Mitochondrial Complexes in *Trypanosoma brucei*

A NOVEL COMPLEX AND A UNIQUE OXIDOREDUCTASE COMPLEX

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African trypanosomes, early diverged eukaryotes and the agents of sleeping sickness, have several basic cellular processes that are remarkably divergent from those in their mammalian hosts. They have large mitochondria and switch between oxidative phosphorylation and glycolysis as the major pathways for energy generation during their life cycle. We report here the identification and characterization of several multiprotein mitochondrial complexes from procyclic form *Trypanosoma brucei*. These were identified and purified using a panel of monoclonal antibodies that were generated against a submitochondrial protein fraction and using tandem affinity purification (TAP) tag affinity chromatography and localized within the cells by immunofluorescence. Protein composition analyses by mass spectrometry revealed substantial divergence of oxidoreductase complex from that of other organisms and identified a novel complex that may have a function associated with nucleic acids. The relationship to divergent physiological processes in these pathogens is discussed. *Molecular & Cellular Proteomics* 7:534–545, 2008.

The *Trypanosoma brucei* group of protozoan parasites causes fatal human African trypanosomiasis or sleeping sickness in large regions of Africa, whereas closely related strains and species that cause important diseases in animals extend into Asia Minor and South Asia. *Trypanosoma cruzi* causes Chagas disease in humans in South and Central America, and *Leishmania* pathogens cause a spectrum of human and animal diseases in tropical and subtropical regions. The genomes of *T. brucei* group trypanosomes, *T. cruzi*, and *Leishmania* pathogens have been sequenced. Despite their early divergence, the genomes of these three groups of pathogens have substantially similar gene content, gene order, and biological processes (1). Notably they all have large polycistronic gene clusters within which the order and content of genes, i.e. synteny, is extensively conserved. However, these genomes also have characteristic differences that tend to occur at breaks in synteny and chromosome ends. These differences may reflect the different diseases that these pathogens cause. e.g. *T. brucei* has numerous minichromosomes, subtelomeric variant surface glycoprotein expression sites, and large gene families that provide for antigenic variation that the other two pathogens lack. Annotation of the completed genome sequences predicts over 7,000 clustered orthologous groups (distinct genes and gene families) of proteins of which ~½ have no known function. The results presented here identify numerous mitochondrial proteins, characterize the complexes within which they occur, and identify the general functions of many genes that were previously annotated as hypothetical.

Biological processes require precise positioning of molecules in time and space (2). This function is often accomplished by their organization into macromolecular complexes. Indeed most cellular proteins exist in multiprotein complexes (3, 4). Proteins that are in the same complex can differ in specific function, but they function in the same overall process and hence have a related general function. Affinity purification of specific proteins and their associated complexes from cellular fractions using monoclonal antibodies (mAbs)¹ and tandem affinity purification (TAP) tag is a useful approach for studying the physical associations and functional properties. The TAP tag procedure, which was originally developed in yeast (5), is widely used to isolate proteins and complexes under native conditions (6). This approach has been applied for large scale screens of prokaryotic and eukaryotic cells to identify a growing collection of cellular complexes (4, 7, 8), and we and others have used it to purify and characterize multiprotein complexes from trypanosomatids (9–12).

The large mitochondrion of *T. brucei* plays crucial roles in diverse biological processes. All of its numerous mitochondrial proteins, except for the 18 that are predicted to be encoded in its mitochondrial genome, are nuclearly encoded, translated in the cytoplasm, and imported into the mitochon-

¹ The abbreviations used are: mAb, monoclonal antibody; mt, mitochondrial; TAP, tandem affinity purification; IP, immunoprecipitation; IFA, immunofluorescence assay; PF, procyclic form; DAPI, 4',6-diamidino-2-phenylindole; OGDC, 2-oxoglutarate dehydrogenase complex; PDC, pyruvate dehydrogenase complex; E3BP, E3-binding protein; BLAST, Basic Local Alignment Search Tool; PSI-BLAST, Position-Specific Iterated BLAST; put-MRB, putative mitochondrial RNA binding; RBZ domain, zinc finger domain in Ran-binding proteins.
The mitochondrion harvests unique processes, including the uridylate insertion/deletion RNA editing that is catalyzed by multiple protein mitochondrial complexes (13). We have used a combination of mAb immunoprecipitation (IP) and TAP tag purification to determine the composition of the mitochondrial complexes that catalyze steps of editing, the 20 S editosomes (10, 14). During the course of these studies we generated a panel of mAbs against a sub mitochondrial fraction containing hundreds of proteins (15). The present proteomics study used these mAbs and TAP tags to identify and characterize the protein compositions of the complexes to which these mAbs bind and in some cases the proteins that they recognize. It reports novel compositions of several T. brucei complexes that have assigned functions, including an oxidoreductase complex in which many proteins have no recognizable relationship to other known proteins. It also identified a novel multiprotein mitochondrial complex with no known function but motifs suggesting a role involving nucleic acids.

**EXPERIMENTAL PROCEDURES**

**Cell Growth**—T. brucei procyclic form (PF) cells strains IsTaR 1.7a and 29.13 were grown in vitro at 27 °C in SDM-79 medium containing hemin (7.5 mg/ml) and 10% fetal bovine serum. The cells were harvested at midlog phase of growth by centrifugation at 6,000 × g for 10 min.

**Immunoprecipitation of Mitochondrial Proteins/Complexes**—Mitochondrial vesicles were isolated from 1.7a cells by hypotonic lysis followed by density gradient floatation in a 20–35% linear Percoll gradient (16). The enriched vesicles were lysed with 1% Triton X-100 and clarified by centrifugation at full speed in a microcentrifuge for 30 min at 4 °C. The cleared supernatant was collected and fractionated on 10–30% glycerol gradients at 38,000 rpm for 5 h (SW40 rotor, Beckman Instruments), and fractions were collected from the top (17).

