Plasmid Vectors for Proteomic Analyses in *Giardia*: Purification of Virulence Factors and Analysis of the Proteasome

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In recent years, proteomics has come of age with the development of efficient tools for purification, identification, and characterization of gene products predicted by genome projects. The intestinal protozoan *Giardia intestinalis* can be transfected, but there is only a limited set of vectors available, and most of them are not user friendly. This work delineates the construction of a suite of cassette-based expression vectors for use in *Giardia*. Expression is provided by the strong constitutive ornithine carbamoyltransferase (OCT) promoter, and tagging is possible in both N- and C-terminal configurations. Taken together, the vectors are capable of providing protein localization and production of recombinant proteins, followed by efficient purification by a novel affinity tag combination, streptavidin binding peptide–glutathione S-transferase (SBP-GST). The option of removing the tags from purified proteins was provided by the inclusion of a PreScission protease site. The efficiency and feasibility of producing and purifying endogenous recombinant *Giardia* proteins with the developed vectors was demonstrated by the purification of active recombinant arginine deiminase (ADI) and OCT from stably transfected trophozoites. Moreover, we describe the tagging, purification by StrepTactin affinity chromatography, and compositional analysis by mass spectrometry of the *G. intestinalis* 26S proteasome by employing the Strep II-FLAG–tandem affinity purification (SF-TAP) tag. This is the first report of efficient production and purification of recombinant proteins in and from *Giardia*, which will allow the study of specific parasite proteins and protein complexes.

*Giardia intestinalis* is a binucleated, amitochondriate protozoan belonging to the order Diplomonadida. The intestinal parasite causes a waterborne, and occasionally food-borne, disease called giardiasis in mammals. The disease is characterized by diarrhea, fatigue, and malabsorption. It is estimated that there are around 280 million cases of giardiasis worldwide annually (23), and the disease has recently been recognized by the WHO as a part of the Neglected Diseases Initiative, raising awareness of its adverse health and socioeconomic impacts in low-income countries (36). *G. intestinalis* has been promoted as one of the earliest diverging protozoans based on phylogenetic studies, as well as its apparent lack of mitochondria, peroxisomes, and prokaryotic-like metabolic features (29). However, the basal eukaryotic position has been called into question by the discovery of mitochondrion-derived genomic sequences and the discovery of vestigial mitochondrial organelles called mitosomes (34, 44). The completion of the *Giardia* genome project (29), along with releases of two draft genomes (9, 14), has made it possible to settle some of the questions regarding *G. intestinalis*. Database mining revealed what seems to be typical eukaryotic machinery, albeit with fewer individual components than a eukaryote with a genome of similar size (29). Nevertheless, this does not mean that *Giardia* can be thought of as a primitive organism; it is highly evolved to parasitize its vertebrate hosts, and it is the most widely distributed enteric protozoa (11, 26).

The vegetative form of *Giardia*, the trophozoite, colonizes the upper small intestine and attaches to the epithelium by a unique cytoskeletal component called the ventral disk (1). *Giardia* cells have a range of different adaptations for coping with the intestinal environment and immune defense. Trophozoites may prevent the production of reactive nitric compounds by host cells by utilizing arginine, the host substrate for production of nitric oxide (NO) by nitric oxide synthase (NOS) (6). Arginine, moreover, is the preferred substrate for energy production via the arginine dihydro-
been extensively demonstrated in *Saccharomyces cerevisiae* (5, 41). *G. intestinalis* is transmitted by cysts that are formed in a developmental process known as encystation. Sorting of cyst wall material into specialized vesicles that subsequently form the highly resistant cyst wall is believed to be essential to the encystation process and appears to be regulated by the proteasome (42). The proteasome inhibitor bortezomib has been identified in drug screenings as an inhibitor of *Giardia* proliferation (4). The development of *Giardia*-specific proteasome inhibitors could potentially block both the proliferation and transmission of the parasite.

The *Giardia* genome projects (9, 14, 29) have paved the way for functional characterization of gene products, proteomics, and localization studies. These approaches have generally employed specially designed vectors, the construction of which can be time-consuming and laborious. Here, we delineate the construction and application of a novel affinity tag combination, streptavidin binding peptide–glutathione S-transferase (SBP-GST)-PreScission protease, in a vector with the capability to facilitate production and purification of recombinant proteins in *Giardia*. We further develop the means to perform protein complex purification by Strept II–FLAG–tandem affinity purification (SF-TAP) tagging and StrepTactin affinity chromatography (10). Either of these vector systems can be utilized as an affinity tag at the N- or C-terminus of proteins. These vectors will advance the understanding of *Giardia* biology and aid in the development of novel drugs and an effective recombinant-protein-based vaccine against giardiasis.

**MATERIALS AND METHODS**

**Plasmid construction.** The starting plasmid for construction was the PAC-pBS plasmid (S. Svärd, unpublished data), which is based on the pBlueScript II KS (+) (Stratagene) backbone. The plasmid carries a puruymycin *N*-acytlytransferase (PAC-pBS) gene that allows selection by 50 μg/ml puromycin in *Giardia*. The PAC-pBS gene is flanked by 47 bp of the giardial glutamate dehydrogenase (GDH) promoter and a 123-bp 3' untranslated region (UTR) from GDH inserted between KpnI and NotI restriction sites (39).

