Proteome Mapping of the Protozoan Parasite *Leishmania* and Application to the Study of Drug Targets and Resistance Mechanisms*

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Leishmania **is a protozoan parasite responsible for significant morbidity and mortality worldwide. Few parasites have been subjected to proteomic analysis to date, but a genome sequencing project for *Leishmania major* is currently underway, making these studies possible. Here we present a high resolution proteome for *L. major* comprising almost 3700 spots, making it the most complete two-dimensional gel representation of a parasite proteome generated to date. We have identified a number of landmark proteins by mass spectrometry and show that several of these are valid for the related species *Leishmania donovani infantum*. We have also observed several forms and fragments of α- and β-tubulins and show that the number and amount of these fragments increase with the age of the parasite culture. Trypanothione reductase (TRYR), which replaces glutathione reductase in trypanosomatid parasites, is an essential protein specific to these parasites and as such is under considerable scrutiny as a drug target. Two-dimensional gel analysis of a *L. major* strain overexpressing TRYR revealed increased amounts of five spots, all at the predicted molecular weight for TRYR and differing by 0.08 pH units in pI. Mass spectrometry identified four of these as TRYR, leading to the novel suggestion that it could be post-translationally modified. Finally quantitative comparative analysis of a methotrexate-resistant mutant of *L. major* generated *in vitro* found that a known primary resistance mediator, the pteridine reductase PTR1, was overexpressed. This constitutes the first proteomic analysis of drug resistance in a parasite and also the clearest identification of a primary drug resistance mechanism using this approach. Together these results provide a framework for further proteomic studies of *Leishmania* species and demonstrate that these tools are valuable for the essential study of potential drug targets and drug resistance mechanisms.** ***Molecular & Cellular Proteomics 2:146–155, 2003.***

Leishmania is a protozoan parasite that can cause a species-dependent spectrum of disease, ranging from self-healing cutaneous lesions (*Leishmania major*) to visceral infections that are fatal if untreated (*Leishmania donovani*). With an estimated 12 million cases of leishmaniasis worldwide and 1.5–2 million new cases reported each year (1), this protozoan has a significant impact on human populations. *Leishmania* is transmitted by a sandfly vector as a motile, elongated promastigote. Upon transmission, the parasite is engulfed by a macrophage where it transforms into a round, non-motile amastigote able to spread within the host. The parasite is primarily found in South America, Asia, southern Europe, and Africa, but recent reports have confirmed the visceral form of the disease in dogs in 21 states in the United States and in Canada (2). In some regions, up to 80% of cases exhibit clinical drug resistance (3) leading to high levels of treatment failure.

While the number of global proteomic expression profiling studies involving infectious disease caused by bacteria and viruses are numerous, the application of these methodologies to protozoan parasites has lagged behind. This phenomenon can be attributed to a number of causes. Protozoan parasites tend to be challenging to culture and manipulate *in vitro*, making it difficult to obtain enough pure material for proteomic analysis. However, *Leishmania* promastigotes can be cultured relatively easily, and the amastigote form of several species can be propagated in cell-free culture (for a review, see Ref. 4). In addition, methods for gene transfection, overexpression, and disruption are available for *Leishmania*. A more substantial impediment is the lack of genome sequences for parasites in general. There are ongoing genome projects for only 20 species of protozoan parasites as opposed to several hundred bacterial and archaeal examples (see wit.integratedgenomics.com/GOLD). Among these are two species of *Leishmania*. An expressed sequence tag database of *Leishmania chagasi* is being created, and the 34-Mb genome of the human pathogen *L. major* Friedlin is being completely sequenced by a consortium of laboratories (www.sanger.ac.uk/Projects/L_major/). To date, the sequences of five of the 36 chromosomes have been completed, a further 21 are nearing completion, and the entire genome is projected to be finished in 2003. It is estimated that the *Leishmania* genome contains 8000 genes, and while numerous post-translational modifications are known to exist in this organism,

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the genes in general have no introns, and there is no alternative mRNA splicing. Thus, unlike the predicted case in higher eukaryotes, the proteome of *Leishmania* should not contain a drastically higher number of proteins than genes.

Its significance as a human pathogen has made *Leishmania* the focus of numerous studies in areas such as host-parasite interactions, cell differentiation, and our area of specialization, mechanisms of drug resistance. The availability of molecular methods, cell-free culture, and genomic sequence makes the application of proteomic methods to the study of these and other aspects of *Leishmania* biology possible and practical. Preliminary efforts in this direction include the generation of a partial two-dimensional (2D)[1] gel map for *Leishmania guyanensis* (5), and in another early analysis proteins differentially expressed in *Leishmania donovani infantum* amastigotes *versus* promastigotes were pinpointed (6). A proteome map of the species being sequenced is required, and the work presented here provides a framework for 2D gel- and mass spectrometry-based approaches to quantitative proteomics in *L. major* and other species. We have also applied these methods to the study of resistance mechanisms and novel drug targets and show that they are effective tools for biological discovery.

EXPERIMENTAL PROCEDURES

Cell Culture—*L. major* Friedlin, LV39, *L. donovani infantum*, and *Leishmania donovani donovani* were grown in M199 medium (Invitrogen) supplemented with 10% fetal calf serum (Wisent) and 5 μg/ml hemin (ICN Biochemicals). *Leishmania tarentolae* TARII and *L. major* MHOM/IL/67/JERICHO II (50122) transfected with the trypanothione reductase (*TRYR*) gene (7) were grown in SDM-79 medium (8) also supplemented with 10% fetal calf serum and hemin. Where appropriate, methotrexate (MTX, Sigma) or G418 (Invitrogen) was added. Cultures were incubated at 25 °C.

2D Sample Preparation and Electrophoresis—Cultures were grown to late log phase as determined by optical density at 600 nm. Cells were harvested by centrifugation at 2500 $\times$ *g*, washed twice in phosphate-buffered saline, and resuspended in 2D lysis buffer (7 M urea, 2 M thiourea, 40 mM Tris, 4% CHAPS, 0.1 mg/ml phenylmethylsulfonyl fluoride). Lysis was allowed to proceed for 2 h at room temperature, and samples were centrifuged to remove insoluble material. Protein concentration was assayed by the Amido Black filter assay or the 2D Quant kit (Amersham Biosciences). Proteins were aliquoted into single-use samples and stored at −80 °C.

