Signal Transduction Triggered by Iron to Induce the Nuclear Importation of a Myb3 Transcription Factor in the Parasitic Protozoan Trichomonas vaginalis*

Received for publication, July 24, 2014, and in revised form, August 27, 2014. Published, JBC Papers in Press, September 2, 2014, DOI 10.1074/jbc.M114.599498

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Background: Iron induces the immediate nuclear influx of Myb3 in T. vaginalis.

Results: Iron triggered a cAMP-mediated signaling that resulted in the phosphorylation and ubiquitination of Myb3 to accelerate its nuclear influx.

Conclusion: Iron triggers signal transduction to activate a rapid nuclear influx of Myb3.

Significance: This work revealed a novel role of iron in poorly studied signal transduction in the parasite.

Iron was previously shown to induce rapid nuclear translocation of a Myb3 transcription factor in the protozoan parasite, Trichomonas vaginalis. In the present study, iron was found to induce a transient increase in cellular cAMP, followed by the nuclear influx of Myb3, whereas the latter was also induced by 8-bromo-cyclic AMP. Iron-inducible cAMP production and nuclear influx of Myb3 were inhibited by suramin and SQ22536, respective inhibitors of the G protein subunit of heterotrimeric G proteins and adenylyl cyclases. In contrast, the nuclear influx of Myb3 induced by iron or 8-bromo-cAMP was delayed or inhibited, respectively, by H89, the inhibitor of protein kinase A. Using liquid chromatography-coupled tandem mass spectrometry, Thr156 and Lys143 in Myb3 were found to be phosphorylated and ubiquitinated, respectively. These modifications were induced by iron and inhibited by H89, as shown by immunoprecipitation-coupled Western blotting. Iron-inducible ubiquitination and nuclear influx were abortive in T156A and K143R, but T156D was constitutively ubiquitinated and persistently localized to the nucleus. Myb3 was phosphorylated in vitro by the catalytic subunit of a T. vaginalis protein kinase A, TvpKAc. A transient interaction between TvpKAc and Myb3 and the phosphorylation of both proteins were induced in the parasite shortly after iron or 8-bromo-cAMP treatment. Together, these observations suggest that iron may induce production of cAMP and activation of TvpKAc, which then induces the phosphorylation of Myb3 and subsequent ubiquitination for accelerated nuclear influx. It is conceivable that iron probably exerts a much broader impact on the physiology of the parasite than previously thought to encounter environmental changes.

With an estimated ~275 million annual new cases worldwide (1), trichomoniasis caused by the infection of the protozoan parasite, Trichomonas vaginalis, in humans is the most prevalent but neglected sexually transmitted disease of nonviral origin (2, 3). Trichomoniasis during pregnancy can cause premature delivery, abortion, low birth weight, or stillbirth, but in most cases the infection is asymptomatic or manifests only mild symptoms (4, 5). Although the disease can be effectively and inexpensively cured with drug treatments, trichomoniasis may have a much greater impact on public health because it is a risk factor in transmission of human immunodeficiency virus and human papillomaviruses (6, 7) and promotes progressive types of cervical and prostate cancers (8–10).

T. vaginalis persistently colonizes human urogenital tracts without an alternate cyst stage to survive outside the human host and has to encounter challenges from immune surveillance; fluctuating levels of oxygen, iron, and hormones; and competition from indigenous microflora in host environments (5, 11). Among these variables, iron has been shown to exert versatile roles in growth and virulence expression of the parasite (12). Iron is an essential element for virtually all organisms. Both ferrous (Fe2+) and ferric (Fe3+) ions exert profound cellular effects through myriad iron-binding proteins (13). The influence of the ferrous versus ferric status in these proteins may provide a way for cells to sense intracellular oxidative stress and cause them to relay signals that elicit downstream cellular responses (14). They also react with oxygen and hydrogen peroxide and generate reactive oxygen species that can be cytotoxic (15). Living in a low oxygen environment, iron overloading is a lesser problem but might still be cytotoxic to T. vaginalis because hydrogen peroxide can be generated locally when the host immune cells encounter microbial pathogens (16). To meet the requirement for iron and avoid its cytotoxicity, cells often exploit specific iron-binding proteins, such as the transcription factors Fur and Aft1p in Escherichia coli and Saccharomyces cerevisiae, respectively, and the RNA-binding protein IRP1 and its upstream regulator FBLX5 in mammals, to sense the level of intracellular iron and regulate the expression of genes involved in iron homeostasis (17–20).

* This work was supported by National Science Council Grant NSC102-2320-B-001-003 and Institute of Biomedical Sciences (IBMS), Academia Sinica.

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TABLE 1
Sequences of oligonucleotides used in this study

| Name                                      | Sequence (5′–3′)        |
|-------------------------------------------|-------------------------|
| pAP65–2.1-ha-myb3–T156A plasmid construct | GAGGATTTCAAGCTTAATCCAAACACAGATCCCTT          |
| Myb3–(T156A)–5′                        | ATTGAATTCAAGCTTAACTCCCTT          |
| Myb3–(T156A)–3′                        | ATTGAATTCAAGCTTAACTCCCTT          |
| pAP65–2.1-ha-myb3–T156D plasmid construct | GAGGATTTCAAGCAATTTCAACTCACAAGATCCCTT          |
| Myb3–(T156D)–5′                        | ATTGAATTCAAGCTTAACTCCCTT          |
| Myb3–(T156D)–3′                        | ATTGAATTCAAGCTTAACTCCCTT          |
| pAP65–2.1-ha-myb3–K143R plasmid construct | AGCAAGAATAGATGGAATTCATCCATCGATCC          |
| Myb3–(K143R)–5′                        | ATTTCCTGAACCATTGCGTCATGCC          |
| Myb3–(K143R)–3′                        | ATTTCCTGAACCATTGCGTCATGCC          |
| pET6H/TvPKAc plasmid construct          | AGGATCCAAGTTAAGAGATCTCCCTT          |
| BamHI–TvPKAc–5′                         | ACTCGAGTTAATAATTTTCAAACTTGATCTT          |
| XhoI–TvPKAc–3′                          | ACTCGAGTTAATAATTTTCAAACTTGATCTT          |

Interestingly, Salmonella typhimurium has evolved an unusual system, the PmrB/PmrA two-component system, to sense the extracellular ferric ion and activate transcription of PmrA target genes for growth in iron-rich environments (21). In these systems, physiological responses to ironoverloading are often monitored a few h or longer post-repletion.