**OCT promoter fragment cloning.** The *Giardia ornithine carbamoyltransferase* gene (GL50803_10311) is one of the most highly expressed genes in *Giardia* (3). We selected the OCT promoter region to be used for strong constitutive expression of transcripts; 206 bp of the OCT promoter region was amplified by PCR from *Giardia* WB-C6 genomic DNA using primers OCT-1 and OCT-2 (see Table S1 in the supplemental material), introducing HindIII and EcoRV restriction sites. The purified DNA was doubly digested with HindIII and EcoRV according to the manufacturer’s recommendations. The resulting 824-bp SBP-GST cassette was recovered from gel-purified PAC-pBS plasmid cut with HindIII and EcoRV, creating the AO4 plasmid. The AO4 plasmid was used as an overexpression vector in *Giardia*, providing strong constitutive expression from the OCT promoter and permitting purification by the C-terminal 6×His tag. The AO4-MinusHis vector allows overexpression without the addition of a C-terminal tag.

**GST fragment cloning.** The glutathione S-transferase coding region and PreScission protease cleavage site were PCR amplified from the pGEX-6P-3 plasmid (GE Healthcare) using primers GST-1 and GST-2 (see Table S1 in the supplemental material), producing a 716-bp fragment that introduces Clal and NotI restriction sites. The purified DNA fragment was doubly digested with Clal and NotI according to the manufacturer’s recommendations. Cleaved DNA was gel purified, ligated into a gel-purified pBlueScript SK (+) plasmid (Stratagene), and cut with Clal and NotI, creating the GST-7 vector.

**Design of affinity tag sequences for gene synthesis.** The SBP tag amino acid sequence (16) was reverse translated with the Entelechon back-translater (8; http://www.entelechon.com/backtranslation), employing a codon usage table from *G. intestinalis* (http://www.kazusa.or.jp/codon/cgi-bin/showcodon.cgi?species=5741). Unique restriction sites present in the AO4 vector were excluded from the resulting nucleotide sequences to permit future cloning. The Web-based program Assembly PCR oligonucleotide maker (35) was used to generate oligonucleotides suitable for PCR-based gene construction. The program was queried with the SBP nucleotide sequence flanked by KpnI and EcoRV N-terminal restriction sites and a Clal C-terminal restriction site totaling 137 bp.

**Design of the SF-TAP cassettes, both C and N terminal, was based on the system developed for use in mammalian cells (10). In short, the SF-TAP polypeptides were reverse translated as described for the SBP tag. Unique restriction sites present in the AO4 vector were excluded from the resulting nucleotide sequences to facilitate future cloning. Finally, a pBlueScript II KS (+) multiple-cloning site was incorporated to allow exchange of inserts between vectors. Assembly primers and flanking primers were created using Assembly PCR oligonucleotide maker.

**SBP tag and SF-TAP cassette construction by simplified gene synthesis.** Simplified gene synthesis (SGS) is a PCR-based approach for construction of synthetic genes (47). The procedure simplifies the process by combining fill-in PCR and amplification of a full-length product in a single PCR. Oligonucleotide assembly primers for the respective assembly PCRs (see Table S1 in the supplemental material) were combined from 20 μM solutions and diluted to 1 μM each primer in sterile H2O. A dilution series to 500 nM, 250 nM, and 100 nM was made using sterile H2O. Amplification primers for the respective assembly PCRs (see Table S1 in the supplemental material) were diluted to 10 μM in sterile H2O. SGS reaction mixtures were prepared by combining 5 μl SBP (SBP 1 to 4), N-SF-TAP (N-SF-TAP A1 to A6), and C-SF-TAP (C-SF-TAP A1 to A6) assembly primer mixture (1 μM, 500 nM, 250 nM, or 100 nM), 2 μl 10 μM respective flanking primer mixture, 1 μl 10 mM deoxynucleoside triphosphates (dNTPs), 5 μl 10X Thermopol buffer (NEB), 0.75 μl Vent DNA polymerase (NEB), and 36.3 μl sterile H2O. SGS was then performed on an Applied Biosystems 2720 thermal cycler with the following profile: 94°C for 5 min, followed by 25 cycles of 94°C for 30 s, 52°C for 30 s, and 72°C for 1 min, ending with a 5-min step at 72°C. The PCR products were analyzed by 1.5% agarose gel electrophoresis. The remaining SGS 100 nM PCR products (40 μl), which gave the most specific product, were purified using the QiAquick PCR purification kit. The SBP tag fragment was doubly digested with KpnI and Clal according to the manufacturer’s recommendations. The digested DNA fragments were gel purified and ligated into gel-purified GST-7 plasmid cleaved with KpnI and Clal, creating the SGS-GST-7-15 vector. The N-SF-TAP and C-SF-TAP fragments were doubly digested using BamHI and Apal, respectively, and ligated into the AS6-Alp vector digested using the respective enzyme combinations. This created the AN (AO4-N-SF-TAP) and AC (AO4-C-SF-TAP) vectors.

**Construction of SBP-GST tagging vectors.** The SBP-GST tagging vectors were constructed by insertion of the SBP-GST cassette of the SGS-GST-7-15 vector into the AO4 vector. The SGS-GST-7-15 plasmid was doubly digested using EcoRV and NotI according to the manufacturer’s recommendations. The resulting 824-bp SBP-GST cassette was recovered.
by gel purification and ligated into a gel-purified EcoRV- and Noti-digested AO4 vector to create the AS6 vector. The C-terminal SBP-GST vector was created by PCR amplifying the SBP tag with primers SBP-C-F and SBP-C-R, followed by amplification of the GST tagging using primers GST-C-F and GST-C-R. The fragments were purified and digested with Clal. The cleaved fragments were ligated, purified, and used as the template in a PCR using the GST-C-F and SBP-C-R primers. The C-terminal SBP-GST fragment (817 bp) was amplified by PCR and digested using Noti and Apal. The purified fragment was ligated into a gel-purified Noti- and Apal-digested AC vector, creating the GST-SBP vector.

