Global Analysis of Protein Palmitoylation in African Trypanosomes\textsuperscript{7,†}

Brian T. Emmer,\textsuperscript{1,‡} Ernesto S. Nakayasu,\textsuperscript{2,‡} Christina Souther,\textsuperscript{1} Hyungwon Choi,\textsuperscript{3} Tiago J. P. Sobreira,\textsuperscript{4} Conrad L. Epting,\textsuperscript{5} Alexey I. Nesvizhskii,\textsuperscript{3,6} Igor C. Almeida,\textsuperscript{2,§} and David M. Engman\textsuperscript{1,*}

\textit{Departments of Pathology and Microbiology-Immunology\textsuperscript{1} and Department of Pediatrics,\textsuperscript{5} Northwestern University, Chicago, Illinois; The Border Biomedical Research Center, Department of Biological Sciences, University of Texas at El Paso, El Paso, Texas 79968;\textsuperscript{2} Department of Pathology\textsuperscript{1} and Center for Computational Medicine and Bioinformatics;\textsuperscript{6} University of Michigan, Ann Arbor, Michigan 48109; and Laboratory of Genetics and Molecular Cardiology, Heart Institute (InCor), University of São Paulo, São Paulo SP 05403-000, Brazil\textsuperscript{§}}

Received 3 October 2010/Accepted 20 December 2010

Many eukaryotic proteins are posttranslationally modified by the esterification of cysteine thiols to long-chain fatty acids. This modification, protein palmitoylation, is catalyzed by a large family of palmitoyl acyltransferases that share an Asp-His-His-Cys Cys-rich domain but differ in their subcellular localizations and substrate specificities. In \textit{Trypanosoma brucei}, the flagellated protozoan parasite that causes African sleeping sickness, protein palmitoylation has been observed for a few proteins, but the extent and consequences of this modification are largely unknown. We undertook the present study to investigate \textit{T. brucei} protein palmitoylation at both the enzyme and substrate levels. Treatment of parasites with an inhibitor of total protein palmitoylation caused potent growth inhibition, yet there was no effect on growth by the separate, selective inhibition of each of the 12 individual \textit{T. brucei} palmitoyl acyltransferases. This suggested either that \textit{T. brucei} evolved functional redundancy for the palmitoylation of essential palmitoyl proteins or that palmitoylation of some proteins is catalyzed by a noncanonical transferase. To identify the palmitoylated proteins in \textit{T. brucei}, we performed acyl biotin exchange chemistry on parasite lysates, followed by streptavidin chromatography, two-dimensional liquid chromatography-tandem mass spectrometry protein identification, and QSpec statistical analysis. A total of 124 palmitoylated proteins were identified, with an estimated false discovery rate of 1.0%. This palmitoyl proteome includes all of the known palmitoyl proteins in procyclic-stage \textit{T. brucei} as well as several proteins whose homologues are palmitoylated in other organisms. Their sequences demonstrate the variety of substrate motifs that support palmitoylation, and their identities illustrate the range of cellular processes affected by palmitoylation in these important pathogens.

The attachment of palmitic acid to a protein substrate can have dramatic effects on its function by targeting it to specific membrane domains, promoting association with lipid microdomains, regulating responsiveness to upstream signaling, and/or protecting it from degradation (22). However, the extent of protein palmitoylation in eukaryotes has long been underappreciated, due in part to the technical difficulty in detecting this modification. Metabolic labeling with \textsuperscript{3}H-palmitate requires immunoprecipitation and prolonged autoradiography exposure. Further complicating palmitoyl protein analysis is the inherent difficulty in candidate identification. While there are a few sequences that are highly predictive of protein palmitoylation (notably, a cysteine in close proximity to an N-terminal myristoylation site or a C-terminal CaaX box prenylation site), these are present in only a minority of palmitoyl proteins. Considerable advances have recently been made by instead applying an unbiased proteomic approach, first in \textit{Saccharomyces cerevisiae} (34) and then in rat neurons (18). These approaches have yielded several important findings. First, detection of palmitoylation for a given protein provides a possible mechanism for the regulation of its activity, trafficking, or expression. Second, an expanded data set allows for pattern detection in substrate sequences that may predict enzyme-substrate relationships. For example, the detection of a FWC carboxy-terminal tripeptide on amino acid permeases in yeast led to the discovery of those proteins all being modified by the same enzyme, Pfa4 (34). Finally, given the many known regulatory roles for palmitoylation in eukaryotic biology, palmitoyl proteome identification provides insight into the specific cellular processes affected by this modification. The discovery of a critical role for palmitoylation in regulating dendritic spine morphogenesis was prompted by the detection of an unusual Cdc42 splice variant in the rat neuron palmitoyl proteome (18).

