Identification and Specific Localization of Tyrosine-Phosphorylated Proteins in Trypanosoma brucei

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Phosphorylation on tyrosine residues is a key signal transduction mechanism known to regulate intercellular and intracellular communication in multicellular organisms. Despite the lack of conventional tyrosine kinases in the genome of the single cell organism Trypanosoma brucei, phosphorylation on trypanosomal protein tyrosine residues has been reported for this parasite. However, the identities of most of the tyrosine-phosphorylated proteins and their precise site(s) of phosphorylation were unknown. Here, we have applied a phospho-tyrosine-specific proteomics approach to identify 34 phosphotyrosine-containing proteins from whole-cell extracts of procyclic form T. brucei. A significant proportion of the phosphotyrosine-containing proteins identified in this study were protein kinases of the CMGC kinase group as well as some proteins of unknown function and proteins involved in energy metabolism, protein synthesis, and RNA metabolism. Interestingly, immunofluorescence microscopy using anti-phosphotyrosine antibodies suggests that there is a concentration of tyrosine-phosphorylated proteins associated with cytoskeletal structures (basal body and flagellum) and in the nucleus of the parasite. This localization of tyrosine-phosphorylated proteins supports the idea that the function of signaling molecules is controlled by their precise location in T. brucei, a principle well known from higher eukaryotes.

Reversible protein phosphorylation by protein kinases and phosphatases is a major regulatory mechanism of most cellular processes in eukaryotic organisms (7). Dysregulation of protein phosphorylation networks is responsible for a myriad of diseases from cancers to immune disorders and neurodegenerative diseases (8, 9). These findings have prompted the characterization of the protein kinase complements (“kinomes”) of a number of organisms (2, 6, 14, 16, 18, 21, 24, 27, 32, 41), and an important turning point came with the realization that par-asite kinomes are substantially different from those of the hosts they infect. One major difference is the absence of genes coding for any recognizable tyrosine-specific kinases in the ge-nome of Trypanosoma brucei (29; I. R. E. Nett, D. M. A. Martin, D. Miranda-Saavedra, D. Lamont, J. D. Barber, A. Mehlert, and M. A. J. Ferguson, submitted for publication). T. brucei causes human African trypanosomiasis (also known as African sleeping sickness) and is responsible for ~30,000 deaths per annum (35). However, despite the lack of conventional tyrosine kinases in this parasite and related trypanosomes, there is evidence that several proteins are phosphorylated on tyrosine residues in these organisms (10, 30). Tyrosine phosphatase activity has also been observed in cell extracts of bloodstream form and procyclic form T. brucei (1). More recently, the identification and biochemical characterization of a T. brucei protein tyrosine phosphatase 1 suggest a role for tyrosine phosphorylation in the regulation of the trypanosome life cycle (36). In addition, the characterization of the T. brucei protein phosphatase complement (“phosphatome”) revealed the presence of 19 dual-specificity protein phosphatases. However, orthologues of the human mitogen-activated protein ki-nase (MAPK) phosphatases are missing in the T. brucei ge-nome (3), although plant-like MAPK phosphatase homologues are present. This is an interesting observation, since our large-scale phosphoproteomics analysis of bloodstream form T. bru-cei cells (Nett et al., submitted for publication) revealed phos-phorylation on tyrosine residues of 13 protein kinases, of which two belong to the MAPK family (GeneDB accession no. Tb927.6.4220 and Tb10.61.0250).

Phosphorylation of protein tyrosine residues regulates impor-tant cell functions in higher eukaryotes, but the role of this posttranslational modification is largely unknown for T. brucei. Here, we used a phosphotyrosine-specific mass spectrometry (MS)-based approach to identify proteins carrying this modification in the procyclic form of the parasite and reveal that phosphorylation of tyrosine residues within canonical se-quence motifs is conserved in T. brucei. However, by using anti-phosphotyrosine-specific antibodies, we show a localization pattern for phosphotyrosine-containing proteins in T. bru-cei that is substantially different from that in mammalian cells.

MATERIALS AND METHODS

Cell preparation and lysis. The procyclic form T. brucei cell line 29-13-6 (44) was cultured in SDM-79 medium (5), containing G418 (Gibco) at 15 μg/ml and hygromycin B (Roche) at 50 μg/ml. Human HeLa cells were grown in Dulbecco modified Eagle medium (Gibco) supplemented with 10% fetal calf serum (Gibco) and 2 mM l-glutamine (Gibco) at 37°C.

For the isolation of phosphotyrosine-containing peptides from trypanosomes,
1 liter of cells was grown to a density of $1 \times 10^{12}$ cells/ml at 28°C in a water-jacketed incubator. Cells were treated with 800 μM hydrogen peroxide for 30 min before lysis. Cell lysis and phosphopeptide preparation were performed using the PhosphoScan P-Tyr-100 kit (Cell Signaling) according to the manufacturer’s instructions.