**Insertion of multiple-cloning site.** A multiple-cloning site introducing PstI, MluI, and XbaI restriction sites was constructed by annealing two oligonucleotides, MCS-1 and MCS-2 (see Table S1 in the supplemental material). The oligonucleotides anneal to create BamHI and EcoRI sticky ends for ligation into a vector cut with 5’ BamHI and 3’ EcoRI sites, keeping the correct frame for the 6XHis tag and stop codon. MCS-1 and MCS-2 oligonucleotides were dissolved to 200 μM each in sterile H2O. The annealing mixture consisted of 20 μl 200 μM MCS-1, 20 μl 200 μM MCS-2, 8 μl 10× One-Phor-All Plus Buffer (GE Healthcare), and 32 μl sterile H2O. The annealing mixture was incubated for 10 min in a heat block at 84°C. The solution was left to cool to room temperature to promote efficient annealing. The annealed oligonucleotides were stored at −20°C.

The annealed oligonucleotides were ligated into AS6- and AO4-His vectors, doubly digested with BamHI and EcoRI, and transformed into One Shot Chemically competent cells (Invitrogen) by heat shock. Positive clones were screened by PstI restriction cleavage ligation. One positive clone from each transformation, ASM and ASM-His, was sent for sequencing with the −40 M13 forward primer.

**Cloning of ADI, OCT, Rpt1, Pre1, and Rpn11.** The ADI (GLS0803_112103), OCT (GLS0803_103111), Rpt1-1 (GLS0803_86683), Pre-1 (GLS0803_3209), and Rpn11 (GLS0803_16823) genes were amplified by PCR from *G. intestinalis* genomic DNA (see Table S1 in the supplemental material). The PCR products ADI (1,753 bp), OCT (1,197 bp), Rpt1 (1,554 bp), Pre1 (696 bp), and Rpn11 (1,021 bp) were gel purified, digested, and ligated into the destination vector. ADI was ligated into the ASM vector as a BamHI and Noti fragment. OCT was ligated in the SBP-GST vector as a HindIII and Noti fragment. Rpt1 was digested with BamHI and Noti for cloning into the AN vector. Pre1 and Rpn11 were digested with EcoRv and Noti and cloned into the AC vector.

**Culturing and transfection of *Giardia* trophozoites.** *Giardia* trophozoites (clone WB-C6-A11) were grown in 10-mL culture tubes at 37°C in TYI-S-33 medium supplemented with bile according to the method of Keister (17). Plasmid DNA was isolated using the Qiagen Plasmid Midi Kit (Qiagen). DNA analysis was performed according to the manufacturer’s recommendations and stored at −20°C. Transfection of *Giardia* trophozoites was performed essentially as described previously (39). Puromycin (50 μg/mL) and gentamicin (100 μg/mL) were added to the transfectants 24 h after electroporation. The medium was exchanged after 2 to 3 days to remove dead unattached cells, and fresh TYI-S-33 with 50 μg/mL puromycin and gentamicin (100 μg/mL) was added. The cells were grown to confluence and were passaged in the same manner as wild-type trophozoites, keeping selection at 50 μg/mL puromycin.

**Harvesting of *Giardia* trophozoites for analysis of expressed protein.** Confluent transfected *Giardia* trophozoites were harvested by keeping them on ice for 20 to 30 min. The cells were further detached by tapping followed by centrifugation (500 × g; 10 min; 4°C). The supernatant was removed by vacuum suction, and the pellet was resuspended with 1 mL ice-cold phosphate-buffered saline (PBS). The absorbance of the suspension was measured at 600 nm with Smart Spec 3000 (Bio-Rad) and then diluted with PBS to get an optical density (OD) value of 0.05 unit (≈5 × 10⁶ cells) per 20 μL solution. The cell suspension was then further processed for analysis of expressed proteins by Western blotting.

**SDS-PAGE.** Proteins were separated by Tris-glycine SDS-PAGE using 5% stacking and 10% resolving gels as described previously (22), with minor modifications. Samples for SDS-PAGE were boiled for 10 min in appropriate amounts of 6× SDS loading dye, and 10-μl of sample was loaded in separate wells. Samples were prepared for downstream mass spectrometry analysis were mixed with an equal amount of 2× SDS loading dye containing 20 mM dithiothreitol (DTT) and boiled for 5 min. After cooling to room temperature, 0.5 M iodoacetamide (IAA) was added to a final concentration of 20 mM, followed by incubation for 20 min in the dark. Samples were neutralized by the addition of 1 μL 1 M Tris-HCl, pH 7.0, if needed (sample had a color other than blue) and then loaded into separate wells (2 to 20 μL), together with either PageRuler Prestained Protein Ladder Plus or Spectra Multicolor Broad Range Protein Ladder for Western blotting and PageRuler Unstained Protein Ladder for silver staining. Subsequently, the gels were prepared for Western blotting. Coomassie staining, or silver staining.

