expressed proteins (Remacha et al., 1995a).

Trypanosoma cruzi P proteins lack the phosphorylable consensus serine in the carboxyl-terminal domain, however, in this work we show strong evidence for the phosphorylation of these proteins, indicating that another residue must be involved in this phosphorylation. These results show a difference between *T. cruzi* and the protozoan *Tetrahymena*, where

the P proteins lack the last serine and experiments suggest that they are not phosphorylated in vivo (Sandermann et al., 1979; Hansen et al., 1991). The presence of phosphatases and kinases in the ribosomal fraction suggests the existence of a complex mechanism involved in the regulation of ribosome activity. The identification of these proteins will provide tools for the understanding of the translation processes. The experiments described for *T. cruzi* raise new questions about the role of other phosphorylation sites in the transduction machinery of eukaryotic cells.

References

Ballesta, J.P.G., Remacha, M., 1996. The large ribosomal subunit stalk as a regulatory element of the eukaryotic translational machinery. Prog. Nucleic Acid Res. Mol. Biol. 55, 157–93.

Ballesta, J.P.G., Remacha, M., Naranda, T., Santos, C., Bermejo, B., Jimenez-Diaz, A., Ortiz-Reyes, B.L., 1993. The acidic ribosomal proteins and the control of protein synthesis in yeast. In: Brown, A.J.P., Tuite, M.F., McCarthy, J.E.G. (Eds.), Protein Synthesis and Targeting in Yeast, Springer, Berlin.

Beltrame, M., Bianchi, M.E., 1990. Sequence of cDNA for one acidic ribosomal protein of *Schizosaccharomyces pombe*. Mol. Cell. Biol. 10, 2341–8.

Bielka, H. (Ed.), 1982. The Eukaryotic Ribosome Springer-Verlag. Berlin, Heidelberg, New York.

Borda, E., Pascual, J., Cossio, P., De la Vega, M., Arana, R., Sterin-Borda, L., 1984. A circulating IgG in Chagas' disease which binds to β-adrenergic receptor of myocardium and modulates their activity. Clin. Exp. Immunol 5, 679–86.

Bravo, T., Ansorge, V., 1982. Proceeding of Electrophoresis. de Gruyter, W. (Ed.). Stathatos, D, Berlin.

Egebjerg, J., Douthwaite, S., Liljas, A., Garret, R.A., 1990. Characterisation of the binding sites of protein L11 and the L10 (L12) pentameric complex in the GTPase domain of 23S ribosomal RNA from *Escherichia coli*. J. Mol. Biol. 213, 275–88.

Elkon, K.B., Skelly, S., Parnassa, A., Möller, W., Danho, W., Weissbach, H., Brot, N., 1986. Identification and chemical synthesis of a ribosomal protein antigenic determinant in systemic lupus erythematosus. Proc. Natl. Acad. Sci. USA 83, 7419–23.

Gómez, M.L., Erijman, L., Arauzo, S., Tones, H.N., Téllez-Iñón, M.T., 1989. Protein kinase C in *Trypanosoma cruzi* epimastigote forms: partial purification and characterization. Mol. Biochem. Parasitol. 36, 101–8.

Hansen, T.S., Andreasen, P.H., Dreisig, H., Hojrup, P., Nielsen, H., Engberg, J., Kristiansen, K., 1991. *Tetrahymena thermophila* acidic ribosomal protein L37 contains an archaebacterial type of C-terminus. Gene 105, 143–50.

Hasler, P., Brot, N., Weisbach, H., Parnassa, A.P., Elkon, K.B., 1991. Ribosomal proteins P0, P1, P2 are phosphorylated by casein kinase II at their conserved carboxil termini. J. Biol. Chem. 266, 13815–20.

Kaplan, D., Ferrari, I., López-Bergami, P., Mahler, E., Levitus, G., Chiale, P., Hoebeke, H., Van Regenmortel, M.H.V., Levin, M.J., 1997. Antibodies to ribosomal P proteins of *Trypanosoma cruzi* in Chagas disease possess functional autoreactivity with heart tissue and differ from anti-P autoantibodies in lupus. Proc. Natl. Acad. Sci. USA 94, 10301–6.

Lavergne, J.P., Conquet, F., Reboud, J.P., Reboud, A.M., 1987. Role of acidic phosphoproteins in the partial reconstitution of the active 60S ribosomal subunit. FEBS Lett. 216, 83–88.

