Parasitol Res (2015) 114:265–271
DOI 10.1007/s00436-014-4188-y

ORIGINAL PAPER

Biosynthesis of very long chain fatty acids in *Trypanosoma cruzi*

Verónica I. Livore · Antonio D. Uttaro

Received: 9 June 2014 / Accepted: 14 October 2014 / Published online: 24 October 2014
© Springer-Verlag Berlin Heidelberg 2014

Abstract *Trypanosoma brucei* and *Trypanosoma cruzi* showed similar fatty acid (FA) compositions, having a high proportion of unsaturated FAs, mainly 18:2Δ9,12 (23–39 %) and 18:1Δ9 (11–17 %). C22 polyunsaturated FAs are in significant amounts only in *T. brucei* (12–20 %) but represent a mere 2 % of total FAs in *T. cruzi*. Both species have also similar profiles of medium- and long-chain saturated FAs, from 14:0 to 20:0. Interestingly, procyclic and bloodstream forms of *T. brucei* lack very long chain FAs (VLCFAs), whereas epimastigotes and trypomastigotes of *T. cruzi* contain 22:0 (0.1–0.2 %), 24:0 (1.5–2 %), and 26:0 (0.1–0.2 %). This is in agreement with the presence of an additional FA elongase gene (*TcELO4*) in *T. cruzi*. TcELO4 was expressed in a *Saccharomyces cerevisiae* mutant lacking the endogenous ScELO3, rescuing the synthesis of saturated and hydroxylated C26 FAs in the yeast. Expression of TcELO4 also rescued the synthetic lethality of a ScELO2, ScELO3 double mutation, and the VLCFA profile of the transformed yeast was similar to that found in *T. cruzi*. By identifying TcELO4 as the enzyme responsible for the elongation of FA from 16:0 and 18:0 up to 26:0, with 24:0 being the preferred product, this work completed the characterization of FA elongases in *Trypanosoma* spp.

Keywords *Trypanosoma cruzi* · Very long chain fatty acid · Elongase · Lipids

V. I. Livore · A. D. Uttaro (✉)
Instituto de Biología Molecular y Celular de Rosario (IBR), CONICET, Facultad de Ciencias Bioquímicas y Farmacéuticas, Universidad Nacional de Rosario, Ocampo y Esmeralda, 2000 Rosario, Santa Fe, Argentina
e-mail: toniuttaro@yahoo.com.ar

Introduction

Trypanosomes are flagellated protozoa responsible for serious human diseases. *Trypanosoma brucei* is the causative agent of the sleeping sickness in Africa, whereas *Trypanosoma cruzi* causes Chagas' disease, the American trypanosomiasis. They belong to the class Kinetoplastea, which comprises organisms extensively studied due to their impact on human and animal health (Barrett et al. 2003) and because of the remarkable biological characteristics they have (Ginger 2005), one of which being related to the fatty acid (FA) biosynthesis (Uttaro 2014).

Short- and medium-chain FAs are usually synthesized by the soluble fatty-acid synthetase (FAS) systems and subsequently elongated to long- and very long-chain FAs (VLCFAs) by the particulate elongase (ELO) system (Leonard et al. 2004; Tehlivets et al. 2007; Vance and Vance 2002). The elongation of FAs involves the addition of two carbon units to their carboxyl end, using malonyl-CoA as two-carbon donor. The system is composed of four enzymes: β-ketoacyl-CoA synthase (KS) or condensing enzyme, β-ketoacyl-CoA reductase, β-hydroxyacyl-CoA dehydrase and trans-2-enoyl-CoA reductase. FAS systems from all organisms have the same four basic reactions, although the enzymes are neither structurally or phylogenetically related; in addition, FAS uses acyl-ACP (acyl-acyl carrier protein) instead of acyl-CoA substrates. KS catalyzes the first and rate-limiting step and is responsible for the substrate specificity in terms of chain length and degree of unsaturation, so ELO is frequently used also to refer to KS (Leonard et al. 2004). Cells contain multiple ELOs, many of them with overlapping specificities. *S. cerevisiae*, for example, has three isoforms, with ScELO1 being responsible for elongating the 14:0 FA de novo synthesized by FAS I, to 16:0 and 18:0. ScELO2 and ScELO3 are involved in the elongation of 16:0/18:0 to 24:0. Additionally, ScELO3 but not ScELO2 elongates 24:0 to 26:0 (Tehlivets

et al. 2007). Yeast sphingolipids are predominantly composed of 26:0-containing phytoceramides which are precursors for the synthesis of complex sphingolipids, such as inositol-phosphoryl-ceramide (IPC), mannosyl-inositol-phosphoryl-ceramide (MIPC), and mannosyl-di-inositol-phosphoryl-ceramide ($M[IP]_2C$). *ScELO3* null mutation causes striking changes in the metabolism of phosphatidyl inositol/inositol phosphate and sphingolipids, which are known to mediate numerous biological and signaling functions (Ponnusamy et al. 2008).

