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Protein Farnesyltransferase from *Trypanosoma brucei*

A HETERODIMER OF 61- AND 65-kDa SUBUNITS AS A NEW TARGET FOR ANTIPARASITE THERAPEUTICS*

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We have previously shown that protein prenylation occurs in the Trypanosomatids *Trypanosoma brucei* (*T. brucei*), *Trypanosoma cruzi,* and *Leishmania mexicana* and that protein farnesyltransferase (PFT) activity can be detected in cytosolic extracts of insect (procyclic) form *T. brucei*. A PFT that transfers the farnesyl group from farnesyl pyrophosphate to a cysteine that is 4 residues upstream of the C terminus of the Ras GTP-binding protein RAS1-CVIM has now been purified 60,000-fold to near homogeneity from procyclic *T. brucei*. By screening a mixture of hexapeptides SSCAL*X* (*X* is 20 different amino acids), it was found that SSCALM binds to *T. brucei* PFT with sub-micromolar affinity, and affinity chromatography using this peptide was a key step in the purification of this enzyme. On SDS-polyacrylamide gel electrophoresis, the enzyme migrates as a pair of bands with apparent molecular masses of 61 and 65 kDa, and thus its subunits are ~30% larger than those of the mammalian homolog. The 61-kDa band was identified as the putative β-subunit by photoaffinity labeling with a ^{32}P-labeled analog of farnesyl pyrophosphate. Mimetics of the C-terminal tetrapeptide of prenyl acceptors have been previously shown to inhibit mammalian PFT, and these compounds also inhibit *T. brucei* PFT with affinities in the nanomolar to micromolar range, although the structure-activity relationship is very different for parasite *versus* mammalian enzyme. Unlike mammalian cells, the growth of bloodstream *T. brucei* is completely inhibited by low micromolar concentrations of two of the PFT inhibitors, and these compounds also block protein farnesylation in cultured parasites. These compounds also potently block the growth of the intracellular (amastigote) form of *T. cruzi* grown in fibroblast host cells. The results suggest that protein farnesylation is a target for the development of anti-trypanosomatid chemotherapeutics.

The attachment of the 15-carbon farnesyl group or the 20-carbon geranylgeranyl group to proteins, termed protein prenylation, was described for mating factor peptides from fungi (1) and for proteins in mammalian cells (2–5). Examples of prenylated proteins include nuclear lamin B (2), many of the small GTPases that are members of the Ras superfamily (6), heterotrimeric G protein γ-subunits (7, 8), and hepatitis delta virus large antigen (9). Evidence is mounting that protein prenyl groups not only anchor proteins in membranes, possibly by insertion of the prenyl group into the membrane, but also that they are recognized as molecular handles by other proteins (10–12).

To date, three enzymes have been identified in mammals and yeast that attach prenyl groups to proteins (13, 14). Protein farnesyltransferase (PFT)[1] catalyzes the transfer of the farnesyl group from FPP to the cysteine residue in the C-terminal motif Caa*X*, where C is cysteine, a is usually but not necessarily an aliphatic residue, and *X* is glutamine, serine, methionine, and probably a limited number of other residues (15). PGGT-I transfers the geranylgeranyl group from GGPP to the cysteine of the C-terminal motif Caa(L/F) (16, 17). Finally, protein geranylgeranyltransferase-II (also known as Rab geranylgeranyltransferase) attaches two geranylgeranyl groups to proteins with C-terminal sequences CC*XX*, C*X*C, and CC (18). PFT and PGGT-I are heterodimeric enzymes that consist of a common α-subunit and of distinct β-subunits (19–21). Protein geranylgeranyltransferase-II activity requires a catalytic core composed of two unique subunits and also a third component called Rep that presents the Rab protein to the catalytic core (22–25).

There is considerable interest in protein prenyltransferase inhibitors because it has been shown that selective inhibition of PFT efficiently suppresses transformation induced by oncogenic forms of Ha-Ras that are farnesylated (26, 27). However, cells transformed by oncogenic K-Ras, which is also normally farnesylated, as well as normal cells show relatively much less sensitivity to PFT inhibitors (28) but are sensitive to a combination of PFT and PGGT-I inhibitors (29). These results have been explained by the facts that in the presence of PFT inhibitors, K-Ras and N-Ras become geranylgeranylated instead of farnesylated in cells (30) and that geranylgeranylation of Ras proteins is sufficient for proliferation of normal and Ras-trans-

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[1] The abbreviations used are: PFT, protein farnesyltransferase; Caa*X*, C-terminal signal sequence of PFT and PGGT-I substrates (C is cysteine; a is usually but not always an aliphatic residue, and *X* is a variety of amino acids); [^{32}P]DATFP-GPP, derivative of geranyl pyrophosphate (^{32}P in both phosphates) containing a 2-diazo-3,3,3-trifluoropropionyloxy group attached to the terminal methyl group (*E* isomer); [^{3}H]FPP, [1-^{3}H]farnesyl pyrophosphate; [^{3}H]GGPP, [1-^{3}H]geranylgeranyl pyrophosphate; Ha-Ras-CVLS and Ha-Ras-CVLL, mutants of human Ha-RAS with C-terminal sequences CVLS and CVLL, respectively, and having an N-terminal 6-histidine tag; [^{3}H]MVL, (*RS*)-[5-^{3}H]mevalonolactone; RAS1-CVIM, a yeast RAS1 mutant produced in *E. coli* and with C-terminal sequence CVIM; PGGT-I, protein geranylgeranyltransferase-I; PAGE, polyacrylamide gel electrophoresis; DTT, dithiothreitol; HPLC, high performance liquid chromatography.

formed cells. By using selective PFT and PGGT-I inhibitors, FTI-277 and GGTI-298, Vogt and co-workers (31) have shown that protein geranylgeranylation but not farnesylation is critical for cell cycle progression from G_0/G_1 to S phase in mouse 3T3 cells. Possible reasons for the ability of PGGT-I inhibitors to block mammalian cell growth have been proposed (32, 33).

Recent studies have shown that protein prenylation occurs in protozoan parasites *Giardia lamblia* (34), *Trypanosoma brucei* (35, 36), *Trypanosoma cruzi* (37), *Leishmania mexicana* (37), and in *Schistosoma mansoni* (38). This is based on the observation that proteins in these parasites become radiolabeled when cells are cultured in the presence of radiolabeled mevalonic acid (MVL), the universal precursor of prenyl groups. None of these prenylated parasite proteins have been identified, but a major group includes those of molecular mass of ~25 kDa, which is typical of small GTP-binding proteins that belong to the Ras superfamily of regulatory proteins. The latter have been shown to be a major group of prenylated proteins in mammalian cells (6, 39, 40). Evidence is accumulating that Trypanosomatids contain heterotrimeric G proteins (41–48), and the γ-subunits of these signaling molecules are either geranylgeranylated (7, 8) or farnesylated (49) in mammalian cells. Rab GTP-binding proteins, which are known to be doubly geranylgeranylated in mammalian cells, have recently been cloned from Trypanosomatids (50–54). These parasite proteins have double cysteine C-terminal motifs (DSCC, GGCC, CAC) that are typical of the mammalian motifs that are recognized by protein geranylgeranyltransferase-II (18).