A panel of mAbs originally generated against a sub mitochondrial fraction containing complex protein mixtures (15) was used to probe alternate gradient fractions by Western blot and native dot blot analysis. The peak reactive fractions for specific mAbs were pooled and purified by calmodulin affinity chromatography by binding to calmodulin binding peptide tag and eluted with EGTA (19). The tagged pyruvate dehydrogenase complex (TAP030) was purified from cell lysate prepared by two-step lysis with Nonidet P-40 detergent as described previously (4). Briefly the cells were lysed with 0.25% Nonidet P-40 and cleared by low speed centrifugation, and the supernatant was further lysed with 1.25% Nonidet P-40 and cleared by high speed centrifugation. The tagged complexes were isolated by sequential IgG affinity and calmodulin affinity columns.

**SDS-PAGE**—The immunoprecipitated protein samples bound to the beads and the TAP-tag-purified samples were denatured in SDS-PAGE loading buffer and resolved by 10% SDS-PAGE. The proteins were stained with SYPRO Ruby stain (Molecular Probes) and detected by UV fluorescence. For mass spectrometry analysis, either individual proteins bands were excised from the full-length gel, or the protein samples were precipitated with 6 volumes of acetone, and the proteins were denatured with 8M urea, 1 mM DTT, diluted 1:4, and digested with sequencing grade modified trypsin (Promega) overnight; the resulting peptides were extracted with 50% acetonitrile, 5% formic acid solution and dried in SpeedVac. The TAP tag-purified protein samples were precipitated with 6 volumes of acetone, and the proteins were denatured with 8 M urea, 1 mM DTT, diluted 1:4, and digested in solution with trypsin. The resulting peptides were purified using C18 beads (magnetic Dynabeads RPC18, Invitrogen). The peptides from gel pieces or complex mixtures were fractionated by nanoflow liquid chromatography using a 10-cm-long × 75-µm-inner diameter C18 capillary column and analyzed on line by electrospray ionization tandem mass spectrometry. The bound peptides were
eluted from the C18 column at a 200 nl/min flow rate with a 45-min linear gradient of 5–40% acetonitrile in 0.4% acetic acid followed by a 5-min linear gradient of 40–80% acetonitrile in 0.4% acetic acid.

Xcalibur 1.4 SR1 version software was used to collect mass spectrometry data, and the mass range for the MS survey scan was m/z 400–1400. The MS/MS data of the five most intense ions were collected sequentially following each MS scan using the dynamic exclusion parameter where a specific ion was sequenced only twice and was excluded from the list for 45 s. The peak lists were generated using the SEQUEST module of Bioworks 3.1, cluster version SR1 (Thermo Electron) using the default parameters (molecular weight range, 400–3500 Da; precursor mass tolerance, 1.4; group scan, 25; minimum ion count, 15). The acquired data were compared with the T. brucei version 4.0 protein database (20) (GeneDB) using the TurboSEQUEST program in the BioworksBrowser 3.1 software package (Thermo Electron). The database contained 9,210 T. brucei nuclearly encoded protein sequences of which 612 are annotated as hypothetical uniquely (20) plus 18 mitochondrially encoded protein sequences. Also mouse immunoglobulin heavy and light chains, bovine serum albumin, and human keratin sequences were included in the database. No enzyme was specified during the SEQUEST search, peptide mass tolerance was set at 1.5, and fragment ion tolerance was set at 0.0. No fixed modification was set for any of the amino acids, but differential modification for “Met” was set at 15.994 Da. The SEQUEST search results were filtered and compiled using PeptideProphet and ProteinProphet programs (21, 22). The data set presented here includes only the doubly tryptic peptides that have minimum peptide identification probability of 0.9 and have a minimum SEQUEST X-correlation value of 1.5 for +1 ions, 1.8 for +2 ions, and 2.5 for +3 ions. We excluded any peptide containing more than one missed trypsin cleavage site in the sequence and any peptide containing a cysteine amino acid because the alkylation step was not carried out during sample preparation. Proteins containing these peptides and with minimum identification probability of 0.9 were considered positive.

Sequence Analysis—The general characteristics of the proteins were predicted by database homology searches. BLASTp and PSIBLAST searches were carried out against the National Center for Biotechnology Information (NCBI) database to identify any homologous or related proteins. The probable functions of the proteins were assigned based on homology searches for motifs and domains in the PROSITE database, InterPro database, and Conserved Domain Database (CDD). If no related protein was identified then the protein was designated as novel.

Immunofluorescence Assay—Subcellular localization of the proteins in the cell was determined by immunofluorescence assay (IFA) using specific mAbs as described previously (15). The expressed tagged proteins were localized by IFA using anti-Myc mAb. Briefly the cells were fixed with 4% paraformaldehyde, permeabilized with 0.2% Triton X-100, blocked with 5% fetal bovine serum, and incubated with specific mAb tissue culture supernatant at 1:2 dilution or anti-Myc mAb in the case of tagged proteins at 1:100 dilution. After washing the cells were incubated with anti-mouse FITC-conjugated antibody (1:250 dilution), washed, and treated with 4′,6-diamidino-2-phenylindole (DAPI) stain to stain DNA. A phase-contrast image of the cells and their fluorescence was captured with a Nikon fluorescence microscope equipped with a camera and the appropriate filters.

