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The C-terminal domain of the *Salmonella enterica* WbaP (UDP-galactose:Und-P galactose-1-phosphate transferase) is sufficient for catalytic activity and specificity for undecaprenyl monophosphate

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Two families of membrane enzymes catalyze the initiation of the synthesis of O-antigen lipopolysaccharide. The *Salmonella enterica* Typhimurium WbaP is a prototypic member of one of these families. We report here the purification and biochemical characterization of the WbaP C-terminal ($WbaP_{CT}$) domain harboring one putative transmembrane helix and a large cytoplasmic tail. An N-terminal thioredoxin fusion greatly improved solubility and stability of $WbaP_{CT}$ allowing us to obtain highly purified protein. We demonstrate that $WbaP_{CT}$ is sufficient to catalyze the in vitro transfer of galactose (Gal)-1-phosphate from uridine monophosphate (UDP)-Gal to the lipid carrier undecaprenyl monophosphate (Und-P). We optimized the in vitro assay to determine steady-state kinetic parameters with the substrates UDP-Gal and Und-P. Using various purified polyisoprenyl phosphates of increasing length and variable saturation of the isoprene units, we also demonstrate that the purified enzyme functions highly efficiently with Und-P, suggesting that the $WbaP_{CT}$ domain contains all the essential motifs to catalyze the synthesis of the Und-P-P-Gal molecule that primes the biosynthesis of bacterial surface glycans.

Keywords: isoprenoid / lipopolysaccharide / membrane protein / O-antigen / sugar transferase

Introduction

The membrane enzyme WbaP of *Salmonella enterica* serovar Typhimurium initiates O-antigen synthesis by catalyzing the transfer of galactose (Gal)-1-phosphate from a nucleotide precursor [uridine monophosphate (UDP)-Gal], onto the membrane-embedded isoprenoid lipid carrier, undecaprenyl monophosphate (Und-P), yielding Und-P-P-Gal (Wang and Reeves 1994; Wang et al. 1996; Patel et al. 2010). WbaP belongs to the polyisoprenyl-phosphate hexose-1-phosphate transferase (PHPT) family (Valvano 2003; Valvano et al. 2011). PHPT members occur only in prokaryotes and initiate the synthesis of various types of glycans such as, for example, colanic acid in *Escherichia coli* K-12 (Stevenson et al. 1996), K30 capsule in *E. coli* 09:K30 (Drummelsmith and Whitfield 1999), type 2 capsule in *Streptococcus pneumoniae* (Cartee et al. 2005) and glycans for S-layer protein glycosylation in *Geobacillus stearothermophilus* (Steiner et al. 2007). Most proteins in this family utilize hexose sugars, but some exceptions exist: PglC in *Campylobacter jejuni* and PglB in *Neisseria* sp. transfer 2,4-diacetamido-2,4,6-trideoxyglucose (bacillosamine)-1-P and a 2(4)-acetamido-4(2)-glyceramido-2,4,6-trideoxyhexose-1-P, respectively, to initiate the synthesis of precursors for protein glycosylation pathways (Power et al. 2000; Glover et al. 2006; Chamot-Rooke et al. 2007; Hartley et al. 2011). Another member of this family, WecP, functions with UDP-*N*-acetylgalactosamine (Merino et al. 2011).

WbaP has five predicted transmembrane (TM) helices and three domains (Saldías et al. 2008; Figure 1). The N-terminal domain, including the first four TMs and their connecting loops, has no assigned function, although it contributes to the overall stability of the protein in the membrane (Saldías et al. 2008). A large predicted soluble loop between TMIV and TMV has been implicated in modulating chain length distribution of O-antigen, while the C-terminal domain that includes TMV and the tail is sufficient for the activity of the enzyme in vivo and in vitro (Wang et al. 1996; Saldías et al. 2008; Patel et al. 2010). Trypsin cleavage and green-fluorescent protein reporter experiments confirmed that the C-terminus of the protein including approximately the last 20 kDa is cytoplasmic (Patel et al. 2010). Most PHPT proteins studied are predicted to contain five complete TMs, but others such as *Caulobacter crescentus* PssY and PssZ contain only the C-terminal domain (Toh et al. 2008). At least some of the highly conserved residues within the cytosolic C-terminal region of WbaP are required for function (Patel et al. 2010). The location of the catalytic domain to a defined region of the protein prompted us to purify and biochemically characterize

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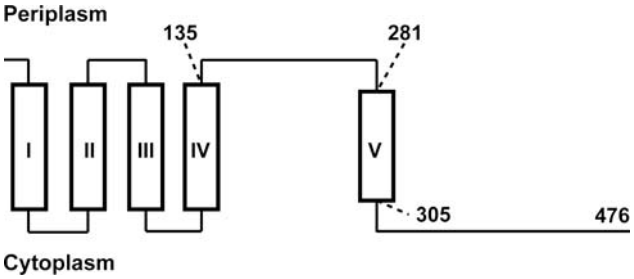

Fig. 1. Predicted topology of *S. enterica* WbaP adapted from Saldías et al. (2008).

