Kinetoplastid Molecular Cell Biology

An International Conference

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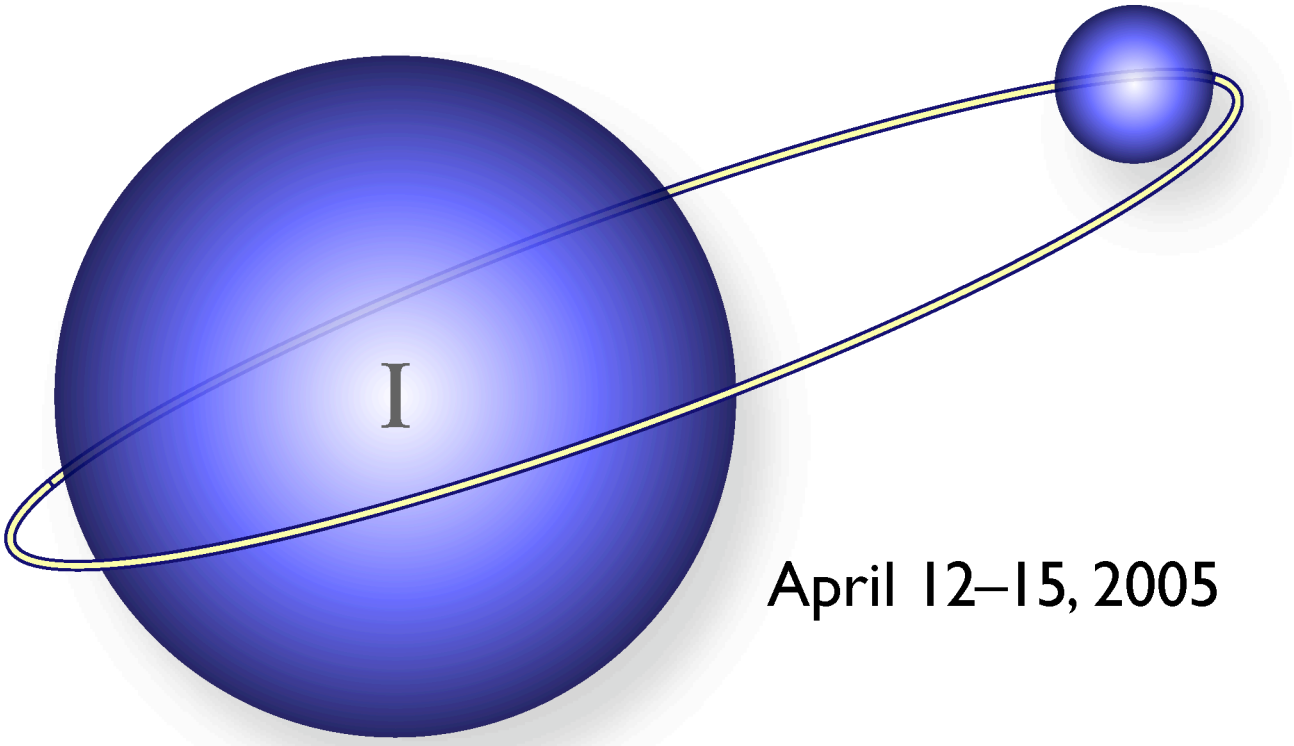

April 12–15, 2005

KMCBM 2005 Acknowledgements

First Kinetoplastid Molecular Cell Biology Meeting, April 12–15, 2005

Organized by George A. M. Cross (The Rockefeller University, New York, USA)

The organizer wishes to thank:

The program committee:

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Laurie Reed (State University of New York School of Medicine, Buffalo, USA)

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TALK SESSIONS: I - VI

April 12 7:00p.m. Chair(s) - S. Beverley

Session I: Cell Biology

1A Biogenesis of the Golgi apparatus. ***Warren, G.***, *He, C., Ho, H., de-Graffenried, C., Yelinek, J.*

1B Too fast or too slow? Implications of the rapid cell surface recycling in *Trypanosoma brucei*. ***Engstler, M.***

1C Morphogenesis and molecular cytology of the flagellum and flagellar pocket of kinetoplastid parasites. ***Gull, K.***

Break or Coffee Break

1D Sorting signals required for trafficking of the Cysteine-Rich Acidic-repetitive transMembrane protein in *T. brucei*. ***Chuang, B.***, *Yang, S., Jin, Y.*

1E GPI valence and secretory trafficking in African trypanosomes. *Schwartz, K., Tazeh, N., Peck, R.,* ***Bangs, J.***

1F Structural requirements for the cell surface routing of the Variant Surface Glycoprotein of *Trypanosoma brucei*. ***Jones, N.***, *Chattopadhyay, A., Berchthold, D., Gabriel, G., Engstler, M., Carrington, M.*

1G Forward transport of GPI-anchored proteins in *Trypanosoma brucei* occurs in the absence of de novo sphingolipid synthesis. ***Sutterwala, S.***, *Menon, A., Bangs, J.*

April 13 8:30 a.m. Chair(s) - E. Ullu

Session II: Genomics and Antigenic Variation

2A Trypanosome telomeres are protected by a homologue of mammalian TRF2. ***Li, B.***, *Espinal, A., Cross, G.*

2B Chromatin remodeling and the regulation of trypanosome DNA glycosylation. ***Sabatini, R.***, *DiPaolo, C., Kieft, R.*

2C Localization of spliced leader gene transcription in a defined nuclear domain of *Trypanosoma cruzi* . ***Schenkman, S.***, *Dossin, F.*

2D SL RNA gene transcription in Trypanosomatids. ***Das, A.***, *Bellofatto, V.*

2E *Trypanosoma brucei* SL RNA gene transcription is directed by a TRF4/SNAPc/?? complex. ***Schimanski, B.***, *Nguyen, T., Günzl, A.*

2F Transcriptional effects of Tbrpb9 RNAi in *Trypanosoma brucei*. ***Lecordier, L.***, *Devaux, S., Pays, E., Vanhamme, L.*

Break or Coffee Break

2G A homologue of the vaccinia virus cap-specific methyltransferase VP39 is involved in cap 4 modification in *Trypanosoma brucei* . ***Arhin, G.***, *Ullu, E., Tschudi, C.*

2H Cytoplasmic degradation of an unstable mRNA in trypanosomes. *Kimblin, N., Webb, H., Ellis, L.,* ***Carrington, M.***

2I Control of mRNA degradation in *Trypanosoma brucei*. ***Clayton, C.***, *Hartmann, C., Colasante, C., Robles, A., Chi-Ho, L., Cristodero, M.*

2J A family of eIF4E homologues from *Leishmania*. *Yoffe, Y., Lerer, A., Zuberek, J., Lewdorowicz, M., Darzynkiewicz, E.,* ***Shapira, M.***

2K A developmentally regulated non-coding RNA in *Leishmania infantum* associates with ribosomal complexes. ***Chow, C.***, *Dumas, C., Papadopoulou, B.*

April 13 7:00 p.m. Chair(s) - C. Clayton

Session III: Genomes and Tools

3A Comparative genome organization of the Trypanosomatids. ***Blandin, G.***, *El-Sayed, N., Myler, P., Hall, N., the, T.*

3B Comparative genomic analysis of *Trypanosoma* species. ***Hertz-Fowler, C.***, *Adlem, E., Hamlin, N., Lennard, N., Berriman, M.*

3C Genome-wide analysis of trypanosomatid protein kinases. ***Parsons, M.***, *Ward, P., Worthey, L., Mottram, J.*

3D RNomics and SnoRomics in *Trypanosoma brucei*: bioinformatic and experimental approaches reveal trypanosome-specific features. ***Michaeli, S.***, *Liang, X., Uliel, S., Barth, S., Hury, A., Doniger, T., Unger, R.*

3E Organization of non-LTR retrotransposons in the trypanosomatid genome. ***Bringaud, F.***, *Ghedin, E., Blandin, G., Bartholomeu, D., El-Sayed, N.*

Break or Coffee Break

3F Interaction of *Trypanosoma brucei* Argonaute 1 with polyribosomes: defining the role of the RGG domain. ***Chamond, N.***, *Shi, H., Djikeng, A., Tschudi, C., Ullu, E.*

3G Evolutionary conservation of the RNAi pathway. ***shi, h.***, *Tschudi, C., Ullu, E.*

3H Genetic tools for *Trypanosoma brucei*. ***Cross, G.***

3I Tools for inducible expression in *Trypanosoma brucei*. ***Alsford, S.***, *Horn, D.*

April 14 8:30 a.m. Chair(s) - K. Gull

Session IV: Biochemistry and Host-Parasite Interactions

4A Acidocalcisome and contractile vacuole enzymes are involved in osmoregulation in *Trypanosoma cruzi*. *Fang, J., Rohloff, P., Ruiz, F.,* ***Docampo, R.***

4B Vitamin C biosynthesis in trypanosomes: a new role for the glycosome. ***Wilkinson, S.****, Prathalingam, R., Horn, D., Kelly, J.*

4C A new mechanism for fatty acid synthesis in *Trypanosoma brucei*. ***Lee, S.****, Paul, K., Englund, P.*

4D Purine sensing in *Leishmania donovani*. ***Carter, N.****, Gessford, S., Ullman, B.*

4E Trypanothione synthetase: biochemical and biological characterization of a suitable drug-target against trypanosomiasis. ***Comini, M.***

4F The pharmacokinetic/pharmacodynamic relationship of the antitrypanosomal drug melarsoprol. ***Bakshi, R.****, Shapiro, T.*

Break or Coffee Break

4G Apolipoprotein L-I lyzes trypanosomes through an anionic pore on the lysosomal membrane. ***Pérez-Morga, D.***

4H Trypanolytic human HDLs contain both Hpr and apoL-I. ***Shiflett, A.****, Bishop, J., Hajduk, S.*

4I Cyclical transmission of procyclin null mutants. *Vassella, E., Urwyler, S., Fragoso, C., Kunz Renggli, C., Oberle, M., Hemphill, A.,* ***Isabel, R.***

4J 'Antigenic variation' in *Leishmania major*. *Dobson, D., Soares, R., Barron, T., Sacks, D., Turco, S., Gu, C.,* ***Beverley, S.***

4K The proteome of *Trypanosoma cruzi* shed vesicles involved in host immunomodulation and cell invasion. ***Nakayasu, E.****, Torrecilhas, A., Nohara, L., Lamont, D., Alves, M., Ferguson, M., Almeida, I.*

April 14 1:00 p.m. Chair(s) - B. Sollner-Webb

Session V: Kinetoplast Replication, Editing and Expression

5A Regulation of replication initiation at the kinetoplast DNA minicircle replication origins. ***Shlomai, J.***, *Milman, N., Zick, A., Sela, D., Kapeller, I., Onn, I., Bezalel, R.*

5B The rotational dynamics of kinetoplast DNA replication. ***Liu, Y.***, *Englund, P.*

5C Mitochondrial DNA ligases of *Trypanosoma brucei*. ***Downey, N.***, *Hines, J., Sinha, K., Bist, P., Ray, D.*

5D Developmental regulation of RNA editing in *Trypanosoma brucei*. ***Ochsenreiter, T.***, *Cipriano, M., Hajduk, S.*

5E Reconstitution of in vitro editing with recombinant proteins. ***Simpson, L.***, *Kang, X., Gao, G., Rogers, K.*

5F Mitochondrial RNA degradosome homologs in *Trypanosoma brucei* have pleiotropic effects on multiple RNA classes. ***Penschow, J.***, *Sleve, D., Read, L.*

5G Multiple ribosomal RNP complexes in kinetoplasts of *Leishmania tarentolae*. ***Maslov, D.***, *Agrawal, R., Bhargava, K., Butler, E., Falick, A., Sharma, M., Simpson, L., Spremulli, L.*

Break or Coffee Break

April 15 8:30 a.m. Chair(s) - C.C. Wang

Session VI: Cell and Developmental Cycles

6A *In vivo* activity of *Trypanosoma brucei* PKA-like kinases changes in response to environmental conditions. ***Kramer, S.***, *Boshart, M.*

6B A protein implicated in polycystic kidney disease is involved in basal body and flagellar biogenesis in *T. brucei*. *Morgan, G., Denny, P., Vaughan, S., Goulding, D., Jeffries, T., Smith, D., Gull, K.,* ***Field, M.***

6C Flagellar motility is essential in *Trypanosoma brucei* and is modulated by an evolutionarily-conserved dynein regulatory system. ***Ralston, K.***, *Lerner, A., Hill, K.*

6D Flagellum force and elongation drives basal body migration. ***Kohl, L.***, *Absalon, S., Branche, C., Rusconi, F., Toutirais, G., Bonhivers, M., Robinson, D., Bastin, P.*

6E MOB1 is essential for cytokinesis in *Trypanosoma brucei*. ***Hammarton, T.***, *Lillico, S., Mottram, J.*

6F Methylation of histone H3 lysine 76 is important for differentiation, virulence and cell cycle regulation in *T. brucei*. ***Janzen, C.***, *Hake, S., Lowell, J., Cross, G.*

6G Variant Surface Glycoprotein is monitored in the cell cycle of *Trypanosoma brucei*. *Sheader, K., Vaughan, S., Minchin, J., Hughes, K., Gull, K.,* ***Rudenko, G.***

POSTER SESSIONS: A-C

A SERIES: April 13 1:00 p.m.

B SERIES: April 13 3:30 p.m.

C SERIES: April 14 3:30 p.m.

200A Forward genetics identifies genes associated with procyclin glycosylation and GPI anchor modification in *T. brucei*. ***Lee, S.***, *Browitt, A., Barry, D., Acosta-Serrano, A.*

200B Every gene in *Leishmania* is subject to strong regulation during metacyclogenesis. ***Akopyants, N.***, *Matlib, R., McPherson, J., Beatty, W., Warren, W., Crosby, S., Stormo, G., Beverley, S.*

200C Analysis of the role of trypanin in the motility of *Trypanosoma brucei*. ***Baron, D.***, *Hill, K.*

201A A library of RNAi mutants to study the multiple functions of flagella. ***Absalon-Benghanem, s.***, *Kohl, L., rusconi, F., Toutirais, G., Bonhivers, M., Robinson, D., Bastin, P.*

201B Reduced expression of flagellar PKA-like kinase leads to a cell cycle dependent motility phenotype in *Trypanosoma brucei*. *Krumbholz, C., Engstler, M., Grünfelder, C., Kramer, S.,* ***Boshart, M.***

201C *Trypanosoma congolense* procyclin: unmasking a cryptic surface glycoprotein in procyclic forms. *Utz, S., Roditi, I., Acosta-Serrano, A., Kunz Renggli, C.,* ***Buetikofer, P.***

202A Investigation of the RNA-associated proteome of *Trypanosoma cruzi*. ***Bundy, B.***, *Atwood, J., Weatherly, B., Cannon, B., Tarleton, R.*

202B A mitogen-activated protein kinase controls proliferation and differentiation of bloodstream forms of *Trypanosoma brucei*. *Domenicali Pfister, D.,* ***Burkard, G.***, *Morand, S., Roditi, I., Vassella, E.*

202C Characterization of a *Trypanosoma brucei* histone acetyltransferase that may be involved in RNA interference. ***Chambers, J.***, *Morris, J.*

203A Differential import of precursor proteins into *Trypanosoma brucei* mitochondria. ***Chaudhuri, M.***, *Williams, S.*

203B Functional analysis of a type II topoisomerase from *Crithidia fasciculata*, a potential drug target. ***Chen, J.***

203C New insights into the characterization of the *Trypanozoon* subgenus. ***Claes, F.***, *Brems, S., Clayton, C., Hoheisel, J., Büscher, P.*

204A Characterization of PBP-2 in *Trypanosoma brucei* SL RNA transcription. ***Conforti, G.**, Bellofatto, V.*

204B Characterization of a putative immunomodulatory factor from *Trypanosoma brucei*. ***De Muylder, G.**, Vincendeau, P., Pays, E., Vanhamme, L.*

204C Induction of differentiation in kinetoplastids by thiazolidindiones. ***Denninger, V.**, Figarella, K., Duszenko, M.*

205A RNA polymerase I and II in *Trypanosoma brucei*: two distinct Rpb5 subunits, each one specific to each polymerase. ***Devaux, S.**, Lecordier, L., Walgraffe, D., Pays, E., Vanhamme, L.*

205B Gene expression profiling detects synergistic pattern of human macrophage responses following *Leishmania* infection. ***Dogra, N.**, McMaster, R.*

205C Consequences of telomere shortening in *Trypanosoma brucei*. ***Dreesen, O.**, Cross, G.*

206A *In vitro* selection for human serum resistance in *Trypanosoma brucei brucei*. ***Faulkner, S.**, Kieft, R., Oli, M., Hajduk, S.*

206B Control of *Aut 1* gene expression by natural RNAi in *T.b.gambiense* ? ***Felu, C.**, Pérez Morga, D., Xong, H., Ullu, E., Pays, E.*

206C Characterisation of GlcNAcPI-de-N-Acetylase, a validated drug target for African sleeping sickness. *Urbaniak, M., Crossman, A., Chang, T., Smith, T., van Aalten, D.,* ***Ferguson, M.***

207A Induction of programmed cell death in the bloodstream form of *Trypanosoma brucei* by PGD_2 and its metabolites. ***Figarella, K.**, Uzcategui, N., Duszenko, M.*

207B 50-bp repeats are not required to maintain the active status of a VSG Expression Site in *Trypanosoma brucei*. ***Figueiredo, L.**, Teng, G., Cross, G.*

207C Proteomic analysis of antimony resistance in *Leishmania infantum*: increased coverage by prefractionation. ***Foucher, A.**, Drummelsmith, J., Ouellette, M.*

208A Inhibiting serine palmitoyltransferase causes cell-cycle and vesicular trafficking defects and lipid raft disruption. ***Fridberg, A.**, Olson, C., Tyler, K., Rogers, L., Engman, D.*

208B Moving cells and finding lost structures: the flagellum of endosymbiont-bearing kinetoplastids. ***Gadelha, C.**, Wickstead, B., de Souza, W., da Cunha-e-Silva, N., Gull, K.*

208C Functional complementation of *Trypanosoma brucei* RNA in vitro editing with recombinant RNA ligase. ***Gao, G.**, Simpson, A., Kang, X., Rogers, K., Nebohacova, M., Simpson, L.*

209A Telomeric sequences and VSG silencing in *T. brucei*. ***Glover, L.**, Horn, D.*

209B Functional and structural properties of guide RNAs are determined by separate rules. ***Golden, D.**, Hajduk, S.*

209C Interactions between the Cyclins and cdc2-related kinases from *Trypanosoma brucei*. ***Gourguechon, S.**, Savich, J., Wang, C.*

210A Procyclins and free GPI glycolipids are not essential for the growth of procyclic form *Trypanosoma brucei*. ***Guther, M.**, Ferguson, M.*

210B The phosphatidylinositol kinases of *Trypanosoma brucei*. ***Hall, B.**, Goulding, D., Field, M.*

210C Centrin is involved in Golgi biogenesis in *Trypanosoma brucei*. ***He, C.**, Warren, G.*

211A Ordered assembly of the Golgi apparatus in *Trypanosoma brucei*. ***Ho, H.**, Graffenried, C., He, C., Warren, G.*

211B Physiological significance of GPI inositol deacylation in *Trypanosoma brucei*. ***Hong, Y.**, Nagamune, K., Morita, Y., Maeda, Y., Kinoshita, T.*

211C Functional genomics relating to the biosynthesis of trypanosome glycoconjugates. ***Izquierdo, L.**, Nakanishi, M., Machray, G., Barton, G., Ferguson, M.*

212A Aurora kinase TbAUK1 is essential for nuclear division in *Trypanosoma brucei*. ***Jetton, N.**, Rothberg, K., Ruben, L.*

212B Functional characterisation of the iron superoxide dismutase gene repertoire in *Trypanosoma brucei*. ***Kelly, J.**, Prathalingham, R., Martin, T., David, H., Wilkinson, S.*

212C A third mitochondrial DNA polymerase, PolID, is essential for kDNA replication in *Trypansoma brucei*. ***Chandler, J.**, Klingbeil, M.*

213A RNA interference of a new type II topoisomerase in *Trypanosoma brucei*. ***Kulikowicz, T.**, Shapiro, T.*

213B Polo-like kinase controls cytokinesis but not the mitosis in *Trypanosoma brucei*. ***Kumar, P.**, Wang, C.*

213C *T. brucei* RNA editing: the role of band-II protein (TbMP81) and key considerations for analysis by RNAi. ***Law, J.**, Huang, C., O'Hearn, S., Sollner-Webb, B.*

214A Brucipain takes a tryp across the blood-brain barrier. ***Lima, A.**, Nikolskaia, O., Kim, Y., Garcia-Garcia, J., Lonsdale-Eccles, J., Scharsftein, J., Grab, D.*

214B *Lok1*: A new kDNA replication protein in *Trypanosoma brucei*. ***Lindsay, M.**, Englund, P.*

214C p38, a *Trypanosoma brucei* protein involved in kinetoplast DNA replication. ***Liu, B.**, Molina, H., Kalume, D., Pandey, A., Griffith, J., Englund, P.*

215A The SR network In trypanosomatids: conservation of the constitutive mechanism in SR protein kinases. ***Lobo, G.**, Flawia, M., Ullu, E., Torres, H., Tschudi, C.*

215B A role for insect laminin in parasite interaction. ***Lopes, A.**, Dias, F., Souza dos Santos, A., Lery, L., Bisch, P., Saraiva, E., Souto-Padrón, T.*

215C Telomere trapping with TAR: update on telomere clones from *Trypanosoma brucei*. ***Louis, E.**, Becker, M.*

216A Down-regulation of nuclear encoded subunits of complexes III and IV disrupts the respiratory chain in procyclic *T. brucei*. *Horvath, A., Horakova, E., Pravdova, E., Slapetova, I., Cuninkova, L., Verner, Z., Kralovicova, S.,* ***Lukes, J.***

216B The role of PUF proteins in gene expression in *Trypanosoma brucei*. ***Luu, V.***, *Clayton, C.*

216C Exploring the RNA binding specificity of RNA Editing-Associated Protein (REAP-1). ***Madison-Antenucci, S.***, *Wood, Z.*

217A Cyclic AMP Export from *Trypanosoma brucei*. ***Marti, G.***, *Seebeck, T.*

217B Proteomic analysis of stage-specific gene expression in *Leishmania infantum*. ***McNicoll, F.***, *Drummelsmith, J., Müller, M., Girard, I., Brochu, V., Ouellette, M., Papadopoulou, B.*

217C Characterization of trypanosome deadenylases. *Milone, J., Wilusz, J., Utter, C., Bellofatto, V.*

218A The downstream effectors of cAMP signaling pathway in *Trypanosoma brucei*. ***Minca, M.***, *Seebeck, T.*

218B Deficiency of GPI-anchored proteins in procyclic *Trypanosoma brucei* and its effect on cell growth. ***Morita, Y.***, *Nagamune, K., Hong, Y., Maeda, Y., Kinoshita, T.*

218C Connecting glycolysis to surface molecule expression: building the glucose sensing pathway in *Trypanosoma brucei*. ***Morris, J.***, *DeBruin, C., Seifert, C., Ridings, C., Morris, M.*

219A TbHKT1, a novel K^+ transporter of *Trypanosoma brucei*. ***Mosimann, M.***, *Luescher, A., Maeser, P.*

219B Over-expression of a cytochrome b5 reductase-like protein causes kDNA loss in *Trypanosoma brucei*. ***Motyka, S.***, *Englund, P.*

219C Highly efficient tandem affinity purification of trypanosome protein complexes based on a novel epitope combination. ***Nguyen, T.***, *Schimanski, B., Gunzl, A.*

220A Global analysis of genes expressed in human brain microvascular endothelial cells in response to *T. brucei* infection. ***Nikolskaia, O.***, *Garcia-Garcia, J., Dumler, S., Grab, D.*

220B Intracellular distribution of cAMP-specific phosphodiesterases of *Trypanosoma brucei* (TbPDEs). ***Oberholzer, M.***, *Seebeck, T.*

220C A cytoskeletal chaperone found in trypanosomes and its role in nutrient uptake. ***Olson, C.***, *Veesenmeyer, J., Engman, D.*

221A Cytidine deaminases in *Trypanosoma brucei*. ***Pastar, I.***, *Janzen, C., Cross, G., Papavasiliou, N.*

221B Taking a ride on the spliced leader RNA promoter: SLACS retroposon expression in *Trypanosoma brucei*. ***Patrick, K.***, *Shi, H., Ullu, E., Tschudi, C.*

221C Acetyl-CoA carboxylase is required for fatty acid synthesis in *T. brucei*. ***Paul, K.***, *Englund, P.*

222A Stage-specific requirement for the lysosomal membrane glycoprotein, p67, in *Trypanosoma brucei*. ***Peck, R.***

222B A highly conserved type I protein arginine methyltransferase from *Trypanosoma brucei*. ***Pelletier, M.***, *Pasternack, D., Read, L.*

222C ARL1 and ARF1 have essential roles in protein trafficking in *Trypanosoma brucei*. ***Price, H.***, *Goulding, D., Smith, D.*

223A Molecular characterization of genes encoding HSP70 protein in *Trypanosoma rangeli*. *Cuervo, C., López, M.*, ***Puerta, C.***

223B cAMP phosphodiesterases in *Trypanosoma cruzi*. ***Rascon, A.***, *Beavo, J., Diaz-enjumea, R.*

223C Phospholipid metabolism by Phospholipase A_1 in *Trypanosoma brucei*. ***Richmond, G.***, *Smith, T.*

224A Essential role of glucose transporters in the life cycle of *Leishmania mexicana*. ***Rodriguez-Contreras, D.***, *Landfear, S.*

224B The tryparedoxins of *Leishmania infantum*: characterization of *Li*TXN5 a tryparedoxin-like protein. ***Romao, S.***, *Castro, H., Sousa, C., Tomás, A.*

224C A conserved nuclear methyltransferase is required for hypermethylation of the cap structure of trypanosome U-snRNAs. ***Ruan, J.***, ***Chamond, N.***, ***Ullu, E.***, ***Tschudi, C.***

225A The effect of anti-actin drugs on the polarity and function of the actin cytoskeleton of *Trypanosoma brucei*. ***Rubotham, J.**, Bane, F., Hughes, D., Garcia-salcedo, J., Pays, E., Derek, N.*

225B Alterations in traffic, lipid biosynthesis and pro-Cruzipain maturation in *T. Cruzi* overexpressing Chagasin. ***Santos, C.**, Sant'Anna, C., Cunha-e-Silva, N., Souza, F., dos Reis, F., Scharfstein, J., Heise, N., Lima, A.*

225C Purification of the 19S proteasomal regulatory complex from *Trypanosoma brucei* by affinity chromatography. ***Savich, J.**, Wang, C.*

226A Inactivation of the dynamin-like protein of *T. brucei* inhibits division of the mitochondrion and cytokinesis. *Chanez, A., Vassella, E., Schneider, A.*

226B An essential topoisomerase IA of *Trypanosoma brucei*. ***Scocca, J.**, Shapiro, T.*

226C Post translational modifications in the universal minicircle sequence binding protein regulate its binding at the kDNA replication origin. ***Sela, D.**, Onn, I., Yaffe, N.*

227B Sequence variations affecting *trans*-splicing efficiency in *Trypanosoma brucei*. ***Siegel, N.**, Tan, K., Cross, G.*

227C Characterization of alanine aminotransferase in *Trypanosoma brucei*. ***Spitznagel, D.**, Henehan, G., Nolan, D.*

228A Multiple roles for acyl carrier protein in the trypanosome life cycle. ***Stephens, J.**, Paul, K., Englund, P.*

228B Thiamine metabolism and Melarsoprol. ***Stoffel, S.**, Schweingruber, A., Schweingruber, M.*

228C UDP-GlcNAc pyrophosphorylase as a potential drug target in African trypanosomes. ***Stokes, M.**, Guther, M., Alphey, M., Ferguson, M.*

229A Developing a one step PCR-based method for gene disruption and *in vivo* epitope tagging in *Crithidia fasciculata*. ***sun, y.**, Hines, J., Ray, D.*

229B Biochemical and functional characterization of serine proteases in *Leishmania donovani*. ***Swenerton, R.**, Kelly, B., Sajid, M., McKerrow, J.*

229C The endoplasmic membrane network associated protein Tb292 is essential for cytokinesis in *Trypanosoma brucei*. ***Tawde, M.**, Jin, Y., Jetton, N., Ruben, L., Lee, M.*

230A Okadaic acid reveals the mechanism regulating G1/S checkpoint in *Trypanosoma brucei*. ***Tu, X.**, Wang, C.*

230B An LC/MS method for identifying sugar nucleotides in Trypanosomes. ***Turnock, D.**, Ferguson, M.*

230C Permeability of *Trypanosoma brucei* aquaglyceroporins for non conventional solutes: As III, Sb III, and dihydroxyacetone. ***Uzcategui, N.**, Carmona-Gutiérrez, D., Bassarak, B., Figarella, K., Duszenko, M.*

231A Determinants of substrate specificity in a nucleoside transporter from *Leishmania donovani*. ***Valdés Angues, R.**, Liu, W., Arendt, C., Ullman, B., Landfear, S.*

231B Host dependent expression of the transferrin receptor in *Trypanosoma brucei*. ***Van Luenen, H.**, Mussmann, R., Engstler, M., Gerrits, H., Kieft, R., Ter Riet, B., Bentin Toaldo, C., Borst, P.*

231C Identification and characterization of a membrane/HDL addressing domain in Apolipoprotein L-I. ***Vanhollebeke, B.**, Pérez-Morga, D., Paturiaux-Hanocq, F., Nolan, D., Lins, L., Vanhamme, L., Tebabi, P., Pays, A., Poelvoorde, P., Jacquet, A., Brasseur, R., Pays, E.*

232A Dissecting the developmental regulation of GPI-PLC expression in *Trypanosoma brucei*. ***Webb, H.**, Burns, R., Carrington, M.*

232B Protein kinases and flagellar length regulation. *Scholz, A., Erdmann, M., Kuhn, D., Bengs, F.,* ***Wiese, M.***

232C Identification and partial characterization of proteins from the tomato lectin binding fraction in *Trypanosoma brucei*. ***Woods, K.**, **Nic A Bhaird, N.**, Rubotham, J., Dierick, J., Pays, E., Nolan, D.*

233A Pyrimidine biosynthesis and salvage in *Leishmania donovani*. ***Yates, P.**, Ullman, B.*

233B Identification of two dimethylases involved in rRNA modifications in*Trypanosoma brucei*. ***Zamudio, J.****, Zeiner, G., Alexander, S., Feder, M., Bujnicki, J., Sturm, N., Campbell, D.*

233C Creation of a *Leishmania donovani hgprt/xprt* conditional null mutant. ***Zarella-Boitz, J.****, Ullman, B.*

234A Functional characterization of DNA polymerase beta and DNA polymerase beta-PAK from *Trypanosoma brucei*. ***Zhao, Z.****, Saxowsky, T., Englund, P.*

ABSTRACTS: SESSION I

Cell Biology

April 12 7:00p.m.

Chair(s) - S. Beverley

Biogenesis of the Golgi apparatus

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The growth and division of cells is accompanied by the duplication and segregation of cellular organelles. For the Golgi apparatus, the duplication process has been difficult to follow because most cells have multiple Golgi stacks. Fortunately, protozoan parasites such as *Trypanosoma brucei* offer the advantage of a single Golgi that can be tagged and followed through the cell cycle by video fluorescence microscopy. The data suggest that the old Golgi seeds the growth of a new Golgi matrix, which then grows in an ordered fashion, acquiring those components needed for it to function in transporting the VSG cargo that leaves the endoplasmic reticulum at the nearby exit site. The new Golgi is constructed near to the old Golgi at a position that is determined by the new basal body.