**Cell lysis for Western blotting.** Before lysis, cells were washed three times in ice-cold phosphate-buffered saline (PBS) buffer and then lysed in ice-cold RIPA lysis buffer (10 mM Tris-HCl, pH 7.5; 1 mM sodium-pyrophosphate; 1 mM sodium-pyrophosphate; 1 mM sodium fluoride; 5 mM EDTA; 0.5% NP-40; 0.2% sodium-deoxycholate; 0.2% sodium dodecyl sulfate [SDS]; EDTA-free protease inhibitor tablet [Roche]; 100 μM activated sodium-orthovanadate) at a ratio of $1 \times 10^{12}$ cells/ml per 1 ml of lysis buffer. The lysate was sonicated two times for 30 s at 80% power of an ultrasonic processor machine (Mecenso) at 4°C and centrifuged at 14,000 rpm for 20 min at 4°C. The supernatant was transferred into a fresh tube, and the protein concentration determined using the Micro BCA protein assay kit (Pierce) according to the manufacturer’s instructions. Approximately 10 μg of proteins were mixed with the appropriate volume of 4X Laemmli sample buffer and 10X sample-reducing agent (Invitrogen), heated, and separated on a precast Novex 4 to 12% Bis-Tris SDS-polycrylamide electrophoresis gel. After electrophoresis, proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane, and the membrane was blocked with 4% bovine serum albumin in Tris-buffered saline (TBS) (150 mM NaCl, 50 mM Tris-HCl, pH 7.2) containing Triton X-100 (0.2%) for 1 h at room temperature. The membrane was incubated with the anti-phosphotyrosine monoclonal antibody 4G10 (1 mg/ml; Upstate) or with a 1:30 dilution in 2% fish gelatin in TBS/0.2% Triton X-100, and separated on a precast Novex 4 to 12% Bis-Tris SDS-polyacrylamide electrophoresis gel. Western blotting detection reagent (GE Healthcare) according to the manufacturer’s instructions and were exposed to Hyperfilm (GE Healthcare).

To examine phosphotyrosine-containing proteins of cytosol and cytoskeletal fractions, aliquots of $3 \times 10^{12}$ procyclic form *T. brucei* cells were washed in PBS buffer and lysed in either 100 μl ice-cold MME buffer (10 mM MOPS, pH 6.9; 1 mM MgSO4) containing EDTA-free protease inhibitor tablet (Roche); 0.2% Triton X-100, and a phosphotyrosine antibody (Upstate) revealed several tyrosine-containing proteins by Western blotting. The 4G10 antibody and 4G10 labeling with either anti-mouse IgG Alexa Fluor 633 (2 μg/ml; Invitrogen) or anti-mouse IgG Alexa Fluor 488 (2 μg/ml; Invitrogen). BBA4 labeling was visualized with anti-mouse IgM Alexa Fluor 488 (2 μg/ml; Invitrogen). Cells were counterstained with anti-mouse fluorescein isothiocyanate- conjugated anti-α-tubulin (1.5 μg/ml; Sigma) and/or 4′,6′-diamidino-2-phenylindole (2 μg/ml; Sigma) before mounting with Vectashield (Vector Laboratories).

For immunofluorescence studies of human HeLa cells, 2 × 10^6 cells were seeded onto a square coverslip (22 mm by 22 mm) and incubated at 37°C for 24 h. The cells were washed twice with 1 ml of PBS and fixed in 4% PFA/PBS. Antibody incubations and mounting of cells were performed as described for trypanosomes.

Images were collected using a DeltaVision Spectris restoration wide-field deconvolution microscope (Applied Precision LLC) equipped with a CoolSnap HQ cooled charge-coupled device camera. Optical sections were processed using SoftWoRx software (Applied Precision LLC) and Adobe Photoshop (Adobe). For the dephosphorylation assay, methanol-fixed trypanosomes were incubated with 2 μl alkaline phosphatase buffer (see above) containing 10 units of alkaline phosphatase in a carbosolv glassette at pH 9.5. The preparation was transferred to a 1-liter Erlenmeyer flask, and the protein concentration determined using the Micro BCA protein assay kit (Pierce) according to the manufacturer’s instructions. Approximately 10 μg of proteins were mixed with the appropriate volume of 4X Laemmli sample buffer and 10X sample-reducing agent (Invitrogen), heated, and separated on a precast Novex 4 to 12% Bis-Tris SDS-polycrylamide electrophoresis gel. After electrophoresis, proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane, and the membrane was blocked with 4% bovine serum albumin in Tris-buffered saline (TBS) (150 mM NaCl, 50 mM Tris-HCl, pH 7.2) containing Triton X-100 (0.2%) for 1 h at room temperature. The membrane was incubated with the anti-phosphotyrosine monoclonal antibody 4G10 (1 mg/ml; Upstate) at a dilution of 1:10,000 for 1 h at room temperature, then washed, and incubated with secondary antibody (anti-mouse horseradish peroxidase; Roche) at 1:5,000. Western blots were developed using the ECL Western blotting detection reagent (GE Healthcare) according to the manufacturer’s instructions and were exposed to Hyperfilm (GE Healthcare).

