Cloning and Expression of Neurotrophic *Trypanosoma cruzi* Ribosomal Proteins

An honors thesis for the Department of Biology

Monica Mugnier

Mercio PereiraPerrin, Research Advisor

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ABSTRACT

Trypanosoma cruzi, the infectious agent of Chagas' Disease, has been found previously to have a mimic of nerve growth factor (NGF), known as parasite-derived neurotrophic factor (PDNF). PDNF binds to the NGF receptor TrkA and promotes the survival and differentiation of neuronal cells, as well as entry into the cell by *T. cruzi*. The neurotrophic effects of PDNF suggested that *T. cruzi* might express other mimics of neurotrophic factors, so a screen for *T. cruzi* proteins that mimic other neurotrophic factors was performed. In this screen, a *T. cruzi* mimic of glial cell-derived neurotrophic factor (GDNF) was identified by its binding to the GDNF family of ligands (GFL) receptors GFRα-1, 2, and 3. GDNF, like NGF, is a neurotrophic factor involved in neural cell growth and survival. TGFL, like the ligands it imitates, appears to promote cell survival and neurite outgrowth. It is a complex of proteins expressed on the surface of *T. cruzi*. Sequencing analyses revealed many of the proteins in TGFL to be *T. cruzi* ribosomal proteins, including ribosomal proteins L13 and L4.

In order to better understand the role of TGFL in *T. cruzi* infection, L13 and L4 were cloned and bacterially expressed. Although attempts at purification were met with some difficulties, the cloned genes will allow for the eventual purification of the recombinant ribosomal proteins, and elucidation of their role in infection. Once purified, we will test their biological activity, specifically whether these ribosomal proteins can bind GFRα-1,2, or 3 individually, and whether they are capable of promoting neuronal survival and differentiation like TGFL.

INTRODUCTION

Trypansoma cruzi is the protozoan parasite that causes Chagas' disease (Figure 1). The parasites, which are transmitted by Triatomine bugs, bloodfeeding insects known as "assassin" or "kissing" bugs, primarily affect those in poor rural areas of South America, Central America, and Mexico (Figure 2). Chagas' disease can be asymptomatic for many years, but in the long term can cause damage to the heart, dilation of the digestive track, and neurological disorders. It can be fatal if left untreated.

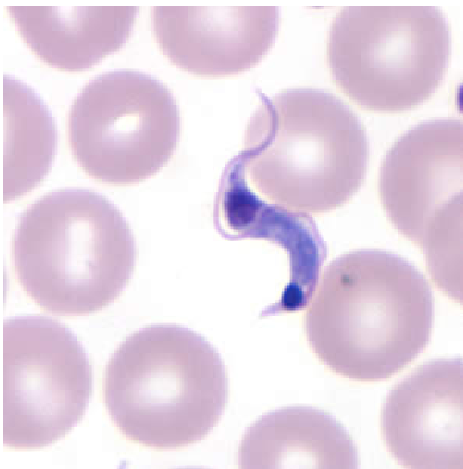

Figure 1. *T. cruzi* trypomastigote in a blood smear.

Figure 2. Areas of the world affected by Chagas'' Disease. The disease primarily affects poor, rural areas of South America, Central America, and Mexico.

The parasite is transmitted into the human host during a bloodmeal of the assassin bug (Figure 3). The trypomastigote form of the parasite resides in the rectum of the insect and is transmitted through its feces. Because of this, *T. cruzi* can only be transmitted through mucosa or abrasions of the skin, often caused by scratching the insect bite. The parasites enter host cells, transform into the amastigote form, and divide. After division, the amastigotes become trypomastigotes again, break out of the cells, and enter the host's blood stream to infect other tissues and divide further. These trypomastigotes in the bloodstream can then be taken up by the

assassin bug at another bloodmeal. Reservoirs of the parasite include opossums, armadillos, and wild rodents (1).