Too fast or too slow? Implications of the rapid cell surface recycling in *Trypanosoma brucei*

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Bloodstream stages of *Trypanosoma brucei* are capable of recycling their plasma membrane with surprising speed. The whole cell surface is internalized and recycled once every 10 minutes [(1)]. Given that endo- and exocytosis only occur at the flagellar pocket, this is an extraordinary example of membrane traffic. While analyzing the intracellular itinerary of the variant surface glycoprotein (VSG), we came across a variety of almost paradox problems: Formation or budding of clathrin-coated vesicles from the flagellar pocket occurs at a rate that cannot be explained by known mechanisms. The kinetics of trafficking and sorting of VSG in endosomes is not compatible with multiple fission and fusion events. The randomization of secreted or exocytosed VSGs on the cell surface cannot be achieved by lateral diffusion alone. Host antibodies are removed from the VSG-coat at a rate that actually surmounts the capacity of the fast endocytosis machinery of *T. brucei*. We have brood over these apparent contradictions and would like to present some recent ideas, experiments and (maybe) conclusions.¡/div¿

[(1)] Engstler, M., et al. (2004) Journal of Cell Science, 117: 1105-1115

Morphogenesis and molecular cytology of the flagellum and flagellar pocket of kinetoplastid parasites

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Our cytological understanding of the kinetoplastid parasites is defined, in the main, by descriptions of cell shape, organelle position and the arrangement of a flagellum emerging from a flagellar pocket. The architecture of the flagellar pocket, defined by emergence and structure of the flagellum, is a critical feature of the pathogenicity of *Trypanosoma brucei* and other kinetoplastid parasites. My laboratory, together with collaborators, has defined a proteomic description of over 300 flagellar components and embarked on a 3D electron tomography reconstruction of the cytskeletal arrangement of the flagellum and flagellar pocket. I will illustrate how functional analysis of a set of known and novel proteins is allowing us to define the molecular cytology for this region of the parasite, to understand how cytoskeletal architecture influences membrane topography in the flagellar pocket, and to understand dependency relationships that operate to ensure morphogenesis of the flagellum and flagellar pocket during cell division.

Sorting signals required for trafficking of the Cysteine-Rich Acidic-repetitive transMembrane protein in *T. brucei*

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The Cysteine-Rich Acidic-repetitive transMembrane protein (CRAM) localizes at the flagellar pocket of *Trypanosoma brucei* and potentially functions as a receptor or an essential catalytic component for lipoprotein particles uptake. We identified and characterized protein sorting signals that are involved in efficient trafficking of CRAM to and from the flagellar pocket in the procyclic form of *T.brucei*. Previous deletional mutagenesis indicated the presence of signals in the CRAM cytoplasmic domain, specific for its localization at the flagellar pocket and for efficient endocytosis. To delineate the functional domains of putative sorting signals in the cytoplasmic extension of CRAM, we further performed (Ala)3 scanning mutagenesis and site directed mutagenesis in CRAM cytoplasmic domain. Subcellular localization of CRAM mutants demonstrated that amino acid sequences between ?5 to ?14, referred to as a transport signal, are essential for export of CRAM from the endoplasmic reticulum to the pocket; and the region spanning from -5 to ?22, which overlaps with the transport signal, is required for efficient endocytosis. Since the clathrin and adaptor complexes are also required for trafficking of CRAM to the flagellar pocket, we addressed whether the CRAM-derived sorting signal interacts with the *T.brucei*u1 adaptin (Tbu1). We showed that the Tbu1 can efficiently interact with the CRAM-derived sorting signal and the tyrosine based signal. Mutations at amino-acids that are essential for the function of the transport signal in CRAM abolished the interaction with the Tbu1. In summary, we characterized the first sorting signal for trafficking a receptor protein to the flagellar pocket in *T.brucei* and hypothesized that interaction of the CRAM transport signal with Tbu1 is essential for the correct trafficking fate of CRAM.

GPI valence and secretory trafficking in African trypanosomes

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A correlation exists between GPI-valence and progression/stability of secretory membrane proteins in bloodstream African trypanosomes: native homodimeric VSG (GPI^2) is a stable surface protein ($t_{1/2,}$ >30 hr); heterodimeric transferrin receptor (TfR, GPI^1, $t_{1/2,}$ ~1.5 hr) is primarily located in the flagellar pocket; and recombinant GPI-minus VSG (GPI^0) is rapidly degraded in the lysosome ($t_{1/2,}$ ~1 hr). We have now overexpressed a series of monomeric GPI^0 and GPI^1 reporters in bloodstream parasites. GPI^1 procyclin (EPMH) is processed by addition of poly-N-acetyllactosamine, and is found prominently in the flagellar pocket and on the cell surface. GPI^0 EPMH-gpi is lysosomally targeted and rapidly degraded. Surprisingly, ~50% of EPMH is released from the cell surface with an intact GPI anchor, the remaining portion being degraded in the lysosome. Two additional GPI^1 reporters were tested, p67HP and BiPNHP, based on the lysosomal and ER proteins p67 and BiP. BiPNHP was released quantitatively (¿80%), while p67HP was turned over by both release (~15%) and lysosomal degradation (¿50%). Finally we investigated the fate of endogenous TfR. The ESAG6 and ESAG7 subunits of TfR were coordinately turned over ($t_{1/2,}$ ~1.5 hr) mainly by lysosomal degradation (¿90%), but also by minor amounts of release (¡5%). There is a decreasing rank order of release for GPI1 reporters (BiPNHP ¿ EPMH ¿ p67HP ¿ TfR), with an inverse order of lysosomal targeting and degradation. These reporters apparently traffick to the flagellar pocket where they either enter the endosomal/lysosomal pathway or egress onto the external plasma membrane and are shed into the media. The degree to which a given reporter partitions between these pathways may be a function of individual physical properties.

Structural requirements for the cell surface routing of the Variant Surface Glycoprotein of *Trypanosoma brucei*

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The VSG is the major surface protein of bloodstream form African trypanosomes and forms a densely packed surface coat that protects underlying surface proteins from the host immune response. The VSGs of *Trypanosoma brucei* consist of an N-terminal domain and one or two C-terminal domains and each VSG contains at least one N-linked glycosylation site, which can be located in either the N-terminal and/or C-terminal domains. The VSG must be routed to the cell surface and pack densely enough to form a protective layer. The features of the VSG necessary to form the protective coat are not well characterised. Here, the structural requirements for cell surface routing of the VSG are investigated. The C-terminal domain was not required for cell surface routing. A deletion mutant removing the structured C-terminal domain of MITat1.1 and two chimeric VSGs in which the C-terminal domain of MITat1.1 was replaced by the C-terminus of the *T. congolense* VSG BeNat1.1 were all routed to the cell surface. The glycosylation of the C-terminal deletion mutant was altered. The requirement for an N-linked glycosylation is VSG specific: the single N-linked glycosylation site in MITat1.4 is not necessary for cell surface localisation. Mutation of both sites in MITat1.1 results in an unstable protein whereas single site mutants are expressed on the cell surface. The N-linked glycosylation appears not to be a structural requirement for folding as adding back an N-linked glycosylation site to the N-terminus of the non-glycosylated mutant restored the ability of the VSG to reach the cell surface. In conclusion, the C-terminal domain and many N-linked glycosylation sites are not necessary for cell surface expression of VSGs.

Forward transport of GPI-anchored proteins in *Trypanosoma brucei* occurs in the absence of de novo sphingolipid synthesis

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The association of glycosylphosphatidylinositol (GPI)-anchored proteins with cholesterol and sphingolipid-rich membrane microdomains, also known as lipid rafts, is thought to play a crucial role in GPI-dependent protein sorting. The first step in sphingolipid biosynthesis, the condensation of L-serine and palmitoyl CoA into 3-ketodihydrosphingosine (3-KDS), is catalyzed by the enzyme serine palmitoyltransferase (SPT). We treated bloodstream-form *Trypanosoma brucei* with myriocin, a highly specific inhibitor of SPT, to investigate the requirement for de novo sphingolipid synthesis in the sorting of GPI-anchored proteins. Similar to previous studies in yeast and mammalian cells, we find that myriocin inhibits the growth of both procyclic and bloodstream trypanosomes and growth can be rescued by addition of 3-KDS. Lipid analysis confirmed that myriocin treatment abolishes incorporation of [^{3}H]serine into base-resistant lipids. The sphingolipid biosynthetic pathway diverges to generate sphingomyelin in higher eukaryotes and inositol phosphorylceramide (IPC) in the majority of lower eukaryotes. Interestingly, unlike yeast, *T. cruzi* and *Leishmania*, our results show that the major sphingolipids synthesized by *T. brucei* appears to be sphingomyelin and an unidentified species that is unlikely to be IPC. Despite the lack of sphingomyelin synthesis in myriocin-treated cells, the forward transport of the major surface coat protein, GPI-anchored variant surface glycoprotein (VSG), is not perturbed. However, myriocin-treated cells show a decrease in receptor-mediated endocytosis. Additionally, myriocin-treated cells have an enlarged lysosomal compartment. Our findings indicate that, unlike the situation in yeast, on-going sphingolipid synthesis is not required for forward transport of GPI-anchored proteins in bloodstream trypanosomes, but may play a role in endocytosis.

ABSTRACTS: SESSION II

Genomics and Antigenic Variation

April 13 8:30 a.m.

Chair(s) - E. Ullu

Trypanosome telomeres are protected by a homologue of mammalian TRF2

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Antigenic variation is crucial for the pathogenesis of *Trypanosoma brucei*, yet its regulation mechanisms remain elusive. The localization of expressed *Variant Surface Glycoprotein* genes to subtelomeric regions, and the observation that expression of reporter genes in silent expression sites is inversely correlated with the distance of the gene from the telomere repeats, suggest that telomere structure might play a role in the antigenic variation. In order to better characterize telomere functions, we aimed to identify telomere-binding proteins in *T. brucei*. Putative TTAGGG repeat-binding factor (TRF) homologues were identified in the genomes of *Trypanosoma brucei, Trypanosoma cruzi* and *Leishmania major*. They have significant sequence similarity to higher eukaryotic TRFs in their C-terminal DNA-binding myb domains, but only weak similarity in their N-terminal domains. *T. brucei* TRF associates with telomeres *in vivo*, and binds to duplex TTAGGG repeats *in vitro*. tbTRF is able to interact with itself, probably as a dimer. tbTRF is essential, and RNAi-mediated knock-down arrested bloodstream cells at G2/M and procyclic cells partly at S phase. Functionally, tbTRF resembles mammalian TRF2 more than TRF1, as tbTRF knock-down diminished telomere single-stranded G-overhang signals. This suggests that tbTRF, like vertebrate TRF2, is essential for telomere end protection, and supports the hypothesis that TRF, rather than Rap1, is the more ancient DNA-binding component of the telomere protein complex. Identification of the first *T. brucei* telomere DNA binding protein and characterization of its function and its partners will provide a new route to explore the roles of telomeres in the pathogenesis of this organism. This work also establishes *T. brucei* as an attractive model for telomere biology.

Chromatin remodeling and the regulation of trypanosome DNA glycosylation

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Beta-D-Glucosyl-hydroxymethyluracil, also called base J, is an unusual modified DNA base present in the genome of bloodstream form *Trypanosoma brucei*. In *T. brucei*, base J results from the glycosylation of specific thymine residues within telomeric DNA and correlates with the bloodstream form specific epigenetic silencing of the variant surface glycoprotein genes involved in antigenic variation. The absence of base J within procylic form trypanosome DNA, is thought to be due to the lack of a complete J-synthesis pathway. We have previously identified a base J specific DNA binding protein (JBP1) that is involved in propagating J-synthesis in the genome. Here, we show that when ectopically expressed in procyclic trypanosomes, JBP1 can propagate and maintain J-synthesis once an artificial 'seed' of J is provided in the genome. Therefore, procyclic cells contain a fully functional J-synthesis pathway, but still lack J. We have identified a homologue of JBP1, JBP2, containing a domain related to the SWI2/SNF2 family of chromatin remodeling proteins. Ectopic expression of JBP2 in procyclic trypanosomes lead to its association with chromatin and de-novo synthesis of base J. This activity is inhibited following mutagenesis of conserved residues critical for SWI2/SNF2 function. Concomitant expression of JBP1 leads to further propagation of J-synthesis. DNA immunoprecipitation analysis indicates that this J is localized to telomeric regions of the chromosome as seen in bloodstream form DNA. We propose a model in which chromatin remodeling by JBP2 regulates the bloodstream form specific and chromosome site-specific synthesis of J with further propagation and maintenance of J by JBP1. The analysis of JBP2 function will allow us to address the function of base J.

Localization of spliced leader gene transcription in a defined nuclear domain of *Trypanosoma cruzi*

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RNA polymerase II of trypanosomes transcribes long polycistronic messages, which are not capped, but are processed by trans-splicing and polyadenylation to form mature mRNAs. The same RNA polymerase II also transcribes the genes coding for the spliced leader RNA, which are capped, exported to the cytoplasm, processed, and re-imported into the nucleus, before they are used as splicing donors to form mRNAs from pre-mRNA polycistronic transcripts. These findings suggest that pre-mRNA and spliced leader transcription by RNA polymerase are uncoupled. To address this question, in this report, we studied how the RNA polymerase is distributed in the nucleus of *Trypanosoma cruzi*. Using specific antibodies specific for the *T. cruzi* RNA polymerase II carboxy-terminal domain, we demonstrated that large amounts of the enzyme are found concentrated in a domain, close to the parasite nucleolus and containing the spliced leader genes. The remaining RNA polymerase II is diffusely distributed in the nucleoplasm. The spliced leader-associated RNA polymerase II localization is dependent on the cell transcriptional state. It disperses when transcription is blocked by alpha-amanitin and actinomycin D. Tubulin genes are excluded from this domain suggesting that it may exclusively be the transcriptional site of spliced leader genes. Trypomastigote forms of the parasite, which have reduced spliced leader transcription, show less RNA polymerase II labeling and the spliced leader genes are more dispersed in the nucleoplasm. These results provide strong evidences that transcription of spliced leader RNAs occurs in a particular domain in the *T. cruzi* nucleus, separated from the pre-mRNA transcription. Supported by FAPESP/CNPq

SL RNA gene transcription in Trypanosomatids

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Spliced Leader RNA (SL RNA) plays a crucial role in trypanosomatid gene expression. In these organisms, protein-coding genes are initially transcribed as polycistronic precursor RNAs that are subsequently processed into mature mRNAs by the trans-splicing of a short 39 nucleotide SL RNA to the 5'-end of the message and by the addition of a poly-(A) tail. The trypanosome SL RNA genes are transcribed from the only defined RNA polymerase II promoter in this group of organisms. About 200 copies of SL RNA genes are organized in tandem in the genome. Each individual repeat unit has its own promoter that lies within 100 bp upstream of the transcription start site. Using *Leptomonas seymouri* as a model organism, we initially characterized a multi-subunit transcription factor involved in SL RNA transcription. Extending our study to infectious *Trypanosoma brucei*, we purified the analogous protein complex (tbPBP-1) using tandem affinity purification scheme. This complex can specifically bind the SL RNA gene promoter in gel shift assays. Furthermore, using an in vitro transcription system we have demonstrated that the purified tbPBP-1 has robust transcription activity. The subunit composition of tbPBP-1 and its function in SL RNA gene transcription will be presented.

Trypanosoma brucei SL RNA gene transcription is directed by a TRF4/SNAPc/?? complex

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In trypanosomatids, the spliced leader (SL) RNA is a key molecule in gene expression donating its 5'terminal region in SL-addition *trans* splicing of nuclear pre-mRNA. While there is no evidence that this process exists in mammals, it is obligatory in parasite mRNA maturation. Hence, throughout their life cycle, trypanosomatid organisms crucially depend on high levels of SL RNA synthesis which are accommodated by approximately 200 SL RNA gene (*SLRNA*) copies per cell. The trypanosomatid *SLRNA* promoter is conserved and consists of a bipartite upstream sequence element (USE) and a putative initiator element recruiting RNA polymerase II. Thus far, a partially characterized small nuclear RNA-activating protein complex (SNAPc) and the TBP-related factor 4 (TRF4) have been identified as putative *SLRNA* transcription factors. By designing a novel epitope combination termed PTP tag, we have developed a tandem affinity purification protocol which facilitates highly efficient purification of lowly expressed transcription factors from crude trypanosome extracts. PTP tagging and purification of both TRF4 and SNAP proteins revealed the same complex of six subunits. Mass spectrometric identification of these proteins showed that the complex consists of TRF4, three SNAPc subunits and two subunits (one putative) of a basal transcription factor. All six proteins bound specifically to the *SLRNA USE* and, most importantly, the complex impressively reconstituted *SLRNA* transcription in an extract in which the factor had been depleted and *SLRNA* transcription was completely abolished.

Transcriptional effects of Tbrpb9 RNAi in *Trypanosoma brucei*

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The Rpb9 subunit is part of the RNA polymerase II complex. In yeasts, the non essential rpb9 protein is involved in 5' splicing of mRNAs and in transcription elongation of RNA PolII. We created mutant trypanosomes in which the *Tbrpb9* mRNA is degraded by induction of *Tbrpb9* dsRNA. This *Tbrpb9* RNAi led to rapid death of procyclic or bloodstream cell lines, showing that the Tbrpb9 protein is essential. *In situ* analysis of transcription revealed that the transcription was abolished in the nuclear matrix, but was still active in the nucleolus. This phenotype could not be rescued by simultaneous expression of the *S. cerevisiae* rpb9 protein. To study the role of Tbrpb9 in transcription, mRNAs were analysed in *Tbrpb9* RNAi procyclic and bloodstream cell lines. As expected, we observed in procyclics a rapid decrease of mRNAs transcribed by PolII after induction of dsRNA. Interestingly, as was observed for the TbTFIIH RNAi, induction of *Tbrpb9* RNAi led to contrasting results for the *procyclin* and *ESAG7* mRNAs, which were respectively down- and up-regulated. In bloodstream forms, ablation of rpb9 led to only a small decrease of mRNAs synthesized by Pol II, in parallel with appearance of uncleaved precursors RNAs, which suggests splicing defects. As observed in procyclics, the RNA PolI transcription was also affected since we observed a decrease of the PolI transcribed *VSG* mRNA. The rpb9 subunit has been expressed in procyclics in fusion with the TAP tag, allowing affinity purification of the major part of the Pol II complex. In addition to the two major Pol II subunits rpb1 and rpb2, the rpb3, rpb4 and rpb5.2 subunits were also copurified.

A homologue of the vaccinia virus cap-specific methyltransferase VP39 is involved in cap 4 modification in *Trypanosoma brucei*

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The spliced leader (SL) RNA plays a key role in the biogenesis of mRNA in trypanosomatids by providing the capped SL sequence for the 5' end of every mRNA. The cap structure of the SL RNA is unique in eukaryotes in terms of its high content of modified nucleotides and by convention was named cap 4. It has been postulated that enzymes involved in cap 4 synthesis play an essential role by regulating the availability of trans-splicing competent SL RNA. However, very little is known about the enzymes responsible for cap 4 modifications. By database mining we have now identified a *T. brucei* polypeptide of 57 kDa (TbMT57) with significant homology to vaccinia virus VP39, a bifunctional protein involved in the maturation of both ends of nascent transcripts. In particular, at the 5' end the protein acts as a cap-specific mRNA nucleoside-2'-O-methyltransferase. TbMT57 downregulation by RNAi did not reveal an obvious growth phenotype. However, analysis of the SL RNA 5' end by primer extension showed a clear defect in cap 4 formation. Direct analysis of the cap 4 nucleotides revealed that modifications at positions 3 and 4 were missing, thus identifying the first enzyme responsible for cap 4 modifications. Fractionation of TbMT57 on a gel filtration column further showed that this protein is in a complex of 200 kDa, laying the foundation for the identification of additional activities involved in cap 4 synthesis. Most interestingly, MT57 homologues are only found in trypanosomatid protozoa that have a cap 4 structure and in poxviruses, of which vaccinia virus is a prototype, thus highlighting a potential therapeutic target.

Cytoplasmic degradation of an unstable mRNA in trypanosomes

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The GPI-specific phospholipase C (*GPI-PLC*) mRNA is 50 fold more abundant in bloodstream form *Trypanosoma brucei* than in the procyclic form. The regulation of this differential expression is post-transcriptional and the *GPI-PLC* mRNA is unstable in the procyclic form with a half life of 3 minutes. At which stage of gene expression does the *GPI-PLC* mRNA become unstable in the procyclic form? To address this question, the effect of blocking discrete steps in gene expression on *GPI-PLC* mRNA steady state levels have been determined. When maturation of mRNA was blocked using sinefungin, equivalent amounts of *GPI-PLC* pre-mRNA accumulated in both the bloodstream form and the procyclic form. Thus, the *GPI-PLC* pre-mRNA is not unstable in the procyclic form. Export of mRNA from the nucleus was blocked by RNAi knock down of expression of the orthologue of the yeast gene *MEX67*. In yeast, Mex67 is necessary for mRNA, but not protein, export from the nucleus. After *MEX67* knockdown in procyclic form trypanosomes there was a stabilisation of the *GPI-PLC* mRNA. This results indicates that the mature *GPI-PLC* mRNA is stable if retained in the nucleus. Translation of the *GPI-PLC* mRNA was blocked by the inclusion of a hairpin in the 5'UTR of the *GPI-PLC* mRNA. This specific block to translation did not stabilise the *GPI-PLC* mRNA in the procyclic form. Thus, in the procyclic form, *GPI-PLC* mRNA turnover is not dependent on its translation. The conclusion of this work is that, in the procyclic form, the unstable *GPI-PLC* mRNA is degraded during or after nuclear export but before translation.

Control of mRNA degradation in *Trypanosoma brucei*

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Stage-specific gene expression in *T. brucei* depends on regulation of mRNA stability and translation. We are investigating the enzymes responsible for mRNA degradation and the RNA sequences that specify the half-life and translation efficiency. The exosome complex has 3′-¿5′ exonuclease activity and is important in degrading very unstable mRNAs. Of four 5′-¿3′ exonucleases, three are confined mainly to the nucleus; the role of the cytoplasmic one in mRNA degradation is being investigated. At the same time, we have mapped the sequences which are responsible for regulating the expression of a procyclic-specific amino-acid transporter and bloodstream-specific glycosomal phosphoglycerate kinase. The regulatory sequences are in the 3′-untranslated regions and affect both translation and mRNA degradation. We are also interested in proteins which bind to the regulatory sequences in mRNAs. *Tc*UBP1 and *Tc*UBP2 are two cytosolic proteins which interact with U-rich sequences and are implicated in the regulation of mucin mRNA levels in *T. cruzi*. The equivalent proteins in *T. brucei* are *Tb*UBP1A and *Tb*UBP1B. Over-expression of *Tb*UBP1A or *Tc*UBP1 in *T. brucei* inhibited growth of bloodstream and procyclic forms and caused increases in several mRNAs in bloodstream forms. One target transcript encoded a protein with a cyclinF box. Simultaneous down-regulation of *Tb*UBP1A and 1B also inhibited growth, but the cyclinF-box mRNAs were unaffected. Instead mRNAs encoding a protein with multiple trans-membrane domains increased in abundance and some ESAG7-like transcripts were reduced.

A family of eIF4E homologues from *Leishmania*

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Eukaryotic mRNAs posses a 5' cap that participates in splicing, transport, stabilization and assembly of the translation initiation complex. The 5' cap of trypanosomatids is complex; in addition to m7GTP, it includes unique modifications on the first four transcribed nucleotides, thus denoted cap-4. A group of eIF4E homologues was identified in *Leishmania*: LeishIF4E-1 is an eIF4E homologue that contains the conserved cap-binding pocket, binds m7GTP and a synthetic analogue of cap-4. Although the mouse eIF4E has a higher association-constant for all cap analogues tested, it differentiates between cap-4 and m7GTP, while LeishIF4E-1 binds both compounds equally well. Sequence alignment shows that LeishIF4E-1 lacks the region derived from the C-terminus in the murine eIF4E. C-truncation of the mouse protein reduces the binding affinity to all cap analogues, and eliminates the difference observed between binding to m7GTP and cap-4. We therefore hypothesize that removal of the C-terminus in the mouse protein stabilizes, at least in part, the interaction with the cap-4. LeishIF4E-1 is distributed in the cytoplasm, and is assumed to be the major translation factor, based on its binding characteristics. However, it cannot substitute the mammalian eIF4E in an in vitro translation system, most probably due to the structural differences. Two other eIF4E isoforms were tested: LeishIF4E-3 binds m7GTP rather weakly, and LeishIF4E-2 fails to bind m7GTP or TMG, although a structure prediction suggests that the cap-binding pocket is mostly conserved. A clue to their possible role of the different eIF4E homologues can be deduced from variations in their ability to bind the human 4E-BP1, a reaction that modulates the interaction with eIF4G. Possible implications of this behavior will be discussed.

A developmentally regulated non-coding RNA in *Leishmania infantum* associates with ribosomal complexes

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The protozoan parasite *Leishmania* is the aetiological agent of human leishmaniasis, worldwide. *Leishmania* has two distinct life-cycle stages which comprise motile promastigotes in the gut of the sandfly vector and amastigotes that live in phagolysosomes of mammalian macrophages. The intracellular survival of amastigotes is obviously dependent on the developmental regulation of specific genes and potentially expressed non-coding genomic sequences. Characterisation of the genomic sequences that are preferentially expressed during the parasite's intracellular growth would enhance our understanding of the mechanisms controlling stage-specific gene regulation for intracellular development of the parasite. Using a genomic DNA library which was differentially hybridised to probes made from total RNA of amastigotes or promastigotes, we identified developmentally regulated non-coding RNAs which are novel and the first of their kind in *Leishmania*. These RNAs were found to be expressed in both orientations specifically in amastigotes. Nuclear run-on experiments showed that regulation of these RNAs occurs at the post-transcriptional level. FISH analyses indicated sense and anti-sense RNAs localised at the nuclear periphery in close contact with the ER suggesting that these non-coding RNAs may associate with ribosomes. Moreover, ribosomal sucrose gradient fractionation experiments confirmed that these RNAs bind to complexes which associate with ribosomes. Taken together, these data suggest that these expressed non-coding RNAs may have a role in the regulation of translation in amastigotes.

ABSTRACTS: SESSION III

Genomes and Tools

April 13 7:00 p.m.

Chair(s) - C. Clayton

Comparative genome organization of the Trypanosomatids

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The Tritryp sequencing consortium has completed the first draft genome sequences of the three reference Trypanosomatids -*Trypanosoma brucei*, *Trypanosoma cruzi* and *Leishmania major*. While these three pathogens share many general characteristics, each organism presents distinct aspects, including transmission by a different insect, unique life cycle features, different target tissues and immune evasion mechanisms, as well as different disease characteristics in their mammalian host, all of which are reflected by differences in their genome sequences. In an effort to characterize the core TriTryp proteome as well as genes that are species-specific, we have identified the orthology relationships between individual genes of the three genomes. Clusters of orthologous genes (COGs) were constructed and used to compare gene content as well as genome architecture. Our results show that regions encoding the Tritryp core proteome present a remarkably high degree of synteny (conservation of gene order) while in *Trypanosoma*, non-syntenic regions have been expanded and harbor many large species-specific gene families, majority of which appear to be surface antigen families. The different numbers of such species-specific genes largely reflect the different strategies of immune evasion used in each organism. Examination of large-scale synteny also sheds some light on chromosome evolution history in Trypanosomatids and emphasizes the role of multigene families, mobile elements or structural RNAs in shaping the genomic landscape of the present day parasites. We will also report on our analysis of the composition and organization of protein domains encoded by each genome, and an analysis of the rates of evolution for selected gene families.

Comparative genomic analysis of *Trypanosoma* species

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Complete genome sequencing of *Trypanosoma brucei* has been followed by shotgun sequencing projects of both the livestock infective *Trypanosoma vivax* and *Trypanosoma congolense* as well as the human infective *Trypanosoma brucei gambiense*. The genome of *T. brucei* is essentially complete and has been manually annotated to a high level. As a consequence of extensive conservation in synteny between the related kinetoplastid organisms *Leishmania major* and *Trypanosoma cruzi*, comparative analyses have further refined gene predictions and product assignments. The related *Trypanosoma* species have been sequenced to a depth of 5x and the *T. congolense* sequence data is currently undergoing automated pre-finishing. Using the *T. brucei* genome as a template, gene models and orthologues have been predicted and product descriptions transferred where appropriate. The data, including extensive links between the four genomes, are available via GeneDB (http://www.genedb.org). Preliminary data on the number and distribution of variant surface glycoproteins and species-specific differences in genes and chromosome architecture will be presented.

Genome-wide analysis of trypanosomatid protein kinases

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Protein kinases (PKs) integrate environmental and intracellular signals to modulate the activity of their protein targets, promoting cellular growth and survival. They are targets of drugs in human diseases. Our analysis showed that *L. major, T. brucei* and *T. cruzi* genomes encode 178, 156 and 160 distinct eukaryotic PKs that are likely to be catalytically active, as well as 22, 20 and 19 atypical PK genes. These kinomes are about one third that of human and twice that of *Plasmodium falciparum*. Most of trypanosomatid PKs have orthologues in all three species. The trypanosomatid kinomes differ in numerous ways from that of the host. They lack catalytic domains belonging to the receptor-linked or cytosolic tyrosine kinase families, and possess only a handful of PKs with predicted transmembrane domains. Some groups of PKs are poorly represented, while others are relatively expanded, particularly those involved in cell cycle control, such as cyclin dependent kinases and NEK family kinases. The parasites also possess many PKs that are related to those involved in MAP kinase cascades, which in other organisms are responsible for transducing signals from a variety of environmental cues. Trypanosomatid PKs generally lack additional Pfam domains, in contrast to the human kinome where half of the kinases possess additional domains, many of which mediate interactions with molecular partners. Numerous trypanosomatid PKs showed little similarity to any of the recognized families of PKs, suggesting they might be targeted without interfering with host signaling modules. Less than 10% of the identified PKs have been studied in detail experimentally; hence the dataset provides numerous opportunities for experimentation.

RNomics and SnoRomics in *Trypanosoma brucei*: bioinformatic and experimental approaches reveal trypanosome-specific features

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Non-coding RNAs have diverse essential functions in eukaryotes including splicing, protein sorting, rRNA processing, RNA modifications and developmental regulation (microRNAs). To investigate the repertoire of such RNAs in trypanosomes, whole genome search was conducted based on comparative Tri-Tryp genome analysis. Several bioinformatic tools were applied in parallel to examine the repertoire of small nucleolar RNAs from the C/D and H/ACA families that guide 2'-O-methylation (Nm) and pseudouridylation, respectively on rRNA and small RNAs. Twenty-one clusters encoding for 57 C/D and 34 H/ACA were identified. Almost all the clusters carry a mixture of H/ACA and C/D snoRNA and are repeated several times in the genome. The number of guide RNAs suggest that trypanosomes contain many modifications relative to their genome size. The data led us to reconstruct a map of these modifications on the rRNA. Interestingly, 40% of the Nms are trypanosome-specific and 40% of those lie outside the conserved domains which are rich in modifications also in other eukaryotes. To investigate the role of small RNAs and the modification they guide, we silenced by RNAi the pseudouridine synthase, an H/ACA binding protein, and fibrillarin, a C/D snoRNP protein. Both pathways are essential for life. The H/ACA pathway was found to be essential not only for rRNA processing and modification but also for *trans*-splicing. The destabilization of SLA1 abolished modification at pseudouridine 28 of the SL RNA and elicited defects at the cap-4 structure, supporting the role of SLA1 as a chaperone of the SL RNA. The large repertoire of RNA modifications suggest that these may play a central role in coping with temperature shift during cycling between the two hosts.

Organization of non-LTR retrotransposons in the trypanosomatid genome

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The non-LTR retrotransposons are the most abundant mobile elements characterized so far in the trypanosomatid genome. The long retrotransposition-competent elements (T. brucei: ingi; T. cruzi: L1Tc) share their 5'-extremity with the short non-autonomous elements (T. brucei: RIME; T. cruzi: NARTc). The latter uses the machinery encoded by ingi and L1Tc for retrotransposition. In addition, trypanosomatids (including Leishmania) contain highly degenerate retrotransposons related to ingi/L1Tc, called DIRE. We have analyzed all the non-LTR retrotransposons and DIREs present in the T. brucei chrI-XI, in L. major Chr1-36 and in T. cruzi contigs larger than 10-kb (which represents 1x coverage of the T. cruzi nuclear genome). This analysis provides new insights regarding abundancy, distribution, preferential sites of insertion, mechanism of retrotransposition and evolutionary relationship between these different retroelements.