**Immunolocalization.** Detection of tyrosine-phosphorylated proteins by immunofluorescence using 4G10 anti-phosphotyrosine antibody. To initiate an analysis of tyrosine-phosphorylated proteins in *T. brucei*, we investigated the usefulness of commercially available anti-phosphotyrosine antibodies to bind to trypanosomal phosphotyrosine-containing proteins by Western blotting. The 4G10 anti-phosphotyrosine antibody (Upstate) revealed several protein bands, especially high-molecular-weight proteins, in whole cell lysates of both procyclic (Fig. 1, lane 1) and bloodstream form *T. brucei* (see Fig. S1 in the supplemental material). The association of phosphotyrosine-containing proteins with microtubule structures, induced by immunofluorescence microscopy (see below), was also analyzed by Western blotting using whole-cell, TX-100-soluble and cytoskeleton extracts of procyclic form *T. brucei* cells (Fig. 1). Anti-phosphotyrosine reactive proteins were detected in both fractions to different degrees.

RESULTS

**Detection of tyrosine-phosphorylated proteins by immunoblotting using 4G10 anti-phosphotyrosine antibody.** To initiate an analysis of tyrosine-phosphorylated proteins in *T. brucei*, we investigated the usefulness of commercially available anti-phosphotyrosine antibodies to bind to trypanosomal phosphotyrosine-containing proteins by Western blotting. The 4G10 anti-phosphotyrosine antibody (Upstate) revealed several protein bands, especially high-molecular-weight proteins, in whole cell lysates of both procyclic (Fig. 1, lane 1) and bloodstream form *T. brucei* (see Fig. S1 in the supplemental material). The association of phosphotyrosine-containing proteins with microtubule structures, induced by immunofluorescence microscopy (see below), was also analyzed by Western blotting using whole-cell, TX-100-soluble and cytoskeleton extracts of procyclic form *T. brucei* cells (Fig. 1). Anti-phosphotyrosine reactive proteins were detected in both fractions to different degrees.
A higher proportion of tyrosine-phosphorylated proteins was found in the cytoskeleton extract (Fig. 1, lane 3) than for TX-100-soluble proteins obtained from an equal amount of cells (Fig. 1, lane 2). To assess the possibility of nonspecific antibody binding, parallel incubations of PVDF membranes containing the same amount of procyclic cell lysate were performed in the absence (Fig. 1, control) and presence of alkaline phosphatase (alk. phosphatase, lanes 4 to 6) or SHP-1 phosphotyrosine-specific phosphatase (SHP-1, lanes 7 to 9) treatment of the blot. Following the 4G10 Western blotting, the membranes were stripped and probed with anti-tubulin antibodies as a loading control (lower panels). α-pTyr, anti-phosphotyrosine.

Tyrosine phosphorylation is hydrogen peroxide sensitive. We next examined the level of tyrosine phosphorylation after treating the cells with different concentrations of hydrogen peroxide (Fig. 2). Hydrogen peroxide has been shown to be involved in the regulation of redox signaling pathways by inactivating protein tyrosine phosphatases and, as a result, increasing tyrosine phosphorylation of target proteins (33). In procyclic trypanosomes, first responses to the hydrogen peroxide treatment were detected at concentrations of 200 μM, and detection levels reached their maximum in cell lysates obtained from cells that had been incubated with 800 μM hydrogen peroxide for 20 min or 30 min before cell lysis. An aliquot of 15 μg protein was loaded in each lane. Lane 1, protein lysate of untreated cells. Bands that show a clear response to the hydrogen peroxide treatment are indicated with an asterisk. α-pTyr, anti-phosphotyrosine.

In order to identify T. brucei tyrosine-phosphorylated proteins, we immunoprecipitated tyrosine-phosphorylated peptides from tryptic digests of whole-cell lysates of the procyclic form and analyzed the immunoprecipitate by LC-MS/MS. Procyclic trypanosomes were treated with 800 μM hydrogen peroxide for 30 min before cell lysis to increase the levels of phosphorylation on tyrosine residues and thus improve the detection of phosphorylated proteins by MS. In total, 45 phosphotyrosine-containing peptide isoforms from 34 proteins were identified in two separate experiments using approximately 40 mg starting material (see Table S1 in the supplemental material). An example of a fragment mass spectrum of a tyrosine-phosphorylated peptide is shown in Fig. 3. A mass increment of 243 Da, which corresponds to a phosphorylated tyrosine residue (pY), was ob-
served for residue Y188 in the phosphopeptide sequence GV
GVNVTSpYVVTR of GeneDB accession no. Tb927.6.1780.
The loss of a phosphate group in the form of phosphoric acid
(H3PO4) was observed for T186, suggesting this residue as a
second site of phosphorylation in the same peptide.
The majority of proteins found to be phosphorylated on
tyrosine residues were protein kinases belonging to the CMGC
kinase group, and one kinase was designated a NEK group
member (Table 1).