How iron homeostasis is maintained in T. vaginalis remains elusive, yet iron overload is beneficial to the parasite and induces the expression of genes involved in nutrient acquisition, energy metabolism, cytoadherence, and immune evasion in sustaining cell growth and survival (12, 22, 23). Studies on transcription regulation of an iron-inducible ap65–1 gene in the parasite showed that upon overnight exposure, iron could exert differential effects on three transcription factors, Myb1, Myb2, and Myb3, which are not iron-binding proteins, at the levels of gene expression, nuclear translocation, and promoter entry (24–26). In contrast, iron was recently shown to induce a nuclear influx of Myb3 in T. vaginalis within several min (27), implying that the parasite might elicit immediate cellular responses right after a sudden iron overload. With an estimated 46000–60000 protein genes and a vast array of >900 protein kinase genes in the genome of T. vaginalis (28, 29), the signal transduction network might be crucial for the unicellular parasite to quickly adapt to and thrive in the ever-changing host environments. Given its complex proteome and kinome, the study of rapid cellular responses of the parasite upon environmental stimuli and host-parasite interactions can be challenging.

In this report, the mechanism underlying the iron-inducible nuclear influx of Myb3 was studied. We found that iron might trigger a Ga-mediated signal transduction pathway that relays the upstream signal to the production of cAMP and activates protein kinase A (PKA), which induces phosphorylation and ubiquitination of Myb3 in accelerating its nuclear influx. As an initial study on signal transduction in T. vaginalis, our work provides a useful model to decipher signaling molecules involved in the regulatory network and to reveal the physiological relevance of iron overloading to the parasite.

MATERIALS AND METHODS

Cultures—T. vaginalis T1 cells were maintained in TYI-S-33 medium supplemented with 10% calf serum (30). Iron depletion and depletion were achieved with the addition of ferrous ammonium sulfate at concentrations specified throughout and 50 μM 2,2′-dipyridyl, respectively, in growth medium. Serum starvation was achieved by transferring a 1-ml overnight culture into a 13-ml TYI-S33 medium.

DNA Transfection and Selection of Stable Transfectants—Plasmids were electroporated into T. vaginalis, and transfectants were selected by paromomycin and cloned as described (30).

Inhibitors and Activators of Signal Transduction—Cells depleted of iron were treated with suramin (31), SQ22536 (32), H89 (33), and KT5823 (34), the respective inhibitors of Ga, adenyl cyclases, PKA, and protein kinase G, for 30 min prior to iron repletion at concentrations specified throughout. In some experiments, iron-depleted cells were stimulated with 50 μM 8-bromo-cAMP.

Oligonucleotides—Sequences of the oligonucleotides used in the present study are listed in Table 1 unless otherwise reported elsewhere (26, 27).

Plasmid Construction—The HA-Myb3 expression plasmid, pAP65–2.1-ha-myb3, was obtained from a previous study (27). To produce a point mutation in HA-Myb3, a 5′-DNA fragment was amplified by polymerase chain reaction (PCR) from pAP65–2.1-ha-myb3 using a primer pair, tub90f and Myb3-(XnXY′)-3′ (where X indicates the amino acid to be mutated, n indicates the location of the residue, and X′ is the mutated residue), and a 3′-DNA fragment was amplified by PCR using a primer pair, Myb3-(XnXY′)-5′ and SP6. Gel-purified PCR products were mixed, denatured, and annealed for a second PCR using a primer pair, tub90f and SP6. The PCR product digested by BglII and XhoI was cloned into a BglII/XhoI restricted pAP65–2.1-ha-myb3 backbone (27) to generate pAP65–2.1-ha-myb3-T156A, pAP65–2.1-ha-myb3-T156D, and pAP65–2.1-ha-myb3-K143R.

The coding sequence of the TvPKAc (accession number TVAG_177140; Fig. 8)2 gene was amplified from T. vaginalis genomic DNA by PCR using a primer pair, BamHI–TvPKAc–5′ and XhoI–TvPKAc–3′. Gel-purified PCR product digested by BamHI and XhoI was cloned into a pET6H backbone restricted by BamHI and XhoI to generate pET6H/TvPKAc.

Expression and Purification of Recombinant Proteins—pET6H/TvPKAc, pET30/Myb2 (25), and pET28b/Myb3 (26) each transformed into E. coli BL21 (DE3). A colony from each transformation was inoculated into LB broth containing 50 μg

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ml⁻¹ ampicillin and incubated at 37 °C with constant shaking until an A₆₀₀ of ~0.6 was reached. Expression of the recombinant protein was induced with 0.4 mM isopropyl-1-thio-β-D-galactopyranoside for 2 h and purified using a His-bind nickel column as described by the supplier (Novagen).

**Antibody Production**—Purified His-TvPKAc was used to immunize rats and mice following a standard protocol (35), and antisera were collected and purified by protein A-affinity chromatography as described by the supplier (Sigma).

**Immunofluorescence Assay (IFA)**—Cells on a slide were fixed in methanol at −20 °C for 30 min. Sequential immunoreactions were performed using a mouse monoclonal anti-hemagglutinin (HA) antibody (HA-7; Sigma) (300× or 1000×, as specified throughout) and FITC-conjugated anti-mouse IgG (Jackson ImmunoResearch) (200×). Nuclei were stained with DAPI. Fluorescent images were recorded with confocal microscopy and cell morphology by phase-contrast microscopy (SLM700, Zeiss). Relative intensity of fluorescence detected in the nucleus in a total of 300 cells randomly selected from five microscopic fields was quantified by Metamorph software (Molecular Devices).