Interaction of *Trypanosoma brucei* Argonaute 1 with polyribosomes: defining the role of the RGG domain

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The classical arm of RNA interference (RNAi) regulates gene expression through mRNA degradation. Endonucleolytic cleavage of mRNA by the RNAi nuclease or slicer is the first step in mRNA degradation and is carried out by a member of the Argonaute protein family in a ribonucleoprotein complex termed the RNA-induced silencing complex (RISC). Although several reports associate RISC with ribosomes, we have little understanding of how this interaction is mediated and whether it is a prerequisite for target mRNA degradation. In *Trypanosoma brucei* the slicer homologue TbAGO1 is in part associated with polyribosomes via the N-terminal RGG-rich domain, whose deletion results in severe defects in mRNA cleavage. RGG motifs have been implicated in protein-protein interactions as well as in RNA binding and may be modified by arginine methylation. Indeed, mass spectrometry analysis of TbAGO1 indicated that some arginine (R) residues in the RGG domain are dimethylated. Thus, to test whether R residues are relevant for TbAGO1 function, we generated RGG-domain mutants containing lysine (K) or alanine (A). R to K substitutions resulted in an approximately five-fold decrease in RNAi and reduced co-sedimentation with polyribosomes, whereas R to A mutations nearly abolished RNAi and TbAGO1-polyribosome association. These results provide evidence that arginines are key residues for the functioning of the TbAGO1-RGG domain. Furthermore, the RGG domain of TbAGO1 consists of five almost similar repeats. Progressive reduction of the number of repeats led to a striking correlation between the level of the RNAi response and TbAGO1 association with polyribosomes, thus supporting the hypothesis that RISC association with ribosomes is critical for RNAi.

Evolutionary conservation of the RNAi pathway

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RNA interference (RNAi) is a conserved mechanism that regulates gene expression in response to the presence of double-stranded (ds)RNAs. This effect is mediated by an RNA-induced silencing complex (RISC), which contains two signature components: small interfering RNAs (siRNAs) and the RNAi endonuclease, a member of the Argonaute (AGO) protein family. We have previously identified by genetic and biochemical analysis TbAGO1 as an essential component of the RNAi pathway in *Trypanosoma brucei* . AGO1-knockout cells are RNAi-deficient leading to a striking reduction in the accumulation of siRNAs and an increase in retroposon transcript levels. To gain further insight into the RNAi mechanism and to gauge the degree of evolutionary conservation of Argonaute function in the RNAi pathway, we expressed two human Argonaute proteins (hsAGO1 and hsAGO2) in the *T. brucei* AGO1-KO cells. Although multiple Argonaute proteins are present in mammals, only hsAGO2 is competent for mRNA cleavage. The expression of hsAGO1 and hsAGO2 resulted in the accumulation of retroposon siRNAs, similar to what we observed with a TbAGO1 complementation. However, the average size of the siRNAs that accumulated in both cell lines, were a few nucleotides longer than that in wild-type or TbAGO1-complemented cells. Similar to TbAGO1, a proportion of hsAGO1 and hsAGO2 associated with polyribosomes. Most interestingly, hsAGO2 restored the RNAi response to about 30%, whereas expression of hsAGO1 did not, thus mimicking the situation in mammalian cells. Taken together, our experiments demonstrate that the function of Argonaute proteins and the mechanism of RNAi are conserved from *T. brucei* to man. In addition, our results show potential for the reconstruction of the RNAi pathway in RNAi-negative organisms.

Genetic tools for *Trypanosoma brucei*

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Investigators are usually in an understandable rush to get results, and few investigators can afford the time or resources that are needed to develop more robust genetic tools that can be relied upon in the longer term, and for more complex experiments requiring multiple genetic manipulations in a stable background. Although the available tools for genetically manipulating *Trypanosoma brucei* have been responsible for many advances in dissecting gene function, they have many limitations that should be avoidable. Others, such as the low transfection efficiency of bloodstream forms, may be more difficult to overcome. I will briefly review the systems that are currently used for genetic manipulation in *T. brucei* and their deficiencies. I will propose approaches for improving these systems in three areas: (1) developing positive/negative selectable vectors and recombination-based excision methods for gene mutation and knockout; (2) improving regulated expression vectors and cell lines, particularly in the areas of stability, reproducibility, and low background expression that are sorely needed, especially for RNAi-based knockdown; (3) developing forward genetic methods based either on RNAi or transposon-mediated insertional mutagenesis.

Tools for inducible expression in *Trypanosoma brucei*

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Tetracycline inducible expression is established as a powerful tool for *Trypanosoma brucei* analysis, particularly for RNA interference and expression of recombinant proteins. However, current methods for the generation of the required recombinant cells can suffer from problems with leaky expression, downstream effects resulting from prolonged expression of T7 RNA polymerase and variable position effects. Bloodstream form cells also suffer from low transfection efficiency. We have developed solutions or improvements in all four of these areas. We report a new inducible *rDNA* promoter that we use to drive T7 RNA polymerase transcription and have also generated cells with a tagged locus that eliminates position effects and allows significantly increased transfection efficiency. We are specifically interested in variant surface glycoprotein (VSG) gene-silencing so we also developed a system that allows tetracycline-regulation of single active *VSG*. *VSG* switching can be triggered at a rate ~250,000 fold higher than reported previously in this strain. New cells and vectors have been constructed that incorporate all the features outlined above. These tools will facilitate increased throughput and studies on VSG regulation.

ABSTRACTS: SESSION IV

Biochemistry and Host-Parasite Interactions

April 14 8:30 a.m.

Chair(s) - K. Gull

Acidocalcisome and contractile vacuole enzymes are involved in osmoregulation in *Trypanosoma cruzi*

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Acidocalcisomes are dense, acidic organelles with a high concentration of phosphorus present as pyrophosphate and polyphosphate complexed with calcium, and other cations. The acidocalcisome membrane contains a number of pumps (Ca^{2+}-ATPase, V-H^+-ATPase, H^+-PPase), exchangers (Na^+/H^+, Ca^{2+}/H^+), and channels (aquaporins), while its matrix contains enzymes related to pyrophosphate and polyphosphate metabolism. Acidocalcisomes have been found in several pathogenic microorganisms as well as in the green alga *Chlamydomonas reinhardtii*, and the slime mould *Dictyostelium discoideum*. The identification of acidocalcisomes in bacteria and the finding that human platelet dense granules are homologous to acidocalcisomes, indicate that these are the only known organelles that have been conserved from bacteria to humans. Acidocalcisomes function in the storage of cations and phosphorus, pyrophosphate and polyphosphate metabolism, calcium homeostasis, maintenance of intracellular pH homeostasis, and osmoregulation. Acidocalcisomes have been linked to the contractile vacuole complex in *C. reihnardtii*, *D. discoideum* and *T. cruzi*. A microtubule- and cyclic AMP-mediated fusion of acidocalcisomes to the contractile vacuole complex in *T. cruzi* results in translocation of aquaporin and the resulting water movement, which, in addition to swelling of acidocalcisomes, is responsible for the volume reversal not accounted for by efflux of osmolytes. Polyphosphate hydrolysis occurs during hypo-osmotic stress and we found an exopolyphosphatase present in the contractile vacuole complex of this parasite. This is in agreement with the hypothesis that, when cells are submitted to hypo-osmotic stress, acidocalcisome polyphosphate is hydrolyzed after fusion to the contractile vacuole increasing its osmotic pressure and facilitating water movement. The gene encoding this exopolyphosphate has been cloned and the recombinant enzyme has been shown to hydrolyze preferentially short-chain polyphosphate.

Vitamin C biosynthesis in trypanosomes: a new role for the glycosome

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Vitamin C (L-ascorbate) is a powerful antioxidant that functions as a free radical scavenger. In mammals, it is also involved in several important physiological reactions, all of which depend on its property as an electron donor. In *Trypanosoma cruzi*, an ascorbate-dependent haem peroxidase is localised in the ER. This plant-like enzyme plays a major role in H_2O_2 detoxification. As a first step to investigating the functional importance of ascorbate to antioxidant defence in trypanosomes, we have cloned a homologue of gulonolactone oxidase, the mammalian enzyme that catalyses the last step in ascorbate biosynthesis. Humans lack this activity, a deficiency that makes us susceptible to scurvy. In *T. brucei*, the 57 kDa enzyme uses FAD as a cofactor and synthesises ascorbate from arabinolactone and galactonolactone (the usual fungal/plant substrates), but not from gulonolactone (the mammalian substrate). Intriguingly the enzyme is localised to the glycosome, thus identifying a new role for this parasite-specific organelle. Double gene knockout had no effect on the viability or infectivity of bloodstream *T. brucei* although mutant parasites exhibited a growth phenotype that was exacerbated in medium treated with ascorbate oxidase. This implies that the parasite has an ascorbate transporter capacity that is sufficient to partially compensate for disruption of the biosynthetic pathway.

A new mechanism for fatty acid synthesis in *Trypanosoma brucei*

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T. brucei BSF GPI anchors contain exclusively myristate, the 14-carbon fatty acid end product of a specialized fatty acid synthesis (FAS) pathway. In contrast, PCFs make longer fatty acids, up to stearate (C18). The genome encodes no type I FAS but predicts a type II FAS system. However, several observations suggested it was not involved in the FAS system described above: (1) The type II system is mitochondrial. (2) Type II systems are soluble, but trypanosome FAS activity is membrane-associated. (3) Triclosan, a type II inhibitor, and (4) RNAi knockdown of acyl carrier protein, a key component of type II systems, have little if any effect on FAS (see J. Stephens abstract). (5) Finally, cerulenin inhibition of FAS suggests at least two ketoacyl synthases (KAS), but only one type II KAS is predicted. The trypanosome genome encodes four fatty acid (FA) elongases (TbELO1-4) thought to be involved in a microsomal FA elongation pathway. In mammals and yeast, FA elongases function to extend stepwise, via CoA-linked intermediates, the end product–usually palmitate (C16)–of cytosolic type I FAS. We study FAS in a cell-free assay containing trypanosome membranes, a butyryl-CoA (C4) primer, radiolabeled malonyl-CoA (the two carbon donor), and NADPH. Using membranes prepared from RNAi and homozygous knockouts, we now have data that indicate trypanosome elongases are responsible for all of the FAS activity detected in our cell free assay. They extend butyryl-CoA stepwise to myristate (C14) in BSF and stearate (C18) in PCF lysates. This is the first example of microsomal elongases functioning in *de novo* fatty acid synthesis.

Purine sensing in *Leishmania donovani*

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Leishmania donovani, like all parasitic protozoa, are obligatory scavengers of host purines. While the components of purine metabolism and transport have been extensively studied, the mechanisms by which these parasites adapt to changes in the extracellular purine milieu and regulate their intracellular purine environment are unknown. Withdrawal of purines from the cultivation media of *L. donovani* leads to a rapid augmentation of both nucleobase and nucleoside uptake, indicating that *L. donovani* are able to respond swiftly to changes in their purine environment. Studies with nucleoside transport-deficient *L. donovani* indicate that this augmentation is contingent upon the presence of the nucleoside transporters, LdNT1 (enhanced adenosine/uridine uptake) and LdNT2 (augmented inosine/guanosine transport). Analyses of LdNT1 and LdNT2 activities from purine-replete and purine-starved cells reveal striking differences in their kinetic parameters. Both LdNT1 and LdNT2 exhibit significantly increased V_{max} values (several orders of magnitude) for their respective purine ligands under purine-deplete conditions. However, whereas the apparent affinity and substrate-specificity of LdNT1 remains unchanged, the affinity and specificity of LdNT2 for its ligands is significantly altered. While under purine-replete conditions LdNT2 is a high affinity, specific transporter for inosine and guanosine, under purine-starved conditions its affinity for these ligands significantly diminishes by at least two orders of magnitude. Remarkably, LdNT2 substrate-specificity is also modified to accommodate the nucleobases, hypoxanthine and guanine, in addition to the nucleosides, inosine and guanosine. The mechanism by which these changes are implemented is currently under investigation in our laboratory.

Trypanothione synthetase: biochemical and biological characterization of a suitable drug-target against trypanosomiasis

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The presence of thiols in living systems is critical for the maintenance of cellular redox equilibrium, as well as for the protection of cells against reactive oxygen species. In trypanosomatids, these biochemical capacities are mainly fulfilled by trypanothione [$T(SH)_2$], a bis-glutathionyl conjugate of spermidine. Since this dithiol is absent in the mammalian host, the enzymes involve either in its synthesis or utilization arose special interest as potential drug targets. While the metabolic pathways using $T(SH)_2$ are being elucidated at considerable speed, the equally important biosynthesis has remained a matter of debate and confusion since its discovery. Therefore, our studies focused on: i) elucidation of the biosynthetic pathway operating in several Kinetoplastida genus (*Trypanosoma cruzi*, *T. brucei*, and *Crithidia fasciculata*), ii) assessment of the biological significance of $T(SH)_2$-biosynthesis by means of inverse genetics (RNAi), and iii) biochemical and molecular analysis of trypanothione synthetase. These investigations were undertaken with the long-term perspective to design trypanocidal drugs on the basis of in-depth characterized and validated drug targets. References: - Comini *et al.* (2003) Biol. Chem. 384: 653-656. - Comini *et al.* (2004) Free Radic. Biol. Med. 36: 1289-1302. - Comini *et al.* (2005a) J. Biol. Chem. 280: 6850-60. - Comini *et al.* (2005b) J. Biol. Chem. 280: 7407.

The pharmacokinetic/pharmacodynamic relationship of the anti-trypanosomal drug melarsoprol

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It is well known that the efficacy of anti-bacterial agents depends on the concentration-time course achieved *in vivo*. The influence of these kinetics (pharmacokinetics, or PK) on the effects of the drug (pharmacodynamics, or PD) varies between drug classes: efficacy of some antibacterials is dependent on maximum concentration (C_{max}) in blood, while the success of others is governed by time above minimum effective concentration (Time above MEC). Elucidating the PK/PD relationship for a drug is vital for optimal dosing of existing drugs, and is essential for streamlining first-in-human studies on experimental agents. There is limited information on the PK/PD relationship of melarsoprol, the most widely used drug against late-stage human African trypanosomiasis. Current dosing regimens were empirically determined and have significant toxicity. We have utilized a novel *in vitro* perfusion system (cartridge system) that exposes parasites to continuously varying drug concentrations, such as those that occur *in vivo* after drug administration. We have manipulated melarsoprol PK parameters in this system in an effort to elucidate the governing determinants of anti-trypanosomal activity. We demonstrate that the antiparasitic effect of melarsoprol is C_{max}-dependent. In contrast, the effect of melarsoprol on mammalian cells is not governed by C_{max}. These results provide a rationale for designing more efficacious and less toxic dosing strategies for this frontline anti-trypanosomal agent.

Apolipoprotein L-I lyzes trypanosomes through an anionic pore on the lysosomal membrane

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David Pérez-Morga*, Benoit Vanhollebeke*, Françoise Paturiaux-Hanocq*, Derek P. Nolan[1], Laurence Lins[2], Fabrice Homblé[3], Luc Vanhamme*, Patricia Tebabi*, Annette Pays*, Philippe Poelvoorde*, Alain Jacquet[4], Robert Brasseur[2] & Etienne Pays* * Laboratory of Molecular Parasitology, IBMM, Université Libre de Bruxelles, 12, rue des Profs Jeener et Brachet, B6041 Gosselies, Belgium. [1] Dept. Biochemistry, Trinity College, Dublin 2, Ireland. [2] Centre de Biophysique Moléculaire Numérique, Université de Gembloux, Belgium. [3] Structure et Fonction des Membranes Biologiques, Université Libre de Bruxelles, Belgium. [4] Laboratory of Applied Genetics, IBMM, Université Libre de Bruxelles, Belgium ApoL-I is a human-specific serum apolipoprotein bound to HDL particles that is able to lyze the African trypanosome *Trypanosoma brucei*, but not the parasite subspecies adapted to humans (*T. b. rhodesiense, T. b. gambiense*). Trypanosome lysis results from uptake of apoL-I in the lysosome. We show that apoL-I contains a membrane pore-forming (pf) domain functionally similar to that of bacterial colicins, flanked by a pH-sensitive amphipatic alpha-helix domain that allows binding to HDL or targeting to a membrane (see Vanhollebeke *et al.*). In planar lipid bilayer membranes or liposomes the pf domain of apoL-I forms ion channels with preference for anions. In trypanosomes, recombinant apoL-I, but not mutants impaired in either pf or membrane-addressing activity, mimics normal human serum in triggering considerable osmotic swelling of the lysosome linked to depolarisation of the lysosomal membrane and continuous influx of chloride into the cell. Blocking this flux by the anion channel inhibitor DIDS prevented trypanolysis. These observations suggest that apoL-I kills trypanosomes by forming lysosomal membrane pores causing uncontrolled chloride influx in the lysosome.

Trypanolytic human HDLs contain both Hpr and apoL-I

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Trypanosoma brucei brucei is unable to establish infection in humans because of the presence of a serum factor that fractionates with HDLs. HDLs are composed mainly of phospholipids, neutral lipids, and apolipoproteins. Two apolipoproteins have been shown to possess trypanolytic properties, haptoglobin-related protein (Hpr) and apolipoprotein L1 (apoL-1). Antibodies antagonistic to either protein inhibit lysis of *T. b. brucei* by total human HDL, though high quantities of each antibody alone are unable to abolish lysis, indicating that each protein is capable of inducing lysis independently of the other. Purified Hpr and apoL-I share similar specific activities of 0.10 and 0.14 units/μg when tested in lysis assays using *T. b. brucei*. Reconstitution of the two proteins into an HDL particle gave an increase in specific activity (1.10 units/μg) as compared to incubation together without reconstitution and reconstitution singly into HDL particles. Native human HDL particles were physically partitioned into four subclasses based on their Hpr and apoL-I composition. The vast majority of HDLs (99%) does not contain either protein and is entirely deficient in lytic activity. The two subclasses of HDL containing only Hpr or apoL-I alone were found in low abundance (0.1% and 0.05% of the total HDL respectively), had low specific activities of 0.5 and 0.8 units/μg, and accounted for only 0.4% and 0.3% of the total HDL population. By far the most important particle for providing lytic activity was that containing both Hpr and apoL-I, with a specific activity of 83.3 units/μg. This single class of HDL, while comprising only 0.13% of the total HDL population, was responsible for 99% of the recoverable activity present in human serum.

Cyclical transmission of procyclin null mutants

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Procyclic form trypanosomes in the midgut of the fly are covered by several million copies of GPI-anchored proteins known as EP and GPEET procyclins. Functions that have been proposed for procyclins include protection against the protease-rich environment of the midgut and/or a role in determining the route of the parasite through the digestive tract of the fly. We have generated a procyclin null mutant in a fly-transmissible strain of *Trypanosoma brucei*, AnTat 1.1. Unexpectedly, this mutant can complete the entire life cycle in the fly, from infection with bloodstream forms to the production of metacyclic forms in the salivary glands. The null mutant is only slightly impaired in its ability to establish midgut infections (~2-fold less than the wild type) but the rate of salivary gland infections is only 2%, compared with 12-14% for the wild type. In addition, the number of trypanosomes in the salivary glands is reduced considerably and they do not associate as tightly with the salivary gland epithelia. Introducing a single, highly expressed copy of GARP (a surface glycoprotein expressed by late procyclic forms and epimastigote forms of *T. congolense*) did not re-route the trypanosomes from the salivary glands to the proboscis. When flies were infected with a mixture of null mutant and CFP-tagged wild type trypanosomes, the mutant was overgrown by the CFP-tagged cells, even though they grew at similar rates when they were co-cultured. We conclude that procyclins are neither essential for protection against proteases nor for determining tropism. Nevertheless, any trypanosome without them would be outcompeted very rapidly by procyclin-positive parasites.

'Antigenic variation' in *Leishmania major*

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Leishmania major procyclic promastigotes attach to sand fly midguts through interactions involving the stage-specific surface adhesin lipophosphoglycan, to avoid being excreted with the digested bloodmeal. For the Friedlin strain, attachment involves monogalactosyl modifications of the LPG [Gal-Man-P] repeating units, binding to the *Phlebotomus papatasi* galectin receptor PpGalec. Procyclic LPG modifications vary in other strains, ranging from unmodified to poly-galactosylation. Genetic and biochemical studies show that these modifications affect the ability of strains to be transmitted by different sand fly species. PG side-chain galactosylation arises through the activity of the seven members of the *SCG* family of telomeric galactosyltransferases. Different *SCG*s encode different scGalT activities, including initating, elongating and both scGalT specificities, while several genes appear nonfunctional. The expression and specificity of scGalTs in a given *Leishmania* strain is then integrated to give the final LPG galactosylation pattern. Changes in expression appear to be mediated by 1) gene conversion, including ones bringing appropriate SCG activities into (or out of) a quasi-expression site accounting for 50% of SCG mRNAs, 2) smaller changes affecting mRNA levels, and 3) point mutations that alter, activate or inactivate SCG catalytic activity. These studies highlight unique features in the 'logic' of the parasite surface variation involving carbohydrate ligands. *SCG* heterogeneity and simultaneous expression is favored since carbohydrate synthesis occurs through the action of many enzymes and does not require exclusivity. The genetic flexibility of telomeric localization would be expected to facilitate rapid shifts in the LPG modification pattern. Potentially, evolutionary change may be driven by selection involving shifts in sand fly hosts as well as changes in the LPG receptor which may reduce colonization by *Leishmania*.

The proteome of *Trypanosoma cruzi* shed vesicles involved in host immunomodulation and cell invasion

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Cell-derived trypomastigote forms of *Trypanosoma cruzi* release antigen-rich vesicles to the culture medium (Exp. Parasitol.72:43,1991). Here we have further fractionated these vesicles by gel-filtration, followed by affinity chromatography using anti-alpha-galactosyl antibodies, purified from chronic Chagasic patients. We found that alpha-Gal-containing vesicles (alpha-GalVs) were rapidly internalized by phagocytic and non-phagocytic mammalian cels. Furthermore, alpha-GalVs induced a potent proinflammatory host immune response and greatly enhanced host cell invasion, both by Toll-like receptor 2 (TLR2)-dependent mechanisms. For proteomic analysis, alpha-GalVs were digested using different strategies: a) trypsin in urea-containing buffer; b) double digestion with trypsin and endoproteinase Glu-C; and c) trypsin in methanol-containing buffer. To increase protein coverage, peptides were fractionated by strong cation-exchange chromatography followed by LC-MS/MS. We reliably identified 131 proteins, including several members of the trans-sialidase (TS) superfamily, such as active and inactive TS, and Tc85, proteins known to play a key role in host cell adhesion and invasion by the parasite. Interestingly, we also found several polypeptides frequently present in mammalian cell exosomes, and others related to the exosomal/vacuolar formation. Taken together, our data strongly support an exosomal-like biogenesis of alpha-GalVs. Thus far this study is the most comprehensive proteome analysis of a *T. cruzi* cell compartment. Supported by BBRC (Grant#2G12RR008124), FAPESP, and The Wellcome Trust.

ABSTRACTS: SESSION V

Kinetoplast Replication, Editing and Expression

April 14 1:00 p.m.

Chair(s) - B. Sollner-Webb

Regulation of replication initiation at the kinetoplast DNA minicircle replication origins

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Kinetoplast DNA (kDNA) minicircles in the trypanosomatid *C. fasciculata* contain two copies of their replication origin (*OriA* and (*OriB*). Within the origin site, minicircles replication initiates at two conserved sequences, a dodecamer (UMS) and a hexamer, located at the proposed origins of the minicircle L and H-strands (*oriL* and *oriH*, respectively). A UMS binding protein (UMSBP) binds the 12-mer UMS and a 14-mer sequence containing the conserved hexamer. UMSBP binding, as well as its oligomerization, is regulated *in vitro* by the protein redox state. Yeast two hybrid, and co-immunopercipitation analyses demonstrated the interaction of UMSBP with other proteins involved in the replication and shaping of kDNA, such as pol beta, SSE1 and KAP3. *In vivo* cross-linking analyses reveal the binding of UMSBP to kDNA networks in the cell, and ChIP-like analysis *in vitro* demonstrated its binding to the minicircle's native *OriA* and *OriB*. Several lines of evidence suggest that these two minicircle's origins may not be equally (alternately) functional during of kDNA minicircles replication. 2D gel electrophoresis analyses of minicircles' replication intermediates indicated that minicircles replication initiates *in vivo* only (or highly preferably) at the *OriA* and proceeds unidirectionally from this site. Moreover, BIAcore binding analyses revealed an order of magnitude higher binding affinity in the interaction of UMSBP with the *OriA* site versus its interaction with *OriB*. Within the *OriA* site, the nucleoprotein complexes generated at the two UMSBP binding sequences (*oriL* and *oriH*) differ in their stability, due to significant structural differences between the two initiation sites, providing a plausible mechanistic model for the sequential initiation of the minicircle's L and H strands replication.

The rotational dynamics of kinetoplast DNA replication

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kDNA synthesis in *T. brucei* or *C. fasciculata* involves release of covalently-closed minicircles from the network, replication of the free minicircles, and reattachment of progeny (containing gaps) at two antipodal sites flanking the kDNA. In *T. brucei*, as expected, gapped progeny minicircles accumulate at the network poles, adjacent to the antipodal sites. In *C. fasciculata*, however, gapped minicircles attach at two antipodal sites but subsequently distribute around the network periphery. This distribution is due to relative movement between the network disk and the antipodal sites. From these results we assumed that *C. fasciculata* has a spinning kinetoplast and *T. brucei* has a stationary kinetoplast. However, current studies indicate that the *T. brucei* kinetoplast is neither stationary nor spinning, but oscillating. We pulse-labeled cultured trypanosomes with BrdU. Its subsequent detection in isolated networks, by immunofluorescence, revealed the minicircle attachment sites. To determine the extent of replication of these isolated networks, we also labeled gapped minicircles using terminal deoxynucleotidyl transferase (TdT) and fluorescent-dUTP. We found two BrdU labeling sites, always 180^0 apart on the network periphery, but unexpectedly, not always at the network poles. Instead the BrdU labels along the periphery within the TdT-labeled regions. We believe these data reflect a relative movement between the T. brucei kinetoplast and the antipodal sites. The kDNA disk appears to oscillate back and forth, distributing newly-replicated minicircles around the network poles. We obtained more evidence for this pattern of movement by pulse-chase and pulse-chase-pulse labeling with BrdU. The function of kinetoplast movement may be to control the final shape of the replicating network, dumbbell-shaped in *T. brucei* and circular in *C. fasciculata*.

Mitochondrial DNA ligases of *Trypanosoma brucei*

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The mitochondrial DNA of *Trypanosoma brucei*, termed kinetoplast DNA or kDNA, consists of thousands of minicircles and a small number of maxicircles catenated into a single network organized as a nucleoprotein disk at the base of the flagellum. Minicircles are replicated free of the network but still contain nicks and gaps after rejoining to the network. Covalent closure of remaining discontinuities in newly replicated minicircles after their rejoining to the network is delayed until all minicircles have been replicated. The DNA ligase involved in this terminal step in minicircle replication has not been identified. A search of kinetoplastid genome databases identified two putative DNA ligase genes in tandem. These genes (*LIG ka* and *LIG kb*) are highly diverged from mitochondrial and nuclear DNA ligase genes of higher eukaryotes. Expression of epitope tagged versions of these genes shows that both LIG ka and LIG kb are mitochondrial DNA ligases. Epitope tagged LIG ka localizes throughout the kDNA whereas LIG kb shows an antipodal localization close to, but not overlapping, that of topoisomerase II, suggesting that these proteins may be contained in distinct structures or protein complexes. Knockdown of the *LIG ka* mRNA by RNA interference led to a cessation of the release of minicircles from the network and resulted in a reduction in size of the kDNA networks and rapid loss of the kDNA from the cell. Closely related pairs of mitochondrial DNA ligase genes were also identified in *Leishmania major* and *Crithidia fasciculata*. We will also present biochemical and enzymatic characterization of the *Crithidia fasciculata* LIG ka.

Developmental regulation of RNA editing in *Trypanosoma brucei*

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RNA editing in the mitochondrion of trypanosomes is a post-transcriptional, protein catalyzed process that modifies primary mRNAs by the insertion or deletion of uridylate residues. The number and site of uridylate insertion or deletion is directed by small non-coding guide RNAs (gRNA). In *Trypanosoma brucei* this process is developmentally regulated. Although several components of the mRNA editing complex have been identified nothing is known about the mechanisms modulating the developmental timing and the extent of RNA editing. Here we present the first comparative analysis of cDNA libraries from the mitochondrial mRNA and gRNA of the bloodstream form as well as the procyclic form of *T. brucei*. A detailed analysis of cytochrome oxidase c subunit III cDNA (COIII) has revealed a significant difference in RNA editing patterns between bloodstream and procyclic parasites. The accumulation of an alternatively edited population of COIII cDNAs in bloodstream trypanosomes might indicate a point of regulation of this gene product during the life cycle of the parasite. We show that alternatively edited COIII mRNAs are stable products and not intermediates of the editing process. We also have identified gRNAs that encode for the alternative editing pattern of COIII mRNAs. Furthermore, bioinformatic analysis shows that the alternatively edited COIII mRNAs encode several large open reading frames (¿360 nt) that contain unique N-terminal sequences contiguous with sequences homologous to the hydrophobic C-terminus of COIII from *T. brucei*.

Reconstitution of in vitro editing with recombinant proteins

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The L-complex in trypanosomatid mitochondria consists of approximately 16 proteins including two RNA ligases and two Exo-Endo_Phos motif proteins. One of the latter, the L. major and T. brucei LC-2 (MP100), was expressed in insect cells. The TAP-tagged proteins were shown to have a 3'-5' exoribonuclease activity and this was therefore renamed RNA editing exonuclease 1 (REX1). Recombinant REX1 specifically trims 3' overhanging U's and stops at a duplex region. Evidence is presented that REX1 is responsible for deletion of the 3' overhanging U's from the bridged mRNA 5' cleavage fragment and that REL1 is responsible for the ligation of the two mRNA cleavage fragments in U-deletion editing. Pre-cleaved U-deletion in vitro editing could be reconstituted with only two recombinant enzymes: rREX1 and rREL1. In a related project, REL1 and REL2 were down regulated in T. brucei procyclic cells by RNAi. Addition of the rREL1 or rREL2 proteins to mitochondrial lysate that was depleted of the cognate endogenous ligase led to integration of the protein into the L-complex. In the case of REL1, there was a functional complementation of in vitro pre-cleaved U-insertion and U-deletion editing activities of the 20S L-complex. Down regulation of REL2 had no phenotype. Integration of the recombinant proteins did not occur or occurred at a very low level with L-complex depleted of the non-cognate ligase or with wild-type L-complex. A C-terminal region of the T. brucei rREL1 protein downstream of the catalytic domain was identified as being involved in integration into the L-complex. The ability to perform functional complementation in vitro provides a powerful tool for molecular dissection of the editing reaction.