In the first dimension, samples were run on 18-cm Immobiline Dry Strips (pH 4.0–5.0, 4.5–5.5, 5.0–6.0, 5.5–6.7, and 6.0–9.0; Amersham Biosciences) on an IPGphor isoelectric focusing system as recommended by the manufacturer with modifications as described in Ref. 9 for samples run on pH 6–9 strips. Strips were equilibrated in equilibration buffer (50 mM Tris-Cl, pH 8.8, 6 M urea, 30% glycerol, 2% SDS, trace of bromphenol blue) containing 10 mg/ml dithiothreitol for 15 min and then in equilibration buffer containing 25 mg/ml iodoacetamide for 15 min and sealed to 12% acrylamide gels using 0.5% agarose in standard Tris-glycine electrophoresis buffer. Second dimension SDS-PAGE was run in a Hoefer DALT apparatus (Amersham Biosciences) at 40 mA/gel and 15 °C until the tracking dye ran off the gel.

Gel Staining, Imaging, and Image Analysis—Proteins were visualized by either Sypro Orange or Sypro Ruby fluorescence. For Sypro Orange fluorescence, staining was carried out by the method of Malone *et al.* (10). Briefly, gels were fixed for 2 h in 30% ethanol, 2% acetic acid, 0.0005% SDS; washed 3 $\times$ 1 h in 2% acetic acid, 0.0005% SDS; and stained in wash solution containing a 1:5000 dilution of Sypro Orange (Molecular Probes) in the dark from 5 h to overnight. Gels were briefly rinsed with 2% acetic acid and scanned using a Typhoon laser scanner (Molecular Dynamics). Scans were produced using a 532 nm laser and 580 nm emission filter and by scanning at 100-μm resolution. Photomultiplier tube voltages were adjusted to obtain images of similar intensity. For Sypro Ruby, gels were fixed for at least 2 h in 10% methanol, 7% acetic acid. Gels were stained from 5 h to overnight and then destained in fixing solution. Gels were imaged with the ProXpress CCD camera-based scanner (PerkinElmer Life Sciences) at 100-μm resolution using a 480 nm excitation wavelength and 620 nm emission filter. To generate the proteome map, 2D gels of three independent samples per pH range were analyzed using PDQuest software, version 6.2.1 (Bio-Rad). For quantitation of TRYR overexpression, gels were analyzed with Progenesis software, version 2002.01 (Nonlinear Dynamics).

Mass Spectrometry—Gel plugs containing the proteins of interest were excised by hand (confirmed by rescanning the gel) and sent for peptide mass fingerprinting (Eastern Quebec Proteomics Centre, Centre Hospitalier de l'Université Laval, Quebec, Canada). Gel plugs were placed in 96-well plates and then washed with water. Tryptic digestions were performed on a MassPrep liquid handling robot (Micromass) according to the manufacturer's specifications using sequencing grade modified trypsin (Promega). After extraction from the gel into 50% acetonitrile, peptides were lyophilized in a speed vacuum and resuspended in 3 μl of 0.1% trifluoroacetic acid solution. The peptide sample solution was then combined with an equal volume of matrix (α-cyano-4-hydroxycinnamic acid, 20 mg/ml in 50% acetonitrile, 0.1% trifluoroacetic acid) and spotted onto a MALDI sample plate. The sample/matrix solution was allowed to air dry at room temperature and was then washed three times with 2 μl of 0.1% trifluoroacetic acid. Mass spectra were acquired on a Voyager-DE PRO MALDI-TOF mass spectrometer (Applied Biosystems) operating in the positive ion reflector delayed extraction mode. Protein identifications were obtained using MASCOT (MatrixScience) and by searching for matching peptide mass fingerprints in a protein database generated by automatic annotation of *L. major* Friedlin sequence information available through the Wellcome Trust Sanger Institute website (www.sanger.ac.uk/Projects/L_major/). The search criteria used were complete carboxamidomethylation of cysteine, partial methionine oxidation, and mass deviation smaller than 60 ppm. A score of greater than 51 was considered significant ($p < 0.05$). We also required at least six matched peaks per protein and considered the accuracy of the experimental to theoretical pI and molecular weight.

Peptide tandem mass spectra were obtained by capillary liquid chromatography coupled to an LCQ DecaXP (ThermoFinnigan, San Jose, CA) quadrupole ion trap mass spectrometer with a nanospray interface. An aliquot of the digested protein sample was diluted to 5 μl with 0.1% formic acid and loaded onto a reversed-phase column (PicoFrit 15-μm tip, BioBasic C18, 10 cm $\times$ 75 μm; New Objective, Woburn, MA). Peptides were eluted from the column with a linear gradient of water/acetonitrile in 0.1% formic acid at a flow rate of ~250 nl/min. Mass spectra were acquired using a data-dependent acquisition mode in which each full scan mass spectrum was fol-

[1] The abbreviations used are: 2D, two-dimensional; DIG, digoxigenin; MTX, methotrexate; PTR1, pteridine reductase 1; TRYR, trypanothione reductase; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; MALDI, matrix-assisted laser desorption ionization; TOF, time-of-flight; MS, mass spectrometry; MS/MS, tandem MS; IPG, immobilized pH gradient.

lowed by collision-induced dissociation of the three most intense ions. The dynamic exclusion function was enabled, and the relative collisional fragmentation energy was set to 35%. Resulting peptide MS/MS spectra were interpreted using the SEQUEST algorithm (11) and searched against proteins in the National Center for Biotechnology Information (NCBI) non-redundant protein database. Partial carboxamidomethylation of cysteine and oxidation of methionine were considered in the search. A protein was considered a good match if at least two peptides were confidently identified. Confident identification of a peptide required a cross-correlation score of 1.9, 2.5, and 3.7 for singly, doubly, and triply charged peptides, respectively. Each peptide identification was confirmed by manual inspection of the spectrum.