Just as the identification of palmitoylation substrates has been slowed by technical obstacles, so too has the identification of enzymatic mediators. Palmitoyl acyltransferase (PAT) activity is tightly associated with cellular membranes and is often unstable upon extraction, hindering purification and identification of these enzymes by conventional biochemical approaches. The observation that certain proteins could spontaneously undergo palmitoylation \textit{in vitro} advanced the argument for enzyme-independent palmitoylation. Ultimately, however, genetic screens in yeast were successful in identifying the first \textit{bona fide} PATs (23, 33). Both of these enzymes, Erf2/Erf4 and

\textsuperscript{*} Corresponding author. Mailing address for I. C. Almeida: University of Texas at El Paso, Dept. of Biological Sciences, The Border Biomedical Research Center, 500 W. University Ave., El Paso, TX 79968. Phone: (915) 637-8266. Fax: (915) 747-5808. E-mail: icalmeida@utep.edu.
\textsuperscript{‡} Mailing address for D. M. Engman: Northwestern University, Dept. of Pathology, 303 E. Chicago Ave., Ward 6-175, Chicago, IL 60611. Phone: (312) 503-1267. Fax: (312) 503-1265. E-mail: d-engman@northwestern.edu.
\textsuperscript{§} Supplemental material for this article may be found at http://ec.asm.org/.
\textsuperscript{†} B. T. Emmer and E. S. Nakayasu contributed equally to this work.
\textsuperscript{‡} Published ahead of print on 30 December 2010.
Akr1p, were found to share an Asp-His-His-Cys Cys-rich domain (DHHC-CRD) motif, which was subsequently shown to play a direct role in PAT activity. The DHHC-CRD gene family has since been shown to be responsible for most, if not all, protein palmitoylation in yeast (34). This gene family is highly conserved among eukaryotes, with 7 members in yeast, 22 in mice, 23 in humans, and 12 in T. brucei (10). A comprehensive analysis of DHHC-CRD protein localization in human embryonic kidney 293T cells demonstrated these enzymes to have distinct and restricted subcellular localizations (25), a property that might underlie the differential trafficking effects on individual protein substrates. We previously identified the T. brucei calflagin PAT, TbPAT7, and demonstrated its role in calflagin trafficking to the flagellar, rather than pellicular, membrane (9, 10). Here, we expand on these earlier investigations of T. brucei protein palmitoylation to assess both the enzymes that catalyze this modification and the substrates that comprise the entire palmitoyl proteome.

MATERIALS AND METHODS

Parasites. All T. brucei strains described in this study are derivatives of the procyclic 29-13 strain or the bloodstream-form single-marker cell line, both of which have been engineered to coexpress the bacteriophage T7 polymerase and tet repressor (38). Parasite culture methods and generation of tetracycline-resistant parasites has been previously described (10) with the following modifications: a total of 3.2 ml TRIzol reagent (Invitrogen) were used to amplify a 250- to 350-bp region outside the RNAi target sequence in order to avoid amplification from the induced double-stranded RNA (dsRNA). For each set of PCR primers, a pilot experiment was conducted to determine an optimal number of cycles that enabled detection in the linear range (dsRNA). Primers were used to generate cDNA from 0.5 to 1.0 µl SuperScript first-strand synthesis for reverse transcription-PCR (RT-PCR) system (Invitrogen). Gene-specific primers (sequences are available upon request) were used to amplify a 250- to 350-bp region outside the RNAi target sequence in order to avoid amplification from the induced double-stranded RNA (dsRNA). For each set of PCR primers, a pilot experiment was conducted to determine an optimal number of cycles that enabled detection in the linear range of the assay.

RNA analysis. Total RNA was isolated from 1 × 10⁷ parasites by extraction with 1 ml TRIzol reagent (Invitrogen, Carlsbad, CA) and treated with amplification-grade DiNAse I (Invitrogen) for 15 min at 50°C. The raw LC-MS/MS data files and protein sequence database are available at the Proteome Commons Tranche network (https://proteomecommons.org/dataset.jsp?i=5cbNNwJmf3snjQAAAAAAAACZg%3D%3D). The identified proteins were deposited in TriTrypDB (tritrypdb.org). The identified proteins were deposited in TriTrypDB (tritrypdb.org).