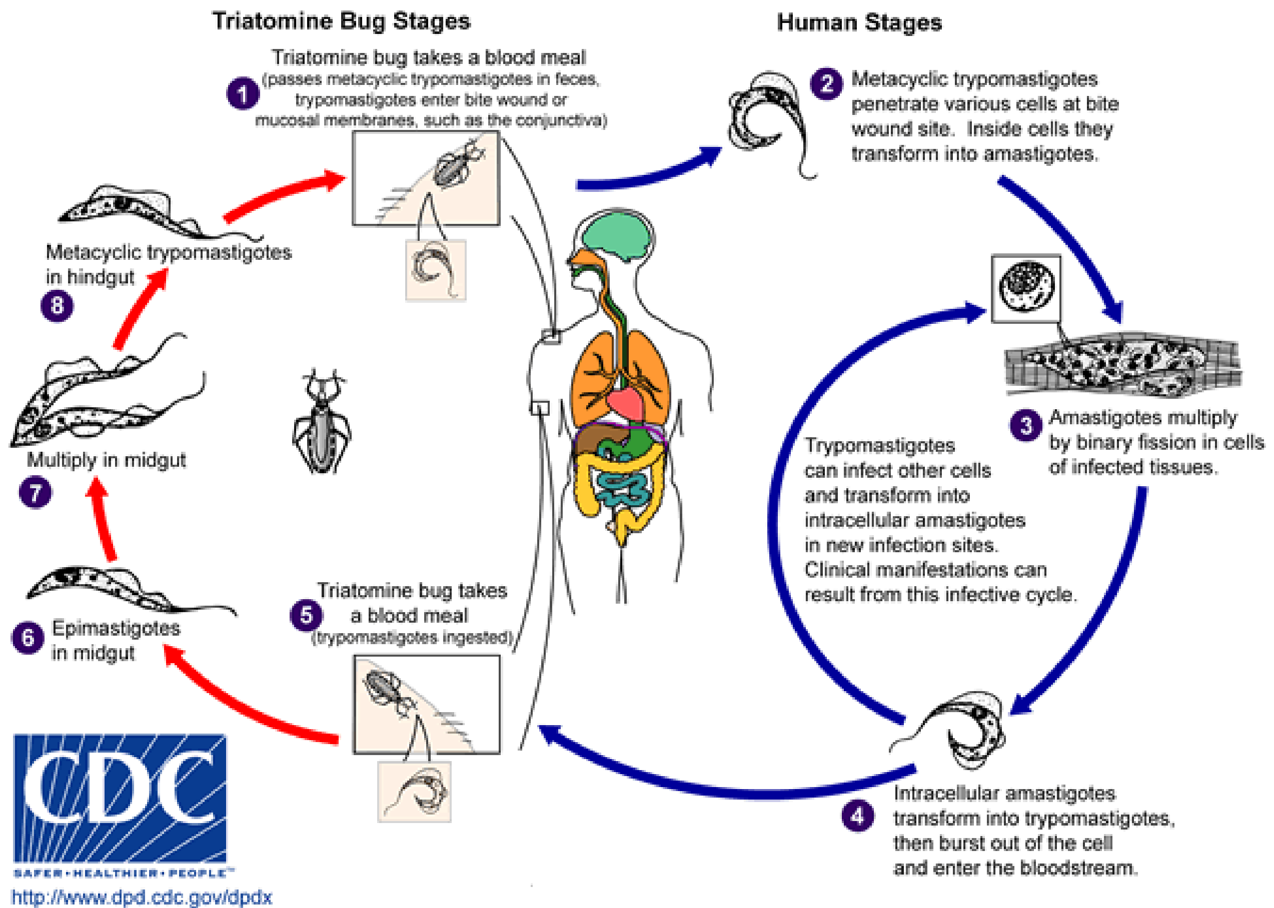

Figure 3. Life cycle of *T. cruzi.*

Following infection, most patients are asymptomatic, although some experience an acute phase of the disease with symptoms including chagoma, a local reaction at the point of parasite entry, "general malaise, with fever, lymphadenopathies, hepatomegaly and splenomegaly" (2). After the acute phase, patients enter the chronic phase of the disease. This begins with the asymptomatic "indeterminate form", which the majority of patients remain in for the rest of their lives. A minority of patients enter the cardiac or digestive forms of the disease, and about 10% of patients experience symptoms of the peripheral nervous system. The cardiac form of the

disease usually appears 10-20 years after infection and symptoms include heart failure, cardiac arrhythmias and thromboembolism. The digestive form of the disease, also appearing long after infection, is typically identified by megaesophagus and megacolon, although other abnormalities are sometimes observed.

The large number of asymptomatic cases of *T. cruzi* infection is interesting, and raises the question of whether there is some kind of host- or parasite-derived protection from disease. One example of possible parasite-derived protection is PDNF (parasite-derived neurotrophic factor). PDNF, a GPI-anchored protein on the surface of *T. cruzi*, is a mimic of the neurotrophin nerve growth factor (NGF), which is involved in neural development and repair in mammals (3). PDNF protects various types of neuronal cells from injury caused by starvation or toxicity from various compounds, including a neurotoxin that causes Parkinson's disease (4). PDNF activates at least two survival signaling pathways – phosphatidyl 3-kinase/Akt protein kinase and mitogen-activated protein kinase—that underlie neuron survival and differentiation (5,6). These properties could be important for *T. cruzi* to promote nervous tissue repair in the very site it invades, protecting the host's cells from the damage the parasitic infection would otherwise cause.

Following the discovery of PDNF, a screen was performed using affinity chromatography to other growth factor receptors in order to determine whether any *T. cruzi* proteins could bind other host neurotrophic receptors. This screen identified a mimic of the glial-cell-line derived neurotrophic factor (GDNF) family of ligands (GFL), named TGFL (*T. cruzi* mimic of GFL). Like NGF, GFLs are important in neural development and maintenance. There are four GDNF family receptors , GFRα-1, 2, 3, and 4. The proteins are GPI anchored to the outer membrane of many cell types and trigger RET tyrosine kinase signaling by binding one of the four GFLs: GDNF, neurturin, artemin, and persephin. GDNF binds to GFRα1, neurturin binds to GFRα2,

artemin to GFRα3, and persephin binds to GFRα4. Neurturin and artemin may interact weakly with GFRα1, and GDNF may interact with GFRα2 and GFRα3, but there is generally little crosstalk between the GFLs and GFRs (7).

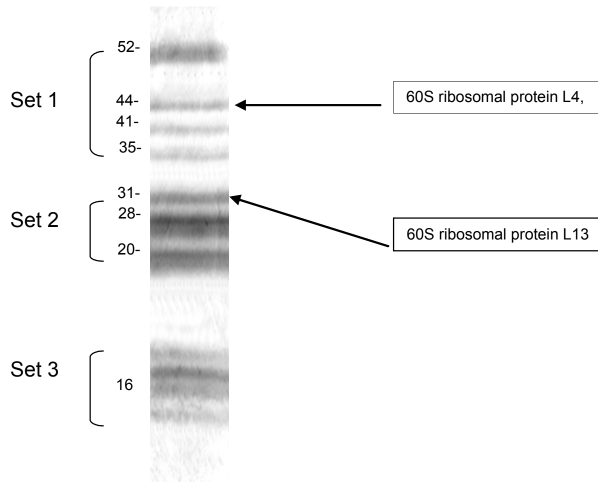

Figure 4. SDS-PAGE of TGFL, affinity purified with GFRα-1. Coomassie staining reveals three sets of proteins. Mass spectrometry analysis identified two ribosomal proteins: one protein in set 1, 60S ribosomal protein L4, and one protein in set 2, 60S ribosomal protein L13.

TGFL, on the other hand, binds the GFL receptors GFRα1, 2, and 3 with equal affinity (GFRα4 was not tested), but does not bind other neurotrophic receptors. Like GDNF, it promotes neurite outgrowth and survival *in vitro* (8). TGFL consists of three sets of proteins. When these proteins were analyzed by mass spectrometry, two of the proteins were identified as the *T. cruzi* ribosomal proteins L13 and L4 (Figure 4).

The eukaryotic 80S ribosome is made up of two subunits, the small 40S subunit and the large 60S subunit. Besides containing ribosomal RNAs, the eukaryotic ribosome contains about eighty ribosomal proteins. These proteins are synthesized in the cytoplasm, and imported into the nucleolus where the ribosome is assembled. Following assembly, ribosomes are transported back into the cytoplasm where they facilitate the translation of mRNA into protein (9).