RESULTS

Monoclonal Antibodies—Twenty-seven of the 81 independent mAbs that had been prepared from a complex protein mixture from T. brucei mitochondrial vesicles, including three that are directed against MRP1 (gBP21) (15), were used to characterize mitochondrial proteins and complexes. The antibodies had been prepared against antigens in a ~20 S glycerol gradient fraction of a sample prepared by differential ammonium sulfate precipitation of non-ionic detergent-lysed mitochondria. Twenty-three mAbs were found to be of the IgG1 isotype, one was of the IgG2b isotype, and one was of the IgM isotype (data not shown), but the isotype of the other two mAbs could not be determined. Further analysis was carried using 19 IgG1 mAbs, and 11 of these reacted with proteins in Western blots of mitochondrial lysates, whereas the other eight mAbs only reacted with proteins on native dot blots indicating they are directed against conformational epitopes. Representative glycerol gradient sedimentation profiles of the target proteins/complexes of the mAbs are shown in Fig. 1.

Protein and Complex Identifications—The peak reactive fractions from glycerol gradients of cleared lysates from floatation-purified mitochondria were pooled, and IP in combination with MS was used to identify their target proteins and/or complexes. All but one (mAb41) of the target proteins that reacted with mAbs in Western blots were identified by analyses of their corresponding protein bands from SDS-PAGE.

![Fig. 1.](image-url) Western blot (A) and dot blot (B) analyses of the glycerol gradient-fractionated cleared mitochondrial lysate showing the sedimentation profile of the mAb-reactive proteins. Fraction 1 is at the top of the gradient. The sizes of the mAb-reactive protein bands are indicated.
The mAb-reactive target proteins are indicated in bold.

| mAb          | Associated proteins                                                                 |
|--------------|-------------------------------------------------------------------------------------|
| 81           | Tb10.70.0280/Tb10.70.0430 (heat shock protein 60)                                    |
| 33, 78       | Tb927.6.3740/Tb927.6.3750/Tb927.6.3800 (heat shock protein 70)                       |
| 68           | Tb11.02.0250 (heat shock protein 84)                                                 |
| 56, 61, 67   | Tb11.55.0009 (MRP1/gBP21)                                                           |
|              | Tb11.01.4860 (MRP2/gBP25)                                                           |
| 55           | Tb10.70.4280 ( Δ1-pyruvate-5-carboxylate dehydrogenase)                              |
| 17, 30, 70   | Tb11.01.1740 (2-oxoglutarate dehydrogenase, E1 component)                           |
|              | Tb11.47.0004 (2-oxoglutarate dehydrogenase, E1 component)                           |
|              | Tb11.01.3550 (2-oxoglutarate dehydrogenase, E2 component)                           |
| 66, 72       | Tb11.01.8470 (dihydrolipoyl dehydrogenase, E3)                                       |
| 43, 53       | Tb927.8.6970 (3-methylcrotonoyl-CoA carboxylase α)                                   |
| 41           | Tb11.02.4480 (3-methylcrotonoyl-CoA carboxylase β)                                   |
| 52, 63, 69   | Tb927.7.2570 (component of put-MRB complex 1; see Table II)                          |
|              | Oxidoreductase complex (see Table III)                                              |

* These mAbs are reactive in native dot blot only, and their target proteins are not known. The proteins are named according to GeneDB, and the predicted or demonstrated functions are in parentheses.

* Two proteins having signature sequence characteristics of E1 component were identified in OGDC.

In the case of mAb41, two proteins were identified in the gel piece, and we have not attempted to separate those by two-dimensional PAGE. This approach does not allow the identification of target proteins that only react with mAbs in native dot blots but allows the identification of proteins in complexes that contain the target protein. Different mAbs that reacted in either Western or dot blots that generated the same glycerol gradient sedimentation profiles consistently contained the same proteins. For example, mAb56 and mAb67 only reacted in dot blots and had sedimentation profiles that were similar to that of mAb61 that is specific for MRP1 (previously gBP21), and MS detected MRP1 and MRP2 (previously gBP25). This indicates that mAb56 and mAb67 recognize MRP1 or MRP2. Similarly mAb30 and mAb70 revealed similar gradient profiles, and MS detected the same proteins indicating that both mAbs detect proteins in 2-oxoglutarate dehydrogenase complex (OGDC) (Fig. 1 and Table I). The mAbs directed against mitochondrial heat shock proteins 60, 70, and 84 and Δ1-pyruvole-5-carboxylate dehydrogenase protein pulled down only their respective target proteins; this is consistent with their overall sedimentation at top of the gradient (Tables I and Fig. 1). Other mAbs pulled down multiprotein complexes.

All of the proteins reported in this study were identified by multiple peptide matches except two (Tb927.3.1590 and Tb927.2.6070) that were identified by single peptide match (supplemental Tables 1 and 2). The acquired MS/MS spectra matching the peptides from these two proteins are shown in supplemental Fig. 1. All of the assigned peptides were unique to the matched proteins only, and few of these proteins are encoded by multicopy genes (as indicated in Tables I and II). However, the encoded amino acid sequences by the gene copies are virtually the same for the specific protein.

**Compositions of Multiprotein Complexes**—The compositions of six different multiprotein complexes, five of which were pulled down by multiple mAbs, were determined (Tables I-III). The SDS-PAGE protein profile of the immunoprecipitated complexes are shown in Fig. 2. The incubation and washes during IP were carried out in buffer containing 200 mM salt, which yielded consistent results. Lowering the salt to 150 mM often resulted in pulldown of additional proteins, most of which are likely contaminants, probably as a result of abundance and/or general adsorption to proteins because they were detected in multiple different complexes (results not shown).