RESULTS

Growth inhibition of T. brucei by 2-bromopalmitate. We first employed a pharmacologic approach to inhibit palmitoylation in T. brucei. Procyclic and bloodstream-form cells were incubated for 48 h in the presence of a range of concentrations of the nonmetabolizable palmitoyl analogue 2-bromopalmitate. Growth was analyzed relative to the vehicle control (Fig. 1). Both life cycle stages of the parasite were sensitive to 2-bromo-...
mopalmitate treatment, with a calculated IC\textsubscript{50} of 197 \(\mu\)M (95% confidence interval, 127 to 304 \(\mu\)M) for the procyclic life cycle stage and 226 \(\mu\)M (95% confidence interval, 147 to 348 \(\mu\)M) for the bloodstream life cycle stage. Growth was completely inhibited at concentrations consistent with the dose required in mammalian cells to inhibit palmitoylation (31). In contrast, 2-bromopalmitate had no effect on the growth of \textit{E. coli}, in which protein palmitoylation is not known to occur.

**Inhibition of specific PATs does not affect in vitro growth cell morphology, or motility.** To investigate the requirement of individual palmitoyl acyltransferases for the viability and growth of \textit{T. brucei}, we analyzed a series of cell lines containing an integrated, drug-inducible construct for RNAi inhibition of each PAT. The target genes were selected on the basis of their containing a DHHC-CRD motif (Pfam ID 01529). Target sequences were chosen to avoid off-target inhibition (29). These cell lines were previously used to identify the PAT that palmitoylates the flaggins, a necessary step in their trafficking to the flagellar membrane (10). We measured the transcript levels of each PAT in the parental cell line and in the RNAi line by semiquantitative RT-PCR (Fig. 2). Primers were selected to amplify transcripts in regions outside that targeted by RNAi to avoid detection of dsRNA. For each cell line, RNAi inhibition was successful in reducing transcript levels. While there were low levels of transcription following RNAi in a few of the lines, our experience with TbPAT7 indicates that these residual levels are not sufficient to maintain palmitoylation (10).

We performed phenotypic analysis on the 12 PAT RNAi lines. We first tested for an effect on parasite viability and/or proliferation by analyzing growth curves. Both procyclic-stage and bloodstream-stage parasites were seeded at a density that supports logarithmic growth. RNAi was induced by the addition of tetracycline to the culture medium. Cell counts were determined for induced and uninduced cultures over a 10-day period, with cultures diluted as necessary to prevent a shift into stationary phase. In contrast to the potent growth inhibition observed with 2-bromopalmitate treatment, none of the 12 individual RNAi lines, in either life cycle stage, exhibited a growth defect in culture (Fig. 3). Gross observation of parasite morphology and motility likewise did not uncover any defect in these mutants. Thus, while \textit{T. brucei} palmitoylation on a global level appears essential, these cells are not exquisitely sensitive to inhibition of any single enzyme. From a therapeutic standpoint, therefore, it appears more promising to target critical palmitoylation substrates rather than enzymatic regulators. With this in mind, we next sought to identify all of the proteins modified by these enzymes.

**Purification and identification of total cellular palmitoyl proteins.** To purify \textit{T. brucei} palmitoylated proteins, we first performed an acyl biotin exchange reaction on procyclic-stage \textit{T. brucei} whole-cell lysates (Fig. 4) (36). In this reaction, the nucleophilic reagent hydroxylamine (NH\textsubscript{2}OH) is used to cleave protein-palmitoyl bonds, liberating a reactive thiol that is labeled by coincubation with N-[6-(biotinamido)hexyl]-3′-(2′-pyridyldithio)propionamide (biotin-HPDP). To prevent nonspecific labeling, a preincubation step treats lysates with N-ethylmaleimide, which reacts with free thiols to generate an NH\textsubscript{2}OH-insensitive bond. As a control for nonspecific labeling, half of the sample is treated with the labeling reaction mixture minus NH\textsubscript{2}OH. The resulting sample can then be either analyzed directly by probing with streptavidin-horseradish peroxidase (HRP) (Fig. 5A) or purified by streptavidin-agarose chromatography. Elution of biotinylated proteins from streptavidin was performed by incubation with \(\beta\)-mercaptoethanol, which reduces the protein-HPDP linkage. Immunoblotting with a panel of antibodies against control proteins previously demonstrated the sensitivity and specificity of this approach in \textit{T. brucei} (10).