Western Immunoblotting—Cells were solubilized in Laemmli SDS-PAGE sample buffer and separated on 12% acrylamide gels. Gels were blotted to nitrocellulose membranes by the method of Towbin *et al.* (12). The blot was blocked for 1 h in 3% bovine serum albumin in phosphate-buffered saline. A monoclonal anti-α-tubulin antibody directed against an amino-terminal peptide of bovine α-tubulin highly conserved in *Leishmania* (A-11126, Molecular Probes) was diluted 1:2000 in blocking solution and incubated for 1 h with the blot. The blot was washed 3 $\times$ 5 min in phosphate-buffered saline containing 0.02% Tween 20 and incubated with horseradish peroxidase-conjugated sheep-anti mouse IgG (Amersham Biosciences) diluted 1:10,000 in blocking solution. The blot was washed as above, incubated with ECL Plus chemiluminescent substrate (Amersham Biosciences), and exposed to x-ray film.

Southern Hybridization—Genomic DNA was prepared from *Leishmania* cells using DNAzol (Invitrogen). DNA was digested with *Sac*I (New England Biolabs) according to the manufacturer's recommendations, and the resulting fragments were separated on a 0.7% agarose gel using digoxigenin (DIG)-labeled DNA Molecular Weight Marker II (Roche Applied Science) as a size standard. A *PTR1* probe was generated by PCR and random prime-labeled with DIG-High Prime (Roche Applied Science) according to the manufacturer's directions. The DNA gel was blotted to positively charged nylon membrane (Roche Applied Science), hybridized with the *PTR1* probe, detected with anti-DIG-AP Fab fragments and CSPD (Roche Applied Science), and exposed to x-ray film, all according to the manufacturer's directions.

RESULTS

Two-dimensional Map of the L. major Friedlin Proteome—Proteins were extracted from late log phase *L. major* Friedlin promastigotes with a solution of urea, thiourea, and CHAPS. After 2D gel electrophoresis, the resulting images of triplicate gels for each pH range were analyzed using PDQuest software, edited, and merged into two images. The first image (Fig. 1*A*), generated from gels spanning the pH ranges 4–5, 4.5–5.5, 5–6, and 5.5–6.7, contains 3113 spots, while the second image (Fig. 1*B*) is of a single pH range of 6–9 and contains ~583 spots. Determination of the number of spots on the pH 6–9 gel was challenging due to significant streaking, a problem commonly encountered with IPG strips in the basic range (13). As there appears to be little, if any, overlap between the basic end of Fig. 1*A* and the acidic end in Fig. 1*B*, probably due to poor resolution in these areas, we therefore estimate that we have resolved 3696 distinct protein spots.

Our next step was to identify a number of these spots, both to serve as landmark proteins and to determine the likelihood of obtaining identifications by MALDI-TOF MS, given the incomplete state of the genome sequencing project and the fact that few curated translations from the *Leishmania* genome project have been submitted to public databases. 62 spots of varying intensity were excised and digested with trypsin, and the resulting peptides were subjected to MALDI-TOF analysis. Of the 55 good quality spectra acquired, 37 identifications were obtained (Table I). There was a correlation between spot intensity and spectrum quality, although some intense spots gave few peaks by MALDI-TOF. In all, 29 different proteins were found among the 37 spots identified. α- and β-tubulins were identified in four and six different spots, respectively (Fig. 1*A* and Table I) with spots 616 and 611 most likely corresponding to the full-length α- and β-tubulins, respectively, and HSP70 and 3-hydroxy-3-methylglutaryl-CoA synthase were identified in two spots each. Several related HSP70 proteins are present in the *Leishmania* genome, and while there are no reports in the literature of post-translational modification of 3-hydroxy-3-methylglutaryl-CoA synthase, we may have identified a covalent enzyme intermediate or a modified form.

Tubulins Are Extensively Processed and Modified in Leishmania—The number of α- and β-tubulin species identified in our survey surprised us, and we were concerned that this variability was excessive. Tubulins are known to be extensively post-translationally modified (by polyglutamylation, polyglycylation, acetylation, phosphorylation, etc.; for a review, see Ref. 14), and we believe that these modifications account for the long trails of intense spots found in the 50-kDa region (see spots 611 and 616 in Fig. 1*A*). However, several spots identified as tubulins but smaller than the expected size were also apparent in our 2D gels (Table I). The solubilization solution we use contains protease inhibitors as well as thiourea, which has been shown to effectively inhibit proteolysis (15), but we were concerned that the number of tubulin fragments identified was the result of proteolysis during sample preparation. To determine whether this processing occurs *in vivo* or *in vitro*, cells were lysed directly in SDS-PAGE sample buffer and immediately electrophoresed. Western immunoblotting with an anti-α-tubulin antibody directed against the amino terminus of the protein showed that numerous smaller fragments of α-tubulin were indeed present in cells (Fig. 2). Many of the bands seen on the Western immunoblot correspond in mass to α-tubulin fragments identified on our 2D gels. While small amounts of the fragments are present in mid-logarithmic phase cells (Fig. 2, *lane 1*), some degradation products seem to increase significantly in amount with the age of the culture as shown by the number and intensity of smaller bands revealed using an early stationary phase sample (Fig. 2, *lane 2*). Our samples for 2D gels are taken in late log phase, which could explain the number and amount of fragments detected.

2D Protein Maps for Other Leishmania Species—We were interested in whether 1) MALDI-TOF would be able to identify

