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Identification of a novel UDP-sugar pyrophosphorylase with a broad substrate specificity in *Trypanosoma cruzi*

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The diverse types of glycoconjugates synthesized by trypanosomatid parasites are unique compared with the host cells. These glycans are required for the parasite survival, invasion or evasion of the host immune system. Synthesis of those glycoconjugates requires a constant supply of nucleotide-sugars (NDP-sugars), yet little is known about how these NDP-sugars are made and supplied. In the present paper, we report a functional gene from *Trypanosoma cruzi* that encodes a nucleotidyltransferase, which is capable of transforming different types of sugar 1-phosphates and NTP into NDP-sugars. In the forward reaction, the enzyme catalyses the formation of UDP-glucose, UDP-galactose, UDP-xylose and UDP-glucuronic acid, from their respective monosaccharide 1-phosphates in the presence of UTP. The enzyme could also convert glucose 1-phosphate and TTP into TDP-glucose, albeit at lower efficiency. The enzyme requires bivalent ions (Mg^{2+} or Mn^{2+}) for its activity and is highly active between pH 6.5 and pH 8.0, and at 30–42 °C. The apparent K_m values for the forward reaction were 177 μM (glucose 1-phosphate) and 28.4 μM (UTP) respectively. The identification of this unusual parasite enzyme with such broad substrate specificities suggests an alternative pathway that might play an essential role for nucleotide-sugar biosynthesis and for the regulation of the NDP-sugar pool in the parasite.

Key words: nucleotide-sugar salvage pathway, Sloppy, *Trypanosoma cruzi*, UDP-sugar pyrophosphorylase.

INTRODUCTION

The trypanosomatid parasites are the cause of human and animal trypanosomiasis, which are fatal diseases. The trypanosomatid cell surface consists of various structurally complex and diverse carbohydrates that are critical for their survival and for the infection of the host cells and virulence [1,2]. Major components of those glycoconjugates (glycans), including galactopyranose, mannose, glucosamine, glucose and *N*-acetylglucosamine, are commonly found in *Leishmania major*, *Trypanosoma cruzi* and *Trypanosoma brucei*. However, the structures and sugar compositions of some glycoconjugates are fundamentally diverse among different types of trypanosomatid parasites. For example, *T. cruzi* exclusively produces a wide range of unique glycan structures: the protein O-linked glycan that contains *N*-acetylneuraminic acid and galactose residues [3], N-linked glycans that are decorated with L-fucose residues [4], and the protein phosphodiester-linked glycan that contains rare L-rhamnosyl and xylosyl residues [5,6]. To date, these glycan structures appear to be particular to *T. cruzi* and have not been reported in other parasites such as *T. brucei* and *L. major*. The specific glycosyl residues D-xylose, L-rhamnose, D-galactofuranose and L-fucose in *T. cruzi* were reported to be linked to the phosphoserine/phosphothreonine-linked carbohydrate chain of the glycoprotein gp72 [6], which is found to be associated with flagellal attachment. *T. cruzi* mutants lacking gp72 have reduced virulence in both stages of their life cycle: the insect and the mammalian host [7]. The function of these sugar residues within gp72 is lacking, as are the metabolic pathways leading to their formation.

The common glycosyl donors for the synthesis of glycoconjugates are nucleotide-sugars (NDP-sugars). Different types of NDP-sugars have been recently identified in the cell extracts of *L. major*, *T. cruzi* and *T. brucei* [3]. Some of these NDP-sugars are unique and not found in humans, such as UDP-galactofuranose, making the enzymes that generate them (e.g. UDP-galactopyranose mutase [8]) attractive targets for the development of anti-parasite drug. Although many genes involved in NDP-sugar biosynthesis have been identified in many species, less is known in *T. cruzi* regarding the synthesis of UDP-xylose, UDP-rhamnose or GDP-fucose, and whether their synthesis is analogous to other enzymes belonging to the interconversion pathway described in other organisms remained elusive. Some of the NDP-sugar biosynthetic genes were shown to be essential for the parasite invasion. UDP-galactose, for example, synthesized via UDP-glucose 4-epimerase is indispensable for both bloodstream-form and procyclic-form *T. brucei* [9–11] and is likely to be essential for epimastigote-form *T. cruzi* [12].

In addition to the NDP-sugar interconversion pathway, the role of the salvage pathway in supplying NDP-sugars to different metabolic processes remains largely unknown. In this pathway, free sugars generated from the degradation of polysaccharide, glycoprotein and glycolipid could be recycled into the cell by specific sugar transporters. These sugars can be phosphorylated at the C-1 position by specific kinases to form sugar 1-phosphates. Subsequently, a group of nucleotidyltransferases, also known as NDP-sugar PPases (pyrophosphorylases), could transfer a nucleotidyl residue to form NDP-sugars. Two functional PPase genes have been identified in trypanosomatid parasites: the UGP (UDP-glucose PPase) from *L. major* [13], and UAP (UDP-*N*-acetylglucosamine PPase) from *T. brucei* [14]. Knockout of the latter gene is lethal; however, a conditional mutant of this gene significantly reduces the amount of poly-*N*-acetylglucosamine structure and leads to underglycosylation of *T. brucei* glycoprotein, suggesting that other NDP-sugar pathways (i.e. interconversion) cannot substitute in forming UDP-*N*-acetylglucosamine. We are interested in identifying potential

Abbreviations used: AtSloppy, *Arabidopsis thaliana* Sloppy; LB, Luria–Bertani; LmSloppy, *Leishmania major* Sloppy; PPase, pyrophosphorylase; TcSloppy, *Trypanosoma cruzi* Sloppy; UAP, UDP-*N*-acetylglucosamine pyrophosphorylase; UGP, UDP-glucose pyrophosphorylase.

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enzymes that contribute to the synthesis of NDP-sugars to better understand how the formation of diverse glycans provides the organism fitness with its surrounding. This led, among others, to the isolation of a plant gene fondly named *Sloppy* [15], a unique PPase enzyme that utilizes diverse sugar 1-phosphates and UTP to form UDP-sugars [15–17]. On the basis of genome data to date, few organisms carry such a PPase. In the present paper, we first report the identification and characterization of a functional broad range UDP-sugar PPase in *T. cruzi* that could metabolize various sugar 1-phosphates: glucose 1-phosphate, galactose 1-phosphate, glucuronic acid 1-phosphate and xylose 1-phosphate into their corresponding UDP-sugars. The TcSloppy (*T. cruzi* Sloppy) may explain alternative pathways for the synthesis of UDP-xylose, UDP-galactose, UDP-glucuronic acid and perhaps UDP-rhamnose in this organism.

EXPERIMENTAL

cDNA cloning of TcSloppy

Total genomic DNA of *T. cruzi* was a gift from Dr Robert Sabatini (University of Georgia). The coding sequence of TcSloppy was amplified by PCR using 1 unit of high-fidelity proofreading Platinum DNA polymerase (Invitrogen) and 0.2 μM each of forward and reverse primers, 5′-TCatgaagatggtgcctgacg-3′ and 5′-GGATCctaaagcttcgcatgatg-3′ respectively (where upper case represents the nucleotides added for cloning purposes), using genomic DNA of *T. cruzi* as template. The PCR product was cloned to generate plasmid pCR4-topoTA:TcSloppy, and DNA was sequenced (GenBank® accession number GU443973, Tc00.1047053511761.10). The BspHI/KpnI fragment containing the full-length *Sloppy* gene without the stop codon was subcloned into an *Escherichia coli* expression vector derived from pET28b [18], generating Sloppy with an extension of six histidine residues at its N-terminus. Expression of the *Sloppy* gene is under the T7 promoter, and the plasmid was transformed into BL21(DE3)pLysS-derived *E. coli* strain (Novagen) for gene expression.

Protein expression and purification

E. coli cells harbouring the plasmid construct or an empty vector were cultured for 16 h at 37 °C in LB (Luria–Bertani) medium (20 ml) supplemented with kanamycin (50 μg/ml) and chloramphenicol (34 μg/ml). A portion (8 ml) of the cultured cells was transferred into fresh LB liquid medium (250 ml) supplemented with the same antibiotics, and the cells then grown at 37 °C at 250 rev./min until the D_{600} reached 0.6. The cultures were then transferred to 18 °C and gene expression was induced by the addition of isopropyl β-D-thiogalactoside to a final concentration of 0.5 mM. After 24 h of growth while shaking (250 rev./min), the cells were harvested by centrifugation at 6000 ***g*** for 10 min at 4 °C, resuspended in 10 ml of lysis buffer [50 mM Tris/HCl (pH 7.6), containing 10 % (v/v) glycerol, 50 mM NaCl, 1 mM $MgCl_2$, 0.5 mM EDTA, 1 mM DTT (dithiothreitol) and 0.5 mM PMSF] and lysed in an ice bath by 24 sonication cycles each of 10 s pulse and 20 s rest using a Misonix S-4000 sonicator equipped with microtip probe. The lysed cells were centrifuged at 20 000 ***g*** for 30 min at 4 °C, and the supernatant (termed s20) was recovered and kept at −20 °C. His_6-tagged proteins were purified on a column (10 mm internal diameter × 150 mm length) containing Ni^{2+}-Sepharose (2 ml, Qiagen) equilibrated with 50 mM sodium phosphate buffer (pH 7.6) containing 0.3 M NaCl. The bound His_6-tagged protein was eluted with the same buffer containing increasing concentrations of imidazole. The fractions containing Sloppy activity were stored in aliquots at −80 °C. The concentration of protein was determined using BSA as a standard. The molecular mass of the recombinant protein was estimated by size-exclusion chromatography using a Waters 626 LC HPLC system equipped with a photodiode array detector (PDA 996) and a Waters Millennium32 workstation. Separate solutions (0.5 ml) of TcSloppy or a mixture of standard proteins [10 mg each of alcohol dehydrogenase (157 kDa), BSA (66 kDa), carbonic anhydrase (29 kDa) and cytochrome *c* (12.4 kDa)] were chromatographed separately at 0.5 ml · min^{-1} on a Superdex 200 column (10 mm internal diameter × 300 mm length; GE Healthcare) equilibrated with 0.1 M sodium phosphate buffer (pH 7.6) containing 0.1 M NaCl. The eluant was monitored at A_{280} and fractions were collected every 15 s. Fractions containing enzyme activity were pooled and kept at −80 °C.

