Glycobiology vol. 16 no. 3 pp. 230–236, 2006
doi:10.1093/glycob/cwj054
Advance Access publication on November 4, 2005

Demonstration by heterologous expression that the *Leishmania SCA1* gene encodes an arabinopyranosyltransferase

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Received on September 30, 2005; revised on November 1, 2005; accepted on November 1, 2005

In part of the life cycle within their sand fly vector, *Leishmania major* parasites first attach to the fly's midgut through their main surface adhesin lipophosphoglycan (LPG) and later resynthesize a structurally distinct LPG that results in detachment and eventual transmission. One of these structural modifications requires the addition of α1,2-D-arabinopyranose caps to β1,3-galactose side chains in the phosphoglycan repeat unit domain of LPG. We had previously identified two side chain arabinose genes (*SCA1/2*) that were involved in the α1,2-D-Ara$_p$ capping. *SCA1/2* exhibit canonical glycosyltransferase motifs, and overexpression of either gene leads to elevated microsomal α1,2-D-Ara$_p$T activity, resulting in arabinopyranosylation of β1,3-Gal side chains in LPG (hereafter called side chain D-arabinopyranosyltransferase [sc-D-Ara$_p$T]). Heterologous expression in a null arabinose background was used to determine whether the *SCA1* gene encodes the actual sc-D-Ara$_p$T. *SCA1* expression constructs introduced into both mammalian COS-7 cells and the baculovirus-sf9 cell system exhibited considerable expression of the protein. However, functional sc-D-Ara$_p$T activity was observed only in the latter. In *in vitro* assays incubated with guanidine 5′-diphosphate (GDP)-D-[^{3}H]Ara$_p$ as the sugar donor and utilizing exogenous LPG as an acceptor, significant sc-D-Ara$_p$T activity was observed when microsomes from the baculovirus-sf9 cells were incubated in presence of the LPG acceptor. No activity was observed in the absence of LPG. These results demonstrate that *SCA1* encodes a sc-D-Ara$_p$T and provide the first example of heterologous expression of a D-Ara$_p$T gene.

Key words: arabinose/arabinosyltransferase/*Leishmania major*/lipophosphoglycan/metacyclogenesis

Introduction

Leishmania parasites are the causative agents of leishmaniasis, a disease that afflicts millions of people throughout the tropics and subtropics. The parasites spend the extracellular phase of their life cycle as flagellated promastigotes in the alimentary tract of their sand fly vectors. Following their inoculation into the human host, *Leishmania* exist as intracellular amastigotes in macrophages. Lipophosphoglycan (LPG) is an abundant molecule on the surface of *Leishmania* promastigotes (Turco and Descoteaux, 1992). The glycoconjugate consists of a small oligosaccharide that caps a backbone structure of repeating -6Gal(β1,4)-Man(α1)-PO_4 units (n = 15–30), which are linked by an oligosaccharide core to a phosphatidylinositide anchor. Species-specific polymorphisms in LPG structure occur in the structure of the oligosaccharide cap and in the composition and the number of oligosaccharide side chains that branch off from the repeat units (Thomas *et al*., 1992; Turco and Descoteaux, 1992; McConville *et al*., 1995).

LPG has been shown to serve as an adhesion molecule that mediates the interaction of promastigotes within the midgut epithelium of the sand fly (Sacks and Kamhawi, 2001). To maintain infection in the fly, the promastigote must attach to the epithelial cells during elimination of the digested bloodmeal. Subsequently, detachment facilitates movement of the parasite from the midgut to the mouthparts for eventual transmission. In *Leishmania major* Friedlin V1 strain (LmFV1), the β1,3-Gal-terminated side chains of LPG are critical for the attachment process which involves binding to a recently identified galectin (PpGalec) (Kamhawi *et al*., 2004) in the fly's midgut epithelium. During the process of metacyclogenesis (Sacks, 1989), the differentiation of weakly infective procyclic promastigotes into highly infective metacyclic promastigotes, *L. major* detach from the midgut to facilitate transmission. Detachment is mediated by structural changes in LPG in which the procyclic LPG that is involved in attachment is replaced by a metacyclic LPG that cannot attach to the midgut (Figure 1). In one of the modifications, there is an increase in size of LPG because of an approximate doubling in the number of repeat units. Second, there is a pronounced decrease in surface LPG with β1,3-Gal-terminated side chains and a corresponding increase in β1,3-Gal side chains terminating in α1,2-D-Ara$_p$ (McConville *et al*., 1992). Importantly, this side chain arabinose (SCA) (sc-D-Ara$_p$) structural modification is not recognized by any midgut lectin, and the parasite disengages.