Elongases have been found in the endoplasmic reticulum as integral membrane proteins with five to seven predicted transmembrane helices and a characteristic HX_2HH histidine motif. They are involved in the elongation of saturated, monounsaturated, and polyunsaturated acyl-CoAs in eukaryotes, from protozoa, algae, and fungi to mammals (Leonard et al. 2004; Uttaro 2006). Trypanosomatids lack the classic eukaryotic or FAS I system but conserve a mitochondrial FAS II (Stephens et al. 2007). This latter system is mainly involved in the production of octanoate, for lipoic acid synthesis, and palmitate, both for local use in the mitochondrion. The main FA pool is synthesized by a specialized ELO system that appears to be an adaptation to the parasitic lifestyle. *T. brucei* has four ELOs; three of them, encoded in tandem array on chromosome 7 (Lee et al. 2006; Livore et al. 2007), are responsible for the successive elongation of butyryl-CoA to decanoyl-CoA (TbELO1), then to myristoyl-CoA (TbELO2) and finally to stearoyl-CoA (TbELO3) (Lee et al. 2006). This modular synthesis allows the parasite to regulate the production of different intermediates as required throughout its life cycle (Lee et al. 2007). Additionally, TbELO5 is involved in the elongation of polyunsaturated FAs (Lee et al. 2006; Livore et al. 2007). *T. brucei* contains medium- and long-chain saturated FAs, up to 20:0, and polyunsaturated FAs from 18 to 22 carbons. We have previously described that C22-polyunsaturated FAs are synthesized by elongation (Livore et al. 2007) and further desaturation (Tripodi et al. 2006) of C20-intermediates taken up from the host or culture media.

The content and biosynthesis of polyunsaturated FAs in *T. cruzi* are similar to those found in *T. brucei*, having also an ELO5 orthologue (Livore et al. 2007). However, in addition to medium- and long-chain saturated FAs, we have detected 26:0 and mainly 24:0 VLCFAs (this work). 24:0 was found previously as fatty acyl moieties in the glycosylphosphatidylinositol (GPI) anchors of membrane components of *T. cruzi*, like mucins and glycoinositol-phospholipids (GIPLs) (Serrano et al. 1995; Almeida et al. 2000). Based on experimental evidence, Lee et al. (2006) proposed the presence of a similar pathway for de novo biosynthesis of 18:0 in *T. cruzi* to that found in *T. brucei*, carried out by TcELO1, TcELO2, and TcELO3. It implies that *T. cruzi* synthesize VLCFAs by means of an additional ELO with a substrate profile similar to ScELO2/ScELO3. An in silico analysis of the genome revealed the presence of a fourth gene (*TcELO4*) in the equivalent cluster found on an unidentified chromosome of *T. cruzi* (Livore et al. 2007). After the expression of TcELO4 in *S. cerevisiae ELO* mutants, impaired in the synthesis of VLCFAs, we were able to characterize the substrate and product profiles of this elongase. These results indicate that TcELO4 is the enzyme involved in the biosynthesis of VLCFAs in *T. cruzi*.

Materials and methods

Materials

Culture media and fetal calf serum were obtained from Gibco (Gibco-Life Technologies). Sodium methoxide, yeast nitrogen base, 5-fluoroorotic acid (5-FOA), glucose, and amino acids were obtained from Sigma (Sigma-Aldrich, St Louis, MI, USA). All solvents were purchased from Merck (Whitehouse Station, NJ, USA).

Trypanosomes growth conditions

Epimastigotes of *T. cruzi*, CL Brener strain, were grown at 28 °C in brain–heart infusion tryptose medium supplemented with 10 % heat-inactivated fetal calf serum (FCS) and hemin (Cazzulo et al. 1985). Cell-derived trypomastigotes were harvested from the supernatant of Vero-infected cells grown in minimal essential medium (MEM), as described by Andrews and Colli (1982). Cell line was from the A.T.C.C. (Manassas, VA, U.S.A.) and was grown at 37 °C in 5 % CO_2 in the indicated medium supplemented with 10 % FCS, 0.292 g/l L-glutamine, 100 IU/ml penicillin, and 100 µg/ml streptomycin. Bloodstream form (BSF) of *T. brucei* Lister 427, cell line 90–13, was cultured in HMI-9 medium containing 10 % FCS at 37 °C under water-saturated air with 5 % CO_2 (Wirtz et al. 1999). Procyclic form (PCF), cell line 29–13, was cultured in SDM-79 medium supplemented with 15 % FCS at 28 °C under water-saturated air with 5 % CO_2 (Brun et al. 1979; Wirtz et al. 1999). Cultures were always harvested in the exponential growth phase, i.e., at densities lower than 2×10^6 cells/ml for BSF and 2×10^7 cells/ml for PCF and *T. cruzi*, by centrifugation at 1,000×*g* for 10 min.