It is certainly unusual that *T. cruzi* expresses ribosomal proteins on its surface, but it is not the first instance of membrane-bound ribosomal proteins. Despite their primary function in the cytoplasm of both eukaryotes and prokaryotes, ribosomal proteins can occasionally be

expressed on the cell surface. Heparin/heparan sulfate interacting protein/ribosomal protein L29 (HIP/RPL29), first discovered on uterine epithelial cells, is found on the surface of many human cells and tissues. RPL29 is up-regulated in 70% of cancer cells. Liu, et al discovered that RPL29 has an anti-apoptotic effect on colon cancer cells, and that knocking down expression of this cell surface ribosomal protein both increases the number of apoptotic cells and renders the cells more susceptible to sodium butyrate-induced apoptosis. RPL29 may play a role in cell-cell and cell-matrix interactions or may be involved in regulating interactions between growth factors and their receptors, either of which might explain its anti-apoptotic effect (10-15). Alternatively, a decrease in RPL29 expression may trigger the apoptotic pathway directly. Although its function is not well understood, it is clear that this ribosomal protein is both expressed on the cell surface and playing a role in cellular activities besides translation.

Similarly, another group examined the total integral and peripheral membrane proteins in *Yersinia pestis,* the bacterium that causes the plague. Their analysis identified a number of ribosomal proteins as “true peripheral membrane proteins” although they designated the proteins as contaminants because their presence on the cell surface seemed so unlikely (15).

The human ribosomal protein L13 is also known to have non-ribosomal functions in human cells. Also known as BBC1, breast basic conserved protein, this ribosomal protein is differentially expressed in some tumors, and is thought to play a role in a number of cancers, including breast, pancreatic, and prostate cancer (16,17). These are just a few examples of the many functions ribosomal proteins have been found to have (9,18-23). In this study, we hope to elucidate the possible non-ribosomal, neurotrophic functions of the *T. cruzi* ribosomal proteins L13 and L4.

The surface-bound TGFL may activate neurotrophic receptors in a novel way in order to repair infected nervous tissue, not unlike PDNF. Furthermore, it is possible that these ribosomal proteins might neutralize RNA and DNA, particularly unmethylated CpG motifs, in order to reduce inflammation and, consequently, promote parasitism. The discovery that ribosomal proteins make up this neurotrophic complex, TGFL, was totally unexpected, but provides a unique opportunity to study the function of ribosomal proteins expressed on the surface of a cell. The goal of this project was, time permitting, to clone and purify *T. cruzi* ribosomal proteins L13 and L4, and characterize their biochemical and biological role in host-parasite interactions.

MATERIALS AND METHODS

Subcloning

The L13 and L4 genes amplified from *T. cruzi* genomic DNA by PCR. *T. cruzi* lacks introns, so genomic DNA could be used instead of cDNA for this reaction. A number of primers were designed to amplify the genes based on published sequences. The primers amplified the entire sequence of the gene, excluding the stop codon. By excluding the stop codon, a C-terminal tag could be included on the recombinant proteins. The primers also incorporated restriction endonuclease sites, so that the PCR products could be cloned into the final vector. Directional cloning was used, so each primer pair incorporated two different restriction sites to ensure that the gene would be inserted into the vector in the right orientation. The primers designed were then used in a PCR reaction, and the product of the primer pair that produced the most DNA was purified and used for further cloning steps. L13 was amplified using the forward primer 5'-ATGAATTCATGCCGAAGGGAAAAAACGCG-3' incorporating a EcoRI site, and the reverse primer 5'-GCCTCGAGCTTCTTCTCGTTCTCTTTG-3' incorporating a XhoI site. L4 was amplified using the forward primer 5'-TTTCCCGGGTATGACTGCTCGTCCGT-3' incorporating a SmaI site, and the reverse primer 5'-CCGCTCGAGCTTCTTCTTGGC-3' incorporating an XhoI site. The PCR conditions were a denaturation for 3 min at 93°C followed by thirty cycles of denaturation for 30s at 93°, annealing for 30s at 55°C, and extension for 90s at 68°C, and finished with an extension for 30 min at 68°C.

The PCR products from each reaction were subcloned into the pCR 2.1-TOPO vector (Invitrogen) using TA cloning. This type of subcloning takes advantage of the 3' adenosine overhangs produced by Taq polymerase during PCR, and allows PCR products to be ligated directly into the TOPO vector. 4 µL of the PCR products were added to 1µL of salt solution (1.2

M NaCl, 0.06 M $MgCl_2$) and 1 μL TOPO vector. This mixture was incubated at room temperature for 30 minutes. 2μL of the product of this ligation reaction were added to TOP10 competent cells (Invitrogen), incubated on ice for 30 minutes, and then heat-shocked at 42°C for 30 seconds. 250μL of SOC media was added to the bacteria and they were incubated with shaking (225 rpm) for 1 hour at 37°C. The bacteria were then plated onto kanamycin/X-gal plates. Positive clones contain the plasmid, which confers kanamycin resistance, but presence of the insert interrupts the lacZ gene, allowing clones containing the insert to be selected by blue/white screening. White colonies were selected, and the TOPO plasmid was isolated by miniprep and digested with the appropriate restriction endonucleases. The plasmids from clones that showed an excised band of the right size were then sequenced and aligned with the published L13 or L4 sequence. Only clones with silent mutations were used in later steps.

Cloning into pGex-6p-1 Vector

The PCR product was excised from the TOPO vector and re-ligated into a pGex-6p-1 vector with a 3x-Flag sequence 3' to the multiple cloning site. This vector produced recombinant proteins with a C-terminal Flag tag, as well as the N-terminal GST tag that is normally included in the pGex plasmid. Dephosphorylation of the vector DNA and ligation was performed using Rapid DNA Dephos and Ligation Kit (Roche) following manufacturer's instructions. 2 μL of the ligation reaction were transformed into TOP10 competent cells following the procedure used for the subcloning products. Ampicillin resistant clones were selected, and the presence of the appropriate insert was confirmed by restriction digest.

The pGex plasmid for each protein was then purified and transformed into BL21 bacteria, a strain of *E. coli* that lacks proteases that could potentially degrade any fusion protein. 1 μL of

plasmid was added to the bacteria, incubated on ice for 30 minutes and heat shocked at 42°C for 30 seconds. 250 µL of SOC medium was added to the cells, and the cells were incubated at 37°C for 1 hr with shaking (225 rpm). The cells were then selected for ampicillin resistance by plating on ampicillin plates.