Enzyme assays

The typical forward HPLC-based reactions for the formation of NDP-sugars were carried out in a final volume of 50 μl and consisted of 1 mM sugar 1-phosphate, 1 mM UTP (or other NTPs), 5 mM $MgCl_2$, 100 mM Tris/HCl (pH 7.6), 1 unit of yeast inorganic pyrophosphatase (Sigma) and 45 ng of recombinant TcSloppy. After 15 min of incubation at 37 °C, reactions were terminated by heating for 1 min at 100 °C, an equal volume of chloroform was added, and the products were analysed by anion-exchange chromatography using a TSK-DEAD-5-PW column (7.5 mm internal diameter × 75 mm length; Bio-Rad Laboratories) and ammonium formate HPLC gradient system [15]. Nucleotides and nucleotide-sugars were detected by their UV absorbance using the photodiode array detector that was connected to the HPLC system. The maximum absorbance for uridine nucleotides and UDP-sugars was 261.8 nm in ammonium formate. The peak area of analytes was determined based on standard calibration curves. HPLC-based reverse reactions were carried out in a similar manner and included 1 mM PP_i, 1 mM UDP-sugar, 5 mM $MgCl_2$, 100 mM Tris/HCl (pH 7.6) and 45 ng of TcSloppy. After 15 min at 37 °C, reactions were terminated, and the amount of UTP produced was determined from a standard calibration curve.

Real-time ^{1}H-NMR analysis of NDP-sugar pyrophosphorylation

Individual pyrophosphorylation reaction mixtures in final volumes of 180 μl with $^2H_2O/H_2O$ at 8:1 (v/v) consisted of 100 mM sodium phosphate (pH 6), 5 mM $MgCl_2$, 1 mM UTP, 1 mM sugar 1-phosphate and enzyme: 0.9 μg of recombinant TcSloppy was supplied in water/buffer. Immediately upon addition of enzyme, the reaction mixture was transferred to a 3 mm NMR tube. In the combined UDP-sugar reaction, assays were as described above, but included 4 mM UTP and 1 mM of each UDP-sugar and 1 unit of yeast inorganic pyrophosphatase. Real-time ^{1}H-NMR spectra were obtained using a Varian Inova 600 MHz spectrometer equipped with a cryogenic probe. Data acquisition was not started until approx. 2 min after the addition of enzyme to the reaction mixture due to spectrometer set-up requirements (shimming). Sequential one-dimensional proton spectra were acquired over the course of the enzymatic reaction. All spectra were referenced to the water resonance at 4.765 p.p.m. downfield of DSS (2,2-dimethyl-2-silapentane-5-sulfonate). Processing of the data as covariance matrices was performed with Matlab (Mathworks).

Enzyme properties and inhibition assays

The forward PPase activity of TcSloppy was measured with various buffers, at different temperatures, different ions or

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different potential inhibitors. For the optimal pH experiments, 45 ng of recombinant enzyme was first mixed with 5 mM $MgCl_2$ and 100 mM of each individual buffer (Tris/HCl, phosphate, Mes, Mops or Hepes). The optimal pH assays were initiated after the addition of specific sugar 1-phosphate and UTP. Inhibitor assays were performed under standard assay conditions except for the addition of various additives (sugars or nucleotides) to the reaction buffer. These assays were incubated for 15 min at 37 °C, and were subsequently terminated by heating for 1 min at 100 °C. The amount of UDP-sugar formed was calculated from a calibration curve of HPLC UV spectra of standards. For the experiments aimed at defining the optimal temperature, assays were performed under standard assay conditions, except that reactions were incubated at different temperatures for 15 min. Subsequently, the activities were terminated by heating for 1 min at 100 °C. For the experiments aimed at determining whether TcSloppy required metals, assays were performed with UTP, specific sugar 1-phosphate (glucose 1-phosphate) and a variety of ions. After incubation at 37 °C for 15 min, the PPase activity was terminated by heating for 1 min at 100 °C. The amount of UDP-sugar formed was calculated from HPLC UV spectra of standards.

For the experiments aimed at determining the ability of TcSloppy to utilize other sugar 1-phosphates, Sloppy assays were performed under standard assay conditions, except for replacing the glucose 1-phosphate with different sugar 1-phosphates (glucuronic acid 1-phosphate, galactose 1-phosphate, etc.). These assays were incubated at 37 °C for 60 min (unless indicated otherwise), and were subsequently terminated by heating for 1 min at 100 °C. The amount of UDP-sugar formed was calculated from HPLC UV spectra.

Kinetics

The forward pyrophosphorylation catalytic activity of TcSloppy was determined at 37 °C for 5 min. Reactions contained 100 mM Tris/HCl (pH 7.6), 5 mM $MgCl_2$, 9 ng of recombinant enzyme (0.13 pmol) and various concentrations of UTP (40 μM–8 mM) and 1 mM glucose 1-phosphate or with 1 mM UTP and various concentrations of glucose 1-phosphate (40 μM–8 mM). The forward reaction was also carried out with various concentrations of galactose 1-phosphate or xylose 1-phosphate (40 μM–8 mM) and 1 mM UTP, using 74 and 220 ng of recombinant TcSloppy respectively. Kinetics for the forward reaction of TcSloppy with glucose 1-phosphate and TTP were carried out the same way as described for UTP, except for using 880 ng of recombinant TcSloppy. The kinetic assays were also carried out with 2 units of yeast inorganic pyrophosphatase to deplete PP_i. Kinetics for the reverse reactions were performed under the same conditions as above, with a fixed concentration of PP_i (1 mM) and various concentrations of UDP-Glc (40 μM–8 mM) and 9 ng of recombinant TcSloppy (0.13 pmol). In a separate series of reverse reaction experiments, assays were performed with a fixed amount of UDP-glucose (1 mM) and various concentrations of PP_i (40 μM–8 mM). Enzyme velocity data of the amount of UDP-sugar produced (μM/s), as a function of substrate concentrations were plotted. The Solver tool (Excel version 11.5) was used to generate a best-fit curve calculated by non-linear regression analyses, and for the calculation of V_{max} and apparent K_m.

RESULTS

Identification, cloning and characterization of TcSloppy

NTP-sugar PPases, also known as NTP:sugar-1-phosphate nucleotidyltransferases, are very specific for their nucleotides. Whereas some utilize ATP [19], others are specific for GTP, UTP, CTP or TTP [20]. Until recently, most PPases were shown to be specific for their sugar 1-phosphates as well. Some recognize mannose 1-phosphate; others recognize glucose 1-phosphate or *N*-acetylglucosamine 1-phosphate [21–23]. A few of the PPases, most notably the plant and bacterial ADP-glucose PPase, are allosterically regulated by intermediates of the carbon assimilation pathways [24]. The discovery of a 'Sloppy' UDP-sugar PPase in plants [15–17], an enzyme that can utilize diverse sugar 1-phosphates with UTP to form UDP-sugars, provides an alternative route to explain how NDP-sugars can be made. The existence of such an enzyme supports the essential role of the glycan-salvage pathway for normal cellular function. The cellular regulation of each specific NDP-sugar PPase compared with the Sloppy PPase remained unclear.

Although all NDP-sugar PPases belong to the same family of nucleotidyltransferases, they are very diverse with low amino acid sequence identity, although, on the basis of structural analyses [23,25,26], they appear to have a conserved fold. The nucleotidyltransferases also vary in length: in eukaryotes, UGP ranges in size across species from 470 to 510 amino acids, whereas TDP-glucose PPase in prokaryotes is much shorter at ~ 300 amino acids, and is more closely homologous with GDP-mannose PPase. The eukaryote UAP is 505 amino acids, and the plant UDP-sugar PPase Sloppy is 614 amino acids long. CMP-*N*-acetylneuraminic acid PPase and CMP-2-keto-3-deoxymanno-octulosonic PPase are also members of the family [27,28]. What the structural alterations in the ancestral PPase are that provide this class of enzymes their strict NTP and sugar specificities as opposed to being 'sloppy' remained unclear, since only plant Sloppy-like enzymes were characterized.

To identify other potential Sloppy-like PPases across species, we compared the AtSloppy (*Arabidopsis thaliana* Sloppy) protein sequence (GenBank® accession number ABC55066.1) with NR (non-redundant) sequence database. BLAST analyses of homologous proteins from different species revealed that Sloppy shares overall a low sequence identity with UGP and UAP (23 and 26 % respectively), suggesting they may share a similar protein fold and conserved catalytic residues. Sequence alignment of three PPases found two consensus motifs (Figure 1A): the N-terminal region of Sloppy consisted of a putative nucleotide-binding motif 'GG(L/Q)G(E/T)(R/T)(L/M)GX_3(I/P)K' (starting at residue 136) and the 'PXGHGD(V/I)HX_2(L/I)' (starting at residue 251) motif probably involved in uracil binding. Interestingly, and unexpectedly, was the identification of a relatively close Sloppy-like homologous protein in *T. cruzi*, an organism that is evolutionarily far removed from the plant kingdom. The *T. cruzi* Sloppy-like gene encodes a protein with 35 % amino acid identity with AtSloppy. Phylogenetic analysis (Figure 1B) indicated that the Sloppy-like proteins from different species are distinguished from UGP and UAP. To determine, however, whether the *T. cruzi* gene encodes a PPase and more specifically to establish whether it has a different or similar range of sugar 1-phosphates and NTP specificity, the gene was cloned and the recombinant protein, expressed in *E. coli*, was analysed.