T. brucei protein kinases were annotated
according to the classification described in the work of Nett et
al. (submitted for publication). Of the CMGC kinases, we
identified 10 putative T. brucei mitogen-activated protein ki-
nases (TbMAPK) homologous to the 15 putative MAPKs en-
coded in the Leishmania genome (42), including the experi-
mentally characterized TbMAPK2 (28) (Table 1). We also
detected the T. brucei homologue of LmjMPK15, which falls
into the T. brucei CDK kinase family (Tb10.329.0030).
The signature TXY motif in the activation loop of MAPKs
(25) was found fully phosphorylated in 8 of the 10 TbMAPKs
(Table 1), and the remaining 3 kinases (including the identified
TbCDK) were phosphorylated only on the tyrosine residue.
The TXY motif present in the putative TbMAPKs showed a
rather variable central amino acid; five TbMAPKs were found
to contain a TEY motif, three TbMAPKs displayed a TDY
motif, and the remaining two TbMAPKs had either THY or
TSY. Another variation of the TXY motif was found in the T.
brucei homologue of LmjMPK15, which showed a TFY signa-
ture motif in the activation segment.

Other tyrosine-phosphorylated proteins identified in our
study are involved in protein synthesis (GeneDB accession
no. Tb927.6.1780 translation initiation factor, putative; ri-
bosomal protein S27, putative), energy metabolism (glyco-
somal phosphoenolpyruvate carboxykinase; ATP-dependent
phosphofructokinase), and RNA metabolism (ATP-depen-
dent DEAD/H RNA helicase, putative). Tyrosine-contain-
ing peptides were also found for nonclassified proteins (10
“hypothetical proteins”) (see Table 2).

Localization studies of tyrosine-phosphorylated proteins in
T. brucei. We next investigated the localization pattern of ty-
rosine-phosphorylated proteins using the anti-phosphotyrosine
monoclonal antibodies 4G10, PY-20, and PY-100 on PFA-
fixed procyclic trypanosomes (Fig. 4). Phosphotyrosine-
containing proteins appeared to localize specifically to punctate
structures in the posterior of the cell (Fig. 4A) and to the
nucleus (Fig. 4A to C). A phosphotyrosine signal was also
observed along the flagellum (Fig. 4A to C), which faded out
toward its distal tip, particularly in the case of 4G10 and PY-
100 staining. It is worth noting that the fixation method (4%
PFA versus 100% methanol) had no effect on the overall stain-
ing pattern; however, the recognition of phosphotyrosine-con-

FIG. 3. Mass spectrometric analysis of T. brucei phosphotyrosine-containing peptides. Shown is the fragmentation spectrum of the diphos-
phorylated peptide GVGVNVTSpYVVTR (p indicates phosphorylated residue) of a putative TbMAPK (GeneDB accession no. Tb927.6.1780)
measured on an LTQ-Orbitrap mass spectrometer. Phosphorylation at the threonine and tyrosine residues of the TSY motif could be deduced due
to the neutral loss of phosphoric acid starting from the y4 and b7 ions (−P) and the observed mass increment of 243 Da (+P) between the y4 and
y3 ions, respectively.

![Mass spectrometric analysis of T. brucei phosphotyrosine-containing peptides](image-url)
taining structures using methanol-fixed procyclic form cells seemed to be weaker compared to that using PFA fixation (data not shown). A no-primary antibody control was also included to demonstrate that the staining was a consequence of the primary antibody and not a nonspecific background (Fig. 4D). The specificity of the anti-phosphotyrosine antibody 4G10 was tested by treating coverslips with alkaline phosphatase overnight and then with 4G10 staining and immunofluorescence signal, demonstrating the selectivity of the 4G10 antibody after cell fixation and ruling out the possibility of cross-reactivity.

In order to investigate whether the proteins visualized by the anti-phosphotyrosine antibodies were associated with microtubular structures, procyclic form T. brucei total cytoskeletons were extracted with detergent, fixed in 4% PFA, and immunostained with the anti-phosphotyrosine antibodies 4G10 and 21F3, respectively, followed by secondary antibody conjugated to fluorescent dyes. The immunofluorescence images of the cytoskeletal proteins stained with 4G10 and 21F3 antibodies showed overlapping localization patterns, indicating a potential interaction between phosphotyrosine-containing proteins and microtubules.