**Cellular Fractionation**—Cell lysates were fractionated into cytosolic and nuclear fractions using a cellular fractionation kit, NE-PER™ as prescribed by the supplier (Pierce).

**Immunoprecipitation (IP)**—Agarose bead-conjugated mouse anti-HA antibody (Sigma) was added to protein samples, and reactions were incubated at 4 °C for 2 h with constant agitation. Agarose beads recovered from low speed centrifugation were washed three times in PBS containing 0.1% Triton X-100 for 10 min, and proteins were eluted with 50 μl of 50 mM acidic glycine (pH 2.8) and immediately neutralized with 3 μl of 1 M Tris base (pH 9.0). The elution was repeated three times, and eluates were combined.

**Western Blotting**—Proteins were separated by SDS-PAGE in 12% gel. The gel was stained with SyproRuby as described by the supplier (Invitrogen) or blotted to a polyvinylidene difluoride membrane, IMMOBILON-P (Millipore), by a semidry electrophoretic transfer. Sequential immunoreactions were performed, and the ECL system was used for signal detection as instructed by the supplier (Pierce). The chemiluminescence signal was recorded at multiple exposures by an image acquisition system (LAS-3000, Fujifilm), and the intensities of each band in the linear range of detection from three separate experiments were digitized and quantified by Metamorph software (Molecular Devices). Reaction conditions for antibodies from commercial sources, including mouse anti-α-tubulin (5000×) (DM1A; Sigma), rat anti-HA (2000×) (3F10; Roche Applied Science), rabbit anti-acetylation histone H3K9 (3000×; Upstate Biotechnology, Inc.), rabbit anti-ubiquitin (1000×; Santa Cruz Biotechnology, Inc.), rabbit anti-phospho-(Ser/Thr)PKA substrate (1000×; Cell Signaling), rabbit anti-PKAα (catalytic subunit (phospho-Thr¹⁹⁷) (3000×; Abcam), mouse anti-phospho-Thr/Pro¹⁰¹ (2000×; Cell Signaling), and anti-His₆ (5000×; Clontech) were performed as described by suppliers. Myb1, Myb2, Myb3, TvCyP1, TvHsp70-1, and TvPKAc were detected by mouse anti-Myb1 (1000×) (24), rabbit anti-Myb2 (2000×) (25), rabbit anti-Myb3 (2000×) (26), rat anti-TvCyP1 (3000×) (27), rat anti-TvHsp70-1 (5000×) (36), and rat anti-TvPKAc (1000×), respectively.

**Detection of Intracellular Reactive Oxygen Stress**—Cells harvested at post-iron repletion time intervals were washed in PBS three times, resuspended in PBS containing 1% BSA, and incubated with 5 μM acetyl ester of 5-(and 6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (CM-H₂DCFDA) (Molecular Probes) at 37 °C in the dark for 30 min with constant agitation. Cells were washed in PBS three times, and fluorescence was recorded by confocal microscopy.

**Detection of Cellular cAMP**—A total of 5 × 10⁷ T. vaginalis cells were resuspended in 1 ml of ice-cold 6% TCA with vigorous vortexing for 1 min. Supernatants were recovered by centrifugation at 10,000 g at 4 °C for 15 min and extracted with 1 ml of water-saturated ether four times to collect the aqueous phase. Samples were dried in a speed vacuum and dissolved in distilled water, and cAMP was detected by liquid chromatography-coupled tandem mass spectrometry (LC-MS/MS). The assay was performed with a UPLC system (ACQUITY UPLC, Waters, Milford, MA) coupled to a hybrid Q-TOF mass spectrometer (Synapt HDMS G1, Waters, Manchester, UK). The UPLC system, the mobile phase A was 2% acetonitrile in water, and B was 100% acetonitrile. The sample was separated online with a hybrid-phase column (ACQUITY UPLC HSS T3, 1.7 μm, 2.1 × 150 mm) at a flow rate of 0.45 ml/min using a 6-min 2–99% acetonitrile/water gradient. The temperature of the separation column was maintained at 40 °C. For the MS analysis, the LC column was coupled online to the LockSpray electrospray ion source of the hybrid Q-TOF mass spectrometer, and 0.035 ppm sulfadimethoxine was continuously infused to the LockSpray emitter with a flow rate of 40 μl/min. The MS was switched to acquire the reference sulfadimethoxine signal from the LockSpray emitter every 6 s for mass error correction. The electrospray ionization voltage was −2.5 kV, with a cone voltage of 40 V. The cone and desolvation gas flows were 50 and 800 liters/h, respectively. The source and desolvation temperatures were 80 and 250 °C, respectively. The LC-MS data were collected in MS or MS/MS mode with an m/z range of 50–350 Th and 0.2-s scan time. For the MS/MS mode, the precursor m/z 328 Th was selected, and the collision energy was set to 25 eV. To quantify the cAMP using LC-MS/MS data, a cAMP collision-induced dissociation fragment m/z 134.05 Th with an extraction m/z window of 0.015 Th was selected to plot the extracted ion chromatogram and calculate the peak area using MassLynx version 4.1 (Waters, Manchester, UK).