In the present study, we have purified PFT from insect stage (procyclic) *T. brucei* to near homogeneity. Since the sequences of Caa*X*-containing farnesyl acceptors from Trypanosomatids are not known, we prepared a mixtures of Caa*X*-containing peptides in order to find one that has high affinity for *T. brucei* PFT. Such a peptide formed the basis for affinity chromatography of the parasite enzyme, which was a key step in the overall ~60,000-fold purification. Photoaffinity radiolabeling of *T. brucei* PFT with a FPP photoprobe analog clearly identifies the *T. brucei* homolog of the mammalian PFT β-subunit. We also found that Caa*X* mimetic-based inhibitors of mammalian PFT are also inhibitors of *T. brucei* PFT. Unlike mammalian cells, sub to low micromolar concentrations of PFT inhibitors are lethal to cultured Trypanosomatids. The development of inhibitors of mammalian PFT is an advanced area of medicinal chemistry, and the Caa*X* substrate specificity of *T. brucei* PFT is very different from that of the mammalian enzyme (36). These results suggest that trypanosomatid PFT is an ideal target for the development of chemotherapeutics.

EXPERIMENTAL PROCEDURES

Materials—[^{3}H]FPP (20 Ci/mmol), [^{3}H]GGPP (15 Ci/mmol), and [^{3}H]MVL (15 Ci/mmol) were purchased from American Radiolabeled Chemicals. The radiochemical purity of the prenyl pyrophosphates was periodically assessed by thin layer chromatography (55), and the compound was used only if its purity was >80%. [^{32}P]Orthophosphoric acid (carrier-free) was obtained from NEN Life Science Products. Saponified simvastatin was obtained as a generous gift from Prof. A. Corsini (University of Milan). Ellman's reagent ([5,5′-dithiobis-(2-nitrobenzoic acid)) was from Pierce. Recombinant rat PFT and PGGT-I were produced in an Sf9/baculovirus expression system and purified as described (56). Recombinant baculoviruses that express the α- and β-subunits of rat PFT and PGGT-I were obtained as generous gifts from Prof. Y. Reiss (University of Tel Aviv). RAS1-CVIM and Ha-Ras-CVLL are generous gifts from Dr. C. Omer (Merck) and Prof. G. James (University of Texas). PFT and PGGT-I inhibitors were obtained as generous gifts from the following sources: L-745,631 from Dr. J. B. Gibbs (Merck) and FTI-276, FTI-277, GGTI-297, and GGTI-298 from Prof. A. D. Hamilton (Yale University).

The PFT inhibitor SCH-44342 was prepared as follows. Loratidine was obtained by extracting an aqueous suspension of the prescription drug Claritin with $CHCl_3$. To remove the ethoxycarbonyl group, the extract was concentrated to dryness, and the residue was refluxed for 6 h in concentrated aqueous HCl. After cooling, the mixture was neu-

TABLE I
Purification of T. brucei PFT

Purification step	Protein	Activity	Specific activity	Purification
	mg	*microunits*	*microunits/μg*	*-fold*
Cytosol	7,429	1,705	0.00023	1
$(NH_4)_2SO_4$ fractionation	1,328	1,820	0.0014	6.1
Q-Sepharose	133	1,155	0.0087	37.8
Phenyl-Sepharose	14.1	634	0.045	196
SSCALM-Sepharose	0.167	319	1.91	8,300
Superdex 200	0.012[a]	161.6	13.5	58,700

[a] Estimated from the intensity of protein bands in the silver-stained gel relative to standard amounts of rat PFT.

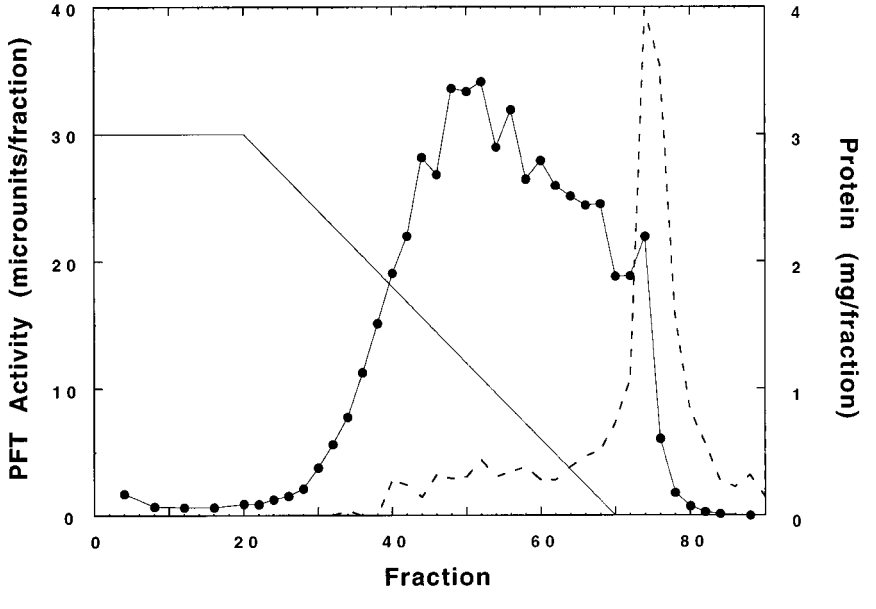


FIG. 1. **Phenyl-Sepharose chromatography of *T. brucei* PFT.** The material containing PFT obtained from Q-Sepharose chromatography (133 mg of protein, 1155 microunits) was mixed with 4 M KCl to give a final KCl concentration of 1.2 M, and the mixture was chromatographed on a phenyl-Sepharose 6 Fast Flow (low substitution) column (2.6 × 14 cm) as described under "Experimental Procedures." The gradient elution of decreasing concentrations of KCl (*solid line*) from 1.5 to 0 M starts at fraction 21 and ends at fraction 70. Fractions of 16 ml were collected. Aliquots of fractions were assayed for PFT (●) and protein (*dashed line*). *Fractions 38–70* were pooled.

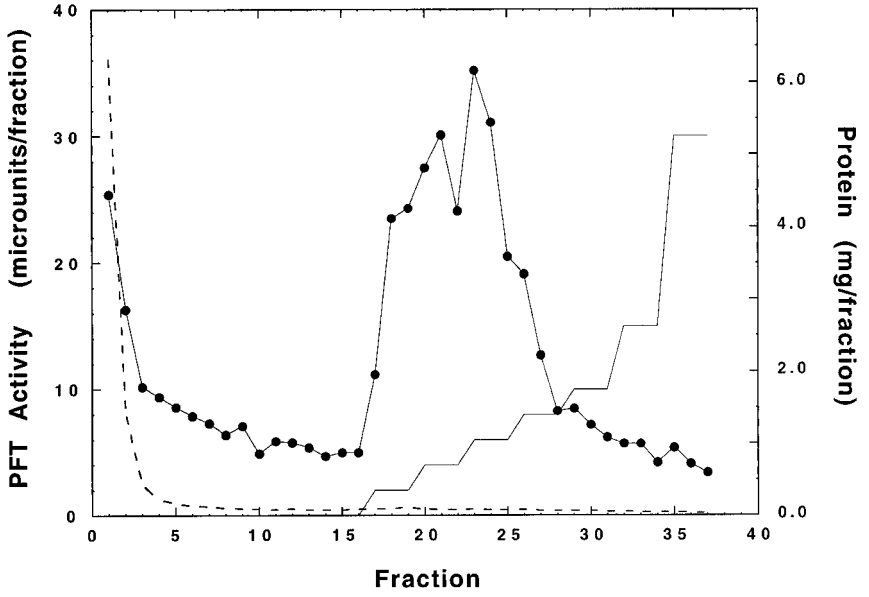
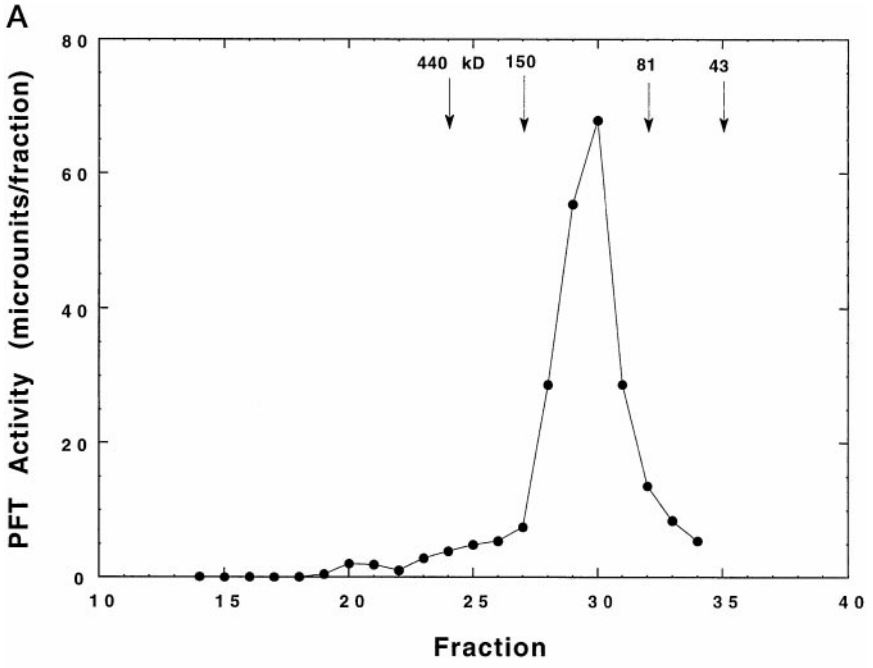