Mitochondrial RNA degradosome homologs in *Trypanosoma brucei* have pleiotropic effects on multiple RNA classes

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Posttranscriptional gene regulation is of major importance in the mitochondria of *Trypanosoma brucei*. In *Saccharomyces cerevisiae*, the mitochondrial degradosome functions in multiple aspects of mitochondrial RNA processing and turnover. The yeast degradosome is comprised of two proteins, DSS-1 exoribonuclease and SUV3 RNA helicase. Through database mining, we identified *T. brucei* homologs of these proteins, TbDSS-1 and TbSUV3. To begin to assess their function, we created cell lines that are down-regulated in TbDSS-1 and TbSUV3 by RNA interference (RNAi). Both TbDSS-1 and TbSUV3 RNAi cell lines exhibit a growth defect and TbDSS-1 depletion leads to decreased levels of never edited COI mRNA along with both unedited and edited COIII mRNAs. We also observed a considerable reduction in the level of edited CYb mRNA and a corresponding increase in unedited CYb mRNA. The abundance of both gCYb[560] and gA6[149] guide RNAs is reduced upon TbDSS-1 depletion although the reduction in gCYb[560] is much greater. Similar experiments in S. cerevisiae DSS1 and SUV3 null mutants revealed a large accumulation of mRNA and rRNA precursors. To determine if the decrease in specific mature mRNAs and gRNAs in TbDSS-1 RNAi cells results from impairment of precursor processing, we used cRT-PCR to analyze one of the only known processing precursor RNAs, 12S 5'-flanking region. cRT-PCR and northern blot analysis revealed a dramatic increase in multiple 12S 5'-flanking precursor products upon TbDSS-1 depletion. We are currently characterizing TbSUV3 RNAi cells to determine if they have a phenotype similar to TbDSS-1 RNAi cells. Coexpression of recombinant proteins is also being performed to determine whether TbDSS-1 and TbSUV3 are associated and demonstrate enzymatic activity.

Multiple ribosomal RNP complexes in kinetoplasts of *Leishmania tarentolae*

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We have discovered several ribonucleoprotein complexes containing ribosomal 9S (SSU) and 12S (LSU) RNAs in detergent lysates of kinetoplasts isolated from *L. tarentolae*. The 9S rRNA sedimentation profile denotes an abundant 50S complex and less abundant 20-25S and 66S complexes. The profile of the 12S rRNA denotes a 45S complex and a 65S complex. In addition to these complexes, a 52S complex was detected by peaks of both ribosomal RNAs. Insight into the nature of these complexes was obtained by their partial purification, analysis of their protein composition by tandem mass spectroscopy and cryo-EM. The 9S RNA-containing 50S complex contains at least 43 polypeptides including ten homologs of the small ribosomal subunit proteins from other organisms. The 12S RNA-containing 45S complex contains at least twelve homologs of the large ribosomal subunit proteins, as well as additional polypeptides. The 65S and 66S complexes represent homodimers of the 45S and 50S complexes, respectively. A preliminary cryo-EM investigation of the 52S fraction reveals a two-subunit complex with characteristic ribosomal features. The larger of its subunits is consistent with the parameters of the 45S complex, while the smaller subunit appears to correspond to the 20-25S complexes. The main peak of the poly(U)-directed phenylalanine polymerization activity was found sedimenting in the 52S region. Further characterization of the putative 52S monosomes including identities of its large and small subunits and their relationships to the identified rRNP complexes are in progress.

ABSTRACTS: SESSION VI

Cell and Developmental Cycles

April 15 8:30 a.m.

Chair(s) - C.C. Wang

In vivo activity of *Trypanosoma brucei* PKA-like kinases changes in response to environmental conditions

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It is largely unknown how trypanosomes perceive changes of their environment, particularly changing host environments in the life cycle. Since the abundance of adenylate cyclases suggests an involvement of the cAMP pathway, we have investigated a family of PKA-like kinases. Surprisingly, cAMP does not act as an activator of these kinases but can competitively inhibit under certain conditions. These properties were confirmed using a newly established *in vivo* kinase assay. This assay is based on the immunological detection of the phosphorylated form of the heterologous PKA reporter substrate VASP (mammalian vasidolator stimulated phosphoprotein) that was transgenically expressed in BSF cells. The evidence that VASP phosphorylation represents the activity of the *T. brucei* PKA-like kinases is based on the effects of PDE inhibitors, specific kinase inhibitors and membrane permeable cyclic nucleotide derivatives that reflect the *in vitro* properties. The *in vivo* assay for PKA-like kinases was used to detect activity changes under different environmental conditions. Most interestingly, activity transiently increased upon cold shock treatment, mild acid stress and hypoosmotic stress. These results provide the first hints to an intracellular signaling pathway mediating the environmental information.

A protein implicated in polycystic kidney disease is involved in basal body and flagellar biogenesis in *T. brucei*

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Trypanosoma brucei is a flagellated protozoan with a highly polarised cellular structure. TbLRTP is a trypanosomal protein containing multiple SDS22-class leucine-rich repeats and a coiled-coil domain with high similarity to a mammalian testis-specific protein of unknown function. Homologues are present in a wide range of higher eukaryotes including zebrafish, where the gene product has been implicated in polycystic kidney disease. Western analysis and immunofluorescence with antibodies against recombinant TbLRTP indicate that the protein is expressed throughout the trypanosome life cycle and localises to distal zones of the basal bodies. Overexpression and RNA interference demonstrate that TbLRTP is important for faithful basal body duplication and flagellum biogenesis. Expression of excess TbLRTP suppresses new flagellum assembly whilst reduction of TbLRTP protein levels often results in the biogenesis of additional flagellar axonemes and paraflagellar rods that, most remarkably, are intracellular and fully contained within the cytoplasm. The mutant flagella are devoid of membrane and are often associated with four microtubules in a similar arrangement to that observed in the normal flagellar attachment zone. Aberrant basal body and flagellar biogenesis in TbLRTP mutants also influences cell size and cytokinesis. These findings demonstrate that TbLRTP suppresses basal body replication and subsequent flagellar biogenesis and indicate a critical role for the LRTP family of proteins in the control of the cell cycle. These data further underscore the role of aberrant flagellar biogenesis as a disease mechanism.

Flagellar motility is essential in *Trypanosoma brucei* and is modulated by an evolutionarily-conserved dynein regulatory system

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The flagellum of *Trypanosoma brucei* is a multifunctional organelle with critical roles throughout the trypanosome lifecycle. However, our understanding of flagellum structure and function in these parasites is currently limited. Trypanin (TPN) is a flagellar protein that is required for directional cell motility but its molecular function is unknown. Recently, the trypanin homolog in *Chlamydomonas reinhardtii*, PF2, was demonstrated to be part of a dynein regulatory complex (DRC) that regulates axonemal dynein in response to signals from central pair/radial spoke complexes. In *C. reinhardtii*, the DRC is defined by the ability of DRC mutants to suppress flagellar beat defects of central pair/radial spoke mutants. To determine if an analogous dynein regulatory system operates in *T. brucei*, we used RNAi to knockdown expression of radial spoke (*rsp3*) and central pair (*pf16* and *pf20*) components individually or in combination with trypanin. We find that *rsp3*, *pf16* and *pf20* single mutants are immotile with severely defective flagellar beat patterns. Surprisingly, these mutants ultimately cease cell division, suggesting that flagellar motility is essential in *T. brucei*. Detailed examination of *rsp3* mutants demonstrates they are defective in the progression of cytokinesis. Finally, a direct comparison of *pf16* single mutants and *pf16*/*tpn* double mutants demonstrates that loss of trypanin suppresses *pf16* flagellar beat defects. Recent studies have established that the *T. brucei* flagellar apparatus provides critical positional and directional information for cleavage furrow formation and our data provide the first evidence that flagellar beat plays an active role in cytokinesis. Additionally, our results indicate that the DRC is an evolutionarily-conserved dynein regulatory system and that trypanin functions as part of this system in *T. brucei*.

Flagellum force and elongation drives basal body migration

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Cilia and flagella play essential and multiple functions in many organisms, especially in protists. In order to study flagella functions, we have identified close to 100 *Trypanosoma brucei* flagellar genes and analysed their role by RNA interference. Combining the results obtained by different mutants, we were able to demonstrate the involvement of the motile flagellum in basal body migration. It has been shown previously that the tip of the new flagellum is anchored to the side of the existing, old flagellum by the flagella connector in procyclic trypanosomes (Moreire-Leite et al.,2001, Science 294, 610). Here we show that the new flagellum exerts a pressure on the connector, but since the latter is firmly anchored, this pressure produces a reaction force at the other end of the flagellum that drives basal body migration. Specific inhibition of new flagellum motility prevents basal body migration and also results in new flagellum detachment. Absence of flagella connection leads to formation of a new motile flagellum that is no longer attached to the cell body and is accompanied by failure in basal body migration. Moreover, interfering with motility and adhesion of the new flagellum to the cell body without affecting flagella connection impairs basal body migration. This is a striking situation where the use of physical force and topological organisation of cytokeletal elements allows re-positioning of organelles during the cell cycle.

MOB1 is essential for cytokinesis in *Trypanosoma brucei*

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To divide successfully, a cell must replicate and segregate its organelles and partition them correctly between daughter cells during cytokinesis. In *Trypanosoma brucei*, cytokinesis occurs via the unidirectional ingression of a cleavage furrow along the longitudinal axis of the cell from the anterior to the posterior end, a method that is distinct from the actomyosin ring constriction that is seen in yeast and animal cells. The arrangement of replicated nuclei and kinetoplasts at the beginning of cytokinesis differs between bloodstream and procyclic trypanosomes which is probably how these morphologically distinct cells achieve the required symmetry for successful division. To investigate cytokinesis at the molecular level, functional studies were carried out on the trypanosome MOB1 proteins. MOB1 is evolutionarily conserved and has been shown to be essential for cytokinesis in yeast, as well as being required to exit mitosis in *Saccharomyces cerevisiae*. Two highly similar MOB1 proteins exist in *T. brucei*. Using RNAi, we demonstrate that these proteins are essential for cytokinesis in both bloodstream and procyclic form trypanosomes, but unlike their counterpart in yeast, they are not required for mitotic exit. In the bloodstream form, down-regulation of MOB1 delays cleavage furrow ingression during cytokinesis, and may also have an inhibitory effect on kinetoplast replication. In contrast, in procyclic trypanosomes, MOB1 appears to be primarily required for accurate positioning of the cleavage furrow, rather than for the timing of cytokinesis. This may indicate that *T. brucei* differentially regulates cytokinesis in different life cycle stages. Current work is focussing on identifying the proteins MOB1 interacts with, and establishing their localisation in the trypanosome.

Methylation of histone H3 lysine 76 is important for differentiation, virulence and cell cycle regulation in *T. brucei*

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The amino-terminal tails of core histones are subject to multiple covalent modifications including acetylation, methylation, and phosphorylation. As with acetylation, histone methylation has emerged as an important modification that regulates chromatin structure and gene activity. Recently, a new family of histone methyltransferase was defined by the discovery of DOT1 in a genetic screen for proteins that affect telomeric silencing in yeast. DOT1 is an evolutionarily conserved protein that methylates lysine 79 in the core of histone H3 in humans and yeast. H3 K79 methylation is involved in a variety of nuclear functions, including transcriptional silencing, meiotic checkpoint control, and response to DNA double-strand breaks. We have identified two orthologs of DOT1 in *Trypanosoma brucei*, which we name KMT1 and KMT2. Using modification-specific antibodies, gene deletion and mass spectrometry, we show that KMT1 is responsible for the di-methylation of H3 K76 (the positional equivalent of yeast H3 K79), whereas KMT2 exclusively directs the tri-methylation of the same amino acid. These are the first specific histone modifications described in trypanosomes. *KMT1* appears to be essential. *KMT2*-null bloodstream-form parasites grow normally *in vitro* but are slower to re-adapt to growth *in vivo* and are unable to complete developmental differentiation to procyclic forms *in vitro*. *KMT2*-null procyclic forms, but not bloodstream forms, exhibit defects in cell cycle regulation. Di-methylation of H3 K76 is only detectable during mitosis. To our knowledge, this is the first time that histone methylation has been described as a mitosis-specific marker. We propose that the coordination of KMT1 and KMT2 activities regulates cell cycle progression by restricting H3 K76 di-methylation to mitosis.

Variant Surface Glycoprotein is monitored in the cell cycle of *Trypanosoma brucei*

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Trypanosoma brucei multiplies extracellularly in the bloodstream, relying on antigenic variation of a dense Variant Surface Glycoprotein (VSG) coat to escape antibody-mediated lysis. We investigated the role of VSG in proliferation and pathogenicity by using inducible RNA interference (RNAi) to ablate *VSG* transcript. Induction of *VSG* RNAi resulted in reduction of *VSG* transcript down to 1-2% normal levels. Inhibiting VSG synthesis *in vitro* triggers a rapid and specific cell-cycle checkpoint blocking cell division. Parasites arrest at a discrete precytokinesis stage with two full length flagella and opposing flagellar pockets, without undergoing additional rounds of S-phase and mitosis. A subset of the stalled cells (less than 10%) have internal flagella, indicating that the progenitors of these cells were already committed to cytokinesis when VSG restriction was sensed. Although there was no obvious VSG depletion *in vitro* after 24 hours induction of *VSG* RNAi, there was rapid clearance of these cells *in vivo*. We propose that a stringent block in VSG synthesis produces stalled trypanosomes with a minimally compromised VSG coat, which can be targeted by the immune system. Our data indicate that VSG is monitored during cell-cycle progression in bloodstream form *T. brucei*, and describes the first precise precytokinesis cell-cycle arrest. This checkpoint before cell division provides a link between the protective VSG coat and progression through the cell-cycle, and could function as a novel parasite safety mechanism preventing extensive dilution of the protective VSG coat in the absence of VSG synthesis.

ABSTRACTS: POSTER SESSIONS

A SERIES: April 13 1:00 p.m.

B SERIES: April 13 3:30 p.m.

C SERIES: April 14 3:30 p.m.

Forward genetics identifies genes associated with procyclin glycosylation and GPI anchor modification in *T. brucei*

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The *Trypanosoma brucei* procyclic form expresses both procyclin and free GPIs on its surface. Procyclin, the major procyclic surface molecule, is a GPI-anchored glycoprotein made up almost entirely of amino acid repeats. There are two major forms: EP procyclin, which contains 18-30 Glu-Pro (EP) repeats, and GPEET procyclin, which has five to six Gly-Pro-Glu-Glu-Thr (GPEET) repeats. Some EP-procyclin isoforms can be glycosylated at the N-terminus by a $Man_5GlcNAc_2$ glycan. The GPI anchor of procyclin and the less abundant cell surface free GPIs, are also modified by a heterogeneous side chain of branched poly-*N*-acetyllactosamine (pNAL) repeats. This NAL side chain serves as a sialic acid acceptor for the cell surface trans-sialidase, and previous work suggests that sialylation may be essential for the parasite to survive in the tsetse fly. It is generally thought that both free GPIs and procyclin glycans play a role in parasite protection in the tsetse midgut. In this work, forward genetics was used to identify genes associated with procyclin *N*-glycosylation and GPI-anchor modification. Two random mutant pools, generated by an RNAi library and a *Mos1 Mariner* transposon system, were screened with several trypanocidal, procyclin-binding lectins. Several ConA- and potato lectin-resistant mutants were obtained. Work is in progress to identify the genes affected and to characterise the glycosylation defects.

Every gene in *Leishmania* is subject to strong regulation during metacyclogenesis

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Metacyclogenesis is a key step in the *Leishmania major* infectious cycle. We developed long (70-mer) oligonucleotide-based microarrays to assess changes in transcript abundance during this developmental transition. Of 5702 ORFs present on our arrays, 375 (6.6%) were differentially expressed, based on 3 biological x 12 technical replicas and combined statistical and 2-fold change criteria. This estimate exceeds those using shotgun or EST clone arrays, suggesting that oligonucleotide arrays have broader utility. We were surprised to find that the yield of total RNA declined ~10-fold in purified PNA- metacyclics relative to logarithmically growing procyclic promastigotes. This was accompanied by a comparable change in cell volume, and thus the ratio of RNA/cell volume remains constant during development. Nuclear run-on analysis showed the decline in RNA arose from decreased transcriptional rates across all polymerase classes. EM analysis suggests that this is accompanied by changes in chromatin structure. Notably, typical array or Northern blot estimates compare RNAs normalized to the total mass of RNA. If as in other organisms one defines differential gene regulation based on the levels of RNA/gene, our data imply that ~94% of *Leishmania* genes undergo 10-fold transcriptional down-regulation during development. Superimposed on this background, only a small proportion of genes (~6%) are subject to other regulatory mechanisms, which thereby lead to differential abundance. That metacyclic *Leishmania* differ morphologically from procyclic promastigotes is well known but the consequences of the extreme decline in cellular volume to models of gene regulation has not been fully appreciated. One consequence is that selective escape from global down-

regulation must now be considered as a potential regulatory mechanism, in addition to RNA stability and translation.

Analysis of the role of trypanin in the motility of *Trypanosoma brucei*

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African trypanosomes are digenetic, having both a mammalian host and a tsetse fly insect vector, and motility is thought to be important in both stages. However, there is very little information about proteins that contribute to motility. Trypanin (TPN) is a 54-kDa protein component of the *Trypanosoma brucei* flagellar cytoskeleton required for regulating flagellar beat, thus, TPN may be part of the T. brucei dynein regulatory complex (DRC). We are examining TPN for possible characteristics of a DRC protein, e.g. phosphorylation and interaction with the radial spoke, as well as identifying other TPN-interacting proteins. In 2D westerns of trypanosome flagellar cytoskeletons TPN migrates as a series of spots more acidic than its predicted pI, indicative of phosphorylation. Interaction between TPN and the radial spoke is currently being tested by directed yeast two-hybrid assays and suppressor mutant analysis. To identify other TPN-interacting proteins, 2D-PAGE of wild type and TPN(-) extracts, immunoblotting, yeast two-hybrid, and cross-linking are employed. Though successful in *C. reinhardtii*, 2D analysis of TPN(-) profiles revealed no clear differences relative to wild-type. Immunoblotting of four known flagellar cytoskeleton components shows that their association with the flagellar cytoskeleton does not require TPN. Interestingly, yeast two-hybrid screening has yielded eight novel genes, one of which is a predicted Ser/Thr phosphatase. This is consistent with our DRC hypothesis, as phosphorlyation is thought to control DRC activity. EDC treatment results in specific cross-linking of TPN into $\sim$100kDa, $\sim$110kDa, $\sim$130kDa, and $>$250kDa complexes, which is consistent with TPN interacting with other proposed DRC proteins.

A library of RNAi mutants to study the multiple functions of flagella

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Cilia and flagella are present in most eukaryotic organisms and their structure is well conserved throughout evolution. We have developed a flagellar gene database after genomic comparaison of organisms with or without flagella. The role of these novel genes is being analysed by inducible RNA interference in Trypanosoma brucei. First, we have identified several candidate genes involved in IntraFlagellar Transport (IFT), a process conserved in ciliated organisms that is required for assembly and maintenance of the organelle. We have demonstrated the involvement of these genes in IFT, including novel genes so far of unknown function. After induction of RNAi silencing, cells first exhibit a shorter flagellum, demonstrating the importance of IFT for flagellum length control. Second, we have identified genes coding for proteins involved in trypanosome motility. Third, another function of flagella is their involvement in basal body migration. It has been shown that the tip of the new flagellum is anchored to the old flagellum by a specific structure called the flagella connector. RNAi silencing of a protein localized in the basal body leads to defects in flagella connexion, detached flagella and inhibition of basal body segregation. Detached flagella and poor basal body migration are also observed in mutant cells with reduced flagellum motility. In conclusion, such a library of mutants provides a great tool for analysis of flagellum functions in general.

Reduced expression of flagellar PKA-like kinase leads to a cell cycle dependent motility phenotype in *Trypanosoma brucei*

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The PKA-like kinase of *T. brucei* is preferentially located within the flagellum in all life cycle stages. High resolution fluorescence microscopy, colocalisation analysis, and electron microscopy revealed that the regulatory subunit (PKAR) was associated with an axoneme proximal substructure of the paraflagellar rod (PFR). The absence of colocalisation with the minor ROD1 antigen of the PFR was supplemented by the finding that PKAR remained within the flagellum after ablation of the major component PFR-A by RNAi. This shows for the first time that defined substructures of the trypanosoma PFR can be involved in anchoring/tethering of signal transduction proteins. RNAi-mediated ablation of PKA-like kinase subunits caused severe impairment of cytokinesis and lethality. In contrast, stable expression of a reduced level of the R subunit, achieved by gene replacement with an N-terminally truncated mutant, led to a motility phenotype with a higher fraction of tumbling versus swimming cells. The consistent phenotype of independent mutants was fully rescued by ectopic wild type level expression of the R subunit. The tumbling subpopulation was enriched in cells with a dividing kinetoplast. Selective analysis of cells in the respective cell cycle window confirmed a high fraction of tumbling cells. Quantitative assessment of the parallelity of the old and new flagellum in this cell cycle window revealed a less ordered pattern in the mutants. We suggest that PKA-like kinase is important for synchronisation of the beat phase of the emerging daughter flagellum

Trypanosoma congolense procyclin: unmasking a cryptic surface glycoprotein in procyclic forms

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Trypanosoma congolense procyclic (insect midgut) forms are covered with a dense layer of glycosylphosphatidylinositol (GPI)-anchored molecules that includes a previously characterized glutamic acid and alanine-rich protein, GARP, and a protease-resistant surface molecule, PRS. However, proteins consisting of internal peptide repeats, corresponding to the well known EP and GPEET procyclins in *T. brucei* procyclic forms, have not been demonstrated in *T. congolense*. We now partially purified a 58 kDa GPI-anchored protein from *T. congolense* Kilifi procyclic forms and obtained an N-terminal amino acid sequence that was used to generate oligonucleotides to screen a corresponding cDNA library. This led to the identification of a gene encoding a protein of 163 amino acids with a putative N-terminal signal sequence and a C-terminal GPI addition site. The mature protein consists of a repetitive sequence of 13 heptapeptides (EPGENGT) and is heavily *N*-glycosylated. Similar proteins were subsequently identified in *T. congolense* Savannah procyclic forms and they were named *T. congolense* procyclins to reflect their relationship to the *T. brucei* EP and GPEET procyclins. Using an antiserum raised against the repeat unit, we identified the proteins on *T. congolense* procyclic forms in culture and in the tsetse fly and found that, in contrast to PRS and GARP, which are only transiently expressed during midgut infection, the *T. congolense* procyclins are present at all stages in the fly midgut.

Investigation of the RNA-associated proteome of *Trypanosoma cruzi*

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Recent genome sequencing and annotation of *Trypanosoma cruzi*, *Trypanosoma brucei* and *Leishmania major*, along with whole-organism proteome analysis of the 4 life cycle stages of *T. cruzi*, has paved the way for better understanding of basic kinetoplastid parasite biology. One interesting facet of kinetoplastid biology is that regulation of gene expression is almost exclusively post-transcriptional, but the basic components and interactions of the regulation machinery largely remain a mystery. We have used two approaches to identify transcript-associated proteins which may be part of the regulatory machinery of *T. cruzi*: motif searching of annotated genes and experimental identification of proteins associated with transcripts. The most common RNA-binding domain, the RNA recognition motif (RRM), is present in approximately 70 non-redundant proteins in *T. cruzi*. Nearly all of these proteins have orthologues in both *T. brucei* and *L. major*, but two appear to be unique to *T. cruzi*. Genome annotations are inherently limited to the identification of functional domains that have been previously characterized. In order to identify RNA-associated proteins in *T. cruzi* without any bias toward known RNA-binding domains or expected protein-protein interactions, we have utilized analysis of transcript-associated proteins by MS/MS. Samples enriched for mRNA-associated proteins with oligo(dT) conjugated magnetic beads contained expected proteins including polyA-binding protein, mitochondrial RNA-binding protein, and a variety of ribosomal proteins. To date, we have also identified 5 additional proteins not previously detected in our whole-organism proteome analysis. None of these proteins were present in RNA-free control samples. A comprehensive analysis of the RNA-associated proteins of the epimastigote, trypomastigote, and amastigote life-cycle stages of *T. cruzi* is on-going.

A mitogen-activated protein kinase controls proliferation and differentiation of bloodstream forms of *Trypanosoma brucei*

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African trypanosomes undergo complex developmental differentiation steps to adapt to different environments in the mammalian host and tsetse fly vector. In other eukaryotic organisms these processes are often mediated by mitogen-activated protein kinases (MAPK). To characterise the biological role of *Tb*MAPK3, a MAPK homologue in *T. brucei*, we constructed a knockout in procyclic forms. The knockout proliferated normally in culture and could be transmitted by tsetse, indicating that *Tb*MAPK3 is not essential for other life cycle stages in the fly. It was also able to infect immunosuppressed mice, but the peak parasitaemia was 16-fold lower than that of the wild type. The difference in parasite density between the wild type and the knockout decreased in immunocompetent mice, owing to an 10-fold reduction in the peak parasitaemia of the wild type. In contrast, the host immune response did not appear to play a significant role for the knockout. Differentiation of the long slender to the non-proliferating short stumpy bloodstream form is triggered by accumulation of stumpy induction factor(s) (SIF) released by the parasite. Diaphorase assays revealed that the knockout had undergone premature differentiation at a much lower density, suggestive of increased sensitivity to SIF. The differentiation phenotype was partially rescued by complementation of the knockout with wild type *Tb*MAPK3, but exacerbated by introduction of a non-phosphorylatable mutant form, suggesting that the latter exerts a dominant negative effect on other kinases. Accelerated differentiation and growth arrest of the knockout were also apparent in culture. Our results indicate a regulatory function for *Tb*MAPK3 in the differentiation of bloodstream form trypanosomes, possibly by influencing the production of SIF or the response to it.

Characterization of a *Trypanosoma brucei* histone acetyltransferase that may be involved in RNA interference

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In a genome-wide screen to identify genes involved in RNA interference (RNAi) in the protozoan parasite, *Trypanosoma brucei*, we have identified a putative histone acetyltransferase (TbHAT) that, when silenced, enhances RNAi. TbHAT contains a conserved acetyltransferase domain and shares greatest homology with the HAT elongator-like protein 3. To explore the activity of the protein, we have expressed recombinant TbHAT (rTbHAT) in *E. coli*, and the partially purified protein has demonstrated histone acetyltransferase activity on calf thymus histones. To initiate characterization of TbHAT activity in *T. brucei*, we have expressed TbHAT as a fusion protein to C-terminal GFP (TbHAT-GFP). This fusion localizes to the nucleus, suggesting that TbHAT may function to modify histones *in vivo*. We propose that TbHAT may prevent RNAi-dependent chromatin silencing in two ways - first by blocking methylation of histones by acetylation, and second by preventing RNAi-dependent recruitment of histone methyltransferases by inhibition of RNAi.

Differential import of precursor proteins into *Trypanosoma brucei* mitochondria

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During its digenetic life cycle *T. brucei* modifies their mitochondrial activities dramatically to adopt in two different host systems. Like in other eukaryotes, 90% of mitochondrial proteins in *T. brucei* are nuclear encoded and need to be imported after their synthesis on the cytosolic ribosomes. Thus, mitochondrial protein import is an essential step for mitochondrial function and is possibly involved in controlling mitochondrial activities. *T. brucei* mitochondrial precursor proteins possess two different types of presequences. One is shorter in length, about 8-9 amino acid long, and is similar to the presequences found in hydrogenosomal precursor proteins. The other is longer, about 15-35 or more amino acids and resembles the presequences usually found in fungi, and other higher eukaryotes. Here, we compare the import characteristics of two such proteins. One is polymerase beta (Pol beta), with a presequence of 9 amino acids, and the other is trypanosome alternative oxidase (TAO), which has a predicted presequence of 22 to 23 amino acids. We have found that import of both proteins required membrane potential, an ATP generating system and proteinaceous material on the outer surface of the mitochondria. However, Pol beta was imported at a much faster rate than TAO. Import maxima occur within 5-10 min for Pol beta, in contrast to 20-25 min for TAO. Moreover, we have found that TAO precursor protein is cleaved at different positions in bloodstream and procyclic mitochondria under *in vitro* conditions.

Functional analysis of a type II topoisomerase from *Crithidia fasciculata*, a potential drug target

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Parasite infection is a severe threat to public health in many developing countries due to the increasing drug resistance of some parasite strains to classical drugs, and co-infection of Leishmania with HIV. Recently, parasite topoisomerases has been a growing focus of interest, particularly as target of chemotherapy. CfTOP2 is a type II topoisomerase that plays a key role in the replication of the unusual mitochondrial DNA (kinetoplast DNA) of protozoan parasite Crithidia fasciculata. We have purified the recombinant CfTOP2, and shown that a classic anti-cancer drug, m-AMSA, and some traditional antitrypanosomal drugs including ethidium bromide, pentamidine, and berenil inhibit Cf TOP2's decatenation activity of kDNA network in vitro. However, pentamidine does not inhibit E. coli Topo IV's decatenation activity. Among these drugs only m-AMSA seems to act as a topo II poison, and the rest act by affecting the Cf TOP2's interaction with the DNA substrate. We also tested and found four newly synthesized dicationic compounds can inhibit Cf TOP2's catalytic activity. The results indicate the recombinant topoisomerase from trypanosome (CfTOP2) can be used to screen current and newly synthesized topoisomerases inhibitors or poisons, and served as the target for much-needed new anti-parasitic chemotherapy.

New insights into the characterization of the *Trypanozoon* subgenus

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African trypanosomes are unicellular eukaryotic parasites which cause sleeping sickness in human and various diseases in cattle. We intend to differentiate the (sub)species of the *Trypanozoon* subgenus by means of DNA microarrays, which allows to query gene expression levels of whole genomes in a single experiment. To investigate the expression pattern we used a *Trypanosoma brucei* microarray containing 24,567 different PCR products spotted on glass slides. The arrayed fragments were generated from a shotgun clone library, which had been prepared from randomly sheared and size fractionated genomic DNA. Labeled cDNA was generated from blood stream forms *T. b. brucei*, *T. b. gambiense* types I and II, *T. b. rhodesiense*, *T. evansi* and *T. equiperdum* total RNA, and subsequently spotted on the chip to examine the parasites' respective transciptomes. Results shows that the *T. brucei* array can be used for the analysis of other closely related species. Correspondance analysis from the obtained expression profiles show that (i) *T. b. gambiense* type I and *T. b. rhodesiense* are closely related to the *T. b. brucei* control , (ii) *T. b. gambiense* type II behaves as a hybrid between *T. b. gambiense* type I and *T. b. rhodesiense*, (iii) *T. equiperdum* is more closely related to *T. b. brucei* than to *T. evansi*. Annotation and identification of the differentially hybridising genes is in progress. Subsequently we will verify selected results by Northern blot or real-time PCR, to check whether results are due to differences in the genome or in gene expression. The results obtained will lead to a better insight into the differences in gene expression and metabolism between the different taxa.

Characterization of PBP-2 in *Trypanosoma brucei* SL RNA transcription

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Transcriptional processes in trypanosomatids appear to be unique among eukarya, as each messenger RNA is produced through a trans-splicing reaction of polycistronic precursors with a capped spliced leader RNA (SL RNA). In contrast to the long primary mRNA transcripts that form mature mRNA, each SL RNA gene has a discrete transcriptional start site. Transcriptional analysis of the SL RNA promoter in *Leptomonas seymouri* revealed a tripartite structure that requires two DNA-binding proteins, PBP-1 and PBP-2. These are the first sequence-specific DNA binding proteins discovered by our lab that function to promote SL RNA transcription. Amino acid sequence of PBP-2 revealed that it is a member of the DEAD/H box family of DNA/RNA helicases. We have identified the *Trypanosoma brucei* homologue of PBP-2 and show that this protein contains all of the motifs needed for helicase activity. Furthermore, we have generated a TAP-tagged PBP-2 cell line in *T. brucei* procyclic parasites. Purified PBP-2 protein exhibits ATPase activity in an in vitro TLC-based ATPase assay. All DEAD/H box helicase proteins require ATPase activity for nucleic acid unwinding. Therefore, we have begun to characterize the functional enzymatic properties of PBP-2 as a helicase that is involved in SL RNA transcription.

Characterization of a putative immunomodulatory factor from *Trypanosoma brucei*

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African trypanosomes are extracellular parasites that live in the bloodstream of their hosts. They evade the immune response by two major mechanisms, antigenic variation and immunomodulation. Regarding the second mechanism, they release factors acting on cells of the immune system including macrophages. A fraction released by *Trypanosoma brucei* in the culture medium was observed to trigger alternative activation of macrophages. This is characterized by induction of arginase activity as opposed to the classical activation pathway monitored by NO production. Arginase activation antagonizes NO production thereby creating a favourable environment for the parasite development. A candidate active component of this fraction released by *T. brucei* was identified as a kinesin heavy chain. The expression of the gene coding for this trypanosome kinesin heavy chain (TbKHC) was knocked down by conditional RNAi. In vitro, the knocked-down trypanosomes are not able to activate arginase in mouse peritoneal macrophages supporting TbKHC as the active factor in alternative activation of macrophages.