Arabinosyl-containing glycoconjugates are plentiful in microbes (Brennan and Nikaido, 1995), plants (Fincher *et al*., 1983), and protozoan parasites (Previato *et al*., 1982; Xavier Da Silveira *et al*., 1998; Guha-Niyogi *et al*., 2001), but are absent in mammalian cells. Unlike other monosaccharides, arabinose can occur naturally in glycoconjugates in pyranose or furanose conformations and in D- and L-stereoisomers. For example, in the mycobacterial lipoarabinogalactan and arabinogalactan (Brennan and Nikaido, 1995), D-arabinofuranose is abundantly present, whereas in

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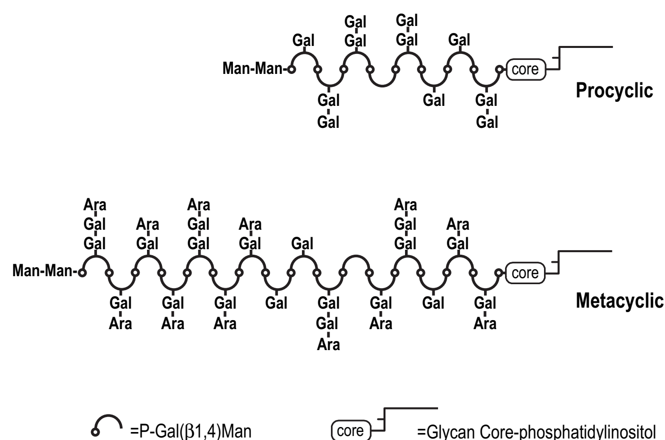

Fig. 1. Schematic diagram of LPG structures of procyclic and metacyclic *Leishmania major* FV1. The structure of the glycan core is Gal(α1,3)Gal(α1,3)Gal_f(β1,3)[Glc(α1-PO_4)-6]-Man(α1,3)Man(α1,4)GlcN(α1,4) linked to 1-*O*-alkyl-2-*lyso*-phosphatidylinositol anchor. The repeat units contain -PO_4-6Gal(β1,4)Man(α1) as a backbone structure. The precise location of the various side chains in the repeat units is not known. The β-Gal side chain-terminating LPG from procyclic *L. major* was used in the sc-D-Ara_pT assays.

Leishmania LPG, the pentose exists as D-arabinopyranose (McConville *et al.*, 1992). The anomeric configuration of the D-Ara_p in the latter glycoconjugate was initially reported as α1,2- based on nuclear magnetic resonance (NMR) analysis (McConville *et al.*, 1990) and later as β1,2- as suggested by susceptibility of the β-configuration to mild-acid hydrolysis with trifluoroacetic acid (McConville and Ferguson, 1993; Schneider *et al.*, 1996), but recently reconfirmed as α1,2- (Malcolm McConville, personal communication). Besides LPG, *Leishmania* are reported to express other Ara-containing glycoconjugates (Wyder *et al.*, 1999), indicating that the parasites do have ample GDP-D-Ara_p precursors available.

Recently, we used an intraspecies expression cloning technique to identify two side chain D-Ara_p genes (*SCA1/2*) that, when overexpressed in *L. major* strain LV39, led to increased α1,2-D-Ara_p capping of galactosylated LPG repeat units during metacyclogenesis (Dobson *et al.*, 2003). The predicted open-reading frame (ORF) of the *SCA1* gene encodes a 92.5-kDa protein containing 832 amino acids, whereas *SCA2* (99.8%) differed by four conservative amino acid substitutions. Both SCA proteins possess hallmarks of eukaryotic glycosyltransferases, including the topology of a type II membrane protein containing a single transmembrane domain preceded by an N-terminal signal anchor sequence. Furthermore, the proteins contain "DXD" catalytic sequence motifs (Wiggins and Munro, 1998; Unligil and Rini, 2000) and are localized to the Golgi apparatus (Dobson *et al.*, 2003). Importantly, microsomes from either *SCA1*- or *SCA2*-transfected *L. major* strain LV39 resulted in elevated arabinosylation of LPG in *in vitro* assays.

Although homologous expression of either of the *SCA* genes in *Leishmania* resulted in elevated side chain D-arabinopyranosyltransferase (sc-D-Ara_pT) activity, these results did not prove experimentally that the genes encode the actual sc-D-Ara_pT. In this article, we establish that the SCA1 protein is the sc-D-Ara_pT that attaches arabinose residues to galactosylated LPG and not an activator or accessory protein of the sc-D-Ara_pT. The evidence that supports this conclusion was obtained by heterologous expression of the *SCA1* gene in host cells with null arabinose backgrounds followed by the demonstration of sc-D-Ara_pT activity in *in vitro* assays.