FIG. 2. **Peptide affinity chromatography of *T. brucei* PFT on SSCALM-Sepharose.** The PFT-containing material from the phenyl-Sepharose column (14.1 mg of protein, 634 microunits) was chromatographed on a 0.9-ml column of the peptide affinity gel. Stepwise elution with increasing NaCl concentrations (*solid line*) was carried out with 20 mM (*fractions 17–19*), 40 mM (*fractions 20–22*), 60 mM (*fractions 23–25*), 80 mM (*fractions 26–28*), 100 mM (*fractions 29–31*), 150 mM (*fractions 32–34*), and 300 mM NaCl (*fractions 35–37*) in the buffer. Three-μl aliquots of the fractions (3.6 ml) were assayed for PFT activity (●), and amount of protein (*dashed line*) was measured by Bradford assay. *Fractions 18–26* were pooled.



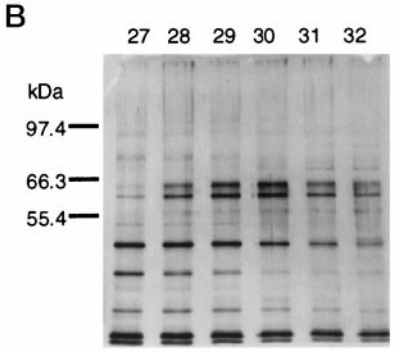


FIG. 3. **Superdex 200 chromatography of *T. brucei* PFT.** *A,* the peptide affinity column-purified PFT (167 μg of protein, 319 microunits) was chromatographed on a Superdex 200 HR10/30 column (1 × 30 cm). Aliquots (0.25 μl) of fractions (0.5 ml) were assayed for PFT activity (●). *B,* SDS-PAGE of Superdex 200 fractions. Six-μl aliquots of *fractions 27–32* were analyzed by SDS-PAGE using a 10% gel, and the gel was silver-stained.

tralized with concentrated NH_4OH and extracted with two portions of CH_2Cl_2. Solvent was removed to give the amine which was used without additional purification. Conversion of the amine to SCH-44342 was achieved as described (57), and the PFT inhibitor was purified by HPLC on a reverse-phase C18 column (Vydac 218TP1010) with a 30-min gradient from water with 0.1% HCO_2H to methanol with 0.1% HCO_2H. The proton NMR is identical to the published spectrum (57).

Preparation of [^{32}P]DATFP-GPP—The photoaffinity analog of FPP was prepared by a modification of the reported procedure (58, 59). In our hands, oxidation of chloroacetyl geraniol with H_2SeO_3/*t*-butyl hydroperoxide/salicylic acid yielded much more aldehyde than alcohol. Thus the mixture of products was reduced by treatment with 0.2 M $NaBH_4$ in absolute ethanol for 15 min at room temperature. Then, ~10 ml of 250 mM Hepes, pH 7.5, was added followed by ~10 ml of water.

The mixture was extracted with ethyl acetate, and the organic layer was washed with brine and dried over anhydrous Na_2SO_4. (*E,E*)-1,8-Dihydroxy-3,7-dimethyl-2,6-octadiene was purified by flash chromatography on silica gel using ethyl acetate in low boiling petroleum ether. The R_f value on a silica TLC plate was 0.6 with ethyl acetate. The diol was reacted with 1 eq of 2-diazo-3,3,3-trifluoroacetyl chloride as described (58, 59). Additional small portions of acyl chloride were added as needed to convert most of the diol to the mono-acetylated product with minimal production of the diester (followed by TLC). Flash chromatography with ethyl acetate in low boiling petroleum ether afforded the desired isomer (*E, E*)-8-DATFP-1-hydroxy-3,7-dimethyl-2,6-octadiene, which eluted slightly earlier than the isomer with the acyl group on the other hydroxyl.

The alcohol was submitted to Cramer phosphorylation as follows. Alcohol (2.9 mg) was dissolved in 38 μl of CH_3CN containing 5.2 μl of CCl_3CN. Carrier-free [^{32}P]orthophosphate (10 mCi) was dried *in vacuo* and mixed with 9.3 mg (47 μmol) of $[(CH_3)_3NH]_2HPO_3$ (final specific activity 0.21 Ci/mmol) in 720 μl of CH_3CN. This solution was added dropwise with stirring to the reaction mixture over 3 h at room temperature. After further stirring for an additional 24 h, 1.5 ml of 1 mM aqueous NH_4OH and 3.5 ml of ether were added. After vortexing, the aqueous phase was concentrated to dryness, and the residue was dissolved in 0.5 ml of 1 mM aqueous NH_4OH and chromatographed on an Amberlite XAD-2 column (1 ml). After washing three times with 1 ml of 1 mM aqueous NH_4OH, phosphorylated products were eluted with 1 mM aqueous NH_4OH/CH_3OH (1:1), and fractions of 1 ml were collected. The second and third fractions contained about 9.3×10^9 cpm and were combined. Analysis of these fractions on silica gel TLC with 2-propanol/NH_4OH/H_2O (6:3:1) followed by autoradiography showed that approximately 50% of the radioactive material in these fractions was [^{32}P]DATFP-GPP (it co-migrated with FPP), with most of the remaining radioactive material at the origin.

Preparation of Peptide Affinity Gel—The peptide affinity gel SSCALM-Sepharose was prepared by coupling 10 mg of the peptide SSCALM (SynPep) with 1.5 ml of pre-swollen activated CH-Sepharose 4B (Amersham Pharmacia Biotech) in 10 ml of 50 mM succinate NaOH, 0.5 M NaCl, pH 6.0, as described (17). Prior to coupling, the peptide was purified on a C18 reverse phase HPLC column (Vydac 218TP1010). The amount of peptide coupled to the gel was estimated to be 3.2 μmol/ml gel by measuring the SH group content using Ellman's reagent.