Induction of differentiation in kinetoplastids by thiazolidindiones

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Thiazolidindiones are synthetic agonists for PPARg which has been shown to function as a transcription factor in higher eukaryotes. By activation of this receptor, they are able to increase lipid and glucose uptake, to enforce metabolism and to induce differentiation [Willson *et al.* 2001; Hihi *et al.* 2002]. The latter also occurs in kinetoplastids like *Trypanosoma* and *Leishmania*. The bloodstream form of *T. brucei* undergoes a differentiation of the dividing long slender into the non-dividing short stumpy form within its mammalian host. Incubation with the thiazolidindiones troglitazone, rosiglitazone and ciglitazone led to a dose-dependent inhibition of growth. This is obviously due to the thiazolidindione-moiety of these substances, as a-tocopherol, which shares the other residue with troglitazone, did not have any effect up to higher μM-concentrations. Additionally, we have also found changes in morphology, glucose metabolism and mitochondrial membrane potential, as well as an elevated transformation rate from the bloodstream into the the procyclic form of treated cells compared to control cells. Likewise, *L. major* promastigotes differentiate within the midgut of the sandfly into the form infective to mammals. In this case, after troglitazone treatment we observed a longer but more slender cell body and an increase of the flagellum length, accompanied by a decrease of PNA-agglutination. All these are indicators of metacyclogenesis [Sacks *et al.* 1985; Zakai *et al.* 1998]. These results show that thiazolidindiones are able to induce differentiation of both *Trypanosoma brucei* and *Leishmania major* and suggest the existence of a target protein with similar properties to PPARg, which is still to be isolated and characterized.

RNA polymerase I and II in *Trypanosoma brucei*: two distinct Rpb5 subunits, each one specific to each polymerase

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The RNA polymerase I (Pol I) of *T.brucei* is involved in the transcription of rRNA but also in the transcription of genes coding for surface antigens (VSG in the bloodstream form and procyclin in the procyclic form). Despite the use of this polymerase, transcripts are spliced and polyadenylated exactly as mRNAs synthesised by a RNA polymerase II (Pol II). In order to explore a possible link between these two transcriptional machineries, we purified the polymerase II complex and we compared it with the polymerase I complex in procyclic forms. Our results suggest that the overal composition of these polymerases is conserved when compared to those of other eukaryotic cells. By contrast, we found two different Rpb5-like subunits. We named these two subunits TbRpb5.1 and TbRpb5.2 in function of their association to the Pol I or Pol II complexes. We show that the down regulation of TbRpb5.1 by RNAi does not affect the transcription of genes transcribed by Pol II. In contrast, the down regulation of TbRpb5.2 by RNAi led to a strong transcriptional inhibition of these same genes.

Gene expression profiling detects synergistic pattern of human macrophage responses following *Leishmania* infection

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Infection with L. major triggers several pathways in the host cell that are crucial to elementary infection as well as those that are used by Leishmania to enhance its replication and disease. IFN-γ activates macrophage by driving JAK/STAT dependent induction of transcription which is unresponsive in L. major infected host cells. To identify the genetic mechanism of pathogenesis of Leishmania, we studied the synergistic effect of gene expression on human monocytic cell line THP1 cells exposed to IFN-γ and/ or L. major infection using high density human oligonucleotide microarray. Among the 21000 target genes, 1272 statistically significant (p¡0.05) genes were regulated by L. major infection. A set of genes regulated individually by IFN-γ and L. major overlapped showed synergistic effect on host cells. The analysis identified transcripts for proinflammatory leukocyte chemoattractants, adhesive molecules and complement that were upregulated and may account for the inflammatory pathology that is hallmark of Leishmania infection. Interestingly, alterations in the transcription of several genes in the ubiquitin and endosomal protein trafficking pathways in survival and multiplication within the host. The persistence of high L. major loads despite an extensive IFN-γ response suggest that L. major can resist IFN-γ treatment. In conclusion, these data suggest that L. major can alter macrophage pathways and simultaneously inhibit apoptosis and innate immune mechanisms within macrophage, permitting intracellular survival of the parasite. The transcriptional suppression of genes regulating innate immunity may allow L. major to evade host responses and establish infection and may reduce innate host defense against. L. major infection will provide a foundation for understanding the infection process of L. major.

Consequences of telomere shortening in *Trypanosoma brucei*

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Telomere maintenance plays a critical role in human ageing and cancer. Telomere-associated proteins are involved in transcriptional silencing of subtelomeric regions. Due to the end-replication problem, telomeres shorten at each replicative cycle. Telomerase is a specialized ribonucleoprotein that can extend and maintain telomere length. Telomerase consists of a reverse transcriptase (TERT) and an RNA component, which provides a template to add hexameric repeats to the terminal single-strand G-overhang. *T. brucei* evades the immune response by frequently changing its surface coat, which consists of a homogeneous layer of variant surface glycoproteins (VSG). Silent *VSG* genes are dispersed around the genome, and many are located immediately upstream of the telomeric repeats, on the megabase, intermediate and mini chromosomes. The single actively transcribed *VSG* is located in one of ∼20 similarly organized sub-telomeric expression sites (ES) on intermediate and megabase chromosomes. Identification and deletion of TERT allowed us, for the first time, to manipulate telomere length in *T. brucei* and study its consequences. Telomerase deficiency leads to a 3-6 bp/generation progressive shortening of the telomere tract. In many organisms, the rate of telomere decline correlates with the length of the G-overhang. In *T. brucei*, G-overhangs are undetectable (¡30 nt) by in-gel hybridization. The rate of telomere erosion therefore correlates with the shortening due to the end replication problem, and is consistent with our observation that G-overhangs are short. The average telomere length in the *T. brucei* Lister 427 strain is ∼15kB. After extensive in vitro culturing, telomeres have now dramatically shortened and we are studying the consequences of critically short telomeres on transcriptionally active and silent expression sites.

In vitro selection for human serum resistance in *Trypanosoma brucei brucei*

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The animal pathogen *Trypanosoma brucei brucei* is a close relative to the human pathogen *Trypanosoma brucei rhodesiense* that causes African sleeping sickness. *T. b. brucei* is lysed by a human high-density lipoprotein (HDL) particle termed the trypanosome lytic factor (TLF). However, *T. b. rhodesiense* is resistant and can thus infect humans. Human serum resistance in *T. b. rhodesiense* is conferred by the expression of a serum resistance associated gene (SRA). We have experimentally selected human serum resistant *T. b. brucei* in order to understand the evolutionary relationship between the human and animal pathogens. We present here the first *in vitro* study, yielding human serum resistant *T. b. brucei*. After repeated exposure to increasing concentrations of human lytic HDLs, we obtained a stable population that exhibits 160-fold increased serum resistance than the parental population. Serial Analysis of Gene Expression (SAGE), protein analysis and immunofluorescence microscopy showed that the only change associated with selection for resistance was the expressed variant surface glycoprotein (VSG). The parental 221 VSG is lost during selection with human HDLs and a new VSG (800) is moved into the VSG 221 expression site by gene conversion. Experiments to directly test whether the VSG 800 confers human serum resistance are underway.

Control of *Aut 1* gene expression by natural RNAi in *T.b.gambiense* ?

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T.b.rhodesiense and *T.b.gambiense* are infectious to humans due to the resistance of these parasites to lysis by normal human serum (NHS). The resistance of *T.b.rhodesiense* has been shown to be due to a unique truncated VSG gene *SRA*. In *T.b.gambiense*, we have identified a single analogue of *SRA*, named *TgsGP*. This gene is specific to *T.b.gambiense*. The telomeric locus containing *TgsGP* was the result of a translocation event interrupting a gene homologous to the *S.cerevisiae Aut 1* gene. This DNA rearrangement is also a conserved characteristic of *T.b.gambiense*. In other eukaryotes, *Aut I* is a gene essential for autophagocytosis, a process by which cells recover essential nutrients in response to starvation by triggering the formation of vesicles that engulf cytoplasmic proteins and delivers them to the lysosome. Even though *T. b. gambiense* still retains a wild type allele of *Aut 1 (aut1/Aut1)* , this gene is neither expressed at the protein or at the mRNA level. We are currently characterizing the mechanism by which this inhibition occurs based on the working hypothesis of a natural RNAi effect. By Northern Blot analysis using probes directed against regions of the targeted *Aut 1* gene we were able to detect a small RNA of approximately 50nt. We are presently attempting to isolate this miRNA which could potentially target the degradation of *Aut 1* in *T.b.gambiense*. To demonstrate that the inhibition of *Aut 1* gene expression is indeed due a natural RNAi mechanism, we intend to restore *Aut 1* expression in a *T.b.gambiense* strain where the RNAi pathway has been annihilated.

Characterisation of GlcNAcPI-de-N-Acetylase, a validated drug target for African sleeping sickness

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A conditional-null mutant of the second step of the glycosylphosphatidylinositol pathway (GlcNAcPI de-N-acetylase) has shown that this enzyme is essential for the bloodstream form of Trypanosoma brucei [1]. However, the structure and mechanism of the GlcNAcPI-de-N-acetylase are unknown, precluding rational drug design. We present recent results of the characterisation of the parasite and mammalian GlcNAcPI-de-N-acetylase enzymes. The recently reported structure of the de-N-acetylase of mycothiol biosynthesis, MshB, revealed an unusual Zn2+ binding motif at the active site which, despite low overall homology (¡10% identity), is conserved in the GlcNAcPI-de-N-acetlyases [2]. We have recently demonstrated that GlcNAcPI-de-N-acetylase is inhibited by M2+ chelating agents in the trypanosome cell-free system. We also examined the M2+ dependence of the rat de-N-acetylase with a new mass spectrometry-based assay that uses a truncated soluble recombinant enzyme and a synthetic GlcNAc-PI substrate analogue. The results strongly suggest that the GlcNAcPI-de-N-acetylases are Zn2+-dependent amidases. Finally, we have produced a model of the GlcNAcPI-de-N-acetylase active site and identified active site resides by site-directed mutagenesis. The latter experiments involved a semi-quantitative FACS assay of GlcNAcPI-de-N-acetylase activity using alanine mutants of the rat and trypanosome enzymes fused to GFP and transiently transfected into a CHO cell reporter system. Further structural studies are ongoing. We acknowledge the financial support from Wellcome Trust Programme Grant 071463. [1] Chang, T.; Milne, K. G.; Guther, M. L. S.; Smith, T. K.; Ferguson, M. A. J. J. Biol. Chem. 2002, 277, 50176-50182. [2] McCarthy, A. A.; Paterson, N. A.; Knijff, R.; Baker, E. N. J. Mol. Biol. 2004, 335, 1131-1141.

Induction of programmed cell death in the bloodstream form of *Trypanosoma brucei* by PGD_2 and its metabolites

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Trypanosoma brucei is the causal agent of African sleeping sickness which is characterized by an up-regulation of prostaglandin production in serum and cerebrospinal fluid, especially the PGD_2 concentration is selectively and markedly elevated in these patients (Pentreath et al., 1990). We have already demonstrated that African trypanosomes, as well as mammals, produce PGF_{2a}, PGE_2, and PGD_2 (Kubata et al., 2000). Recently, we reported that this latter prostanoid induces an apoptosis-like programmed cell death [PCD] in the bloodstream form of *Trypanosoma brucei* (Figarella et al., 2005). Since this compound is very unstable in media containing albumin we were prompted to investigate if the PGD_2 metabolites could also induce PCD in this form of the parasite. Only the metabolites of the J series, PGJ_2 and delta12PGJ_2, led to a significant inhibition of thymidine-incorporation, a cell cycle arrest in the G1 phase and were able to induce PCD more rapidly and efficiently than PGD_2, as demonstrated by TUNEL test and FACS analysis. However, the use of a stable PGD_2 analog [17-phenyl trinor PGD_2] led to the same phenotype as the natural PGD_2, indicating that PGD_2 it self induces PCD. Interestingly, stumpy cells were more sensitive to prostaglandins than slender ones, which suggest that the effect is specific for the stumpy form. In conclusion, we propose that PGD_2 production by *Trypanosoma brucei* bloodstream form and formation of its metabolites, PGJ_2 and delta12PGJ_2, are involved in cell cycle regulation and PCD induction, as part of the population regulation mechanism of the parasite in the host.

50-bp repeats are not required to maintain the active status of a VSG Expression Site in *Trypanosoma brucei*

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Trypanosoma brucei expresses *VSG* genes from one of ~20 bloodstream Expression Sites (ES). An ES is delimited by telomeric repeats at the distal end and by 10-50 kb arrays of 50-bp repeats upstream of the promoter. Some ES contain two tandem promoters. We wish to determine the role of the 50-bp repeats in regulating ES *in situ* switching. To be able to study trypanosomes that undergo an ES switch, we inserted a Puromycin-resistance gene (*PUR*) immediately downstream of the active ES promoter (221-ES), and a Neomycin-resistance gene (*NEO*) immediately downstream of the promoters of random inactive ESs. Restriction analysis and PFGE identified clones containing 15 independent *NEO* insertions, at different inactive ES promoters. Surprisingly, 6 distinct *NEO* insertions mapped to ESs on Intermediate Chromosomes (IC). *In situ* switchers were obtained *in vitro* from two of these *PUR-NEO* reporter clones (17.13 and 17.21) at a frequency of 10^{-5}-10^{-6}. Switching was reversible, but at a 10-fold lower frequency. *NEO* was also targeted to random inactive ESs in a clone in which the 50-bp repeats upstream of the *PUR*-tagged 221-ES had been deleted. Analysis of one such clone showed no change in the growth rate *in vitro* or virulence in mice. FACS analysis of a population of cells grown without drug pressure showed that the expression of VSG221 remained high for at least 2 weeks, suggesting that the 50-bp repeat array is not required for the maintenance of the active state of the ES. The consequences of 50-bp repeat deletion on ES switching frequency are being addressed.

Proteomic analysis of antimony resistance in *Leishmania infantum*: increased coverage by prefractionation

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Proteomics contained powerful and recognised techniques that enable the analysis of differential expression of proteins. However, the large dynamic range of complex biological samples is a challenge to proteomic research. To achieve a better proteome representation with a reasonable coverage, the sample needs to be fractionated prior to separation by 2DE. We developed a fractionation protocol based on the serial extraction of protein by step-wise increase concentration of digitonin. The technique is reproducible, fast, simple and efficient. Using this technique, we obtained four fractions (F1 to F4). F1 and F2 contained cytosolic proteins and we identified 28 proteins and 7 hypothetical proteins present in F2. F3 contained 16 proteins corresponding to mitochondrial, glycosomal and nuclear proteins. In addition, we identified 8 hypothetical proteins in this fraction and leader peptides could be detected by sequence softwares. The protein complement in F4 is being investigated. The fractionation technique was also employed to perform a comparative 2D analysis of *L. infantum* sensitive and antimony resistant mutants. The differential expression of some antimony resistance associated proteins was confirmed, particularly for proteins involved in cysteine and thiol biosynthesis. In addition, proteins that were not previously found to be associated with antimony resistance, including one hypothetical protein, were discovered. Their role in resistance is curently being study by gene transfection.

Inhibiting serine palmitoyltransferase causes cell-cycle and vesicular trafficking defects and lipid raft disruption

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Lipid rafts are transient areas of increased membrane order in cell membranes enriched in sphingolipids and 3-beta-hydroxysterols. The flagellar membrane of *Trypanosoma brucei* is enriched in lipid rafts. Serine palmitoyltransferase (SPT) catalyzes the joining of L-serine with palmitoyl-CoA into 3-ketodehydrosphingosine, the first committed step in sphingolipid biosynthesis. In yeast, SPT is essential for cell growth, survival, and the delivery of raft-associated proteins to the cell membrane; however, in *Leishmania*, SPT is not required for survival or raft formation. We examined the effects of sphingolipid reduction in T. brucei by using inducible RNAi to knock down SPT2 expression in procyclic cells. RNAi induction resulted in SPT2 RNA degradation within 24 hours. Cell division was greatly reduced by 72 hours, and a variety of abnormal cell types were produced, characterized by the loss of normal cell shape, polyploid DNA content (up to 8n8k) and multiple flagella. In a number of cells, two flagella were observed to emerge from a single flagellar pocket. Many cells undergoing division failed to complete cytokinesis, with daughter cells remaining connected via cytoplasmic tethers. A variety of abnormal lipid inclusions and multilamellar structures were present in most cells. These results suggest that normal synthesis and distribution of sphingolipids are essential to appropriate vesicular trafficking, as well as normal cellular morphogenesis and cell cycle progression. SPT RNAi and myriocin treatment disrupt detergent-resistant membranes, suggesting that in *T. brucei*, unlike *Leishmania*, sphingolipids are vital for lipid raft formation. Current studies are focused on confirming the loss of sphingolipid biosynthesis in RNAi lines, further characterizing the secretory trafficking defects, and determining the role of SPT2 in bloodform stages and cell differentiation.

Moving cells and finding lost structures: the flagellum of endosymbiont-bearing kinetoplastids

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Cilia and flagella are central to many biological processes in diverse organisms. The main component of cilia/flagella is the 9+2 axoneme - a structure which formed very early in eukaryotic evolution. Kinetoplastids are very appealing models for the study of flagellar function, particularly given the availability of extensive genome information. In many organisms, the axoneme is augmented by extra-axonemal structures - the fibrous sheath in mammalian spermatozoa, R-fibre in dinoflagellates, and the paraflagellar rod (PFR) in Euglenozoa. The best characterized of these is the kinetoplastid PFR which is composed of two major proteins, PFR1 and PFR2, and several minor ones, most of which are uncharacterised. The PFR is necessary for full motility in the kinetoplastids and provides support for metabolic regulators that may influence flagellar beating. However, there is an intriguing puzzle: one clade of endosymbiont-containing trypanosomatids apparently lacks a PFR yet are as motile as other species that possess a PFR. We investigated how these organisms locomote and whether the apparent absence of the PFR reflects a loss of the PFR genes within their genomes. We have identified a single-copy *PFR1* gene in the endosymbiont-bearing trypanosome *Crithidia deanei*. This gene is expressed in *C. deanei* and is able to partially rescue a *PFR1* null mutation in *Leishmania* cells, demonstrating that the encoded protein is functional. Moreover, antibodies against PFR1 recognise a small extra-axonemal structure in *C. deanei* and careful re-examination of the flagellar ultrastructure reveals the existence of a PFR that is greatly reduced in comparison to non-endosymbiont bearing kinetoplastids. This demonstrates the existence of a universal kinetoplastid motility structure that had been missed by previous reports.

Functional complementation of *Trypanosoma brucei* RNA in vitro editing with recombinant RNA ligase

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The ~20S RNA ligase-containing L-complex in trypanosomatid mitochondria interacts via RNA linkers with at least two other multiprotein complexes to mediate the editing of mitochondrial cryptogene transcripts. The L-complex contains approximately 16 proteins, including the two RNA ligases, REL1 and REL2. Leishmania tarentolae REL1 and REL2 and Trypanosoma brucei REL1 were expressed as enzymatically active TAP-tagged proteins in a Baculovirus system. When these proteins were added to mitochondrial lysates from T. brucei procyclic cells that were depleted of the cognate endogenous ligase by RNAi down regulation of expression, the added proteins were integrated into the L-complex, and in the case of REL1, there was a complementation of in vitro pre-cleaved U-insertion and U-deletion editing activities of the 20S L-complex. Integration of the recombinant proteins did not occur or occurred at a very low level with non-cognate ligase-depleted L-complex or with wild-type L-complex. A C-terminal region of the T. brucei rREL1 downstream of the catalytic domain was identified as being involved in integration into the L-complex. The ability to perform functional complementation in vitro provides a powerful tool for molecular dissection of the editing reaction.

Telomeric sequences and VSG silencing in *T. brucei*

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Antigenic variation in *Trypanosoma brucei* requires monogenic variant surface glycoprotein (*VSG*) expression and the silencing of ~1000 *VSG* genes, ~250 immediately adjacent to telomeres and the remainder in sub-telomeric arrays. We are investigating the mechanism that limits *VSG* expression to a single telomere. We have demonstrated telomere-proximal gene silencing which appears to require active suppression as demonstrated by chemical disruption and *VSG* expression from a second recombinant telomere. To examine the potential role of conserved (sub-) telomeric sequences we have used targeted chromosome fragmentation to place reporters adjacent to de novo telomeres with different sub-telomeric sequences. The *VSG* 3'UTR is not required for silencing but rather appears to be required for active *VSG* expression. The function of a sub-telomeric T_2AG_3-related repeat is under investigation and *de-novo* telomere formation in TElomerase Reverse Transcriptase (TERT) knock-out cells has been used to manipulate the very end of the chromosome. This approach should allow us to determine the role of the terminal T_2AG_3 repeats.

Functional and structural properties of guide RNAs are determined by separate rules

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RNA editing is a unique post transcriptional event through which uridine residues are specifically added or deleted from mRNA in the mitochondrion of kinetoplastids. Guide RNAs are predominately transcribed from minicircles of the kinetoplast DNA network and interact through the formation of an anchor duplex with a pre-edited mRNA and direct the correct number of uridine insertions or deletions. An endonuclease for cleavage, terminal uridyl-transferase for uridine addition, and a ligase to rejoin the mRNA fragments are required for the reaction. Previous work from our lab has shown the ability for the 3′ UTR of cytochrome oxidase II mRNA to guide an editing event in cis but not in trans. Mutational analysis of the 3′ UTR of COII showed a direct relationship between the length of the anchor duplex, with the amount of edited product formed when the 3' UTR was placed in trans. The efficiency of editing is not due to the increased stability of the gRNA/mRNA binary complex, but was a consequence of anchor length. Limiting ribonuclease digestion of the 3' UTR of COII predicts a secondary structure that is not similar to the most energetically favorable structure. Mutational analysis of the COII 3' UTR allowed us to map residues potentially involved in tertiary interactions. Presumably the RNA structures of gRNAs are mediated primarily by tertiary interactions and not the secondary structure of the molecules. We propose that the functional requirements of gRNAs, anchor duplex and guiding uridine insertion, are mediated by simple Watson Crick interactions. Whereas the 3D fold of a gRNA is predominately determined by tertiary interactions, minimally influenced by its secondary structure.

Interactions between the Cyclins and cdc2-related kinases from *Trypanosoma brucei*

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There are four PHO80 homologs (CycEs), three cyclin B homologs and five cdc2-related kinases (CRKs) identified in Trypanosoma brucei. Results from previous RNA interference (RNAi) experiments indicated that CycE1/CYC2, CRK1 and CRK2 are involved in regulating the G1/S transition, whereas CycB2/CYC6 and CRK3 play a pivotal role in controlling the G2/M checkpoint. In order to search for potential interactions between the cyclins and CRKs that may not be revealed by the individual RNAi knockdown assays, we used the yeast two-hybrid system and identified interactions between CycE1and CRK1, CRK2 and CRK3, between CycB3 and CRK2 and between CRK1 and CycE3 and CycE4. The interactions between CycE1 and CRK1, CRK2 and CRK3 were further confirmed using an in vitro GST pull-down assay, which pulled down also CycB2 and CRK3 together, though such binding was not demonstrated in the yeast two-hybrid assay. RNAi analysis of CycE1/CRK1 and CycE1/CRK2 double knockdowns indicated that the cells were arrested more extensively in the G1 phase beyond the cumulative effect of individual knockdowns. The results support the conclusion that CycE1 forms a complex with CRK1 and CRK2 in promoting the passage of T. brucei cells across the G1/S checkpoint. It will be interesting to learn the biological significance in the binding between CycE1, a G1 cyclin, and CRK3, a mitotic kinase, which will be, hopefully, revealed prior to the upcoming Meeting in April.

Procyclins and free GPI glycolipids are not essential for the growth of procyclic form *Trypanosoma brucei*

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GPI anchors are essential for the survival of bloodstream form *Trypanosoma brucei* [1-3]. Procyclin-free mutants of procyclic form have been generated by disrupting GPI biosynthesis [1,3] and by deleting all procyclin genes [4]. In all cases, the procyclin surface coat was replaced by a coat of free GPI glycolipids, suggesting that free GPI glycolipids might be essential for parasite growth. Here, we show that procyclins and free GPI glycolipids are not essential for the growth of procyclic form *T. brucei*. We generated conditional (tetracycline-inducible [5]) null mutants of TbGPI12, the GlcNAc-PI de-N-acetylase. The growth of these cells is not affected by the withdrawal of tetracycline. Analysis of total cell lysates by SDS-PAGE and Western blotting suggest that, under non-permissive conditions, these cells are devoid of procyclins and free GPIs. Work is in progress to identify molecules that might have taken the place of procyclins and free GPIs in these mutants. Supported by Wellcome Trust Grant 071463. 1. Nagamune, K., Nozaki, T., Maeda, Y., Ohishi, K., Fukuma, T., Hara, T., Schwarz, R. T., Sutterlin, C., Brun, R., Riezman, H., and Kinoshita, T. (2000) Proc Natl Acad Sci U S A 97, 10336-10341 2. Chang, T., Milne, K. G., Guther, M. L., Smith, T. K., and Ferguson, M. A. (2002) J Biol Chem 277, 50176-50182 3. Lillico, S., Field, M. C., Blundell, P., Coombs, G. H., and Mottram, J. C. (2003) Mol. Biol. Cell 14, 1182-1194 4. Vassella, E., Butikofer, P., Engstler, M., Jelk, J., and Roditi, I. (2003) Mol. Biol. Cell 14, 1308-1318 5. Wirtz, E., Leal, S., Ochatt, C., and Cross, G. A. (1999) Molecular and Biochemical Parasitology 99, 89-101

The phosphatidylinositol kinases of *Trypanosoma brucei*

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Localised generation of phosphoinositides by phosphatidylinositol kinases (PIKs) is an important cellular mechanism for targeting proteins to membrane subdomains. Proteins containing phosphoinositide-binding domains recognise distinct phosphorylation patterns within phosphatidylinositol head groups produced by different PIKs. Mammalian PIKs involved in signal transduction and membrane trafficking are under investigation as targets for anti-cancer therapy; thus PIKs in *Trypanosoma brucei* are potential drug targets for trypanosomiasis. We have identified eight putative PIKs in the genome of *T. brucei*, including a single Class III PI3-kinase, TbVPS34. Mammalian and yeast VPS34 kinases are downstream effectors of Rab5 and act primarily at the endosome, regulating endosome to lysosome traffic and retrograde endosome to Golgi transport. Inhibition of TbVPS34 expression in bloodstream form *T. brucei* by RNAi leads to marked inhibition of cell growth and distorted cellular morphology. Ultrastructural analysis reveals abnormalities in both the flagellar pocket and Golgi apparatus. Further, transport of internalised cargo to the lysosome and export of newly synthesised VSG are impaired by TbVPS34 RNAi. The results confirm that TbVPS34 is an essential gene and has similar functions to homologues in other eukaryotic systems. However, the distinct effects of TbVPS34 RNAi on cell morphology suggest that this kinase may have additional roles in *T. brucei*.

Centrin is involved in Golgi biogenesis in *Trypanosoma brucei*

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The accurate duplication of cellular organelles is essential to ensure propagation through successive generations. While the semi-conservative replication of DNA and DNA-containing organelles has been well studied, the mechanisms used to duplicate other organelles including the centrioles/basal bodies and membrane-bound organelles such as the Golgi apparatus, remain elusive. In *Trypanosoma brucei*, the basal body seeds the growth of the single flagellum, and divides mitochondrial DNA during cell division. Previous work also revealed a close association between the Golgi and the basal body. Replication of the basal body is immediately followed by the appearance of a new Golgi near to the old. While the old Golgi stays close to the old basal body, the new Golgi is built next to the new basal body and this association is maintained throughout the cell cycle. Most interestingly, our recent studies have shown that a centrosomal component – centrin –is present not only on the basal bodies, but also on a structure adjacent to the Golgi. Centrin belongs to a highly-conserved family of small Ca^{2+}-binding proteins that are important for the duplication of centrosomes in mammalian cells. The presence of centrin next to the Golgi raises the intriguing possibility that this protein is also involved in Golgi duplication. Using molecular cloning, fluorescent tagging and specific antibodies, we have found that only one of several putative centrins in the *T. brucei* genome localizes to the cis-side of the Golgi stack, in addition to the basal bodies. Conditional RNAi of this centrin leads to defects not only in the duplication of the basal bodies but also to defects in the generation of the new Golgi.

Ordered assembly of the Golgi apparatus in *Trypanosoma brucei*

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The Golgi apparatus is composed of stacks of flattened cisternae that process and sort the cargo in the secretory pathway. Accurate inheritance of the Golgi apparatus, which is comprised of organelle duplication and segregation, is essential for successful cell propagation. While studies on Golgi inheritance have focused mostly on the segregation process, Golgi duplication is not well understood. Two non-exclusionary models for Golgi duplication have been considered. One requires the ER to produce a new Golgi, while the other suggests that the old Golgi plays an important role. We have employed *T. brucei*, which contains a single Golgi stack, to study the duplication of the organelle. We have shown previously that the old Golgi supplies at least one enzyme for building the new and are now determining the order in which the new Golgi is constructed. To examine the order of TGN and Golgi stack assembly, Tb-GRIP70 (TGN marker) and Tb-GRASP (Golgi stack marker) were tagged and followed simultaneously using time-lapse fluorescence microscopy. We observed that Tb-GRASP is recruited first, suggesting that the new Golgi is built by ordered addition of materials, with the Golgi stack preceding the TGN. We have also examined epsilon-COP (a subunit of COPI coatomer) recruitment to the new Golgi. Our data show that epsilon-COP is recruited after Tb-GRASP, suggesting that the Golgi is not fully functional when Tb-GRASP is first recruited. Furthermore, a time lag between Tb-GRASP recruitment and entry of a secreted form of YFP into the new Golgi has also been observed, arguing that new Golgi is initially not competent for processing cargo.

Physiological significance of GPI inositol deacylation in *Trypanosoma brucei*

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Trypanosoma brucei has a dense coat of glycosylphosphatidylinositol (GPI) anchored proteins. Bloodstream form, which causes sleeping sickness, is covered by variant surface glycoproteins (VSG) whereas procyclic form, which resides in tsetse fly, is covered by procyclins. During GPI biosynthesis, inositol moiety of phosphatidylinositol becomes acylated. The inositol is deacylated prior to the attachment to VSG in bloodstream form whereas it remains acylated in procyclins. To examine the role of inositol acylation and deacyaltion in trypanosomes, we have identified and characterized a *T. brucei* GPI inositol deacylase (GPIdeAc2) based on homology to mammalian inositol deacylase (PGAP1, Tanaka *et al*., JBC 2004). Real-time PCR analysis revealed that GPIdeAc2 was expressed in bloodstream form but not in procyclic form, as expected. We then examined the roles of GPIdeAc2 in the GPI metabolism. First, we induced the expression of GPIdeAc2 in procyclic form. Analysis of GPI biosynthesis *in vitro* using cell lysates revealed generation of a large amount of GPI species lacking inositol-linked acyl chain. Second, we suppressed GPIdeAc2 expression by RNAi in the bloodstream form. The induction of double-stranded GPIdeAc2 RNA by doxycycline led to significant decrease in GPIdeAc2 mRNA level by 48 h after induction. GPIdeAc2 knockdown was accompanied by accumulation of inositol-acylated GPI intermediate, glycolipid C'. These results indicate that GPIdeAc2 is involved in GPI inositol deacylation. GPIdeAc2 knockdown cells showed a decreased level of VSG expression on the cell surface and a slower growth rate, suggesting an essential role for GPIdeAc2 in the bloodstream form.