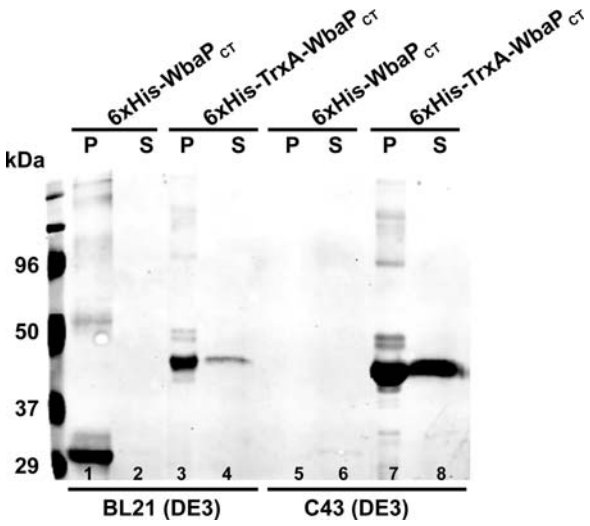

Fig. 2. Solubilization of 6xHis-WbaP$_{CT}$ and 6xHis-TrxA-WbaP$_{CT}$ from total membranes of BL21(DE3) and C43(DE3) cells (lanes 1–4 and 5–8, respectively). After IPTG induction and solubilization with 2.5% DDM, the insoluble pellet (P) and soluble supernatant (S) fractions were recovered as described in *Materials and methods*. Samples were separated by 14% SDS–PAGE and probed with anti-His antibodies. kDa, molecular mass markers.

the C-terminal domain of WbaP (spanning residues 258–476, WbaP$_{CT}$) in the absence of contaminating membrane proteins or lipids. Using an in vitro assay with chemically defined components, we demonstrate that WbaP$_{CT}$ is sufficient for enzymatic activity and also carries high specificity for the Und-P lipid acceptor.

Results and discussion

Overproduction and solubilization of the C-terminal domain of WbaP with an N-terminal thioredoxin fusion

Salmonella enterica Typhimurium *wbaP*$_{R274-Y476}$ was amplified from LT2 DNA and cloned into pET28a generating the plasmid pKP18. This construct encoded a product with an N-terminal 6xHis tag to facilitate detection by immunoblot and protein purification by Ni^{2+}-affinity chromatography. Expression of the recombinant protein was under the control of the Isopropyl β-D-1-thiogalactopyranoside (IPTG)-inducible *lacZ* promoter. After induction of BL21(DE3)/pKP18 with IPTG, total membranes were prepared and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE). Immunoblotting revealed a 29-kDa band corresponding to 6xHisWbaP$_{CT}$ (data not shown). We next attempted to solubilize 6xHis-WbaP$_{CT}$ from the total membrane fractions for purification. To retain the activity of the protein, we tested non-ionic detergents, like Triton X-100 and *n*-dodecyl-β-D-maltoside (DDM), and the zwitterionic detergent, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) at over 10× their critical micelle concentrations. None of the detergents were successful in extracting 6xHis-WbaP$_{CT}$ from the membrane fractions (data not shown). Varying growth and induction conditions, such as temperature, time, IPTG concentration and aeration, did not improve solubilization, suggesting that BL21(DE3) could produce 6xHisWbaP$_{CT}$ as inclusion bodies. Inclusion bodies are typically described for proteins that accumulate in the periplasm or in the cytosol, but can also be membrane associated and therefore detected in the total membrane fraction (Geertsma et al. 2008). An N-terminal fusion of the 12 kDa thioredoxin (TrxA) to a target protein prevents the formation of inclusion bodies (LaVallie et al. 1993). Indeed, we reported previously that an N-terminal TrxA fusion enhanced membrane localization and folding of WbaP$_{CT}$ (Patel et al. 2010). We reasoned that if this fusion reduced the production of inclusion bodies, it would improve solubilization by detergents. Plasmid pKP41 expresses *wbaP*$_{R258-Y476}$ with an N-terminal 6xHis tag and TrxA fusion (Patel et al. 2010). We solubilized total membranes from BL21(DE3) cells expressing 6xHis-TrxA-WbaP$_{CT}$ and the insoluble 6xHis-WbaP$_{CT}$ in 2.5% DDM. As before, 6xHis-WbaP$_{CT}$ was not solubilized from total membranes (Figure 2, lanes 1 and 2). In contrast, 6xHis-TrxA-WbaP$_{CT}$ was well expressed and readily soluble in the detergent suspension (Figure 2, lanes 3 and 4). To improve the solubilization, we repeated the experiments using the C43(DE3) strain. This strain derives from BL21(DE3) and allows for improved expression of membrane proteins (Miroux and Walker 1996). Expression and solubilization of 6xHis-TrxA-WbaP$_{CT}$ was more efficient in C43(DE3) bacteria (Figure 2, lanes 7 and 8), whereas 6xHis-
WbaPCT was not well expressed in these cells (Figure 2, lanes 5 and 6). Similar results were obtained using 6% CHAPS and 7% Triton X-100 (data not shown).