Screening for a Peptide That Binds Tightly to T. brucei PFT—The peptide mixture SSCAL*X*, where *X* is a mixture of 20 different naturally occurring amino acids, was prepared by SynPep Inc. using a mixture of amino acid loaded resins and extending this with SSCAL by standard coupling methods. After cleavage from the resin, SSCAL*X* was fractionated by HPLC on a C18 reverse phase column (Vydac 218TP1010) using a solvent gradient from 98% A (0.06% trifluoroacetic acid in water) and 2% B (0.054% trifluoroacetic acid in acetonitrile) to 60% B over 60 min. Absorbance at 230 nm was monitored, and 13 fractions, each containing 1–4 discernible peaks, were collected. After lyophilization of each fraction, residues were solubilized in water, and the concentration of SH-containing peptides was measured with Ellman's reagent. Each mixture was tested at a total peptide concentration (based on moles of SH) of 5 μM for inhibition of *T. brucei* PFT activity using partially purified enzyme (from the Q-Sepharose column, see below) and 5 μM RAS-CVIM substrate under the standard conditions (see below). The fraction eluting from the HPLC column at 28–33 min was found to contain the most potent PFT inhibitor: 73% inhibition. This fraction was submitted again to HPLC using a solvent gradient of 98% A, 2–10% B over 10 min and then to 50% B over 60 min. Of the ~4 UV peaks, only the material eluting at 38.2 min inhibited PFT (94% inhibition at 5 μM). This material was further purified by HPLC using a gradient of 98% A, 2–15% B over 10 min and then to 40% B over 60 min. The peptide eluted as a single peak at 33.2 min and was shown to have a mass of 611.2 by electrospray mass spectrometry, which is consistent with the SSCALM peptide (calculated 610.7). This peptide was synthesized on a 100-mg scale and shown to inhibit *T. brucei* PFT with an IC_{50} of 0.2 μM using the standard assay (see below).

Purification of T. brucei PFT—T. brucei brucei EATRO 140 (procyclic form) was cultured at 27 °C in SDM79 medium containing 10% fetal calf serum. The cells from 8–12-liter culture were collected, washed, and lysed in 1 mM Tris-HCl, 1 mM EDTA, pH 8.0, using a Dounce homogenizer as described (36). The post-mitochondrial fraction was obtained by centrifuging the lysate in 0.25 M sucrose at $15,800 \times g$ for 10 min at 4 °C. To the resulting supernatant were added the following components at the indicated final concentrations: 20 mM Tris-HCl, pH 8.0, 5 mM DTT, 5 μM $ZnCl_2$, and freshly added protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 30 μM each of tosyl-lysine chloromethylketone and tosyl-phenylalanine chloromethylketone, and 10 μg/ml each of aprotinin, leupeptin, and pepstatin A). The mixture was centrifuged at $120,000 \times g$ for 80 min at 4 °C. The supernatant (cytosol fraction) was subjected to protein precipitation with 55% saturated ammonium sulfate at 0 °C. After collecting the precipitate by centrifugation, the pellet was stored at −80 °C for up to 4 months.

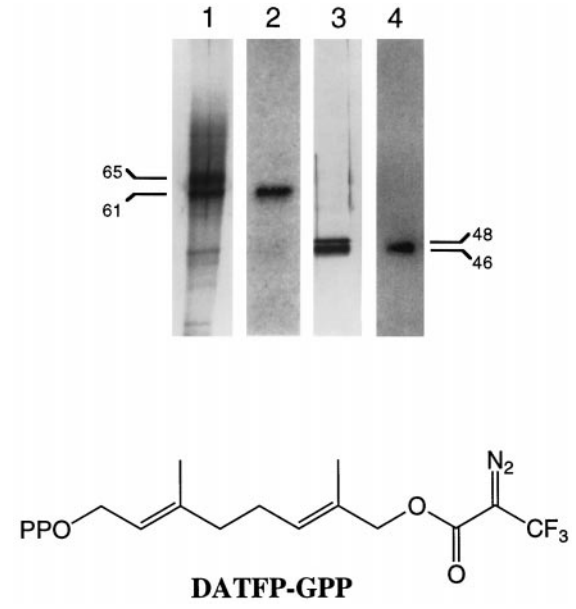


FIG. 4. **Photoaffinity labeling of *T. brucei* PFT by [^{32}P]DATFP-GPP.** An aliquot (16 μl) of Superdex 200 fraction 30 (Fig. 3) was labeled with 1 μM (4.2 μCi) [^{32}P]DATFP-GPP as described under "Experimental Procedures." The sample was analyzed by SDS-PAGE using a 10% gel. The gel was subjected to silver staining (*lane 1*) followed by autoradiography (*lane 2*). Two major silver-stained protein bands with molecular masses of 61 and 65 kDa are indicated. For comparison, recombinant rat PFT (20 ng) was also labeled with [^{32}P]DATFP-GPP in the same way. The silver stain (*lane 3*) and autoradiograph (*lane 4*) of the SDS-PAGE gel are shown. Migration positions of α- and β-subunits (48 and 46 kDa) of rat PFT are indicated. The structure of [^{32}P]DATFP-GPP is shown at the *bottom*.

Purification of PFT started from frozen ammonium sulfate precipitate that was obtained from a total of 44 liters of culture. Ice-cold buffer A (20 mM Tris-HCl, pH 8.0, 50 mM NaCl, 1 mM DTT) was added to the frozen precipitate, and after thawing at 4 °C, the material was dialyzed at 4 °C overnight against 4 liters of buffer A (three exchanges). All steps except affinity chromatography were carried out at 4 °C. The dialyzed 0–55% ammonium sulfate fraction (1.33 g of protein, Bradford dye binding assay from Bio-Rad) was loaded onto a column (4.8×22 cm) of Q-Sepharose Fast Flow (Amersham Pharmacia Biotech) previously equilibrated with buffer A. After washing the column with 2.5 liters of buffer A at a flow rate of 8 ml/min, a gradient of buffer A and buffer B (buffer A containing 1 M NaCl) was applied as follow: 0–12.6 min, 0–15% buffer B; 12.6–390.6 min, 15–60% buffer B; 390.6–403.2 min, 60–100% buffer B; 403.2–441 min, 100% buffer B. Fractions of 50.4 ml (6.3 min) were collected. Fractions comprising the major PFT peak (36) were pooled (535 ml).

This material was mixed with 4 M KCl to give a final salt concentration of 1.2 M and loaded onto a column (2.6×14 cm) of phenyl-Sepharose 6 Fast Flow (low substitution, Amersham Pharmacia Biotech) that was previously equilibrated with 20 mM Tris-HCl, 1 mM DTT, 1.5 M KCl, pH 7.4. After washing with 320 ml of the same buffer at a flow rate of 4 ml/min, a gradient of KCl from 1.5 to 0 M in 20 mM Tris-HCl, 1 mM DTT, pH 7.4 (total volume 800 ml), was applied. The column was washed with 320 ml of 20 mM Tris-HCl, 1 mM DTT, pH 7.4. Fractions of 16 ml were collected. The major PFT peak were pooled, and octyl-β-glucoside was added to give a final concentration of 0.1%. The solution was concentrated to about 300 μl by ultrafiltration using a stirred cell and a PM30 membrane followed by a Centricon 30 (Amicon). The buffer of the concentrate was replaced with 1.2 ml of 30 mM potassium phosphate, 1 mM DTT, 0.1% octyl-β-glucoside, pH 7.7 (buffer C), by two cycles of concentration and dilution with this buffer.