Results

Heterologous expression of SCA1

To provide the necessary evidence that *SCA* encodes an sc-D-Ara_pT, our strategy was to heterologously express an epitope-tagged construct of the gene in cells having null arabinose backgrounds and then determining whether the expressed protein has sc-D-Ara_pT activity. For this purpose, mammalian COS-7 cells and sf9 insect cells of the baculovirus system were utilized as recipient cells because they lack arabinose-containing glycoconjugates (Altmann *et al.*, 1999). A C-terminal polyhistidine (His6) epitope-tagged *SCA1* was prepared to facilitate detection of expressed protein (~93 kDa) in western blots using an anti-His monoclonal antibody. The *SCA1His* gene was subcloned in the mammalian expression vector pCDNA3 for transfection of COS-7 cells and also into the baculovirus expression vector pFASTBAC1 to prepare recombinant bacmid for the transfection of insect sf9 cells.

Transfection of COS-7 cells with the *SCA1His* construct resulted in the expression of the expected 93-kDa protein as detected by western blotting of microsomal preparations (Figure 2A, lane 2); the signal was absent in microsomal

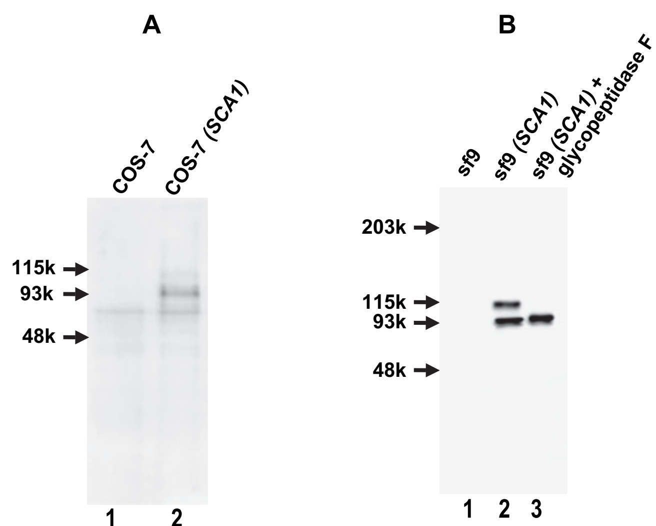

Fig. 2. Western blotting of SCA1His in microsomal preparations. Microsomes (1 μg) from pCDNA3 or pCDNA3-SCA1His-transfected COS-7 cells (panel **A**) and sf9 cells or sf9 cells harboring *SCA1His* (panel **B**) were subjected to western blotting using anti-His monoclonal antibody (1:5000 anti-His conjugated with horseradish peroxidase [HRP]). In lane 3 of panel B, microsomes harboring *SCA1His* were pretreated with glycopeptidase F before electrophoresis.

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membranes from cells transfected with the empty pCDNA3 vector (Figure 2A, lane 1). Microsomes from sf9 cells infected with baculovirus harboring the *SCA1His* gene resulted in the expression of two proteins with molecular weights of 93 and 115 kDa compared with uninfected sf9 cells (Figure 2B, lanes 1 and 2). The larger 115-kDa band was suspected as being a N-glycosylated form of the 93-kDa protein. This possibility was confirmed by pretreatment of the detergent-solubilized microsomes from *SCA1His*-haboring sf9 cells with glycopeptidase F before western blotting. The upper 115-kDa band disappeared after the glycopeptidase-F treatment of microsomes, whereas only the 93-kDa band was detectable (Figure 2B, lane 3).

SCA1 activity in microsomes

To determine whether SCA1His expressed heterologously functioned as an sc-D-Ara$_p$T, an *in vitro* assay for the enzyme was developed. The basic principle of the assay was to use purified LPG from procyclic LmFV1 (Figure 1) as an exogenous acceptor since the glycoconjugate possesses many terminal β1,3-Gal residues as potential acceptor sites for sc-D-Ara$_p$T. Microsomal membranes were prepared from the *SCA1His*-transfected cells, solubilized with detergent, and incubated with the exogenous LPG acceptor along with GDP-D-[^{3}H]Ara$_p$ as the arabinose donor. After a 1-h incubation at 30°C, the LPG was extracted, purified, and radioactivity incorporated into LPG was measured. No sc-D-Ara$_p$T activity was measured using microsomal preparations from *SCA1His*-transfected COS-7 cells (data not shown) even though there was substantial expression of the 93-kDa protein (Figure 2A). A probable explanation to account for the failure to obtain active sc-D-Ara$_p$T activity might be because of the inability to efficiently *N*-glycosylate SCA1 (Figure 2A).

