A DnaJ-like Protein Homologous to the Yeast Co-chaperone Sis1 (TcJ6p) Is Involved in Initiation of Translation in *Trypanosoma cruzi*

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In eukaryotes, proteins homologous to the bacterial DnaJ protein are involved in regulation of the Hsp70 molecular chaperones, which are implicated in a variety of protein biogenesis pathways. We report here the molecular characterization of a *T. cruzi* DnaJ gene, termed *TcJ6*, encoding a protein that displays high sequence homology with the *Saccharomyces cerevisiae* Sis1 co-chaperone required for the initiation of translation. *TcJ6* protein was expressed as a polypeptide of 36.5 kDa at a constant level during parasite differentiation and was associated to the cytoplasmic fraction. We showed that overexpression of *TcJ6* complemented a temperature-sensitive yeast *sis1* mutant. In addition, a sucrose gradient sedimentation analysis of polysomes from *T. cruzi* and a yeast mutant overexpressing *TcJ6* showed that the trypanosomal co-chaperone was closely associated with ribosomal subunits, 80 S monosomes and the smaller polysomes, as observed for Sis1p. Furthermore, in *T. cruzi* *TcJ6* was also found to be preferentially concentrated around the nucleus, giving a speckled staining pattern. This suggests that *TcJ6* is associated with the endoplasmic reticulum. Taken together, these data suggest that the trypanosomal DnaJ is involved in initiation of translation.

In *Escherichia coli* the DnaK, DnaJ, and GrpE genes encode heat shock proteins that are essential for growth at temperatures above 42 °C (reviewed in Ref. 1). DnaJ stimulates the weak intrinsic ATPase activity of DnaK (2) and can directly interact with specific substrates of the chaperone machinery (3). Various DnaJ homologs have been discovered in eukaryotic cells. Eukaryotic DnaJ-like proteins belong to the conserved heat shock protein 40 (Hsp40) family and are in direct interaction with specific substrates of the chaperone machinery (3). Various DnaJ homologs have been discovered in eukaryotic cells. Eukaryotic DnaJ-like proteins belong to the conserved heat shock protein 40 (Hsp40) family and are involved in regulation of the Hsp70 molecular chaperones (DnaK-like proteins), mediating the biogenesis of proteins. Some Hsp40s may be considered to be true molecular chaperones in that they prevent aggregation by binding directly to unfolded polypeptide substrates (4, 5). All DnaJ-like proteins contain a J domain of about 70 amino acids, generally N-terminal, which represents the signature of the family (6) and is required for interaction with the ATPase domain of Hsp70 (7). The basic mechanism of action of the Hsp70 proteins is sequential binding and release in an ATP-dependent manner of polypeptides in non-native conformations (protein folding). The subcellular compartmentalization of different Hsp70 members and their specific interactions with various DnaJ-like proteins allows these chaperones to be involved in a variety of protein biogenesis pathways including the assembly and disassembly of protein complexes, proteolysis, the translocation of proteins into organelles and translation initiation (reviewed in Ref. 8). The *Saccharomyces cerevisiae* genome data base (genome-www.stanford.edu/Saccharomyces) contains 20 putative Hsp40 homologous to *E. coli* DnaJ, but only half have been characterized and localized to major cellular compartments (4, 8, 9). The Hsp40 family has been divided into three distinct subgroups based on the presence of conserved functional domains in addition to the J domain (7, 9). Class I Hsp40s have a glycine-phenylalanine-rich (G-F) region followed by a cysteine-rich region (CRR domain), which forms a zinc finger motif with four repeats of a CXXCXXGX motif and a weakly conserved C-terminal domain. The archetypal *E. coli* DnaJ and the *S. cerevisiae* Ydj1 protein are class I Hsp40s. Class II Hsp40s include the *S. cerevisiae* Sis1 protein and its mammalian homolog, the co-chaperone Hdj1, and lack the CRR domain. Finally, in class III Hsp40s, only the conserved J domain is present. The J domain is thought to be involved in interactions with Hsp70s via its HPD tripeptide loop (10, 11), whereas the G-F region is a critical determinant required for the specificity of *S. cerevisiae* Sis1p (9). In class I Hsp40 proteins, the zinc finger motif and the poorly conserved C-terminal domain have been shown to act as binding sites for proteins in denatured state (12, 13). It has also been suggested that the J domain and G-F region may be sufficient for the basic functions of class I and class II Hsp40s in vivo, whereas the distal C-terminal regions are important in yeast in suboptimal growth conditions (9).

The unicellular protozoan, *Trypanosoma cruzi*, the etiological agent of Chagas’ disease, has a complex life cycle where the parasite passes through three differentiation forms and two hosts, a reduvid insect vector (triatomine) and a mammalian

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* The abbreviations used are: Hsp, heat shock protein; kb, kilobase(s); RT-PCR, reverse transcriptase polymerase chain reaction; ORF, open reading frame; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; YEP, yeast episomal plasmid; YCP, yeast centromeric plasmid.

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host (14). During this cycle, the parasite encounters drastic environmental changes (e.g., in temperature and pH), and these changes are accompanied by major morphological changes. The epimastigotes (insect forms) replicate in the insect host midgut and differentiate to produce metacyclic trypomastigotes (by a process known as metacyclogenesis). These metacyclic trypomastigotes are non-proliferative and infectious forms released in the excreta of the bug. Although the insect feeds on the blood of the mammalian host, metacyclic trypomastigotes invade the bloodstream of the host and infect various cell types (mainly macrophages and muscle cells) in which the trypomastigotes differentiate to produce amastigotes (proliferative forms). Metacyclogenesis can be simulated in vitro using a chemically defined differentiation medium (15).