To identify total proteins purified under experimental (with
and control (without NH$_2$OH) conditions, acyl biotin exchange samples were subjected to protein identification by LC-MS/MS. Peptides mapping to each protein under each condition from triplicate analyses were then converted to normalized spectral abundance factors (40) (Fig. 5B) and analyzed for significance by QSpec, which uses a hierarchical Bayes estimation of a generalized linear mixed-effects model to assess significance (3). A total of 1,672 proteins were identified, and most of them were present in similar proportions under control and experimental conditions (Fig. 5B; see also Tables S1 and S2 in the supplemental material). These likely represent proteins for which N-ethylmaleimide blockade of free thiols was incomplete or proteins that nonspecifically associated with streptavidin-agarose beads. A subset, however, was present in significantly greater abundance under experimental conditions. A total of 132 proteins met the threshold for significance for being palmitoylated, with a false-discovery rate cutoff of 1% (Fig. 5C; see also Table S3 in the supplemental material). These included all of the known procyclic-stage \textit{T. brucei} palmitoyl proteins—calflagins Tb17, Tb24, and Tb44 (10), CAP5.5 (15), and PPEF phosphatase (24), as well as three proteins, phosphoinositide-specific phospholipase C and two calpain-like protein fragments, which have homologues in the closely related \textit{Trypanosoma cruzi} (12) and \textit{Leishmania major} (35), respectively, that are palmitoylated at Cys residues conserved in the \textit{T. brucei} proteins. To cull for false-positive identifications, we next removed eight noteworthy proteins for which an alternative hydroxylamine-sensitive thioester linkage has been reported. These included ubiquitination enzymes,

FIG. 3. \textit{T. brucei} growth is not affected by RNAi depletion of any single PAT mRNA. Tetracycline-inducible PAT RNAi cell lines were cultured at an initial density of 5 \times 10^3 cells/ml and propagated for 10 days in the presence (closed squares) or absence (open circles) of 1 \mu g/ml tetracycline. Every 48 h, cell density was quantified with an automated cell counter and cells were diluted as necessary to prevent entry into stationary phase.
FIG. 5. The T. brucei palmitoyl proteome. (A) Acyl biotin exchange samples prepared from procyclic-stage T. brucei were fractionated by SDS-PAGE, transferred to nitrocellulose membranes, and probed with streptavidin-HRP to detect total lysate labeling under each condition. (B) Individual proteins purified by streptavidin chromatography from acyl biotin exchange samples were identified by LC/MS-MS. Their relative abundances were quantified and normalized for experimental and control conditions. In this graphical representation, each point represents an individual protein, with its coordinates indicating its abundance under each condition. Thus, proteins clustered along the x-axis show high NH2OH dependence for their purification and, by extension, palmitoylation. Gray dots represent those proteins deemed insignificant by statistical analysis, while black dots represent proteins that met the threshold for significance. (C) The T. brucei palmitoyl proteome (n = 124 proteins) is listed, with corresponding NCBI accession numbers and functional groupings.
which form a transient thioester link with ubiquitin (14, 27), and pyruvate dehydrogenase, whose lipoic acid prosthetic group accepts acetyl moieties via thioester linkage. We also detected two palmitoyl acyltransferases, TbPAT2 and TbPAT11, which are expected to form a transient DHHC-palmitate intermediate during catalysis. After removing these proteins, we were left with a list of 124 high-confidence palmitoylated proteins (Fig. 5C; see also Table S3).

Characteristics of the \textit{T. brucei} palmitoyl proteome. Among the 124 palmitoylated proteins identified in this study (Fig. 5C; Table S3), a variety of sequence contexts for palmitoylation were identified (Fig. 6A). A total of 21 palmitoylated proteins (17%) have sequences predictive of N-myristoylation. In each case, a cysteine residue could be found within five residues of the myristoylated glycine. These are highly likely to be dually acylated. Another 15 (12%) and 47 (38%) palmitoylated proteins in \textit{T. brucei} have single or multiple predicted transmembrane domains. These are proteins for which palmitoylation is likely to serve a secondary role in modulating protein sorting or association with lipid microdomains. The remaining 41 (33%) proteins, on the other hand, are otherwise hydrophilic. Palmitoylation for this subset likely serves as the main anchor for membrane attachment.