In significant contrast to the mammalian system, heterologous expression of *SCA1His* in the baculovirus system resulted in robust sc-D-Ara$_p$T activity (Figure 3). As a positive control, microsomes from wild-type LmFV1 exhibited substantial sc-D-Ara$_p$T activity in the presence (44 pmol arabinose/h/mg protein) or absence (37 pmol arabinose/h/mg protein) of exogenously added LPG. The incorporation of radiolabel in the absence of the exogenous substrate results from the presence of endogenous LPG in the microsomal preparation which serves as an acceptor in the reaction mixture. In contrast, microsomes from sf9 cells failed to incorporate radioactive arabinose into exogenous LPG. Importantly, microsomes from sf9 cells infected with viral stock containing recombinant *SCA1His* displayed substantial enzymatic activity (73 pmol arabinose/h/mg protein) which was entirely dependent on the addition of exogenous LPG. When no LPG was added, no radioactive product was formed.

Product characterization of sc-D-Ara$_p$T assays

To characterize the radioactive material synthesized in the sc-D-Ara$_p$T assays containing exogenously added LPG, the [^{3}H-Ara]-labeled product was extracted from the assay mixtures of the control LmFV1 cells and sf9 cells transfected

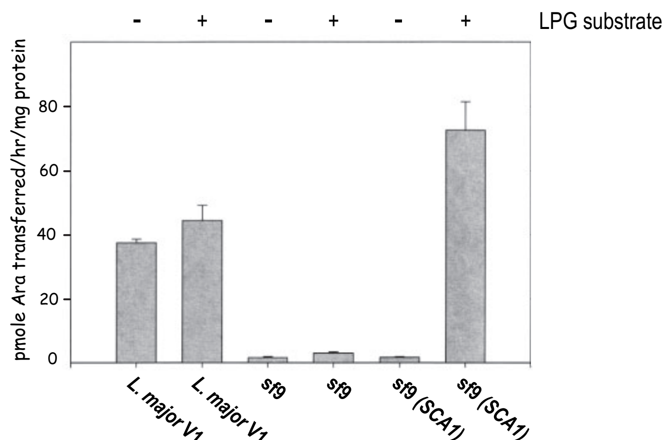

Fig. 3. *In vitro* sc-D-Ara$_p$T activity of recombinant *Leishmania* SCA1. Microsomes (1 mg) were solubilized with 0.02% Triton X-100 and incubated with GDP-D-[^{3}H]Ara$_p$ in the presence or absence of exogenous LPG (10 μg) for 1 h at 30°C. The microsomes were then extracted for LPG, and the radioactivity was quantitated by scintillation counting to determine sc-D-Ara$_p$T-specific activity. The left two bars are activities of microsomes from control *Leishmania major* FV1 cells; the middle two bars from sf9 cells; and the right two bars from sf9 cells harboring the *SCA1His* construct. The top of the figure indicates whether the LPG substrate was added to the assay.

with *SCA1His*. The putative [^{3}H]Ara-LPGs were purified using the typical protocol to extract LPG (Orlandi and Turco, 1987). Aliquots were then subjected to strong acid hydrolysis, and the products analyzed by paper chromatography. The [^{3}H]arabinose label of both samples was quantitatively recovered as [^{3}H]arabinose (Figure 4), indicating that the pentose was not metabolized to another substituent.

Another aliquot of each of the purified [^{3}H]Ara-LPGs was depolymerized into phosphorylated repeat units by treatment with 0.02 N HCl at 60°C for 15 min. These mild-acidic conditions cleave the labile Man(α1)-PO_4 linkages in LPG, yielding a mixture of phosphorylated oligosaccharide repeat units. The [^{3}H-Ara]-oligosaccharide fragments were resolved by Dionex HPAEC (Figure 5). The left panels of Figure 5 show the pulsed amperometric detector (PAD)-detection profile and the corresponding radioactive profile of the phosphorylated oligosaccharides derived from LPG from the control LmFV1. The two main peaks in both profiles coeluted with the standard Ara$_p$-containing phosphorylated tetrasaccharide (Ara-Gal as side chains attached to Gal of the PO_4-Gal-Man repeat unit) and the Ara$_p$-containing phosphorylated pentasaccharide (Ara-Gal-Gal as side chains attached to Gal of the PO_4-Gal-Man repeat unit). The structures of the standard phosphorylated oligosaccharides were obtained by procedures discussed previously (McConville *et al.*, 1992). As shown on the right panels of Figure 5, the analogous chromatographic profiles revealed the identical phosphorylated tetrasaccharide (peak 1) and phosphorylated pentasaccharide (peak 2) repeat units from fragmented [^{3}H-Ara]LPG generated in sf9 cells transfected with *SCA1His*. From these results, we have concluded that *SCA1* encodes an sc-D-Ara$_p$T.