In T. cruzi, 5 members of the DnaJ-like family (TcDnaJ) have been partially characterized (16, 17) and shown to have a highly conserved N-terminal J domain. The proteins tcj2, tcj3, and tcj4 belong to Hsp40s class I, whereas tcj1 and TcDJ1 belong to class III. Except for the putative mitochondrial DnaJ-like protein, TcDJ1, the function and subcellular location of the other family members are still unknown. A highly conserved gene encoding a novel TcDnaJ was recently sequenced in five species of trypanosomatids (Trypanosoma b. brucei, Trypanosoma congolense, Trypanosoma vivax, T. cruzi, and Leishmania donovani) in a downstream region flanking the glucose transporter gene cluster (18). In T. cruzi, glucose transporters (TcrHT1) are encoded by a multigene family that is organized into several clusters, all contained within a genomic fragment no larger than 150 kb (18, 19). In the T. cruzi CL strain, two clusters of TcrHT1 genes, separated by several dozen of kilobases, flank several duplicates of a 20-kb region composed of genes encoding an adenylate cyclase homologous to the T. b. brucei pESAG4 (20), a DnaJ protein homologous to human DNAJ1, and two small G-proteins homologous to human Rab1 and yeast YPT7, which belong to the Rab family. This kind of repetition in tandem of large stretches of DNA is characteristic of intrachromosomal amplifications, which are frequently observed in T. cruzi. Run-on experiments showed that in T. b. brucei the battery of genes flanking the THT (trypanosome hexose transporter) gene cluster are transcribed by an RNA polymerase II on a distance of at least 50 kb, suggesting that these genes belong to the same transcription unit (18). Similar large transcription units have been described in T. brucei that contained various genes grouped in an active expression site where they are co-transcribed by a polymerase I-like RNA polymerase (21). This is the case of the variant surface glycoprotein (VSG) genes and a number of co-expressed linked genes, which may be involved in optimization of parasite adaptation to different environmental conditions (22), suggesting that the parasite contains functional units similar to prokaryotic operons (23).

In this study, we mapped a DNA fragment of 14.3 kb homologous to the downstream region of the glucose transporter gene cluster isolated from a T. cruzi genomic library. This gene unit differs from the repeat region of 20 kb previously described in the TcrHT1 locus by the presence of a pseudogene of the large trans-sialidase multigene family (reviewed in Ref. 24). To determine if these genes form a functional unit similar to the VSG polycistronic unit in T. brucei bloodstream forms, we decided to investigate the function of the novel TcDnaJ-encoded protein, termed TcJ6p, on the differentiation process in T. cruzi. Surprisingly, the trypanosomal co-chaperone displayed a high level of similarity to various eukaryotic class II Hsp40s (Caenorhabditis elegans protein homologous DnaJ, human DNAJ1 protein, Sis1p), whereas only 21–40% of sequence conservation was found with the other five members of the T. cruzi DnaJ-like family. The yeast Sis1p is the only class II Hsp40 protein that has been functionally characterized. This protein is required for the initiation of translation in S. cerevisiae (25). These observations suggested that the Sis1 and TcJ6 proteins might have similar functions. In this study, we used heterologous functional complementation and polysome sucrose gradient sedimentation to demonstrate that TcJ6p has a function similar to that of the Sis1 co-chaperone, which is essential for translation initiation in yeast.

**EXPERIMENTAL PROCEDURES**

**Growth and Differentiation of Parasites and Isolation of Nucleic Acids**

_T. cruzi_ clone Dm28c (26) epimastigotes were cultured in liver infusion tryptose medium (27). In vitro metacyclogenesis of _T. cruzi_ (Dm28c) was performed under chemically defined conditions as previously described (15). Recovering of parasites at different stages of in vitro differentiation was carried out as previously described (28). Total parasite DNA was extracted as described elsewhere (29). Total parasite RNA was prepared after the LiCl-urea method as previously described (30).

**Strains**

E. coli strains XL1-Blue and TOP10F were used for plasmid construction. _S. cerevisiae_ strain FY833 (MATa, his3Δ1 110, ura3-52, leu2Δ21, lys2Δ202, trp1Δ63, GAL12+). Complementation of Sis1 function by TcJ6 protein was carried out on the control strain CY736 expressing wild type Sis1p and on the temperature-sensitive strain CY732, carrying a sis1-85 allele. The genotypes of the strains are CY736 (MATa, ura3-1, leu2-3, 112, his3-11, 15, trp1-1 ade-2 1 ssd1-d2 can1-100 Δsis1::His3 (SIS1 on LEU2/CEN plasmid)) and CY732 (MATa, ura3-1, leu2-3, 112, his3-11, 15, trp1-1 ade-2 1 ssd1-d2 can1-100 Δsis1::His3 (NH2-hemagglutinin-tagged sis1-85 on LEU2/CEN plasmid)). These strains were kindly donated by Dr. Kim T. Arndt (31).

**Media**

Rich media contained 1% yeast extract, 2% bacto-peptone, and 2% glucose or 2% galactose. Synthetic media contained 0.67% yeast nitrogen base without amino acids, 2% glucose, or 2% galactose and the supplements to satisfy auxotrophic requirements.