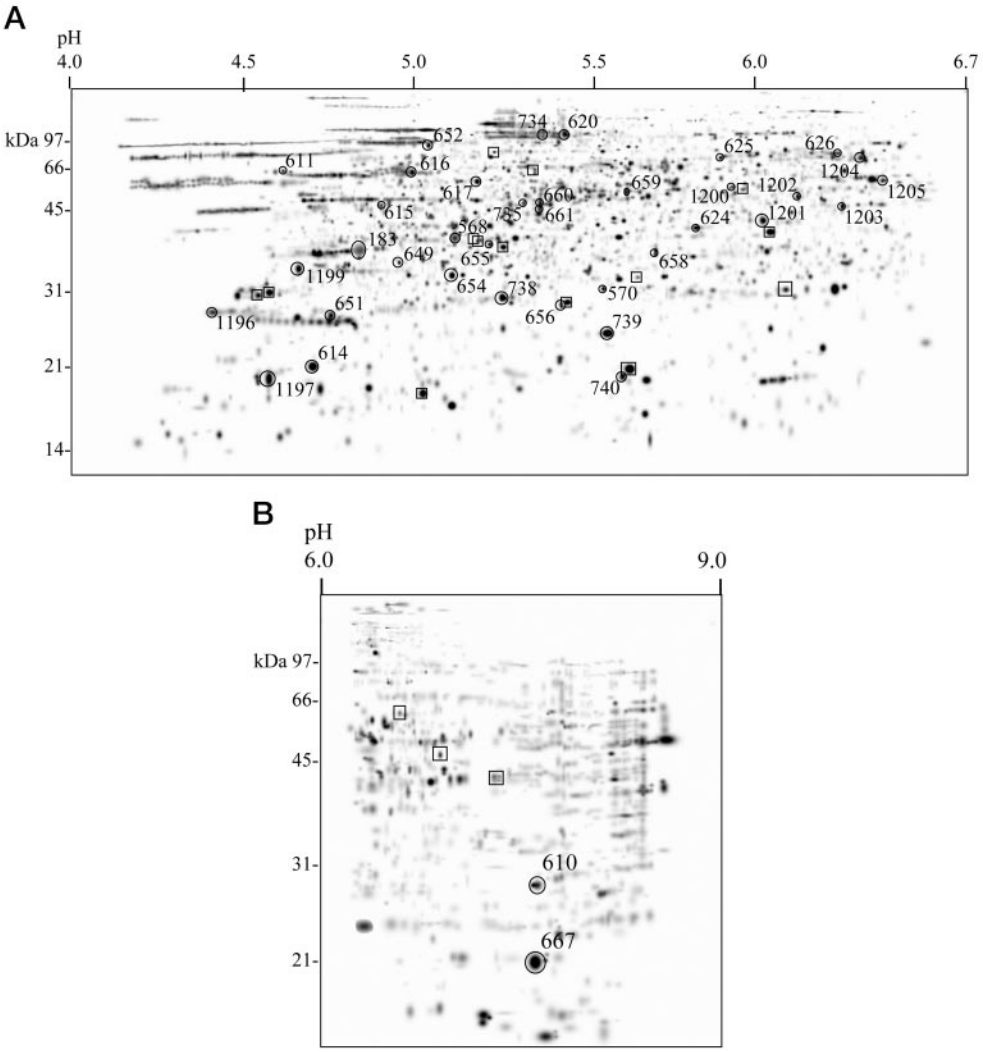

FIG. 1. **2D map of the urea/thiourea/CHAPS-soluble proteome of *L. major* Friedlin.** In *A*, images covering narrow range IPG strips of pH 4–5, 4.5–5.5, 5–6, and 5.5–6.7 were merged using PDQuest software. In *B*, a medium range IPG strip of pH 6–9 was used. Marked spots were excised and sent for MALDI-TOF protein identification. *Circled* and *numbered* spots were identified and are listed in Table I, while a spectrum, but no identification, was obtained for those marked with *squares*. A browsable version of this data is available at www.cri.crchul.ulaval.ca/proteome.

L. donovani proteins in the absence of a genome sequence for this species and 2) the *L. major* 2D protein map could be used as a basis for 2D gel studies of other species, such as the related *L. donovani.*

We performed *in silico* tryptic digests using 10 proteins whose sequences were known in both *L. major* and *L. donovani* and calculated the number of theoretical peptides between 500 and 4000 Da that matched in mass. The percentage of matching peptides varied from a high of 96% for α-tubulin to a low of 27% for the thiol-specific antioxidant TSA with an average of 71% peptide mass identity over 333 peptides considered. This suggested that it would be realistic to attempt MALDI identification of *L. donovani* proteins provided 2D gel patterns were similar.

We ran 2D gels of pH 4–7 of *L. major* Friedlin, *L. major* LV39, *L. donovani infantum*, *L. donovani donovani*, and *L. tarentolae* strains. Inspection of the protein profiles of these samples (Fig. 3) suggests that the degree of apparent similarity in protein profiles can be correlated to the relatedness of the species in question. Second, while there are similarities in the positioning of many protein spots, the variability between even closely related strains precludes the option of creating a "master" 2D protein map for differential expression studies of *Leishmania* in general. In fact, attempts at detailed spot matching between the five species were difficult, and the results were somewhat dubious. However, the degree of apparent similarity, particularly among the more highly expressed proteins, suggested that if similarly placed spots were the same protein they could be used as common landmarks. While we did not attempt to identify similarly placed

TABLE I

Mass spectrometric identifications of spots derived from the L. major proteome

Spot IDs are as in Fig. 1. Accession numbers are from the GeneDB_protein_database_270103 (ftp://ftp.sanger.ac.uk/pub/databases/L.major_sequences/LEISHPEP/). Apparent pI and molecular weight (MW) were calculated using PDQuest software, while predicted values were calculated using software available at www.up.univ-mrs.fr/~wabim/d_abim/compo-p.html. MASCOT scores above 51 were considered significant ($p < 0.05$). Low scores or low coverage (less than six peptides) are marked with an asterisk, and identifications of two spots (614 and 616) with both of these parameters were confirmed by MS/MS. Two spots unidentified by MALDI (618 and 651) were also subjected to MS/MS, resulting in one identification. expt, experimental; pred, predicted.