**Western blotting.** SDS gels were transferred by semidry blotting to polyvinylidene difluoride (PVDF) membranes using standard techniques. Blocking was performed for 1 h at room temperature (RT) or overnight at 4°C in 5% dry milk in PBS-0.1% Tween 20 or, alternatively, in 3% dry milk in Tris-buffered saline (TBS)-0.1% Tween 20 for the FLAG tag. Polyclonal mouse antibodies raised against the 20S proteasome alpha subunit 4 (GLS0803_15099), Giardia Rpt1 (GLS0803_86683) (42), polyclonal goat anti-GST (GE Healthcare), and FLAG tag monoclonal anti-FLAG M2 from mouse (Sigma) were diluted 1:1,000 in blocking buffer and incubated for 1 h at RT with gentle agitation. The membrane was washed with PBS-0.1% Tween 20 or with TBS-0.1% Tween 20 for FLAG tag for 15 min, followed by 3 5-min washes. Secondary antibodies, rabbit anti-goat peroxidase (Zymed), and rabbit anti-mouse IgG peroxidase (Dako), were added, diluted 1:10,000 in 5% dry milk in PBS-0.1% Tween 20, and incubated for 1 h at RT with gentle agitation. The membrane was washed as described above, and proteins were detected with the Amersham ECL Plus Western Blotting Detection System (GE Healthcare) and developed on Amersham Hyper film ECL (GE Healthcare).

**Staining of SDS-PAGE gels.** For Coomassie staining, the gel was soaked in Coomassie brilliant blue solution (40% MeOH, 10% acetic acid [HAc], 0.1% Coomassie brilliant blue) and destained by soaking in 10% ethanol (EtOH).

For silver staining, a protocol compatible with mass spectrometry was adapted from Shevchenko et al. (38). Stained gels were stored in 1% HAc at 4°C.

**Preparation of *Giardia* genomic DNA PCR template.** Genomic DNA for PCR was isolated from *Giardia* trophozoites (clone WB-C6) grown in TYI-S-33 medium. Confluent trophozoites grown in a 50-mL culture flask were harvested and pelleted by centrifugation at 500 × g for 10 min at 4°C. The cells were resuspended in 400 μL PBS and split into two tubes. Isolation of genomic DNA was then performed according to the instructions for the DNeasy tissue kit (Qiagen), adhering to the protocol for cultured animal cells. DNA was recovered in 400 μL H2O.

**PCR conditions.** PCR amplifications for cloning purposes (except for SBD tag) were performed using PuReTaq Ready-To-Go PCR Beads (GE Healthcare, Little Chalfont, United Kingdom). Each bead was dissolved in 1 μL 10 ng/μL DNA template (Giardia genomic WB-C6 DNA or plasmid DNA), 1 μL 10 μM primer pair mixture, and 25 μL sterile H2O. The tube containing the PCR components was spun briefly and then loaded into an Applied Biosystems 2720 Thermal Cycler. PCR was performed using the following PCR program: 96°C for 5 min, followed by 30 cycles of 96°C for 30 s, 59°C for 30 s, and 72°C for 1 min/kb of amplification product, ending with 10 min at 72°C. After PCR amplification, the sample was kept at 4°C in the machine. The remainder was either processed immediately or stored at −20°C.

**Plasmid sequence construction.** Plasmid sequences were constructed with the help of the freeware programs BioEdit v7.0.5.3 (1) and Ape v1.10.4. *Giardia* gene reference sequences were obtained from the GiardiaDB v2.5 database, which contains the genome sequence of the WB-C6 clone (ATCC 50803).
Sequencing. Sequencing was performed at Uppsala Genome Centre (UGC) using BigDye Terminator v3.1 (Applied Biosystems) and ABI 3730 and capillary electrophoresis for sequence separation and detection. Chromatograms were processed with Sequence scanner v1.0 (Applied Biosystems) and analyzed with BioEdit v7.0.5.3 (1), ClustalW (http://www.ebi.ac.uk/clustalw/), and ApE v1.10.4.

(i) Purification of recombinant \textit{Giardia} proteins. Large-scale cultures, harvesting, and purification of ADI and OCT from transfected \textit{Giardia} trophozoites. For production of recombinant \textit{G. intestinalis} ADI (GiADI) and OCT (GiOCT), 2 liters of transfectant cultures were set up in 50-ml tubes. After having reached confluence, cultures were harvested by GiADI and OCT (GiOCT), 2 liters of transfectant cultures were set up in 50-ml tubes. After having reached confluence, cultures were harvested by (GiADI) and OCT (GiOCT), 2 liters of transfectant cultures were set up in 50 ml PBS. In the last washing step, the pellet was taken up in cold PBS containing 1 M Complete Mini Protease Inhibitor Cocktail (Roche Diagnostics, Bromma, Sweden). The cells were pelleted, snap-frozen in liquid nitrogen, and stored at -80°C until further purification. Trophozoite pellets were thawed in 10 ml PBS containing 2 Complete Mini Protease Inhibitor Cocktail and 5 mM DTT and sonicated 4 times for 25 s at an intensity of 5 to 6. Triton X-100 was added to 1.5%, and debris was removed by centrifugation at 19,000 g for 12 min at 4°C. The supernatant was incubated with 660 µl of equilibrated gluthionate Sepharose beads (GE Healthcare, Uppsala, Sweden) for 3 h at 4°C with end-over-end rotation. Unbound protein was removed by centrifugation at 500 g for 3 min at 4°C, and the resident pellet was washed 3 times in cold PBS and 2 times in cold phosphate buffer (PB) (20 mM, pH 7.4) containing 1 mM DTT. Protein elution was performed in cold PB containing 1 mM DTT and 20 U PreScission protease (GE Healthcare) overnight at 4°C with end-over-end rotation. The eluate was recovered by centrifugation at 19,000 × g for 1 min at 4°C, and BSA was added to 1 mg/ml for protein stabilization. Aliquots of the proteins were stored at -20°C. One aliquot left without bovine serum albumin (BSA) was used for concentration determination by the Bio-Rad Protein Assay (Bio-Rad, Sundbyberg, Sweden) according to the manufacturer’s instructions. The purity and correct size of recombinant GiADI and GiOCT were assessed by SDS-PAGE and silver staining.