Affinity chromatography using SSCALM-Sepharose was carried out at room temperature using a glass wool-plugged Pasteur pipette containing 0.9 ml of settled gel. Prior to use, the gel was equilibrated with

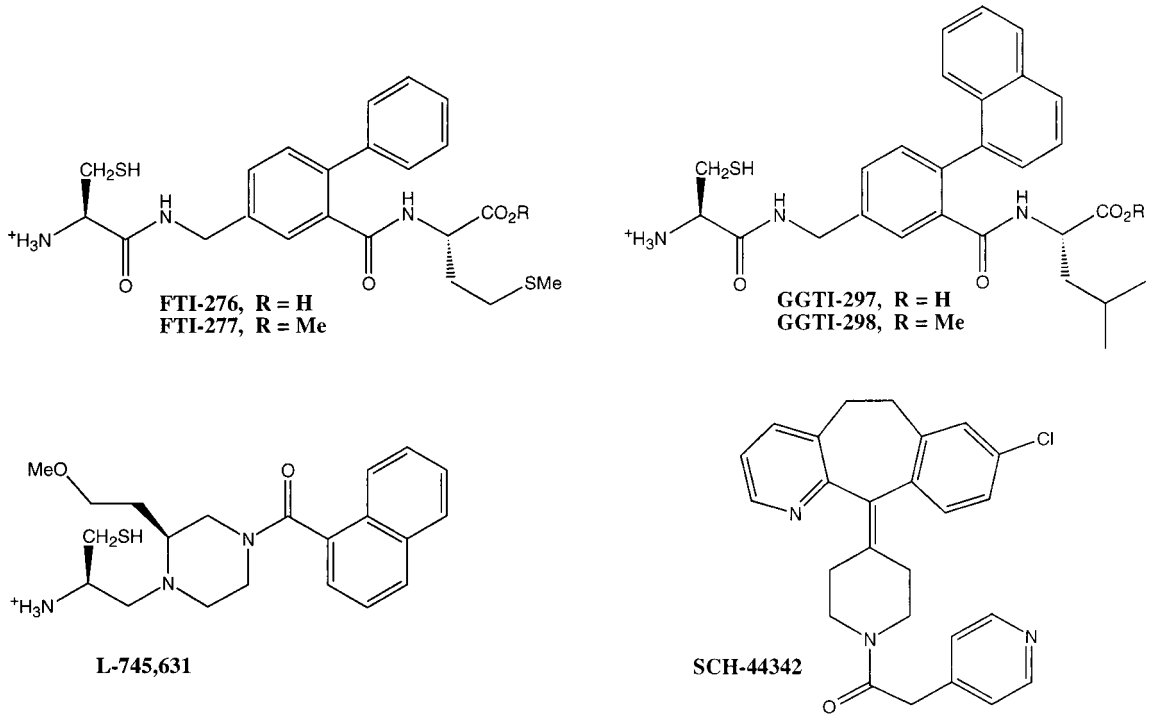


FIG. 5. **Structures of the *CaaX* mimetic inhibitors of PFT used in this study.**

buffer C. The sample was loaded onto the column, and the eluant was collected and re-loaded (gravity flow). This loading and re-loading cycle was repeated seven more times. The column was washed with multiple portions of buffer C (total 54 ml). Then the column was washed successively with 10.8 ml each of buffer C containing 20, 40, 60, 80, 100, 150, and 300 mM NaCl. The eluants were collected in 3.6-ml fractions. Fractions 18–26 (20–80 mM NaCl) that contained most of the PFT activity were pooled and concentrated to ~300 μl using a Centricon 30.

To the concentrated sample was added 1.7 ml of buffer D (20 mM Tris-HCl, 150 mM NaCl, 1 mM DTT, 0.2% octyl glucoside, pH 8.0), and the mixture was concentrated to 70 μl using a Centricon 30. This sample was subjected to gel filtration chromatography on a Superdex 200 HR10/30 column (1 × 30 cm, Amersham Pharmacia Biotech) that was previously equilibrated with buffer D. The column was eluted at a flow rate of 1 ml/min with the same buffer, and 0.5-ml fractions were collected.

PFT Assay—The standard assay for PFT enzymatic activity was carried out with 5 μM RAS1-CVIM, 0.75 μM [^{3}H]FPP (0.3 μCi), 30 mM potassium phosphate, 5 mM DTT, 0.5 mM $MgCl_2$, and 20 μM $ZnCl_2$, pH 7.7, in a total volume of 20 μl. After incubation at 30 °C for 15 min, the reaction was terminated by adding 200 μl of 10% HCl in ethanol, and the amount of ^{3}H-farnesylated protein was quantified by the glass fiber filter method (60). One microunit of PFT is defined as the amount of enzyme that produces 1 pmol of product per min.

For *in vitro* inhibition studies with Caa*X* mimetics, inhibitor stock solutions were prepared with 10 mM DTT in Me_2SO. Stock solutions were stored at −20 °C. Inhibitor solutions were diluted with the same solvent so that the final amount of Me_2SO in the PFT assay was 5% for all reactions.

Photoaffinity Labeling—A mixture of *T. brucei* PFT (16 μl of Superdex 200 fraction 30) and 1 μM [^{32}P]DATFP-GPP (0.21 Ci/mmol) in a total volume of 20 μl containing 16 mM Tris-HCl, 120 mM NaCl, 0.8 mM DTT, 0.16% octyl glucoside, pH 8.0, was placed in a porcelain dish on ice. The sample was illuminated with UV light for 10 min using a germicidal lamp (8 watts, General Electric G8T5) (bulb to sample distance of ~2 cm). The sample was treated at 40 °C for 30 min with Laemmli sample buffer containing β-mercaptoethanol (2% final concentration) and subjected to SDS-PAGE on a 10% gel. The gel was silver-stained, dried, and subjected to autoradiography using BioMax MS2 film with a BioMax intensifying screen (Kodak) at −80 °C for 2–4 days.

Experiments with Cultured Trypanosomes—Caa*X* mimetics were tested for their ability to block the growth of bloodstream and procyclic forms of *T. brucei* as described (37). All inhibitor stock solutions were prepared in 25% Me_2SO, 2.5 mM DTT in water. The final concentration of Me_2SO in culture media was held constant at 0.25%, which suppressed cell growth by <25%.

TABLE II
Inhibition of T. brucei PFT by CaaX mimetics

In vitro inhibition studies with PFT and PGGT-I inhibitors were carried out with *T. brucei* PFT (post-Superdex 200, 20 nanounits), with recombinant rat PFT (0.01 μg), and with rat PGGT-I (0.05 μg). Assays were carried out using 5 μM RAS-CVIM and 0.75 μM [^{3}H]FPP for PFT or using 5 μM Ha-Ras-CVLL and 1 μM [^{3}H]GGPP for PGGT-I as substrates. The slight differences in the IC_{50} values of some inhibitors for rat PFT measured in the present study *versus* previously reported values may be due to different protein substrates (and thus different K_M values) used in the various studies.

Inhibitor	IC_{50} (nM)[a]		
	T. brucei PFT	Rat PFT	Rat PGGT-I
L-745,631	52	23 (5)[b]	8,500 (10,000)[b]
FTI-276	1.7	4 (0.6)[b]	100 (50)[b]
FTI-277 (prodrug)	40	405	4,500
GGTI-297	3.2	35 (190)[b]	50 (50)[b]
GGTI-298 (prodrug)	46	500	305
SCH-44342	158,000	(250)[b]	(>114,000)[b]

[a] Estimated error for all IC_{50} values is <20%.

[b] Values in parentheses are previously reported IC_{50} values for L-745,631 (67) and for FTI-276 and GGTI-297 (70) (using Ha-Ras-CVLS and Ha-Ras-CVLL as substrates for human PFT and PGGT-I, respectively), and for SCH-44342 using Ha-Ras-CVLS and Ha-Ras-CVLL as substrates and rat PFT and PGGT-I, respectively (57).

Inhibition of growth of the intracellular form (amastigote) and insect form (epimastigote) of the Tulahuen strain of *T. cruzi* was studied using the β-galactosidase-based assay as described previously (37, 61). The effect of PFT inhibitors on the growth of noninfected 3T3 fibroblast host cells was quantified with Alamar Blue as described (61). The maximum concentration of Me_2SO and DTT in the culture medium was 0.1% and 0.01 mM, respectively, which alone had no effect on parasite or fibroblast cell growth.