**Gene Cloning and Genomic Mapping**

A densely arrayed _T. cruzi_ Dm28c AEMBL3 Sau3A genomic library (29) was screened at high stringency with a probe corresponding to the 5′ conserved region of a gene related to the sialidase family. The cDNA probe was obtained by reverse transcriptase polymerase chain reaction (RT-PCR). A reverse oligonucleotide, 5′-CCCTAAAGCAATCTG-CAGC-3′, corresponding to the 5′-conserved region of a sialidase-like gene was used as a primer using the Superscript II enzyme (Life Technologies, Inc.) as indicated by the manufacturer, on total RNA from the parasites pretreated by RQ1 RNase-free DNase (Promega). The first-strand cDNA was subsequently amplified by PCR according to the manufacturer’s instructions (Life Technologies) using a forward miniexon oligonucleotide BomHII (5′-CGGGCGGTATCACAGTTCTGTAC-TATTTG-3′ (BomHII site is underlined) and the reverse oligonucleotide with the following cycle parameters: 94 °C, 30 s; 52 °C, 30 s, 72 °C, 1 min for 3 cycles and 94 °C, 30 s; 55 °C, 30 s; 72 °C, 1 min for 30 cycles. The purified PCR product was cloned and sequenced (GenBank database accession number AY017371). All PCR reactions were performed on a PerkinElmer DNA Thermal Cycler (GenAmp PCR System 9600), and PCR products were separated in an agarose gel, purified on glass beads (Gene-clean, Bio 101) or directly purified on QIAquick column (Qiagen, Inc.), and subcloned into PCR2.1 or pCR-Blunt vectors (Invitrogen). Sequencing was performed manually using the Thermosequenase sequencing kit (Amersham Pharmacia Biotech) with [α-32P]dNTPs (1500 Ci/mmol) as terminators, according to the manufacturer’s instructions. Sequences were determined by sequencing on both strands of DNA. From 200,000 recombinant phages hybridized with the cDNA probe, 14 phages were isolated after three consecutive screenings. Analysis of DNA inserts of each phage were characterized with the cDNA probe, 14 phages were isolated after three consecutive screenings. Analysis of DNA inserts of each phage were characterized with the cDNA probe, 14 phages were isolated after three consecutive screenings. Analysis of DNA inserts of each phage were characterized with the cDNA probe, 14 phages were isolated after three consecutive screenings. Analysis of DNA inserts of each phage were characterized with the cDNA probe, 14 phages were isolated after three consecutive screenings. Analysis of DNA inserts of each phage were characterized with the cDNA probe, 14 phages were isolated after three consecutive screenings. Analysis of DNA inserts of each phage were characterized with the cDNA probe, 14 phages were isolated after three consecutive screenings. Analysis of DNA inserts of each phage were characterized with the cDNA probe, 14 phages were isolated after three consecutive screenings.
c33t81g in bacteriophage M13 (mp18 and mp19) or in plasmid pBluescript Sk+ (Stratagene, La Jolla, CA) to be sequenced. The GenBank™ data base accession number of a 4.3-kb SalI-c3263g genomic subclone containing the sialidase pseudogene, TcJ6, and 3′-coding region of Rod1 homolog is AF245536. The relative position of DNA fragments was confirmed by sequencing subclones 4.3-kb c3263g genomic DNA was confirmed by sequencing extremities of each cloned fragment by PCR. The complete genetic map of c3263g and the relative positions of adenylate cyclase and small G-protein YPT7 homolog genes were determined with probes derived from the corresponding genes contained in the CosTer1 cosmid of the CL strain, kindly donated by Dr. F. Bringaud and T. Baltz (18). The authenticity of these subclones and transfections was confirmed by sequencing the 5′- and 3′-flanking regions of the 4.3-kb SalI fragment of c3263g. The 3′ splice site of TcJ6 transcript was determined by RT-PCR using the forward mini-exon oligonucleotide BamHI and a reverse oligonucleotide specific to the 3′ transcript was determined by RT-PCR using a forward oligonucleotide (Instituto Oswaldo Cruz, Departamento de Bioquímica) and fixed through a Seph- adeg G-50 column. A small subunit ribosomal RNA probe used as a loading control in Southern analysis was obtained using the forward oligonucleotide, 5′-CCGATGATCCTTTTTTTTGACATTGG-3′, and the reverse oligonucleotide, 5′-CCTCTATTTTTTTTTTTCATGTTG–3′, as primers for RT-PCR on 5 μg of T. cruzi total RNA (33). The conditions for RT-PCR reaction were identical, as described above but using the following cycle parameters: 94 °C, 1 min; 55 °C, 1 min; 72 °C, 2 min, 30 cycles. The purified PCR fragment of 168 base pairs was cloned and used as a template for a HindIII and BamHI restriction sites immediately upstream of TcJ6 ATG, 5′-GGAAAGCTTGGATCC- GTTGAACATCTG-3′ (the HindIII and BamHI sites are underlined), and a reverse primer with a SacI restriction site at position +11 of TcJ6 stop codon, 5′-CAAGCTTGTTTAAACAACAT- AACTG-3′ (SacI site and stop codon are underlined). 1-kb HindIII– SacI and BamHI/SacI DNA fragments were subcloned, respectively, into HindIII–SacI-digested pYES2 and BamHI/SacI-digested pRN93 vectors. Yeast were transformed with the resulting plasmids by the lithium acetate method (38). Total yeast RNA was isolated as described elsewhere (39).

**Pulsed-field Gel Electrophoresis**

Chromosome separation (2 × 10⁶ parasites/slot) was performed using the Pulsaphor system (Amersham Pharmacia Biotech) with 1.2% agarose gel (37). Total yeast RNA was isolated as described (33). The conditions for total RNA (33). The conditions for RT-PCR on 5 μg of T. cruzi total RNA (33). The conditions for RT-PCR reaction were identical, as described above but using the following cycle parameters: 94 °C, 1 min; 55 °C, 1 min; 72 °C, 2 min, 30 cycles. The purified PCR fragment of 168 base pairs was cloned and sequenced. The 5′-tubulin probe was obtained from an incomplete cDNA clone of 1.3 kb sub-cloned in M13, kindly provided by Dr. Mônica Carreiro (Instituto Oswaldo Cruz, Departamento de Bioquímica e Biologia Molecular). Sequence analysis was performed using the GCG/Wisconsin software (34).
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**Fig. 1. Southern analysis of the TcJ6 gene.** Panel A, restriction map and genomic organization of the genes flanking the gene TcJ6 in a 14.3-kb SalI fragment cloned from a genomic Sau3A λEMBL3 T. cruzi library (cl263g). B, BamHI; E, EcoRI; HII, HindIII; HII, HincII; H, HaeIII; P, PstI; Sac, SacII; Sal, SalI; S, Smal. Panel B, TcJ6 gene organization. T. cruzi (Dm28c) genomic DNA was digested with HaellI (I), HaeIII (II), HincII (III), HindIII (HII), PstI (P), SacI (S), Stul (Stu), Stul + HaeIII (Stu + Hae), Stul + HincII (Stu + Hinc), and XbaI (Xba) and analyzed by Southern blot hybridization with the TcJ6 ORF as genomic probe (probe A), indicated by a solid bar under the map in panel A. Panel C, chromosomal location of the TcJ6 gene and sialidase pseudogene (Δ-sialidase) in three different T. cruzi strains. T. cruzi chromosomes from the strain CL, Y, and Dm28c (Dm) were separated by pulsed-field gel electrophoresis and analyzed by Southern blot hybridization with a sialidase pseudogene cDNA probe (SacI-Smal, 0.94 kb, probe C) and a TcJ6 genomic probe (Stu1-HincII, 0.89 kb, probe B), represented by a solid bar under the map in panel A. S. cerevisiae chromosomes were included as size standards (molecular mass in megabases).