(ii) Assays for detection of ADI and OCT activities. The activity of recombinant GiADI was determined according to the method of Knodler et al. (19) by incubating the enzyme for 10 min at 37°C in a total reaction volume of 100 µl with 1 mM arginine in HEPES (1 M, pH 7.0). Recombinant GiOCT was measured according to the method of Vassef et al. (45), incubating the enzyme samples for 60 min at 37°C in a total volume of 60 µl containing 6 mM L-ornithine and 10 mM carbamoyl phosphate in HEPES (45 mM). Citrulline formed by recombinant GiADI or GiOCT was detected by boiling the reaction mixtures in chromogenic reagent (16.6% H$_2$SO$_4$, 13.3% H$_3$PO$_4$, 0.016% FeCl$_3$, 0.167% 2,3-butanedione monoxime, 0.0034% thiosemicarbazide) for 10 min and measuring the OD at 530 nm in a SmartSpec 3000 reader (Bio-Rad). The values were compared to a citrulline dilution series (0.01 to 0.7 mM), boiled in chromogenic reagent, and measured at 530 nm, as well, and final enzyme activities were calculated in U/mg.

(iii) Purification with Strept II tag. Cell pellets from harvested transfected \textit{Giardia} trophozoites were thawed or resuspended in a double volume of ice-cold buffer A (50 mM Tris-HCl [pH 7.5], 100 mM NaCl, 5 mM MgCl$_2$, 1 mM EDTA, 10% glycerol, 0.5% Nonidet P-40, 1 mM ATP, 0.5 mM DTT, 20 mM NaF, 1 mM Na$_2$VO$_4$, 2 Complete Mini Protease inhibitor tablet [Roche]) and incubated for 20 min on ice. The cells were lysed by Dounce homogenization, and the cell debris was spun down with a Sorvall RC5C Plus centrifuge at an SS34 rotor for 10 min at 4°C. The cleared lysate was transferred to a 15-ml Falcon tube and placed on ice. Purification of the lysate was performed with StreptTactin Superflow beads (IBA). The beads were prepared as follows: 200 µl of 50% suspended (giving a 100-µl bed volume) StreptTactin Superflow beads was pipetted into a 15-ml Falcon tube and washed with 1 ml (10 bed volumes) of ice-cold buffer A. The cells were centrifuged (950 × g; 5 min; 4°C), with removal of the supernatant. Washing of the beads was repeated twice. The cleared lysate was added to the washed beads and incubated for 1 h with end-over-end rotation on a nutator (Sky Line Intelli-Mixer; ELM1) at 20 rpm at 4°C. The beads and lysate were centrifuged (950 × g; 5 min; 4°C), and the supernatant was removed. The beads were mixed with 400 µl wash buffer Strep (buffer A with 0.1% Nonidet P-40) and moved to a Pierce Snap Cap Spin Column (Pierce), followed by centrifugation (950 × g; 1 min; 4°C). The beads were washed with 400 µl wash buffer Strep with centrifugation (950 × g; 1 min; 4°C). An additional 400 µl of wash buffer Strep was mixed and incubated with end-over-end rotation and agitation on a nutator (Sky Line Intelli-Mixer; ELM1) at 20 rpm at 4°C and centrifuged (950 × g; 1 min; 4°C). Washing was repeated 7 times. Elution from the StreptTactin beads was accomplished by resuspending the beads in 500 µl desthiobiotin elution buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 5 mM MgCl$_2$, 1 mM EDTA, 10% glycerol, 1 mM ATP, 2 mM o-desthiobiotin [IBA]), followed by 10 min of incubation (end over end on a nutator) at 20 rpm at 4°C, ending with centrifugation (950 × g; 1 min; 4°C). The elution procedure was repeated once. Eluates were concentrated with Microcon Centrifugal Filter Devices (Millipore) according to the manufacturer’s instructions. Aliquots of the flowthrough, washes, and eluates were analyzed by Western blotting and/or silver staining.

Identification of proteins using MALDI-TOF mass spectrometry. Bands (1 to 18) were cut out with a scalpel and processed for identification by matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectrometry. Proteins were digested using trypsin (porcine, modified and sequence grade; Promega) overnight at 30°C to reduce autodigestion. Trypsin was inactivated by the addition of 1% trifluoroacetic acid (TFA), and the peptide mixture was collected directly from the digestion tube on a C$_{18}$-µZipTip (Millipore) after dilution with 0.1% TFA to bring down the acetonitrile (ACN) concentration from the 10% used during digestion. The bound peptides were washed with 0.1% TFA and eluted with 75% ACN-0.1% TFA with half-saturated matrix (alpha-cyano-4-hydroxy-cinnamic acid) solution directly onto the target plate. Crystallization was accomplished by incubation at room temperature for 1 min. Mass spectra were recorded using an Ultraflex III TOF/TOF (Bruker, Bremen, Germany) instrument. The MALDI mass range was set to 600 to 4,500 m/z. Postsource decay was performed in a few cases on very intense peaks.