MRP1 or MRP2 mAb56, mAb61, and mAb67 (15) pulled down complexes in which only MRP1 and MRP2 proteins were observed upon MS analysis. This is the same result obtained with purified complexes in which MRP1 or MRP2 proteins are TAP-tagged. This confirms the association of these two proteins in *T. brucei* (23) as was reported for *Leishmania tarentolae* (24), but in contrast to the *L. tarentolae* report, no additional proteins were detected in MRP1/2 complexes from *T. brucei*.

Similar analyses with mAb66 and mAb72 pulled down the 3-methylcrotonoyl-CoA carboxylase complex that contained two proteins. One was identified as the β subunit of this

* A. Zíková, J. Kopečná, M. A. Schumacher, K. Stuart, L. Trantírek, and J. Lukeš, manuscript submitted.
complex and was found to react with mAb66. The other protein, which is currently annotated as a putative protein, was found by sequence comparisons with public databases to correspond to the \(/H9251\) subunit. Western blot analysis showed that the complex sediments in the ~20 S region in the glycerol gradient (Fig. 1, mAb66).

Western blot analysis of glycerol gradient fractions revealed that dihydrolipoyl dehydrogenase protein (Tb11.01.8470) sedimented in two peaks (Fig. 1, see mAb70). IP from the upper peak (5–10 S region) with this mAb pulled down only dihydrolipoyl dehydrogenase (E3 subunit) protein that along with its size in Western blots showed it to be the target of mAb70. IP from the lower peak (40 – 60 S) pulled down dihydrolipoyl dehydrogenase protein (E3) along with 2-oxoglutarate dehydrogenase E1 (Tb11.01.1740) and E2 (Tb11.01.3550) components and another protein currently annotated as a putative component of this complex (Tb11.47.0004). Sequence analysis showed that both Tb11.01.1740 and Tb11.47.0004 have a SucA (COG0567.2) domain indicating they are E1 components of OGDC. Hence two E1 components and the E2 and E3 components of OGDC were identified in the mAb affinity-purified complex. Similar results were obtained in IPs with mAb17 and mAb30 (data not shown).

The complex reacting with mAb41 sedimented at the bottom of the gradient, and thus IP was carried out using cleared mitochondrial lysate, and it pulled down the pyruvate dehydrogenase complex (PDC). In addition to PDC α and β subunits dihydrolipoamide acetyltransferase proteins as well as dihydrolipoyl dehydrogenase subunit E3 were identified in the complex (Table I). The composition of PDC was further characterized by analysis of complexes purified by TAP tagging the PDC β subunit (Fig. 3). Tagged PDC β subunit pulled down PDC α and dihydrolipoamide acetyltransferase precursor (Tb10.6k15.3080) and dihydrolipoamide acetyltransferase (Tb10.70.5380), but unlike in the mAb IP, dihydrolipoyl dehydrogenase (Tb11.01.8470) was not detected in the tagged complex perhaps due to the effect of the tag or dissociation during purification. Interestingly the dihydrolipoyl dehydrogenase protein (Tb11.01.8470) was also not detected by Western blot in gradient fraction 23 (Fig. 1A, mAb70) where PDC sediments (mAb41). This is probably due to its dissociation from PDC in the gradient, or PDC containing all of the subunits as described below goes to the pellet at the bottom of the tube that we did not analyze by Western blot. So we carried out IP from cleared mitochondrial lysate without any gradient fractionation that resulted in isolation of PDC containing all subunits. Reciprocally the mAb against dihydrolipoyl dehydrogenase protein pulled down both OGDC and PDC from unfractionated cleared mitochondrial lysate (results not shown) further validating its association with both complexes.

In PDC the E2 subunit corresponds to dihydrolipoamide acetyltransferase protein. The presence of an AceF domain (COG0508.2) along with a 2-oxoacid_dh catalytic domain in Tb10.6k15.3080 (which is currently annotated as dihydrolipoamide acetyltransferase precursor) indicates that this protein corresponds to the E2 subunit of PDC. Tb10.70.5380 (which is annotated as dihydrolipoamide acetyltransferase) contains an AceF domain but lacks the catalytic domain and may correspond to Protein X/E3-binding protein (E3BP), which is related to the E2 component and found in PDC in other eukaryotes (25). A targeted BLAST search against a yeast protein database showed that the N terminus of this protein

| Table II | Proteins identified in put-MRB complex 1 |
|----------|----------------------------------------|
| Protein  | Motifs/domains       | mIP43 | mIP53 | TAP022 | TAP013 | TAP015 |
| Tb927.7.2570^TAP022^a | — | 7 | 9 | 10 | 2 | 2 |
| Tb927.2.3800^a | — | 9 | 8 | 11 | 2 | 1 |
| Tb927.5.3010^TAP013 | — | 6 | ND | 2 | 18 | 11 |
| Tb11.02.5390 | — | 10 | ND | 4 | 2 | 5 |
| Tb11.01.8620 | — | 4 | ND | 4 | 2 | 3 |
| Tb927.8.8180/Tb927.4.4150 | — | 6 | ND | 1 | ND | 3 |
| Tb927.7.800 | — | 3 | ND | ND | 1 | 3 |
| Tb927.4.1500 | ATP-dependent RNA helicase | 21 | ND | 1 | ND | ND |
| Tb927.2.1860 | — | 6 | ND | ND | ND | 3 |
| Tb927.8.8170^b | — | 5 | ND | 1 | ND | ND |
| Tb927.4.4160^b | — | 2 | ND | ND | ND | ND |
| Tb927.6.1680 | C_2H_2 zinc finger | 2 | 1 | ND | ND | ND |
| Tb10.6k15.0150^TAP015 | — | 1 | ND | 1 | 2 | 13 |
| Tb10.406.0050 | RNA binding | 1 | ND | 1 | ND | 2 |
| Tb927.3.1590 | ATPase of the ABC class | 1 | ND | 1 | ND | ND |
| Tb927.2.6070 | RBZ zinc finger | 1 | ND | ND | ND | ND |

^a Protein pair with sequence homology.
^b Protein pair with sequence homology.
has significant sequence homology to both E2 and E3BP of PDC. Thus overall, the *T. brucei* PDC appears to contain five different proteins, the \( /H9251 \) and \( /H9252 \) E1 subunits, the dihydrolipoamide acetyltransferase E2 subunit, the dihydrolipoyl dehydrogenase E3 subunit, and an E3-binding protein.