|   | TcJ1 | tcl2 | tcl3 | tcl4 | TcDJ1 | TcJ6 |
|---|------|------|------|------|-------|------|
| tcj1 | 29.2 | 31.4 | 33.4 | 22.5 | 29.8  |
| tcj2 | 29.2 | 56.6 | 43.7 | 21.3 | 39.6  |
| tcj3 | 31.4 | 56.6 | 45.7 | 35.2 | 21.2  |
| tcj4 | 33.3 | 43.7 | 45.7 | 25.3 | 35.2  |
| TcDJ1 | 22.5 | 24.1 | 21.3 | 25.3 | 21.2  |
| TcJ6 | 29.6 | 36.5 | 39.6 | 35.2 | 21.2  |

**Table I.** Percentage of amino acid sequence similarity among DnaJ-like proteins from T. cruzi

The highest DnaJ pair homologies are in boldface (percentage similarity ≥40%).

A region of a gene related to the sialidase family (24) was used as a probe to screen a phage genomic library of T. cruzi. Two types of recombinant phage were found based on the SalI digestion pattern and contained 14.3- and 19.8-kb inserts; two of them designed, respectively, cl263g and cl381g, were selected for further analysis. The genomic clone cl263g was partially sequenced (Fig. 1A). Analysis of the complete nucleotide sequence of the sialidase-like gene, with sequence similarity extending over about 1 kb (boxed in Fig. 1A), revealed that this gene was interrupted by multiple stop codons in each frame and contained a small ORF (196 nucleotides). This ORF encodes a putative 66-amino acid polypeptide presenting similarities to other members of the gp85/sialidase family. However, the very small size of the polypeptide, the absence of the aspartate consensus essential for sialidase enzymatic activity, and the presence of stop codons suggest that this sialidase-like gene is probably a pseudogene, as already described for other members of the gp85/sialidase family (42). The nucleotide sequence of the 3'-flanking region of the sialidase pseudogene revealed the presence of two other ORFs encoding homologs of DnaJ, termed TcJ6 and Rab1. The nucleotide sequences of these genes were almost identical (99%) to that of the downstream flanking region of the TcrHT1 gene cluster, recently described by Bringaud et al. (18). A more detailed analysis of the flanking sequences identified other conserved copies present at the TcrHT1 locus, an ORF encoding an adenylate cyclase localized 5' to the sialidase pseudogene and an ORF localized 3' to the Rab1 gene and encoding another small G-protein homologous to yeast YPT7 (Fig. 1A). Thus, the 14.3-kb genomic fragment is highly homologous to the duplicated region flanking the glucose transporter gene cluster and may therefore be an alternative genomic rearrangement of the downstream-flanking region of the THT gene cluster in T. cruzi. The nucleotide sequence of the TcJ6 gene predicts an open reading frame of 1014 base pairs encoding a putative protein of 338 amino acids with a molecular mass of 36.5 kDa. The 3' splice site and the poly(A) addition site were determined by RT-PCR using a 5' reverse internal primer with a mini-exon-derived primer and a 3' forward internal primer with a oligo-dT primer, respectively. This analysis predicted a small 5'-untranslated region (UTR) of 20 nucleotides and a 3'-UTR of 191 nucleotides. Southern blotting with a restriction enzyme cutting outside the gene (PstI (P) in Fig. 1B) was used to determine the number of copies of the TcJ6 gene in the genome. Several copies were detected using a...
**Fig. 2. Amino acid sequence homology between TcJ6 and yeast Sis1 proteins.** The sequence alignment was performed using the GAP program of GCG (34). The bars indicate identity, and double and single dots refer to frequent and infrequent conservative substitutions respectively. Gaps have been introduced to maximize the alignment and are represented by a dotted line. The conserved J and G-F domains, and the glycine/methionine-rich sequence are indicated by solid bars above the sequence alignment. The highly conserved HDP motif is boxed, and the glycine and phenylalanine residues of the G-F region and glycine residues of the G-M region are in bold.

|    | J’ domain |
|----|-----------|
| Sis1p | XVKTDELLGYGPKRANQAEKLQGYKALKHERPGPTSDLQ...KFK | G-F region |
| TcJ6p | MGHDYKVLGVRKNAIPKD...KKYAHRRCYKHFQKTNCRSSLRPP | Gly/Met rich sequence |

probe spanning the entire ORF. We investigated whether these members of the new DnaJ-like subfamily of genes were, like other *T. cruzi* genes encoding Hsps, organized as direct tandem repeats by digestion with two restriction enzymes, one with a single site in the 5’ extremity of the ORF (StuI) and one with a single site in the 3’ extremity of the ORF (HincII). Low stringency hybridization of *T. cruzi* genomic DNA digested with StuI alone or with HincII alone to a probe covering the entire ORF gave no hybridizing fragment common to both digests. This suggests that the various copies of the gene encoding the TcJ6 protein are not organized in tandem repeats but are instead spread throughout the genome of *T. cruzi*. Southern blot hybridization of a pulse-field gel of three strains of *T. cruzi* showed that the gene copies encoding TcJ6p are located on a chromosome or a pair of homologous chromosomes of ~1,300 kb (Fig. 1C). In addition, probes for the sialidase pseudogene (Fig. 1C), Rab1, and TcrHT1 (data not shown), labeled the same set of chromosomes. These results suggest that, despite the difference in chromosome size between the various strains of *T. cruzi*, the TcJ6 gene copies are present on a subset of chromosomes of the megabase range that is conserved between various strains of *T. cruzi*. Altogether these data are consistent with the study by Bringaud et al. (18), showing the existence of various duplications of a large area characteristic of the 3’-end of the TcrHT1 gene cluster.