To find an efficient detergent for solubilization of 6xHis-TrxA-WbaP$_{CT}$ and compatibility with enzymatic activity, we also tested the non-ionic detergent octylglucoside (OG) at 0.5 and 1% concentrations. After solubilization and high-speed centrifugation, 2.5% of the insoluble pellet and the soluble supernatant fractions were analyzed by SDS–PAGE and western blotting and the soluble fractions were also tested for enzymatic activity with 20 mM $MgCl_2$, 100 μM Und-P and 0.33 μM ^{14}C-labeled UDP-Gal. Although DDM was most effective at solubilizing 6xHis-TrxA-WbaP$_{CT}$ (Figure 3), the protein was most active in CHAPS at a concentration of 1% (Table I). We concluded that DDM was preferred to solubilize and purify 6xHis-TrxA-WbaP$_{CT}$ while CHAPS would be optimal to assay the protein in vitro.

Purification of an active 6xHis-TrxA-WbaP$_{CT}$ protein and cleavage of the 6xHis fusion partner

6xHis-TrxA-WbaP$_{CT}$ was purified as described in *Materials and methods*. Total membranes from a 150 mL culture of C43

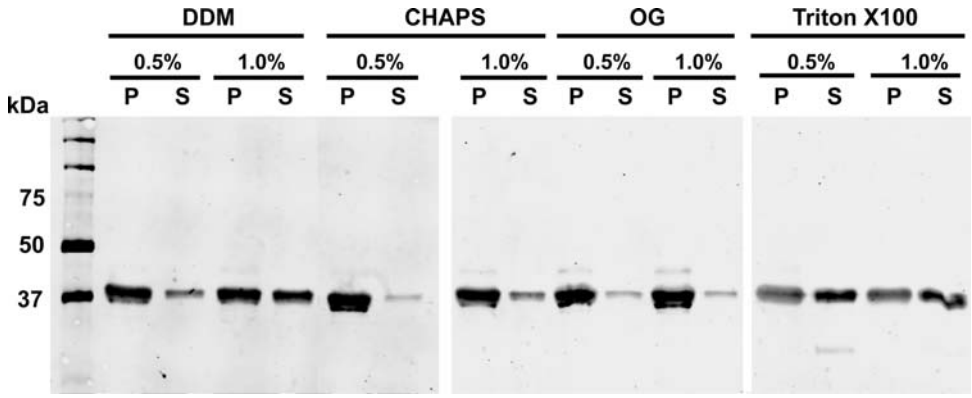

Fig. 3. Detergent screening for solubilization of 6xHis-TrxA-$WbaP_{CT}$. Total membranes from IPTG-induced C43(DE3)/pKP18 cells were solubilized in 0.5 and 1% DDM, CHAPS, Triton X-100 and OG. The insoluble pellet (P) and soluble supernatant (S) fractions were recovered and 2.5% of each fraction was separated by 14% SDS–PAGE and probed with anti-His antibodies.

Table I. In vitro activity of 6xHis-TrxA-$WbaP_{CT}$ solubilized with non-ionic detergents

Sample	Activity relative to total enzymatic activity in crude membranes (%)[a]
Crude membranes with no detergent[b]	100.0
Membrane solubilized (detergent; %)	
CHAPS (0.5)	62.4
CHAPS (1.0)	91.8
DDM (0.5)	81.5
DDM (1.0)	44.5
OG (0.5)	65.5
OG (1.0)	76.5
Triton X-100 (0.5)	10.0
Triton X-100 (1.0)	3.5

[a]Total membrane protein (200 µg) was solubilized in buffer with detergents to a final volume of 200 µL; 50 µL of the soluble fraction corresponding to 50 µg of solubilized membrane protein was tested for in vitro enzymatic activity as described in *Materials and methods*.
[b]50 µg of crude membranes were tested for in vitro enzymatic activity.

(DE3)/pKP41 were solubilized in buffer containing 1% DDM. Following high-speed centrifugation, the supernatant was recovered and applied to $Ni2^{+}$-loaded resin. After washing the resin, 6xHis-TrxA-$WbaP_{CT}$ was eluted with 250 µM imidazole. SDS–PAGE and Coomassie staining showed that 6xHis-TrxA-$WbaP_{CT}$ efficiently bound to the column and was eluted at high levels of imidazole (Figure 4A). After overnight dialysis, the protein concentration was quantified and the purified enzyme remained active as assayed with exogenous Und-P and ^{14}C-labeled UDP-Gal (data not shown).