A highly expressed protein band (67 kDa) was detected after SDS/PAGE analysis of *E. coli* cells expressing TcSloppy (see Supplementary Figure S1, lane 2 and 4, at http://www.BiochemJ.org/bj/429/bj4290533add.htm; indicated by the arrow). The mass of the column-purified protein is in agreement with the calculated mass of the translated gene product fused at the N-terminal portion to His_6. Preliminary experiments have shown that, in the presence of Mg^{2+}, the recombinant *T. cruzi* protein converts glucose 1-phosphate and UTP into a new UDP-sugar peak that was eluted at 12.3 min (see peak #1, in Figure 2, trace 1). This UDP-sugar

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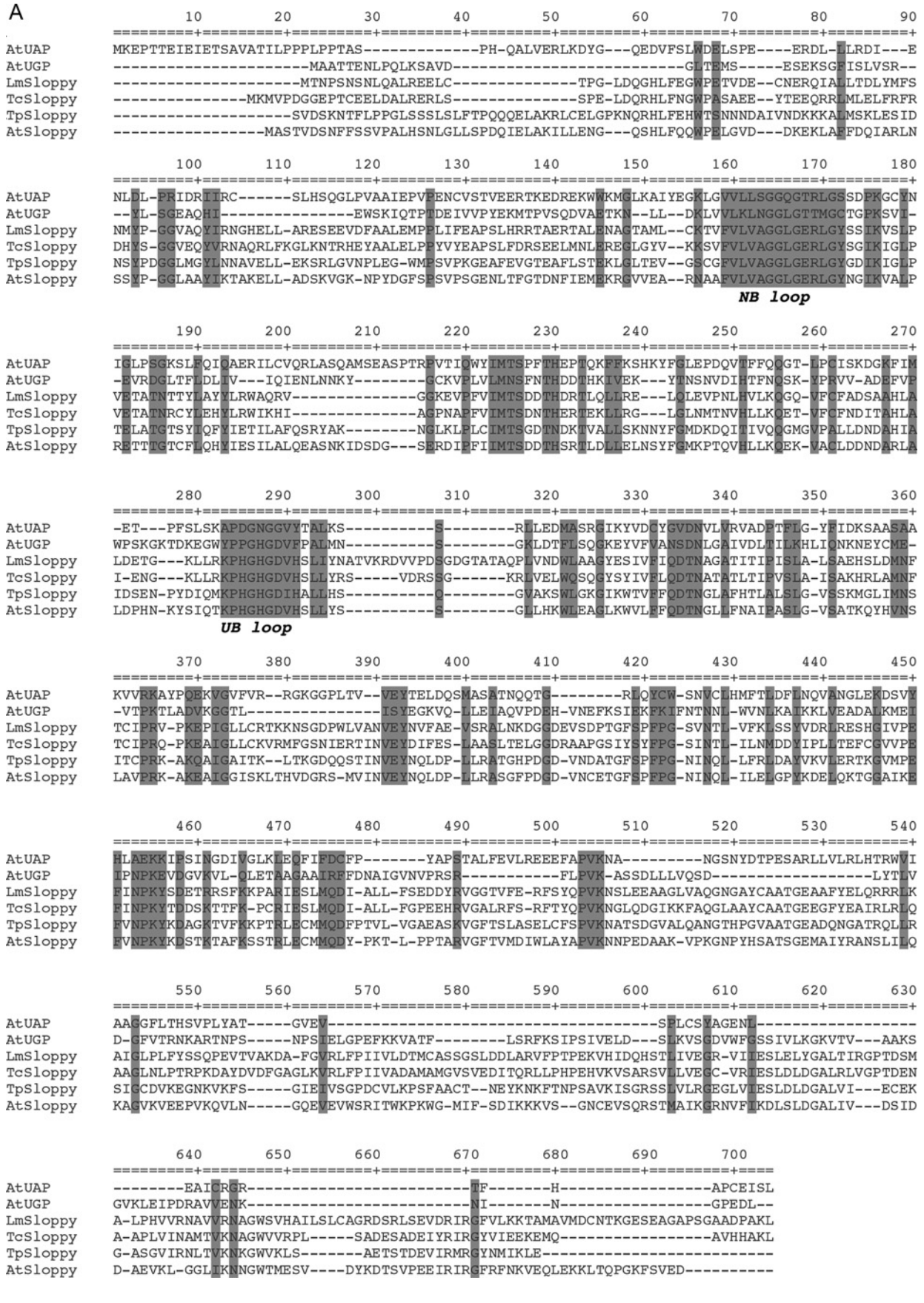

Figure 1 **For Legend see facing page**

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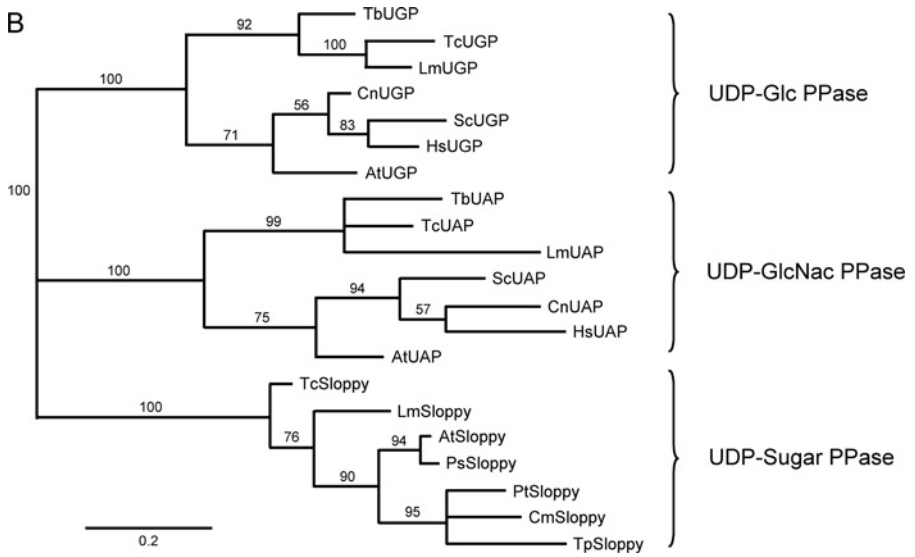

Figure 1 Sequence alignments and phylogenetic relationships of UGP, UAP and UDP-sugar PPases (Sloppy) from different organisms

(**A**) Sequences of UGP, UAP and Sloppy (see gene names below) were aligned using T-coffee [30] software with G-block [31]. The conserved motifs presumably involved in nucleotide-sugar binding (NB) and uracil binding (UB) are labelled in bold. Potential amino acids that are conserved in UGP, UAP and Sloppy are highlighted in grey, on the basis of sequence alignment. (**B**) Phylogenetic relationships of UGP, UAP and Sloppy in different species. Protein sequences (see name and gene accession numbers below) were aligned and analysed using T-coffee [30] software with G-block [31] and the phylogenetic tree was created using MrBayes 3.1.2 software [32,33]. Branch support values (more than 50 %) are shown. The bar represents 0.2 protein substitutions per site. AtSloppy (ABC55066.1), TcSloppy (GU443973, Tc00.1047053511761.10), *Thalassiosira pseudonana* Sloppy (TpSloppy, XP_002291538), LmSloppy (GU443974, LmjF17.1160), *Pisum sativum* Sloppy (PsSloppy, Q5W915), *Paramecium tetraurelia* Sloppy (PtSloppy, XP_001430540) and *Cryptosporidium muris* Sloppy (CmSloppy, XP_002141351); UGP from *Homo sapiens* (HsUGP, NP_006750), *A. thaliana* (AtUGP, NP_186975), *Saccharomyces cerevisiae* (ScUGP, NP_012889), *T. cruzi* (TcUGP, XP_808700), *T. brucei* (TbUGP, XP_827798), *L. major* (LmUGP, XP_001682505) and *Cryptococcus neoformans* (CnUGP, XP_569599); UAP from *H. sapiens* (HsUAP, NP_003106), *A. thaliana* (AtUAP, NP_564372), *S. cerevisiae* (ScUAP, CAY78406), *T. cruzi* (TcUAP, XP_820911), *T. brucei* (TbUAP, XP_828335), *L. major* (LmUAP, XP_001686013) and *C. neoformans* (CnUAP, XP_571302).

was eluted with the same retention time as the UDP-glucose standard. To determine the identity of the UDP-sugar peak, it was collected from the column and analysed by ^{1}H-NMR. The NMR spectrum (Figure 3, trace 1, and Supplementary Figure S2A at http://www.BiochemJ.org/bj/429/bj4290533add.htm) provided chemical shifts consistent with UDP-α-D-glucose. The diagnostic $J_{1,2}$ value of 3.5 Hz and $J_{2,3}$, $J_{3,4}$, $J_{4,5}$, $J_{5,6}$ and $J_{6a,6b}$ values of 9.7, 9.7, 9.7, 3 and 12 Hz respectively indicate an α-glucopyranose configuration, along with the distinct chemical shift of H1 (5.59 p.p.m.). The linkage of the sugar moiety to the phosphate is given by the coupling constant value of 7 Hz for $J_{1,P}$ of the proton anomeric glucose residue and a coupling value of 3 Hz for $J_{2,P}$. These data confirmed that the *T. cruzi* enzyme is a PPase.