**TcJ6 Co-chaperone Homologous to Yeast Sis1p—**Data base searches showed similarity between TcJ6 and *E. coli* DnaJ proteins (60% similarity for the first 76 amino acids, which define the J domain), and only 21–40% sequence similarity to the other five members of the *T. cruzi* family (tcj1-4 and TcDJ1) (Table I) (16, 17). TcJ6p was most similar (~40% similarity) to the putative cytosolic DnaJ protein tcj3. Similar characteristics can be observed for tcj1 and the putative mitochondrial TcDJ1, two class III Hsp40 members from the *T. cruzi* family lacking the GF domain and the zinc finger region. Surprisingly, the TcJ6 protein displays its highest score for sequence similarity with two eukaryotic class II Hsp40s, 54% similarity to a *C. elegans* hypothetical DnaJ-like protein (F54D5.8) and 50% similarity to the yeast Sis1p protein, which is required for normal initiation of translation in *S. cerevisiae* (Fig. 2) (25, 31). Particularly well conserved were the N-terminal sequence containing the highly conserved 70-amino acid J domain with its HDP tripeptide motif (boxed in Fig. 2) and the glycine-phenylalanine-rich “spacer” region of about 50 amino acids (G-F region), which is not followed by the zinc finger motif and is therefore a typical class II Hsp40. In the middle third of the sequence, Sis1p contains a striking glycine/methionine-rich sequence (G-M region), which in the case of TcJ6p, is replaced by a glycine-rich sequence (with a conservation of almost all the glycine residues of Sis1p). In a previous study (9), chimeric fusion proteins in which the various functional domains (J, G-F, G-M, C terminus) had been exchanged between the two yeast cytosolic proteins Sis1p and Ydj1p were used in experiments to rescue the Δsis1 yeast mutant. The G-F region was found to be essential for the function of Sis1p and redundant to the function of the G-M and C-terminal regions. These structural features suggest that the trypanosomal DnaJ may have a function similar to that of yeast Sis1p. In addition, analysis of the predicted amino acid sequence of TcJ6p suggests that, like Sis1p, the trypanosomal co-chaperone is cytosolic. Indeed, its hydrophilic character and the absence of both a detectable N-terminal putative peptide signal and a C-terminal CAAX motif (substrate for prenylation) of some DnaJ-like proteins, which allows association with cell membranes) suggest that this protein is probably cytosolic.

**TcJ6 Is Constitutively Expressed during the Parasite Development—**The expression pattern of TcJ6 was determined during the *in vitro* development of *T. cruzi* by both Northern blot analysis and immunoblotting (Fig. 3). Total RNA was extracted at various stages of the *in vitro* differentiation of *T. cruzi* epimastigotes and analyzed with probes for the TcJ6 gene and the nuclear gene encoding the small subunit ribosomal RNA, as a loading control for constitutively expressed genes (33). Analysis of the relative intensities of the bands corre-
responding to the TcJ6 transcript and small subunit ribosomal RNA (ssrRNA) showed that the amount of the 1.4-kb mRNA increased during in vitro metacyclogenesis, reaching a maximum in metacyclic forms (Met in Fig. 3A and arrow). Similar results were obtained by semi-quantitative RT-PCR (data not shown). A larger RNA species (the asterisk in Fig. 3A) was also detected (sensitive to RNaseA-DNase-free, data not shown), reaching a maximum 24 h after the start of the differentiation.

Fig. 3. Analysis of the expression pattern of TcJ6. T. cruzi epimastigotes exponentially growing in complete medium (Epi) were subjected to a nutritional stress for 1 h (Str) and then allowed to differentiate into infectious metacyclic cells (Met). 6 and 24 h after the start of in vitro differentiation (6H, 24H), most of the parasites had adhered to the plastic culture flask, and the process was complete within 96 h (28). Panel A, regulated expression of TcJ6 mRNA during metacyclogenesis in vitro and during a heat shock. 10 μg of total RNA from each differentiation stage of T. cruzi were analyzed by Northern blotting with a TcJ6 genomic probe (StuI-HinII, 0.89 kb, probe B in Fig. 1, panel A) or a nuclear small subunit ribosomal RNA (ssrRNA) cDNA fragment. A similar Northern blot analysis was carried out using 10 μg of total RNA from epimastigotes subjected to a heat shock at 37 °C for 6 and 24 h (6H and 24 H). The arrow indicates the TcJ6 mRNA, and the asterisk indicates the putative polycistronic transcript of high molecular mass. Panel B, Western blot analysis of the TcJ6 protein expression during metacyclogenesis in vitro and heat shock at 37 °C and comparison with the recombinant expression of TcJ6ΔJp in E. coli (ΔJ represents the difference of molecular mass due to the J domain between the truncated recombinant protein and the trypanosomal DnaJ). Total protein was extracted from T. cruzi during metacyclogenesis in vitro (5 × 10^6 cells/lane), separated by SDS-PAGE in a 13% acrylamide gel, electroblotted, and probed with an anti-TcJ6ΔJp rabbit polyclonal antibody (anti-TcJ6p) or a preimmune serum (PI). Panel C, cross-reactivity of the anti-TcJ6ΔJp antibody with other species. Cell extracts from L. major promastigotes (Lm, 10^7 cells, CC1 strain), T. b. brucei bloodstream forms (Tbb, 10^7 cells, AnTat1.1 clone). T. cruzi epimastigotes (Tc, 10^7 cells, Dm28C clone), and cytoplasmic (Cyto) and membrane (Mb) fractions of T. cruzi epimastigotes (equivalent to 5 × 10^6 cells) were separated by SDS-PAGE in a 13% acrylamide gel and immunoblotted with anti-TcJ6ΔJp antibody (anti-TcJ6p) or preimmune serum (PI).