Functional genomics relating to the biosynthesis of trypanosome glycoconjugates

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The African trypanosome *Trypanosoma brucei* causes African sleeping sickness and the cattle disease nagana. The human and socio-economic impact of these diseases is considerable. The available drugs for humans are inadequate and difficult to administer. Parasites are very dependent on glycoconjugates for their survival and infectivity. The main goal of this project is to determine the function of genes involved in galactose-containing glycoconjugate biosynthesis and processing in *T. brucei*. The emerging *T. brucei* genome is being systematically mined, and 26 candidate genes have been classified in different glycosyltransferase (GT) groups. Representative examples of these putative GTs are being experimentally examined by gene knockout, or by construction of tetracycline-inducible conditional null mutants. Mutant cells are assessed for growth in vitro and are biochemically phenotyped in both the bloodstream and procyclic lifecycle forms of the parasite. In this way, we should be able to assign precise functions to genes and thus, identify sequence motifs/patterns that discriminate between different families of parasite enzymes. Some preliminary results show that one of these putative GTs, TbGT8, may be involved in the glycosylation of procyclins, in the procyclic form of the parasite.

Aurora kinase TbAUK1 is essential for nuclear division in *Trypanosoma brucei*

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African trypanosomes propagate as they invade the fluid spaces of the host. To understand the process of cell replication, we have begun to study signal events associated with later stages of the cell cycle, including chromatin segregation and cytokinesis. Here, we focus on an aurora kinase from Trypanosoma brucei (TbAUK1). The sequence of TbAUK1 shares around 31% identity with aurora kinases from other eukaryotes, and this increases to nearly 70% identity in the characteristic signature motif and degradation box. An AU1-epitope tagged TbAUK localized to the nucleus in dividing cells. RNAi knockdown of TbAUK1 in both procyclic (PF) and bloodstream (BF) trypanosomes produced cells whose nuclei became elongated but did not divide. Flow cytometry of these cells revealed an accumulation of DNA in these arrested cells, indicating that cell cycle progression continued despite the inhibition of mitosis. Other phenotypic changes were distinct between the PF and BF forms. In PF trypanosomes, the posterior end became elongated and the kinetoplast segregated into this region. However, despite multiple rounds of nuclear DNA synthesis, no further kinetoplast replication was observed. By contrast, BF trypanosomes lost their overall architecture and began to replicate multiple kinetoplasts, each associated with a flagellum. In both PF and BF trypanosomes, the growth rate became rapidly inhibited upon induction with RNAi. Since inhibitors of aurora kinase are currently being developed as anti-proliferation drugs, these might prove useful in halting infection by T. brucei.

Functional characterisation of the iron superoxide dismutase gene repertoire in *Trypanosoma brucei*

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Superoxide dismutases (SOD) are a family of antioxidant enzymes that function by removing superoxide anions from the cellular environment. Here, we show that the African trypanosome, Trypanosoma brucei expresses four SOD isoforms, three of which we have validated biochemically as iron-dependent, a feature normally associated with prokaryotic SODs. Localisation studies reveal that two of the enzymes are found predominantly in a parasite-specific organelle, the glycosome (TbSODB1 and TbSODB2), while the other two are targeted to the mitochondrion (TbSODA and TbSODC). Functional analysis of the SOD repertoire in bloodstream form parasites was performed using an inducible RNA interference (RNAi) approach. Down-regulation of the glycosomal SOD transcripts corresponded with a significant reduction in the corresponding proteins and a dramatic level of cell death within the population. The importance of one of the mitochondrial enzymes (TbSODA) only became apparent when parasites were exposed to the superoxide generating agent paraquat following induction of RNAi. These experiments therefore identify essential components of the superoxide metabolising arm of the T. brucei oxidative defence system and validate these enzymes as parasite-specific targets for drug design.

A third mitochondrial DNA polymerase, PolID, is essential for kDNA replication in *Trypansoma brucei*

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A unique feature of *Trypanosoma brucei* and related parasites is their mitochondrial genome that is a catenated network of minicircles (thousands) and maxicircles (tens) called kinetoplast DNA (kDNA). Additionally, the single mitochondrion contains at least SIX DNA polymerases (Pol) for kDNA replication and repair processes. Previous RNAi studies on four of these Pols (related to bacterial Pol I) indicated essential roles for PolIB and PolIC in kDNA replication while PolIA might be a repair protein (1). This study using the pZJM vector did not clarify a role for PolID, but we hypothesized that PolID might also be a repair protein based on a similar localization pattern to PolIA. However, the formal possibility existed that PolID might play a role in kDNA replication. Therefore, to study the cellular role of PolID we used an inducible stemloop RNAi construct as an alternative. Stemloop silencing resulted in a $\sim$75% reduction of PolID mRNA following 2 days of tetracycline induction, and growth inhibition within 5 days. Additionally, induced cells lost their kDNA (58% dyskinetoplastic) or contained small kDNA networks (36%) following 10 days of PolID silencing. These data indicate that PolID also has an essential role in replicating the kDNA network. Now it appears that three Pol I-like enzymes are essential for replication of the unique kDNA network, compared to just a single enzyme, pol gamma, required for mitochondrial DNA replication in yeast and animals. We are further characterizing this silenced phenotype to determine the specialized role that PolID plays in kDNA replication by examining the relative loss of minicircles vs. maxicircles as well as kDNA replication intermediates. 1. Mol Cell (2002) 10:175-186.

RNA interference of a new type II topoisomerase in *Trypanosoma brucei*

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Topoisomerases manage the topological state of DNA without altering its sequence, and are proven targets for a variety of antimicrobial and anticancer agents. Our previous studies with topoisomerase inhibitors suggested trypanosomes might be unusual among lower eukaryotes in having more than one type II enzyme. The expressed sequence tag screen of *T.brucei* reported by Djikeng *et al.* included a single tag with significant homology to *Saccharomyces cerevisiae* topoisomerase IIA, and markedly different from the *TbTOP2mt* gene reported by Strauss and Wang. Based on this tag, we identified two genes encoding new type IIA topoisomerases. These genes, termed *TbTOP2A*α and *TbTOP2A*β, encode predicted polypeptides of 165 and 167 kDa, respectively. Unlike TbTOP2mt, which localizes to the mitochondrion, predicted products of these new genes have multiple nuclear localization signals. Furthermore, phylogenetic analysis reveals that the new genes are clearly distinct from type II topoisomerase sequences previously reported for kinetoplastids, all of which are homologs of *TbTOP2mt*. Southern and northern blot analyses indicate single copies positioned $\sim$1.8 kb apart with a distinct transcript for each gene: 5.7 kb for *TbTOP2A*α and 6.5 kb for *TbTOP2A*β. RNA interference of *TbTOP2A*α, using the stem/loop system, leads to depletion of cognate mRNA and protein; cell growth arrest; giant, multiflagellated parasites; and pleomorphic nuclear abnormalities. RNAi of *TbTOP2A*β results in no detectable phenotype. These studies describe, for the first time, a satisfying source of type II topoisomerase activity for nuclear DNA metabolism, and provide a basis for the differing drug susceptibilities in nuclear and mitochondrial compartment.

Polo-like kinase controls cytokinesis but not the mitosis in *Trypanosoma brucei*

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Polo-like kinase (Plk) plays multiple roles in mitosis and cytokinesis among all eukaryotes examined thus far. Its down-regulation interferes with spindle formation during mitosis leading to cell cycle arrest and apoptosis. Here we report that in an ancient eukaryote *Trypanosoma brucei* the only Plk homolog, tbPlk, controls only cytokinesis. This is regulated primarily by the kinetoplast cycle instead of the nuclear cycle. A knockdown of the expression of tbPlk by RNA interference in the procyclic form of *T. brucei* resulted in apparently normal spindle formation, chromosome segregation and nuclear division. There was accumulation, in each individual cell, of multiple nuclei kinetoplasts, basal bodies and flagella all in equal numbers, indicating that duplication and segregation of all the cellular organelles necessary for cell division proceeded in a normal manner. But the cells were completely blocked from dividing. Since the procyclic-form *T. brucei* is known to continue cell division under mitotic arrest to produce anucleated cells (the zoids), tbPlk probably does not play a role in mitosis at all but is, instead, associated more closely with the kinetoplast cycle in regulating the initiation of cytokinesis. Indeed, no zoid was detectable among the tbPlk knockdown cells. This is for the first time that a polo-like kinase is found unassociated with mitosis and has been found to play a crucial function only in initiating cytokinesis. It provides a powerful tool to dissect the precise role of Plks in initiating cytokinesis in all eukaryotes and delineate the course of evolution of cell cycle regulation among them.

T. brucei RNA editing: the role of band-II protein (TbMP81) and key considerations for analysis by RNAi

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RNA editing, the massive processing of trypanosome mitochondrial transcripts, involves insertion and deletion of uridylate (U) residues through cycles of cleavage, U-addition or U-removal, and ligation. These U-insertion and U-deletion cycles are catalyzed by an ~20S complex that we purified to seven major proteins (bands-I through -VII), although additional proteins are also important, and RNA interference (RNAi) is proving useful in elucidating roles of these proteins. We recently showed (MCB in press) that depletion of band-II by RNAi has no direct effect on U-deletion but markedly impairs each step of U-insertion. Interestingly, band-II is none of the U-insertional enzymes but rather appears required for their recognition(s) of the RNA substrate and/or editing complex. Thus, band-II is a KRERi (kinetoplastid RNA editing recognition for insertion). These KRERi studies also yielded generalizable finding about extract preparation. We find that activities of the KRERi-depleted editing complex that are retained in a rapidly prepared extract (~1.5 hrs) are lost during traditional mitochondrial extract preparation (~8-10 hrs). Thus, depleted editing complexes can be relatively unstable, and rapid extracts can help distinguish direct effects from indirect effects due to such diminished stability. Our KRERi studies additionally show the value of assessing both (i) the individual editing steps and (ii) the basic activities of those enzymes. These analyses define which steps are affected and affords distinction between loss of action in editing versus loss of the basic enzymatic activity. In conclusion, band-II/KRERi has a unique recognition role specific for U-insertion and that when using RNAi to assign protein function within a complex, it is critical to consider the complex's stability and the basic activities of its enzymes.

Brucipain takes a tryp across the blood-brain barrier

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In classical human sleeping sickness the African trypanosomes invade the central nervous system (CNS) and the infected individuals suffer from neurological degeneration with concomitant psychiatric disorders and death. Using a model of the human blood-brain barrier (BBB) consisting of human Brain microvascular Endothelial Cells (BMEC) it was found that contact and/or cellular adhesion of African trypanosomes to human BMEC are coupled to signaling responses leading to rapid changes in $[Ca^{2+}]_I$, resulting in endothelial cell contraction. Studies using small molecular weight synthetic protease inhibitors have indicated that proteases may play a fundamental role in the virulence and/or survival of these pathogens. We now show that synthetic cysteine protease inhibitors affect the ability of *T. b. gambiense* and of *T. b. rhodesiense* to cross the BBB model in vitro, suggesting that these enzymes may also play an important role in BBB transversal. The synthetic inhibitor N-Pip-Phe-homoPhe-Vinylsulfone also prevented *T. b. gambiense* induced $[Ca^{2+}]_I$ responses in BMECs. The use of the biotinylated inhibitor allowed the identification of the parasite's papain-like cysteine protease, brucipain, as the main target of these compounds. Furthermore, the bloodstream forms of *T. b. gambiense*, *T. b. rhodesiense* and *T. b. brucei* were found to secrete enzymatically active brucipain to the extracellular milieu. *T. b. gambiense* and *T. b. rhodesiense*, which cross the BBB more efficiently than *T. b. brucei*, secrete significant higher levels of brucipain than the latter, supporting the notion of a putative role of brucipain in the crossing of the brain-barrier.

Lok1: A new kDNA replication protein in *Trypanosoma brucei*

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In a search for new kDNA replication proteins, we screened an RNAi library for genes whose silencing caused loss of kDNA in *T. brucei*. We identified a gene that we call *Lok1* for loss of kDNA. While *Lok1* homologs exist in *T. cruzi* and *Leishmania* species, we found no homologous protein in any other organism. Its presence only in kinetoplastids indicates that *Lok1* may have a kDNA-specific function. *Lok1* is annotated as a hypothetical protein of 19 kDa. It has no functional domains or motifs that can be predicted by web-based bioinformatics software and there is no obvious mitochondrial targeting signal. In a cloned cell line, northern hybridization showed greater than 95% loss of the *Lok1* mRNA 3 days after RNAi induction. RNAi of *Lok1* inhibits cell growth after 5 days, at which point the kDNA starts to shrink in size. Unlike RNAi of other genes involved in kDNA replication, loss of kDNA is not dramatic, with small kDNAs persisting even after 10 days of RNAi. At day 10 about 30% of cells have lost kDNA. Expression of a *Lok1*-GFP fusion reveals that this protein is localized in the mitochondrion, highly concentrated within or very near the kDNA disk. Analysis of free minicircle replication intermediates by Southern hybridization shows that covalently closed minicircles accumulate after 4 days of RNAi whereas the nicked/gapped minicircle progeny decrease. This finding suggests that *Lok1* knockdown does not affect minicircle release from the network, but that it does affect their replication. Our current hypothesis is that *Lok1* is required for initiation of minicircle replication.

p38, a *Trypanosoma brucei* protein involved in kinetoplast DNA replication

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One unusual feature of the kDNA network is that its minicircles are not supertwisted. Neither are the covalently-closed minicircles released from the network for replication. Therefore we were surprised to discover a highly supertwisted minicircle species when we conducted RNAi on a *T. brucei* protein named p38. We had first identified p38 by mass spectrometry of mitochondrial DNA binding proteins. RNAi knockdown of p38 causes rapid loss of kDNA, suggesting a role in replication. Consistent with that role, p38-GFP appears to localize within or near the antipodal sites flanking the kDNA disk. Gel electrophoresis of free minicircle replication intermediates after RNAi revealed an abundant novel minicircle species that ran as a smear. Two-dimensional neutral/alkaline gel electrophoresis, and Southern blotting, indicated that the smear was not composed of theta structures or circles with a D-loop. The smear was converted to relaxed covalently-closed minicircles by treatment with topoisomerase I or II, raising the possibility that it consisted of highly supertwisted minicircles. Supertwisting was confirmed by comparison of its electrophoretic mobility with that of gyrase-treated minicircles, and by EM. In fact, electrophoresis suggested that these minicircles are so underwound that they may contain unpaired sequences. The absence of metabolic labeling with BrdU demonstrates that these supertwisted minicircles are not a replication product. All these data are consistent with the possibility that minicircles are partly unwound prior to replication. RNAi knockdown of p38 could prevent replication initiation on these underwound templates. Therefore, p38 may function in replication initiation.

The SR network In trypanosomatids: conservation of the constitutive mechanism in SR protein kinases

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The regulation of the gene expression in trypanosomatid protozoa is exerted mainly at the post-transcriptional level, including RNA processing, RNA stability and mRNA turnover. It is well established in higher eukaryotes that SR proteins and the specific SR kinases, which constitute the SR Network, are critical for pre-mRNA processing. We have identified and characterized components of this network in *Trypanosoma cruzi* and *Trypanosoma brucei*: the serine/arginine-rich protein (TcSR) and the specific SR kinases, TcSRPK and TbSRPK. These proteins were functionally characterized using *in vitro* phosphorylation assays, *in vivo* alternative splicing reactions in HeLa cells and complementation of *Schizosaccharomyces pombe* mutant strains. Our results show that the two trypanosome proteins form an SR network and suggest an involvement in the trans- and/or cis- splicing processes. The functional conservation of the trypanosome SR kinases was further substantiated by modeling the proteins on the crystal structure of Sky1p, the SR protein kinase of *S. cerevisiae*. Based on this modeling wild-type and mutant kinase proteins were analyzed in wild-type and kinase-deficient *S. pombe* strains. This revealed that the constitutive activity mechanism is conserved and that the kinase activity is essential to arrest the cell growth in G2/M checkpoint. This arrest is a consequence of defects in pre-mRNA processing, thus again highlighting a possible role of the trypanosome SR network in gene expression. Our findings also suggest that the function of the SR network has been conserved during the evolution of the eukaryotic lineage.

A role for insect laminin in parasite interaction

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Trypanosomatids parasitize all classes of vertebrates, as well as some invertebrates, preferentially insects from the orders Diptera and Hemiptera, and also plants. Often recognized trypanosomatid genera, only two - pathogenic *Trypanosoma* and *Leishmania* - have been actively investigated for any length of time while the plant flagellates - *Phytomonas* - have recently begun to attract attention due to their role as agricultural parasites. *Phytomonas* species are parasites of both plants and insects. The hemipteran *Oncopeltus fasciatus* is not only the natural host for *Phytomonas elmasiani*, but it is also capable of hosting different species of trypanosomatids by experimental infection. The invasion of the vectors salivary glands is one of the most important events for the life cycle of *Phytomonas species*. In the present work we observed, by means of scanning electron microscopy, the *ex vivo* interaction between *P. serpens* and the external face of salivary glands of *O. fasciatus*. This binding seems to occur through a 130 kDa insect protein. The association of the parasites with the salivary glands was strongly inhibited by N-acetyl-D-glucosamine, N-acetyl-D-galactosamine and α-methyl-D-mannoside, as well as by the 130 kDa purified protein. Polyclonal antibodies raised against human laminin were able to recognize this protein, through immunoblotting. The mass spectrometry of the trypsin-digest of this protein matched 23% of human laminin beta-3 chain precursor sequence by the digested peptides.

Telomere trapping with TAR: update on telomere clones from *Trypanosoma brucei*

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Collections of bloodstream form expression site containing clones have successfully been obtained for the genome project strain of *T. brucei*, 927, as well as for strain Lister 427 using transformation associated recombination. These are in the process of being sequenced. We have continued developing vectors for the cloning of specific telomeres as well as a more general targeting vector for all telomeres. A progress report of the status of the analysis of BES clones, the completion of telomeres for the genome project, and the cloning of telomeres from other strains and species will be made. Both specific telomere cloning using unique targets as well as generic cloning using **ingi** have been successful. This work is supported by The Wellcome Trust and is done in collaboration with Dave Barry and Gloria Rudenko. Contributions from The Sanger Institute (Matt Berriman and Mike Quail) and TIGR (Najib El-Sayed and Elodie Ghedin) have been essential for the success of this project as is the Trypanosome community.

Down-regulation of nuclear encoded subunits of complexes III and IV disrupts the respiratory chain in procyclic *T. brucei*

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In *Trypanosoma brucei* procyclics, the functions of nuclear-encoded subunits of respiratory complexes III and IV, their stability, turnover, and mutual interactions were studied using RNA interference (RNAi). The growth rates of clonal cell lines of knock-downs for Rieske protein and apocytochrome c1 (apoc1) of complex III, and cytochrome oxidase subunit 6 (cox6) of complex IV were significantly decreased 3 to 4 days upon RNAi induction and ceased around day 9. The mRNA of the silenced genes was virtualy undetestable within 24 hrs of RNAi interference, and remained so throughout the entire experiment. Western analysis of mitochondrial lysates using specific polyclonal antibodies revealed almost complete elimination of the target proteins 4 to 6 days after downregulation. Importantly, the level of Rieske protein was significantly reduced in the apoc1 knock-downs and vice versa, indicating mutual dependence of these components of complex III. However, the cox4 protein remained at the wild type level in the cox6 knock downs. Analysis of mitochondrial lysates of apoc1 silenced cells by 2D blue native electrophoresis showed the disruption of complex III, while no changes were observed for complex IV. Due to depletion of the targeted subunits, the activities of complex(es) III and/or IV were drastically reduced and membrane potential significantly decreased. Using SHAM and KCN as specific inhibitors of alternative oxidase (TAO) and complexes III and IV, respectively, we have shown that as a consequence of downregulation of the targeted proteins terminal electron transfer to oxygen is exclusively mediated by TAO.

The role of PUF proteins in gene expression in *Trypanosoma brucei*

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Control of gene expression in trypanosomes is mainly post-transcriptional and heavily dependent on mechanisms affecting transcript stability. Most known *cis*-regulatory elements determining mRNA stability are located in the 3'-untranslated regions (3'-UTRs). *Trans*-acting factors that bind these, modulate transcript stability and largely mediate the response of mRNA molecules to the intracellular environment. Members of the PUF family are 3'-UTR regulatory proteins that generally repress expression of multiple target transcripts by control of mRNA-degradation or interference with translation. In addition to the previously characterized TbPUF1-protein (Cross *et al*., 2002) we have identified eight undocumented PUF genes in the *T. brucei* genome: Tbpuf2-Tbpuf9. Each of the TbPUF protein has a homologue in *T. cruzi* and their similarity range between 36% and 62%. TbPUF1 was shown to be essential in bloodstream (Cross *et al*., 2002) but is dispensable in procyclic forms (our lab). However we observed that overexpression and RNAi-knockdown of these proteins did not affect growth in both bloodstream and procyclic cells. So far our microarray studies have failed to identify RNA targets. We currently focus on the role of the PUF proteins on translation using 2D gel electrophoresis. Tethered-function analysis together with CAT-assay reveal that TbPUF9 enhances translation. We will further identify putative PUF-interacting partners using the Tandem Affinity Purfication (TAP) method and co-immunoprecipitation approaches.

Exploring the RNA binding specificity of RNA Editing-Associated Protein (REAP-1)

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Within the mitochondria of *Trypanosoma brucei* and related kinetoplastids a number of genes are post transcriptionally modified by RNA editing, involving the addition, and to a lesser extent deletion, of uridines at specific sites in pre-mRNAs. Editing is directed by guide RNAs (gRNAs) and carried out by a ribonucleoprotein (RNP) complex. Through the work of several labs the core ~20S editing complex has been purified and the components identified. However the larger 35-40S complex that carries out editing *in vivo* has not been purified nor well described. We previously identified one component of the larger RNA editing complex, REAP-1. Earlier work demonstrated that REAP-1 is an RNA binding protein and that the binding activity maps to the amino-terminal third of the protein. This domain does not share homology with any known RNA binding motifs suggesting that REAP-1 represents a new type of RNA binding protein. REAP-1 binds preferentially pre-edited RNAs. Using three different pairs of pre-edited and edited substrates we showed that REAP-1 binds consistently and preferentially to the pre-edited RNA over the edited RNA, non-edited RNAs or guide RNAs. We have begun to explore in more detail both preferences among editing substrates and the boundaries of the RNA binding domain of the protein. Using an amino-terminal truncation of recombinant REAP-1 we evaluated several pre-mRNAs in gel shift experiments and find that there are differences in their ability to act as a binding substrate for REAP-1.

Cyclic AMP Export from *Trypanosoma brucei*

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cAMP signalling has traditionally been studied in the context of intracellular signal transduction. Over the last two or three decades, our understanding of this aspect of cAMP signalling in eukaryotes, mostly in mammalian cells and selected unicellular eukaryotes, has been expanded considerably. In contrast, the exploration of cAMP signalling in protozoan pathogens has only recently been initiated. Most investigations so far were done in the frame of mind of intracellular signalling, and processes such as cAMP formation by adenylyl cyclases and cAMP hydrolysis by various phosphodiesterases have been studied. An important aspect of cAMP signalling is the export of cAMP into the extracellular space. In bacteria, this mechanism appears to be important for the regulation of intracellular cAMP concentrations. In eukaryotic cells, cAMP export as a control mechanism for steady-state cAMP concentrations has largely been superseded by the various phosphodiesterases. These allow a much faster downregulation of cAMP levels than does export. Nevertheless, the capacity to export cAMP (and cGMP) has been maintained in many (all?) eukaryotic cells. All currently available evidence indicates that this export represents more than just a way of disposing of excess cAMP. In several cell types and tissues, such as the kidney or in fat tissue, a cAMP-mediated, extracellular signalling pathway has been established. While many aspects of trypanosome cell biology have been extensively studied, nothing is known about cAMP export. The aim of this project is to explore if *Trypanosoma brucei* is capable of exporting cAMP, and, if so, if this extracellular cAMP exerts a physiological function.

Proteomic analysis of stage-specific gene expression in *Leishmania infantum*

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The ability of *Leishmania* to survive within the acidic phagolysosomes of mammalian macrophages is heavily dependent on the developmental regulation of a variety of genes. A large-scale identification of stage-specific genes and the study of their regulatory mechanisms could significantly improve our understanding of the molecular determinants controlling the parasite's intracellular survival, and could eventually lead to the development of novel therapeutic targets. We used a combination of two-dimensional gel electrophoresis, LC/MS-MS mass spectrometry, and in silico screening to identify developmentally expressed proteins in *L. infantum*. We have also established conditions that allow the generation of proteome maps of total soluble proteins of *Leishmania* or subfractionated extracts using differential protein precipitation to enrich for less abundant proteins. Using ammonium sulfate fractionation, we increased protein representation by at least 7-fold compared to total cell extracts for the same pH range. More than 3000 proteins were visualized. Among those, over 9% were unique in amastigotes and $\sim$3% were overexpressed. These proteins belong to different categories, including carbohydrate and energy metabolism, stress response (endoplasmic reticulum and oxidative stresses), proteolytic pathways, translation and signalling. Using total and polysomal RNA preparations, we are currently evaluating the ratio of steady-state RNA levels versus translatable mRNAs in both developmental stages of the parasite. The degree of correlation of the protein expression data to the RNA expression will be discussed. Proteomic studies should contribute to a better understanding of the biology of an organism like *Leishmania* that does not use transcription regulation as its main mode of gene expression control.

Characterization of trypanosome deadenylases

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The stability of mRNAs has emerged as a major control point in the regulation of eukaryotic gene expression. Trypanosomatids are unicellular protozoans that are obligatory eukaryotic parasites. These organisms have a system of gene expression that varies widely from the eukaryotic paradigm. Most protein-encoding genes in trypanosomatids are transcribed as part of long polycistronic pre-mRNAs that are subsequently processed into mature monocistronic mRNAs. Each of the component open reading frames of the original pre-mRNA can exhibit differential steady state levels as mature messages. These findings suggest a reliance on post-transcriptional regulatory steps in these organisms. Therefore, our laboratory has aimed to define the enzymatic processes of mRNA turnover in trypanosomatids to better understand the mechanisms of their gene expression. We have previously developed an in vitro mRNA turnover system in trypanosomes to facilitate the dissection of mRNA turnover processes. This system has allowed us to identify and describe several important mRNA turnover activities such as decapping, scavenger decapping, deadenylation and AU-rich element-mediated 3' to 5' exonucleolytic decay. In this current study, we will present data that describes the first trypanosomal deadenylase, TbPARN (*Trypanosoma brucei* poly(A) ribonucelase). This protein was identified based on similarity to human, *Xenopus*, and plant PARN. A Tandem Affinity Purification (TAP)-tagged version of TbPARN co-purifies with a poly(A)-specific deadenylation activity that is distributive and is divalent cation-dependent. In addition to functional studies, the subcellular localization pattern of this protein has been examined and will be presented.

The downstream effectors of cAMP signaling pathway in *Trypanosoma brucei*

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Cyclic AMP is likely to play an important role at different key steps in the life cycle of *T. brucei*. Differentiation of short-stumpy to procyclic forms coincides with the appearance of two distinct peaks of adenylyl cyclases activity. On the other hand, the differentiation of long slender to short-stumpy forms can be easily induced *in vitro* by treatment with membrane permeable cAMP-analogs. Surprisingly, basically nothing is known about the downstream mechanisms in the cAMP-signaling pathway of *T. brucei*. A canonical cAMP-effector protein, the regulatory subunit of protein kinase A, was identified and characterized in *T. brucei* although it does not seem to bind cAMP. A blast search in *T. brucei* genome database for putative cAMP-binding domains revealed twelve hits. RT-PCR and Northern Blotting studies showed that most of these genes are expressed both in procyclic and in bloodstream trypanosomes. By using affinity chromatography, RNAi, HA-tagging and fusion protein expression methods, we are in the process of identifying and characterizing several of these putative cAMP-binding proteins of *T. brucei*.

Deficiency of GPI-anchored proteins in procyclic *Trypanosoma brucei* and its effect on cell growth

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Glycosyl phosphatidylinositol (GPI)-anchored proteins of *Trypanosoma brucei* are essential for the parasite's survival in the host bloodstream as well as in the tsetse fly vector. Block of GPI biosynthesis in the procyclic form parasites results in the loss of the major surface coat procyclins and a reduced growth rate, but how the deficiency of GPI-anchored proteins affects the cell proliferation is largely unknown. In this study, we artificially expressed GPI inositol deacylase (GPIdeAc2) in the procyclic form, which does not normally express this bloodstream form-specific enzyme. The transformation resulted in two populations of cells: cells expressing normal levels of procyclins (Pro+), and cells expressing reduced levels of procyclins (Pro-). The growth rate of the transformant was comparable to that of the wild type for the first few divisions (up to $\sim$30 h) after the dilution of stationary phase cell culture, but was slightly reduced for the rest of the growth phase. This growth phase-specific reduction of growth rate was associated with prolonged time required for cytokinesis. Furthermore, we found a growth phase-dependent fluctuation of the ratio of Pro+ to Pro- populations, and a dynamic Pro+ to Pro- shift at the $\sim$30 h time point, suggesting that the expansion of Pro- population was associated with the block of cytokinesis and reduction of growth rate. We discuss how the GPIdeAc2-induced loss of procyclins could be associated with growth rate reduction.

Connecting glycolysis to surface molecule expression: building the glucose sensing pathway in *Trypanosoma brucei*

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Silencing glycolytic enzymes in procyclic form (PF) *T. brucei*, including hexokinase 1 (TbHK1), triggers changes in expression of procyclins that are similar to the developmentally coordinated expression of the proteins. Similarly, growth of PF or BSF parasites in low glucose medium causes developmental changes. These observations suggest that *T. brucei* monitors its environment by measuring glycolytic flux in order to regulate development. We have found that RNAi of *T. brucei* homologs of the beta or gamma subunit of mammalian AMP-activated kinase (AMPK), which regulates cellular responses to changes in glycolysis, also causes parasites to alter surface molecule expression. This phenotype is enhanced by growth of parasites in low glucose medium, suggesting an interaction with the glycolytic machinery. Supporting a potential interaction, RNAi of TbAMPKbeta causes an increase in cellular hexokinase (HK) activity. Confounding our understanding of the regulation of HK activity by TbAMPK subunits (and the role of HK in surface molecule expression) in PF trypanosomes is the presence of two expressed, nearly identical HKs (TbHK1 and TbHK2, 97% identical at the amino acid level). Interestingly, single allele knockouts (SAKO) of the genes have distinct phenotypes. Glucose is toxic to TbHK1SAKO cells while TbHK2SAKO are unaffected. Cellular HK activity in TbHK1SAKOs is increased $\sim$ 4-fold compared to parental cells, while TbHK2SAKOs hexokinase activity is unchanged. Unlike parental or TbHK2SAKO cells (but similar to cells in which glycolytic enzymes were silenced), TbHK1SAKO cells have altered surface molecule expression. Taken together, these data suggest that TbHK1 and TbHK2 serve different functions, with TbHK1 possibly acting to connect glucose metabolism and surface molecule expression, possibly coordinated by TbAMPK.

TbHKT1, a novel K^+ transporter of *Trypanosoma brucei*

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Cellular uptake of K^+ is mediated by a variety of transporters including ATPases, antiporters, carriers, and channels. Yet despite their pharmacological potential, nothing is known about the molecular nature of K^+ channels from *Trypanosoma brucei*, although as extracellular parasites, they need to actively concentrate K^+. A genome wide search, outgoing from profiles of known K^+ transporters of other organisms, revealed no classical K^+ channels. This apparent lack is in agreement with experimental data, because *T. brucei* in culture tolerates millimolar amounts of the K^+ channel blocker tetraethylammonium (TEA) and of the toxic K^+ channel substrate caesium (Cs^+). Interestingly, one trypanosomal member of the HKT/Trk family, named TbHKT1, was found. HKT/Trk K^+ transporters are so far only known from bacteria, fungi, and plants. TbHKT1 was functionally expressed in the yeast *Saccharomyces cerevisiae*, and expression of TbHKT1 rescued a K^+ uptake deficient yeast mutant, indicating its function as a K^+ transporter. Thus TbHKT1 is the first K^+ transporter identified from a protozoan parasite. Nevertheless, knocking down TbHKT1 did not show a clear phenotype, indicating the presence of other K^+ transporters in *T. brucei*.