We found that the recombinant enzyme also converts UTP and galactose 1-phosphate into UDP-galactose (12.2 min, see Figure 2, trace 2), UTP and glucuronic acid 1-phosphate into UDP-glucuronic acid (16.0 min, Figure 2, trace 3), UTP and xylose 1-phosphate to UDP-xylose (12.5 min, Figure 2, trace 4), on the basis of the retention time of standards. Control cells expressing empty vector had no detectable activity. To unambiguously determine the identity of each enzymatic product, the individual peaks marked #2 and #4 (Figure 2) were collected from the column, and their structures were confirmed by ^{1}H-NMR as UDP-α-D-galactose, and UDP-α-D-xylose respectively (Figure 3, traces 2 and 4, and Supplementary Figures S2B and S2D). Since the enzymatic product of UTP and glucuronic acid 1-phosphate co-eluted with UTP (Figure 2, trace 3), the reaction mixture was chromatographed on a Q_{15} column as it separates UDP-glucuronic acid from UTP. The peak eluted from the Q-column was analysed by ^{1}H-NMR (Figure 3, trace 3, and Supplementary Figure S2C) and was confirmed as UDP-α-D-glucuronic acid. Interestingly, and unlike AtSloppy, the *T. cruzi* enzyme was capable of converting TTP and glucose 1-phosphate into TDP-glucose (12.3 min, Figure 2, trace 5, and Figure 3, trace 5) as well, albeit at lower rate. Therefore we propose that the *T. cruzi* enzyme is a PPase and displays, like the plant Sloppy, a broad uridylyltransferase activity with various sugar 1-phosphates as substrates. Just like the plant Sloppy, TcSloppy was unable to convert *N*-acetylglucosamine 1-phosphate and UTP.

Characterization and properties of TcSloppy

Crude recombinant TcSloppy is stable when stored at −20 °C. Purified TcSloppy could be stabilized for several months at −80 °C when flash-frozen in liquid nitrogen. TcSloppy requires Mg^{2+}, although Mn^{2+} can substitute for Mg^{2+} (Table 1), and the activity, as expected, was almost completely abolished in the presence of EDTA. The recombinant Sloppy is active between pH 3.3 and pH 9.0 (Figure 4), with maximum activity at pH 7.5–7.8 in Tris buffer, or at pH 6 in phosphate buffer. The enzyme is also active when reactions were performed in Hepes, Mops or Mes buffers. TcSloppy is active between 25 and 55 °C, with maximum activity at 30–42 °C (Table 2).

We next investigated the NTP specificity of TcSloppy. ATP, CTP, GTP and ITP are not substrates for TcSloppy when using glucose 1-phosphate as substrate. Several commercially available sugar 1-phosphates were tested as substrates for TcSloppy with different nucleotides (e.g. ATP, CTP, GTP, ITP and UTP). No activity was observed even when the standard *T. cruzi* assay had a longer incubation time (up to 1 h). To determine whether TcSloppy may recognize other NTPs, we performed standard assays in the presence of competing nucleotides such as ATP, CTP, GTP, ITP and UTP (at 0.5 mM each). In all cases, glucose 1-phosphate was readily uridylated, suggesting that, with the

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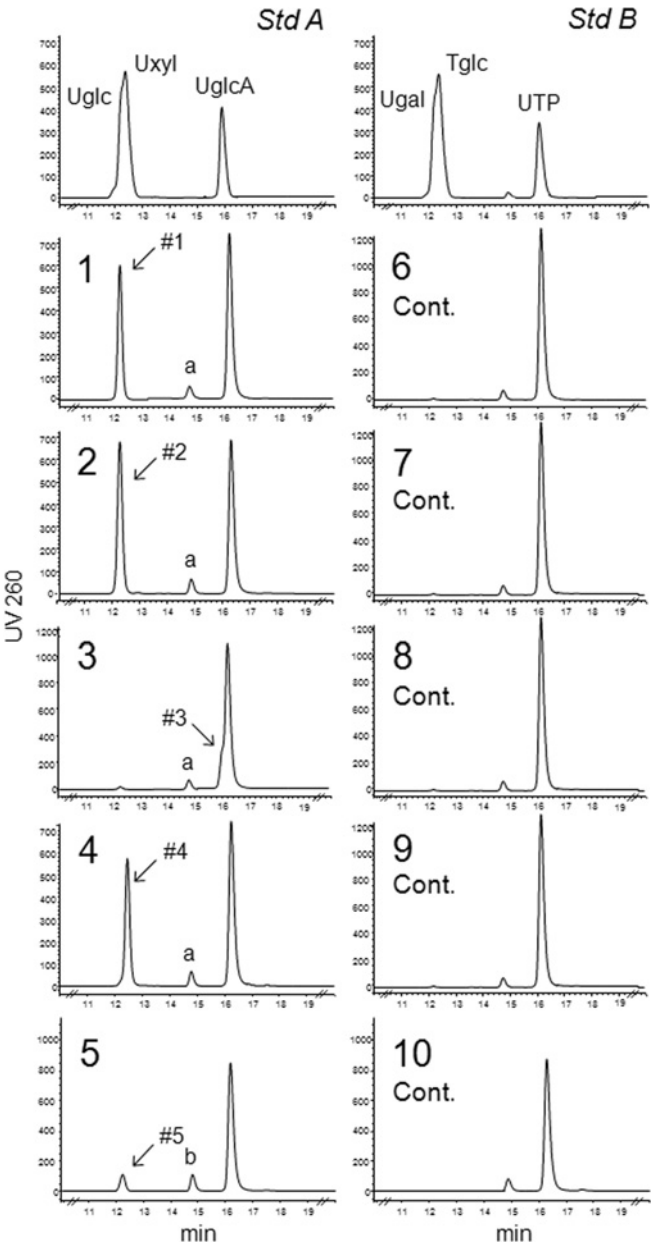

Figure 2 HPLC-based assays of recombinant TcSloppy activity

The assays for the formation of NDP-sugars included UTP and different sugar 1-phosphates with either TcSloppy (traces 1–4) or with control protein (empty vector control cells, traces 6–9). Trace 1 shows the formation of UDP-glucose (marked by arrow #1); trace 2 shows the formation of UDP-galactose (marked by arrow #2); trace 3 shows the formation of UDP-glucuronic acid (marked by arrow #3); trace 4 shows the formation of UDP-xylose (marked by arrow #4). TcSloppy was also incubated with TTP and glucose 1-phosphate; TDP-glucose was formed (marked by arrow #5 in trace 5) when compared with control (Cont.; empty vector control cells, trace 10). The HPLC peaks (traces 1–5) are UTP (16.3 min), TTP (16.3 min), UDP-glucose (12.3 min), UDP-galactose (12.2 min), UDP-glucuronic acid (16.0 min), UDP-xylose (12.5 min) and TDP-glucose (12.3 min). The minor peak marked as 'a' at 14.8 min is UDP contamination in the UTP reagent. Std A and Std B traces show the elution time of standards: UDP-galactose (Ugal; 12.2 min), UDP-glucose (Uglc; 12.3 min), TDP-glucose (Tglc; 12.3 min), UDP-xylose (Uxyl; 12.5 min), UDP-glucuronic acid (UglcA; 16.0 min) and UTP (16.3 min).

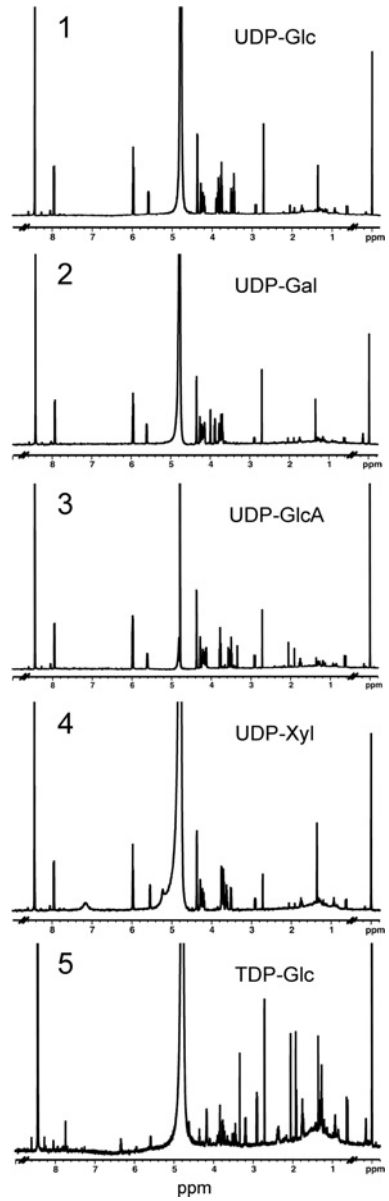

Figure 3 Product analyses of HPLC-based assays by NMR, confirming that recombinant *T. cruzi* enzyme has Sloppy NDP-sugar PPase activity

^{1}H-NMR spectra of TcSloppy enzymatic products. Each peak eluted from the column (see Figure 2, arrows #1, #2, #4 and #5) was collected, freeze-dried, dissolved in ^2H_2O and analysed by ^{1}H-NMR. The assay shown in trace 3 (Figure 2, arrow #3) was chromatographed on a Q_{15} column as it separates UDP-glucuronic acid from UTP. The UDP-glucuronic acid peak was collected and analysed by NMR. The NMR spectra (0–9 p.p.m.) of each individual TcSloppy reaction product are shown and the identity of the product is labelled on the upper right of each panel. Detail NMR data that cover the sugar anomeric region (5.5–6 p.p.m.) and an expansion spectrum of the NDP-sugar ring protons (3.4–4.4 p.p.m.) are provided in Supplementary Figure S2 at http://www.BiochemJ.org/bj/429/bj4290533add.htm.

exception of UTP, the enzyme does not recognize the above NTPs. In addition to UTP, the enzyme could also convert glucose 1-phosphate into TDP-glucose in the presence of TTP, but at a much lower rate (Figure 2, trace 5). To determine whether the enzyme recognizes and binds NDPs (such as UDP or ADP), before the standard assay, the enzyme was incubated with NDPs. These NDPs, as well as other nucleotides tested (e.g. NMP, NAD and NADH), had no effect on Sloppy activity. In contrast, enzyme activity was reduced by 65 % in the presence of 0.5 mM PP_i (see Supplementary Table S1 at http://www.BiochemJ.org/bj/429/bj4290533add.htm) when assays were conducted for the forward reaction.