**Identification of Protein Post-translational Modifications**—Protein bands stained with SyproRuby as specified throughout were excised from gel and processed for trypsin digestion as described (37). Tryptic peptides were analyzed by nanoflow LC-MS/MS. Mass spectral data were acquired using a Thermo LTQ-FIN ion trap mass spectrometer (Thermo Fisher) equipped with a nanoelectrospray ion source (New Objective, Woburn, MA), an Agilent 1100 series binary high performance liquid chromatography...
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Iron-inducible Nuclear Influx of Myb3—Iron was previously shown to induce the rapid nuclear influx of HA-tagged and endogenous Myb3 protein in T. vaginalis (27). In this report, this phenomenon was re-examined in greater detail. HA-Myb3 was localized to the nucleus of T. vaginalis under normal growth conditions but barely detected in iron-depleted cells by IFA using a 300× dilution of the anti-HA antibody (Fig. 1A). When iron-depleted cells were replete with 250 μM iron, HA-Myb3 was detected in the nucleus at 3 min and exhibited an increasing level of peaking at 15 min but returned to baseline at 30 min, probably due to nuclear efflux of the protein (27). Similar results were obtained when cells in normal medium were replete with iron using a 1000× dilution of the anti-HA antibody in the IFA (Fig. 1B). The level of inducible nuclear influx as monitored at 15 min post-repletion was dependent on the iron concentration being in the range of 75–750 μM (Fig. 1C). Rapid nuclear influx of HA-Myb3 was also detected in serum-starved cells replete with 250 μM iron but not with 10% serum (Fig. 1D). Inducible Myb3 nuclear influx similar to controls was also detected in cells pretreated with 10 μM thiourea, scavenger of hydroxyl radicals (41) (Fig. 1E). Oxidative stress detected by CM-H2DCFDA staining was apparent in cells treated with 1 mM hydrogen peroxide for 30 min, but it was detected only at a much lower level in cells at 30 min post-iron reploation (Fig. 1F), implying that iron overloading under our test conditions may generate a slight oxidative stress that is insufficient for triggering a nuclear influx of Myb3. These results suggest that iron overloading alone can trigger a rapid nuclear influx of Myb3. Cells depleted of iron were used in the following experiments to study the effects of 250 μM iron and 8-bromo-cAMP and inhibitors of a few signaling molecules on the nuclear influx of HA-Myb3, and the IFA was performed using a 300× dilution of the anti-HA antibody.

Signal Transduction in the Iron-inducible Nuclear Influx of Myb3—The sequence of Myb3 has a predicted PKA phosphorylation motif (KRXSTI) residing within a small hairpin next to helix 6 in the DNA-binding R2R3 domain (42). To test whether the iron-inducible nuclear influx of Myb3 is mediated through a PKA-centered signal transduction pathway, cells were treated with various inhibitors for 30 min prior to the reploation with iron, 50 μM 8-bromo-cAMP, or the same volume of water and fixed at intervals for the IFA. The nuclear influx of HA-Myb3 induced by iron peaked at 15 min and subsided at 30 min (Fig. 2A). In contrast, the extended nuclear influx of HA-Myb3 was induced in cells stimulated with 50 μM 8-bromo-cAMP. Water had no effect on inducible nuclear influx of Myb3. The inducible nuclear influx of Myb3 peaking at 15 min post-repletion of iron was largely inhibited in cells pretreated with 20 μM suramin and 10 μM SQ22536, the respective inhibitors of the Ga subunit of heterotrimeric G proteins (31) and adenyl cyclases (32), but the nuclear influx was peaking at 30 min in cells pretreated with 42 nM H89, the inhibitor of PKA (33) (Fig. 2B). The nuclear influx of Myb3 induced by 8-bromo-cAMP was not affected by suramin or SQ22536 but was inhibited by H89 at the same dose that only delayed the nuclear influx induced by iron to a later time point.

Iron-inducible Changes in Cellular cAMP Concentration—The effect of iron on the level of cellular cAMP was assayed by LC-MS/MS. The MS/MS spectra and the mass chromatograms targeted on the ion transition m/z 328–134 for cAMP standard and the samples from iron-depleted T. vaginalis and those from iron reploation for 3 min are shown in Fig. 3A. Based on the characteristic cAMP fragmentation ion transition, only one chromatography peak was detected in each T. vaginalis sample, and the MS/MS spectrum within the detected chromatographic peak was matched to the one observed in the cAMP standard. When the time course of intracellular cAMP level post-iron reploation was examined (Fig. 3B), a small quantity of cAMP was detected in iron-depleted samples, and its levels in iron-reploate samples were 7- and 13-fold higher than baseline at 1 and 3 min, respectively, and subsided to baseline at 5–10 min post-iron reploation. The increase in cellular cAMP induced by iron at the peak level was reduced from ~12-fold to ~3- and 2-fold by 20 μM suramin and 10 μM SQ22536, respectively. The basal level of cAMP as detected in these experiments was not affected by suramin or SQ22536.

Post-translational Modifications of Myb3—To identify the post-translational modification sites in HA-Myb3, cell lysates from cells overexpressing HA-Myb3 were enriched by IP using the anti-HA antibody and separated by SDS-PAGE. When
stained by SyproRuby (Fig. 4A, lane 1), several protein bands were detected in gel. A duplicate gel was assayed by Western blotting (Fig. 4A, lane 2), and a major ~34 kDa band and a slower migrating doublet band were detected by the anti-Myb3 antibody. Myb3-related bands were excised from the gel and trypsin-digested. Extracted peptides were analyzed by LC-MS/MS. The phosphorylation at Thr156 and ubiquitination at Lys143 in Myb3 were identified in the peptides 153RISTNSNHKEILLPDR168 (Fig. 4B) and 133LIPGRTDNAIK143 (Fig. 4C), respectively.

 Ubiquitination of Lys143 in Myb3 and Inducible Nuclear Influx—To study the potential role of Lys143 in Myb3, K143R was generated and overexpressed in *T. vaginalis*. As revealed by the IFA (Fig. 5A), K143R was localized to the nucleus to a level similar to that of HA-Myb3 under normal growth conditions, but iron-inducible nuclear influx as seen in controls was not apparent in K143R. Cell lysates harvested before and after iron repletion were separated into cytosolic and nuclear fractions for Western blotting (Fig. 5B). Similar amounts of HA-Myb3 and K143R were detected by the anti-HA antibody in total cell

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overexpressed proteins were enriched by IP and assayed by lysates were separated into cytosolic and nuclear fractions. The HA-Myb3 and K143R were replete with iron for 15 min, and ubiquitinated. A band is nonubiquitinated, and those of higher sizes are seen in iron depletion samples, indicating that the 34 kDa Myb3 samples from nuclear fractions to higher levels at 15 min than at 70, and 130 kDa were also detected upon longer exposure in 30 min. Additional bands with higher molecular sizes at 45, 55, 85, and 130 kDa and smearing signals were detected primarily in nuclear samples by the anti-ubiquitin antibody. Ubiquitination signals were detected to much higher levels in nuclear than cytosolic samples. The ubiquitination signals were observed in HA-Myb3, but not T156A, Myb1, and Myb2, indicating that iron induces the ubiquitination of Myb3 at Lys143 to facilitate its nuclear influx.