### TABLE 1. Tyrosine-phosphorylated protein kinases of whole-cell procyclic trypanosomes

| GeneDB accession no. | GeneDB annotation | Kinase group | Kinase family | MW (in thousands) | Phosphopeptide | Reference |
|----------------------|-------------------|--------------|---------------|------------------|----------------|-----------|
| Tb010.01.4230        | PK, putative      | CMGC         | MAPK          | 53.1             | KVT_{Y}APL_{N}OSKR | 109       |
| Tb010.329.0030       | PK, putative      | CMGC         | CDK           | 64.4             | DAQ_{A}S_{D}T{IP}_{Y}VCTR | 246       |
| Tb09.2.3770          | PK, putative      | CMGC         | MAPK          | 46.9             | ED{T}_{I}Q{D}{P}NKT{H}_{p}P_{Y}VTHR | 196       |
| Tb09.211.0960        | PK, putative      | CMGC         | MAPK          | 41.7             | GL{H}_{V}_{S}_{O}_{P}_{L}_{T}_{E}_{P}_{Y}_{V}_{S}_{T}_{R} | 166       |
| Tb110.70.2210        | CRK3              | CMGC         | CDK           | 35.0             | M{D}_{I}_{L}_{I}_{G}_{E}_{G}_{T}_{P}_{Y}_{V}_{V}_{Y}_{R} | 39        |
| Tb110.02.0640        | PK, putative      | CMGC         | DYRK/class II | 51.5             | L{F}_{T}_{P}_{Y}_{I}_{Q}_{S}_{R} | 273       |
| Tb110.01.5500        | ECK1              | CMGC         | MAPK          | 72.1             | G{N}_{Y}_{T}_{E}_{P}_{T}_{E}_{P}_{Y}_{A}_{V}_{T}_{R} | 164       |
| Tb10.61.1500         | MAPK9, putative   | CMGC         | MAPK          | 42.7             | SRRP{P}_{E}_{T}_{E}_{P}_{Y}_{V}_{Y}_{S}_{T}_{R} | 169       |
| Tb10.61.0250         | MAPK2             | CMGC         | MAPK          | 41.8             | D{D}_{O}_{C}_{T}_{Q}_{T}_{S}_{A}_{L}_{T}_{E}_{P}_{Y}_{V}_{Y}_{T}_{R} | 196       |
| Tb10.61.7780         | PK, putative      | CMGC         | MAPK          | 46.1             | G{O}_{V}_{G}_{V}_{S}_{V}_{T}_{E}_{P}_{Y}_{V}_{Y}_{T}_{R} | 192       |
| Tb10.61.7140         | PK, putative      | CMGC         | MAPK          | 41.7             | GL{H}_{V}_{S}_{O}_{P}_{L}_{T}_{E}_{P}_{Y}_{V}_{S}_{T}_{R} | 196       |
| Tb110.61.3140        | PK, putative      | CMGC         | MAPK          | 40.3             | L{A}_{A}_{D}_{E}_{P}_{N}_{V}_{N}_{A}_{P}_{Y}_{I}_{C}_{S}_{R} | 191       |

* a Shown are proteins identified in our phosphotyrosine-specific proteomics study with at least one tyrosine-phosphorylated residue.
* b Phosphorylated tyrosine residue. Numbers preceding and following the sequences specify the start and the end of the phosphopeptide sequence.
* c TbMAPKs with a fully phosphorylated TXY motif are indicated with an asterisk.
* d MW, molecular weight.

### TABLE 2. Non-protein kinase tyrosine-phosphorylated proteins of whole-cell procyclic trypanosomes

| GeneDB accession no. | Protein description | MW (in thousands) | Phosphopeptide | Reference |
|----------------------|---------------------|------------------|----------------|-----------|
| Tb09.27.5.3120       | Translation initiation factor, putative | 34.9          | 287 SV{Q}_{A}_{V}_{G}_{S}_{A}_{T}_{P}_{Y}_{S}_{A}_{Q}_{V}_{G}_{K}_{R} | 303       |
| Tb11.01.1475         | Ribosomal protein S27, putative | 9.6           | 25 LV{Q}_{G}_{P}_{N}_{S}_{P}_{Y}_{M}_{D}_{V}_{K}_{Y} | 37       |
| Tb27.2.4.210         | Glycosomal phosphoenolpyruvate carboxykinase; glycosomal protein P60 | 58.5         | 172 EQ{V}_{I}_{L}_{G}_{T}_{E}_{P}_{Y}_{A}_{G}_{M}_{E}_{K}_{M} | 185       |
| Tb27.3.3.270         | TbPFK ATP-dependent phosphofructokinase; f-phospho-1-fructokinase | 53.5         | 54 DK{D}_{T}_{Y}_{I}_{M}_{P}_{Y}_{N}_{P}_{R}_{F}_{R} | 66       |
| Tb09.21.1.3510       | ATP-dependent DEAD/H RNA helicase, putative | 82.7         | 138 FD{V}_{D}_{V}_{P}_{F}_{Y}_{D}_{R}_{P}_{R}_{Y}_{R}_{D}_{P} | 147       |
| Tb09.21.1.070        | Hypothetical protein, conserved | 28.8         | 96 F{T}_{G}_{G}_{P}_{Y}_{S}_{T}_{Y}_{P}_{Y}_{T}_{S}_{D}_{T}_{A}_{R}_{A}_{R} | 111       |
| Tb27.4.2.040         | Hypothetical protein, conserved | 20.8         | 2 P{S}_{P}_{Y}_{P}_{R}_{P}_{D}_{E}_{Y}_{R}_{Y} | 11       |
| Tb10.61.2400         | Hypothetical protein, conserved | 97.3         | 53{A}_{E}_{A}_{E}_{A}_{A}_{Q}_{L}_{P}_{Y}_{V}_{G}_{K}_{G} | 549       |
| Tb11.02.0420         | Hypothetical protein, conserved | 21.4         | 7 C{I}_{C}_{A}_{G}_{N}_{S}_{A}_{H}_{P}_{Y}_{R} | 18       |
| Tb10.70.3380         | Hypothetical protein, conserved | 52.4         | 34{S}_{I}_{L}_{G}_{K}_{P}_{Y}_{P}_{R} | 42       |
| Tb09.16.1.1100       | Hypothetical protein, conserved | 85.9         | 19 {I}_{G}_{D}_{P}_{Y}_{E}_{W}_{S}_{N}_{T}_{L}_{A}_{R}_{I}_{R} | 31       |
| Tb11.01.2800         | Hypothetical protein, conserved | 41.6         | 287 TH{D}_{Y}_{D}_{E}_{P}_{O}_{E}_{L}_{L}_{I}_{R}_{R} | 298       |
| Tb27.4.3.310         | Hypothetical protein, conserved | 30.6         | 178 {V}_{G}_{V}_{T}_{S}_{G}_{P}_{Y}_{A}_{N}_{T}_{Q}_{R}_{G} | 190       |
| Tb27.3.1.400         | Hypothetical protein, conserved | 37.2         | 279 Y{L}_{Q}_{S}_{O}_{C}_{N}_{R}_{P}_{T}_{G}_{N}_{A}_{G}_{P}_{Y}_{G}_{G}_{N} | 396       |
| Tb10.61.3460         | Hypothetical protein, conserved | 284.7        | 385 {H}_{M}_{E}_{Q}_{H}_{P}_{Y}_{G}_{T}_{I}_{A}_{K} | 396       |