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Table 1 TcSloppy requires metal for activity

TcSloppy was mixed with different metal salts, EDTA or water as a control for 10 min on ice. Subsequently, UTP and appropriate sugar 1-phosphates were added, and the PPase assay was carried out under standard conditions. Each value is the mean for duplicate reactions, and the values varied by no more than ±5%.

Additive (5 mM)	Relative TcSloppy activity (%)
$MgCl_2$	100
$MnCl_2$	104
$CaCl_2$	4
$ZnSO_4$	2
EDTA	3
Water	2

Table 2 Effect of temperature on TcSloppy activity

Enzymatic reactions were performed under standard conditions for each enzyme activity, except for the reaction temperature. Each value is the mean for duplicate reactions, and the values varied by no more than ±5%.

Temperature (°C)	Relative TcSloppy activity (%)
4	5
25	34
30	102
37	100
42	115
55	26
65	7

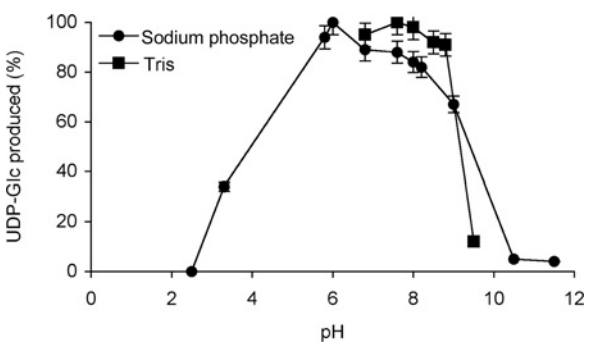

Figure 4 Effects of different buffers and pH on TcSloppy activity

TcSloppy activity was determined in different buffers at different pH values. Each value is the mean for duplicate reactions, and the values varied by no more than ±5%.

Real-time NMR analysis of TcSloppy

To monitor the dynamics of the enzymatic reaction and the substrate preference of TcSloppy, we used real-time ^{1}H-NMR spectroscopy (Figure 5). These assays were carried out in phosphate buffer to avoid the proton signals from Tris. In the NMR reactions presented in Figure 5, all sugar 1-phosphates were combined along with four molar equivalents of UTP. As shown in the time-dependent enzymatic progression, a faster conversion of the glucose 1-phosphate (5.46 p.p.m.) into UDP-α-D-glucose (5.59 p.p.m.) and galactose 1-phosphate (5.50 p.p.m.) into UDP-α-D-galactose (5.63 p.p.m.) were observed when compared with the conversion of glucuronic acid 1-phosphate (5.48 p.p.m.) to UDP-α-D-glucuronic acid (5.61 p.p.m.) and xylose 1-phosphate (5.41 p.p.m.) to UDP-α-D-xylose (5.54 p.p.m.) (Figure 5). After peak deconvolution, the rate order of

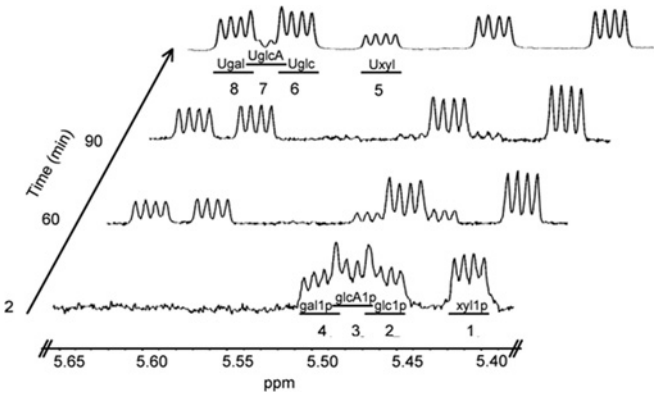

Figure 5 Real-time ^{1}H-NMR-based Sloppy assays

In the real-time NMR assay, recombinant Sloppy was mixed with all sugar 1-phosphates (glucose 1-phosphate, galactose 1-phosphate, glucuronic acid 1-phosphate and xylose 1-phosphate, 1 mM each), buffer and 4 mM UTP. Approx. 2 min after enzyme addition and NMR shimming, data were collected. Progressions of enzyme activity covering the anomeric region of the ^{1}H-NMR spectrum are shown. The signal for the anomeric proton of the sugar 1-phosphate and the UDP-sugar has a quadruplet peak form: peak 1 (5.41 p.p.m.) is xylose 1-phosphate (xyl1p), peak 2 (5.46 p.p.m.) is glucose 1-phosphate (glc1p), peak 3 (5.48 p.p.m.) is glucuronic acid 1-phosphate (glcA1p), peak 4 (5.50 p.p.m.) is galactose 1-phosphate (gal1p), peak 5 (5.54 p.p.m.) is UDP-α-D-xylose (Uxyl), peak 6 (5.59 p.p.m.) is UDP-α-D-glucose (Uglc), peak 7 (5.61 p.p.m.) is UDP-α-D-glucuronic acid (UglcA), and peak 8 (5.63 p.p.m.) is UDP-α-D-galactose (Ugal). Note: glucose 1-phosphate and galactose 1-phosphate peaks overlap in part with glucuronic acid 1-phosphate. The horizontal line shows the p.p.m. spanning the quadruplet shape peak for each of the substrate and the enzymatic product.

specific sugar 1-phosphate conversion was UDP-glucose = UDP-galactose > UDP-xylose > UDP-glucuronic acid.

We also performed similar real-time NMR assays with the individual sugar 1-phosphate in the absence of the pyrophosphatase, to obtain equilibrium between the forward and reverse reactions. The results of these assays are shown in Figure 6 and Supplementary Figures S3(A)–S3(C) (at http://www.BiochemJ.org/bj/429/bj4290533add.htm), and summarized in Supplementary Figure S3(D). At equilibrium, the ratios of sugar 1-phosphate to UDP-sugar were as follows: 1.8 for UDP-glucose, 1.3 for UDP-galactose, 2.8 for UDP-glucuronic acid and 2.8 for UDP-xylose. These data clearly show the preference for the reverse reaction, i.e. conversion of UDP-sugar to sugar 1-phosphate. This preference is common to almost all PPases, such as UAP, UGP and TDP-glucose PPase. In the forward reaction, the sugar 1-phosphate preference of TcSloppy is in contrast with AtSloppy, where UDP-glucuronic acid was the preferred substrate and the conversion rate order was UDP-glucuronic acid > UDP-glucose > UDP-galactose > UDP-galacturonic acid > UDP-xylose ([15] and T. Yang, unpublished work).

Kinetic and catalytic properties of TcSloppy

Kinetic analyses of the enzyme are summarized in Table 3(a). The apparent K_m values for the forward reaction were 177 μM (glucose 1-phosphate) and 28.4 μM (UTP), with V_{max} values of 0.07 $\mu M \cdot s^{-1}$, and k_{cat}/K_m ($s^{-1} \cdot \mu M^{-1}$) of 0.15 (glucose 1-phosphate) and 0.92 (UTP). The kinetics for the reverse reaction had apparent K_m values of 26 μM (UDP-glucose) and 134 μM (PP_i), with V_{max} values of 0.08 $\mu M \cdot s^{-1}$, and k_{cat}/K_m ($s^{-1} \cdot \mu M^{-1}$) of 1.15 (UDP-glucose) and 0.22 (PP_i). Further kinetic data for the forward reaction of TcSloppy with different sugar 1-phosphates and nucleotides are summarized in Table 3(b). The relatively lower activity of TcSloppy for the conversion of TTP and glucose 1-phosphate into TDP-glucose can be explained by the affinity for the nucleotide. The K_m values of TcSloppy for UTP and