Radiolabeling studies with [^{3}H]MVL were carried out with *T. brucei* bloodstream form, and radioactive prenylated proteins were visualized by fluorography of SDS-PAGE gels as described (37). Bloodstream form *T. brucei* (1 × 10^7 cells) was cultured for 24 h in 1 ml of medium containing 6.7 μM (100 μCi) [^{3}H]MVL and 40 μM saponified simvastatin in the presence and absence of FTI-277 or GGTI-298.

RESULTS AND DISCUSSION

Purification of T. brucei PFT—Our previous study of protein prenylation in *T. brucei* showed that PFT activity was detected

in cytosol of the procyclic form (36). In this earlier study, three proteins known to be substrates for mammalian prenyltransferases, Ha-Ras-CVLS, Ha-Ras-CVLL, and RAS1-CVIM were tested as substrates for partially purified *T. brucei* PFT. Surprisingly, Ha-Ras-CVLS, which is a good substrate for mammalian PFT, was not farnesylated by the parasite enzyme. The mammalian PGGT-I substrate Ha-Ras-CVLL was also not utilized by the parasite enzyme. Only the mammalian PFT substrate RAS1-CVIM was farnesylated by the *T. brucei* enzyme (36), and this protein was used to assay parasite PFT during purification. Following Q-Sepharose chromatography (36) (Table I), *T. brucei* PFT was further purified on phenyl-Sepharose (Fig. 1, Table I). Although parasite PFT eluted from this column as a broad peak, it was well separated from the major protein peak.

Affinity chromatography using peptides with Caa*X* C termini as ligands has been extremely useful for the purification of mammalian PFT and PGGT-I (15–17). *T. brucei* PFT did not bind to peptide affinity gel containing TKCVIM, which was used to purify rat PFT (15). Since the sequences of Caa*X*-containing proteins in Trypanosomes are not known, we decided to examine a mixture of peptides of the type SSCAL*X* (*X* is all 20 amino acids) in order to find an optimal peptide for use in affinity chromatography. Fractionation of this peptide mixture by reverse phase HPLC resulted in the identification of a

TABLE III

Inhibition of T. brucei and T. cruzi growth by CaaX mimetics

Parasites were cultured in the presence of Caa*X* mimetics or vehicle for 3 days (*T. brucei* bloodstream and procyclic forms) or 7 days (*T. cruzi* amastigotes in mouse 3T3 cells and epimastigotes).

Inhibitor	EC_{50}			
	T. brucei bloodstream form	*T. brucei* procyclic form	*T. cruzi* amastigote form	*T. cruzi* epimastigote form
		μM		
L-745,631	25	36	11	80
FTI-277	0.7	17	8	~100
GGTI-298	1.7	18	3	45
SCH-44342	30	ND[a]	ND	ND

[a] ND, not determined.

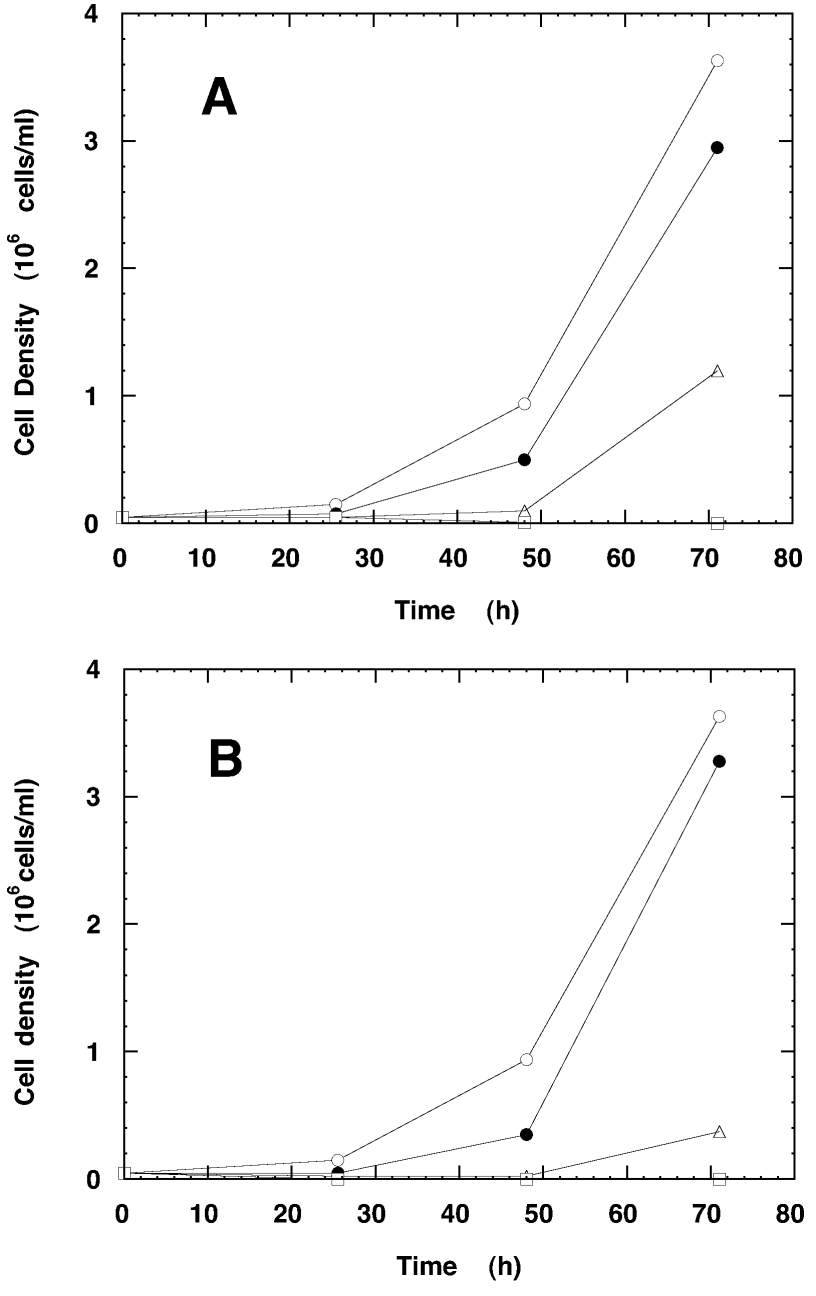


FIG. 6. **Growth inhibition of bloodstream form *T. brucei* by *CaaX* analogs.** *A,* bloodstream form *T. brucei* (5×10^4 cells/ml) was cultured with 0 (○), 0.5 (●), 0.75 (Δ), 1 μM (□) FTI-277 and cells were counted daily. *B,* same as *A* except with GGTI-298, 0 (○), 1 (●), 2.5 (Δ), or 5 μM (□).