Small-scale Expression Screens and Optimization

BL21 clones were screened for expression of the L13 or L4 fusion protein. To screen clones, a small culture (2 mL) of each clone was grown in Luria-Bertani broth (LB) with ampicillin (0.1 mg/mL) at 37°C to OD_{600}= 0.6, at which point expression was induced by addition of Isopropyl β-D-1-thiogalactopyranoside (IPTG) to a concentration of 1mM. Expression was induced overnight at 25°C. After induction, bacterial pellets were resuspended in 1x SDS sample buffer, and analyzed by western blot with an anti-Flag or anti-GST antibody. The clones with the highest expression of fusion protein were used in further expression studies.

Expression conditions were then optimized for each clone using the same procedure as the expression screen, changing only IPTG concentration or OD_{600}. Expression was either induced with varying concentrations of IPTG (from 0.2 mM to 1.0 mM) or at different optical densities (OD_{600} from 0.6-1.3) in order to determine the optimal conditions. To determine if the expressed protein was soluble, bacterial pellets were resuspended in 1x PBS with 1 mM phenylmethylsulphonyl fluoride (PMSF) and sonicated on a setting of 8 for one minute (1 second on/1 second off) with a Fisher 550 Sonic Dismembrator. Lysate was centrifuged in a microfuge at maximum speed for 15 minutes, and the soluble and insoluble fractions were analyzed by western blot.

Large-scale Expression and Purification

Once optimal conditions for expression were determined, large-scale fusion protein purification was performed. A culture of BL21 cells (from 250 mL to 1 L) containing the plasmid of interest was grown in LB with ampicillin (0.1 mg/ml) to the optimal optical density. Then expression was induced with the appropriate concentration of IPTG overnight at 25°C. The bacteria were centrifuged at 6500 RPM for 15 minutes with a Beckman JLA 10.500 rotor, and the pellet was frozen at -80°C overnight. The pellet was then thawed and resuspended in 1x PBS with 1 mM PMSF and 0.1% Triton X-100. Cells were sonicated in a Fisher 550 Sonic Dismembrator at a setting of 8 for one minute (one second pulse followed by one second off). Lysis was confirmed by observation under a microscope. Lysate was then centrifuged for 15 min at 10,000 rpm with a Beckman A-20 rotor. The supernatant was filtered with a 0.45 μm filter. The filtered supernatant was then applied to a glutathione sepharose column containing 0.5 mL Glutathione Sepharose 4 Fast Flow. The column was washed with at least 20 column volumes of 1x PBS, and the fusion protein eluted with 5 mL elution buffer (150mM NaCl, 50 mM Tris-HCl, 10 mM reduced glutathione). The eluate was collected and then concentrated, typically to 250 μL, and washed with 1x PBS in order to remove reduced glutathione in the eluate.

In some cases, PreScission Protease (GE Life Sciences) was used to remove the GST tag from purified proteins. The amount of GST-tagged protein was approximated by absorbance at 280nm, based on the extinction coefficient of GST. In this case 1 A_{280} = 0.5 mg/mL. 1 μL of protease was used for every 100 ng in solution, and the reaction was conducted overnight at 4°C.

DNA and Protein Analysis

PCR products and restriction digests were electrophoresed on 1.5% agarose gels with 0.5 μg/mL ethidium bromide at 80V.

Proteins were analyzed by SDS-PAGE or western blot. Gels were 10-15% (w/v) polyacrylamide, depending on the desired resolution. For western blot analysis, proteins were transferred onto a nitrocellulose membrane by wet transfer at 0.4 A for 60 minutes. Membranes were blocked with 5% milk in TBST for 1 hour at 37°C with gentle shaking (70 rpm). Primary antibody was added in 5% milk at 1:2000 dilution. A monoclonal mouse anti-Flag antibody or a polyclonal rabbit anti-GST antibody was used. The primary antibody was applied at 4°C overnight, followed by washing with TBST. Conjugated secondary antibody, anti-mouse or anti-rabbit HRP, was added for 1 hour at room temperature, followed by washing with TBST. Finally, blots were incubated with chemiluminescence reagents in equal volumes for 1 minute at room temperature. The blots were developed on photographic film. For SDS-PAGE, gels were stained with Coomassie Brilliant Blue R-250 for 1 hour and destained overnight.

RESULTS

Cloning of L13 and L4

PCR was performed on *T. cruzi* genomic DNA using primers based on published sequences for the L13 and L4 proteins. Genomic DNA, rather than cDNA, was used because *T. cruzi* lacks introns. Stop codons were not amplified in the PCR reaction so that a C-terminal Flag-tag could be expressed on the recombinant proteins. The PCR products were checked by gel electrophoresis and were the right sizes based on published sequences, 1125 bp for L4 and 657 bp for L13 (Figure 5).

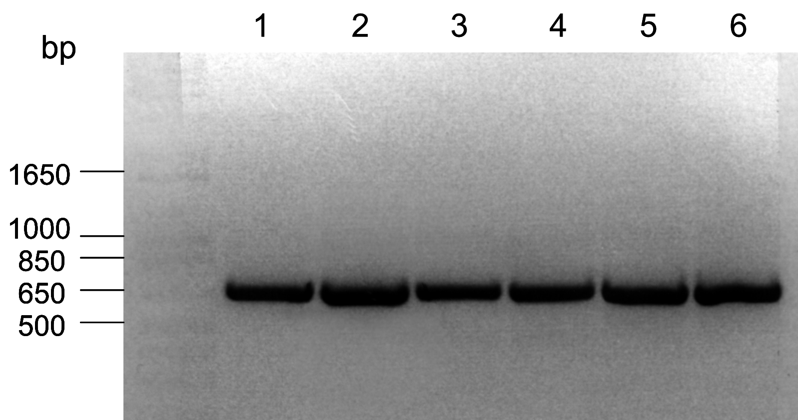

Figure 5. Agarose gel electrophoresis of L13 PCR products. Lanes are PCR products from PCR reactions using different combinations of primers. The product in lane 2 used the primers detailed in the methods section, and was purified and used for further cloning.

The PCR products were purified and subcloned into a pCR 2.1 TOPO vector (Figure 6A). Clones were selected by kanamycin resistance and by blue/white screening. The primers for L4 incorporated SmaI and XhoI sites, and the L13 primers incorporated EcoRI and XhoI sites, so the insertion of the PCR product into the vector was checked by restriction digest of purified plasmid DNA with these sets of restriction endonucleases(Figure 6B). Clones showing inserts of the appropriate size were then sequenced. The clones chosen for further cloning contained only silent mutations.