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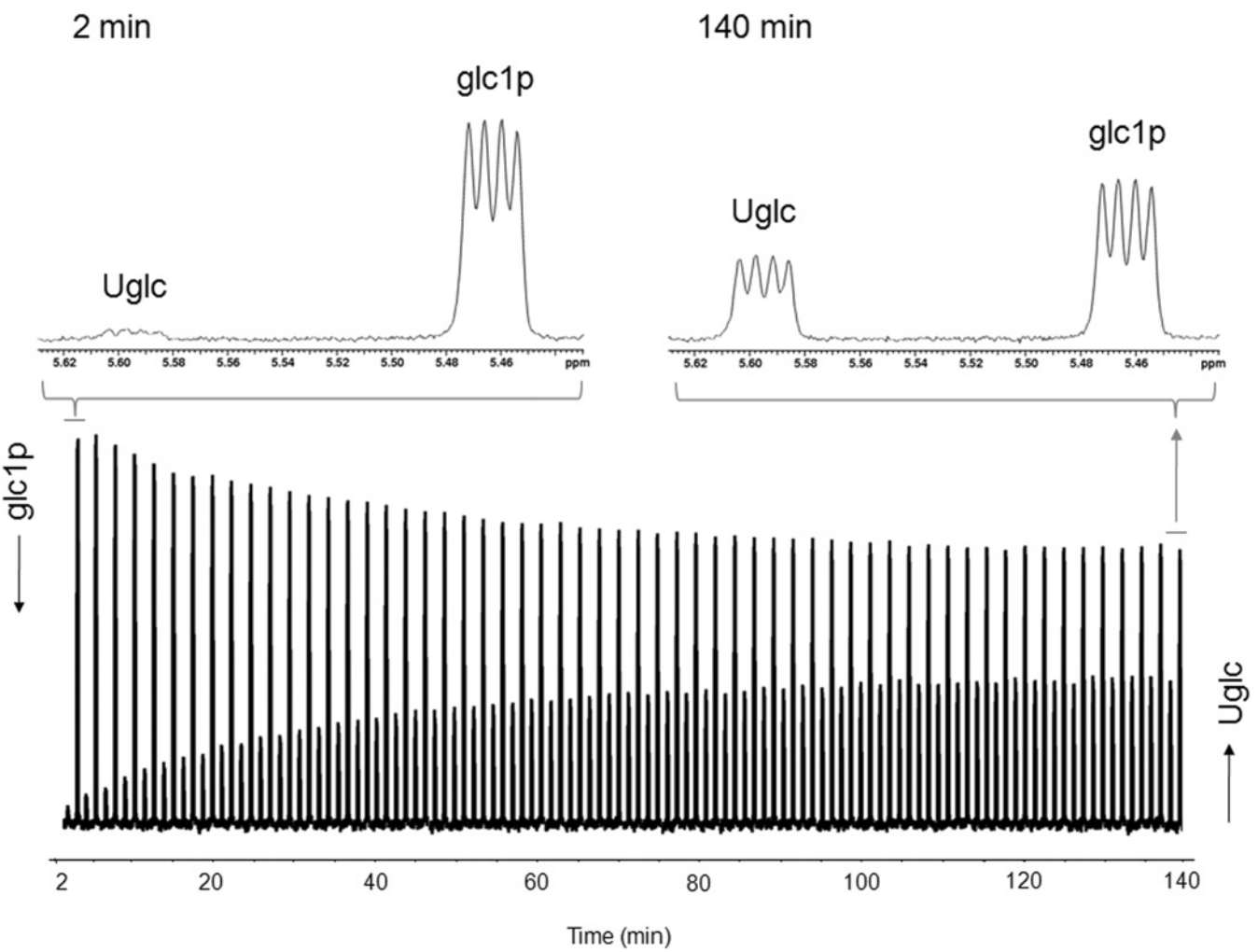

Figure 6 Real-time ^{1}H-NMR-based Sloppy assays with glucose 1-phosphate

Time-dependent ^{1}H-NMR spectrum arrays for the formation of UDP-glucose by TcSloppy. Glucose 1-phosphate (1 mM) was reacted with 1 mM UTP and TcSloppy. Approx. 2 min after enzyme addition and NMR shimming, data were collected. The progression of enzyme activity monitored by following the changes in the spectrum of the sugar anomeric proton region (from 5.4 to 5.7 p.p.m.) is shown. To visualize changes in product over time, each spectrum at a given time is plotted sequentially. Each time point reflects the amount of UDP-glucose formed (Uglc, ↑) in the forward reaction, and the decreased amount of glucose 1-phosphate (glc1p, ↓).

TTP are different (28.4 and 2540.8 μM respectively). In addition, the difference in catalytic efficiency (k_{cat}/K_m) between the two substrates of the enzyme towards UDP-glucose and TDP-glucose reflects in part the different affinities for the respective NDP-sugar, but more significantly was due to the actual rate of catalysis. The kinetic data of TcSloppy are comparable with that of AtSloppy [17] and *L. major* (LmSloppy).

DISCUSSION

We have cloned and biochemically characterized a *T. cruzi* UDP-sugar PPase that, in the presence of Mg^{2+} and UTP, specifically uridylates a broad range of sugar 1-phosphates with higher efficiency towards glucose 1-phosphate and galactose 1-phosphate and decreased efficiency for xylose 1-phosphate and glucuronic acid 1-phosphate. We have also cloned and expressed the Sloppy-like gene from *L. major* (GenBank® accession number GU443974, LmjF17.1160). The LmSloppy is active towards UDP-glucose (Table 3), UDP-galactose, UDP-xylose and UDP-glucuronic acid, but, unlike TcSloppy, it is also active *in vitro* with UDP-galacturonic acid (T. Yang and M. Bar-Poled, unpublished work).

The *T. cruzi* PPase enzyme is reversible and, in the presence of UDP-glucose and PP_i for example, will form glucose 1-phosphate and UTP. The physiological significance of the reverse reaction is unclear, as PP_i in normal cells is thought to be readily hydrolysed by pyrophosphatase to $2P_i$ [29], hence preventing the hydrolysis of NDP-sugar to sugar 1-phosphate. Comparing the activities of Sloppy from different organisms shows altered specificities. AtSloppy can form at least six different UDP-sugars, and it is possible that TcSloppy may have other sugar 1-phosphates as substrates, such as rhamnose 1-phosphate. The analyses of these different Sloppy-like proteins also illustrate that functional biochemical analysis is essential, and that homology is an insufficient criterion to infer functional specificity.

Sloppy-like sequences are found in the genomes of several protozoan parasites such as *L. major*, *Cryptosporidium muris*, *Paramecium tetraurelia* and in the marine phytoplankton *Thalassiosira pseudonana.* Interestingly, the genome of the parasite *T. brucei* does not harbour a Sloppy-like gene and, so far, no xylose, rhamnose or arabinose residues have been reported

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Table 3 Enzyme kinetics of TcSloppy

(a) The forward kinetic TcSloppy reactions were measured for 5 min with different concentrations of glucose 1-phosphate and UTP. (b) In the reverse reaction, the enzymatic activity was measured with different concentrations of UDP-glucose and PP_i. (c) TcSloppy kinetics using various sugar 1-phosphates, UTP and TTP. Reactions with glucose 1-phosphate, galactose 1-phosphate and xylose 1-phosphate were carried out with UTP. The reactions labelled glucose 1-phosphate (TTP) were carried out with a fixed concentration of TTP as the nucleotide. The K_m for TTP was measured with various concentrations of the nucleotide and fixed concentration of glucose 1-phosphate. Enzyme velocities were plotted and Solver software was used to generate the best-fit curve and for calculation of V_{max} and apparent K_m. Each value is the mean for triplicate reactions, and the values varied by no more than $\pm 10\,\%$. The gene encoding LmSloppy (GenBank® accession number GU443974, LmjF17.1160) was cloned, expressed and purified, and the kinetic data were carried out as described for TcSloppy. The kinetics of *Arabidopsis* Sloppy are also provided for comparison [17].

(a) Forward reaction

	K_m (glucose 1-phosphate) (μM)	K_m (UTP) (μM)	k_{cat}/K_m (glucose 1-phosphate) ($\mu M^{-1} \cdot s^{-1}$)	k_{cat}/K_m (UTP) ($\mu M^{-1} \cdot s^{-1}$)
LmSloppy	3605	322	0.02	0.05
TcSloppy	177	28.4	0.15	0.92
AtSloppy	420	190	0.16	0.57

(b) Reverse reaction

	K_m (UDP-glucose) (μM)	K_m (PP_i) (μM)	k_{cat}/K_m (UDP-glucose) ($\mu M^{-1} \cdot s^{-1}$)	k_{cat}/K_m (PP_i) ($\mu M^{-1} \cdot s^{-1}$)
LmSloppy	657	659	0.1	0.1
TcSloppy	26	134	1.15	0.22
AtSloppy	720	160	0.08	0.35

(c) Forward reaction

	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1} \cdot \mu M^{-1}$)
Glucose 1-phosphate	177	26	0.15
Galactose 1-phosphate	818.8	35.2	0.04
Xylose 1-phosphate	2538.8	8.6	0.034
Glucose 1-phosphate (TTP)	1760	0.12	6.8×10^{-5}
TTP	2540.8	0.085	3.34×10^{-5}