Fig. 4. Analysis of TcJ6 expression in transformed S. cerevisiae. Panel A, Northern blot analysis was carried out with 10 μg of total RNA from CY732-YEPTcJ6 grown in the presence of glucose or galactose. Hybridization was carried out with a probe derived from the TcJ6 gene (probe A, Fig. 1). Panel B, immunoblot of protein extracts from CY732-YEPTcJ6 grown in the presence of glucose or galactose and from T. cruzi (Tc) epimastigotes (5 × 10^6 cells), probed with an anti-TcJ6ΔJp antibody depleted on yeast lysate.
DNA probes corresponding to the flanking genes (adenylate cyclase, Rab1) recognized apart from their individual mRNAs the same high molecular weight transcripts, hence suggesting that this RNA is polycistronic (data not shown). The unusual detection of polygenic transcripts might result from the inhibition of processing of primary transcripts due to the stress treatment (acidic and nutritional) required for in vitro differentiation of *T. cruzi*, as already described for the tubulin unit of heat-shocked *T. brucei* cells in which were accumulated high molecular weight mRNA precursors containing both the α- and β-tubulin-coding region (43). Epimastigotes subjected to a heat shock at 37 °C for 6 h (6F in Fig. 3A) accumulated about 2–3 times more mRNA than did untreated epimastigotes (28 °C). This probably reflects an increase in stability of the co-chaperone mRNA during heat shock, as reported for other trypanosomal Hsps (44).

To analyze protein expression during parasite differentiation, polyclonal rabbit antibodies were raised against a recombinant His-tagged TcJ6p lacking its J domain (ΔJ in Fig. 3B) to prevent the cross-reactivity with other members of the TcDnaj family. In Western blot analysis of T. cruzi cell extracts, this antisera recognized a polypeptide with an apparent molecular mass of 36.5 kDa, which corresponds to the molecular mass predicted from analysis of the primary polypeptide sequence (Fig. 3B). This suggests that this protein is unmodified (not prenylated nor glycosylated). No major changes in protein level were observed during differentiation in vitro, and only a modest reduction was observed in metacyclic forms. Thus, the increase in abundance of steady-state RNA observed in metacyclic cells was not concomitant with an increase in the amount of protein. Heat shock at 37 °C for 6–24 h increased the amount of TcJ6p protein by about 2-fold, possibly due to a delay in translation of the mRNA that accumulated during the first 6 h of heat shock.

The antiseraum detected a protein of similar electrophoretic mobility in other trypanosomatid species (*T. b. brucei*, Leishmania major) (Fig. 3C). The increase in molecular weight as observed in L. donovani against 338 and 336 amino acids, respectively, in *T. cruzi* and *T. b. brucei*, (18). Therefore, the cross-reactivity of the anti-TcJ6ΔJp antiseraum with the Dnaj of other trypanosomatids species (*T. b. brucei*, L. major) corroborates a high epitope conservation of the Dnaj-like protein among these species, which share with TcJ6p more than 81% sequence similarity (18). In contrast, in yeast, although some short stretches of peptidic sequence are found conserved between Sis1p and TcJ6p (Fig. 2), the anti-TcJ6ΔJp antiseraum did not react with the protein immunoprecipitated Sis1p (data not shown). Cytoplasmic and membrane fractions of T. cruzi epimastigotes were probed with antisera against TcJ6p, and it was found that the protein was associated exclusively to the cytosolic fraction (Cyt in Fig. 3C). Taken together these results demonstrate that the highly conserved trypanosomal cytosolic co-chaperone is constitutively expressed during metacyclogenesis in vitro.

TcJ6 Suppresses the Temperature-sensitive Phenotype of a SIS1 Mutant (sis1-85)—In yeast, SIS1 is essential for viability and encodes a Dnaj homolog required for normal initiation of translation. To determine whether overexpression of the trypanosomal Dnaj could substitute for Sis1 function, we used a yeast strain in which the SIS1 chromosomal copy has been deleted and contains a plasmid with a temperature-sensitive mutation, sis1-85 (which results in the absence of 22 amino acid residues (255–276) of 352 total residues of Sis1p). This strain is temperature-sensitive for growth (31). We transformed a CY732 strain containing the sis1-85 temperature-sensitive mutation with a high copy vector (pYES2 termed YEP for yeast episomal plasmid) or a CEN vector (pRN93 termed YCP for yeast centromeric plasmid) containing the TcJ6p gene under the control of a GAL promoter, which allows the induction of the heterologous protein in the presence of galactose but not of glucose. As control we used a CY736 strain in which the SIS1 chromosomal copy was been deleted and contained the wild-type SIS1 on a centromeric plasmid. We checked that the trypanosomal Dnaj was synthesized correctly in yeast by Northern blot analysis of total RNA from the CY732-YEPtCJ6-transformed strain grown in the presence of glucose or galactose. As shown in Fig. 4A, the expression of the TcJ6 mRNA was induced by galactose and tightly repressed in its absence. Immunoblotting detected TcJ6p in the presence of galactose.
A. T. cruzi DnaJ-like Protein Involved in Initiation of Translation