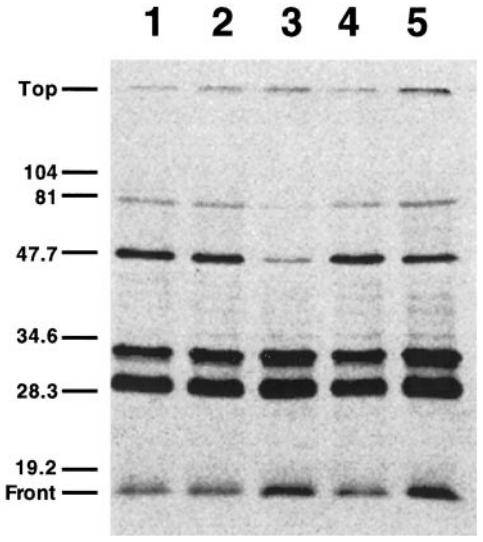


FIG. 7. **Inhibition of *in vivo* prenylation by *CaaX* analogs in bloodstream form *T. brucei*.** Bloodstream form *T. brucei* (1×10^7 cells) was labeled for 24 h with 6.7 μM (100 μCi) [^{3}H]MVL and 40 μM simvastatin in the absence (*lane 1*) and presence of 0.2 (*lane 2*) or 5 μM FTI-277 (*lane 3*), or 0.2 (*lane 4*) or 5 μM GGTI-298 (*lane 5*). Radiolabeled proteins were analyzed on SDS-PAGE using a 12.5% gel followed by fluorography. The gel was exposed to x-ray film at −80 °C for 7 days.

single peptide that inhibited *T. brucei* PFT with an IC_{50} of 0.2 μM. Mass spectrometry identified the active peptide as SSCALM. This peptide binds 25-fold tighter to *T. brucei* PFT than does RAS1-CVIM (IC_{50} of 0.2 μM in the presence of 5 μM RAS1-CVIM).

Use of SSCALM-Sepharose resulted in 40-fold purification of *T. brucei* PFT (Fig. 2, Table I). Interestingly, whereas mammalian PFT and PGGT-I remain bound to peptide affinity columns in the presence of 100 mM NaCl and elute when the pH is dropped below 6 (15–17), *T. brucei* PFT was eluted from SSCALM-Sepharose with buffer containing >20 mM NaCl at pH 7.7. Elution with buffer at low pH and without salt resulted in poor recovery and purification of *T. brucei* PFT.

After affinity chromatography, further purification was carried out by gel filtration on Superdex 200, and *T. brucei* PFT eluted as a single peak (Fig. 3*A* and Table I). Analysis of column fractions by SDS-PAGE with silver staining revealed a pair of bands of nearly equal intensity with apparent molecular masses of 61 and 65 kDa (Fig. 3*B*). Although other protein bands are seen, only the 61- and 65-kDa bands track with PFT enzymatic activity (Fig. 3, *A* and *B*), suggesting that these proteins are the subunits of parasite PFT. The overall purification starting from *T. brucei* procyclic form cytosol is about 60,000-fold with an overall yield of about 10%. The specific activity of the final preparation of 13.5 microunits/μg is comparable to the value for recombinant mammalian PFT of 10 microunits/μg using the same substrate RAS1-CVIM.

Photoaffinity Labeling of T. brucei PFT with [^{32}P]DATFP-GPP—In order to convincingly identify the *T. brucei* PFT subunit, photoaffinity labeling experiments were carried out with the FPP analog [^{32}P]DATFP-GPP (structure shown at the *bottom* of Fig. 4). First we showed that the presence of 10 μM non-labeled DATFP-GPP completely inhibited *T. brucei* PFT-catalyzed RAS1-CVIM farnesylation in the standard assay containing 0.75 μM [^{3}H]FPP. UV irradiation of a mixture of *T. brucei* PFT (fraction 30 from the Superdex 200 column) and 1 μM [^{32}P]DATFP-GPP resulted in the radiolabeling of a single band, the 61-kDa protein as shown by autoradiography of the silver-stained gel (Fig. 4). Control experiments carried out with rat PFT led to radiolabeling of the 46-kDa β-subunit (Fig. 4) as

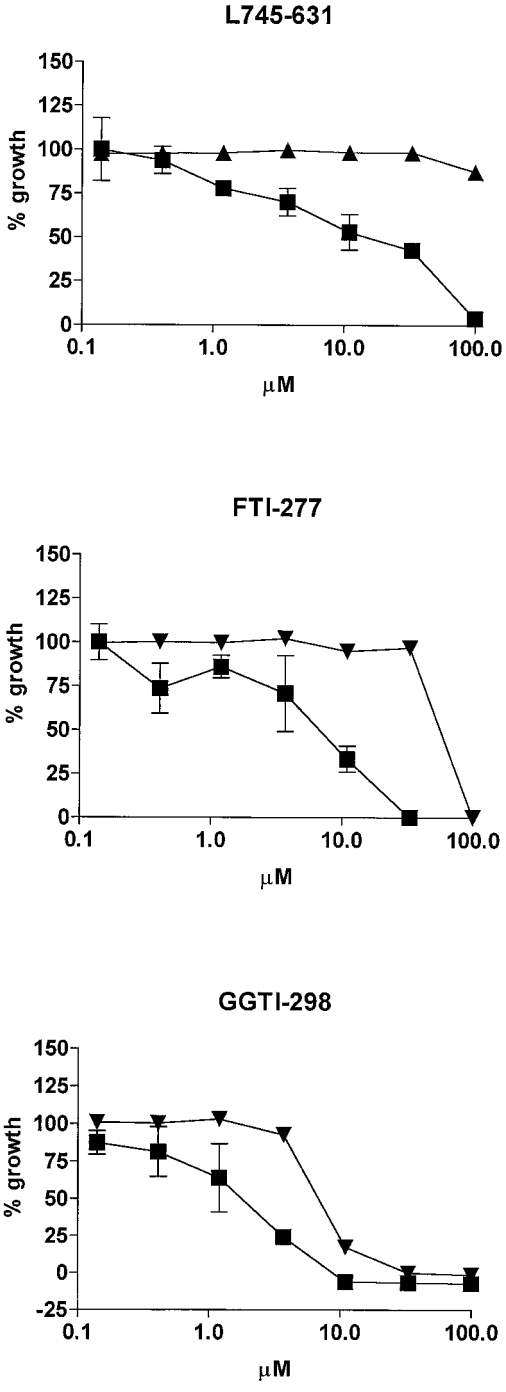


FIG. 8. **Growth inhibition of *T. cruzi* amastigotes and of murine 3T3 fibroblasts by *CaaX* mimetics.** Amastigotes (■) in 3T3 host cells were cultured in the presence of the indicated inhibitors for 7 days, and growth was measured by the colorimetric assay of β-galactosidase (37, 61). Separately, 3T3 fibroblasts (▲) were cultured in the presence of the inhibitor, and growth was measured with Alamar Blue after 5 days.

previously reported (62). It is highly likely that the 61-kDa protein is the β-subunit of *T. brucei* PFT and that the 65-kDa band is the α-subunit, and thus it appears that both subunits of the *T. brucei* enzyme are ~30% larger than the mammalian homologs.

Inhibition of T. brucei PFT by CaaX Mimetics—A number of inhibitors of mammalian PFT have been developed over the past few years since such compounds are potentially useful as anticancer chemotherapeutics (63–66). In this study we tested six Caa*X* mimetics as inhibitors of *T. brucei* PFT (Fig. 5). The

concentrations of inhibitors required for 50% inhibition of PFT activity *in vitro* (IC_{50}) are listed in Table II. FTI-277 and GGTI-298 are the methyl ester prodrugs of the free acids FTI-276 and GGTI-297, respectively. FTI-277, L-745,631, SCH-44342, and GGTI-298 apparently penetrate into cells because they block Ras farnesylation or Rap 1A geranylgeranylation in mammalian cells (31, 67, 68). As reported previously, the Merck compound L-745,631 inhibits mammalian PFT in the low nanomolar range and is highly selective for PFT over PGGT-I (Table II). L-745,631 is similarly potent against *T. brucei* PFT and the rat enzyme. The methionine-containing Caa*X* mimetic FTI-276, which is 25–100-fold more selective for mammalian PFT *versus* PGGT-I, is about 2-fold more potent on parasite PFT than on mammalian PFT. GGTI-297 is 2-fold less potent than FTI-276 toward parasite PFT, whereas it is at least 10-fold less potent than FTI-276 against mammalian PFT.