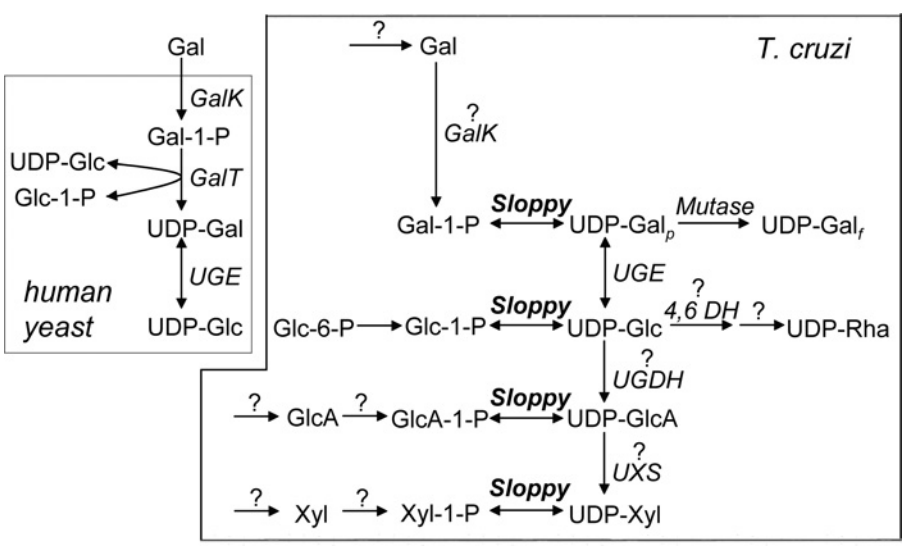

Figure 7 A model for sugar recycling, salvage and NDP-sugar formation in *T. cruzi*

Enzymes and candidate genes are shown in italics. Note: the human or yeast GalT (galactose-1-phosphate uridylyltransferase)-like sequences were not found in the *T. cruzi* genome. The plant UDP-glucose 4,6-dehydratase-like sequences (4,6 DH) were found in the *T. cruzi* genome; however, the subsequent UDP-6-deoxyglucose-3,5-epimerase/4-reductase-like sequences for the formation of UDP-rhamnose were not found in the *T. cruzi* genome. Whether galactose or another sugar (glucuronic acid, xylose, etc.) is internalized into the cell or a by-product of glycan degradation in the cell remains to be determined. GalK, galactokinase; UDP-Gal_f, UDP-galactofuranose; UDP-Gal_p, UDP-galactopyranose; UGDH, UDP-glucose dehydrogenase; UGE, UDP-glucose 4-epimerase; UXS, UDP-glucuronic acid decarboxylase-like.

to exist in the glycans of that organism. What advantage Sloppy affords *L. major* and *T. cruzi*, but not *T. brucei*, remains to be investigated. It would be of interest to express Sloppy in *T. brucei* and examine whether it alters invasion, host preference or survival. Alternatively, the lack of Sloppy in *T. brucei* could be an advantage and allow it to escape the immune system of the host.

Amino acid sequence alignment of several Sloppy-like sequences along with UGPs and UAPs shares very low sequence identity. Yet most of the PPases appear to have conserved binding and catalytic motifs, suggesting that these enzymes have maintained a conserved fold throughout evolution, but the sequences were altered and evolved. Common consensus motifs

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in PPases are the glycine-rich motif for nucleotide binding (on the basis of the nomenclature of Steiner et al. [26]) and a consensus motif for uracil binding. Some amino acids outside of the two motifs, presumably involved in substrate binding and catalytic function are also highly conserved in those PPases. It is likely that insertion of loops between the conserved structural domains generated many changes during evolution to allow the specific enzyme to be either strict to the substrate or to accept different substrates. The functional groups attached to the sugar carbon atoms (C-2, C-4 and C-6) appear to be critical for the recognition of PPases. For example, Peneff et al. [25] pointed to Asn^{223} in UAP, and suggested its involvement in the interaction with the acetyl group linked to C-2 of *N*-acetylglucosamine. Sequence alignment indicates this amino acid is replaced by His^{230} in TcSloppy, and this may explain the inability of Sloppy to uridylate *N*-acetylglucosamine 1-phosphate. In UGP, the recognition of the C-6 hydroxy group of glucose probably occurs via Lys^{380} and Asn^{219}. Sequence alignment indicates that several loops were inserted in this region in TcSloppy, and this may explain the ability of Sloppy to uridylate pentose, hexose and uronate 1-phosphates. The contribution of these loop elements within Sloppy remained to be determined. In addition to the above examples, a significant difference among UGP, UAP and Sloppy is also found in the C-terminal region. Sloppy has several additional domains between amino acids 500 and 603. These domains may be structural, regulatory or other functional elements. Insertion of loops between structural domains may provide alternative binding towards different sugar 1-phosphate substrates or perhaps different nucleotides. Current work is underway to mutagenize and to crystallize TcSloppy with different ligands (e.g. glucose 1-phosphate, glucuronic acid 1-phosphate and galactose 1-phosphate) and UTP to identify how subtle changes in amino acids with certain loops contribute to its 'sloppiness' and be able to accept not only sugar 1-phosphate with difference at the C-4 epimer (i.e. gluco compared with galacto configurations), but also sugar 1-phosphate with altered groups attached to C-5: carboxy (COOH), primary alcohol (CH_2OH), proton (H) and, conceivably, methyl (CH_3) groups. On the basis of the sloppiness of this enzyme, it would suggest that functional groups at the C-2, C-3 and the C-1-phosphate portion of the sugar 1-phosphate are likely to be the only regions that are critical for recognition by these Sloppy enzymes. Interestingly, TcSloppy, like many PPases, is not inhibited by UDP and UMP (Supplementary Table S1), suggesting that the γ-phosphate group of UTP is essential for binding of the nucleotide and perhaps inducing the conformational change of the enzyme.

Unlike *L. major*, *T. cruzi* consists of both the UDP-glucose dehydrogenase-like gene (*UGDH*) and the UDP-glucuronic acid decarboxylase-like gene (*UXS*) with high sequence identity with these functional genes isolated in other organisms. Although the specific functions of these *T. cruzi* genes have not yet been determined, it suggests that *T. cruzi* can convert UDP-glucose into UDP-glucuronic acid and subsequently to UDP-xylose (Figure 7), as proposed originally by Turnock and Ferguson [3]. The presence of TcSloppy in *T. cruzi* is therefore complex, suggesting that different metabolic pathways may be involved in the production of these NDP-sugars in different stages of the parasite life cycle. Another possibility is that recycling of monosaccharides released from the catabolism of glycans is mediated by the salvage pathway, and requires Sloppy. One cannot discount the possibility that TcSloppy, rather than synthesizing UDP-sugars, is actually depleting them by converting them into sugar 1-phosphate.

As Sloppy-like genes do not appear in humans and other animals, it would be worth perusing knockout of the genes and examine whether inhibition of Sloppy could be an effective drug to clear the parasite from its host.

Note added in proof (received 26 May 2010)

While this work was in progress, Damerow et al. [34] recently reported similar UDP-sugar PPase activity in *Leishmania*.

AUTHOR CONTRIBUTION

Ting Yang was involved in all aspects of the study, including experimental design, performing the research, data analysis and manuscript preparation. Maor Bar-Peled directed the study and was involved in all aspects of experimental design, data analysis and manuscript revision.

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online data

SUPPLEMENTARY ONLINE DATA

Identification of a novel UDP-sugar pyrophosphorylase with a broad substrate specificity in *Trypanosoma cruzi*

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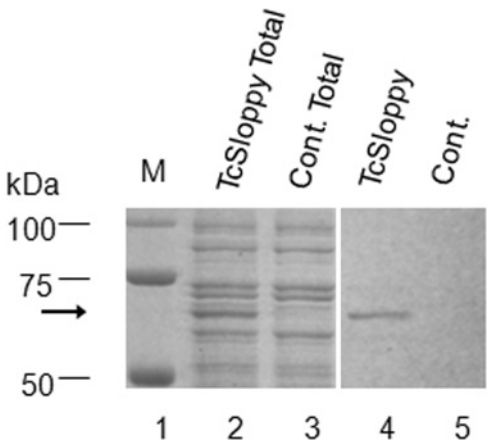

Figure S1 Expression of recombinant TcSloppy

SDS/PAGE of total soluble protein isolated from *E. coli* cells expressing recombinant TcSloppy (lane 2), empty vector control (Cont.) (lane 3), Nickel-column-purified recombinant TcSloppy (lane 4), Nickel-column purified empty vector control (Cont.) (lane 5). Lane 1 contains molecular mass standards with sizes indicated in kDa. The arrow indicates TcSloppy.

Table S1 Effect of potential inhibitors on TcSloppy activity

Inhibitors (at 0.5 mM) or control (water) were mixed with TcSloppy in 100 mM Tris/HCl (pH 7.6) for 10 min on ice before performing the enzymatic reactions. Each value is the mean for duplicate reactions, and the values varied by no more than ±10 %.