Over-expression of a cytochrome b5 reductase-like protein causes kDNA loss in *Trypanosoma brucei*

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We identified from an RNAi library a *T. brucei* cell line with inducible loss of kinetoplast DNA (kDNA). Further analysis revealed that the RNAi vector had aberrantly integrated into a site homologous to its insert, resulting in inducible over-expression of downstream genes. We over-expressed each gene individually and found that one, which encodes a protein with homology to cytochrome b5 reductase (and we call CBRL), caused kDNA loss. Cytochrome b5 reductase in other organisms is a membrane-anchored flavoprotein used for various cellular reductions. We localized a GFP-tagged CBRL to the mitochondrion which is consistent with kDNA loss. To understand the basis for kDNA loss we examined UMSBP, which binds the minicircle replication origin and triggers replication. The Shlomai lab had shown that UMSBP might be regulated by REDOX; monomeric forms are competent to bind the origin but disulfide-linked dimeric forms are not. We found by western blotting of wild type *T. brucei* proteins that UMSBP is monomeric. However, upon CBRL over-expression, UMSBP exists as disulfide-linked oligomers. Analysis of free minicircle replication intermediates in CBRL over-expressing cells revealed an increase in covalently-closed species, as expected for loss of a protein required for replication initiation. Unexpectedly, we also found that over-expression of a mitochondrial tryparedoxin peroxidase causes UMSBP oligomerization and kDNA loss. Our current goal is to understand the biochemical mechanism explaining how over-expression of these two proteins induces UMSBP oligomerization.

Highly efficient tandem affinity purification of trypanosome protein complexes based on a novel epitope combination

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Tandem affinity purification (TAP) allows for rapid and efficient purification of epitope-tagged protein complexes from crude extracts under native conditions. The TAP tag consists of a duplicate protein A epitope, a tobacco etch virus protease cleavage site and the calmodulin-binding peptide (CBP). Our attempts to purify TAP-tagged transcription factors from crude *Trypanosoma brucei* extracts, however, failed due to the inefficiency of the calmodulin affinity chromatography step. To overcome this problem, we created a new epitope combination, termed PTP tag, by replacing CBP with the protein C epitope (ProtC). ProtC binds with high affinity to the monoclonal antibody HPC4 which has the unique property of requiring calcium for target recognition. Thus, analogous to the calcium-dependent CBP-calmodulin interaction, ProtC-tagged proteins can be released from immobilized HPC4 by EGTA. While this feature was maintained, PTP purification has several advantages. The method is reliable because it is not affected by endogenous calmodulin which may block the interaction of CBP and the calmodulin column. It is also very efficient resulting in coomassie-stainable bands of transcription factors from 2.5 liter of a procyclic cell culture. In cases where EGTA inactivates protein, ProtC-tagged proteins can alternatively be eluted with ProtC peptide. Moreover, HPC4 is a highly sensitive and specific tool for the detection of ProtC-tagged proteins in trypanosome protein pools. To facilitate N- and C-terminal PTP tagging and purification, we have generated vectors for stable in-allele integration which allow the fusion of a protein coding sequence to the PTP tag in a single cloning step. Thus far, we have successfully PTP-tagged and purified *T. brucei* TRF4, SNAPc, RNA polymerase I, and the U1 snRNP.

Global analysis of genes expressed in human brain microvascular endothelial cells in response to *T. brucei* infection

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African trypanosomiasis, commonly called sleeping sickness has been claimed to be more deadly than other vector-borne diseases such as malaria. More than 300,000 people are infected and about 55 million are at risk of infection with trypanosomes. The mechanism of invasion of T. brucei into the human brain remains unknown. Microarray-based approaches allowed us to identify which genes or pathways might be involved in a disease process. The context in which these genes are differentially expressed provides insights into functional responses of both host cells and pathogens. Analysis of gene expression in human brain microvascular endothelial cells in response to T. brucei infection showed that different sets of genes were expressed at 1, 3, 6 and 18 h after infection. The number of genes differentially expressed with respect to uninfected cells peaked at 6 h post-infection. We postulate that the time lag may be due to parasite-dependent events that are required before the host cell recognizes the invading parasite and responds to the invasion with differential gene expression, thus showing the use of microarray in advancing biological hypotheses. Knowledge that a gene is expressed under particular conditions is not an answer in itself but only the step in understanding mechanism of disease.

Intracellular distribution of cAMP-specific phosphodiesterases of *Trypanosoma brucei* (TbPDEs)

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In mammalian cells proteins of the cAMP signaling pathway are often located at very specific sites. There is increasing evidence that most or all of the phosphodiesterases are bound to various intracellular structures, where they can interact with other signaling proteins. This binding is often very dynamic and changes with the functional and metabolic state of the cell. Similarly intracellular cAMP concentrations vary widely between different cellular compartments. In *Trypanosoma brucei* at least 2 phosphodiesterase families (TbPDEs) are found. We are interested in the intracellular localization of the TbPDEs and are trying to find possible TbPDE binding proteins. With a PCR based in situ tagging system we are able to fuse various epitop tags to the C-terminus of the TbPDEs in different cell lines. By immunofluorescence and electron microscopy the intracellular distribution of the tagged proteins can be visualized. Our results show that the phosphodiesterase TbPDE2C and TbPDE2B exhibit striking different intracellular localization. TbPDE2C is confined to the paraflagellar rod (PFR), as demonstrated by immunofluorescence analysis of wild type trypanosomes and the PFR-deficient snl-2 mutant (Bastin et al, Journal of Cell Science 113, 3321-3328. 2000). TbPDE2C appears to be an intrinsic component of the PFR and can not be solubilized by detergent extraction.

A cytoskeletal chaperone found in trypanosomes and its role in nutrient uptake

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Molecular chaperones are involved in protein targeting, degradation and translocation across membranes, and in protein folding and assembly in all cellular compartments. Trypanosomes possess four heat shock protein 70 (Hsp70) family members and several dozen unique Hsp40 proteins, some of which are organelle specific. Tcj1 is a member of the *T. cruzi* Hsp40 family that is specifically associated with the flagellar cytoskeleton via its unique C-terminal substrate-binding domain. The tcj1 mRNA and protein are expressed at very low levels in normal cells and visualization of protein production and localization requires expression of a transgene. Tcj1 is encoded by a single copy gene and deletion of the two allelic copies by homologous recombination yields viable cells having at least two interesting phenotypes. Scanning electron microscopy revealed tcj1-/- epimastigotes to have an invagination in the surface membrane in the exact location near the flagellar pocket in all cells, which is not visible upon complementation by a tcj1-expressing episome. This invagination fits the description of the cytostome, a specialized region in *T. cruzi* epimastigotes involved in nutrient uptake. Uptake of several small molecules is reduced by approximately 50 percent in the tcj1-/- mutant compared to wildtype or complemented lines, further linking the invagination with the process of nutrient uptake. Although *T. brucei* is not known to possess a cytostome, it does contain a tcj1 homologue having 64 percent identity with the *T. cruzi* protein. Inhibition of tbj1 by RNA interference is underway to determine the role of this protein in the biology of *T. brucei*.

Cytidine deaminases in *Trypanosoma brucei*

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In higher eukaryotes, cytidine deamination of nucleic acids plays an important role in a variety of processes such as mRNA editing, or gene conversion, class switching, and somatic hypermutation of immunoglobulin genes. The AID/APOBEC deaminase family contains members that can deaminate cytidine in RNA and/or DNA. For example, apolipoprotein B RNA-editing cytidine deaminase (APOBEC1) targets mRNA, whereas activation-induced cytidine deaminase (AID) and APOBEC3 deaminate DNA to trigger pathways in adaptive and innate immunity. We asked if there are cytidine deaminases in *Trypanosoma brucei* involved in specific cytidine to uridine editing. Searching the *Trypanosoma brucei* genome database, we identified two potential cytidine deaminases located on chromosomes 9 and 8. The cytidine deaminase on chromosome 9 (CD09) is 180 amino acids long and shows homology to yeast and human metabolic cytidine deaminases. The second cytidine deaminase (CD08) is located on chromosome 8 and is composed of 225 residues. CD08 contains cytidine and deoxycytidylate deaminase zinc-binding regions and a bipartite nuclear localization signal. Molecular modeling suggests that CD08 is an AID analog. Overexpression of CD08 in *E. coli* increased the mutational frequency, similarly to overexpressed AID. Both CD08 and CD09 were able to deaminate dCTP to dUTP *in vitro*. Depletion of CD08 mRNA by RNAi causes growth arrest in bloodstream-form cells, suggesting that *CD08* is an essential gene. By indirect immunofluorescence, we showed that CD08 is detectable in the cytosol, while CD09 is located in the mitochondrion of *T. brucei*.

Taking a ride on the spliced leader RNA promoter: SLACS retroposon expression in *Trypanosoma brucei*

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The RNA interference (RNAi) machinery has been implicated in the regulation of numerous cellular processes, including modification of histones, chromatin remodeling and the silencing of mobile elements. We have previously shown that retroposon-derived siRNAs are constitutively expressed in *Trypanosoma brucei* and that RNAi deficiency leads to an increase in retroposon transcript abundance via mechanisms operating at the transcriptional and at the RNA stability level. To begin to understand how RNAi regulates the levels of retroposon transcripts, we used permeable cells to investigate how one class of retroposons, known as SLACS (Spliced Leader Associated Conserved Sequence) is expressed. SLACS is a site-specific non-LTR retroposon that inserts exclusively into the spliced leader (SL) coding sequence. Using alpha amanitin, we demonstrate that SLACS are transcribed by an RNA polymerase II machinery also responsible for the synthesis of the SL RNA. The conclusion that SLACS are expressed from the SL promoter was confirmed by primer extension and RT-PCR, which detected SLACS transcripts initiating at the +1 position of the SL coding sequence. Since RNAi deficiency in *T. brucei* leads to upregulation of retroposon transcripts, it is possible that mobilization of retroelements is occurring in these cells. To address this hypothesis, RNAi-deficient cells were selected under drug pressure for about a month and re-cloned. Southern blot analysis revealed dramatic changes in restriction fragments hybridizing to a SLACS probe in several RNAi-deficient clones as compared to wild type cells. This result may be attributable to chromosomal instability, leading to rearrangement events, or to an activation of retroposition via the SLACS-encoded reverse transcriptase. Experiments are in progress to test these possibilities.

Acetyl-CoA carboxylase is required for fatty acid synthesis in *T. brucei*

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Acetyl-CoA carboxylase (ACC) catalyzes the first committed step in fatty acid synthesis: the synthesis from acetyl-CoA of the 2-carbon donor, malonyl-CoA. *T. brucei* ACC is expressed in both BSF and PCF. TbACC is structurally similar to yeast and mammalian ACCs in that all the sub-activities reside on a giant multifunctional polypeptide. To examine the function of TbACC, we knocked down its mRNA by RNAi in both BSF and PCF. There was no growth phenotype in either stage, although RNAi was effective ($\sim$80% knock-down of mRNA and $>$90% knock-down of protein). Interestingly, RNAi of TbACC did inhibit BSF growth in lipid-depleted medium, suggesting a requirement when exogenous lipids are limiting. We were unable to generate homozygous knock-outs in BSF, suggesting that TbACC may be essential. Finally, labeling experiments revealed that fatty acid elongation is inhibited in TbACC RNAi cells, indicating that TbACC is required for fatty acid synthesis *in vivo*. ACCs in other eukaryotes are usually cytoplasmic, but some are membrane-associated. In addition, some ACCs are regulated by polymerization, with the polymer form being active. TbACC is predicted to be soluble and cytosolic. However, localization experiments with TbACC raise the possibility of its polymerization and/or membrane localization. For example, immunofluorescence localized TbACC to numerous cytoplasmic puncta, distinct from the expected diffuse cytosolic pattern. We are now investigating the nature of these puncta and determining the polymeric state of TbACC under conditions known to promote or inhibit polymerization. Given ACC's role in fatty acid synthesis, determining the localization and regulation of TbACC should enhance our understanding of how fatty acid synthesis is modulated in response to the environment.

Stage-specific requirement for the lysosomal membrane glycoprotein, p67, in *Trypanosoma brucei*

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p67 is a lysosomal type I membrane glycoprotein of *Trypanosoma brucei*. The processing, turnover, and targeting of p67 have been characterized, but its function remains unknown. There are 8 copies of the *p67* gene in the *T. brucei* genome, making deletion analysis technically challenging. Therefore, we have used a tet-inducible RNA-interference (RNAi) system to explore the role of p67. Induction of p67 RNAi for 24 hours results in an 80-95% reduction in p67 protein production in both bloodstream and procyclic stage trypanosomes. The reduction of p67 results in a severe growth defect in the bloodstream stage but has very little effect on growth in the procyclic stage. In both lifecycle stages, p67 production is restored upon extended induction of the dsRNA transcript, suggesting a loss of the ability to effect RNAi. In bloodstream stage trypanosomes, this reversion to normal levels of p67 protein production corresponds to a recovery of cells to normal growth. We have focused on the effects of p67 RNAi in bloodstream cells. Gross cellular morphology is clearly affected within 24 hours post-induction and is very pronounced after 48 hours. Surprisingly, p67 ablation has no discernable effect on processing or localization of the major lysosomal protease, trypanopain, as demonstrated by pulse-chase labeling and immunofluorescence microscopy. Receptor-mediated endocytosis is also unaffected as shown by uptake of a fluorescently-labeled tomato lectin. Immunofluorescence microscopy reveals no effects on subcellular architecture, but closer examination by electron microscopy suggests that lysosomal integrity may be compromised by the loss of p67. Collectively these data are most consistent with a structural/protective role in the intensely hydrolytic lysosome of bloodstream trypanosomes.

A highly conserved type I protein arginine methyltransferase from *Trypanosoma brucei*

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In mammals and yeasts, arginine methylation, catalyzed by protein arginine methyltranferases (PRMTs), has been implicated in regulation of diverse processes such as protein-protein interaction, protein localization, signal transduction, and transcription. Two distinct classes of PRMTs have been identified. Type I PRMTs catalyze the formation of monomethylarginine and asymmetric dimethylarginine, while type II enzymes form monomethylarginine and symmetric dimethylarginine. In *T. brucei*, gene regulation is controlled at the levels of RNA processing and translation, and likely involves RNA-binding proteins. As the majority of type I PRMT substrates are RNA-binding proteins, arginine methylation is likely to play a key role in trypanosome gene regulation. We have identified and cloned a type I PRMT from *T. brucei*, which we named TbPRMT1. TbPRMT1 is 51% identical to human PRMT1 and possesses common and absolute features associated with other PRMTs. Recombinant TbPRMT1 is able to methylate an artificial RG-rich peptide, as well as TBRGG1, a mitochondrial RNA-binding protein, and exhibits slightly different substrate specificity and biochemical features compared to rat PRMT1. TbPRMT1 is constitutively expressed during the trypanosome life cycle. Disruption of TbPRMT1 gene expression by RNA interference results in a modest growth defect. In addition, we observe a dramatic decrease in the levels of asymmetric dimethylarginine in the cell upon TbPRMT1 knockdown, indicating that TbPRMT1 catalyzes robust protein arginine methylation *in vivo*. We are currently analyzing the phenotype of TbPRMT1 RNAi cells in detail. Finally, proteins interacting with TbPRMT1 have been identified by yeast two hybrid screening. The interaction of one of those proteins, a previously unidentified Zn finger-containing protein, is being further characterized.

ARL1 and ARF1 have essential roles in protein trafficking in *Trypanosoma brucei*

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The enzyme myristoyl-CoA: protein N-myristoyltransferase (NMT) catalyses the *N*-myristoylation of target proteins and is essential for viability in *Leishmania major* and *Trypanosoma brucei*. In our investigations to define the essential downstream targets of NMT, we have identified over 60 potentially *N*-myristoylated proteins in the *T. brucei* genome, including 5 members of the ADP-ribosylation factor (ARF) family of GTPases, which have roles in protein trafficking, vesicle formation and receptor-mediated endocytosis. The related ARF-like proteins are largely uncharacterized except ARL1, a trans-Golgi localizing protein with a role in the maintenance of Golgi structure and function. The *T. brucei* ARL1 orthologue is localized to the Golgi apparatus and is differentially expressed only in the mammalian bloodstream form of the parasite. RNA interference has been used to demonstrate that ARL1 depletion results in abnormal morphology, including disintegration of the Golgi structure, followed by cell death. The parasites are endocytically active but the exocytosis flux of VSG to the parasite surface is inhibited. In contrast, depletion of a putative ARF protein results in gross enlargement of the flagellar pocket, appearance of intracellular flagella and severe inhibition of endocytosis.

Molecular characterization of genes encoding HSP70 protein in *Trypanosoma rangeli*

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Trypanosoma rangeli and *Trypanosoma cruzi* are sympatric parasites that cause American trypanosomiosis. While *T.cruzi* is the etiological agent of Chagas disease, *T.rangeli* infection in humans is harmless. These parasites share common antigens. Moreover, *T.rangeli* mice infection induces an immune response that leads to protection against *T.cruzi* challenger. Beside the potential of HSPs as adjuvants in subunit vaccines, recent studies have suggested that *T.cruzi* HSP70 antigen alone or fused to *T.cruzi* KMP-11 protein could be a good candidate for the induction of immunoprotective cytotoxic responses against *T.cruzi* natural infection. Therefore, in this work we have studied the HSP70 genes from Tre, a KP1 (-) strain of *T.rangeli*. These genes are encoded by a multi-copy gene of 2500 bp in length. This tandem array is located in a single chromosome of approximately 1.1 kb in the Tre and C23 KP1(-) strains and in Choachi, a KP1(+) strain whereas it is located in two chromosomes in the KP1(+) H14 strain. The amplification and sequencing of these genes showed polymorphism between the two copies cloned. The alignment of the *T.rangeli* HSP70 deduced amino acid sequence with the HSP70 protein from *T.cruzi* revealed an identity of 91 %. *T.rangeli* HSP70 protein is 17 aminoacids shorter than *T.cruzi* protein and it only has 5 of the 11 repetition of the C-ter motif GGMP present in *T.cruzi* HSP70 protein. Interestingly, the T cell epitopes A72 (TLLTIDGGI) and A79 (RIPKVMQLV) from *T.cruzi* protein are conserved in *T.rangeli* deduced amino acid sequence.

cAMP phosphodiesterases in *Trypanosoma cruzi*

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Cyclic AMP is involved in vital processes such as proliferation and differentiation in kinetoplastid protozoa. The molecules responsible for the degradation of this second messenger, the cyclic nucleotide phosphodiesterases (PDEs) are important pieces of the intricate network responsible for cAMP signaling in these parasites. According to their respective genome projects, several PDEs are encoded by *Trypanosoma cruzi*, *T. brucei* and *Leishmania mayor*. Some of the annotated genes correspond to functional PDE enzymes, but a few of them can be identified as pseudogenes, since they lack key amino acids responsible for enzymatic activity. In this work, two genes located in tandem in *T. cruzi* choromosome VI, coding for enzymes designated as TcPDE2C and TcPDE2B, were expressed in HEK-293 cells. The recombinant enzymes are specific for cAMP as substrate, and show a remarkable resistance to inhibition by classic mammalian PDE inhibitors. They share a modular structure similar to that of mammalian PDEs including two GAF domains (regulatory domains). An ORF annotated in the *T. cruzi* Gene DB as a PDE is a pseudogen differentially expressed during the parasite life cycle, as well as the two active TcPDE genes, indicating a possible functional role. GAF domains highly homologous to those found in these proteins were also identified in *T. rangeli*, *Leishmania mexicana*, *L. amazonensis*, *L. braziliensis* and *Crithidia fasciculata*, suggesting that the members of kinetoplastid PDE2 family are likely to have the same regulatory mechanism, as TbPDE2B, which we have recently described as regulated by cAMP. Complete characterization of PDEs will increase the knowledge in the signalling mechanisms undergoing in kinetoplastids, allowing a rational drug design.

Phospholipid metabolism by Phospholipase A_1 in *Trypanosoma brucei*

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Phospholipids play important roles in signal transduction, GPI anchor biosynthesis, and as fatty acid depositories. The biological significance surrounding phospholipid metabolism in trypanosomatids is exemplified by the approval of Miltefosine, a phospholipid analogue that exhibits antiprotozoal activity, as a drug for oral therapy of Visceral Leishmaniasis. Ironically, the mechanisms of action of phospholipid analogues against trypanosomatids remain unknown due to a lack in understanding about how trypanosomatids synthesize and metabolize phospholipids; and our interest lies in identifying and targeting the enzymes and pathways involved in these processes in *Trypanosoma brucei*. Various phospholipases metabolize phospholipids by each specifically hydrolyzing ester bonds on the lipid molecule. Whereas phospholipases C, D, and A_2 have been well studied and their roles in cell signaling understood, much less is known about phospholipase A_1, which has a disparate character in eukaryotic cells. We have identified and cloned a phospholipase A_1 ($TbPLA_1$) of prokaryotic origin from *T. brucei* and expressed the protein recombinantly both *in vitro* and *in vivo*. The purified enzyme is monomeric, Ca2+-independent, and requires interfacial activation with optimal activity at pH 6.0-8.5. $TbPLA_1$ exclusively catalyzes the hydrolysis of acyl groups at the *sn*-1 position of a broad range of phospholipid substrates, including lysophosphatidylcholine. The enzyme contains the serine active site lipase motif GXSXG, but it is insensitive to phenylmethylsulfonyl fluoride and diisopropyl flourophosphate. $TbPLA_1$ accounts for all of the observed phospholipase A_1 activity in trypanosome lysate since lysate from a double knockout cell line has lost its phospholipase A_1 activity, whereas an ectopic copy of $TbPLA_1$ inserted into the double knockout restores activity.

Essential role of glucose transporters in the life cycle of *Leishmania mexicana*

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While glucose is a major source of metabolic energy for *Leishmania* promastigotes, intracellular amastigotes transport much less glucose and derive metabolic energy primarily from oxidation of fatty acids. Despite this down-regulation of glucose metabolism in amastigotes, glucose transporter null mutants of *L. mexicana* (the *Dlmgt* line) are viable as promastigotes but not as amastigotes. In addition to their role as metabolic fuel, hexoses are precursors for the biosynthesis of a variety of glyconjugates in these parasites, so that the essential role of glucose transporters might be explained by the requirement for glycoconjugate biosynthesis. To address this question, we have examined glycoconjugates in glucose transporter null mutant promastigotes and determined that the parasites continue to synthesize all glycoconjugates examined, including lipophosphoglycan, proteophosphoglycan, glycosylinositolphospholipids, glycoproteins, and the storage glycoconjugate mannan. Continued synthesis of glycoconjugates in the absence of hexose transport is apparently explained by activity of gluconeogenesis, as gluconeogenic precursors can be incorporated into complex carbohydrates in the *Dlmgt* null mutant. The non-viability of the *Dlmgt* null mutant as amastigotes is likely due to down-regulation of gluconeogenic enzymes in amastigotes, thus making the parasites dependent upon glucose uptake for synthesis of essential glycoconjugates. These results may explain why glucose transporters are essential in the life cycle stage that down-regulates glucose transport and metabolism.

The tryparedoxins of *Leishmania infantum*: characterization of *Li*TXN5 a tryparedoxin-like protein

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Tryparedoxins (TXNs) are trypanosomatid-specific redox-active proteins which, among other potential functions, are involved in peroxide detoxification in these organisms. TXNs up to now described are reduced only by trypanothione and are considered promising drug targets. *L. infantum* presents five tryparedoxin-like sequences. Two of them encode typical tryparedoxins, *Li*TXN1, cytosolic, and *Li*TXN2, mitochondrial. Work underway is addressing the essentiality of these enzymes and defining their function along the parasite life-cycle, namely whether they are important for *Leishmania* resistance towards exogenous and endogenous oxidative and/or nitrosative stresses. Regarding the other *L. infantum* TXN like sequences, *Li*TXN4 is a pseudogene, however *Li*TXN3 and *Li*TXN5 have the potential to encode functional proteins, albeit with substantial differences from the active tryparedoxins studied before. Both possess a hydrophobic tail in their C-terminus and present a number of changes in amino acids considered important for tryparedoxin activity. The question therefore arises whether these are true tryparedoxins. Data will be presented concerning the characterization of *Li*TXN5. We show that this protein is expressed mainly in dividing promastigotes and in amastigotes. To determine subcellular localization, *L. infantum* expressing an epitope-tagged form of *Li*TXN5 were produced. *Li*TXN5 was found predominantly around the nucleus, but not exclusively, in a pattern suggesting ER localization. TritonX-114 detergent and Na2CO3 fractionation indicated that *Li*TXN5 is an integral membrane protein. Enzymatic analysis confirmed that this protein has no activity in the enzymatic cascade that leads to peroxide detoxification, that is, it has no tryparedoxin activity. Other potential functions for this protein in the parasite are being explored and will be discussed.

A conserved nuclear methyltransferase is required for hypermethylation of the cap structure of trypanosome U-snRNAs

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Many U-snRNAs contain a distinctive hypermodified 2,2,7-trimethylguanosine (TMG) cap structure, which is formed by post-transcriptional methylation of m^7G caps. Recently, the *Saccharomyces cerevisiae* methyltransferase Tgs1 (trimethylguanosine synthase 1) was shown to be required for TMG cap formation. Its localization to the nucleolus suggested a trafficking pathway of U-snRNAs through this compartment, whereas in mammals the hypermethylase is present both in Cajal bodies and in the cytoplasm. At present, little is known about the maturation of U-snRNAs in trypanosomes. The current evidence is consistent with the primary transcript containing an m^7G moiety, but it is not clear whether the conversion of m^7G to TMG takes place in the cytoplasm or in the nucleus. To address this issue, we have characterized the *T. brucei* Tgs1 homologue (TbTgs1), a 28 kDa protein, which is mainly composed of the conserved catalytic domain and lacks a large N-terminal domain present in higher eukaryotes. A GFP fusion with TbTgs1 revealed that this protein localizes predominantly in the nucleus, and in particular in a distinct subcompartment reminiscent of Cajal bodies. RNA interference of TbTgs1 resulted in a slow-growth phenotype and demonstrated an essential role in cap hypermethylation of both snRNAs and snoRNAs. Since we were not able to generate a double-knockout of TbTgs1, we concluded that the cap hypermethlyase is encoded by an essential gene. Our results suggest that m^7G cap hypermethylation in trypanosomes is restricted to the nuclear compartment.

The effect of anti-actin drugs on the polarity and function of the actin cytoskeleton of *Trypanosoma brucei*

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Actin is expressed at similar levels but is distributed differently in bloodstream and procyclic forms of *Trypanosoma brucei*. In bloodstream forms the actin cytoskeleton is highly polarized and colocalizes with the endocytic pathway, whereas in procyclic forms actin is uniformly distributed. Actin is essential in bloodstream forms and is required for the formation of coated vesicles from the flagellar pocket membrane. However, procyclic forms continued to grow when actin expression was repressed. We have employed drugs that interact with actin to investigate further the actin cytoskeleton in *T. brucei*. Evidence will be presented that indicates: (i) Polarity of actin distribution in bloodstream forms depends on formation of F-actin. (ii) The effect of actin RNAi can be mimmicked using inhibitors of actin polymerization. (iii) Procyclic forms do not appear to form F-actin. (iv) Trypanosome F-actin appears to be insensitive to typical drugs that interact with F-actin from higher eukaryotes The significance of these results will be discussed.

Alterations in traffic, lipid biosynthesis and pro-Cruzipain maturation in *T. Cruzi* overexpressing Chagasin

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Chagasin, a protein recently characterized in *Trypanosoma cruzi* , is the prototype of a new family of inhibitors of papain-like cysteine proteases. These inhibitors were also identified in other parasitic protozoa, as well as in *Pseudomonas aeruginosa*. In *T.cruzi*, we have recently shown that chagasin is able to interact with cruzipain, the major cysteine protease of this parasite, forming tight binding complexes and regulating the activity of the enzyme. In addition, these parasites displayed lower capability to differentiate into trypomastigotes and increased resistance to a trypanocidal synthetic CPI, as compared to the wild-type. Furthermore, tissue culture trypomastigotes were less infective than wild type in vitro, due to lower membrane-associated CP activity. Here, we demonstrate that increased chagasin expression interferes with the intracellular traffic and maturation of cruzipain zymogens, which we show to be present at the Golgi apparatus of epimastigotes. The reduction on the cruzipain activity in these parasites leads to an accumulation of pro-cruzipain and increase secretion of these precursors to the flagellar pocket. The uptake of transferrin-FITC and the subsequent recycling of the fluorofore, but not of BSA-FITC, were compromised in chagasin overexpressing epimastigotes, suggesting an alteration of receptor-mediated endocytosis. In agreement with that, sub-cellular fractionation followed by biochemical and ultra-structural characterization of the endo-lysosomal compartments indicated alterations in the density of the microssomes of the transfected parasites. In addition, logarithmic growing chagasin overexpressing epimastigotes showed a modified rate of [3H]palmitate-labeled glycolipids during time-course incorporation experiments. Taken together, these results indicate that chagasin contributes to the control of pro-cruzipain activation and sorting, ultimately influencing the traffic in the secretory compartments.

Purification of the 19S proteasomal regulatory complex from *Trypanosoma brucei* by affinity chromatography

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Three-dimensional structure of the 19S regulatory complex in the 26S proteasome remains unresolved presumably due to the presence of isomeric subunits in the complex and the lack of structural integrity in vitro. The 19S complex of *Trypanosoma brucei* is composed of six Rpt and 11 Rpn subunits like the others. A knockdown of each of the 17 individual subunits by RNA interference, however, invariably leads to accumulation of polyubquitinated proteins and growth arrest in *T. brucei*. The 19S complex is also readily dissociated from the 20S proteasome upon cell lysis but remains intact under a variety of *in vitro* conditions. It is thus a stable protein complex of irreplaceable individual subunits and is an ideal candidate for three-dimensional structural analysis. One of the 19S subunit tbRpt6 tagged with a triple HA was expressed in transfected procyclic-form *T. brucei* and incorporated into the 19S complex. The latter was purified to near homogeneity by affinity chromatography and found to contain all its subunits in SDS-PAGE and Western blot. We are now aiming at scaling up the purification procedure to provide sufficient pure *T. brucei* 19S complex for X-ray crystallographic analysis.

Inactivation of the dynamin-like protein of *T. brucei* inhibits division of the mitochondrion and cytokinesis

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The parasitic protozoon *Trypanosoma brucei* has a single mitochondrion only and no fission or fusion events are observed during the interphase of the cell cycle. However, extensive mitochondrial fission can be induced by ectopic expression of the human pro-apoptotic protein Bax. Mitochondrial fission generally requires the activity of a member of the dynamin protein family. Interestingly *T. brucei* possesses only a single dynamin (TbDLP), whose sequence is most similar to the dynamin-like proteins which in other organisms are involved in mitochondrial fission. Conditional inactivation of TbDLP by RNA interference or by expression of a dominant-negative TbDLP variant leads to a growth arrest. Furthermore, if DLP is inactivated in Bax-expressing *T. brucei*, mitochondrial fission is abolished, indicating that the trypanosomal DLP is required for mitochondrial division. Further analysis of the cell lines lacking functional DLP by DAPI staining and by flow cytometry shows an approx. 3,5-fold accumulation of cells having two mitochondrial genomes and two nuclei (2K2N). Among these 2K2N cells we find a subtype that is approx. 60 fold enriched. These cells show a quasilinear order of Nuclei (N) and kDNAs (K) which is N-K-K-N and thus corresponds to a late stage of cytokinesis. In order to better understand the phenotypes the lack of TbDLP causes, we also performed immunofluorescence analysis by using hemaglutinine-tagged TbDLP. Interestingly, these experiments revealed a dual localization of the protein in (i) a punctate pattern associated with the mitochondrion and (ii) at the basal bodies. - In summary our results suggest that TbDLP has multiple functions and may be involved in the coordination of mitochondrial fission and cytokinesis.