Expression of the elongase gene

Based on information provided by the genomic resource for the Trypanosomatidae (TriTrypDB, http://tritrypdb.org/tritrypdb/), we designed the primers Tc1 (5′-CCGGATCCATGGACTTCGTTCTAAATACAAT) and Tc2 (5′-CCAAGCTTCTACGCCATCTTCTCGCCA). They were used in the PCR amplification of the gene on *T. cruzi* CL Brener genomic DNA. The amplified, cloned sequence (*TcElo4*)

was ligated into the *Bam*HI and *Hind*III sites of p426GPD and p425GPD, the 2-μm yeast expression vectors containing a glyceraldehyde-3-phosphate dehydrogenase promoter (Mumberg et al. 1995). These vectors contain selectable marker genes, which confer uracil and leucine prototrophies in the host, respectively. The plasmid construct p426-TcElo4 and the vector alone (p426GPD) were introduced by electroporation into *S. cerevisiae* strain CSY3H (*MATa*, *elo3Δ::HIS3*, *leu2–2*, *leu2–112*, *can1–100*, *ura-3–1*, *ade2–1*, *his3–11*, *his3–15*) (Oh et al. 1997). Transformed yeasts were selected on minimal agar plates lacking uracil and supplemented with leucine and adenine. To determine the enzyme activity, wild-type strain DTY10A (*MATa*, *leu2–2*, *leu2–112*, *can1–100*, *ura-3–1*, *ade2–1*, *his3–11*, *his3–15*) and transformed yeasts were cultured overnight at 30 °C in 0.67 % (*w*/*v*) yeast nitrogen base (Sigma), 2 % (*w*/*v*) glucose and leucine, adenine, and histidine (all at 20 mg/l). These cultures were diluted to a D_{600} value of 0.2 and grown for 72 h in a shaking incubator at 30 °C. Plasmids p425GPD and p425-TcELO4 were introduced into the *S. cerevisiae Elo2Δ*, *Elo3Δ* double mutant, strain TDY7005 (*MATa*, *lys2*, *ura3-52*, *trp1Δ*, *leu2Δ*, *elo2::KAN*, *elo3::TRP1/pRS316-ScELO3*) (Paul et al. 2006). The double mutant is viable due to the ectopic expression of *ScELO3*. Transformed yeasts were selected on minimal agar plates lacking uracil and leucine and supplemented with lysine. Selected clones were plated in the same media containing uracil and 5-FOA. Viable colonies were obtained only with yeasts electroporated in the presence of the p425-TcELO4 plasmid. These clones were proved to have lost uracil prototrophy, indicating the loss of the pRS316-ScELO3 plasmid and the capacity of TcELO4 to bypass the synthetic lethality of the double mutation.

Fatty acid analysis

Cells from 20 ml cultures were collected by centrifugation at 500×*g* for 5 min, and the pellets washed twice with 20 ml of distilled water. Lipids were extracted according to Bligh and Dyer (1959). The organic phase was reduced to dryness under N_2, and fatty acid methyl esters were prepared by adding 1 ml of 0.5 M sodium methoxide in methanol and incubating for 20 min at room temperature. After neutralization with 6 M HCl and extraction with 2 ml hexane, the organic solvent was evaporated to dryness under a N_2 stream. The composition of fatty acid methyl esters was analyzed by running samples through a polyethylene glycol column (SUPELCOWAX, 30 m×0.25 mm inside diameter, Sigma) in a Shimadzu GC-2010 Plus gas chromatograph (Shimadzu Corp., Kyoto, Japan). Column temperature was programmed to increase from 160 to 320 °C at a rate of 3.5 °C/min. Helium was the carrier gas at a constant flow rate of 1 ml/min. The GC-MS was carried out using a GCMS-QP2010 Plus detector (Shimadzu) operated at an ionization voltage of 70 eV with a scan range of 40–600 Da. The retention time and mass spectrum of any new peak obtained was compared with that of standards (Sigma) and those available in the data base NBS75K (National Bureau of Standards). Percentages of FAs were calculated after integration of the chromatogram peaks. Results represent the mean±SD values of three independent experiments.

Results and discussion

Fatty acid composition of trypanosomes

The FA profiles of trypanosomatids were already previously analyzed by several laboratories. However, these were obtained by different (not always comparable) methods, or the analysis focused on medium- and long-chain fatty acids, which represent the main components of membrane lipids. Table 1 shows a comparison of the FA profiles we obtained by GC-MS, including those present at low percentages, between cultured BSF and PCF of *T. brucei* and epimastigotes and trypomastigotes of *T. cruzi*. Figure 1 shows a schematic representation of VLCFA percentages in each trypanosome