Methyl ester prodrugs FTI-277 and GGTI-298 retain high affinity for *T. brucei* PFT with potencies 10-fold higher than those against mammalian PFT and PGGT-I (Table II). The non-peptide Caa*X* mimetic SCH-44342 is 2 orders of magnitude less potent on *T. brucei* PFT *versus* the rat enzyme. All together, these results show substantial mammalian *versus T. brucei* species differences in the structure-activity relationship for a series of PFT inhibitors. Distinct Caa*X* recognition by *T. brucei* and mammalian enzymes was also observed in our earlier substrate specificity studies (36).

Growth Inhibition of T. brucei by CaaX Mimetics—The methyl ester prodrugs FTI-277 and GGTI-298, and L-745,631 and SCH-44342 were tested for their effect on the growth of the bloodstream and insect (procyclic) forms of *T. brucei*. Cells were cultured for 3 days in the presence of various amounts of inhibitors at which time potencies of the inhibitors were determined. The concentrations of inhibitors required to reduce the cell number relative to control culture by 2-fold (EC_{50}) are listed in Table III. Both FTI-277 and GGTI-298 are highly potent anti-parasite agents (EC_{50} = 0.7 and 1.7 μM, respectively). Growth curves for bloodstream form *T. brucei* in the presence of different concentrations of these compounds are shown in Fig. 6. Growth of bloodstream parasites was completely blocked with 1 μM FTI-277 and with 5 μM GGTI-298. GGTI-298 has been shown to be useful for selective inhibition of mammalian PGGT-I *in vivo* and for studying the consequence of this inhibition (31). However, growth inhibition of *T. brucei* caused by GGTI-298 might be due to inhibition of protein farnesylation but not geranylgeranylation since both this compound and FTI-277 are potent inhibitors of *T. brucei* PFT *in vitro* (Table II). The bloodstream form is more sensitive to these compounds than the procyclic form (Table III). L-745,631 and SCH-44342, which are considerably less potent at inhibiting *T. brucei* PFT *in vitro*, are also less potent at stunting parasite growth. For all tested inhibitors, cell shape deformation was observed within 24 h after adding inhibitor, and significant cell lysis occurs during the course of the treatment. Thus, these Caa*X* mimetics seem to be cytocidal rather than cytostatic.

Inhibition of in Vivo Protein Prenylation in T. brucei by CaaX Mimetics—The effect of PFT inhibitors FTI-277 and GGTI-298 on protein prenylation in bloodstream *T. brucei* was examined in cells labeled with [^{3}H]MVL in the presence of simvastatin (to block endogenous mevalonic acid production). As shown in Fig. 7, 5 μM FTI-277 significantly blocked tritium incorporation into a specific set of *T. brucei* proteins, with molecular masses of 48 and 77 kDa (as well as others). FTI-277 produced a stronger effect than did GGTI-298, which is consistent with its higher potency on *T. brucei* PFT activity *in vitro* and on cell growth. Radiolabeling of the prominent bands in the 28–35-kDa region was not significantly affected by these inhibitors. At least some

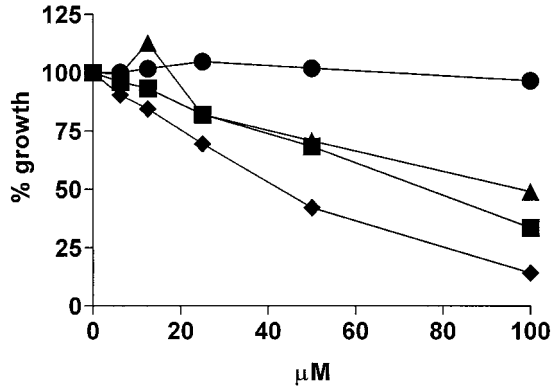


FIG. 9. **Growth inhibition of *T. cruzi* epimastigotes by *CaaX* mimetics.** Parasites were cultured in the presence of inhibitors (■, L745–631; ▲, FTI-277; ◆, GGTI-298) or in the presence of vehicle only (●) for 7 days, and growth was quantified by the colorimetric assay of β-galactosidase (37, 61).

of these proteins may be Rab proteins, which are present in Trypanosomatids, and which do not have Caa*X* sequences and are most likely doubly geranylgeranylated by a distinct protein prenyltransferase (see Introduction). Caa*X* mimetics are not expected to inhibit Rab protein geranylgeranyltransferase since the mammalian form of this enzyme does not recognize short peptides (14).

Growth Inhibition of Amastigote and Epimastigote Forms of T. cruzi by CaaX Mimetics—The effect of Caa*X* mimetics on the growth of *T. cruzi* amastigotes inside of 3T3 host cells was assayed using the Tulahuen strain of this parasite that is stably transfected with the *Escherichia coli* β-galactosidase gene (*lacZ*) (61). This procedure permits the number of amastigotes to be readily quantified using an enzyme-linked immunosorbent assay plate reader and a colorimetric substrate for *E. coli* β-galactosidase. Fig. 8 shows that FTI-277, GGTI-298, and L-745,631 block growth of *T. cruzi* amastigotes in a dose-dependent manner (measured after 7 days of culture). The EC_{50} values are listed in Table III. Of the three Caa*X* mimetics tested, only GGTI-298 was toxic at low micromolar amounts to noninfected 3T3 host cells. On the other hand, concentrations of L-745,631 and FTI-277 sufficient to completely block amastigote growth did not affect the growth of noninfected host cells. The mammalian PGGT-I inhibitor GGTI-298 but not the PFT inhibitor FTI-277 has been reported to block cell cycle phase progression from G_0/G_1 to S phase in 3T3 cells (31). As shown in Fig. 9 and Table III, the Caa*X* mimetics also inhibit the growth of insect form *T. cruzi* (epimastigote) in a dose-dependent manner. The compounds are roughly an order of magnitude less potent against epimastigotes compared with amastigotes.

Protein Farnesylation as a Target for Anti-trypanosomatid Chemotherapeutics—The following observations suggest that trypanosomatid PFT is an ideal target for the development of anti-parasite agents. 1) PFT inhibitors including Caa*X* mimetics and FPP analogs (37) are cytocidal to trypanosomatids at concentrations that are not cytocidal or cytostatic to mammalian cells. In fact two inhibitors of human PFT are currently undergoing clinical trials for the treatment of cancer, which indicates that these compounds are tolerated in humans. If trypanosomes do not have PGGT-I (36), some proteins which are mono-geranylgeranylated in mammalian cells may be farnesylated in trypanosomatids, and this could contribute to selective toxicity of PFT inhibitors for parasites. Further work on this issue is in progress. 2) There is an enormous medicinal chemical effort to develop mammalian PFT inhibitors as antineoplastic agents, and several classes of highly potent com-

pounds have been reported (63). Since resources for development of anti-trypanosomatid agents are very limited, making use of this wealth of medicinal chemical information is advantageous. 3) The specificity of mammalian and *T. brucei* PFTs with respect to Caa*X* recognition are very different (Table II and Refs. 36 and 37) and thus it should be possible to develop parasite-selective PFT inhibitors. For example SCH-44342 is more than 100-fold selective for the mammalian *versus T. brucei* PFT, and a compound that shows the reverse preference can presumably be obtained. The identification of the *T. brucei* PFT subunits as 61- and 65-kDa SDS-PAGE bands will allow their partial amino acid sequences to be determined. Molecular cloning and overexpression of the genes will allow crystallization trials to begin. Since the x-ray structure of mammalian PFT is available (69), the structure of the parasite enzyme would provide a framework for structure-based drug design of trypanosomatid-selective PFT inhibitors.

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