An essential topoisomerase IA of *Trypanosoma brucei*

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The completed sequencing of the *T. brucei* genome allowed identification of three genes with open reading frames which could encode topoisomerases with homology to the type IA topoisomerases. One of these located on chromosome 10 would encode an 806 amino acid protein with homology to the bacterial topo IAs. We have found that this gene is transcribed to produce a 4 Kb mRNA. In cultured procyclic *T. brucei* cells ablation of the mRNA by RNAi causes growth arrest after four days. The growth arrest is accompanied by disappearance of the kinetoplast DNA. Southern analysis of kinetoplast minicircle DNA reveals the accumulation of structures which may be intermediates in the replication of the minicircles. Although topoisomerases of types IB and II have been reported and found essential to *T. brucei*, to our knowledge this is the first recognition of an essential bacterial-like topoisomerase IA in these organisms.

Post translational modifications in the universal minicircle sequence binding protein regulate its binding at the kDNA replication origin

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Replication of kinetoplast DNA (kDNA) minicircles of trypanosomatids initiates at conserved origin sequences that are bound by the universal minicircle sequence binding protein designated UMSBP. Post-translational modifications of UMSBP were examined as a plausible mechanism that controls its binding at the origin during kDNA replication initiation. UMSBP was found to be phosphorylated *in vivo*, exclusively in serine residue(s). Protein phosphorylation was inhibited *in vivo*, as well as in partially fractionated *C. fasciculata* cell extracts, by a specific protein kinase C (PKC) inhibitor. Furthermore, purified UMSBP served as a specific substrate for PKC *in vitro*. Both UMSBP dimerization and its DNA binding activity are redox-sensitive. Oxidation of UMSBP results in the protein dimerization, mediated through its N-terminal domain, with a concomitant inhibition of its DNA-binding activity. UMSBP reduction yields monomers that are active in the binding of DNA, through the protein C-terminal region. Tryparedoxin activates the binding of UMSBP to UMS *in vitro*. Remarkably, phosphorylation of UMSBP inhibits the protein binding to UMS *in vitro* and was found to be associated mainly with UMSBP dimers *in vivo*. It appears that both UMSBP phosphorylation and its redox state play a functional role in the regulation of UMSBP binding onto the origin site. Analysis of UMSBP activity in synchronized *C. fasciculata* cell cultures revealed no significant changes in the protein binding activity throughout the cell cycle, when assayed under reducing assay conditions. However, UMSBP binding activity, assayed under non-reducing conditions peaks during the S and M phases, implying the cell cycle control of the protein redox state.

Sequence variations affecting *trans*-splicing efficiency in *Trypanosoma brucei*

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mRNA maturation in *Trypanosoma brucei* is dependent on *trans*-splicing, and it has long been suggested that variations in *trans*-splicing efficiency could be an important step in controlling the levels of individual mRNAs dissected out from polycistronic transcription units. Early experiments showed that successful splicing requires specific sequence elements such as a conserved 5' splice site, a branch point sequence, a pyrimidine rich region (polyY tract), a 3' splice site (3'SS), and possibly exon-internal enhancer elements. In order to explore the essential elements of the *trans*-splicing signal more systematically and in greater depth, we constructed a luciferase-beta-galactosidase double-reporter system to analyze sequence requirements for efficient *trans*-splicing, in the polyY tract and surrounding the 3'SS. By testing more than 90 constructs, we demonstrated that the optimal polyY tract length is $\sim$25 nt. Interspersing a purely Uridine-containing polyY tract with Cytidine resulted in an increase in *trans*-splicing efficiency, whereas interspersing a few purines led to a large decrease. We demonstrated that the position of the polyY tract relative to the 3'SS is important and that the nucleotides in the -3 and -4 positions can have very large inhibitory effects on *trans*-splicing. Finally, we showed that a suboptimal 3'SS requires a second AG site further downstream, which may aid in recruitment of the splicing machinery. These systematic findings should assist the development of improved algorithms for identifying 3'SS and help to discriminate non-coding open reading frames (ORFs) from coding ORFs in current efforts to annotate the *T. brucei* genome.

Characterization of alanine aminotransferase in *Trypanosoma brucei*

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Alanine aminotransferase (AAT) is one of the most abundant enzymes in the cytoplasm of bloodstream forms of *Trypanosoma brucei* (1 unit/mg). AAT catalyses the following reversible reaction: ALANINE + KETOGLUTARATE → PYRUVATE + GLUTAMATE In most cells transaminases can function in the direction of amino acid synthesis or degradation depending on the precise concentrations of the substrates and products. The role/direction of the high level of AAT activity in bloodstream forms of *T. brucei* is unclear, although the exclusive end product of the highly active glycolytic pathway in these cells is pyruvate under aerobic conditions (typically 220 nmol/min.mg). We have characterized the activity encoded by the single putative AAT gene in *T. brucei* and assessed its function by RNA interference.

Multiple roles for acyl carrier protein in the trypanosome life cycle

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Trypanosoma brucei evades the immune system by antigenic variation of a surface glycoprotein possessing a myristoylated GPI anchor. In studying the source of myristate, our lab discovered a fatty acid synthesis pathway whose major product is myristate. The genome reveals two putative fatty acid synthesis pathways: a type II system in the mitochondrion and an elongase system in the ER. Acyl carrier protein (ACP) plays a critical role in type II systems. Immunofluorescence places *T. brucei* ACP in the mitochondrion of both procyclic forms (PCF) and bloodstream forms (BSF). RNAi (with nearly complete loss of ACP mRNA) suggests that TbACP is essential for growth only in PCF while KO studies indicate an essential role in both forms. We have been unable to rescue growth by the addition of fatty acids to the medium. Unexpectedly, TbACP loss has little or no effect on in vitro fatty acid synthesis demonstrating that the mitochondrial type II system is not a major contributor to these in vitro products (see SooHee Lee's abstract). Presently, we are working to identify the fatty acid products of the mitochondrial pathway. We are also considering alternative roles for TbACP. In some organisms, ACP is a subunit of respiratory Complex I and is essential for its assembly. Oxygen uptake studies indicate that TbACP RNAi inhibited respiration in PCF, supporting a role in Complex I. We are currently trying various biochemical approaches to confirm this observation in PCF. BSF, on the other hand, lack a conventional respiratory chain; thus we believe ACP is required for its role in fatty acid synthesis.

Thiamine metabolism and Melarsoprol

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Thiamine, also known as vitamin B_1, is a water-soluble, B-complex vitamin, mainly necessary for the metabolism of carbohydrates and fats. It is also involved in membrane function. In its diphosphate form (TDP) thiamine functions as a cofactor in more than 24 enzymes. Beriberi is the classical thiamine deficiency syndrome. Experiments in *S. pombe* show that Melarsen Oxide (MelOx), the reactive component of the antitrypanosomal drug Melarsoprol, interferes with thiamine metabolism. Are some antitrypanosomal drugs targetting proteins of thiamine metabolism? For the investigation of this question we study aspects of thiamine metabolism in *Trypanosoma brucei*. We found that *T. brucei* is prototrophic for thiamine and that thiamine diphosphate (TDP) represents the major intracellular form both, in cells grown in the presence and absence of thiamine. Surprisingly, growth of trypanosomes is not abolished or reduced by toxic thiamine analogues such as pyrithiamine or oxithiamine. We show by lysis protection and growth inhibition assays that the toxicity of Melarsen Oxide for trypanosomes can be reduced by high concentrations of thiamine. This shows the interference of vitamin B_1 with the drug. At the molecular level we investigate TDP dependent enzymes, which play an important role in energy production during glycolysis and citrate cycle. Also thiamine biosynthetic enzymes seem to be very interesting, because they are potential drug targets due to their lack in mammals. In summary, the aim of this project is the description of thiamine metabolism in *Trypanosoma brucei*.

UDP-GlcNAc pyrophosphorylase as a potential drug target in African trypanosomes

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Nucleotide sugars serve as high energy donor substrates for the biosynthesis of complex carbohydrates, such as N/O-linked glycans and glycosylphosphatidlylinositol anchors. The formation of certain nucleotide sugars have been shown to be essential for survival in several protozoan parasites, including Trypanosoma brucei, thus validating them as potential drug targets. This study describes the ongoing work of examining the enzyme uridine diphosphate N-acetylglucosamine (UDP-GlcNAc) pyrophosphorylase (EC 2.7.7.23), that catalysed the reaction GlcNAc1-P + UTP -¿ UDP-GlcNAc + PPi, as a potential drug target in *T. brucei*. Thus far, we have cloned and expressed parasite pyrophosphorylase and demonstrated enzymatic activity. Gene knockout and subcellular localisation studies are ongoing. This work is supported by an MRC PhD studentship to MJS and a Wellcome Trust Programme grant.

Developing a one step PCR-based method for gene disruption and *in vivo* epitope tagging in *Crithidia fasciculata*

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An efficient gene targeting system has been developed for analysis of gene function *in vivo* in *Crithidia fasciculata*, in order to take advantage of the *Trypanosoma* and *Leishmania* genome projects, which have provided sequence information of orthologous genes. We first optimized the electroporation conditions for episomal expression of a hygromycin resistance gene to give a transfomation efficiency of 3,000-5,000 cfu/ug plasmid DNA from approximately 40% viable transformed cells. Based on these conditions, we disrupted both alleles of the nonessential *KAP1* gene by introducing a DNA fragment with 0.5 and 0.8 Kb of homologous sequence on 5' and 3' termini in a single electroporation. Western blot results showed that expression of the KAP1 protein was abolished in approximately 20% of the colonies with double drug-resistance while the transfomation efficiency decreased to 10-30 cfu/ug introduced DNA. To investigate whether a PCR based strategy is applicable in *C. fasciculata*, we used PCR fragments with only 200 bp or 60 bp of the homologous region for gene disruption. Using primers complementary to sequence flanking the targeted gene fused to the drug resistance gene, PCR analysis of DNA isolated from drug resistant colonies showed that 20-30% of the resistant colonies had the expected integration. With a transfomation efficiency of approximately 50 cfu/ug of DNA fragment, this result suggests that PCR primers containing 60 bp of homologous sequence for the targeted gene and another 20 bp specific for the drug resistance gene can be used to amplify the desired fragments for the targeted gene disruption. We are currently using this method to disrupt *C. fasciculata* kDNA replication related genes.

Biochemical and functional characterization of serine proteases in *Leishmania donovani*

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Serine proteases are known to be crucial in the infectious lifecycle of parasitic protozoa. Recent chemical inhibition studies by our group suggest that this class of enzymes is critical to the lifecycle of *Leishmania*. For example, treatment of ¡/I¿L. donovani¡/I¿ with the broad serine protease inhibitor, Pefabloc, attenuated growth. Initial biochemical studies of ¡/I¿L. donovani¡/I¿ extracts identified several serine protease activities. One class of important serine proteases in eukaryotes is convertases. In the apicomplexan parasite *Plasmodium*, subtilisin-like convertases have been identified that have a central role in erythrocyte invasion and in the maturation of merozoite surface protein 1 (Barale *et al.*, 1999). Using bioinformatics we have identified an ¡/I¿L. donovani¡/I¿ subtilisin. This enzyme has been deleted by gene targeting and knockout parasites are currently under phenotypic analysis. Initial investigations suggest that these mutant parasites show impaired promastigote to amastigote differentiation. An additional class of serine peptidases that has been identified in the trypanosomatids is the oligopeptidases. *T. cruzi* oligopeptidase B signaling has been shown to mediate host cell invasion (Caler *et al.*, 1998). Through the use of biochemical fractionation and mass spectrometry we have purified two leishmanial oligopeptidases, oligopeptidase B and a prolyl oligopeptidase. These enzymes are currently being biochemically characterized and gene targeting is underway. Our most recent findings on this research will be discussed.

The endoplasmic membrane network associated protein Tb292 is essential for cytokinesis in *Trypanosoma brucei*

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The Tb-29 family contains two members, Tb291 and Tb292, both encoding proteins with a large domain consisting of several repeats of an octapeptide (EARLRAEE). Apparent trans-membrane domains are present in Tb-292 but not in Tb-291. Tb-29 proteins are abundantly distributed in the area surrounding the nucleus, the region between the nucleus and the flagellar pocket in both the procyclic form and the bloodstream-form trypanosomes. This sub-cellular distribution suggests a possible role for Tb29 proteins in the cell's vesicular transport system. Attempts to knock out Tb-29 genes were failed, suggesting that Tb-29 family may serve essential biological function. We further explored the function of Tb-292 protein by RNA interference approach. Induction of Tb-292 RNAi rapidly leads to a reduced growth and cell death. 24 hour after induction of Tb-292 RNAi, most of procyclics showed two kinetoplasts and two nuclei phenotype and were unable to divide. In addition, Tb-292 RNAi also resulted in drastic morphological changes in procyclics including the shortening of flagellum and changing from the polarized elongated shape to the apolarized round shape. Longer induction of Tb292 RNAi led to accumulation of more kinetoplasts as well as nuclei per cell indicating that DNA synthesis machinery is unaffected though cells have severe defects in cytokinesis. At 48h of Tb292 RNAi induction, procyclics started dying. Since Tb-29 proteins may be important components of trypanosome's vesicular transport system, down-regulation of Tb292 may severely affect the membrane trafficking, subsequently leading to severe defects in cytokinesis. Hence, we hypothesize that Tb292 is essential for cytokinesis of trypanosomes.

Okadaic acid reveals the mechanism regulating G1/S checkpoint in *Trypanosoma brucei*

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Mitosis and cytokinesis are highly coordinated in eukaryotic cells. But procyclic-form Trypanosoma brucei under mitotic arrest is capable of cytokinesis and cell division resulting in anucleated daughter cells (zoids). Cytokinesis is thus apparently driven by the kinetoplast cycle in this organism. Okadaic acid (OKA), an inhibitor of protein phosphatases PP1 and PP2A, is known to inhibit kinetoplast segregation and cytokinesis without affecting mitosis in these cells, yielding multinucleated cells with single mitochondrial networks (Das et al., 1994, J. Cell. Sci. 107: 3477-3483). Procyclic-form T. brucei was arrested in G1 phase with extended and/or branched posterior morphology, when expression of its cdc2-related kinases 1 and 2 was knocked down together (Tu and Wang, 2005, Mol. Biol. Cell 16: 97-105). When these G1 cells were treated with OKA, nuclear DNA synthesis and nuclear division were both resumed while kinetoplast segregation and cell division remained blocked. These changes resulted in the formation of multinucleated and single-kinetoplast cells similar to the OKA-treated wild-type except that the aberrant posterior morphology of G1 cells still remains. Thus, inhibition of certain phosphatase(s) PP1 and/or PP2A by OKA can apparently release the G1-arrested cells into normal nuclear cell cycle progression. Seven PP1 and three PP2A homologues are present in T. brucei. We are currently using RNA interference to knock down expression of each of the ten phosphatases or in various combinations to elucidate the mechanism of G1/S transition in T. brucei, which does not possess any CDKI homolog such as sic1, p21 or p27.

An LC/MS method for identifying sugar nucleotides in Trypanosomes

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The protozoan parasites *T. brucei* and *T. cruzi* contain numerous glycoproteins, many of which are essential for growth or pathogenicity. The sugar chains of glycoproteins are synthesized from activated nucleotide sugar donors by glycosyltransferase enzymes. This raises the possibility that sugar nucleotide biosynthesis enzymes and glycosyltransferases may be potential drug targets in these organisms. In fact, this has already been shown for the enzyme involved in the biosynthesis of UDP - galactose in *T. brucei*. There are several examples of glycoproteins which contain sugars that are not routinely found in trypansomatids. For example, the glycoprotein gp72 of *T. cruzi* contains xylose, rhamnose and fucose. The sugar nucleotide biosynthesis pathways of these unusual sugars may provide more specific and novel drug targets. To this end, we have designed a mass spectrometric based method to identify low abundance sugar nucleotides in parasite cell extracts. The method is able to detect UDP - rhamnose, UDP - xylose and GDP - fucose in *T. cruzi* epimastigotes, consistent with the presence of these sugars in the gp72 glycoprotein. GDP- fucose but not UDP - xylose or UDP - rhamnose can be found in both the procyclic and bloodstream forms of *T. brucei*. This is consistent with the presence of GDP-fucose biosynthesis enzyme homologs in the *T. brucei* genome database. These putative biosynthesis enzymes have been over-expressed in *E. coli* and assayed using the mass spectrometric method to show that they are able to convert GDP - mannose to GDP - fucose in vitro.

Permeability of *Trypanosoma brucei* aquaglyceroporins for non conventional solutes: As III, Sb III, and dihydroxyacetone

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The different facilities of water and polyol transport by aquaglyceroporins have been well characterized in several types of living organisms. However, in *Escherichia coli, Saccharomyces cerevisiae, Leishmania* and mammals, aquaglyceroporins are involved in uptake of cytotoxic compounds. Two toxic agents were identified as permeant to these channels: As III and Sb III. Interestingly, arsenical and antimonial drugs are the first choice for treatment of sleeping sickness or leishmaniasis, respectively. Recently, we described aquaglyceroporins of *Trypanosoma brucei*-TbAQPs- as water/glycerol/dihydroxyacetone facilitators (Uzcátegui et al 2004) and now we are currently investigating the selectivity profile of aquaglyceroporins as entry pathways of drugs. Yeast heterologously expressing TbAQPs were hypersensitive to As III, and Sb III, suggesting that these compounds are taken up by TbAQPs. These drugs kill bloodstream form of *Trypanosoma brucei* in a nanomolar range (IC50: 308 nM or 107 nM, respectively). On the contrary, the transformants showed a resistant phenotype in the presence of As V. Since this substance is effective against trypanosomes (IC50: 6.77 μM), another entry pathway into the cell must be assumed. Methylglyoxal was also tested and only yeast cells expressing TbAQP2 were affected, a result that revealed the importance of the structural differences between TbAQP1,3 and TbAQP2 in the pore. The former ones exhibit the classical NPA motifs, whereas TbAQP2 present an NSA and an NPS motif. Additionally, we also demonstrated that dihydroxyacetone (DHA) inhibits *Trypanosoma brucei* growth and causes a cell-cycle arrest in G2/M. DHA kinase, an enzyme involved in detoxification of DHA, is not present in trypanosomes. In conclusion, the newly described TbAQPs could represent important anti-trypanosomal drug vehicles.

Determinants of substrate specificity in a nucleoside transporter from *Leishmania donovani*

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Parasitic protozoa are unable to synthesize purines de novo and must salvage them from their hosts using high affinity nucleoside or nucleobase transporters. The LdNT1 permease from *L. donovani* mediates the uptake of adenosine and the pyrimidine nucleosides, whereas the LdNT2 transporter takes up inosine and guanosine. As part of continuing structure-function studies, we have mutagenized charged amino acid residues in LdNT1 that are located within predicted transmembrane (TM) helices, as such charged residues are often critical for the function of transporters. The mutations E94D (TM2) and K153A (TM4) reduced adenosine transport activity by >95% compared to wild type permease, indicating a crucial role in substrate permeation. Of considerable interest, a K153R mutation was still active in adenosine uptake but acquired the ability to transport inosine, which is not normally a substrate for LdNT1. Hence, this mutation induces a change in substrate specificity, further confirming that K153 is central to transport function and is likely to be a component of the 'permeation pathway' that mediates translocation of substrate across the membrane. Preliminary evidence suggests that the E94D mutant that has lost most of the adenosine transport capacity may also have acquired the ability to transport inosine. Parallel forward genetic studies on the CfNT2 permease from *Crithidia fasciculata* have also implicated the corresponding K residue in TM4 as a determinant in substrate specificity for that inosine/guanosine transporter. Comprehensive studies of this type will allow us to map components of parasite nucleoside permeases that are essential for transport function.

Host dependent expression of the transferrin receptor in *Trypanosoma brucei*

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Trypanosomes express a heterodimeric transferrin receptor (Tf-R) that mediates iron uptake from the bloodstream. The genes encoding the receptor, *ESAG6* and *ESAG7*, are found at the beginning of each VSG expression site (ES). Different ESs encode Tf-Rs that are similar but not identical. These differences determine the binding affinity for transferrins from different hosts. Transfer of *T. brucei* variant 221a from calf to dog serum based medium induces an acute iron starvation, as the Tf-R of variant 221a binds dog Tf poorly. The trypanosomes try to overcome this acute iron shortage by a 5-fold increase in Tf-R mRNA and protein within 8 hours. Since iron stores are still high 8 hours after transfer, we infer that the signal for Tf-R overproduction is the decreased availability of cytosolic iron when cellular iron import drops. Up to 30% of the extra Tf-R spills out of the flagellar pocket onto the pellicular surface and we have shown that this Tf-R is functional and contributes to Tf uptake. We have proposed that the rapid upregulation of Tf-R levels in response to acute Tf starvation helps the trypanosome to accumulate enough cytosolic iron to support growth. After this initial response the trypanosomes can alter the nature of the Tf-R expressed to allow growth in the presence of new host Tf. This involves either switching to another ES and expressing a different Tf-R or by adaptation without switching. This adaptation involves either a very high increase in the expression level of the Tf-R genes (from the active and/or inactive ESs) or a replacement of the *ESAG6* and/or *ESAG7* gene in the active ES.

Identification and characterization of a membrane/HDL addressing domain in Apolipoprotein L-I

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ApoL-I is a human-specific serum apolipoprotein bound to HDL particles that is able to lyze the African trypanosome *Trypanosoma brucei*, but not the parasite subspecies adapted to humans (*T. b. rhodesiense*, *T. b. gambiense*). Trypanosome lysis results from uptake of apoL-I in the lysosome. We have shown that apoL-I kills trypanosomes by uncontrolled osmotic swelling of the lysosome, most probably mediated by Cl- influx through an apoL-I ionic pore (see Pérez-Morga *et al.*). Here we show that the pore forming domain of apoL-I in flanked by a pH-sensitive bifunctional domain. Under (human) serum conditions, the domain appears to be responsible for the attachment of apoL-I to HDL particles, enabling efficient HDL receptor-mediated uptake of the lytic factor by the trypanosome. Acidic conditions (late endocytic compartments) seem to reduce the affinity of the domain for the HDL particles probably through conformational remodeling. The free domain is able to address apoL-I to biological membranes. Functional evidence indicates conserved membrane targeting activity in bacteria, yeast and trypanosomes.

Dissecting the developmental regulation of GPI-PLC expression in *Trypanosoma brucei*

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Differentially expressed mRNAs have been used to investigate the post-transcriptional regulation of gene expression that predominates in *T. brucei*. On differentiation from bloodstream forms (BSF) to procyclics there is a 50-fold down regulation of *GPI-PLC* mRNA, consistent with a 10-fold decrease in message half life. In order to discover which part of the *GPI-PLC* mRNA confers developmental regulation, the *GPI-PLC* gene was modified at its endogenous locus by insertion of drug resistant ORFs and tubulin inter-ORF sequences upstream and/or downstream of the *GPI-PLC* coding sequence. The expression pattern of the resulting chimaeric mRNAs indicates that the *GPI-PLC* 3'UTR is both necessary and sufficient for developmental regulation at the mRNA level. One chimaeric gene generated by this approach encodes a neoR mRNA with a *GPI-PLC* 3'UTR. This message is expressed in BSFs but is absent on differentiation to procyclics, which are consequently sensitive to G418. Two methods have been used to delineate the responsible sequence element(s) within the 2.3 kb 3'UTR. Classical deletion analysis defines a 500 b region at the 3' end of the message which is sufficient to confer developmental regulation. Selection for spontaneous loss of function mutants (gain of G418 resistance) also indicates that removal of the last 800 b of the 3'UTR may be sufficient to achieve procyclic expression. Down regulation of reporter expression in procyclics proved to be very robust; G418 resistant cells only arose in conjunction with alterations in the *GPI-PLC* 3'UTR. In the future, such reporter genes could be used as the basis of a non-presumptive screen for trans-acting factors involved in stage specific message instability.

Protein kinases and flagellar length regulation

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The molecular basis of organelle and cell size control in eukaryotes is largely unknown. As a unicellular model for organelle size, flagella of the green alga *Chlamydomonas reinhardtii* and of the kinetoplastids *Leishmania* and *Trypanosoma brucei* have been used. The flagellum appears to be a stable organelle, however, its internal structures like the microtubules and appended proteins and additionally the components of the paraflagellar rod (PFR), a crosshatched structure running along the axoneme in kinetoplastids, are constantly turning over. Intraflagellar transport (IFT) has been described as the mechanism responsible for the perpetual delivery of material to the flagellar tip for assembly and removal of used material. As such it regulates the growth of a new flagellum and its maintenance. We have found protein kinases classified by amino acid sequence homology as members of the mitogen-activated protein (MAP) kinase family which we could show by deletion analysis to be critically involved in flagellar length regulation. One pair of kinases comprised of a MAP kinase kinase and a MAP kinase is involved in shortening of the flagellum. The other pair is critical for the elongation of the flagellum. In both cases we demonstrated phosphorylation and activation of the MAP kinase by its interacting MAP kinase kinase. This is the first time that partial signal transduction cascades have been described in *Leishmania*. Moreover, related proteins have been found in other eukaryotes indicating that the regulation of flagellar length by protein kinases is a conserved mechanism.

Identification and partial characterization of proteins from the tomato lectin binding fraction in *Trypanosoma brucei*

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Bloodstream forms of African trypanosomes depend on receptor-mediated uptake of host-derived factors, e.g. transferrin and lipoproteins, for growth. Endocytosis in bloodstream forms of the African trypanosomes occurs at very high rates but is restricted to a small specialized region of the cellular surface, termed the flagellar pocket (FP). Only a few proteins from the FP/endocytic pathway have been characterized. Tomato lectin can be used to isolate a sub fraction of proteins from the FP/endocytic pathway since only proteins from this region of the cell contain linear N-linked poly-N-acetyllactosaminyl side chains (pNAL) and bind specifically to the lectin. We have employed a variety of techniques to identify and characterize the proteins present in the TL-binding fraction. To date we have identified 19 different proteins some of which have been described before but most represent potential new candidate proteins for further investigation. A summary of the current situation (function/location/glycosylation and expression status etc) of each proteins will be presented.

Pyrimidine biosynthesis and salvage in *Leishmania donovani*

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Existing information on the pyrimidine biosynthetic and salvage pathways of *Leishmania* is extremely limited. To overcome this dearth of knowledge, we have initiated a molecular and biochemical dissection of the pyrimidine pathway of *Leishmania donovani* by cloning all the genes for both pyrimidine biosynthesis and salvage. A single cosmid containing the five genes that encode the six enzymatic activities of the *de novo* pyrimidine biosynthesis pathway was isolated from a *L. donovani* library using portions of the *L. major* carbamoyl phosphate synthetase (CPS) and dihydroorotate dehydrogenase (DHOD) as probes. Although the organization and genomic structure of the genes has not been rigorously determined, the presence of all five genes on the same cosmid indicates a clustering of the pyrimidine biosynthesis genes in a manner similar to that observed in *L. major* , *Trypanosoma cruzi*, and other trypanosomatids. The *L. donovani* gene encoding uracil phosphoribosyltransferase, an enzyme that is conjectured to be the sole enzyme of pyrimidine salvage, has also been cloned. All of the pyrimidine genes from *L. donovani* have now been sequenced in both directions and inserted into bacterial expression vectors for protein production, purification, and biochemical analysis. In addition, flanks of the CPS gene have been inserted into vectors carrying drug resistance markers in order to create null mutants by targeted gene replacement. These molecular reagents will now provide the cornerstone for a thorough molecular, biochemical, genetic, and cell biological analysis of a parasite metabolic pathway that has potential for both drug and vaccine development.

Identification of two dimethylases involved in rRNA modifications in*Trypanosoma brucei*

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The maturation of rRNA requires nucleotide modifications, including 2'-*O*-methylation, isomerization of uridine to pseudouridine and base modifications, in addition to multiple cleavages. The 3' end of the rRNA associated with the ribosomal small subunit contains a well-characterized $m_2{}^6Am_2{}^6A$ doublet adenosine modification found in all organisms. Using alignments of the known rRNA:m^6A and mRNA:m^6A methyltransferase families, we created position-specific scoring matrices and used them as queries to detect two groups of dimethylase homologs in kinetoplastids. First, we identified a *Trypanosoma brucei* homolog (Dim1A) of *Saccharomyces cerevisiae* Dim1p. Dim1p catalyzes the dimethylation of the 18S rRNA and *T. brucei* possesses the conserved nucleotides A_{2232}-A_{2233} in the rRNA of the ribosomal small subunit. The second homolog (Dim1B) shows similarity to the dual function mitochondrial transcription factor B that doubles as a dimethylase of mitochondrial rRNA. The localizations and functions of these enzymes are being characterized using GFP fusion proteins and RNAi experiments. Preliminary results indicate Dim1A has a nucleolar localization and shows a severe growth phenotype upon RNAi induction. Base methylations of the 18S rRNA and mitochondrial 9S rRNA will be assayed by primer extension and other techniques.

Creation of a *Leishmania donovani hgprt/xprt* conditional null mutant

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Leishmania are obligatory scavengers of host purines and utilize hypoxanthine-guanine phosphoribosyltransferase (HGPRT), xanthine phosphoribosyltransferase (XPRT), adenine phosphoribosyltransferase (APRT), and adenosine kinase (AK) to salvage purines from their hosts. To establish the relative contributions of these enzymes to purine homeostasis and parasite virulence, *îaprt*, *îhgprt* and *îxprt* cells were generated in an infective strain of *L. donovani* (*Ld-Bob*). All of these mutants were viable and virulent. Mutants lacking APRT, HGPRT, XPRT and/or AK activities were successfully created in various combinations. However, it was not possible to generate the *îhgprt*/*îxprt* double knockout in any genetic background, buttressing the hypothesis that either HGPRT or XPRT is required for *L. donovani* viability. Remarkably, a conditional *îhgprt*/*îxprt* double knockout has finally been constructed in the presence of deoxycoformycin (DCF), an inhibitor of adenine deaminase, using adenine as a sole purine source. Unlike wild type *L. donovani*, the *îhgprt*/*îxprt* line is absolutely dependent on the presence of DCF and either adenine or adenosine in the culture medium, and cannot proliferate in any other purine nucleobase or nucleoside tested. Furthermore, although îhgprt/îxprt parasites are capable of transformation into axenic amastigotes, their ability to infect cultured macrophages is severely compromised. The ability of the knockout strains to infect mammals will now be tested. The conditionally lethal phenotype of the *îhgprt*/*îxprt* null mutant validates our central hypothesis that either HGPRT or XPRT is absolutely essential for parasite viability and demonstrates that all exogenous purines are funneled to hypoxanthine and xanthine by *L. donovani* promastigotes and amastigotes.

Functional characterization of DNA polymerase beta and DNA polymerase beta-PAK from *Trypanosoma brucei*

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T. brucei has two mitochondrial DNA pol beta enzymes, pol beta and pol beta-PAK (Saxowsky et al., 2003). Both have dual activities, nucleotidyl transferase and deoxyribosephosphate (dRP) lyase. Localization of these enzymes (antipodal sites for pol beta; kDNA disk for pol beta-PAK) is consistent with a role in gap filling during kDNA replication. Their lyase activity suggests an additional role in DNA repair. To test for these functions, we conducted RNAi on each enzyme separately and on both simultaneously. RNAi on Pol beta using a stem-loop vector arrests growth but has no effect on kDNA. RNAi on Pol beta-PAK using a stem-loop vector causes only a modest growth reduction with no effect on kDNA. RNAi on both genes simultaneously, using pZJM, causes growth arrest and modest kDNA loss ($\sim$ 40% of cells by day 9 have no kDNA). Double knockdown affects the population of free minicircle replication intermediates in two ways. First, it promotes a reduction in gapped/nicked minicircles after day 8. Second, it promotes appearance of a linearized minicircle species starting at day 2. Based on gel electrophoresis of minicircle samples not treated with proteinase K, the linearized species is likely covalently attached to protein (probably a topo II). The linearized species also appears following RNAi of pol beta-PAK alone. These effects on free minicircle intermediates support a role for these enzymes in kDNA replication.

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