Database searches and bioinformatics. Mass spectra were processed and mass lists were exported with mMass software. Removal of keratin, trypsin, and other contaminating masses was accomplished with PeakEazor software (LightHouse Data). Multipoint calibration of mass lists based on contaminant masses was also performed for each spectrum with PeakEazor. The calibrated and decontaminated mass lists were searched against the NCBI nr database limited to other eukaryotes (including \textit{G. intestinalis} using the MASCOT peptide mass fingerprint (PMF) web server. Trypsin was applied as the digestive enzyme, and one miscleavage was allowed. Carbamidomethylation of cysteine was chosen as a fixed modification, and oxidation of methionine was set as a variable modification. The allowed tolerance for a peptide mass mismatch was set as ±0.03 Da. The annotation, accession number, number of matching masses, p.l, molecular weight (MW), sequence coverage of peptides, score, and significance of the top hits were recorded (see Table S2A in the supplemental material). The top-hit protein sequences were downloaded from GiardiaDB, and BLASTP was used to obtain the closest matching sequences in the NCBI nr database (see Table S2A in the supplemental material). The protein sequences were analyzed for domains or signature motifs using the SMART and Integro/Panther databases (see Table S2B in the supplemental material). Data from these searches were compiled, along with the MASCOT data, into Excel spreadsheets for easy viewing (see Table S2A and B in the supplemental material). A list of verified 26S proteasome components from yeast was compiled from the Saccharomyces Genome Database and queried against the GiardiaDB v2.5 proteome of \textit{G. intestinalis} isolate WB-C6 using BLASTP. The top-scoring sequences were downloaded and used in reciprocal analysis against the yeast pro-
Editors, and the lysate was cleared by centrifugation. The cleared lysate was incubated with glutathione agarose beads, followed by elution by PreScission protease cleavage. Fractions collected throughout the purification procedure were resolved by SDS-PAGE and stained with Coomassie brilliant blue (Fig. 2).

Glutathione beads that had been incubated with the cleared lysate and extensively washed showed the presence of a protein correlating well with the expected size of the SBP-GST-ADI fusion (96.7 kDa) and smaller amounts of a protein of the expected size for wild-type ADI (64 kDa) (Fig. 2). Cleavage by PreScission protease was efficient, leading to the disappearance of the SBP-GST-ADI protein and its replacement by protein species corresponding to recombinant ADI (65.5 kDa) and the SBP-GST moiety (31.3 kDa) (Fig. 2). PreScission protease binding to glutathione beads was effective, judging from the protein of around 40 to 45 kDa exclusively seen on the beads after elution. The elution fraction contained a protein corresponding to recombinant ADI (Fig. 2). The purification of the C-terminally tagged OCT was successful as judged from the protein of around 40 to 45 kDa exclusively seen on the beads after elution. The elution fraction contained a protein corresponding to recombinant ADI (Fig. 2). The purification of the C-terminally tagged OCT was successful as judged from the protein of around 40 to 45 kDa exclusively seen on the beads after elution.

Expression and affinity purification of arginine deiminase and ornithine carbamoyl transferase from Giardia transfectants. Two putative Giardia virulence factors, drug and vaccine candidates ADI and OCT, were chosen as targets to investigate whether Giardia proteins can be tagged, overexpressed, and affinity purification were carried out at 4°C, where possible, to preserve the activity of recombinant ADI. Lane 1, Precision PLUS protein ladder; lane 2, ADI-2 transfectant lysate after sonication; lane 3, lysate cleared by centrifugation; lane 4, insoluble cell material after centrifugation; lane 5, glutathione beads after overnight incubation with cleared lysate and washings; lane 6, glutathione bead flowthrough after overnight incubation with cleared lysate; lane 7, washed glutathione beads after overnight incubation with PreScission protease and elution; lane 8, eluate recovered after overnight cleavage with PreScission protease. Samples were resolved by 10% Tris-glycine SDS-PAGE and stained with Coomassie brilliant blue.
FIG 3 Characterization of AC-Rpn11 and AN-Rpt1 transfectants. (A) Western blot of whole-cell lysates of wild-type (WT) (WB-C6) trophozoites and AC-Rpn11 and AN-Rpt1 transfectants with anti-FLAG M2 monoclonal antibody. Expression of proteins corresponding to Rpn11-C-SF-TAP (42.5 kDa) and N-SF-TAP-Rpt1 (61.9 kDa) was detected.