Within these broader classifications of substrates, we identified more specific palmitoylation motifs. As was the case for the yeast palmitoyl proteome (34), we identified multiple SNARE proteins, each containing a single transmembrane domain with a Cys residue next to the cytoplasmic face of the lipid bilayer (Fig. 6B). Also consistent with the yeast palmitoyl proteome, the \textit{T. brucei} palmitoyl proteome contained several amino acid permeases. These, however, lacked the characteristic C-terminal FWC tripeptide that serves as the acceptor site for the DHHC protein Pfa4 in yeast (Fig. 6D). Thus, while palmitoylation of amino acid permeases is shared between these organisms, the mechanism of this modification is divergent.

Previous investigators have noted the presence of an expanded calpain gene family in the \textit{T. brucei} genome (11). These...
proteins lack the C-terminal calmodulin-like calcium-binding domain of mammalian calpains, but most retain a conserved protease motif. We detected five calpain or calpain-like sequences as palmitoylated. Notably, each of these has a sequence predictive of an N-terminal myristoylation/palmitoylation motif. Of the 17 calpain sequences in the genome lacking this motif, we detected none in the palmitoyl proteome (Fig. 6C). These findings both support the sensitivity and specificity of our results and confirm the previous suspicion that this subset of calpains undergoes palmitoylation.

A novel class of proteins with multiple individual hits in the T. brucei palmitoyl proteome consisted of transporters of the folate/bioprotein family. These proteins have implications for drug development, since trypanosomes must take up from the environment either folate or bioprotein, from which they synthesize folate de novo. Such scavenging renders Leishmania resistant to treatment with antifolates such as methotrexate (20). Despite considerable sequence divergence among them, each has a conserved CCCG tetrapeptide within a 75- to 100-amino-acid hydrophilic loop between transmembrane domains (Fig. 6E). None of the other Cys residues of these proteins is similarly conserved. In Leishmania, folate uptake is growth stage regulated (7) through an unknown posttranscriptional mechanism (4). For the main folate transporter in Leishmania infantum, FT1, the switch from logarithmic phase to stationary phase is accompanied by a redistribution of the protein from the plasma membrane to an internal compartment, where it then becomes degraded (32). The presence of these proteins in our palmitoyl proteome offers a possible explanation, in that palmitoylation might regulate this dynamic localization and degradation in a manner analogous to the stabilization and trafficking of several neuronal proteins by the mammalian PAT HIP14 (16, 39). Another multitransmembrane domain transporter in the T. brucei palmitoyl proteome is multidrug resistance protein A. Like the folate/pteridine transporters, this protein has a CCCG peptide in a region between two of its predicted transmembrane domains. Intriguingly, a homologue of this protein in Leishmania similarly appears to undergo growth stage-dependent redistribution to lysosomal compartments for degradation (6). We therefore propose that these proteins are common substrates for a single PAT whose activity confers growth stage-regulated responses.

DISCUSSION

The discovery of enzymatic regulators of protein palmitoylation, coupled with the development of biochemical methods for purifying palmitoylated proteins, has led to a rapid increase in our understanding of this important protein modification. Palmitoylation increases the lipophilicity of a protein, but many palmitoyl proteins are already associated with membranes via other lipid attachments or transmembrane domains. Thus, the secondary effects of palmitoylation in regulating sorting to different membrane domains, association with lipid rafts, binding partner interactions, and protein degradation are perhaps more significant consequences of this modification.

In T. brucei, treatment with 2-bromopalmitate suggested that palmitoylation serves an essential function in these cells. Although the toxicity of 2-bromopalmitate to mammalian cells limits its therapeutic potential, the considerable sequence divergence among Pats indicates the possibility for targeting individual enzymes. With this in mind, we tested individual PAT RNAi lines in an attempt to identify the enzyme(s) whose inhibition by 2-bromopalmitate was responsible for the effect of this compound on T. brucei growth. None of the 12 individual PAT mutants, however, exhibited a growth defect in vitro. If palmitoylation is truly essential for T. brucei growth, as suggested by the effect of 2-bromopalmitate, there are three possible explanations for this finding. First, it may be that incomplete inhibition by our RNAi constructs preserved a small functional pool of enzyme that was sufficient to maintain the viable phenotype. While this same approach for TbPAT7 completely blocked calflagin palmitoylation and flagellar trafficking, there are surely different levels of inhibition achieved by each construct, so that incomplete inhibition of an essential PAT might preserve viability. Second, the survival of each individual PAT mutant could be a consequence of enzymatic redundancy. T. brucei may have evolved functional redundancy for catalysis of protein palmitoylation for those substrates essential for parasite viability and growth. Indeed, in yeast there are several proteins for which inhibition of multiple Pats is required to prevent palmitoylation (34), and none of the DHHC-CRD genes is individually essential (37). Finally, there might exist in T. brucei noncanonical PAT activity for essential palmitoyl proteins. While DHHC-CRD proteins are required for the great majority of palmitoylation in S. cerevisiae, at least one substrate (Bet3 in yeast, homologous to Tb927.8.5030 here) undergoes DHHC-independent palmitoylation. Simultaneous inhibition of all 12 Pats genetically could theoretically help distinguish among these possibilities, but the individual enzymes are too divergent at the sequence level to allow for targeting with a single RNAi construct.