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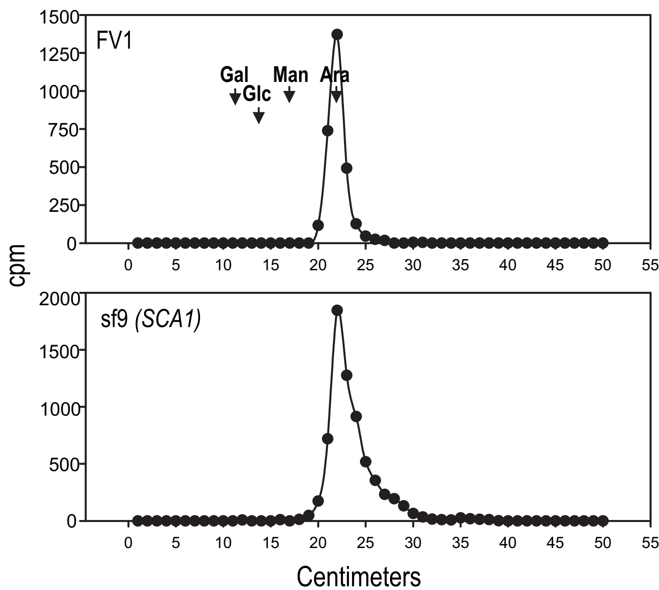

Fig. 4. Paper chromatography of the radioactive products generated by strong acid hydrolysis of LPG. An aliquot of the [^{3}H-Ara]-labeled LPG extracted from sc-D-Ara$_p$T assays from control LmFV1 or sf9 cells containing *SCA1His* (Figure 3) was treated with 2 N trifluoroacetic acid at 100°C for 3 h. Following hydrolysis, the samples were dried by evaporation under N_2 and chromatographed on paper in *n*-butanol/pyridine/water (6:4:3) for 12 h. Quantitation of radioactivity was accomplished by cutting the paper strips into 1-cm segments and counting in a vial containing scintillation fluid with a scintillation counter. Standard sugars were detected by staining with alkaline silver nitrate (Anet and Reynolds, 1954). **Upper** panel, LmFV1; **lower** panel, sf9 harboring *SCA1His*.

Discussion

In contrast to many other sugars, including L-arabinopyranose and D-arabinofuranose, the metabolism of D-arabinopyranose, its activation to the nucleotide level, and its usage as a substrate in glycosylation reactions have not been extensively studied. The activated donor of D-Ara$_p$ in glycosylation reactions in *Leishmania* and other protozoan parasites is GDP-α-D-Ara$_p$ (Schneider *et al.*, 1994). The nucleotide sugar is believed to be generated in trypanosomatids by phosphorylation of arabinose by arabinose 1-kinase to form arabinose 1-phosphate and then activated to the nucleotide level by GDP-D-Ara$_p$ pyrophosphorylase (Schneider *et al.*, 1995; Mengeling and Turco, 1999). The cytoplasmic synthesis of GDP-D-Ara$_p$ necessitates its translocation into the lumen of the Golgi apparatus, which in *Leishmania* occurs via the antiport mechanism of a multispecific GDP-sugar transporter (Hong *et al.*, 2000; Segawa *et al.*, 2005). The intravesicular GDP-D-Ara$_p$ can then be used as a donor substrate by D-arabinopyranosyltransferases in arabinosylation of glycoconjugates.

Compared with the many genes encoding glycosyltransferases in prokaryotic and eukaryotic systems that have been cloned (Field and Wainwright, 1995), very little is known about those that encode an arabinopyranosyltransferase. The *SCA1/2* genes from *Leishmania* were suggested as sc-D-Ara$_p$T-encoding genes based on their ability to enhance α1,2-D-arabinopyranosylation of β-Gal-side chain terminating LPG in *SCA*- or *SCA1/2*-transfected *L. major* LV39 strain and canonical glycosyltransferase structural motifs (Dobson *et al.*, 2003). To authenticate that *SCA1* encodes the actual sc-D-Ara$_p$T and rule out the possibility that the gene encoded an activator protein, chaperone, or auxiliary protein of sc-D-Ara$_p$T, it was imperative to establish the sc-D-Ara$_p$T activity of *SCA1* by heterologous expression in host cells lacking Ara-containing glycoconjugates.