Spot ID	Protein	GeneDB accession number	MW (expt/pred)	pI (expt/pred)	No. peaks matched (percent coverage)	MASCOT score	MS/MS
183	β-Tubulin	CHR32_tmp.502c	34.3/49.7	4.82/4.51	13 (35)	92	
568	α-Tubulin	P1046.33	36.4/49.8	5.11/4.70	5 (13)*	41*	
570	α2 subunit of 20 S proteasome	L7171.14	25.6/25.1	5.53/5.41	5 (20)*	57	
610	STI1 homologue	CHR36_tmp.254c	31.6/29.0	7.67/8.18	13 (41)	171	
611	β-Tubulin	CHR32_tmp.502c	55.0/49.7	4.59/4.51	9 (13)	70	
614	Ribosomal protein S12, putative	CHR34 tmp.454c	17.2/15.6	4.72/4.53	3 (19)*	38*	Yes
615	mNUDC-like protein	P1295.23	44.2/36.9	4.89/4.73	8 (25)	60	
616	α-Tubulin	P1046.33	47.9/49.8	5.01/4.70	6 (10)	31*	Yes
617	Hypothetical protein	CHR36_tmp.205c	50.9/52.4	5.17/5.02	17 (31)	119	
618	Nice spectrum, no ID						No ID
620	Heat shock 70 protein, mitochondrial precursor, probable	CHR30_tmp.17	62.5/67.2	5.41/5.37	15 (19)	156	
624	Prostaglandin F synthase, probable	CHR31_tmp.107	39.0/31.9	5.71/5.90	8 (26)	87	
625	Trypanothione reductase	LM5.34	56.9/53.1	5.79/5.69	14 (18)	93	
626	3-Hydroxy-3-methylglutaryl-CoA synthase, possible	LM24.211	58.0/55.3	6.23/6.37	11 (15)	50*	
649	Monoubiquitin/carboxyl extension protein fusion, probable	CHR36_tmp.479	31.0/27.1	4.95/4.87	6 (31)	58	
651	Kinetochore-related protein, possible	LM5.44	24.1/22.4	4.78/4.60			Yes
652	t-complex protein 1, ε subunit, probable	CHR32_tmp.439c	62.3/59.3	5.03/4.88	10 (18)	66	
654	Hypothetical protein	CHR36_tmp.188c	29.2/29.0	5.10/5.13	9 (38)	100	
655	α-Tubulin	P1046.33	35.5/49.8	5.19/4.70	6 (13)	40*	
656	Hypothetical protein	CHR2_tmp.49c	23.9/17.6	5.42/5.16	9 (63)	117	
658	Cytochrome-*c* oxidase subunit 5, probable	CHR26_tmp.166c	30.9/22.3	5.69/6.00	8 (40)	95	
659	α-Tubulin	P1046.33	44.6/49.8	5.61/4.70	10 (20)	78	
660	β-Tubulin	CHR32_tmp.502c	44.2/49.7	5.34/4.51	18 (34)	179	
661	β-Tubulin	CHR32_tmp.502c	42.5/49.7	5.34/4.51	14 (23)	127	
667	Cyclophilin a, probable	CHR25_tmp.239c	22.7/16.8	7.68/7.94	9 (44)	88	
734	HSP70	CHR30_tmp.17	62.4/71.2	5.35/5.37	14 (24)	72	
735	Hypothetical 38.9-kDa protein	CHR12_tmp.35	43.3/38.1	5.29/5.26	11 (42)	118	
	β-Tubulin	CHR32_tmp.502c	43.3/49.7	5.29/4.51	10 (19)	89	
738	Peroxidoxin precursor, probable	L2581.13	25.6/25.3	5.23/6.39	6 (26)	56	
739	Hypothetical protein	CHR15_tmp.61	20.4/20.3	5.55/5.59	9 (47)	145	
740	Ubiquitin-conjugating enzyme, probable	L232.03	14.9/16.1	5.61/5.76	11 (54)	121	
1196	Translationally controlled tumor protein homologue, probable	LM24.150	25.9/19.4	4.42/4.18	9 (31)	86	
1197	Conserved hypothetical protein	L2230.07	16.2/12.9	4.59/4.49	6 (51)	58	
1199	β-Tubulin	CHR32_tmp.502c	31.6/49.7	4.66/4.51	9 (24)	76	
1200	Hypothetical protein	CHR33_tmp.101c	48.9/37.9	5.83/5.76	11 (44)	76	
1201	LACK protein	CHR28_tmp.11	40.9/34.3	5.94/6.00	6 (22)	78	
1202	Putative nol1-nop2-sun family nucleolar protein, possible	CHR36_tmp.619c	46.2/39.9	6.08/6.00	9 (28)	80	
1203	5′-Methylthioadenosine phosphorylase	L8325.04	44.1/33.4	6.24/6.24	7 (28)	95	
1204	3-Hydroxy-3-methylglutaryl-CoA synthase, possible	LM24.211	56.9/55.3	6.31/6.37	14 (31)	109	
1205	Aspartate aminotransferase	AST	50.3/46.0	6.39/6.54	18 (40)	179	

spots in all the different species, we felt that a more detailed comparison of *L. major* and *L. donovani* was warranted.

Gels of pH 5–6 of total cell extracts of *L. major* and *L. donovani* strains grown under similar conditions were run (Fig. 4). By using a narrow pH range, we were able to compare these gels to our proteome map and select some spots we had previously identified. Gels of *L. major* proteins contained 589 reproducible spots, while those of *L. donovani* contained only 462. Application of gel warping and matching algorithms using Progenesis software (version 2002.01) detected 134

spots as being conserved in location between the two sets of gels. Many of these appeared to be among the more highly expressed proteins, reflecting what was seen in the pH 4–7 gels (Fig. 3). Potential protein pairs were excised from the gels and sent for MALDI-TOF identification. Of 12 protein pairs sent, both members of seven were identified as the same protein (Fig. 4). We did not obtain good spectra for either member of pair 12. It is possible that intrinsic properties of this unknown protein result in few ionizable peptides and thus prevent its identification by MALDI. In one case (pair 3) the *L. donovani* protein was identified as α-tubulin, while the *L. major* protein was not identified. However, two of the major peaks were common between the mass spectra of these proteins. In many cases we obtained only relatively low protein coverage of tubulins by MALDI (Table I), probably due to high levels of modification. It is possible that the number of peptide mass matches was at the threshold required for identification. Of the remaining four pairs, no identifications were obtained in either *L. major* or *L. donovani*, but a number of peaks were common between the spectra in pairs 5 and 7, suggesting that they are the same proteins. All together, the same identification or similar peaks were obtained in nine of 11 cases where spectra were acquired.

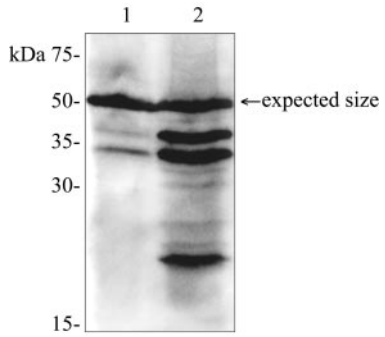

FIG. 2. **Western immunoblot of whole cells lysed in SDS-PAGE sample buffer with an antibody directed against the amino-terminal portion of α-tubulin.** *Lane 1*, *L. major* promastigotes in mid-log phase; *lane 2*, promastigotes in early stationary phase. The band corresponding to the predicted full-size α-tubulin is marked.