* a Shown are proteins identified in our phosphotyrosine-specific proteomics study with at least one tyrosine-phosphorylated residue.
* b Phosphorylated tyrosine residue. Numbers preceding and following the sequences specify the start and the end of the phosphopeptide sequence.
* c MW, molecular weight.
toskeleton preparations gave the same staining pattern that was previously observed with whole cells, except for the nucleus (compare with Fig. 4). Identical results were obtained with bloodstream form T. brucei cytoskeletons using the antiphosphotyrosine antibody 4G10 (see Fig. S4 in the supplemental material). The strong signal for the punctate structures in the posterior of the cell was present in both life cycle stages, and the position and shape of this organelle indicated the labeling of the basal body (mature and immature probasal body). The lack of any gap between the signal for the basal body and the labeling of the flagellum (Fig. 5A), as well as the fact that the signal for the flagellum connects to only one (the mature basal body), strongly pointed to the flagellum signal being axonemal rather than paraflagellar rod or flagellum attachment zone filament (Keith Gull, personal communication). Moreover, the signal did not seem to vary much through the cell cycle, as it could be detected on the old and new flagella (Fig. 5B).

In order to define the individual components within the cytoskeleton that the phosphotyrosine signal was associated with, we performed immunolocalization studies using 4G10 in association with the monoclonal antibodies BBA4 and Rib72. BBA4 is a specific marker for the proximal poles of both the basal and probasal bodies (45) and labels the basal body structure as a two-dot pattern in the posterior of a trypanosome cell (12). Colabeling experiments indicate that phosphotyrosine-containing proteins are localized toward the distal end of both the basal and probasal bodies, as the staining patterns for 4G10 and BBA4 show very close proximity but do not overlap (Fig. 6C). This specific labeling has previously been observed in procyclic trypanosomes when using the anti-phosphopeptide monoclonal antibody MPM2 (12). In addition, the phosphotyrosine signal seems to be on the distal end of the basal body rather than around this organelle, as determined by taking differential interference contrast images of anti-phosphotyrosine 4G10-labeled flagellum extractions (Fig. 6E).

We next investigated the localization of phosphotyrosine-containing proteins observed along the length of the flagellum by using the monoclonal anti-axonemal Rib72 antibody. Rib72 extends from the basal body to the tip of the flagellum but is not present in the basal body itself (Keith Gull, personal communication). Colabeling experiments with 4G10 (Fig. 7B) and

![Image](https://example.com/image1.png)