We next proceeded to remove the 6xHis-TrxA fusion by cleavage at the tobacco etch virus (TEV) site between $WbaP_{CT}$ and the fusion partner. Pure 6xHis-TrxA-$WbaP_{CT}$ was incubated with 6xHis-TEV overnight at 4°C. After proteolysis, the sample was added to Ni^{2+}-charged resin and the flow-through and all fractions were analyzed by SDS–PAGE. The TEV cleavage was complete (Figure 4B, lane 3) affording 10% $WbaP_{CT}$, whereas the reminder of the cleaved protein remained associated with the resin (Figure 4B, lane 4). The protein recovered in the flow-through was concentrated and quantified (Figure 4C). This protocol provided 0.2 mg/l of highly purified and enzymatically active $WbaP_{CT}$, which was sufficient for the biochemical analysis.

Biochemical properties and kinetic parameters of purified $WbaP_{CT}$

We measured the effect of different variables in the in vitro transferase activity of $WbaP_{CT}$. The optimal pH was determined using fresh tris–HCl buffers ranging from pH 6 to 9.5. Transferase activity was only detected between pH 7 and 9, being optimal at pH 8.5 (Figure 5A). Tris–HCl buffer at pH 8.5 was therefore used for all subsequent reactions. NaCl and KCl inhibited $WbaP_{CT}$ activity, particularly at concentrations higher than 200 mM salt that resulted in more than 50% inhibition (Figure 5B). Therefore, upon cleavage of the 6xHis-TrxA, $WbaP_{CT}$ was concentrated by a filtration spin column and rinsed twice with salt-free buffer to prevent the inhibitory effects of NaCl. In vitro assays with crude membranes containing WbaP have previously shown that divalent cations Mg^{2+} or Mn^{2+} are required for activity (Osborn et al. 1962; Patel et al. 2010). To determine the optimal concentration of metal ion cofactor required for the activity of $WbaP_{CT}$, we assayed the protein at concentrations of 0–150 mM $MgCl_2$. Without adding $MgCl_2$, no activity could be detected; activity was optimal at 25 mM, while higher concentrations had an inhibitory effect (Figure 5C).

We next investigated the kinetic parameters of $WbaP_{CT}$. Product formation was assayed over time at various concentrations of protein. The transferase activity profile was linear up to 15 min for concentrations of protein up to 4 ng/µL. To determine the steady-state kinetics of $WbaP_{CT}$, we utilized optimized reaction conditions [50 mM tris–HCl (pH 8.5), 25 mM $MgCl_2$, 1% CHAPS] and tested the substrates UDP-Gal and Und-P at concentrations ranging from 0.2 to 2.5 µM and 10 to 1000 µM, respectively. Typical Michaelis–Menten kinetics were observed for both substrates (Figure 6A and B) with a K_m of 0.55 ± 0.06 µM, a V_{max} of 2448 ± 93 pmol/mg/min and k_{cat} of 4.025 s^{-1} for UDP-Gal, and a K_m of 78.5 ± 0.02 µM and a V_{max} of 1145 ± 65 pmol/mg/min for Und-P.

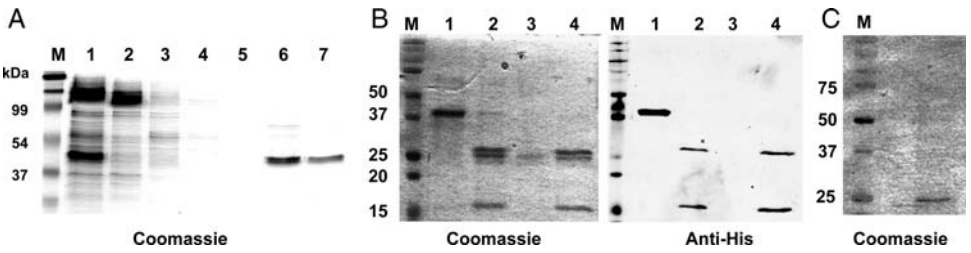

Fig. 4. Purification of TrxA-WbaP$_{CT}$. (**A**) Purification of 6xHis-TrxA-WbaP$_{CT}$ from C43(DE3) total membranes and analysis by 16% SDS–PAGE and Coomassie staining. Lane 1, DDM solubilization; lane 2, flow-through; lane 3, wash 1 (30 mM imidazole); lanes 4 and 5, wash 2 and 3 (60 mM imidazole); lane 6, elution 1 (250 mM imidazole); lane 7, elution 2 (250 mM imidazole). Purified protein migrates at 40 kDa. (**B**) Analysis of TEV cleavage of 6xHis-TrxA-WbaP$_{CT}$ by 16% SDS–PAGE followed by western blot with anti-HIS antibodies (right) and by Coomassie staining (left). Lane 1, purified 6xHis-TrxA-WbaP$_{CT}$ migrates at 40 kDa; lane 2, 6xHis-TrxA-WbaP$_{CT}$ after TEV cleavage. Cleaved 6xHis-TrxA migrates at 17 kDa and WbaP$_{CT}$ migrates at 25 kDa and is not detected by western blot. The 6xHis-TEV protease migrates at 27 kDa. Lane 3, post-TEV-cleavage flow-through. Lane 4, elution with 500 mM imidazole releases all bound protein. (**C**) 12% SDS–PAGE and Coomassie staining of concentrated post-TEV-cleavage flow-through.