Drug Target Characterization and Identification of Differentially Expressed Proteins in Drug-resistant Parasites—One of the proteins mapped in our initial survey was TRYR (Fig. 1*A* and Table I, spot 625). Trypanothione, an abundant reduced thiol responsible for maintaining an intracellular reducing environment, is found exclusively in trypanosomatid parasites (16). It is also involved in detoxification of the first-line drug antimony from the cell and as such is an important mediator of drug resistance in *Leishmania* (17, 18). TRYR is the equivalent of glutathione reductase in other systems (19), and its restricted distribution has put TRYR under considerable scrutiny as a specific parasite drug target (19–21). We overexpressed the *TRYR* gene in a *L. major* strain to see whether we could detect the overexpressed protein in our 2D gels and also to see the effects, if any, of TRYR overproduction on the expression of unrelated proteins. While we did see a predictable increase in the intensity of the spot we had identified as TRYR, we also saw an unexpected increase in the intensity of four other spots in the same general area of the gel (Fig. 5). MALDI-TOF identification confirmed four of the five spots as being TRYR (the most acidic spot contained too little material for identification). These spots have similar molecular weights and a change in pI of ~0.08 pH units. This type of 2D spot pattern is often seen with proteins carrying post-translation modifications, but the MALDI-TOF spectra obtained from trypsin digestion did not suggest possible post-translation modifications or locations for these. No other proteins in the pH range tested (pH 4–7) appeared to be changed following *TRYR* overexpression.

The primary purpose of our foray into proteomics is to study mechanisms of drug resistance in *Leishmania* parasites. One of the drug classes we are interested in includes antifolates such as MTX. Studies on MTX resistance mechanisms have increased our understanding of folate and pterin metabolism in *Leishmania* and have pinpointed novel potential drug targets (for a review, see Ref. 22). To facilitate proteomic approaches, resistant *L. major* mutants were generated *in vitro* by serial passage in increasing concentrations of MTX. Com-

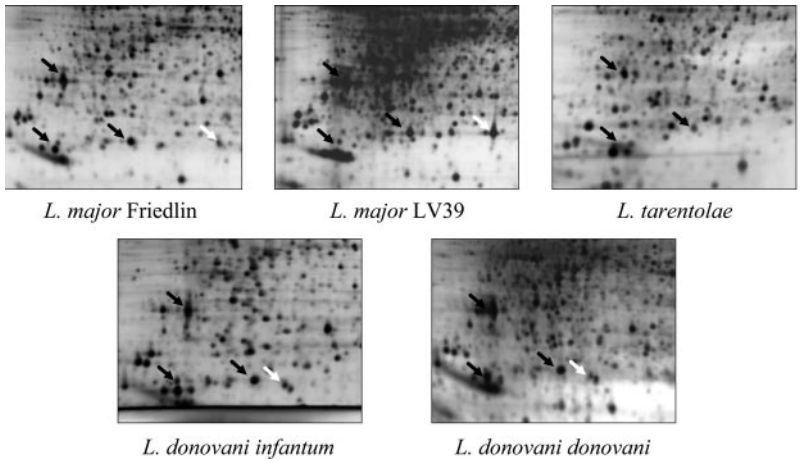

FIG. 3. **2D gel protein profiles of various species of *Leishmania*.** *Black arrows* denote areas of similar spot pattern in all species analyzed, while *white arrows* indicate species-specific spot patterns.

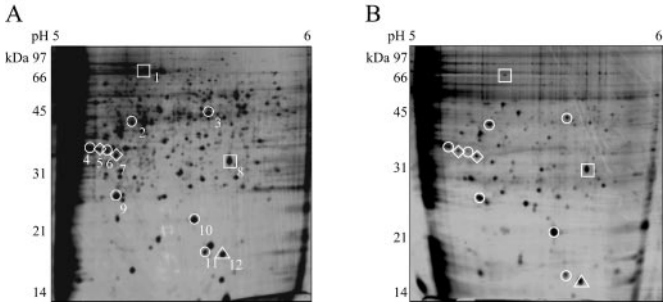

FIG. 4. **Comparison of protein profiles and MALDI-TOF identifications in *L. major* (*A*) and *L. donovani infantum* (*B*).** *Circles*, spots identified as the same protein; *diamonds*, unidentified spots with similar peak profiles; *squares*, unidentified spots with different peak profiles; *triangles*, no spectrum obtained. Protein identifications are as follows: *1*, HSP70; *2*, hypothetical 38.9-kDa protein and β-tubulin; *3*, *4*, and *6*, α-tubulin; *9*, peroxidoxin precursor; *10*, Chr15_tmp.61; *11*, ubiquitin-conjugating enzyme.

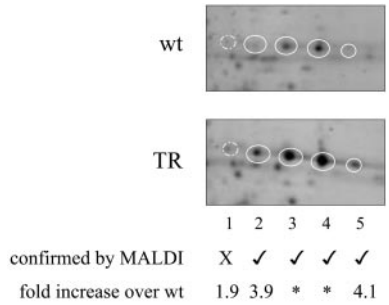

FIG. 5. **Overexpression of the *TRYR* gene in *L. major*.** 2D gel analysis using Progenesis software shows an increase in normalized volume of five spots in the *TRYR* transfectant *versus* the wild type. MALDI analysis identified four of these as TRYR. Quantitative analysis shows a 4-fold increase in normalized volumes of spots 2 and 5. Spots 3 and 4 are saturated in the transfectant, preventing accurate comparison (*). *wt*, wild type; *TR*, TRYR.

parative analysis of 2D gels of sensitive and resistant strains revealed a number of differentially expressed spots. The most obvious one is an intense spot present in *L. major* MTX 60.2 but absent in the sensitive parent (Fig. 6*A*). MALDI-TOF MS identified this protein as the pteridine reductase PTR1 (MASCOT score 87, eight matched peptides, and 33% sequence coverage), overexpression of which is a known primary MTX resistance mechanism (23, 24). Frequently drug resistance in *Leishmania* is brought about by gene amplification (25, 26), and we tested whether the massive overproduction of PTR1 was due to this phenomenon. The level of amplification was such that it was possible to detect a gene amplification event by simply comparing ethidium bromide-stained gels of the digested DNA of wild type and resistant cells (Fig. 6*B*). Southern hybridization of genomic DNA digests of the *L. major* sensitive parent and strain MTX 60.2 with a *PTR1* probe revealed that this gene was indeed present on the amplicons (Fig. 6*B*), suggesting a mechanism for the observed increase in protein. Several amplification events seem to have taken place as several bands hybridized to the *PTR1* probe.