DISCUSSION

ADI and OCT catalyze the first and second steps in the ADH pathway, a metabolic route predominantly found in prokaryotes (19, 20). Arginine conversion is an important energy source for Giardia trophozoites grown in vitro and serves as a good drug target due to the absence of an ADH pathway in animals (20). ADI and OCT have been found to be secreted from the parasite, with elevated levels being released upon interaction with intestinal epithelial cells (33). It has been proposed that this is a defense mechanism for the parasite that deprives epithelial cells of arginine, the substrate used to produce highly reactive NO as a defense against microbial challenge (6, 19). ADI and OCT have also been detected as two of the most immunogenic Giardia proteins in patient sera.
During acute giardiasis (31) and have received interest as vaccine candidates (13). OCT is known to be immunogenic in humans (31), but it did not induce any antibody response in immunized mice, nor did it confer protection (13). ADI could not be expressed successfully in the \textit{Salmonella} vaccine strain and remains unexplored as a vaccine candidate (13), but it has been promoted as a drug candidate for therapeutic treatment of giardiasis due to the essentiality of ADI in trophozoites (25). Therefore, we aimed to answer the question of whether both \textit{Giardia} proteins could be produced in and purified from the parasite, allowing subsequent interaction experiments with human epithelial cell lines \textit{in vitro}, drug screenings, and vaccine trials \textit{in vivo}.

Due to these reasons, ADI and OCT were tagged using the newly developed SBP-GST fusion vectors (Fig. 1) and recombinantly produced in \textit{Giardia} trophozoites. It has been postulated that ADI expression must be tightly regulated, since its high activ-
ity rapidly diminishes the intracellular arginine pool for protein synthesis (19). Despite this, stable ADI transfectants were generated, with cells showing no signs of toxicity or morphological abnormalities. Initial attempts to tag OCT at the N terminus failed to produce transfectants, indicating problems with toxicity of the construct. A C-terminal affinity tag, however, led to the establishment of transgenic parasites that grew without noticeable defects. Purification by glutathione affinity chromatography and elution by PreScission protease resulted in active ADI and OCT in sufficient amounts to allow activity measurements. The activity of recombinant GiADI was slightly lower than what has been reported for conventionally purified (21) and recombinant (19) GiADI produced in E. coli. However, recombinant GiADI expressed and purified by the same protocol from E. coli by GST and PreScission protease cleavage displayed 10 times lower activity than recombinant GiADI purified from transgenic trophozoites (B. Stadelmann, unpublished data). A protein corresponding to the size of endogenous ADI could be seen to copurify on the glutathione beads before protease cleavage (Fig. 2). Since ADI in Giardia forms homodimers (19), this most probably represents native ADI that can form dimers with recombinant GiADI. OCT showed up as a double band as well, with both bands being recognized by

**TABLE 1** Summary of proteins detected by MALDI mass spectrometry from StepTactin-purified AN-Rpt1 transfectant Giardia cells

| Subunit yeast | Subunit | GiardiaDB accession no. | MW | Band(s) in which detected | Peptide sequence coverage (%) |
|---------------|---------|-------------------------|----|---------------------------|------------------------------|
| CP subunits Giardia | α1 | 20S proteasome alpha subunit 1 | 16924 | 27.3 | 12 | 44 |
| | α2 | 20S proteasome alpha subunit 2 | 11434 | 27.5 | 13, 14 | 56, 42 |
| | α3 | 20S proteasome alpha subunit 3 | 14497 | 26.7 | 14 | 33 |
| | α4 | 20S proteasome alpha subunit 4 | 15099 | 23.2 | 16 | 55 |
| | α5 | 20S proteasome alpha subunit 5 | 2980 | 26.7 | 12, 13 | 63, 53 |
| | α6 | 20S proteasome alpha subunit 6 | 7962 | 28.2 | 11, 15 | 64 |
| | α7 | 20S proteasome alpha subunit 7 | 11486 | 31.8 | 11 | 35 |
| | β1 | Proteasome subunit beta type 9 precursor | 9824 | 24.1 | 18 | 27 |
| | β2 | Proteasome subunit beta type 7 precursor | 27059 | 29.3 | 17 | 35 |
| | β3 | Proteasome subunit beta type 3 | 13756 | 23.0 | 16, 17 | 32, 47 |
| | β4 | Proteasome subunit beta type 2 | 3209 | 23.8 | Not detected |
| | β5 | Proteasome subunit beta type 5 precursor | 12949 | 32.3 | Not detected |
| | β6 | Proteasome subunit beta type 1 | 13127 | 28.4 | 14, 15 | 48, 53 |
| | β7 | Proteasome subunit beta type 4 precursor | 1995 | 23.4 | 14 | 48 |
| RP subunits Giardia | Rpn1 | 26S proteasome non-ATPase regulatory subunit 2 | 33166 | 135.9 | 2 | 15 |
| | Rpn2 | 26S proteasome regulatory subunit, putative | 91643 | 147.6 | 1 | 30 |
| | Rpn3 | Hypothetical protein | 15454 | 53.2 | 5, 6 | 49, 27 |
| | Rpn5 | Hypothetical protein | 16929 | 58.9 | 4 | 43 |
| | Rpn6 | Hypothetical protein | 16659 | 46.2 | 7 | 40 |
| | Rpn7 | 26S proteasome non-ATPase regulatory subunit 6 | 4331 | 46.5 | 7 | 24 |
| | Rpn8 | 26S proteasome non-ATPase regulatory subunit 7 | 7896 | 31.3 | 11 | 38 |
| | Rpn9 | Hypothetical protein | 9099 | 45.6 | 7 | 49 |
| | Rpn10 | Hypothetical protein | 15604 | 28.4 | 8 | 35 |
| | Rpn11 | Non-ATPase subunit MPRI of 26S proteasome | 16823 | 37.3 | 8 | 41 |
| | Rpt1 | 26S protease regulatory subunit 7 | 86683 | 56.4, 61.9 | 4, 9, 10 | 41, 26, 25 |
| | Rpt2 | 26S protease ATPase subunit S4, putative | 113554 | 50.2 | 5 | 52 |
| | Rpt3 | 26S protease regulatory subunit 6B | 7950 | 43.4 | 7 | 44 |
| | Rpt4 | 26S protease regulatory subunit 7 | 21331 | 44.2 | 6 | 37 |
| | Rpt5 | 26S protease regulatory subunit 6A | 4365 | 56.0 | 4 | 45 |
| | Rpt6 | 26S protease regulatory subunit 8 | 17106 | 45.0 | 6 | 42 |
| Various proteins detected | Dynamin-related protein | 14373 | 79.5 | 3 | 19 |
| | Bip | 17121 | 74.4 | 3 | 15 |
| | Ubiquitin carboxyl-terminal hydrolase 14 | 8189 | 50.5 | 5 | 56 |
| | Ribosomal protein L3 | 16525 | 43.2 | 7 | 36 |
| | Ribosomal protein P0 | 17054 | 35.0 | 8 | 16 |
| | Ribosomal protein L5 | 17395 | 33.9 | 10 | 22 |
| | Ribosomal protein L4 | 17547 | 35.3 | 10 | 41 |
| | Ribosomal protein L7a | 17244 | 25.4 | 12 | 21 |
| | Ribosomal protein SA | 7766 | 28.1 | 12 | 24 |