Although expression of *SCA1His* in mammalian COS-7 cells and insect sf9 cells was successful, sc-D-Ara$_p$T activity was demonstrable only in the baculovirus system. The most likely explanation to account for the lack of transferase activity in *in vitro* assays with COS-7 microsomes is the possibility that SCA1 is active only when it is N-glycosylated (SCA1 has six potential N-glycosylation sites; Dobson *et al.*, 2003). N-Linked glycosylation is often essential for the folding, stability, intracellular transport, secretion, and function of glycoproteins (Rademacher *et al.*, 1988). The predicted molecular weight of the 832 amino acid SCA1 with the polyhistidine tag is ~93 kDa, which was detected in microsomal preparations from both host cells. Only in sf9 microsomes harboring the SCA1His protein, however, was a larger, glycopeptidase-F sensitive 115-kDa protein observed. It has already been demonstrated that the baculovirus insect cell expression system can produce N-glycosylated proteins (Donald *et al.*, 1998; Luckow and Summer, 1988). Since the 93-kDa nonglycosylated form of SCA1His was also detected in the microsomes from *SCA1*-transfected sf9 cells, this suggested that N-glycosylation of SCA1His apparently is not efficient.

In summary, the results of the *in vitro* assays of arabinosyltransferase using baculovirus recombinant microsomes and characterization of the LPG product in these assays indicate that *SCA1* encodes the sc-D-Ara$_p$T. Ultimately, it will be important to understand how the *L. major* up-regulates *SCA* gene expression in the critical process of metacyclogenesis that enables the parasite to detach from the midgut of its sand fly vector, thereby promoting its transmission.

Materials and methods

Molecular construction of pCDNA3-SCA1His

L. major LV39 (MRHO/SU/59/P) transfected with a pXG-$SCA1_{FV1}$ construct (Dobson *et al.*, 2003) were grown at 25°C in M199 medium containing 10% fetal bovine serum (Kapler *et al.*, 1990), biopterin (1 μg/mL), and G418 (200 μg/mL). DNA was prepared from the transfected cells using miniprep DNA kit (Qiagen). Full-length $SCA1_{FV1}$ gene was polymerase chain reaction (PCR) amplified using 10 ng of miniprep DNA as template, 0.25 μM of each SCA1 EcoR1 forward (5′-ccggacttcatgagggggacatcacag-3′) and SCA1 Not1 reverse (5′-ataagaatgcggccgctcaatgatgatgatgatgatggtataagcccgtgtaaaac-3′) primers, *Taq* polymerase (Promega, Madison, WI), and 0.2 mM of all four dNTPs. DNA of amplified *SCA1* was gel purified by Zymoclean DNA recovery kit (Zymoresearch, Orange, CA). Amplified *SCA1* and pCDNA3 (Invitrogen, Carlsbad, CA) vector were digested with EcoRI and NotI (NEB, Beverley, MA) restriction enzymes; the digested vector was dephosphorylated with

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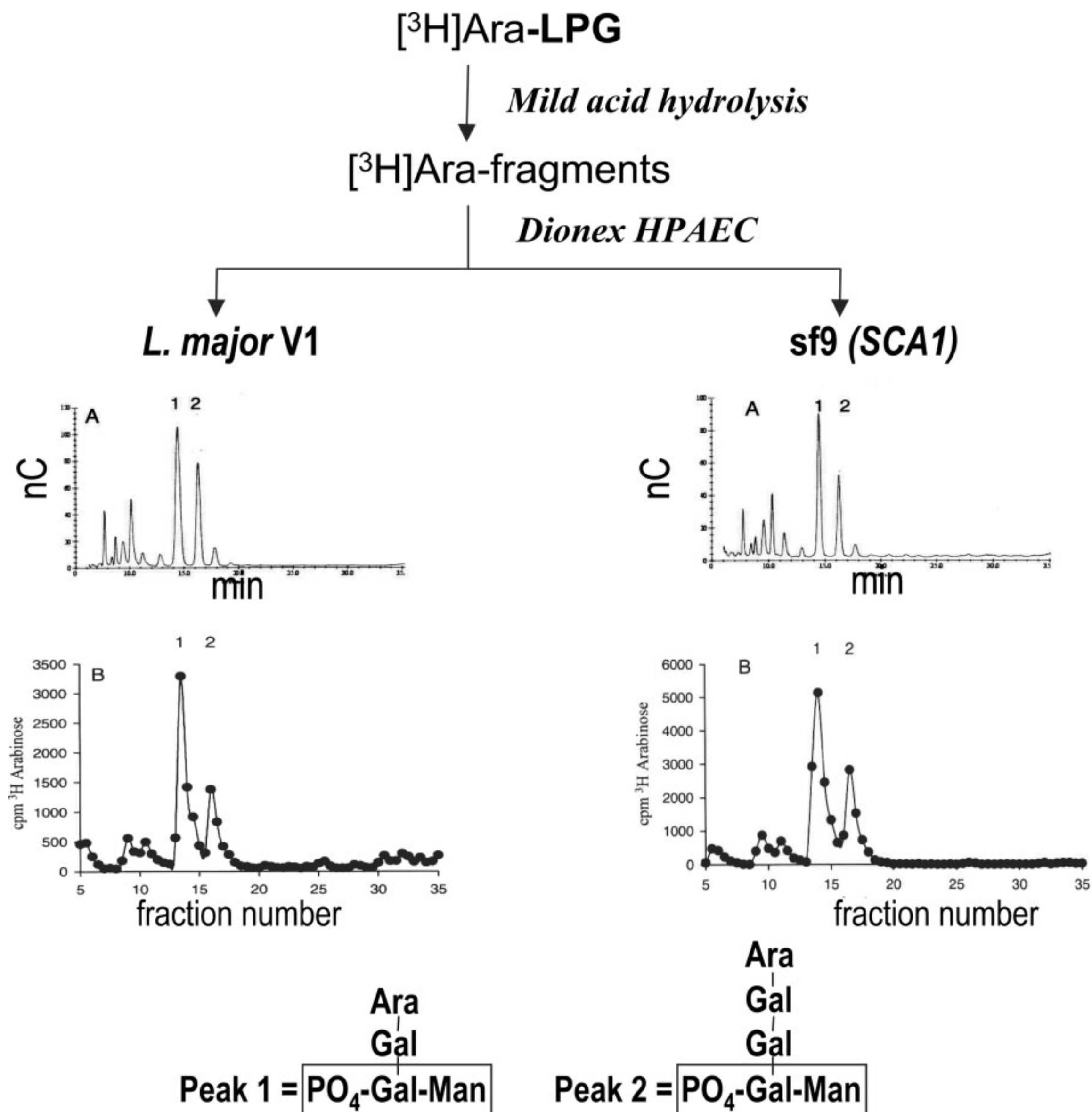