**A Novel Complex**—A novel complex that sediments at \( /H11011 \) 30–40 S was identified and characterized using two monoclonal antibodies and three TAP-tagged proteins (Table II). Sixteen proteins, none of which have a known function, were identified in samples pulled down with mAb43. These include \( \text{Tb10.389.1140} \), a \( /H11011 \) 50-kDa protein to which mAb53 is directed, a related protein \( \text{Tb927.5.450} \), and a third protein, all of which were identified in pulldowns with mAb53. The two related proteins were identified in pulldowns of TAP-tagged Tb927.7.2570 (TAP022) and two other TAP tag pulldowns, TAP013 and TAP015, along with other proteins. Overall of the 16 proteins identified with mAb43 IP, 14 were detected in at least one other pulldown. Six proteins were detected in all tagged complexes, and more were identified in at least two different tagged complexes. The differential detection with the different antibodies probably reflects conformational and perhaps exposed versus linear and buried epitopes. Similarly detection of more proteins with tagged Tb927.7.2570 versus pulldowns with mAb53 against the same protein suggests that the tag is more accessible than the mAb epitope resulting in isolation of a larger assembled complex and the mAb pulling down a partial complex. The consistent co-detection of Tb927.7.2570 and Tb927.2.3800 implies that they may

| Protein ID       | mIP52 | mIP63 | mIP69 | TAP055 | Domains/motifs                              | Homology with human/bovine proteins               |
|------------------|-------|-------|-------|--------|---------------------------------------------|--------------------------------------------------|
| Tb10.389.1140    | 1     | 2     | 1     | 4      | NADH:ubiquinone oxidoreductase               | NDUF51/NADH-ubiquinone oxidoreductase 75-kDa subunit |
| Tb927.5.450      | 6     | 3     | 3     | 3      | NADH:ubiquinone oxidoreductase, Complex1 51K | NDUFV1/NADH-ubiquinone oxidoreductase 51-kDa subunit |
| Tb927.7.6350     | 1     | 2     | 1     | 2      | NADH:ubiquinone oxidoreductase, Complex1 24K | NDUFV2/NADH-ubiquinone oxidoreductase 24-kDa subunit |
| Tb10.61.1790     | 10    | 9     | 9     | 19     | NADH dehydrogenase                          | NDUFA6/NADH-ubiquinone oxidoreductase B14 subunit |
| Tb11.01.8630     | 1     | ND    | 2     | 2      | L51/S25/Cl-B8                               | NDUFA2/NADH-ubiquinone oxidoreductase B8 subunit |
| Tb927.3.860      | 2     | ND    | 1     | 2      | Acyl carrier protein                        | NDUFAB1/NADH-ubiquinone oxidoreductase 9.6-kDa (SDAP) subunit |
| Tb09.160.5260TAPOSS a | 5     | 5     | 3     | 13     | NADPH:quinone reductase and related zinc-dependent oxidoreductase | trans-2-Enoyl-CoA reductase (4e-27) crystallin, \( \zeta \) (3e-04) |
| Tb927.7.7410 a   | 9     | 9     | 9     | 11     | NADPH:quinone reductase and related zinc-dependent oxidoreductase | trans-2-Enoyl-CoA reductase (2e-20) crystallin, \( \zeta \) (5e-05) |
| Tb10.70.5510     | 5     | 4     | 1     | 5      | NADPH-dependent glutamate synthase \( \beta \) chain and related oxidoreductase | Ferredoxin reductase |
| Tb927.5.3350b    | 4     | 1     | 2     | 4      | Iron/manganese-superoxide dismutases        | Manganese-superoxide dismutase |
| Tb11.01.7480b    | 2     | 2     | 1     | 5      | Iron/manganese-superoxide dismutases        | Manganese-superoxide dismutase |
| Tb09.211.0330    | 1     | 2     | 1     | 3      | DnaJ class molecular chaperone               | DnaJ (Hsp40) homolog, subfamily A |
| Tb927.6.2010     | 7     | 7     | 10    | 14     | Acyl-CoA synthetases                        | Hypothetical protein |
| Tb10.6k15.3040   | 11    | 8     | 5     | 19     | Vacuolar H\(^\text{+}\) -ATPase V1 sector, subunit C | — |
| Tb927.8.4250     | 5     | 4     | 3     | 8      | —                                            | Tetratricopeptide repeat domain 6 |
| Tb927.1.730      | 2     | 1     | 1     | 2      | NB-ARC domain                               | — |
| Tb11.02.4810     | 12    | 9     | 12    | 18     | —                                            | — |
| Tb927.7.7330     | 7     | 4     | 3     | 7      | —                                            | — |
| Tb927.2.4380     | 6     | 2     | 2     | 9      | —                                            | — |
| Tb927.6.1410     | 3     | 3     | 3     | 2      | —                                            | — |
| Tb927.3.3660     | 2     | 1     | 3     | 1      | —                                            | — |
| Tb09.160.0760    | 2     | 2     | 2     | 5      | —                                            | — |
| Tb09.244.2670c   | 2     | 3     | 3     | 7      | —                                            | — |
| Tb11.01.5480c    | 1     | ND    | 2     | 1      | —                                            | — |

\( a \) Protein pair with sequence homology.