To confirm the site of ubiquitination, cells overexpressing HA-Myb3 depleted of iron were treated with 250 μM iron, 50 μM 8-bromo-cAMP, or water. In B, iron-depleted cells were pretreated with DMSO, 20 μM suramin, 10 μM SQ22536, or 42 nM H89 for 30 min prior to repletion with 250 μM iron (left) or 50 μM 8-bromo-cAMP (right). Cells were fixed at intervals as indicated above each panel for IFA using the anti-HA antibody and anti-IgG-conjugated with FITC. The intensity of the fluorescence signal in the nuclei was measured under confocal microscopy and quantified by Metamorph software, and the values are listed below each panel with S.E.

**FIGURE 2.** Mapping the signal transduction pathway of Myb3 nuclear influx. In A, *T. vaginalis* cells overexpressing HA-Myb3 depleted of iron were treated with 250 μM iron, 50 μM 8-bromo-cAMP, or water. In B, iron-depleted cells were pretreated with DMSO, 20 μM suramin, 10 μM SQ22536, or 42 nM H89 for 30 min prior to repletion with 250 μM iron (left) or 50 μM 8-bromo-cAMP (right). Cells were fixed at intervals as indicated above each panel for IFA using the anti-HA antibody and anti-IgG-conjugated with FITC. The intensity of the fluorescence signal in the nuclei was measured under confocal microscopy and quantified by Metamorph software, and the values are listed below each panel with S.E.

lysates regardless of iron. The cytosolic and nuclear HA-Myb3 were detected to lower and higher levels, respectively, in samples from iron repletion than in samples from iron depletion. In contrast, the level of cytosolic K143R remained similar, but a slight increase of nuclear K143R was observed post-iron repletion. The subcellular distribution of Myb1 and Myb2 varied little. Consistent with previous findings (27), *T. vaginalis* cells overexpressing HA-Myb3 but not K143R, indicating the purity of each fraction.

To study whether iron induces ubiquitination in Myb3, cells overexpressing HA-Myb3 replete with iron and cell lysates harvested at intervals were fractionated into cytosolic and nuclear fractions, and the overexpressed protein was enriched by IP. When these samples were assayed by Western blotting (Fig. 5C), a major 55 kDa band and several weaker signals at ~36, ~45, ~70, and ~130 kDa and smearing signals were detected primarily in nuclear samples by the anti-ubiquitin antibody. Ubiquitination signals were detected to much higher levels in samples harvested at 15 min than at 7.5 and 30 min after iron repletion, but no such signals were seen in samples depleted of iron. In a duplicate blot, a major 34 kDa band was detected by the anti-Myb3 antibody to similar levels in cytosolic samples harvested over time, but slightly increasing amounts of nuclear Myb3 were detected that reached a peak at 15 min post-iron repletion and declined to a level slightly lower than baseline at 30 min. Additional bands with higher molecular sizes at 45, 55, 70, and 130 kDa were also detected upon longer exposure in samples from nuclear fractions to higher levels at 15 min than at 7.5 and 30 min after iron repletion, but these bands were not seen in iron depletion samples, indicating that the 34 kDa Myb3 band is nonubiquitinated, and those of higher sizes are ubiquitinated.

To confirm the site of ubiquitination, cells overexpressing HA-Myb3 and K143R were replete with iron for 15 min, and lysates were separated into cytosolic and nuclear fractions. The overexpressed proteins were enriched by IP and assayed by Western blotting (Fig. 5D). Ubiquitination signals were detected in HA-Myb3 but not K143R after iron repletion to higher levels in nuclear than cytosolic samples. The ubiquitinated bands were also detected by the anti-Myb3 antibody in samples from cells overexpressing HA-Myb3 but not K143R, suggesting that iron induces the ubiquitination of Myb3 at Lys143 to facilitate its nuclear influx.

**Phosphorylation of Thr<sup>156</sup> in Myb3 and Inducible Nuclear Influx**—Two mutant proteins, T156A and T156D, were generated and overexpressed in *T. vaginalis*. Under normal growth conditions, T156A and T156D are each localized to the nucleus to a level similar to that of HA-Myb3 by the IFA (Fig. 6A), indicating that the phosphorylation of Thr<sup>156</sup> in Myb3 is not crucial for steady state nuclear translocation. The typical iron-inducible nuclear influx seen in HA-Myb3 was aborted in T156A, whereas T156D was persistently localized to the nucleus at a level independent of iron, suggesting that the phosphorylation at Thr<sup>156</sup> of Myb3 may account for its facilitated nuclear influx.