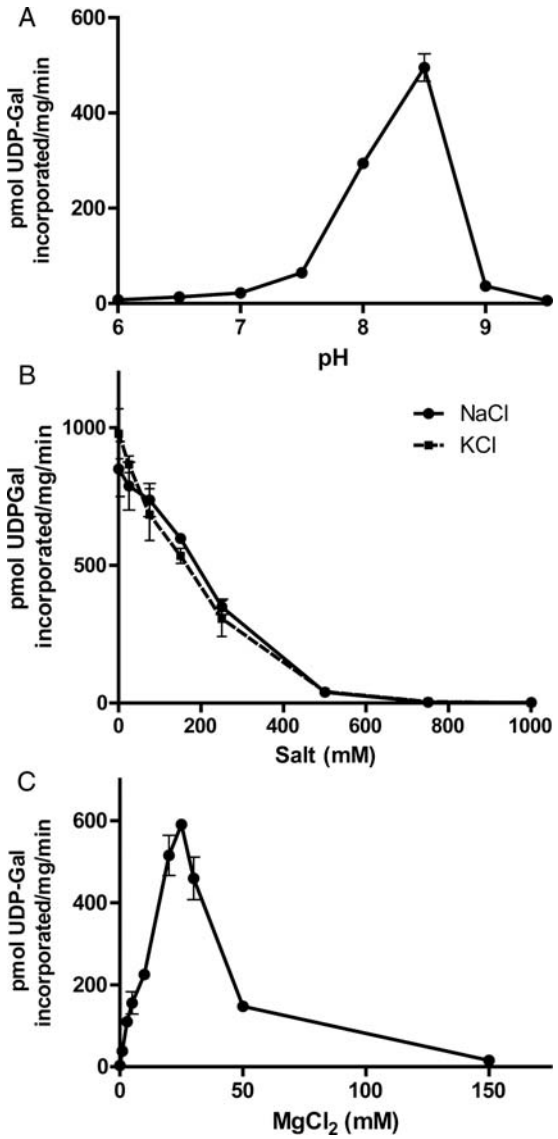

Fig. 5. Biochemical properties of pure WbaP$_{CT}$. The effects of (**A**) pH, (**B**) the salts NaCl and KCl and (**C**) $MgCl_2$ were investigated. Each data point represents the mean of three experiments with standard deviations.

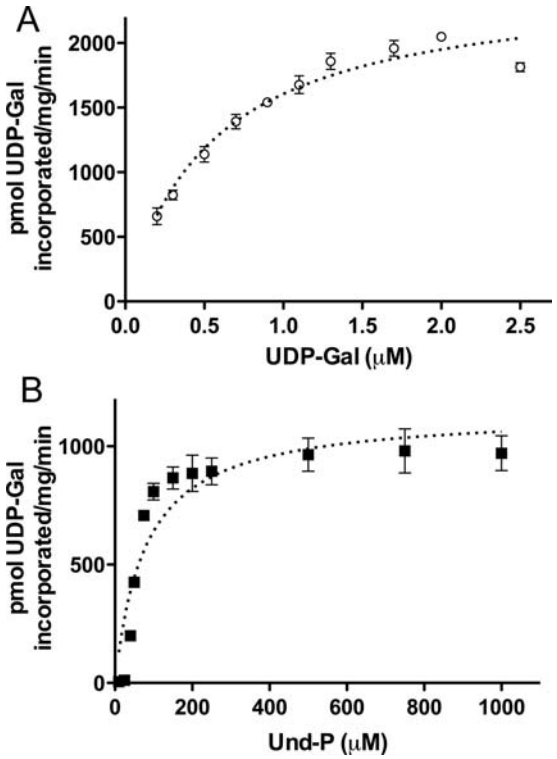

Fig. 6. Steady-state kinetics of WbaP$_{CT}$. A range of (**A**) 0.2–2.5 μM for ^{14}C-labeled UDP-Gal and (**B**) for 10–1000 μM Und-P was assayed under optimized reaction conditions and the reactions were carried out for 15 min at 37 μC in triplicate as described in *Materials and methods*. Each point represents the mean of three experiments with standard deviations.