Table 1 Fatty acid composition of trypanosomes

Fatty acid	Percentage of total fatty acids			
	T. brucei PCF	*T. brucei* BSF	*T. cruzi* EF	*T. cruzi* TF
14:0	0.3±0.1	1.0±0.1	0.6±0.1	1.2±0.2
16:0	8.2±1.2	14.9±2.3	15.6±3.3	20.7±4.2
16:1	1.7±0.1	1.7±0.4	3.5±0.9	1.1±0.3
17:1	3.0±0.4	1.4±0.1	1.4±0.4	–
18:0	10.4±2.1	14.8±3.0	11.8±2.9	25.2±2.2
18:1Δ9	16.5±3.5	11.1±2.8	16.2±3.3	14.0±3.4
18:1Δ11	4.2±0.9	3.8±0.5	3.4±1.0	10.4±2.9
18:2Δ9,12	30.7±3.6	34.0±4.1	38.6±4.7	23.3±1.1
20:0	0.2±0.1	0.6±0.1	0.6±0.1	0.3±0.1
20:2	0.7±0.1	0.4±0.1	0.2±0.1	–
20:3n-6	1.6±0.2	0.6±0.2	–	–
20:3n-3	0.2±0.1	0.6±0.1	0.9±0.2	0.2±0.2
20:4n-6	2.0±0.3	1.9±0.3	1.1±0.4	1.0±0.2
22:0	–	–	0.2±0.1	0.1±0.1
22:4n-3	1.7±0.1	0.7±0.1	0.5±0.2	0.4±0.3
22:5n-6	5.4±0.9	3.8±0.8	–	–
22:5n-3	1.2±0.2	1.1±0.2	–	–
22:6	11.7±1.5	6.3±1.0	1.0±0.2	0.5±0.2
24:0	–	–	2.0±0.8	1.5±0.3
26:0	–	–	0.2±0.2	0.1±0.1

PCF procyclic form, *BSF* bloodstream form, *EF* epimastigote form, *TF* trypomastigote form

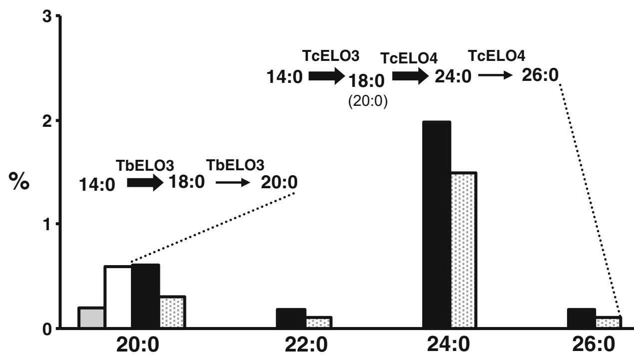

Fig. 1 Very long-chain fatty acids composition of *Trypanosoma* spp. Percentages of VLCFAs from total fatty acids in *T. brucei* procyclic (*gray bar*) and bloodstream (*white bar*) forms and in *T. cruzi* epimastigote (*black bars*) and trypomastigote (*punctuated bars*) forms. Enzymes putatively involved in the synthesis of each FA are indicated in the pathways included as *insets*

developmental form and the putative elongases involved in their synthesis. The longest saturated FA found in both forms of *T. brucei* was 20:0, most probably due to the residual activity of TbELO3 on its myrystoyl-CoA (14:0) primer. The main product of TbELO3 is 18:0 (Lee et al. 2006). It is in agreement with the absence of additional elongase genes (Livore et al. 2007). A contribution of an uptake from the culture medium on the 20:0 content cannot be ruled out, although it is expected to be not relevant as this FA is only present in low amount in the fetal calf serum used to supplement the medium. By contrast, epimastigotes and cell-derived trypomastigotes of *T. cruzi* showed C20–C26 saturated FAs, with 24:0 being the main VLCFA. As far as we know, this is the first report of 26:0 in *T. cruzi*, whereas 24:0 was previously detected only as fatty acyl moieties of GPI anchors of mucins in metacyclic trypomastigotes and GIPLs in epimastigotes (Serrano et al. 1995; Almeida et al. 2000). It could be speculated that these surface macromolecules may be involved in the interaction with the insect vector, with 24:0-GPIs having an important role. In addition, C24–C26 VLCFAs are most probably involved also in the synthesis of sphingolipids, in both the insect and mammalian stages of *T. cruzi*.