When examined by Western blotting, T156A was detected to a level substantially lower than HA-Myb3 in total cell lysates (Fig. 6B). Overall expression levels of these proteins were not affected by iron. The amounts of nuclear and cytosolic HA-Myb3 were higher and lower, respectively, in samples from iron repletion than in samples from iron depletion, but the amounts of T156A, Myb1, and Myb2 changed little. When overexpressed proteins in nuclear lysates were enriched by IP for Western blotting (Fig. 6C), both the smear and distinct ubiquitination signals were observed in HA-Myb3, but not T156A, to a much higher level in samples from iron repletion than in samples from iron depletion. Similar signals plus a 34 kDa band, probably nonubiquitinated Myb3, were detected by the anti-Myb3 antibody in a duplicate blot. Interestingly, a band referred to as Myb3(Thr(P)<sup>156</sup>) that migrated slightly slower than the 34 kDa Myb3 was detected by the anti-phospho-(Ser/Thr)PKA substrate antibody in samples from iron-replete cells overexpressing HA-Myb3 but not T156A, implying that Myb3 is probably phosphorylated at Thr<sup>156</sup> upon iron repletion.
In contrast, T156D was overexpressed to a level only slightly lower than HA-Myb3 in total cell lysates (Fig. 6D). In nuclear lysates from iron-depleted cells, T156D was detected at a higher level than Myb3. An iron-inducible increase in nuclear samples was detected in HA-Myb3 but not T156D (Fig. 6D). When overexpressed proteins in nuclear lysates were enriched by IP for Western blotting (Fig. 6E), the ubiquitination of T156D but not HA-Myb3 was detected. Iron only slightly enhanced the ubiquitination of T156D, probably due to its higher basal level. In the 34-kDa nonubiquitinated form,
T156D migrated slightly faster than HA-Myb3, probably due to an extra negative charge from Asp. In both blots, a ~20 kDa band was detected at a much higher level in T156D than HA-Myb3 and was also higher in samples from iron repletion than in samples from iron depletion, suggesting that this band is probably a degraded form of each protein and implying that the phosphorylation at Thr156 might accelerate protein degradation. This possibility remains to be studied. In a duplicate blot, Myb3(Thr(P)156) was detected only in samples from iron-replete cells overexpressing HA-Myb3 but not T156D. These
observations suggest that iron may induce sequential phosphorylation and ubiquitination of Myb3 at Thr156 and Lys143, respectively, prior to nuclear influx.

**PKA-dependent Phosphorylation and Ubiquitination of Myb3**—To test whether these post-translational modifications are regulated by PKA, transgenic cells (Fig. 7, A and C) and nontransgenic control (Fig. 7B) were treated with H89 or KT5823, the latter being a PKG inhibitor, for 30 min before iron repletion. Lysates were fractionated into cytosolic and nuclear fractions for Western blotting. The expression of HA-Myb3 in total lysates and cytosolic fractions varied little (Fig. 7A), regardless of drug treatments and iron repletion. Upon iron repletion, a substantial increase of HA-Myb3 in nuclear fractions was seen with a concomitant decrease of cytosolic HA-Myb3 in controls treated with DMSO but to a lesser extent also in KT5823-treated samples. The change was marginal in samples treated with H89. The amounts of Myb1 and Myb2 in these samples remained constant. When the subcellular distribution of endogenous Myb3 was examined in nontransgenic cells (Fig. 7B), H89 and KT5823 also exerted similar inhibitory effects, as observed in overexpressed Myb3, suggesting that iron inducible nuclear translocation of both endogenous and overexpressed Myb3 is activated by PKA and possibly also by PKG to a lesser extent. HA-Myb3 in nuclear lysates from transgenic cells was enriched by IP for Western blotting (Fig. 7C). Iron-inducible ubiquitination of HA-Myb3 that was largely inhibited by H89 was only slightly inhibited by KT5823. In a duplicate blot, the doublet 34 kDa bands along with several higher molecular...
weight bands were detected by the anti-Myb3 antibody. The major and faster migrating band in the doublet was present at similar levels in control and treatments. The slower migrating band in the control was detected at much higher levels in samples from iron repletion than in samples from iron depletion. This band was also seen in samples from iron-replete cells pretreated with KT5823 but not H89, with an increasing level lower than that in control. Moreover, Myb3(Thr(P)156) was detected at similar levels in control and samples from KT5823-treated cells only at 15 min after iron repletion. These results suggest that Myb3 can be phosphorylated at Thr\textsuperscript{156} by a PKA upon iron repletion and that under the same conditions, Myb3 may also be phosphorylated by other protein kinases like PKG.2

Identification and Characterization of a TvPKAc—In our preliminary study,\textsuperscript{2} a unique phosphopeptide with a differential level of phosphorylation before and after iron repletion was identified by LC-MS/MS (Fig. 8A). The peptide, which is phosphorylated at Thr\textsuperscript{164} of a T. vaginalis AGC protein kinase (28) and referred to as TvPKAc, is nearly identical in sequence to that spanning an autophosphorylation active site, Thr\textsuperscript{197}, of mouse PKA catalytic subunit (Fig. 8B).

TvPKAc shares \textfrac{11011}{32}\% of its sequence identity with the \textfrac{11021}{925}\% subtype of mouse PKAc. To study the role of the TvPKAc during the inducible nuclear influx of Myb3, recombinant His-TvPKAc was produced and purified. When separated by SDS-PAGE, a single 43 kDa band was stained by Coomassie Blue (Fig. 8C, lane 1). When the purified protein was analyzed by Western blotting, the 43 kDa band was detected by both the anti-His and anti-PKA (catalytic subunit) (phospho-Thr\textsuperscript{197}) (Fig. 8C, lane 3) antibodies, implying that His-TvPKAc is probably autophosphorylated at Thr\textsuperscript{164}. When total lysates from T. vaginalis T1 cells were examined by Western blotting, a major \textfrac{11011}{40}-kDa TvPKAc band and a minor band of smaller size were identified by the anti-TvPKAc antibody (Fig. 8D). These bands were also