but not in the presence of glucose (Fig. 4B). The fact that heterologous protein migrated in SDS-PAGE with an apparent molecular mass similar to the endogenous T. cruzi DnaJ (TcDnaj in Fig. 4B) suggested that TcDnaj protein is properly expressed in S. cerevisiae. We have therefore investigated whether the expression of TcDnaj could abolish the temperature-sensitive phenotype of the sis1-85 mutant (CY732 strain). As shown in Fig. 5A, at the permissive temperature (30 °C) in the presence of galactose, the growth phenotype was the same for all strains, except for the sis1-85 strain transformed with YEPTcDnaj, which grew more slowly (small colonies). At the nonpermissive temperature (39 °C, Fig. 5B) in the presence of galactose, sis1-85 cells did not grow, but when they expressed TcDnaj, they displayed the same slow-growth phenotype as the control strain expressing the wild-type SIS1. Thus, the heterologous expression of the trypanosomal DnaJ gene complemented the sis1-85 strain at nonpermissive temperatures. The accentuated slow-growth phenotype of the mutant strain overproducing the TcDnaj protein (CY732-YEPTcDnaj) grown in galactose reached a plateau at a level about half that of the wild-type strain (CY736), expressing SIS1 or the sis1-85 mutant (CY732-YEP) transformed with the insert-less vector (Fig. 5C). This confirms that overexpression of TcDnaj protein limits yeast growth. As expected, at the nonpermissive temperature, sis1-85 mutant transformed with the insert-less vector did not grow (the asterisk in Fig. 5C). The growth of CY732 strain transformed with a low copy number plasmid carrying TcDnaj (CY732-YCPTcDnaj in Fig. 5D) was absolutely normal at 30 °C and similar to that of strain CY732 transformed with the insert-less plasmid (comparable with the wild-type, CY736, in Fig. 5C). At a restrictive temperature, CY732-YCPTcDnaj grew slowly. The adaptation period (lag phase) was longer in these cells expressing low levels of TcDnaj than in cells overexpressing the co-chaperone from a multicopy plasmid. This suggests that complementation depends on the number of copies of TcDnaj, with excessive expression limiting growth.

Close association of TcDnaj with ribosomal subunits, 80 S monosomes, and polysomes—In S. cerevisiae, a large fraction of Sis1p is associated with 40 S ribosomal subunits, 80 S initiation complexes, and smaller polysomes. At nonpermissive temperatures, the sis1-85 mutant rapidly accumulates high levels of 80 S ribosomes, and the amount of polysomes decreases. These data indicate that the Sis1 co-chaperone is required for the normal initiation of translation (25). The complementation by TcDnaj of a SIS1 mutant suggests that the trypanosomal protein may also be associated with ribosomes. We investigated this possibility by sedimentation on a sucrose density gradient of cytoplasmic extracts of yeast cells overproducing the heterologous protein. Western blot analysis showed that, according to our fractionation procedure, the cytoplasmic trypanosomal protein sedimented throughout most of the gradient, including positions corresponding to ribosomal subunits, 80 S monosomes, and polysomes (Fig. 6A). However, a great proportion of the protein remained at the top of the sucrose gradient (T in Fig. 6A), which contains free cytosolic proteins. This may be due either to the presence of a large excess of overexpressed protein competing for binding factors required by the translation machinery (e.g. certain mammalian-like Hsp70 proteins containing RNA-binding sites that might be involved in the regulation of translation (45)) or to the trypanosomal co-chaperone having other physiological functions in the cytosol, such as a role in the regulation of protein degradation catalyzed by proteases through its chaperone activity, as has been demonstrated for Sis1p (46).

In T. cruzi, the cosedimentation of TcDnaj protein with ribosomes/small polysomes depended on the in vitro growth conditions. Indeed, the polysome profile of trypanosomes is developmentally regulated (37). In proliferating cells (epimastigotes in logarithmic growth phase) the secondary polysome peak was more pronounced in the gradient because of the dense loading of mRNA with ribosomes (Fig. 6C). In stationary cells, this peak decreased, whereas the peak of ribosomal subunits and monosomes increased because the cells were arrested in G0/G1 phase (Fig. 6B). The sedimentation profile of the trypanosomal
co-chaperone followed similar pattern changes. In epimastigotes in stationary phase (Fig. 6B), the sedimentation profile of the trypanosomal chaperone showed peaks corresponding to ribosomal subunits/80 S monosomes, whereas in exponentially growing parasites (Fig. 6C) it was spread over the smaller polysomes. As for the transformed yeast, a large excess of the protein was found associated with the top of the sucrose gradient (T in Fig. 6, B and C). Similarly, in yeast, the most important fraction of Sis1p and Ssap, which functions as the Hsp70 partner for translational function of Sis1p, is found associated to the cytoplasm (47). Whether this result confirmed the possibility that a cytosolic pool of TcJ6p might be involved in other biogenesis pathways, we cannot rule out that this cytosolic TcJ6p was stripped off the ribosomes during the harvest procedure. We noted that two additional upper bands with apparent molecular masses of around 40 and 45 kDa were associated with 80 S ribosomes (Fig. 6B, open arrow). The nature of these bands is still unknown, but they may correspond to hyperphosphorylated forms of the protein (see “Discussion”).

**Localization of Trypanosomal DnaJ by Indirect Immunofluorescence—**The subcellular distribution of TcJ6p was determined by confocal microscopy at two stages of parasite differentiation, dividing epimastigotes and quiescent metacyclic trypomastigotes. In the case of epimastigotes (Fig. 7A), the staining was distributed throughout the cytosol of the cell and was preferentially concentrated in speckles in the peri-nuclear region (Fig. 7B). The overall distribution of the trypanosomal protein was similar in metacyclic cells, but more speckles were observed close to the kinetoplast and in the peri-nuclear region (Fig. 7C). The preferential TcJ6p location in the peri-nuclear region suggests a protein association with the endoplasmic reticulum.