Fig. 2 Sequence alignment of fatty acid elongases. Amino acid sequences of *S. cerevisiae* ELO3 (NCBI accession number P40319), *T. brucei* ELO3 (TriTryp accession number Tb927.7.4160), *T. cruzi* ELO4A (Tc00.1047053510989.10), *T. cruzi* ELO4B (Tc00.1047053511245.160), and cloned enzyme (TcELO4) were aligned by CLUSTALW (Thompson et al. 1994). The characteristic HXXHH motifs of elongases (in *italics*) are *boxed*. Amino acids different between both TcELO4 alleles and cloned TcELO4 are in *bold* and *underlined*

```
ScELO3   MNTTTSTVIAAVADQFQSLNSSSSCFLKVHVPSIENPFGIELWPIFSKVFEYFSGYPAEQFEFIH  65
TbELO3   -------------------------------------MLMNFGGSYDAYINNFQG--------TF  20
TcELO3   -------------------------------------MAIAWMDSYTRWAADFRG--------EH  20
TcELO4A  -----------------------------------MDFVLNTIQWLREVPRNFKG--------EV  22
TcELO4B  -----------------------------------MDFVLNTIQWLREVPHNFKG--------EF  22
TcELO4   -----------------------------------MDFVLNTIQWLCEVPHNFKG--------EF  22

ScELO3   NKTFLANGYHAVSIIIVYYIIIFGGQAILRALNASPLKFKLLFEIHNLFLTSISLVLWLLMLEQL 130
TbELO3   LAEWMLDHPSVPYIAGVMYLILVLYVPKSIMASQPPLNLRAANIVWNLFLTLFSMCGAYYTVPYL  85
TcELO3   LRSWMRDHTEVPAVAVVLYLILVLYVPENVMAHRNPIKLRFLNMLWNLLLTVFSICGAYYCLPRL  85
TcELO4A  ATVVFDDSADILVYCCVLYILLVFMVPEHIMKNREPFNLRLPFVVWNIGLCLFSICGAYSCVKNM  87
TcELO4B  ATVVFDDSADILVYCCVLYILLVFMVPEHIMKNREPFNLRLPFVFWNIGLCLFSFCGAYSCVKNM  87
TcELO4   ATVVFDDSADILVYCCVLYILLVFMVPEHIMKNREPFNLRLPFVFWNIGLCLFSICGAYSCVKNM  87

ScELO3   VPMVYHN----------------------GLFWSICSKEA---FAPKLVTLYYLNYLTKFVELID 170
TbELO3   VKAFMNPEIVMAASGIKLDANTSPIITHSGFYTTTCALADSFYFNGDVGFWVALFALSKIPEMID 150
TcELO3   WEVLTSPRISGLMADPNLGPKAPPPKLPGSFYNSACAWNDKIFFDGFVGLWVAAFVLSKIPEMID 150
TcELO4A  TALYWER----------------------GFYRTTCFFDSSVAYDGEFAFWVFYFILSKIPEMID 130
TcELO4B  IALYWER----------------------GFYRTTCFFDSSVAYDGEFAFWVFYFILSKIPEMID 130
TcELO4   TALYWER----------------------GFYRTTCFFDSSVAYDGEFAFWVFYFILSKIPEMID 130

ScELO3   TVFLVLRRKKLLFLHTYHHGATALLCYTQLIGRTSVEWVVILLNLGVHVIMYWYYFLSSCGIRVW 235
TbELO3   TAFLVFQKKPVIFLHWYHHLTVMLFCWFAYVQKISSGLWFASMNYSVHSIMYLYYFVCACGHRRL 215
TcELO3   TVFLVFQKKPVIFLHWYHHATVMLFCWHAYAYTISSGLWFATMNYCVHSIMYFYYFICACGMRKV 215
TcELO4A  TVFLVFQKKPVIFLHWYHHLTVAIFCWHAGHALIPSGLWFATMNYCVHSIMYFYYFMCACGMRKV 195
TcELO4B  TVFLVFQKKPVIFLHWYHHLTVAIFCWHAGHALIPSGLWFATMNYCVHSIMYFYYFMCACGMRKF 195
TcELO4   TVFLVFQKKPVIFLHWYHHLTVAIFCWHAGHALIPSGLWFATMNYCVHSIMYFYYFMCACGMRKV 195

ScELO3   WKQWVTRFQIIQFLIDLVFVYFATYTFYAHKYLDGILPNKGTCYGTQAAAAYGYLILTSYLLLFI 300
TbELO3   VRPFAPIITFVQIFQMVVGTIVVCYTYTVKHVLG------RSCTVTDFSLHTGLVMYVSYLLLFS 274
TcELO3   IRPIAPLITMMQILQMVAGTLIVLYTYVKKQIMG------EFCAVNNPSLRMGLLMYVSYLFLFS 274
TcELO4A  IRPIAPFITMMQLLQMVAGTLIVLYTAYHSYLSE------SGCEVDRTSIRLGLVMYGSYFFLFA 254
TcELO4B  IRPIAPFITMMQLLQMVAGTLIVLYTAYHSYLGE------SGCEVDRTSIRLGLVMYGSYFFLFA 254
TcELO4   IRPIAPFITMMQLLQMVAGTLIVLYTAYHSYLGE------SGCEVDRTSIRLGLVMYGSYFFLFA 254

ScELO3   SFYIQSYKKGGKKTVKKESEVSGSVASGSSTGVKTSNTKVSSRKA 345
TbELO3   QLFYRSY-------LSPRDKASIPHVAAEIKKKE            301
TcELO3   QLYYRSY-------ISPAAARTLRMANGEKKGK             300
TcELO4A  VLFGKLY-------LKKQVKPSGTASAYAMSKKRNGEKMA      287
TcELO4B  VLFGKLY-------LKKQVKPSGTASAYAMSKKRNGEKMA      287
TcELO4   VLFGKLY-------LKKQVKPSGTASAYAMSKKRNGEKMA      287
```