Substrate specificity of pure WbaP$_{R258\text{-}Y476}$ for the lipid carrier

We also determined whether WbaP$_{CT}$ retains specificity for C55-Und-P. The in vitro activity of purified WbaP$_{CT}$ was tested with polyisoprenyl (PI) phosphates of varying lengths ranging from C10-P to C95-P (Figure 6). Minimal activity was detected with the shorter PIs C10-P, C15-P, C20-P and C35-P at 0.28, 0.91, 1 and 1.8% of C55-P activity, respectively. A similar result was also obtained with the eukaryotic

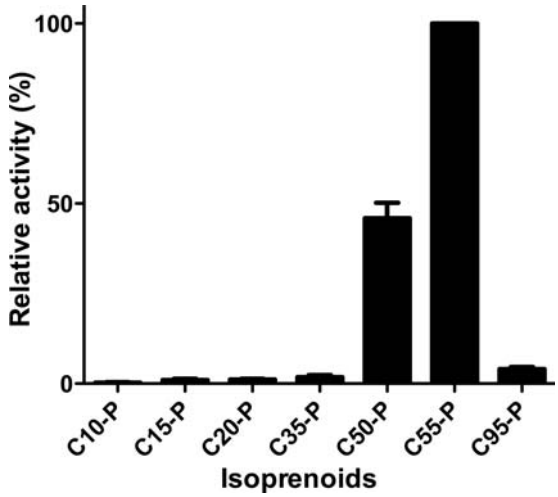

Fig. 7. Specificity of $WbaP_{CT}$ for the lipid carrier. A concentration of 100 μM was used for each PI tested in optimized in vitro assay conditions as described in *Materials and methods.* Data represent the mean of three independent experiments with standard deviations.

lipid carrier, C95-P, at 4.02% activity of C55-P (Figure 7). C50-P, however, resulted in 49.53% activity of C55-P. The synthetic C55-P and C50-P have three internal *trans* residues in their molecules, whereas the endogenous Und-P has two. Since the measurement of the enzymatic activity of purified $WbaP_{CT}$ is related to the exogenous C55-P, we conclude that the differences in the number of *trans* residues are not relevant for substrate recognition. However, C35-P and C95-P have two internal *trans* units like authentic Und-P, and C95-P is hydrogenated at the OH of the terminal of the isoprene unit (α-isoprene). These results indicate that $WbaP_{CT}$ can recognize the length of the PI chain and the unsaturation of the α-isoprene and therefore is highly specific for Und-P.

Concluding remarks

To our knowledge, in vitro studies on WbaP as well as other hexose-1-transferases, such as CpsE and WsaP, have relied on crude membrane preparations (Osborn et al. 1962; Cartee et al. 2005; Steiner et al. 2007; Saldías et al. 2008; Patel et al. 2010). Here, we have purified the $WbaP_{CT}$ domain, which has allowed us characterize the transferase reaction in vitro using chemically defined components. A striking observation in our study was the strong specificity of $WbaP_{CT}$ for Und-P. Kinetic analysis of purified PglC with PI phosphates varying in length, double-bond geometry and degree of saturation has also revealed a preference for the native substrate (Chen et al. 2007). An interaction of the enzyme with the Und-P (C55-P) acceptor presumably occurs in the inner membrane via regions of the protein within or at the boundaries of TM helices. In previous work, we showed the cytosolic tail of WbaP fused to TMI resulted in a protein without function, suggesting that the TMV is important for the catalytic activity of the enzyme (Saldías et al. 2008). A closer examination of the TMV helix, which is present in the purified $WbaP_{CT}$ protein, reveals the sequence LIIASPLMIYLWY. This sequence is strikingly similar to the 13 amino acids consensus sequence LL(F/I)IXFXXIPFXFY, which was described to be important for the recognition of PI phosphates (Albright et al. 1989; Zhou and Troy 2003). NMR and molecular modeling of peptides containing the consensus sequence suggest that one TM region would be sufficient to interact with Und-P (Albright et al. 1989; Zhou and Troy 2003, 2005). Structural studies of the $WbaP_{CT}$ domain, currently underway in our laboratory, will provide more detailed information on the mechanism of catalysis as well as the residues that make contact with substrate and cofactor molecules.

Materials and methods

Bacterial strains and growth conditions

Escherichia coli strains DH5α (laboratory stock), BL21(DE3) (Invitrogen) and C43(DE3) (Invitrogen, Burlington, Ontario, Canada) were used for the overexpression of proteins. *Salmonella enterica* Typhimurium LT2 was used to prepare chromosomal DNA. Bacteria grew aerobically at 37°C in Luria–Bertani (LB) medium (Difco Laboratories, Sparks, MD, USA) (10 mg/mL tryptone, 5 mg/mL yeast extract, 5 mg/mL NaCl). Media were supplemented with 100 μg/mL ampicillin, 30 μg/mL chloramphenicol or 40 μg/mL kanamycin as appropriate.