FIGURE 7. PKA-dependent phosphorylation and ubiquitination of Myb3. Cells depleted of iron overnight were treated with DMSO, H89, and KT5823 for 30 min and replete with 250 μM iron. Transgenic cells overexpressing HA-Myb3 (A) or nontransgenic control (B) were harvested at intervals, and cell lysates were fractionated into cytosolic (Cy) and nuclear (N) fractions for Western blotting detection by antibodies as indicated. The intensity of HA signal (L and S, longer and shorter exposures of the same blot, respectively) and endogenous Myb3 (B) in the cytosolic and nuclear fractions was quantified from three experiments and statistically analyzed as shown in the histograms, with \( p < 0.05 \) (*) and \( p < 0.01 \) (**) indicated. C, HA-Myb3 was enriched by IP. Samples were separated by SDS-PAGE for Western blotting using the anti-Myb3, anti-ubiquitin, and anti-phospho-(Ser/Thr)PKA substrate antibodies to detect overall Myb3, ubiquitinated Myb3, and Myb3(Thr(P)156), respectively. Arrowheads, bands detected by both the anti-ubiquitin and anti-Myb3 antibodies. Arrow, band detected only by the anti-Myb3 antibody. H and L, heavy and light chains of IgG, respectively. To show the linear range of detection among samples in the middle panel of B, the 34 kDa band, as indicated by the arrow, was taken from a very short exposure. H, heavy chain of IgG. Error bars, S.E.
detected by the anti-PKA γ (catalytic subunit) (phospho-Thr197) antibody, indicating that TvPKAc is probably phosphorylated, possibly at Thr164, the conserved autophosphorylation site referred to as TvPKAc(Thr(P)164). In an in vitro protein kinase assay (Fig. 8E), His-TvPKA and [γ-32P]ATP were co-incubated with His-Myb2 or His-Myb3, and the reaction products were separated by SDS-PAGE for detection by the autoradiogram. The radioisotopic 32P signal was detected at a much greater level in Myb3 than in Myb2 but not in His-TvPKAc itself. When a duplicate reaction was performed using only non-isotopic ATP as a substrate, Myb2 was detected at a much higher level than Myb3 by Western blotting using the anti-His6 antibody, indicating that the His-TvPKAc-specific phosphorlyates Myb3, but it may also nonspecifically phosphorlyate Myb2 to a much lesser extent. When iron-depleted cells were replete with iron or stimulated with 8-bromo-cAMP, the level of TvPKAc(Thr(P)164) increased continuously and peaked at 15 min but returned to baseline at 30 min post-repletion (Fig. 8F). During this period, the expression levels of TvPKAc and α-tubulin changed only slightly. These observations suggest that iron and 8-bromo-cAMP might exert similar effects on the phosphorylation of TvPKAc.

Inducible Complex Formation between TvPKAc and Myb3—To study the effects of iron on TvPKAc and Myb3, cells over-expressing HA-Myb3 were replete with iron and harvested at 0, 7.5, 15, and 30 min. Cell lysates were fractionated into cytosolic and nuclear fractions for Western blotting (Fig. 9A). In total cell lysates, increasing levels of TvPKAc(Thr(P)164) were detected post-iron repletion, although a slightly lower level of TvPKAc was detected at 7.5 min than at other time points. Other proteins detected in the same blot varied little. HA-Myb3 was detected to similar levels in cytosolic fractions at various time points. It was detected in increasing amounts in nuclear fractions and peaked at 15 min post-iron repletion, but returned to baseline within 30 min. TvPKAc was only detected in cytosolic fractions at similar levels regardless of iron, but a transient increase in TvPKAc(Thr(P)164) was observed. In contrast, TvCyP1 and H3K9-Ac were detected in cytosolic and nuclear fractions, respectively, and TvHsp70-1 was in both fractions at similar levels.

HA-Myb3 in these samples was enriched by IP for Western blotting (Fig. 9B). Cytosolic HA-Myb3 was detected at a lower level at 7.5 than at 0 min, but the signal increased at later time...
points and reached a maximum at 30 min. By contrast, Myb3(Thr(P)156) was first detected at 7.5 min with decreasing levels thereafter. Much higher levels of nuclear HA-Myb3 were detected at 7.5 and 15 min than at 0 and 30 min, and Myb3(Thr(P)156) in these samples was observed to peak at 15 min post-repletion. 

TvPKAc and TvPKAc(Thr(P)164) were detected post-repletion at 7.5 and 15 min at similar levels only in samples from cytosolic fractions. TvHsp70-1 was detected in samples only from cytosolic fractions at similar levels over the entire time course.

To compare the effects of iron and cAMP on the interaction of TvPKAc and Myb3, cells overexpressing HA-Myb3 were replete with iron or stimulated with 8-bromo-cAMP for 15 min. Cell lysates were fractionated into cytosolic (Cy) and nuclear (N) fractions, as indicated above each panel. Samples were directly analyzed by Western blotting (A and C) or enriched by IP before Western blotting (B and D) using the anti-Myb3, anti-phospho-(Ser/Thr)PKA substrate, and anti-phospho-Thr/Pro, anti-TvPKAc, anti-PKAcγThr(P)197, anti-TvHsp70-1, anti-TvCyP1, and anti-H3K9-Ac antibodies to detect overall Myb3, Myb3(Thr(P)156), Myb3(pTP), TvPKAc, TvPKAc(Thr(P)164), TvHsp70-1, TvCyP1, and H3K9-Ac, respectively. Relative amounts of HA-Myb3 in cytosols (Cy) and nuclei (N) are each quantified from three independent experiments and statistically analyzed as shown in the histogram, with p < 0.05 (*) and p < 0.01 (**) indicated. Error bars, S.E.

These samples were also enriched by IP for Western blotting (Fig. 9D). Cytosolic HA-Myb3 was detected at similar levels in samples from iron repletion and 8-bromo-cAMP stimulation, slightly lower than that in control. In contrast, nuclear HA-Myb3 was detected at similar levels in samples from iron repletion and 8-bromo-cAMP stimulation, much higher than that in control. Myb3(Thr(P)156) was only detected in nuclear samples at similar levels in samples from iron repletion and 8-bromo-cAMP stimulation, much higher than that in control. In a duplicate blot, a phosphorylated Myb3 detected by the anti-phospho-Thr/Pro antibody was mostly in nuclear samples at a much higher level with iron repletion than 8-bromo-cAMP stimulation or control to phosphorylate Myb3 and facilitate its nuclear influx. TvPKAc was detected at similar levels in all cytosolic samples, but TvPKAc(Thr(P)164) was detected at similar levels in samples from iron repletion and 8-bromo-cAMP stimulation, much higher than that in control. TvHsp70-1 was detected in all cytosolic samples at similar levels. These results suggest that the interactions between TvPKAc and Myb3 and subsequent consequences induced by iron are probably in part mediated via its effect on the production of cellular cAMP.
Iron-activated Signal Transduction in T. vaginalis

It is notable that the magnitude of iron-inducible increase in nuclear Myb3 as detected by the IFA (Figs. 1, 2, 5, and 6) and Western (Figs. 5–9) differs greatly, and this incoherence is probably due to the fact that the volume of the nucleus in a typical cell is much smaller than that occupied by the cytoplasm, given that the diameter of the former is ~1 μm, and the latter is ~10 μm in length and ~5 μm in width. Thus, a seemingly slight change in the quantity of nuclear Myb3 by Western blotting can result in substantial change of its concentration in the nucleus. Although the amount of cytosolic Myb3 is always in excess of that of nuclear Myb3, as shown in the Western blots (Figs. 5–9), its concentration in the cytoplasm is low and probably below the detection limit of the IFA. The concentration effect thus may account for the incoherence in the magnitude of induction levels assayed by the IFA and Western blot.