Expression of *T. cruzi* ELO4 in a *S. cerevisiae Elo3Δ* mutant

In a previous work, we have identified clusters of genes encoding putative ELOs in trypanosomatids (Livore et al. 2007). *T. brucei* presents a cluster of three genes on chromosome 7. They are ordered as *ELO1*, *ELO2*, and *ELO3*, which correspond to the same order of action of the encoded proteins in the synthesis of saturated FAs from 4:0 to 18:0 (Lee et al. 2006). *T. cruzi* presents a syntenic cluster but with an additional fourth gene. Experimental evidence suggests that *T. cruzi* ELO1 to ELO3 are most probably involved in the same set of reactions found in *T. brucei*, although it was not conclusively determined (Lee et al. 2006). The fourth gene, *TcELO4* (loci Tc00.1047053510989.10 or TcELO4A and Tc00.1047053511245.160 or TcELO4B in Fig. 2), was then selected as the one encoding the putative enzyme involved in the elongation of 18:0 to VLCFAs. The encoded protein sequences of both loci share 98 % identity and showed 51 % identity with TcELO3, 48 % with TbELO3, and 25 % with ScELO3. It was suggested that TcELO4 is probably the result of a duplication of an ancestral ELO3 gene in the trypanosomatid lineage (Livore et al. 2007). Primers Tc1 and Tc2 were used in the PCR amplification of *TcELO4* on *T. cruzi* CL Brener genomic DNA. Several clones with the amplicon were isolated and checked by sequencing. All of them showed the same sequence which appears to be a hybrid between the two reported loci. The deduced amino acid sequence presents four changes compared to TcELO4B: R12C, F77I, I88T, and F195V. The characteristic histidine box of elongases is highly conserved. Interestingly, TcELO4 presents a 22-amino-acid deletion, compared with both *T. brucei* and *T. cruzi* ELO3 (see Fig. 2). The gene was cloned into the yeast expression vector p426GPD and used to transfect a *S. cerevisiae Elo3Δ* mutant. This mutant grew at similar rates as the wild-type strain at 30 °C but was unable to grow at 37 °C (Paul et al. 2006). *TcELO4* rescued growth at 37 °C, although at rates slower than wild-type yeasts (not shown). The FA profiles of both the yeast mutant transfected with the vector alone and the one containing the *TcELO4* gene are shown in Table 2. In agreement with a previous report (Oh et al. 1997), the mutant presented an increased content of saturated and hydroxylated C20–C24 VLCFAs but was impaired in the synthesis of any C26 FA. Expression of TcELO4 rescued the synthesis of C26 VLCFAs and increased the content of C24 FAs. It indicates that *TcELO4* encodes an active enzyme able to elongate FAs up to 26 carbons, although its expression in a *ScELO2* background precluded the identification of putative primers for elongation as well as the main product of the enzyme (Fig. 3).

Expression of *T. cruzi* ELO4 in *S. cerevisiae Elo2ΔElo3Δ* mutant

The yeast strain TDY7005 harbors an ectopic copy of *ScELO3* which rescues the synthetic lethality of the *Elo2Δ*,

Table 2 Fatty acid composition of *S. cerevisiae* mutants transfected with the vector alone or the vector containing the *T. cruzi ELO4* gene

Fatty acid	Percentage of total fatty acids			
	elo3Δ/p426	*elo3Δ/TcELO4*	*elo2Δ, elo3Δ/ScELO3*	*elo2Δ, elo3Δ/TcELO4*
12:0	0.2±0.1	–	–	–
14:0	1.0±0.2	1.5±0.1	0.95±0.2	2.61±0.2
14:1	0.4±0.1	0.2±0.1	0.1±0.1	0.65±0.2
16:0	13.4±1.2	19.2±3.5	20.1±1.3	13.8±1.1
16:1	52.9±4.1	42.6±4.0	41.7±3.1	42.9±4.2
HO-16:0[a]	0.3±0.1	*t*	0.58±0.2	0.3±0.1
18:0	3.0±0.2	5.1±1.0	6.6±0.4	5.35±0.2
18:1Δ9	30.0±3.9	32.5±1.8	31.83±2.1	38.0±3.3
HO-18:0[a]	*t*	*t*	*t*	*t*
20:0	0.5±0.2	0.6±0.1	*t*	0.1±0.1
HO-20:0[a]	*t*	*t*	–	–
22:0	1.4±0.4	1.6±0.6	*t*	0.1±0.1
HO-22:0[a]	0.2±0.1	0.2±0.1	–	–
24:0	*t*	0.6±0.2	*t*	0.3±0.1
HO-24:0[a]	0.1±0.1	0.2±0.1	–	0.1±0.1
26:0	–	0.1±1.0	0.3±0.1	*t*
HO-26:0[a]	–	0.1±1.0	0.2±0.1	0.1±0.1