Levin, M.J., Mesri, E., Benarous, R., Levitus, G., Schijman, A., Levy-Yeyati, P., Chiale, P.A., Ruiz, A.M., Kahn, A., Rosembaum, M.B., Tones, H.N., Segura, E.L., 1989. Identification of major *Trypanosoma cruzi* antigenic determinants in chronic Chagas heart disease. Am. J. Trop. Med. Hyg. 41, 530–9.

Levin, M.J., Vazquez, M., Kaplan, D., Schijman, A.G., 1993. The *Trypanosoma cruzi* ribosomal P protein family: classification and antigenicity. Parasitol. Today 9, 381–4.

Levitus, G., Hontebeyrie-Joskowicz, M., Van Regenmortel, M.H.V., Levin, M.J., 1991. Humoral autoimmune response to ribosomal P proteins in chronic Chagas' heart disease. Clin. Exp. Immunol. 85, 413–7.

Levitus, G., Levin, M.J., 1998. Autoinmunidad en la enfermedad de Chagas. Rev. Argent. Med. 1, 12–16.

Liljas, A., 1991. Comparative biochemistry and biophysics of ribosomal proteins. Int. Rev. Cytol. 124, 103–36.

Lin, A., Wittman-Leibold, B., McNelly, T., Wool, I.G., 1982. The primary structure of the acidic phosphoprotein from rat liver 60S ribosomal subunit. J. Biol. Chem. 257, 9189–97.

Mesri, E.A., Levitus, G., Hontebeyrie-Joskowicz, M., Dighiero, G., Van Regenmortel, M.H.V., Levin, M.J., 1990. Major *Trypanosoma cruzi* determinant in Chagas' heart disease shares homology with the systemic lupus erythematosus ribosomal P protein epitope. J. Clin. Microbiol. 28, 1219–24.

Mitsui, K., Tsurugi, K., 1988. cDNA and deduced amino acid sequence of acidic ribosomal A2 from *Saccharomyces cerevisiae*. Nucleic Acids Res. 16, 3575.

Naranda, T., Remacha, M., Ballesta, J.P.G., 1993. The activity-controlling Phosphorylation site is not the same in the four acidic ribosomal proteins from *Saccharomyces cerevisiae*. J. Biol. Chem. 268, 2451–7.

Ogueta, S.B., Solari, A., Téllez-Iñón, M.T., 1994. *Trypanosoma cruzi* epimastigote forms possess a Ca2 + /calmodulin-dependent protein kinase. FEBS Lett. 337, 293–7.

Qian, S., Zhang, J.Y., Kay, M.A., Jacobs-Lorena, M., 1987. Structural analysis of the Drosophila rPA1 gene, a member of the eukaryotic 'A' type ribosomal protein family. Nucleic Acids Res. 15, 987–1003.

Remacha, M., Jimenez-Diaz, A., Bermejo, B., Rodriguez-Gabriel, M.A., Guarinos, E., Ballesta, J.P.G., 1995a. Ribosomal acidic phosphoproteins P1 and P2 are not required for cell viability but regulate the pattern of protein expression in *Saccharomyces cerevisiae*. Mol. Cell. Biol. 15, 4754–62.

Remacha, M., Jimenez-Diaz, A., Santos, C., Briones, E., Zambrano, R., Rodriguez-Gabriel, M.A., Guarinos, E., Ballesta, J.P.G., 1995b. Proteins P1, P2 and P0, components of the eukariotic ribosome stalk. New structural and functional aspects. Biochem. Cell Biol. 73, 959–68.

Rich, B.E., Steitz, J.A., 1987. Human acidic ribosomal phosphoproteins P0, P1 and P2: analysis of cDNA clones, in vitro synthesis, and assembly. Mol. Cell. Biol. 7, 4065–74.

Rodriguez-Gabriel, M.A., Remacha, M., Ballesta, J.P.G., 1998. Phosphorylation of ribosomal protein P0 is not essential for ribosome function but affects translation. Biochemistry 37, 16620–6.

Saenz-Robles, M.T., Remacha, M., Vilella, M.D., Zinker, S., Ballesta, J.P.G., 1990. The acidic ribosomal proteins as regulators of the eukaryotic ribosomal activity. Biochim. Biophys. Acta 1050, 51–55.

Sanchez-Madrid, F., Ballesta, J.P.G., 1979. An acidic protein associated to ribosomes of *Saccharomyces cerevisiae*. Changes during cell cycle. Biochem. Biophys. Res. Commun. 91, 643–50.

Sandermann, J., Kruger, A., Kristiansen, K., 1979. Characterization of acidic 60S ribosomal proteins in *Tetrahymena pyriformis*. FEBS Lett. 107, 343–7.