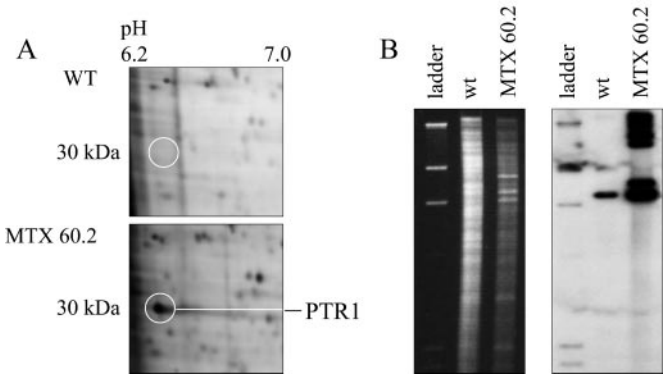

FIG. 6. **Proteomic and genomic analyses of *L. major* wild type and the MTX-resistant mutant MTX 60.2.** In *A*, one of the differentially displayed protein spots identified by 2D gel analysis is *circled* and was identified as PTR1 by MALDI-TOF analysis. *B* shows *Sac*I digestion of total DNA and Southern hybridization with a *PTR1* probe. *Lane 1* contains a DIG-labeled DNA ladder. Amplification of *PTR1* is observed in MTX 60.2. *WT* or *wt*, wild type.

DISCUSSION

Herein we present a high resolution 2D gel map of *L. major*. This map, containing 3700 protein spots, comprises the most complete 2D gel representation of a parasite proteome created to date. A browsable version of this map is available through our website, www.cri.crchul.ulaval.ca/proteome. Previous work resolved about 2000 protein spots using *L. donovani infantum* cells (6), while a recent study using broad range gel analysis of *Leishmania viannia guyanensis* resolved 719 spots (5). In another study, ~1000 protein spots from *Toxoplasma gondii* tachyzoites were separated using medium range gels (pH 4–7 and 6–11), and tests with narrow range gels suggested that it should be possible to resolve between 3000 and 4000 spots (27). Based on our results, this estimate is realistic considering the similarity in genome size between *Toxoplasma* and *Leishmania* (30 and 34 Mb, respectively). Recently two comparative proteome analyses of the life stages of *Plasmodium falciparum* were carried out using a mass spectrometry-based approach. In one, 1300 unique proteins were identified, covering 23% of the open reading frames predicted from the genome sequence (28), while in the second, 2400 unique proteins were detected (29). In a best case scenario, our proteome representation covers about 46% of the predicted 8000 genes in the *Leishmania* genome, although the consideration of post-translational modification and processing will significantly reduce this estimate.

Comparison of the overall 2D gel spot pattern generated using different species and subspecies of *Leishmania* reflects the relatedness of these strains. Phylogenetic analyses using DNA and RNA polymerases (30, 31) or EF1a[2] show that *L. major* and *L. donovani*, both human pathogens, are quite closely related, while the lizard pathogen *L. tarentolae* is placed in a different subgenus. The profiles of the two *L. major*

[2] E. Leblanc, personal communication.

strains are quite similar overall as is the case with the two *L. donovani* strains. Between the *L. major* and the *L. donovani* species there is more variability, and the relatively unrelated *L. tarentolae* species shows little clear similarity to the human pathogens. The landmarks on these maps may often be the same protein, but these results also raise the possibility that some protein spots not found in the same location on 2D gels could be useful as species-specific markers. We attempted to identify a highly expressed example of such a protein (Fig. 4*A*, spot *above* spots 11 and 12), but no identification was obtained from the mass spectrum acquired. A more important finding, however, is that the *L. major* genome will be valuable in the proteomic analysis of other species of *Leishmania*. The success of our attempts at *L. donovani* protein identification reflect the prediction made during our *in silico* analysis of *L. donovani* tryptic peptides. If MALDI-TOF MS is successful at least 60% of the time, currently a reasonable expectation at least for abundant proteins, it would be expected that in many more cases MS/MS methods would serve to confirm tentative MALDI identifications or would succeed where this method had failed. What is perhaps a more tantalizing possibility emerging from these preliminary studies is the prospect of being able to carry out MS-based studies, either similar to those described above using *P. falciparum* (28, 29) or comparative in nature, with various species of *Leishmania*. A recent review (32) summarizes the benefits and drawbacks of the two approaches. MS-based proteomic methods have numerous advantages and are becoming more effective and widespread, but 2D gel analysis is a relatively simple and reproducible method with clear advantages in quantitation and the analysis of protein modifications. The two approaches are complementary, and each is sure to reveal novel aspects of parasite biology.

TRYR regenerates reduced trypanothione, which is the major molecule responsible for maintaining an intracellular reducing environment in trypanosomatids and replaces glutathione in these protozoans. Attempts to disrupt both alleles of the *TRYR* gene in *Leishmania* have been unsuccessful (7, 33), indicating that the protein is essential for parasite survival, and reduced levels of TRYR activity correlate to reduced survival in macrophages (7). Trypanothione is also implicated in the resistance mechanism to antimony, the current drug of choice for the treatment of leishmaniasis. The importance of this enzyme to the survival and infectivity of trypanosomatid parasites and its absence in the mammalian host has led to significant interest in the possibility of using TRYR as a drug target.