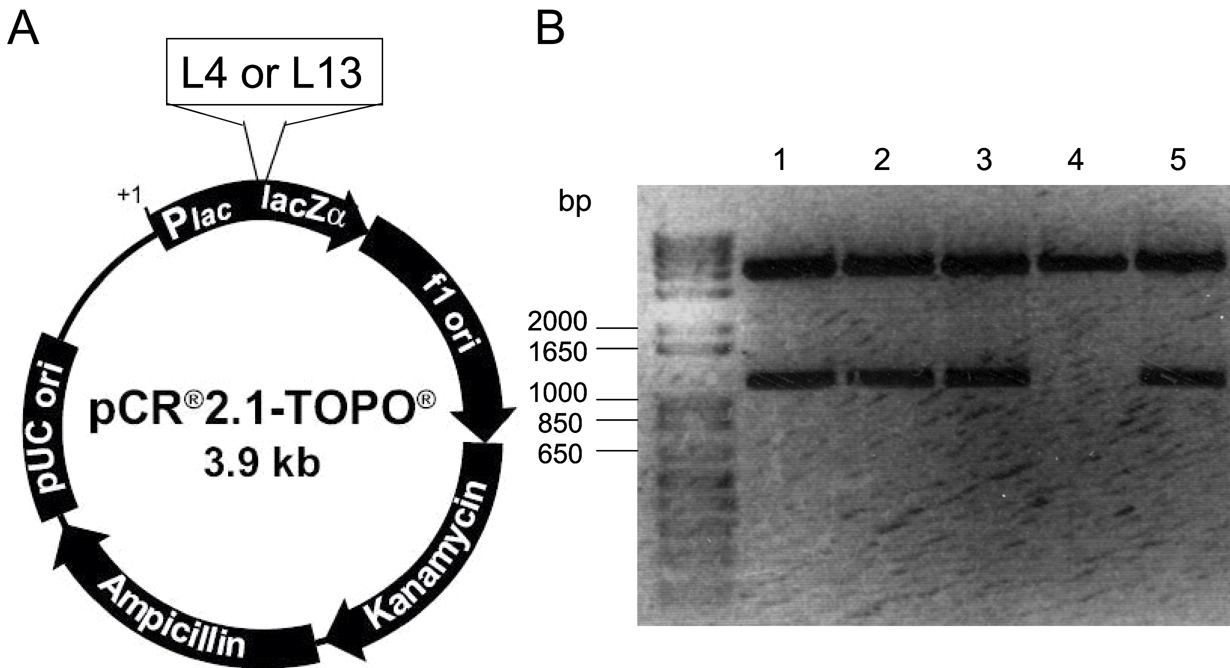

Figure 6. TOPO cloning. A) Map of the TOPO vector. PCR products were ligated directly into TOPO vector, interrupting the lacZ gene, using TA Cloning. Bacteria were transformed with the product of this reaction, and clones were selected for kanamycin resistance and by blue/white screening. B) Plasmid from L4 TOPO clones digested with XhoI and SmaI. Clones 1,2,3, and 5 have the L4 insert at 1150 bp.

The L13 and L4 genes were then excised from the TOPO clones and religated into a pGex-6p-1 vector. This vector contains an N-terminal GST tag to aid in purification of recombinant proteins, and a C-terminal Flag-tag to aid in identification of the proteins (Figure 7A). Clones were identified by ampicillin resistance and confirmed by restriction digest (Figure 7B). pGex plasmid DNA was purified and transformed into BL21 competent cells, a strain of *E. coli* lacking proteases that might degrade the recombinant protein.

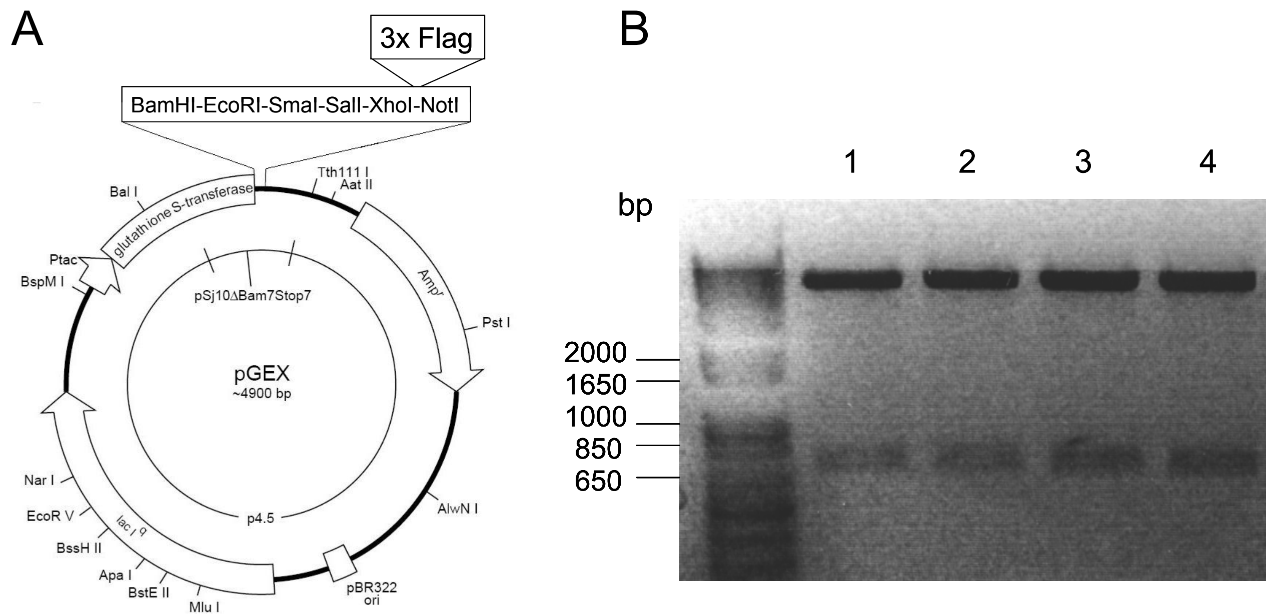

Figure 7. Cloning into pGex vector. A) pGex-6p-1 Vector. The GST gene is 5' to multiple cloning site (MCS) so proteins have a C-terminal GST tag, which aids in purification of recombinant proteins. A 3x Flag-tag sequence is inserted 3' to the MCS so that recombinant proteins can be identified with an anti-Flag antibody. B) pGex plasmid from L13 clones digested with XhoI and EcoRI. All four clones contain the insert at 657 bp.