Plasmid construction and sequencing

Plasmid DNA was isolated using the Qiagen miniprep kit (Qiagen Inc., Mississauga, Ontario, Canada). Digestion with restriction enzymes, ligation with T4-ligase and transformation were carried out as described by Maniatis et al. (1982). DNA sequences were determined using an automated sequencer at the York University Core Molecular Biology and DNA Sequencing Facility, Toronto, Ontario, Canada. Plasmid pKP18 was constructed by polymerase chain reaction (PCR) amplification of a 624 bp fragment using primers 2882 (5′-CTGGTCGACATTATTCAGTACTTCTCG-3′) and 2907 (5′-CTAGTTAGGATCCAGGTCGTCCCGTTTTCTC-3′) and LT2 DNA as template. This fragment was digested with *Sal*I and *Bam*HI and ligated into these sites in pET28a (Novagen, EMD Biosciences, Mississauga, Ontario, Canada). Plasmid pKP41 is described elsewhere (Patel et al. 2010).

Chemicals

All reagents unless otherwise specified were supplied by Sigma-Aldrich (St. Louis, MO, USA). ^{14}C-labeled UDP-Gal (Specific activity of 300 mCi/mmol) was purchased from American Radiolabeled Chemicals Inc. (St Louis, MO). Purified neryl monophosphate ($C_{10}H_{25}N_2O_4P$), farnesyl monophosphate ($C_{15}H_{33}N_2O_4P$), geranylgeranyl monophosphate ($C_{20}H_{41}N_2O_4P$), heptaprenyl monophosphate ($C_{35}H_{65}N_2O_4P$), decaprenyl monophosphate ($C_{50}H_{89}N_2O_4P$), Und-P ($C_{55}H_{97}N_2O_4P$) and dolichyl monophosphate ($C_{95}H_{163}N_2O_4P$) were chemically phosphorylated using phosphoramidite chemistry (Branch et al. 1999; Ye et al. 2001). The purity of the PI phosphates was evaluated by thin layer chromatography and determined to be >95% in all cases. To prepare the PI phosphates, prenols (C35, C50 and C55) were isolated from diverse plant sources (Wellburn and Hemming 1966), whereas dolichol (C95) was isolated from the

mammalian liver and purified from the natural extracts by chromatographic methods (Chojnacki et al. 1975). Oligoprenols (C10, C15 and C20) were from Sigma-Aldrich.

Membrane preparation and immunoblotting

Bacteria grew overnight in 5 mL of LB. The culture was diluted to an initial OD_{600} of 0.2 and incubated at 37°C for 2 h until reaching an OD_{600} of 0.6. At this point, IPTG was added to a final concentration of 100–400 μM. Cells were incubated for 5 h at 30°C. Cells were then harvested by centrifugation at 10,000 × *g* for 10 min at 4°C. The bacterial pellet was suspended in lysis buffer [20 mM Tris–HCl (pH 8.5) + 300 mM NaCl, unless otherwise specified] and protease inhibitor cocktail (Roche Diagnostics, Laval, Quebec, Canada) and the suspension lysed using a French Press cell (Thermo Scientific, Rockville, MD, USA). Cell debris were removed by centrifugation (15,000 × *g* for 15 min at 4°C), and the clear supernatant was centrifuged at 40,000 × *g* for 30 min at 4°C. The pellet, containing total membranes, was suspended in lysis buffer. The protein concentration was determined by the Bradford assay (Bio-Rad, Hercules, CA). Staining was performed with Coomassie brilliant blue R250 (Life Technologies Inc., Carlsbad, CA, USA). SDS–PAGE, protein transfers to nitrocellulose membranes and immunoblots were performed as described (Pérez et al. 2008). For detection of 6xHis proteins, membranes were incubated with a 1:10,000 dilution of anti-His IgG2a monoclonal antibodies (Amersham, Piscataway, NJ).

Membrane protein solubilization

For initial solubility detergent screening, total membranes from BL21(DE3) and C43(DE3) cells expressing pKP18 and pKP41 were solubilized in buffer [20 mM tris–HCl (pH 8.5), 150 mM NaCl, 2 mM 2-mercaptoethanol, 10% glycerol] containing 2.5% DDM, 7% Triton X-100 or 6% CHAPS in a final volume of 100 μL. The mixture was incubated at 4°C for 2 h with rotation in a Barnstead Thermolyne LABQUAKE (Barnstead International, Dubuque, IA). After centrifugation at 40,000 × *g* for 30 min at 4°C, the supernatant, representing the soluble fraction, was collected. 10% of the soluble and pellet fractions were used for SDS–PAGE analysis. For screening to find an optimal detergent for enzymatic activity, the equivalent of 200 μg of total membrane protein was solubilized in buffer [25 mM tris–HCl (pH 8), 150 mM NaCl] and 0.5 or 1% of DDM, CHAPS, OG or Triton X-100 to a final volume of 200 μL and the soluble fraction was recovered as described above. 2.5% of the insoluble and soluble fractions were used for SDS–PAGE analysis, and 50 μL of the soluble fraction was used to determine in vitro enzymatic activity. For protein purification, total membranes were resuspended in buffer [20 mM tris–HCl (pH 8.5), 300 mM NaCl, 10 mM 2-mercaptoethanol, 10% glycerol] with 1% DDM and the soluble fractions was collected as described above.