Neither TRYR nor the related glutathione reductase is known to be enzymatically modified *in vivo*, although it is clear that numerous isoforms of glutathione reductase are present in plant tissues. In one study, the authors were able to distinguish six isoforms of the enzyme in cucumber leaves by native PAGE (34). While some of these isoforms could be the products of different genes and target different subcellular compartments, these observations could also be consistent with the presence of post-translation modifications on the enzyme. The *Leishmania* and *Trypanosoma brucei* genome sequencing projects have identified only a single *TRYR* gene in each organism, and in our case the increases in spot intensities were all brought about by the overexpression of a single gene. An analysis of the effects of oxidized and reduced glutathione levels on glutathione reductase activity in pine needles showed that, while glutathione reductase activity could be significantly increased by various treatments, there was no change in the amount of glutathione reductase protein or of *Gor* gene transcripts (35). Further direct evidence for regulation by modification of glutathione reductase appeared in a recent study (36) where it was shown that upon water stress in maize a series of events occurred resulting in several effects including the increased activity of glutathione reductase. While 38% more enzyme activity was detected, no change in protein amount was observed, suggesting that the activity of the existing protein was being modified.

Using a post-translational method of regulation as opposed to regulation by transcription or translation allows for a quick adjustment of enzyme activity. For trypanosomatids, protection from oxidants is essential for the survival of the parasite in macrophages. Post-translational regulation of TRYR would allow large amounts of the enzyme to be present in the cell in an inactive form, allowing redox levels to be maintained in normal conditions. When the oxidative environment within the macrophage is encountered, the enzyme stockpile could be quickly activated, allowing the cell to survive. It is also possible that non-enzymatic or nonspecific modification is occurring. The activity of the glutathione reductase enzyme is decreased in mammalian tissues by a number of non-enzymatic reactions, including alkylation (37), oxidation (38), and glycosylation (39). Sequence pattern searches have revealed a number of possible modifications, including potential sites of phosphorylation, sulfation, and glycosylation (40, 41).[3] Another possibility is small amino- and carboxyl-terminal truncations, which have been shown to modify the activity of lens crystallins (43) and the structure of prions (44) and could theoretically cause pI shifts similar to those observed for the TRYR spots. We are currently attempting to clarify the identity and the provenance of the purported modification(s) revealed by our experiments using MS and enzymatic techniques.

The primary goal of our proteomic effort is to identify the proteins directly or indirectly involved in resistance. These studies could identify ways to make current treatments more effective, perhaps by identifying adjunct therapies that will reduce the ability of the parasite to resist chemical attack. One benefit of a global analysis of this nature is that important proteins might be identified that would not have been analyzed otherwise, particularly among the estimated 70% of *Leishmania* proteins of unknown function or with no homologue (45) (www.sanger.ac.uk/Projects/L_major/). Finding mediators of resistance or new drug targets among this class

[3] R. Gupta, J. Hansen, and S. Brunak, manuscript in preparation.

of proteins has the added benefit that these are probably unique to the parasite, making inhibitors less likely to cross-react with host proteins and resulting therapies less toxic than current ones. Antifolates comprise a class of molecules that has shown promise in the treatment of various parasitic diseases as well as some cancers (for a review, see Ref. 46). The antifolate MTX is a folate analogue and inhibits the activity of dihydrofolate reductase. Dihydrofolate reductase is responsible for recycling spent dihydrofolate to tetrahydrofolate, the form active in numerous essential biological functions. In this study of a MTX-resistant mutant strain, a highly overexpressed protein was identified as PTR1, a primary resistance mediator (23, 24). PTR1 is able to reduce dihydrofolate to tetrahydrofolate at a low level (47, 48), and overexpression of this activity can thus replace the function of dihydrofolate reductase. The *PTR1* gene is amplified in this mutant (Fig. 6*B*), and this constitutes a clear example that gene amplification can lead to protein overproduction. DNA microarray analysis has since confirmed that this mutant has increased *PTR1* mRNA expression.[4] Since no spot corresponding to PTR1 is visible in the wild type strain, we cannot test how well gene amplification is correlated to protein expression.

Previous proteomic studies of drug-resistant bacteria have reported the differential expression of glyceraldehyde-3-phosphate dehydrogenase in erythromycin-resistant *Streptococcus pneumoniae* (49) and of alkylhydroperoxide reductase in metronidazole-resistant *Helicobacter pylori* (50), but the roles of these proteins in resistance are unclear. A number of differential protein expression studies of drug-resistant cancers have also been carried out (for a review, see Ref. 51), but again the roles of most of the proteins identified by these studies in resistance remain unclear. In one study, the protein MGr1-Ag was found to be overexpressed in multidrug-resistant gastric cancer cells. The function of this protein was correlated to resistance of the cells to several anticancer drugs (52), but the mechanism of this effect is unknown. PTR1 overexpression is the clearest example of a primary antimicrobial resistance mechanism identified using global proteomic methods to date, and although preliminary, this is in fact the first study of drug resistance in parasites using this approach. Resistance to MTX is often multifactorial, and further analysis has identified a number of other proteins differentially displayed between sensitive and resistant strains. We are currently evaluating the roles of these proteins in resistance. Some additional MTX resistance mechanisms are based on defects in membrane transporters such as FT5 (42). Unfortunately membrane proteins are not easily amenable to analysis by 2D gels, but we are exploring other techniques for the proteomic analysis of this class of proteins.

Here we present a high resolution 2D gel map of *L. major* containing a number of landmark proteins identified by mass spectrometry. We have also assessed the feasibility of these approaches to study the as yet unsequenced species *L. donovani*. Our analysis has allowed us to visualize the extensive processing of tubulins *in vivo*. We have obtained evidence that trypanothione reductase, a protein with strong potential as a drug target, may be post-translationally modified, a phenomenon not previously documented. Protein modification can play a significant role in regulation and function of an enzyme, and this discovery, if confirmed, could be critical to the development of inhibitors against this target. Finally, using comparative expression proteomics, we have identified a primary drug resistance mechanism in a methotrexate-resistant strain of *L. major*, thus validating the ability of this method to detect proteins involved in resistance. Taken together, this study provides a framework for the evolution of proteomic analyses in *Leishmania* and other parasites and demonstrates the ability of these investigations to study varied and important aspects of parasite biology.

[4] C. Guimond and M. Ouellette, unpublished observations.

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