L4 Expression and Purification

A number of clones containing the L4 plasmid DNA were screened for expression of the recombinant protein. Small cultures of bacteria were grown up to OD_{600}=0.6 and expression was induced with 1 mM IPTG for 2 hours at 25°C. A western blot of the bacterial lysate from each clone using an anti-Flag antibody revealed two clear bands (Figure 8). These bands were not present in the empty pGex vector expressing only Flag-tagged GST, and the bands were significantly stronger than those from bacteria grown without IPTG. The expected molecular weight of GST-tagged L4 is about 70 kD, and these bands appeared to run between 70-75 kDa. These results indicated that the clones were expressing L4 and that addition of IPTG induces expression.

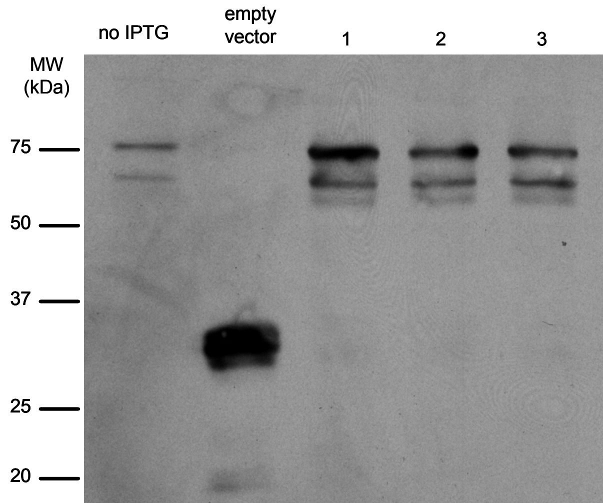

Figure 8. Screen of clones for expression of L4. Small cultures of each clone were grown to OD600=0.6 and expression was induced with 1 mM IPTG for 2 hours at 25°C. Bacteria were resuspended in SDS and expression was examined by western blot using an anti-Flag antibody. No IPTG: clone grown without IPTG. Empty vector: pGex vector with flag tag but no L4 insert. 1, 2, 3: clones tested for expression.

After expression was confirmed, optimal conditions for expression of soluble L4 were determined by testing induction at different optical densities and concentrations of IPTG (data not shown). Once conditions were determined, large scale purification was attempted. A 500mL culture was grown to OD= 0.6 and expression was induced with 0.4 mM IPTG overnight at 25°C, and the soluble fraction of the bacterial lysate was applied to a Glutathione Sepharose column. Recombinant L4 was eluted and concentrated. A portion of the purified protein was digested with PreScission protease to see if the GST tag could be successfully removed, as well as to further confirm the identity of the protein. Western blot with anti-Flag antibody showed the expected bands around 75 kDa, and when cut with the protease, a new band at 50 kDa (Figure 9A). GST is approximately 26 kDa, so the size of this new band corresponds to the size of the L4 protein alone. This confirmed that the L4 protein was expressed and purified. However, a western blot with anti-GST antibody showed a myriad of bands besides the ones seen with the anti-Flag antibody, indicating that the purified protein was not, in fact, pure. The protein could not be visualized by Coomassie staining (not shown). Not only was the protein impure, but its concentration too low for any useful test of biological activity. Examination of the bacterial lysate by Coomassie staining showed that L4 was clearly not being overexpressed by the BL21

E. coli (Figure 9B). Typically, recombinant GST-tagged proteins show a very prominent band in the bacterial lysate, but in this case, no strong bands were visible around 75 kDa.

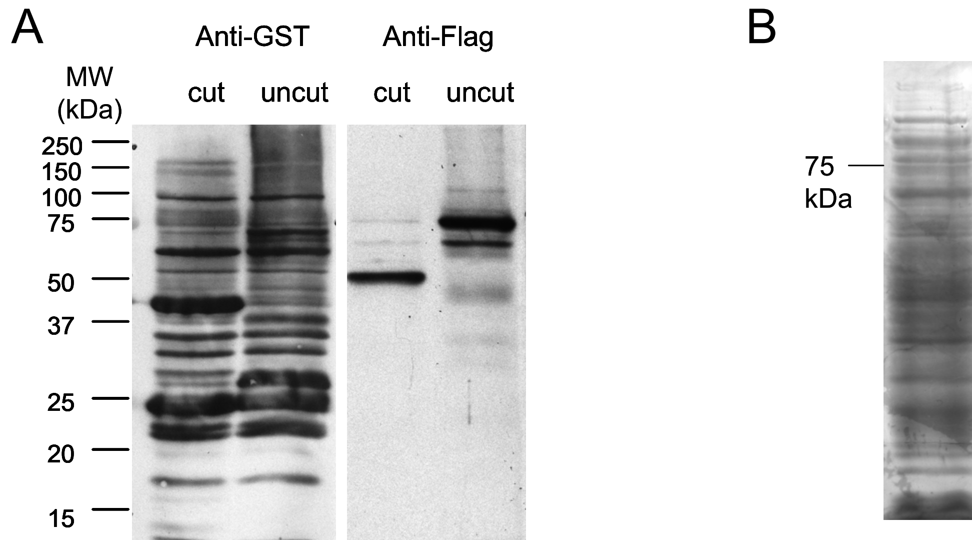

Figure 9. Analysis of L4 purification. A) Western blot of purified L4. Purified L4 was digested with PreScission Protease overnight at 4°C in order to remove the GST tag. Purified L4 (uncut) and digested L3 (cut) were analyzed by western blot with anti-Flag and anti-GST antibodies. B) SDS-PAGE of the soluble fraction of the bacterial lysate used for L4 purification.

L13 Expression and Purification

Clones containing the L13 plasmid DNA were screened for expression of the recombinant protein. Small cultures of bacteria were grown up to OD_{600}=0.6 and expression was induced with 1 mM IPTG for 2 hours at 25°C. A western blot of the bacterial lysate from each clone using an anti-GST antibody revealed a clear band above the 50kDa marker that was not present in the empty pGex vector expressing only Flag-tagged GST. This band was significantly stronger in the clones screened than in a clone grown without IPTG (Figure 10). The expected molecular weight of GST-tagged L13 is approximately 50 kDa, so the 50kDa band observed was L13, indicating that the clones were expressing the recombinant protein. Similarly, addition of IPTG induced expression of L13.