*a* Identified proteins were sorted against proteasome components assigned by reciprocal BLASTP searches against yeast (subunit yeast).

*b* Annotation in GiardiaDB v.2.5.

*c* Detected by Western blotting.

*d* MW of epitope-tagged protein.
an anti-OCT antibody (data not shown). No data are available regarding the quaternary structure of *Giardia* OCT, but copurification of endogenous OCT with recombinant OCT could be a possible explanation. The recombinant GiADI and GiOCT purifications lend credence to the prospect of copurifying interacting proteins in *Giardia* by TAP technology.

Previous protein-tagging attempts with the ASM vector experiments concluded that the large size of the SBP-GST tag (31.4 kDa) might lead to steric hindrance and inefficient incorporation in many protein complexes. With that in mind, vectors were constructed to make use of the small SF-TAP tags (4.6 kDa) that have no dimerization capability and allow a less time-consuming purification protocol (10). The proteasome was chosen as a target complex to evaluate the efficiency and performance of the SF-TAP tags in *Giardia*, because it had been successfully tagged and purified in other organisms, like budding yeast (24) and humans (46).

We demonstrated affinity purification of the *Giardia* 26S proteasome with an almost complete inventory of subunits identified as being shared by *Giardia* and yeast through bioinformatics (Table 1) using N-terminally tagged Rpt1. The results showed that the elution fraction from AN-Rpt1 contained protein species that showed striking similarities to the pattern of conventionally purified *Giardia* 20S proteasomes (7), as well as the RP subunit pattern of yeast (24). Tagging of Pre1 or Rpn11 subunits did not lead to copurification of the proteasome complex, although the fusion proteins were detected by Western blotting (Fig. 3). The two unidentified components (β4 and β5) both belong to the CP and produce only a few peptides due to their small size, which will make PMF identification difficult. These peptides are also predicted to be acidic (data not shown), which can make ionization inefficient. All the putative regulatory particle components identified through bioinformatics were identified in this data set, confirming that reciprocal BLAST searching is a powerful tool for assignment of homologous proteins, even when protein divergence is extremely high, as in the case of the Rpn3 candidate (GLS0803_15454) (see Table S2A and B in the supplemental material). Three other proteins identified in this study have connections to the proteasome. The ubiquitin carboxyl-terminal hydrolase 14 is a ubiquitin-specific protease whose homolog in yeast, UBP6, is situated in the base subcomplex of the 26S proteasome and can be copurified in stoichiometric amounts (24, 32). BiP is an endoplasmonic reticulum (ER)-resident chaperone that excludes unfolded proteins to the proteasome for degradation (30). Dynamin has been shown to localize to nascent encystation-specific vesicles (ESVs) during encystation (28), similar to proteasome components (42). Ribosomal proteins are often identified in affinity-purified protein complexes and have been considered to be contaminants due to their high abundance in cells. The six ribosomal proteins detected in this study are likely to be contaminants, since they appear to be minor species compared to the proteasome components.

The expression of fusion proteins in the vectors up to this point was provided by the strong constitutive OCT promoter, which can result in overexpression of proteins. This creates nonphysiological conditions in cellular systems. Some overexpression is often necessary to incorporate the tagged protein into its protein complex in systems where the native protein is still present, but too high a level of expression can cause adverse effects. Future developments, such as adaptation to the tetracycline-inducible promoter system developed for use in *Giardia* (43) or the utilization of stage-specific promoters (12), could introduce additional flexibility into the present system.

The work presented here has provided the first experimental evidence for the subunit composition of the giardial 26S proteasome, as well as demonstrated efficient production of highly active ADI and OCT, potential virulence factors in giardiasis, from transgenic trophozoites. Purified *Giardia* proteasomes could be used to screen for potential selective inhibitors of proteasome activity and could potentially both eliminate the parasite and block its transmission by inhibiting the encystation process and thereby the production of viable cysts. Purified ADI and OCT could be used for detailed *in vitro* studies on the role of these specific components in the host-parasite interaction in giardiasis and for *in vivo* vaccination trials.

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