**DISCUSSION**

**The TcJ6 Gene Is Highly Conserved in Trypanosomatids—**A novel member of the *T. cruzi DnaJ-like* gene family was found together with a sialidase pseudogene and genes encoding proteins homologous to an adenylate cyclase and two small G-proteins in a 14.3-kb genomic DNA fragment highly homologous to the 3′ region of the glucose transporter gene cluster (18). Southern blot analysis detected at least four copies of TcJ6, which, unlike the Hsp60, Hsp70, and Hsp90 genes of *T. cruzi* (48–50), were not arranged in tandem repeats but were instead dispersed on a single chromosome in the megabase range. The situation was different in *T. brucei* and *L. donovani*, in which two allelic copies of this gene were present (18). This may be accounted for by intrachromosomal amplification of large genomic fragments, typical in *T. cruzi*, even in the absence of drug selection, and resulting in extensive variations in the genome size of *T. cruzi* strains and clones (51). It has been postulated that the organization of the genes flanking the glucose transporter gene cluster has been highly conserved during evolution, because these genes may have related functions (18). In this respect, analysis of expression of the genes detected in the c1263g clone has shown that the transcript level of adenylate cyclase and TcJ6 genes increases in metacyclic cells (Fig. 3A and data not shown). This increase in the mRNA levels of adenylate cyclase and TcJ6 genes observed in metacyclic cells might be a result of processing of the primary transcripts into individual steady state mRNAs, occurring by trans-splicing and polyadenylation, which have been accumulated during the differentiation process. However, at the protein level, the amount of adenylate cyclase increases during metacyclogenesis, but this is clearly not the case for TcJ6p (Fig. 3B and data not shown). These observations reveal no obvious functional coupling between the various genes of the unit.

**TcJ6p, a New Member of the Class II Hsp40 Subfamily—**TcJ6p belongs to the class II Hsp40s, since it has the highly conserved N-terminal J domain and lacks the cysteine-rich region but displays an extensive G-F region. Functional complementation of a class II Hsp40, the yeast Sis1p, and association with ribosomal subunits and translating ribosomes demonstrated that the physiological role of TcJ6p is similar to that of Sis1p. Do all the class II subfamily members have functions similar to Sis1p? A very important piece of evidence was provided by gene swapping experiments with the building domains (J, G-F, G-M, C terminus) of two yeast cytosolic proteins, Ydj1p (class I) and Sis1p (class II), in experiments of rescue of *sis1* yeast mutant. This work showed that although J domains are interchangeable, G-F regions, which are essential for the function of Sis1p, are not (9). Thus, the G-F region is specifically required to discriminate between the function of Ydj1p and Sis1p and not the J domain, as was previously thought. Analysis of the sequence of the trypanosomal DnaJ of *L. donovani* reveals a very interesting feature; the presence of a G-M-rich extension (GGMPGGMPG), which is absent from the other trypanosomatid analyzed (18) and is very similar to the yeast G-M motif, is repeated twice in Sis1p (GGMPGGMPGGMPGG) (31). In this respect, it is noteworthy that the molecular mass of the *L. major* chaperone is close to the 37.5-kDa of Sis1p (see Fig. 3C).

**Does TcJ6p Behaves as a Heat Shock Protein?—**Although the amount of Sis1p has been shown to double after a heat shock from 23 to 39 °C (31), our data provide no clear evidence that heat shock from 28 to 37 °C results in significant overproduction of the trypanosomal co-chaperone. After 6 h of heat shock, we detected a 2-fold enrichment of TcJ6 transcript, which was restored to normal after 24 h. This probably reflects an increase in the stability of co-chaperone mRNA during heat shock, as already observed for Hsp83 from *Leishmania amazonensis* (44). Because the presence of wild-type Sis1p in the *sis1-85* mutant was found to repress the overexpression of Sis1-85 protein (52), we are currently investigating whether TcJ6p could work as a transcriptional regulator in yeast by down-regulating expres-
sion of Sis1p via its cis-element present in the sis1-85 mutant. **Involvement of TcJ6p in Translation Initiation**—Functional complementation of the sis1-85 mutant at nonpermissive temperatures and association with ribosomal subunits and 80 S monosomes are consistent with a direct role for the trypanosomal co-chaperone in translation initiation (25). In most eukaryotes, global changes in translation occur mostly at the level of initiation (53). In *T. brucei*, Brecht and Parsons (37) found that the low level of translation in quiescent cells was probably due to a decrease in translation initiation. In *T. cruzi*, the polysome profiles in the stationary and logarithmic growth phases of epimastigote forms led to the same conclusion. In cells arrested, the blocking of elongation should lead to a depletion of ribosomal subunits, whereas blocking of initiation should lead to the accumulation of these subunits. We observed an accumulation of ribosomal subunits and 80 S monosomes with a small secondary peak of polysomes for cells in stationary phase and the opposite profile in proliferating cells in logarithmic growth phase, which showed a large secondary polysome peak. The preferential association of a fraction of TcJ6p with 80 S monosomes in arrested cells, accounting for less than 10% of total cytosolic protein, might be regulated by phosphorylation of the trypanosomal DnaJ. Indeed, the most likely scenario to explain the detection in Western analysis of two slower-migrating forms (around 40 and 45 kDa) is that they represent differentially phosphorylated forms. In yeast, a band shift of similar magnitude has been reported for the Mcm1 transcription factor (30 kDa, unphosphorylated form), which is present in total cytosolic protein, might be regulated by phosphorylation of Sis1p (19). In most eu-

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1. Georgopoulos, C., and Welch, W. J. (1993) *Annu. Rev. Cell Biol.* 9, 601–634.

2. Liberak, K., Marzalek, J., Ang, D., Georgopoulos, C., and Zylber, M. (1991) *Proc. Natl. Acad. Sci. U. S. A.* 88, 2874–2878.

3. Wickner, S. H. (1990) *Proc. Natl. Acad. Sci. U. S. A.* 87, 2690–2694.

4. Craig, E., Yan, W., and James, P. (1999) in *Molecular Chaperones and Folding Catalysis: Regulation, Cellular Function, and Mechanisms* (Bukau, B., ed.) pp. 139–162, Harwood Academic Publishers, Amsterdam, the Netherlands.

5. Kreibich, S., and Lindquist, S. (2000) *Proc. Natl. Acad. Sci. U. S. A.* 97, 1589–1594.

6. Bukau, B. (1992) *Trends Biochem. Sci.* 17, 129.