Fig. 5. High-performance liquid chromatography (HPLC) analysis of products generated by depolymerization of LPG. Aliquots of the [^{3}H-Ara]-labeled LPG generated in sc-D-Ara$_p$T assays were depolymerized by mild-acid hydrolysis (0.02 N HCl, 60°C, 15 min). The phosphorylated [^{3}H-Ara]-repeat units were extracted by partitioning using water-saturated *n*-butanol and then resolved by Dionex HPAEC. The left panels were profiles from control *Leishmania major* LPG, and the right panels were profiles from sf9 cells harboring the *SCA1His* construct. (**A**) Panels are PAD-detection profiles; (**B**) panels are profiles obtained by scintillation counting at 2-min intervals. In the peak designations, the rectangles represent the basic -PO_4-6Gal(β1,4)Man(α1)-repeat unit backbone.

calf intestinal alkaline phosphatase (NEB). Restricted and dephosphorylated fragments were ligated using the T4 DNA ligase enzyme (NEB) at 16°C for 16 h and transformed using *Escherichia coli* DH10 electrocompetent cells (Invitrogen). Positive clones were verified by restriction endonuclease digestion, and one of them confirmed by sequencing.

Transfection of COS-7 cells

COS-7 cells were maintained in Dulbecco's modified medium with 4 mM L-glutamine adjusted to contain 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, and 10% fetal bovine serum at 37°C with 5% CO_2. Transfection was performed using lipofectamine (Invitrogen) reagent, according to manufacturer's instructions. COS-7 cells (70–80% confluent) were transfected with 12 μg of DNA (pCNA3 or pCDNA3-*SCA1His*) in 100-mm plates. After 24 h of transfection, cells were passaged at a ratio of 1:2, incubated for further 24 h, and harvested (total 48 h after transfection) to perform western blotting and arabinopyranosyltransferase assays.

pFASTBAC1-SCA1His and recombinant bacmid

The *SCA1His* construct was excised from the pCDNA3-*SCA1His* vector using EcoRI and NotI restriction enzymes, gel purified, and cloned into pFASTBAC1 donor plasmid

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between corresponding restriction sites by the same ligation and transformation procedure described above. The DNA of recombinant donor plasmid was transformed into DH10BAC (GIBCO BRL, Invitrogen, Carlsbad, CA) for transposition by the procedure as recommended by the GIBCO and reviewed elsewhere (Davies, 1994; Donald *et al.*, 1998; Jarvis *et al.*, 1998). Positive clones were recognized by blue white selection. Ten white colonies were restreaked on fresh plate containing kanamycin, gentamicin, tetracycline, X gal, and IPTG to verify the phenotype. DNA was isolated from six white recombinant bacmid colonies of 10 by the alkali-lysis method, according to Sambrook *et al.* (1989). Two recombinant bacmid colonies were checked by PCR amplification using 300 ng of pUC forward and reverse primers and also pUC forward and gene-specific reverse primers, 30 ng of recombinant bacmid DNA as template, 10 mM dNTPs, and expand long PCR system (Roche Technologies, Indianapolis, IN). The PCR conditions were as follows: one cycle of 94°C denaturation for 2 min, followed by 20 cycles of 94°C denaturation for 10 s, 57°C annealing for 30 s, 68°C extensions for 4 min, followed by the same 10 cycles with additions of 20 s in each cycle of extension, and finally an extension at 68°C for 7 min.