Santos, C., Ballesta, J.P.G., 1995. The highly conserved protein P0 carboxyl end is essential for ribosome activity only in the absence of proteins P1 and P2. J. Biol. Chem. 270, 20608–14.

Schijman, A.G., Dusetti, N., Vazquez, M.P., Lafon, S., Levy-Yeyati, P., Levin, M.J., 1990. Nucleotide cDNA and complete deduced amino acid sequence of a *Trypanosoma cruzi* acidic ribosomal P protein (P-JL5). Nucleic Acid Res. 18, 3399.

Schijman, A.G., Vazquez, M.P., Ben Dov, C., Ghio, S., Lorenzi, H., Levin, M.J., 1995. Cloning and sequence analysis of the TcP2β cDNA variants of *Trypanosoma cruzi*. Biochim. Biophys. Acta 1264, 15–18.

Shimmin, L.C., Ramirez, G., Matheson, A.T., Dennis, P.P., 1989. Sequence alignment and evolutionary comparison of the L10 equivalent and L12 equivalent ribosomal proteins from archaebacterial, eubacterial and eukaryotes. J. Mol. Evol. 29, 448–62.

Stöffler, G.E, Stöffler-Meilicke, M., 1986. Immunoelectron Microscopy on *Escherichia coli* Ribosomas. In: Hardesty, B., Kramer, G. (Eds.), Structure, Function and Genetics of Ribosomes, Springer-Verlag, New York pp. 28–46.

Szyszka, R., Boguszewska, A., Grankowski, N., Ballesta, J.P.G., 1995. Differential phosphorylation of ribosomal acidic proteins from yeast cell by two endogenous protein kinases; casein kinase-2 and 60S kinase. Acta Bioch. Pol. 42, 357–62.

Szyszka, R., Bou, G., Ballesta, J.P.G., 1996. RAP kinase, a new enzyme phosphorylating the acidic P proteins from *Saccharomyces cerevisiae*. Biochem. Biophys. Acta 1293, 213–21.

Tsurugi, K., Collatz, E., Todokoro, K., Ulbrich, N., Lightfoot, H.Y., Wool, I.G., 1978. Isolation of eukaryotic ribosomal proteins. Purification and characterization of the 60S ribosomal subunit proteins La, Lb, Lf, P1, P2, L13$'$, L14, L20 and L38. J. Biol. Chem. 253, 946–55.

Uchiumi, T., Wahba, A.J., Traut, R.R., 1987. Topography and stoichiometry of acidic proteins in large ribosomal subunits from *Artemia salina* as determined by crosslinking. Proc. Natl. Acad. Sci. USA 84, 5580–4.

Unterkircher, C., Avrameas, S., Ternyck, T., 1993. Autoantibodies in the sera of *Trypanosoma cruzi*-infected individuals with or without clinical Chagas' disease. J. Clin. Lab. Anal. 7, 60–69.

van Agthoven, A., Kriek, J., Amons, R., Möller, W., 1978. Isolation and characterization of the acidic phosphoproteins of 60S ribosomes from *Artemia salina* and rat liver. Eur. J. Biochem. 91, 553–6.

Vard, C., Guillot, D., Bargis, P., Lavergne, J.P., Reboud, J.P., 1997. A specific role for the phosphorylation of mammalian acidic ribosomal protein P2. J. Biol. Chem. 272, 20259–62.

Vidales, F.J., Sáenz-Robles, M.T., Ballesta, J.P.G., 1984. Acidic proteins of the large ribosomal subunit in *Saccharomyces cerevisiae*. Effect of phosphorylation. Biochemistry 23, 390–6.

Vilella, M.D., Remacha, M., Ortiz, B.L., Méndez, E., Ballesta, J.P.G., 1991. Characterization of the yeast acidic ribosomal phosphoproteins using monoclonal antibodies. Proteins L44/45 and L440$'$ have different functional roles. Eur. J. Biochem. 196, 407–14.

Wigboldus, J.D., 1987. DNA and deduced amino acid sequence of *Drosophila* rp21C, another 'A' type ribosomal protein. Nucleic Acids Res. 15, 10064.

Wool, I.G., Chan, Y-L., Glück, A., 1995. Structure and evolution of mammalian ribosomal proteins. Biochem. Cell Biol. 73, 933–47.

Zambrano, R., Briones, E., Remacha, M., Ballesta, J.P.G., 1997. Phosphorylation of the acidic ribosomal P proteins in *Saccharomyces cerevisiae*: a reappraisal. Biochemistry 36, 14439–46.