A number of proteins in kinetoplastids had been previously reported to undergo thioacylation, including SMP-1 (35) and hydrophilic acylated surface proteins (5) of Leishmania major, protein phosphatases with EF hand domains of L. major and T. brucei (24), phosphoinositide-specific phospholipase C (PLC) (12, 26) and FCaBP of T. cruzi (13), and glycoprophosphatidylinositol-PLC (28), calflagins (8, 10), and CAP5.5 (15) of T. brucei. Our previous finding that calflagin palmitoylation was mediated by a single DHHC-CRD protein, TbPAT7, established the conservation in T. brucei of PAT activity by DHHC-CRD proteins (10). Here we identified a total of 124 palmitoyl proteins, of which very few are likely to represent statistical anomalies. Our confidence in these data is strengthened by the identification of each of the known positive controls expressed in the procyclic stage, as well as many proteins very likely to be palmitoylated on the basis of their similarity to known palmitoyl proteins.

It is important to note, however, that the NH2OH-depent labeling and purification of proteins in this study would not distinguish between covalent attachment of palmitate or a different long-chain fatty acid. Although palmitate has been shown to be the predominant such modification in mammalian cells, thioesterification of stearate, palmitoleate, and oleate have also been observed (21a). This consideration is especially salient given the unique features of fatty acid synthesis in T. brucei (21) and the observation in this organism of protein S-myristoylation (1). Therefore, this palmitoyl proteome set might more aptly be termed a “thioacylome.” For the sake of consistency with previously published reports, we nevertheless
use palmitoylation as a more general term referring to Cys thioester-linked fatty acids.

These findings are likely to apply to several other pathogenic kinetoplastid parasites. The PAT family of enzymes is well represented in each of *T. brucei*, *T. cruzi*, *L. major*, *L. infantum*, and *Leishmania braziliensis*. Most PATs retain conservation in gene synten among these species, but lineage-specific variation is also evident, including gene loss and tandem duplication. For a given PAT, sequence variation is highly conserved at the DHHC-CRD catalytic motif and transmembrane domains but divergent for hydrophilic loops of the protein. This pattern likely reflects the underlying function of each region, that while selective pressure on the DHHC-CRD was necessary for retention of catalytic function, the other hydrophilic loops of PATs may serve as selectivity filters for each enzyme. Thus, their divergence probably correlates with divergence of their substrates, possibly with the concordant acquisition and loss of substrates.

This work also lays the groundwork for a comprehensive mapping of individual protein substrates to their cognate PAT enzymes. As was shown for TbPAT7 and the callflagins, purification of each substrate by acyl biotin exchange/streptavidin should depend on the presence of its PAT. The wild-type palmitoyl proteome, therefore, should provide a baseline state from which we can determine the dependence of each palmitoyl protein on individual PAT expression, by acyl biotin exchange proteomics on each of the 12 PAT RNAi lines. Finally, the utility of this data set should only increase as more is learned about the function of individual proteins in *T. brucei*. Given the evolutionary distance of trypanosomes from better-studied eukaryotes, it is not surprising that such a large proportion of palmitoylated proteins are annotated as hypothetical proteins. As more is uncovered about the function of these proteins, palmitoylation will undoubtedly become implicated in an ever broader array of cellular processes.

ACKNOWLEDGMENTS

We thank Amy Roth for advice on the palmitoyl proteome protocol and the Biomolecule Analysis Core Facility at the Border Biomedical Research Center/Biology/UTEP (funded by NIH/NCRR grant 5G12RR008124-16A1) for use of the LC-MS instrument. This work was supported in part by NIH grants R01 AI46781 (D.M.E.), 5G12RR008124-16A1 and 5G12RR008124-16A1S1, and R01 CA126239 (A.I.N.). B.T.E. was supported by predoctoral fellowships from the American Heart Association and the NIH (F30 HL094026). E.S.N. was supported in part by a George A. Krutilek Memorial Scholarship from the Graduate School at UTEP. T.J.P.S. was supported by FAPESP, Brazil.