**FIG. 4.** Indirect immunofluorescence using anti-phosphotyrosine antibodies. Whole-cell procyclic trypanosomes were fixed in 4% PFA and stained with 4G10 (A), PY-20 (B), and PY-100 (C). (D) A no-primary-antibody control was included and demonstrated that the staining was a consequence of the primary anti-phosphotyrosine antibodies. Tyrosine-phosphorylated proteins (in green) localized to punctate structures in the posterior of the cells (white arrows) and were also found along the length of the flagellum (yellow arrows) and in the nucleus (white arrowheads). (E and F) Control HeLa cells labeled with 4G10 showed concentrated anti-phosphotyrosine staining (in red) at focal adhesion regions. DNA (blue) is visualized by 4'-6-diamidino-2-phenylindole (DAPI) staining. Anti-α-tubulin is shown in green. p-Tyr, tyrosine-phosphorylated proteins. White bar corresponds to a length of 5 μm.
Rib72 (Fig. 7C) revealed that the staining pattern of both monoclonal antibodies overlapped in most of the areas (Fig. 7D). Thus, the phosphotyrosine signal occurs as a continuous pattern between the basal body and the tip of the flagellum, indicating that \textit{T. brucei} tyrosine-phosphorylated proteins are associated with, or are immediately adjacent to, the flagellum axoneme. However, part of the phosphotyrosine signal does not colocalize with the Rib72 stain but separates from the axoneme and occurs along the cell body (Fig. 7D). This observation suggested that phosphotyrosine-containing proteins could also be associated with membranous structures close to the flagellum in \textit{T. brucei}. An axonemal location of phosphotyrosine proteins was also supported by flagellum extraction of procyclic form cells and staining with the anti-phosphotyrosine 4G10 antibody (Fig. 6E). The phosphotyrosine signal on the basal body and up the flagellum survived the high-salt treatment applied to obtain these isolated flagellum preparations, indicating that phosphotyrosine-containing proteins are tightly associated with structures forming the flagellum.

To determine the subnuclear localization of phosphotyrosine-containing proteins observed by immunofluorescence microscopy (Fig. 4B), we used a fluorescent marker synthetic peptide [TRITC-D(R10)] that targets the nucleolus (22). This subcompartment of the nucleus was chosen for the colocalization studies because the nucleolar \textit{T. brucei} protein NOPP44/46 (GeneDB accession no. Tb927.8.760) has been identified as having developmentally regulated tyrosine phosphorylation (11, 31). The nucleolus-specific TRITC-D(R10) label colocalized with the anti-phosphotyrosine signal in a distinct region of the nucleus (see Fig. S5 in the supplemental material), thus confirming previous evidence by the Parsons group of tyrosine-phosphorylated proteins in the nucleolus.

**DISCUSSION**

In this study, we used advanced MS-based phosphotyrosine-specific proteomics to shed light on the extent of protein tyrosine phosphorylation in \textit{T. brucei}. We show that protein tyrosine phosphorylation is a relatively abundant posttranslational modification in an organism that lacks conventional tyrosine kinases (Nett et al., submitted for publication). Of the 34 phosphotyrosine-containing proteins we detected in this study, 19 protein kinases and 2 metabolic kinases (phosphoenolpyruvate carboxykinase and 6-phospho-1-fructokinase)
could be identified, which suggests that in *T. brucei* tyrosine phosphorylation preferentially occurs on proteins that possess putative kinase activity. Of the 19 protein kinases, phosphorylation sites on 11 *T. brucei* homologues of *Leishmania* MAPKs could be identified. MAPK family members require phosphorylation of both a threonine residue and a tyrosine residue in the activation loop for full enzymatic activity of the kinase. We found the highly conserved TXY motif in the activation segment of MAPKs fully phosphorylated on eight of the identified putative TbMAPKs. Phosphorylation of the tyrosine residue allows the activation loop to adopt the active conformation, and subsequent phosphorylation of the threonine residue activates the kinase fully by promoting the correct alignment of the catalytic residues (17). Thus, the remaining putative TbMAPKs found to be phosphorylated on only the tyrosine residue, including the previously characterized TbMAPK2 (28), are likely to represent the kinase in an inactive state. These results suggest that the activation of MAPKs in this highly divergent parasite follows the same mechanistic principles as those in higher eukaryotes. The expression of phosphorylated STE kinases, which are thought to represent TbMAPK upstream activator proteins (29) in bloodstream form trypanosomes (Nett et al., submitted for publication), could be evidence for the existence of canonical signal transduction via MAPK in *T. brucei*.

Other protein kinases identified in this study include the cell division cycle 2 (Cdc2)-related protein kinases CRK1 (GeneDB accession no. Tb10.70.7040), CRK2 (GeneDB accession no. Tb927.7.7360), and CRK3 (GeneDB accession no. Tb10.70.2210), all of which were found to be tyrosine phosphorylated in their N terminus (Y<sup>16</sup>, Y<sup>37</sup>, and Y<sup>34</sup>, respectively). All three CRK isoforms have previously been detected phosphorylated on the same residues in our global phosphoproteome study of bloodstream form *T. brucei* (Nett et al., submitted for publication), suggesting that life cycle stage-specific tyrosine phosphorylation of these residues can be excluded. Interestingly, the phosphorylated tyrosine sites are flanked by an S<sup>15</sup> in CRK1, S<sup>56</sup> in CRK2, and T<sup>33</sup> in CRK3, which could correspond to the conserved human CDK1 T<sup>14</sup> and Y<sup>15</sup> residues. Phosphorylation of the conserved Y<sup>15</sup> of CDK1 by the Wee1 dual-specificity tyrosine kinase inactivates the CDK complex (20), and as a Wee1 kinase is encoded by the *T. brucei* genome, it is possible that CRKs in *T. brucei* are regulated by a similar mechanism.