\( b \) Protein pair with sequence homology.

\( c \) Protein pair with sequence homology.

**Table III**

Proteins identified in mAb and TAP tag affinity-purified oxidoreductase complex and their sequence characteristics

The proteins were identified by the indicated number of unique peptide matches in the purified sample, and ND denotes proteins not detected by MS. The tagged protein is indicated. Dashes (—) indicate no predicted motifs or domains. mIP, mAb IP.
Proteomics of Mitochondrial Complexes

A

T. brucei procyclic form cells

Mitochondria

Cleared lysate

Gradient fractions

Western blot

IP with specific mAb

Target protein / complex

hypotonic cell disruption

Triton X-100 lysis

10-30% Glycerol gradient

Pool reactive fractions

IP with specific mAb

Western blot

10% SDS-PAGE gel

Fig. 2. A, schematic of the method used for immunoprecipitation of mitochondrial proteins/complexes using monoclonal antibodies except in the case of mAb41 where cleared mitochondrial lysate was used for IP. B, SYPRO Ruby-stained SDS-PAGE protein profile of immunoprecipitated complexes using the indicated mAbs. Protein bands corresponding to immunoglobulin heavy (hc) and light (lc) chains as well as positions of size standards are indicated.

B

M 70 72 41 52 67 43

10% SDS-PAGE gel

Further study is needed to determine whether the compositional diversity reflects stability of protein association or multiple complexes.

Proteins Tb927.7.2570 and Tb927.2.3800 have 31% sequence identity and 47% similarity over 429 amino acids. In addition, proteins Tb927.7.8.170 and Tb927.4.4.4160 have 78% identity and 86% similarity over 903 amino acids. None of these four proteins or seven other proteins have any known motifs (Table II), and thus we could not ascribe any functions to them. PSI-BLAST search showed that these 11 proteins are unique to kinetoplastids. However, sequence analysis against public databases identified motifs/domains indicative of RNA binding/macromolecular interaction for five of the proteins (Table II), and we thus designated it as the putative mitochondrial RNA binding (put-MRB) complex 1. The Tb927.4.1500 protein from N to C terminus, respectively, has a double-stranded RNA binding motif (DSRM), DEXH box helicases/DEAD-like helicases (DEXHc/DEXDc), helicase superfamily C-terminal domain (HELICc), helicase-associated domain (HA2), and a domain of unknown function (DUF1605) that generally occurs near the C terminus of DEAD box helicases. Tb927.6.1680 has five C2H2 zinc finger motifs, Tb927.3.1590 has a COG3044 domain that has a general predicted function of ATPase of the ATP-binding cassette class, and Tb927.2.6070 has six zinc finger-RBZ domains. The C-terminal region of Tb10.406.0050 has an RNA recognition motif, the N-terminal glycine-rich region has sequence homology to RNA helicase GLH-2 of Caenorhabditis elegans, and this protein (designated as TbRGGm) was also identified in mAb affinity-purified editosomes (26). Additional study is needed to determine the biological role of the put-MRB complex 1.

A Unique Oxidoreductase Complex—mAb52, mAb63, and mAb69 as well as TAP-tagged Tb09.160.5260 pulled down a multiprotein complex containing several components with predicted oxidoreductase function (Table III). Unlike with other mAbs, several proteins were identified in the IP samples that are known to be contaminants based on detection in multiple unrelated complexes, presumably reflecting protein abundance. Purified tagged Tb09.160.5260 complexes had a more well defined SDS-PAGE protein profile compared with immunoprecipitated complexes that necessarily also contain antibody chains (Figs. 2 and 3). Proteins that were identified in pulldowns with at least one mAb and in the tagged complex were assigned as components of the oxidoreductase complex. Those proteins identified only in mAb pulldowns or TAP-tagged sample or identified in both but known to be components of other complexes or other known contaminants are listed in supplemental Table 2.

Three different pairs of related proteins were identified among those assigned to the oxidoreductase complex. Tb09.160.5260 and Tb927.7.7410 both have NADPH:quinone reductase and a related zinc-dependent oxidoreductases domain. Tb927.5.3350 and Tb11.01.7480 both have an iron/manganese-superoxide dismutase domain. Tb09.244.2670 and Tb11.01.5480 are related but have no known motifs/domains. Fourteen of the 24 proteins (58%) assigned as components of the oxidoreductase complex in this study are currently annotated as hypothetical. Of these Tb10.61.1790 and Tb11.01.8630 have low level sequence homology to NADH-ubiquinone oxidoreductase. In addition vacuolar H+-ATPase, acyl-CoA synthetases, and NB-ARC domains were identified in Tb10.6x15.3040, Tb927.6.2010, and Tb927.1.730 proteins, respectively. PSI-BLAST search showed that nine proteins (38%) have no known motifs or domains and are unique to kinetoplastids (Table III).