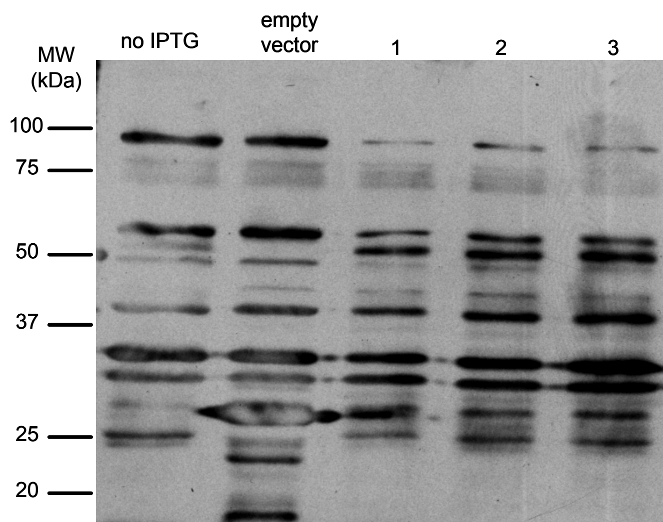

Figure 10. Screen of clones for expression of L13. Small cultures of each clone were grown to OD600=0.6 and expression was induced with 1 mM IPTG for 2 hours at 25°C. Bacteria were resuspended in SDS and expression was examined by western blot using an anti-GST antibody. No IPTG: clone grown without IPTG. Empty vector: pGex vector with flag tag but no L4 insert. 1, 2, 3: different clones tested for expression

Because expression of L4 was so low, expression of L13 was examined by SDS-PAGE before purification. Coomassie staining showed a prominent band above 50 kDa, indicating that the protein was overexpressed (Figure 11). The identity of the protein was further confirmed by western blot with anti-Flag antibody (Figure 11).

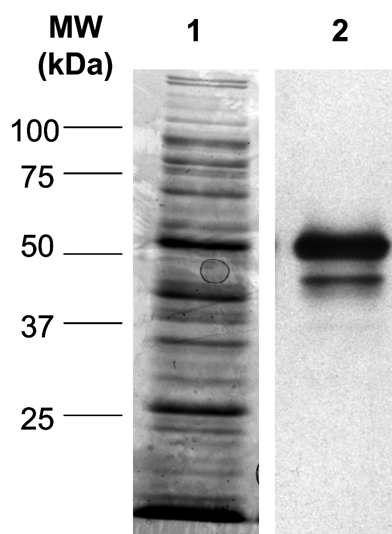

Figure 11. Analysis of expression of L13. Expression was induced in a 2 mL culture at OD600=1.3 with 0.6 mM IPTG at 25°C for 4 hours. 1) SDS-PAGE of soluble proteins after 4 hour induction. 2) Western blot analysis of soluble proteins after 4 hr induction using anti-Flag antibody.

After expression was confirmed, optimal conditions for expression of soluble L13 were determined by testing induction at different optical densities and concentrations of IPTG (data not shown). Once conditions were determined, large scale purification was attempted. A 250mL culture was grown to OD_{600}= 1.3 and expression was induced with 0.6 mM IPTG for 4 hours at 25°C, and the soluble fraction of the bacterial lysate was applied to a Glutathione Sepharose column. Recombinant L13 was eluted and concentrated. Coomassie staining showed the purified protein, but the concentration was extremely low (Figure 12). Binding was not efficient, with the majority of recombinant L13 present in the flow-through. Because the concentration was so low, the protein was washed with PBS to remove free glutathione in the solution, and half the volume was incubated with PreScission Protease in order to confirm its identity. Purified L13 before and after digestion with the protease were examined by SDS-PAGE. The undigested sample shows a clear band above the 50 kDa marker (Figure 13). In the digested sample, this band is lost, and is replaced by two bands, one at 25 kDa which is the cleaved GST tag, and one slightly above this, the L13 protein. The strong band above these is the 47 kDa PreScission protease. A number of other bands can be seen, however, besides the one associated with the recombinant fusion protein. Although the majority of protein eluted appears to be L13, the purification product is not homogeneous.

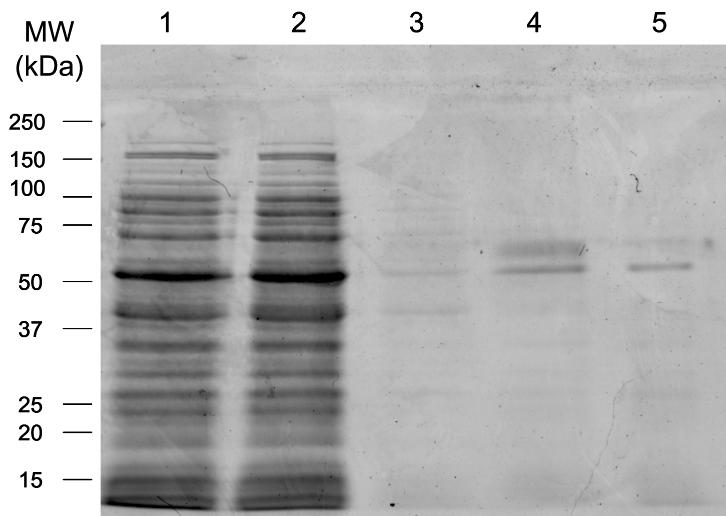

Figure 12. SDS-PAGE analysis of L13 purification. Expression was induced in a 250 mL culture at OD600=1.3 with 0.6 mM IPTG at 25°C for 4 hours. 1) Soluble fraction of bacterial lysate. 2) Flow-through after application to glutathione sepharose column. 3) Flow through after wash with PBS. 4) eluate (15 µL) 5) 20x concentrated eluate (1.5µL)

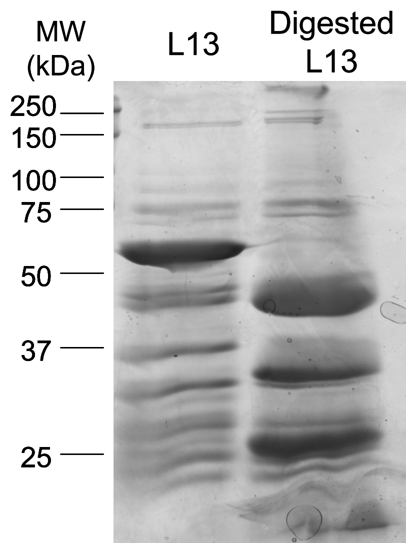

Figure 13. SDS-PAGE analysis of digested L13. The product from the purification of L13 was incubated with PreScission Protease overnight at 4°C. Undigested L13: L13 purification product not incubated with protease. Digested L13: L13 after incubation with protease