t trace amounts, less than 0.05 %

[a] Hydroxylated fatty acid

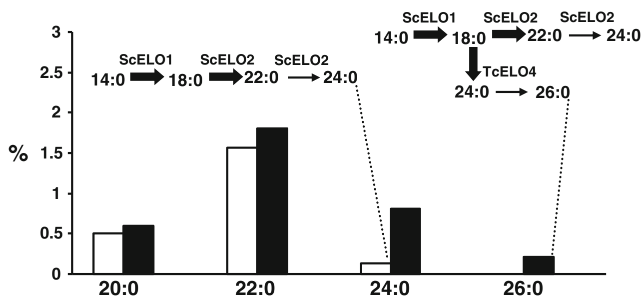

Fig. 3 Very long-chain fatty acids composition of the *Saccharomyces cerevisiae elo3Δ* mutant and the mutant expressing *T. cruzi* ELO4. Shown are the percentages of VLCFAs from the total fatty acids in the yeast mutant harboring the empty vector (*white bars*) or the vector containing the *TcELO4* gene (*black bars*). *Bars* represent the sum of hydroxylated and not hydroxylated VLCFAs. Enzymes involved in the synthesis of each FA are indicated in the pathways included as *insets*

Elo3Δ double mutation (Paul et al. 2006). *TcELO4* was cloned into the p425GPD vector and introduced into TDY7005. The ectopic copy of *ScElo3* was later eliminated, as described in "Materials and Methods," demonstrating that TcELO4 was also able to rescue the lethality produced by the simultaneous lack of ScELO2 and ScELO3. TDY7005 synthesized C26 VLCFAs (0.5 %) by means of the ectopically expressed ScELO3, using palmitoyl- and stearoyl-CoA (16:0 and 18:0) as primers (Table 2). These primers are the products of the other elongase (ScELO1) that remains active in TDY7005 (see Fig. 4) (Paul et al. 2006; Tehlivets et al. 2007). Only trace amounts of other C20-24 FAs were detected. The replacement of ScELO3 by TcELO4 allowed the synthesis of all VLCFAs, most likely using also 16:0/18:0 as primers. C24 FAs were the main products (0.4 %) of the parasite elongase (Fig. 4).

In conclusion, *T. cruzi* has five FA elongase genes. *TcELO1–TcELO4* are arranged in a cluster, whereas *TcELO5* is located in a different chromosome. TcELO5 was previously characterized as the enzyme involved in the elongation of 20:4 and 20:5 FAs taken from the host, to the corresponding C22 polyunsaturated FAs (Livore et al. 2007). The other four genes encode enzymes involved in the synthesis of saturated FAs up to 26:0, acting in the same order in which they appear in the cluster. TcELO1–TcELO3 were involved in the modular elongation of butyrate (4:0) up to 18:0, like that described in *T. brucei* (Lee et al. 2006). In a yeast model, TcELO4 was able to elongate 16:0/18:0 up to 26:0, with 24:0 being the main product of the elongase (Fig. 4). This product profile is in good agreement with the VLCFA profile we found in both stages of *T. cruzi* (Fig. 1). This work completed the characterization of all FA elongases found in trypanosomes, showing the capacity of *T. cruzi* to de novo synthesize FAs up to 26:0.

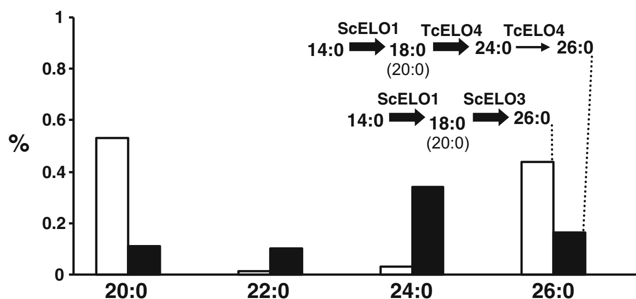

Fig. 4 Percentages of very long-chain fatty acids from total fatty acids composition of the *Saccharomyces cerevisiae elo2Δ/elo3Δ* double mutant expressing ectopically ScELO3 (*white bars*) or TcELO4 (*black bars*). *Bars* represent the sum of hydroxylated and not hydroxylated VLCFAs. Enzymes involved in the synthesis of each FA are indicated in the pathways included as *insets*

Acknowledgments We wish to thank Dr. Paul A. M. Michels for comments and suggestions on the manuscript. We gratefully acknowledge Dr. Charles E. Martin and Dr. Teresa M. Dunn for generously providing us with the yeast strains used in this work and the TriTrypDB genomic resource consortium, the Institute of Genomic Research (TIGR), and the Sanger Institute, for the availability of sequence data. ADU is member of Carrera del Investigador Científico, CONICET, Argentina.

Funding This work was supported by FONCyT, through Grant PICT 2007, No. 00654.