Transfection of sf9 cells

Spodoptera frugiperda (sf9) cells were obtained from GIBCO BRL. Recombinant bacmid DNA (17.5 μg) was used to transfect 1×10^6 cells per 35-mm plate in 2 mL of sf-900 II serum free medium (SFM) media containing 50 units/mL penicillin and 50 μg/mL streptomycin. Transfection was performed using Cellfectin reagent (GIBCO BRL), according to the manufacturer's instructions. The low-speed supernatant ($500 \times g$, 5 min) of SFM media after 72 h of transfection was used to infect sf9 cells at $1–2 \times 10^6$ cells/mL for the first round of amplification in the ratio of 1 to 9 mL (virus-containing supernatant : sf9 cells). The culture media was centrifuged ($500 \times g$, 5 min) after 48 h of infection. The supernatant, containing viral stock, was used for further rounds of amplification at multiplicity of infection at 2.5 and the pellet, containing the recombinant protein, was analyzed for protein expression by western blotting and examined for sc-D-Ara$_p$T activity.

Western blotting

Cells were harvested and resuspended (1:5 weight/volume) in ice-cold lysis buffer containing 50 mM Hepes, pH 7.4, 1 mM ethylene diamine tetraacetic acid (EDTA), and Complete protease inhibitor cocktail (Roche). Cells were lysed by probe sonication (three times at the intervals of 2 min) for 15 s. Microsomal membranes were prepared (Butcher *et al.*, 1996), and aliquots of the microsomes (1 μg of protein) were mixed with 2× loading dye and electrophoresed using 8% sodium dodecyl sulfate (SDS) polyacrylamide gels, transferred onto nitrocellulose membranes, probed with 1:5000 horseradish peroxidase (HRP)-conjugated anti-His monoclonal antibody (Invitrogen) for 1 h, and developed with West Pico Chemiluminescent substrate (Pierce, Rockford, IL). Other aliquots of the microsomes (containing 200 μg of total protein) were incubated with Glycopeptidase F from *Chryseobacterium meiningosepticum* (Sigma, St. Louis, MO) in a reaction volume of 50 μL for 3 h at 37°C (Trimble and Tarentino, 1991).

sc-D-Ara$_p$T assay

The sc-D-Ara$_p$T assay was performed as described elsewhere (Dobson *et al.*, 2003) using microsomes prepared from transfected COS-7 cells or sf9 cells infected with baculovirus (third amplification). Briefly, microsomes were prepared following the method of Butcher *et al.* (1996), and protein was quantitated using the bicinchoninic acid (BCA) reagent kit (Pierce). Microsomes containing 1 mg of total protein were incubated at 30°C for 1 h in the presence of 0.02% Triton X-100 with 4 μM GDP-D-[^{3}H]Ara$_p$ (prepared according to Mengeling and Turco [1999]) as the donor and with 10 μg of β-Gal side chain-terminating LPG purified from logarithmically grown *L. major* FV1 (procyclic stage, Figure 1) as the acceptor (Mahoney and Turco, 1999). Assay reactions were terminated by the addition of chloroform : methanol (3:2). LPG was extracted by solvent E and purified by phenyl Sepharose (Sigma) chromatography, as described elsewhere (Orlandi and Turco, 1987). Incorporation of radiolabeled D-Ara$_p$ into LPG was measured by scintillation counting. For product characterization, the phenyl–Sepharose-purified LPG was depolymerized by mild-acid hydrolysis (0.02 N HCl, 15 min, 60°C), and the aqueous-soluble phosphorylated repeat units from the LPG were separated on a DX-500 HPLC (Dionex Corp., West Chester, OH), as described earlier (Mahoney *et al.*, 1999). Fractions were collected at 2-min intervals.

Acknowledgments

This work was supported by National Institutes of Health grant AI020941 (to S.M.B. and S.J.T.).

Abbreviations

BCA, bicinchoninic acid; D-Ara$_p$, D-arbinopyranose; LPG, lipophosphoglycan; PAD, pulsed amperometric detector; SCA, side chain arabinose; sc-D-Ara$_p$T, side chain D-arabinopyranosyltransferase; SFM, serum free medium.

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