Our detection of previously identified trypanosomal phosphorylation sites within the activation segments of both isoforms of the glycogen synthase kinase-3 (GeneDB accession no. Tb927.7.2420 and Tb10.61.3140) and a DYRK homologue (GeneDB accession no. Tb11.02.0640) (Nett et al., submitted for publication) strongly supports the view that known kinase-
Activating sites are conserved in T. brucei and indicates that several segments of known signal transduction pathways are conserved in this organism.

This study also provided an insight into the localization of tyrosine-phosphorylated proteins in both life cycle stages of T. brucei. Proteins carrying this posttranslational modification were found to be concentrated at the cytoskeleton, specifically with the axoneme and the basal bodies of the flagellum, as well as the nucleolar compartment of the parasite, as determined by immunofluorescence microscopy. This is in stark contrast to the localization pattern of tyrosine-phosphorylated proteins in mammalian cells, in which the signal for this posttranslational modification is concentrated at the plasma membrane in focal adhesion points that are known as locations for high tyrosine kinase activity. In these studies, it is difficult to correlate phosphotyrosine proteome data with localization data, since the former are based on the purification of phosphotyrosine-containing tryptic peptides, whereas the latter are based on antibody recognition in the context of folded and fixed proteins. Therefore, we cannot say what proportion of the phosphoproteome is associated with the cytoskeletal and nucleolar compartments nor what subset of phosphoproteins might be specific for those locations. Nevertheless, the lack of conventional receptor tyrosine kinases and nonreceptor tyrosine kinases in T. brucei might be one aspect of the different staining patterns observed between trypanosomes and mammalian cells. The different locations of phosphotyrosine-containing proteins in the human system and the parasite might also directly reflect different functions of phosphotyrosine-based signaling in the organisms due to their substantially different requirements for survival.

Work by Das et al. has identified a tyrosine-phosphorylated NOPP44/46 protein with a nucleolar localization in procyclic trypanosomes. This observation is consistent with our detection of a phosphotyrosine signal in the nucleolus of procyclic form cells by immunofluorescence. It remains unclear whether our microscopy studies also detected the NOPP44/46 protein, as it was not detected in our focused tyrosine phosphoproteomics screen of whole procyclic form cells. However, analysis of the NOPP44/46 amino acid sequence (GeneDB accession no. Tb927.8.760) reveals that, of the five tyrosine residues it contains, at least four of them are undetectable by MS. One is the C-terminal residue in the sequence RY (the tryptic product would be too small to detect), and three reside in tryptic peptides with masses of 6,947 Da (two sites) and 7,440 Da (one site) that are too large to provide meaningful MS-MS spectra. Only the N-terminal tyrosine residue in the sequence MEGFYGEVSAGQKVK would be potentially detectable by our method. However, there is no guarantee that this is a phosphorylation site in NOPP44/46, and in any phosphoproteome analysis there is no guarantee that every occupied site will be detected. NOPP44/46 has been shown to bind nucleic acid, and RNA interference of the T. brucei-specific protein in theprocyclic form affects large-subunit rRNA processing. This raises the interesting question of whether growth control and development of T. brucei are mediated by nucleolus-localized tyrosine-phosphorylated proteins, with ribosome synthesis as the obvious target of regulation. A more targeted phosphoproteomics study of T. brucei nucleoli could provide answers to the questions of how these nucleolus-localized tyrosine-phosphorylated proteins operate and if there is a connection to known signaling pathways.

The observation of tyrosine-phosphorylated proteins located on the basal body and along the axoneme of the flagellum extending to its distal tip is intriguing, as it suggests that signaling molecules could be associated with microtubules, which has been shown with mammalian cells. In fact, in mammalian cells, many kinases, such as MAPK, MEK1, MEK2, Raf1 (MEKK), CDK5, and CDC2, as well as many phosphatases, such as protein phosphatase 2A (PP2A), protein phosphatase 2B (PP2B), and protein phosphatase 1 (PP1), were detected in microtubule pellets, indicating that microtubule assembly and stability might be regulated by an interplay of these signaling molecules. The same study also revealed that the pool of microtubule-associated MAPK was constitutively active, which would be consistent with our observation that the phosphotyrosine signal along the flagellum and on the basal body was cell cycle independent.

Interestingly, a recent study of the green alga Chlamydomonas reinhardtii has shown that the tyrosine-phosphorylated active form of GSK3 was enriched in the flagellum. RNA interference of Chlamydomonas reinhardtii GSK3 resulted in cells that had no flagella, suggesting a role for GSK3 in assembly and maintenance of flagella, presumably through the regulation of intraflagellar transport. Our study showed that tyrosine-phosphorylated proteins are associated with the flagellum and the basal body, which is an organelle that provides a platform for recruiting proteins involved in intraflagellar transport. In addition, we identified TbGSK3 phosphory-
lated in its activation loop, suggesting that the kinase is active in the parasite. Both flagellar formation and basal body segregation are vital for T. brucei (4, 13), and our findings indicate that tyrosine-phosphorylated proteins might play a role in these essential processes.

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