References

Almeida IC, Camargo MM, Procópio DO, Silva LS, Mehlert A, Travassos LR, Gazzinelli RT, Ferguson MA (2000) Highly purified glycosylphosphatidylinositols from *Trypanosoma cruzi* are potent proinflammatory agents. EMBO J 19:1476–1485

Andrews N, Colli W (1982) Adhesion and interiorization of *Trypanosoma cruzi* in mammalian cells. J Protozool 29:264–269

Barrett MP, Burchmore RJS, Stich A, Lazzari JO, Frasch AC, Cazzulo JJ, Krishna S (2003) The trypanosomiases. Lancet 362:469–480

Bligh EG, Dyer WJ (1959) A rapid method of total lipid extraction and purification. Can J Biochem Physiol 37:911–917

Brun R, Jenni L, Tanner M, Schönenberger M, Schell KF (1979) Cultivation and in vitro cloning of procyclic culture forms of *Trypanosoma brucei* in a semi-defined medium. Acta Trop 36:289–292

Cazzulo JJ, Franke de Cazzulo BM, Engel JC, Cannata JJ (1985) End products and enzyme levels of aerobic fermentation in trypanosomatids. Mol Biochem Parasitol 16:329–343

Ginger ML (2005) Trypanosomatid biology and euglenozoan evolution: new insights and shifting paradigms revealed through genome sequencing. Protist 156:377–392

Lee SH, Stephens JL, Paul KS, Englund PT (2006) Fatty acid synthesis by elongases in trypanosomes. Cell 126:691–699

Lee SH, Stephens JL, Englund PT (2007) A fatty-acid synthesis mechanism specialized for parasitism. Nat Rev Microbiol 5:287–297

Leonard AE, Pereira SL, Sprecher H, Huang Y-S (2004) Elongation of long-chain fatty acids. Prog Lipid Res 43:36–54

Livore VI, Tripodi KE, Uttaro AD (2007) Elongation of polyunsaturated fatty acids in trypanosomatids. FEBS J 274:264–274

Mumberg D, Muller R, Funk M (1995) Yeast vectors for the controlled expression of heterologous proteins in different genetic backgrounds. Gene 156:119–122

Oh CS, Toke DA, Mandala S, Martin CE (1997) ELO2 and ELO3, homologues of the *Saccharomyces cerevisiae* ELO1 gene, function in fatty acid elongation and are required for sphingolipid formation. J Biol Chem 272:17376–17384

Paul S, Gable K, Beaudoin F, Cahoon E, Jaworski J, Napier JA, Dunn TM (2006) Members of the Arabidopsis FAE1-like 3-ketoacyl-CoA synthase gene family substitute for the Elop proteins of *Saccharomyces cerevisiae*. J Biol Chem 281:9018–9029

Ponnusamy S, Alderson NL, Hama H, Bielawski J, Jiang JC, Bhandari R, Snyder SH, Jazwinski SM, Ogretmen B (2008) Regulation of telomere length by fatty acid elongase 3 in yeast. Involvement of inositol phosphate metabolism and Ku70/80 function. J Biol Chem 283:27514–27524

Serrano AA, Schenkman S, Yoshida N, Mehlert A, Richardson JM, Ferguson MA (1995) The lipid structure of the glycosylphosphatidylinositol-anchored mucin-like sialic acid acceptors of *Trypanosoma cruzi* changes during parasite differentiation from epimastigotes to infective metacyclic trypomastigote forms. J Biol Chem 270:27244–27253

Stephens JL, Lee SH, Paul KS, Englund PT (2007) Mitochondrial fatty acid synthesis in *Trypanosoma brucei*. J Biol Chem 282:4427–4436

Tehlivets O, Scheuringer K, Kohlwein SD (2007) Fatty acid synthesis and elongation in yeast. Biochim Biophys Acta 1771:255–270

Thompson JD, Higgins DG, Gibbson TJ (1994) CLUSTALW: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position specific gap penalties and weight matrix choice. Nucleic Acids Res 22:4673–4680

Tripodi KE, Buttigliero LV, Altabe SG, Uttaro AD (2006) Functional characterization of front-end desaturases from trypanosomatids depicts the first polyunsaturated fatty acid biosynthetic pathway from a parasitic protozoan. FEBS J 273:271–280

Uttaro AD (2006) Biosynthesis of polyunsaturated fatty acids in lower eukaryotes. IUBMB Life 58:563–571

Uttaro AD (2014) Acquisition and biosynthesis of saturated and unsaturated fatty acids in trypanosomatids. Mol Biochem Parasitol. doi:10.1016/j.molbiopara.2014.04.001

Vance DE, Vance JE (2002) Biochemistry of lipids, lipoproteins and membranes, 4th edn. Elsevier, Amsterdam

Wirtz E, Leal S, Ochatt C, Cross GA (1999) A tightly regulated inducible expression system for conditional gene knock-outs and dominant-negative genetics in *Trypanosoma brucei*. Mol Biochem Parasitol 99:89–101