Targeted BLAST search against human and bovine reference protein databases showed that six of the component proteins correspond to the subunits of complex 1 of the mitochondrial respiratory chain (Table III). However, a set of other proteins showed homology to proteins with other functions or unrelated complexes. Most of these proteins were also identified in the complex isolated using tagged complex 1 subunit protein Tb10.61.1790 (NDUFA6/B14) (results not shown). Hence the pulled down complex corresponds to NADH-ubiquinone oxidoreductase complex I but may not represent the complete complex because it lacked the known mitochondrionally encoded subunits and six other proteins that were
identified in *T. brucei* database that have significant sequence homology to human/bovine complex I proteins. Five of the latter proteins are currently annotated as hypothetical proteins, but based on sequence homology *Tb10.70.3150* corresponds to NDUFA5/B13, *Tb09.244.2620* and *Tb10.05.0070* correspond to NDUFA9/39 kDa, *Tb11.01.0640* corresponds to NDUFA13 (GRIM19)/B16.6, *Tb11.01.7460* corresponds to NDUFB9/B22 subunits, and *Tb11.47.0017* corresponds to NDUFS7/PSST subunit. All of these proteins are expressed in *T. brucei* PF cells as determined by mass spectrometry analysis of cellular fractions. Overall *T. brucei* complex I proteins are more homologous to human than bovine counterparts, but the preprocessed sizes of these proteins vary considerably compared with their eukaryotic homologs. Although most of these proteins are larger in size in *T. brucei* compared with human, notably A2 (19 versus 11 kDa), A5 (31 versus 13), A6 (83 versus 15), and B9 (36 versus 22 kDa), the S1 subunit is much smaller (34 versus 79 kDa) and lacks the second domain (MopB_Res_Cmplx1_Nad11) of the subunit.

The two related proteins with NADPH:quinone reductase and related zinc-dependent oxidoreductase domains (*Tb09.160.5260* and *Tb927.7.7410*) are more closely related to *trans-2-enoyl-CoA* reductase proteins that are involved in fatty acid synthesis than to crystallin protein, which has NADPH-dependent glutamate synthase β chain and related oxidoreductase domains. NADPH-dependent glutamate synthase β chain and related oxidoreductase domains is closely related to ferredoxin reductase protein that is a mitochondrial flavoprotein involved in initiation of electron transport by cytochromes P450. The two proteins with iron/manganese-superoxide dismutase domains (*Tb927.5.3350* and *Tb11.01.7480*) may bind to the superoxide byproducts of oxidative phosphorylation and convert them to hydrogen peroxide and molecular oxygen. The complex also contains a protein (DnaJ class) predicted to be part of a chaperone (protein folding) system.

**Subcellular Localization of Proteins**—Subcellular localization of proteins/complexes was studied by IFA using mAbs against put-MRB complex 1 and oxidoreductase complex or anti-Myc mAb that recognizes the Myc tag in transgenic cell lines expressing the tagged proteins (Fig. 4). IFA using mAb43 showed staining of mitochondria, indicating the put-MRB complex 1 identified in this study localizes to mitochondria. The results were consistent with that obtained using anti-Myc IFA using two different tagged cell lines, TAP013 and TAP015, representing this complex. IFA using mAb43 showed staining of mitochondria, indicating the put-MRB complex 1 identified in this study localizes to mitochondria.

**DISCUSSION**

This study used a proteomics approach to identify and characterize the target proteins/complexes of a panel of mAbs. It determined the composition of six different *T. brucei* mt complexes including one that is novel and the oxidoreductase complex that has several unique features. The protein assignments to these complexes were done based on the

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results obtained from parallel purifications using mAb immunoprecipitation and TAP tag purification techniques. The localization to mitochondria was validated by immunofluorescence analysis of normal cells using specific mAbs and of transgenic cells using mAbs against tags that were fused to selected proteins.

Of a panel of 19 mAbs that were used in this study 42% reacted only on native dot blots indicating they are directed against conformational epitopes, whereas the remaining 58% also reacted on Western blots indicating that they can recognize linear epitopes. We were able to identify all but one of the target proteins for the latter group by IP followed by LC-MS/MS analysis of the corresponding gel bands of the reactive mAbs. A similar approach using IP and MALDI-TOF enabled characterization of a large subset of mAbs generated against human liver mitochondrial proteins (28). In our study all of the native epitope-reactive mAbs pulled down complexes, and the results obtained with these mAbs were comparable to those with the Western reactive mAbs that recognize the same complex. For example, the sedimentation profile of mAb70 and mAb30 were similar (Fig. 1), and the different mAbs pulled down complexes with comparable protein compositions. Most complexes pulled down with native epitope mAbs had compositions identical to those pulled down by Western reactive mAbs that are specific for the same complex. An exception was mAb43 versus mAb53 where the composition of the pulldowns differed significantly. The oxidoreductase complex was characterized using only native epitope mAbs. The complex composition results obtained from IP experiments were further validated by TAP tag analyses. Thus both types of mAbs were useful tools for proteomics analyses of complexes. We have used similar approaches to isolate and characterize enzymatically active editosomes from T. brucei mitochondria (10, 29). Thus, mAbs for an organism of interest from banks that are generated using shotgun immunization with subcellular fractions or subtractive immunization (30) as well as previously incompletely characterized mAbs can be valuable tools for important proteomics studies.

We identified mAbs against three different mitochondrial heat shock proteins, hsp60, hsp70, and hsp84, in this study. Only the respective target proteins were detected in the immunoprecipitates. Expression of hsp proteins is strongly induced by heat or other stresses, but they also function under normal growth conditions (31). These chaperone proteins often associate with other proteins and complexes, although most of the expressed proteins probably remain free in the cell as indicated by their position at the top of the gradients.

This study shows that the T. brucei 3-methylcrotonoyl-CoA carboxylase complex has two different subunits as in other organisms that is consistent with a heteromeric composition being required for its enzymatic activity (32). We also determined the compositions of OGDC and PDC, which are members of the thiamin diphosphate–requiring 2-oxoacid dehydrogenase complex family and are involved in oxidative decarboxylation of 2-oxoacid substrates to CO2 and acyl-CoA derivatives (33). We showed that in T. brucei, as in other organisms, these two complexes share the dihydrolipoyl dehydrogenase (E3) subunit and that OGDC contains two different proteins with sequence similarity to the E1 component. We identified the α and β E1 subunits and the E2 and the E3 subunits in purified PDC and identified the putative E3BP. Thus, this study provides direct experimental evidence on the composition of these complexes in T. brucei and extends and corrects the annotation for some of their subunits.