DISCUSSION

Many cases of Chagas' disease are asymptomatic for a very long time, if not indefinitely. This suggests that there may be some parasite-derived protection from disease, and tests to discover whether any *T. cruzi* proteins bind neurotrophic receptors, in order to promote neuronal cell survival, revealed an intriguing result. Not only does *T. cruzi* express a set of proteins on its surface that binds a neurotrophic receptor to promote neuronal cell growth and survival, but this complex is made up of ribosomal proteins. In order to more fully examine the role of these proteins and their neurotrophic effects, the genes encoding the ribosomal proteins needed to be cloned so that the proteins could be expressed in *E. coli.* In this project, the genes encoding *T. cruzi* ribosomal proteins L4 and L13 were successfully cloned into the pGex-6p-1 vector. The genes were amplified by PCR, and then subcloned into a TOPO vector. The subclones were sequenced to ensure no mutations in the final clones, and the genes were finally excised from the TOPO vector and ligated into the pGex vector. This vector encoded an N-terminal GST tag, as well as a c-terminal Flag tag. This cloning allowed for the expression of both recombinant proteins in *E. coli*, which will ultimately allow for the purification of these proteins and their use in experiments to determine the importance of L13 and L4 in *T. cruzi* infection.

However, there were a number of problems when large scale purification of L13 and L4 was attempted, which could not be addressed because of time constraints. The problems were in the poor expression of L4, and the low yield of a heterogeneous product upon purification of L13.

Although expression of L4 could be detected with the more sensitive western blot assay, it was not high enough for purification of a significant amount of the recombinant protein. GST-fusion proteins expressed in *E. coli* typically produce an enormous and noticeable band when the

bacterial lysate is visualized using SDS-PAGE. In the case of L4, there was no such band. As a result, when purification was attempted, only a small amount of the purified protein was seen using a western blot with a very sensitive monoclonal anti-Flag antibody. Not surprisingly, this protein could only be visualized by western blot and not by less sensitive assays.

Higher expression of L4 might be attained by altering induction conditions. Although expression was optimized in *E. coli* in LB medium using IPTG, expression might be higher under other conditions. For example, one group, while optimizing the expression of a GST-tagged *E. coli* protein, heat-labile enterotoxin B subunit (HLT-B), found that expression of their recombinant protein was many times higher when bacteria were grown in TB medium, rather than LB. Similarly, they found that expression induced with lactose, rather than IPTG, was nearly twice as high (24). Testing conditions such as growth medium may reveal the optimal conditions for expression of L4. If this does not work, it might be necessary to clone the gene into another vector, perhaps using a different affinity tag.

When L13 was purified, the yield, although higher than that of L4, was too low to perform any tests of biological activity. Because the protein was eluted with its GST tag, to remove the tag would require another round of purification to remove both the cleaved GST and the protease. Judging by the efficiency of the first purification, it is likely that a good deal of the purified recombinant L13 would be lost in this process. Therefore, it is important to improve the yield of this purification.

It is possible that scaling up the entire procedure would improve the yield of purified protein. However, the SDS-PAGE gel analyzing this first L13 purification suggests that the problem is not in the amount of L13 produced by the bacteria, but the binding of L13 to the affinity column; most of the protein seen in the bacterial lysate is also visible in the effluent. It is

unclear why binding is so inefficient. The Glutathione Sepharose 4 Fast Flow beads require dimeric GST for binding to the column, so the expressed protein must be concentrated enough to form these dimers. Improving expression, then, may improve binding. A higher concentration of recombinant protein could also be achieved by simply concentrating the bacterial lysate or lysing the induced bacteria in a smaller volume of binding buffer. On the other hand, it may be that the folding of the recombinant protein makes binding of the GST moiety inefficient in some way, in which case scaling up the procedure may be the only option. On-column cleavage with the PreScission Protease may have a higher yield as well, because it does not require a second round of purification to remove the GST tag.

The purified L13 was also quite heterogeneous, with a number of other *E. coli* proteins appearing in the eluate. It is possible that more extensive washing of the column, or washing under different conditions, such as high salt or higher temperatures, could remove some of these contaminants. Another option would be fast protein liquid chromatography (FPLC), which could provide a very pure sample of recombinant L13. FPLC may reduce the yield of purification, because some of the recombinant L13 would be lost in the process, and, coupled with the repurification after cleavage of the GST tag, it is unlikely that FPLC would yield any recombinant protein under the current conditions. Once the yield is improved, however, FPLC may be necessary to obtain a pure sample.

Once L13 and L4 are successfully purified, we will be able to test their biological activity. TGFL binds GFRα1, 2, and 3, and testing the recombinant proteins for binding to GFRα1,2, and 3 would confirm this result. Through its binding to the GFRs, TGFL promotes the growth and survival of host neuronal cells, so it will be important to determine whether these ribosomal proteins can promote growth and survival as well.

Besides their possible neurotrophic functions, these proteins could participate in the initiation of an anti-inflammatory response by *T. cruzi*. Ribosomal proteins typically bind RNA, so it may be that these *T. cruzi* surface-bound ribosomal proteins bind RNA as well, particularly RNA released when *T. cruzi* exits host cells, causing the cells to burst. If this were the case, TGFL's binding to RNA might prevent RNA-mediated activation of Toll-like receptors, initiating an anti-inflammatory response. This could be tested by determining whether the recombinant proteins bind RNA and DNA, as well as determining if they can neutralize a pro-inflammatory cytokine response *in vitro*.

Certainly, once purification of L4 and L13 is optimized, the role of these proteins in *T. cruzi* infection can be elucidated. *T. cruzi* has developed a very unusual way of communicating with its host: by expressing ribosomal proteins on its surface. Hopefully, a better understanding of the role of these proteins as members of TGFL will lead to a better understanding of TGFL as a whole, *T. cruzi* infection and invasion of cells in general, and, in the long term, perhaps lead to the development of better treatments or vaccines for Chagas' disease.

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