Additive (0.5 mM)	Relative TcSloppy activity (%)
UMP	101
NADH	105
CTP	101
Fructose 1-phosphate	108
Mannose 1-phosphate	98
Fucose 1-phosphate	101
Glucose 6-phosphate	99
Galactosamine 1-phosphate	100
Mannitol 1-phosphate	106
TTP	101
ATP	99
NAD^+	101
GTP	98
ADP	85
AMP	98
$NADP^+$	98
PP_i	64
ITP	99
UDP	109
Glucose 1,6-diphosphate	99
Fructose	100
Control	100

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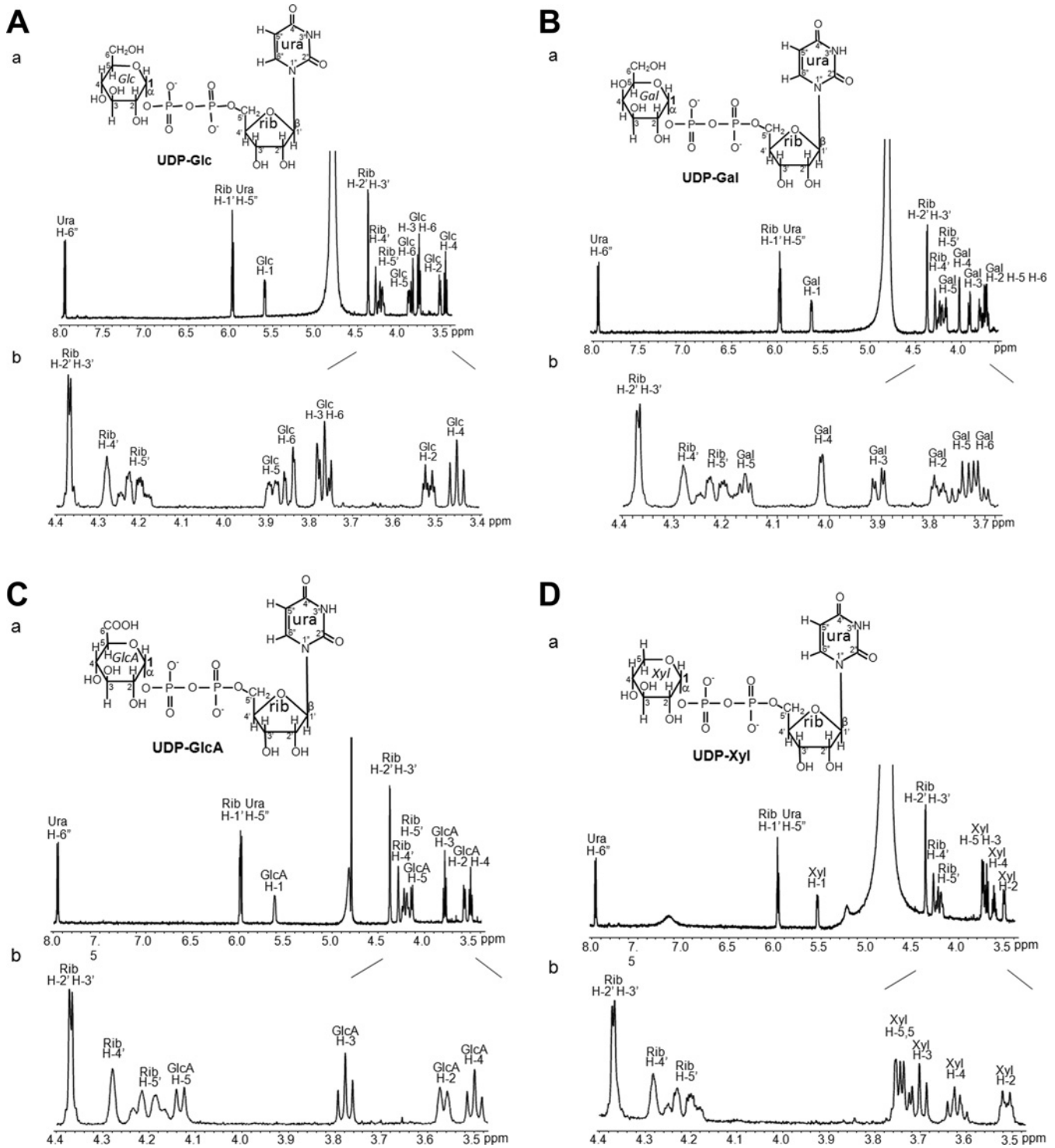

Figure S2 Product analyses of HPLC-based assays by NMR, confirming that the recombinant *T. cruzi* enzyme has Sloppy UDP-sugar PPase activity

^{1}H-NMR spectra of the products formed by TcSloppy. (**A**) ^{1}H-NMR spectrum of UDP-glucose. (**B**) ^{1}H-NMR spectrum of UDP-galactose. (**C**) ^{1}H-NMR spectrum of UDP-glucuronic acid. (**D**) ^{1}H-NMR spectrum of UDP-xylose. Each peak eluted from HPLC column (see Figure 2 of the main text, arrows #1, #2 and #4) was collected, freeze-dried, dissolved in ^2H_2O and analysed by ^{1}H-NMR. The assay in trace 3 of Figure 2 of the main text was chromatographed on a Q_{15} column (as described by Yang et al. [15]), which separates UDP-glucuronic acid from UTP, and the UDP-glucuronic acid was collected and analysed by NMR. The spectra shown are individual UDP-sugars produced by TcSloppy. In each panel, 'a' covers the sugar anomeric region (5.5–6 p.p.m.) and 'b' is the expansion view spectrum of the UDP-sugar carbon ring (3.4–4.4 p.p.m.).

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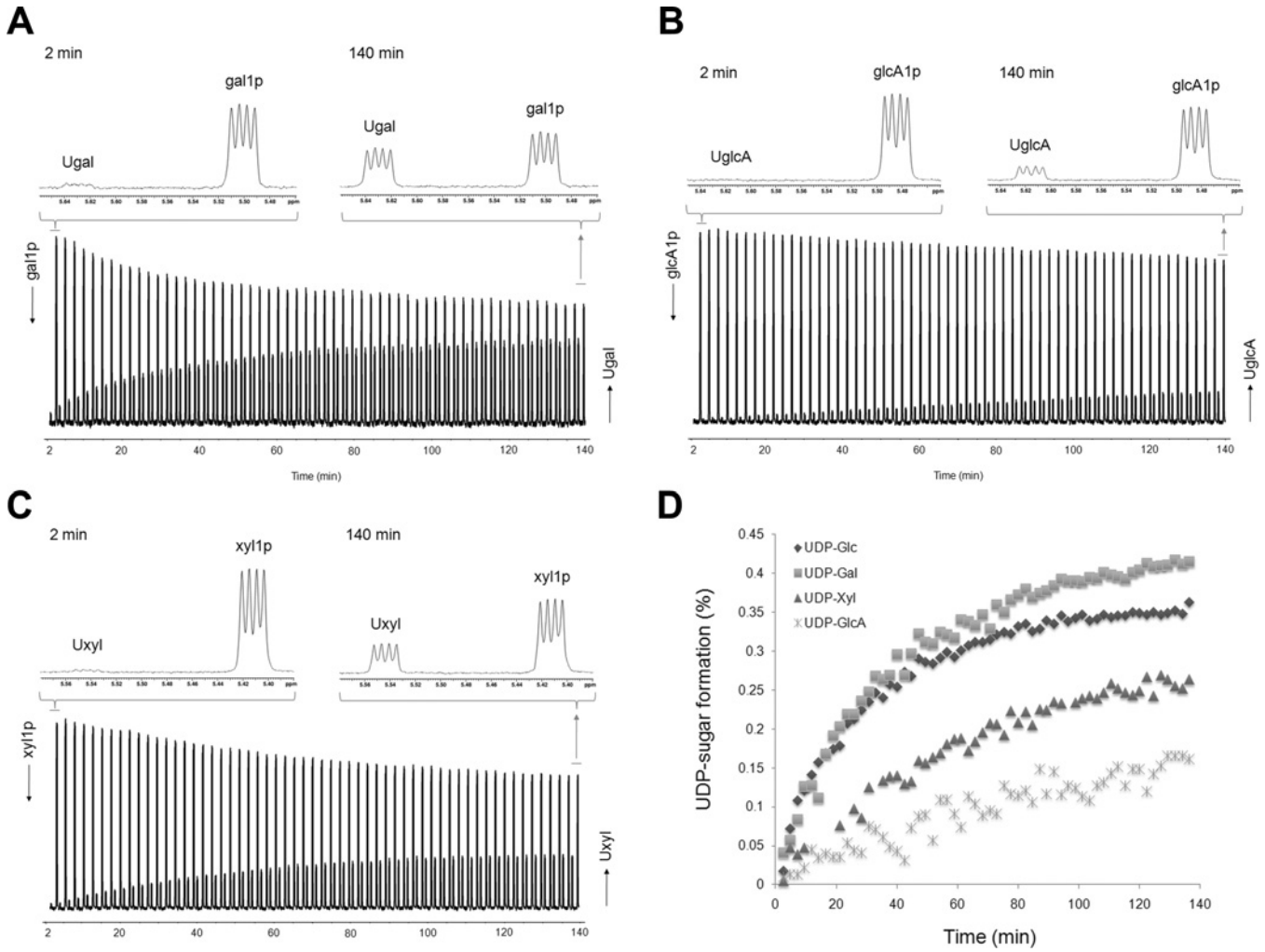

Figure S3 Real-time ^{1}H-NMR-based Sloppy assays with different sugar 1-phosphates

Galactose 1-phosphate, glucuronic acid 1-phosphate or xylose 1-phosphate (1 mM) was separately reacted with 1 mM UTP and TcSloppy. Approx. 2 min after enzyme addition and NMR shimming, data were collected. The progression of enzyme activity, monitored by following changes in the spectrum of the sugar anomeric proton region (from 5.4 to 5.7 p.p.m.), is shown. To visualize changes in product over time, each spectrum at a given time is plotted sequentially. Each time point reflects the amount of UDP-sugar formed (Usugar, where 'sugar' is gal, glc or xyl, ↑) in the forward reaction, and the decreased amount of sugar 1-phosphate (sugar1p, where 'sugar' is gal, glc or xyl, ↓). (**A**) Time-dependent ^{1}H-NMR spectrum arrays for the formation of UDP-galactose by TcSloppy. (**B**) Time-dependent ^{1}H-NMR spectrum arrays for the formation of UDP-glucuronic acid by TcSloppy. (**C**) Time-dependent ^{1}H-NMR spectrum arrays for the formation of UDP-xylose by TcSloppy. (**D**) Integral of the quadruplet peak of the anomeric proton signal of UDP-sugar at each time point was calculated using the NMR software and plotted against time. The relative amount of individual UDP-sugar made during the time course of the enzyme reaction in NMR is shown.

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