In T. brucei the compositions of only a limited number of protein complexes have been determined in detail using proteomics approaches (9, 12, 34–38). This study expands on characterizations of complexes by identifying a large novel complex and also determining the composition of a unique oxidoreductase complex (putative NADH:ubiquinone oxidoreductase; respiratory complex I) for the first time. Large proportions of proteins in both the complexes have no known motifs and domains and are unique to kinetoplastids. The presence of two proteins with double-stranded RNA binding motifs, another with multiple C2H2 zinc finger motifs, and proteins with helicase motifs in put-MRB complex 1 implies a
role for this complex in RNA interaction. Several of its proteins were identified in at least two different pulldowns indicating that they are likely components of this complex. However, although Tb927.2.6070 has six zinc finger-RBZ domains, which are typically associated with protein-protein interaction, it was only identified in mAb43 pulldowns making it uncertain whether it is a stable component of this complex. Although the function of this complex has not been demonstrated the sequence characteristics of its component proteins suggest a role in RNA interaction. Preliminary results from our collaborator\(^4\) and our laboratories\(^5\) support its role in mt RNA processing.

The mt oxidoreductase complex examined in this study (Table III) contains only six proteins that are homologous to the subunits of the NADH:ubiquinone oxidoreductase complex (respiratory chain complex I) and lacks at least an equal number of other identifiable subunits along with those subunits predicted to be encoded by the mt genome. Complex I has been shown to be present in \(T.\) \(brucei\) (39), although its presence in some laboratory-cultivated strains has been debated. Our results further support the presence of complex I in culture-grown PF \(T.\) \(brucei\) cells by mass spectrometry identification of proteins corresponding to 11 different subunits. It is obvious that the oxidoreductase complex isolated here does not represent the complete complex I that contains 14 subunits in prokaryotes and up to 46 subunits in eukaryotes (14 central plus up to 32 accessory subunits) and pumps protons across the plasma or inner mitochondrial membrane, respectively (40). Based on our findings that proteins corresponding to both central (NDUFS1, -S7, -V1, and -V2) and accessory subunits (NDUFA2, -A5, -A6, -A9, -A13, -AB1, and -B9) (40) are expressed in PF \(T.\) \(brucei\) cells, the complex I may be more similar to eukaryotes rather than prokaryotes as has been suggested (39). We have not determined whether the isolated complex is membrane-bound. If it is, its isolation using Triton X-100 lysis might have stripped away membrane-associated proteins. We did not detect proteins that are predicted to be components of complex I that are encoded in mitochondrial DNA and are the products of edited mRNAs. This may reflect their protein sequence characteristics, which are not amenable to detection by standard LC-MS/MS used in this study, or association with the membrane.

The \(T.\) \(brucei\) complex I subunits that have been identified have varying degrees of sequence similarities to their eukaryotic homologs. Although three of the central subunits (S7, V1, and V2) had sizes similar to their eukaryotic counterparts, the fourth subunit (S1) is much smaller and lacks the MopB_Res_Cmplx1_Nad11 domain of the subunit. Interestingly most of the accessory subunits are larger in \(T.\) \(brucei\), and two different proteins with sequence homology to A9/39-kDa subunit were identified in the cell. The homolog of NDUFA6/B14, which has also been demonstrated as a component of trypanosomatid complex I, is 5–6 times larger in trypanosome species (41). Thus, this complex may be quite diverged from typical eukaryotic complex I. Also in \(T.\) \(brucei\) the complex I does not seem to exist in supercomplexes with other respiratory complexes as in other eukaryotes (42). This may reflect the early divergence of trypanosomes from the eukaryotic lineage and/or adaptations to accomplish the respiratory changes that it undergoes during its life cycle.

The oxidoreductase complex identified in this study may be a unique multifunctional complex in trypanosomes that has direct roles in the mitochondrial respiratory chain and fatty acid synthesis both of which use NADH/NADPH (40, 43). It has been demonstrated that complex isolated from bovine heart mitochondria predominantly produces superoxide not hydrogen peroxide (44). Because the complex isolated in this study also contains two proteins with superoxide dismutase domains these proteins may be responsible for converting the superoxide to molecular oxygen and hydrogen peroxide. Interestingly the acyl carrier protein component of complex I (subunit NDUFA) that was also identified in this study in the oxidoreductase complex has been demonstrated to have a role in type II fatty acid synthesis in growing acyl chain (27). Its presence along with two other trans-2-enoyl-CoA reductase proteins in the complex supports the proposed role for these proteins in the reduction steps during the type II fatty acid synthesis that use NADH or NADPH as a reducing agent. Thus, we hypothesize that the mt NADH-ubiquinone oxidoreductase complex in trypanosomes is multifunctional; its isolation from mitochondrial membranes would be expected to reveal its unique characteristics.

The proteomics approach in this study illustrates the value of even uncharacterized mAbs in the isolation and characterization of protein complexes. This approach can be utilized to characterize protein complexes in a high throughput manner. Studies of \(T.\) \(brucei\) mitochondrial protein complexes in our laboratory by this approach have determined the composition of complexes involved in energy metabolism and protein biosynthesis.\(^6\) As seen here, especially in the case of put-MRB complex 1 and the oxidoreductase complex, a significant proportion of components of these complexes is unique to kinetoplastids. This may reflect structural/functional divergence or unique functions, the determination of which will require further study.

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