Purification of 6xHis-TrxA-WbaP$_{CT}$ and cleavage by the TEV protease

Ni^{2+}-bound chelating Sepharose Fast Flow resin (GE Healthcare) equilibrated with wash buffer [25 mM $NaPO_4$ (pH 7.5), 300 mM NaCl, 10 mM 2-mercaptoethanol, 10 mM imidazole, 10% glycerol and 0.03% DDM] was mixed with the cleared supernatant recovered after solubilization with 1% DDM. After 30 min at 4°C with rotation in a Barnstead Thermolyne LABQUAKE (Barnstead International, Dubuque, IA, USA), the resin was centrifuged at 3000 × *g* and the flow-through collected. The resin was washed with wash buffer containing 30 and 60 mM imidazole and the protein was eluted with 250 mM. Elutions were combined and dialyzed overnight against 100× dialysis buffer [25 mM $NaPO_4$ (pH 7.5), 150 mM NaCl, 10 mM 2-mercaptoethanol, 10% glycerol and 0.03% DDM]. To remove the 6xHis-TrxA fusion, the dialyzed protein was concentrated 5× using a Vivaspin 20 centrifugal concentrator (MWCO = 10,000) (Vivaproducts Inc., Littleton, MA). Five microliters of AcTEV protease (Invitrogen) was added to 500 μL of protein and incubated at 4°C overnight. The next day the sample was added to Ni^{2+}-bound resin and the flow-through containing the cleaved protein was collected, concentrated and rinsed with buffer containing no salts. The cleaved protein was quantified using the bicinchoninic acid assay (Pierce, Thermo Scientific, Rockford, IL, USA).

In vitro transferase assay

To test the enzymatic activity of detergent solubilized samples, 50 μL of solubilized protein was added to 50 mM tris–HCl (pH 8), 25 mM $MgCl_2$, 2 mM 2-mercaptoethanol and 0.33 μM ^{14}C-labeled UDP-Gal to a final volume of 100 μL and incubated for 30 min at 37°C. For optimization of in vitro reaction conditions, effects of pH, salt and magnesium ions were tested with 100, 150 and 50 ng of protein, respectively, and PIs were tested with 50 ng. Assays were performed with the addition of 100 μM Und-P in a final volume of 50 μL. Steady-state kinetics were determined using optimized assay conditions (pH 8.5, 25 mM $MgCl_2$, 1% CHAPS). For UDP-Gal, 200 ng of protein was tested in 100 μM Und-P and in the range of 0.2–125 μM for ^{14}C-labeled UDP-Gal. For Und-P, 100 ng of protein was tested in 0.33 μM ^{14}C-labeled UDP-Gal and in the range of 10–1000 μM for Und-P. Reactions were incubated at 37°C for 15 min. Extraction of the lipid fractions was adopted from Schäffer et al. (2002) with modifications. After incubation at 37°C, the reactions were stopped with 200–400 μL of chloroform–methanol (C:M 3:2). To extract the lipid phase, the mixture was shaken vigorously for 3 min and centrifuged for 2 min at 14,000 × *g*. The lower organic phase was collected and 55 μL of 40 mM $MgCl_2$ was added followed by 5 min of vigorous shaking. The mixture was centrifuged as before and the top phase was removed. The organic phase was washed twice with 200–400 μL of pure solvent upper phase (C:M:W:1 M $MgCl_2$ 18:294:293:1). For scintillation counting, the organic phase was added to 5 mL of scintillation fluid (Ecolume, MP Biomedical, Solon, OH) and radioactivity was determined by a Beckman liquid scintillation counter (Beckman Coulter Canada, Inc., Mississauga, Ontario, Canada).

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Conflict of interest

None declared.

Abbreviations

CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; DDM, *n*-dodecyl-β-D-maltoside; Gal, galactose; IPTG, isopropyl β-D-1-thiogalactopyranoside; LB, Luria–Bertani; OG, octylglucoside; PCR, polymerase chain reaction; PHPT, polyisoprenyl-phosphate hexose-1-phosphate transferase; PI, polyisoprenyl; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TEV, tobacco etch virus; TM, transmembrane; TrxA, thioredoxin; UDP, uridine monophosphate; Und-P, undecaprenyl monophosphate; $WbaP_{CT}$, WbaP C-terminal domain.

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