REFERENCES

1. Armah, D. A., and K. Mensa-Wilmot. 1999. S-myristoylation of a glycospbestyldinositol-phospholipase C of *Trypanosoma brucei*. J. Biol. Chem. 274:5931-5936.
2. Bologna, G., C. Yvon, S. Duvaut, and A. L. Veuthey. 2004. N-terminal myristoylation predictions by ensembles of neural networks. Proteomics 4:1626-1632.
3. Choi, H., D. Fermin, and A. I. Nesvizhskii. 2008. Significance analysis of spectral count data in label-free shotgun proteomics. Mol. Cell Proteomics 7:2373-2385.
4. Cunningham, M. L., and S. M. Beverley. 2001. Pteridine salvage throughout the *Leishmania* infectious cycle: implications for antifolate chemotherapy. Mol. Biochem. Parasitol. 113:199-213.
5. Denny, P. W., S. Gokool, D. G. Russell, M. C. Field, and D. F. Smith. 2000. Acylation-dependent protein export in *Leishmania*. J. Biol. Chem. 275:11107-11105.
6. Dodge, M. A., et al. 2004. Localization and activity of multidrug resistance protein 1 in the secretory pathway of *Leishmania* parasites. Mol. Microbiol. 51:1513-1517.
7. Ellenberger, T. E., and S. M. Beverley. 1987. Biochemistry and regulation of folate and methotrexate transport in *Leishmania major*. J. Biol. Chem. 262:10053-10058.
8. Emmer, B. T., M. D. Daniels, J. M. Taylor, C. L. Eping, and D. M. Engman. 2010. Callflagin inhibition prolongs host survival and suppresses parasitemia in *Trypanosoma brucei* infection. Eukaryot. Cell 9:934-942.
9. Emmer, B. T., D. Marie, and D. M. Engman. 2010. Molecular mechanisms of protein and lipid targeting to ciliary membranes. J. Cell Sci. 123:529-536.
10. Emmer, B. T., et al. 2009. Identification of a palmitoyl acyltransferase required for protein sorting to the flagellar membrane. J. Cell Sci. 122:867-874.
11. Ernfled, K., H. Barraclough, and K. Gull. 2005. Evolutionary relationships and protein domain architecture in an expanded calpain superfamily in kinetoplastid parasites. J. Mol. Evol. 61:742-757.
12. Furuya, T., C. Kashuba, R. Docampo, and S. N. Moreno. 2000. A novel phosphatidylinositol-specific phospholipase C in *Leishmania tarentolae*. EMBO J. 19:235-235.
13. Godsel, L. M., and D. M. Engman. 1999. Flagellar protein localization mediated by a calcium-myristoyl/palmitoyl switch mechanism. EMBO J. 18:2057-2065.
14. Herskho, A., and A. Ciechanover. 1998. The ubiquitin system. Annu. Rev. Biochem. 67:425-479.
15. H. Bowler, C., K. Ernsfeld, and K. Gull. 2001. CAP5, a life-cycle-regulated, cytoskeleton-associated protein is a member of a novel family of calpain-related proteins in *Trypanosoma brucei*. Mol. Biochem. Parasitol. 116:25-34.
16. Huang, K., et al. 2004. Huntingtin-interacting protein HIP14 is a palmitoyl transferase involved in palmitoylation and trafficking of multiple neuronal proteins. Neuron 44:977-986.
17. Jurado, J. D., et al. 2007. Complement inactivating proteins and intraspecies protein variation in *Crotalus ornatus* helleri. Toxicon 49:339-350.
18. Kang, R., et al. 2008. Neural palmitoyl-proteomics reveals dynamic synaptic palmitoylation. Nature 456;904-909.
19. Krogh, A., B. Larsson, G. von Heijne, and E. L. Sonnhammer. 2001. Predicting transmembrane protein topology with a hidden Markov model: application to complete genomes. J. Mol. Biol. 305:587-580.
20. Kundig, C., A. Haimer, D. Legare, B. Papadopoulo, and M. Ouellette. 1999. Increased transport of pteridines compensates for mutations in the high affinity folate transporter and contributes to methotrexate resistance in the parasitic protozoan parasite *Leishmania tarentolae*. EMBO J. 18:235-235.
21. Lee, S. H., J. L. Stephens, K. S. Paul, and P. T. Englund. 2006. Fatty acid synthesis by elongases in trypanosomes. Cell 126:691-699.
22. Li, X., Y. Lu, M. Wilkes, T. A. Neubert, and M. D. Redh. 2004. The N-terminal SH4 region of the Src family kinase Fyn is modified by methylation and heterogenous fatty acylation: role in membrane targeting, cell adhesion, and spreading. J. Biol. Chem. 279:8133-8139.
23. Linder, M. E., and J. Deschenes. 2007. Palmitoylation: profiling protein stability and traffic. Nat. Rev. Mol. Cell Biol. 8:374-384.
24. Lobo, S., W. K. Green, T. L. Linder, and J. R. Deschenes. 2002. Identification of a Ras palmitoyltransferase in *Saccharomyces cerevisiae*. J. Biol. Chem. 277:41268-41273.
25. Mills, E., H. P. Price, A. Johner, J. E. Emerson, and D. F. Smith. 2007. Kinetoplastid PPEF phosphatases: dual acylated proteins expressed in the endomembrane system of *Leishmania*. Mol. Biochem. Parasitol. 152:22-34.
26. Ohno, V., A. Kihara, T. Sano, and Y. Igarashi. 2006. Intracellular localization and tissue-specific distribution of human and yeast DHHC cysteine-rich domain-containing proteins. Biochem. Biophys. Acta 1761:474-483.
27. Okura, M., et al. 2005. A lipid-modified phosphoinositide-specific phospholipase C (TcPLC) is involved in differentiation of trypanomastigotes to amastigotes of *Trypanosoma cruzi*. J. Biol. Chem. 280:16235-16243.
28. Passmore, L. A., and D. Barford. 2004. Getting into position: the catalytic mechanism of protein ubiquitylation. Biochem. J. 379:513-525.
29. Pataruio-Hanoq, F., et al. 2000. A role for the dynamic acylation of a cluster of cysteine residues in regulating the activity of the glycospbestyldinositol-phospholipase C of *Trypanosoma brucei*. J. Biol. Chem. 275:12147-12155.
30. Redmond, S., J. Vaidelvis, and M. C. Field. 2003. RNAit: an automated web-based tool for the selection of RNAi targets in *Trypanosoma brucei*. Mol. Biochem. Parasitol. 128:115-118.
31. Ren, J., et al. 2008. CSS-Palm 2.0: an updated software for palmitoylation sites prediction. Protein Eng. Des. Sel. 21:639-644.
32. Redh, M. D. 2006. Use of antibodies and inhibitors to study the functional significance of protein palmitoylation. Methods 40:191-197.
33. Richard, D., P. Leprohon, J. Drummelms, and M. Ouellette. 2004. Growth
phase regulation of the main folate transporter of *Leishmania infantum* and its role in methotrexate resistance. J. Biol. Chem. 279:54494–54501.

33. Roth, A. F., Y. Feng, L. Chen, and N. G. Davis. 2002. The yeast DHHC cysteine-rich domain protein Akr1p is a palmitoyl transferase. J. Cell Biol. 159:23–28.

34. Roth, A. F., et al. 2006. Global analysis of protein palmitoylation in yeast. Cell 125:1003–1013.

35. Tull, D., et al. 2004. SMP-1, a member of a new family of small myristoylated proteins in kinetoplastid parasites, is targeted to the flagellum membrane in *Leishmania*. Mol. Biol. Cell 15:4775–4786.

36. Wan, J., A. F. Roth, A. O. Bailey, and N. G. Davis. 2007. Palmitoylated proteins: purification and identification. Nat. Protoc. 2:1573–1584.

37. Winzeler, E. A., et al. 1999. Functional characterization of the *S. cerevisiae* genome by gene deletion and parallel analysis. Science 285:901–906.

38. Wirtz, E., S. Leal, C. Ochatt, and G. A. Cross. 1999. A tightly regulated inducible expression system for conditional gene knock-outs and dominant-negative genetics in *Trypanosoma brucei*. Mol. Biochem. Parasitol. 99:89–101.

39. Yanai, A., et al. 2006. Palmitoylation of huntingtin by HIP14 is essential for its trafficking and function. Nat. Neurosci. 9:824–831.

40. Zybailov, B., et al. 2006. Statistical analysis of membrane proteome expression changes in *Saccharomyces cerevisiae*. J. Proteome Res. 5:2339–2347.