As shown by the effects of suramin and SQ22536 on nuclear influx of Myb3 (Figs. 2 and 3), iron probably triggers a Gα-mediated pathway that relays upstream signals to adenylyl cyclase, which in turn catalyze the biosynthesis of cAMP, a second messenger for cAMP-binding proteins, including PKA (46), in which the binding of cAMP to the regulatory subunits may release the catalytic subunits from the tetrameric holoenzyme and phosphorylate Myb3 to induce its nuclear influx, as depicted in Fig. 10. Consistent with the proposed pathway, iron induced transient elevation of intracellular cAMP that was inhibited by suramin and SQ22536 (Fig. 3), whereas 8-bromo-cAMP induced a prolonged Myb3 nuclear influx that was inhib-
Iron-activated Signal Transduction in T. vaginalis

The iron-inducible nuclear influx of Myb3 was inhibited by suramin and SQ22536, but it was only delayed to a later time point by H89 (Fig. 2A). This discrepancy is unlikely to be due to an insufficient amount of H89 used in the experiments because the same dosage inhibited 8-bromo-cAMP-induced nuclear influx of Myb3 and the phosphorylation and ubiquitination of Myb3 at Thr\textsuperscript{156} and Lys\textsuperscript{143}, respectively (Figs. 2C and 7). Moreover, the level of phosphorylation of an unknown Thr/Ser residue(s) preceding Pro at Myb3 was only inhibited by iron and not 8-bromo-cAMP (Fig. 9D), implying that iron may also activate a proline-directed protein kinase like MAPK or CDK to phosphorylate Myb3 (16, 49). This possibility is supported by the observation that nuclear translocation of Myb3 is regulated by TvCyP1 (36), a cyclophilin catalyzing the cis-trans interconversion of the peptidyl-prolyl bond (50). Iron-inducible influx of Myb3 was also partially inhibited by KT5823 (Fig. 7), which had little effect on iron-inducible phosphorylation at Thr\textsuperscript{156} and ubiquitination at Lys\textsuperscript{143} of Myb3, whereas 8-bromo-cGMP could also induce the nuclear influx of Myb3.\textsuperscript{2} Together, these observations suggest that iron might simultaneously trigger multiple signaling pathways and that Myb3 imported into the nucleus through those mediated other than by TvPKAc may bind DNA at promoter sites to positively regulate transcription. Presumably, the parasite might switch off transcription of a subset of its target genes before switching on transcription of another subset. Why the parasite should exploit such a complicated mechanism to regulate nuclear import of Myb3 remains an intriguing question.

The proper annotation of protein kinases in T. vaginalis has been difficult, in part due to their complexity and sequence divergence from eukaryotic paradigms. These hurdles were overcome in the identification of a TvPKAc while exploring a phosphoproteomics approach.\textsuperscript{2} The activity and substrate specificity of recombinant His-TvPKAc were confirmed by an in vitro protein kinase assay (Fig. 8D), and its phosphorylation in vivo was induced by iron and 8-bromo-cAMP to similar levels (Fig. 8F). Iron and 8-bromo-cAMP exerted similar roles in inducible interaction between TvPKAc and Myb3, leading to the nuclear influx of Myb3 (Fig. 9, B and D). Because iron but not cAMP induced phosphorylation of Myb3 at some Ser/Thr residues preceding Pro (Fig. 9D), Myb3 might also interact with a Pro-directed protein kinase (16, 51) and form a transient protein complex distinct from that with TvPKAc.\textsuperscript{2} In contrast, Myb3 was persistently associated with TvHsp70-1 in the cytoplasm independently of iron and cAMP. In this regard, TvHsp70-1 may serve as molecular chaperone in maintaining proper conformation of Myb3 for the assembly and disassembly of the protein complexes. Also, the transient change of cellular cAMP that leads to the interaction of TvPKAc and Myb3 is probably regulated by feedback control of the intracellular cAMP level through a phosphodiesterase (32). These possibilities remain to be investigated. A survey of the genome database only revealed a TvPKAc and two regulatory isoforms of TvPKAr in T. vaginalis.\textsuperscript{2} Given such simplicity in the TvPKA system, its global roles in iron overloading can be investigated. TvPKAc might also be activated by other extracellular stimuli or intrinsic factors because cAMP in T. vaginalis can be produced from numerous adenylyl cyclases (47). To the best of our knowledge, this is the first protein kinase in T. vaginalis to be biochemically and functionally defined.

T. vaginalis is a successful parasite that rarely causes severe symptoms in humans. Unlike most other parasites, it survives solely in the human host as simple asexual trophozoites without apparent cell differentiation or a dormant stage. With a remarkably complex proteome and kinome for a unicellular organism, the parasite may have evolved intricate cellular machinery to adapt to hostile host environments. In this regard, the signal transduction network can be a frontline for the parasite to counteract host defenses. Our observations suggest that T. vaginalis uses a conserved signaling pathway to relay cellular
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signals triggered by iron from plasma membrane to the nucleus. It would be interesting to see whether this phenomenon is also present in other organisms. Given the nature of signal transduction for amplifying upstream signals at each subsequent step, it is conceivable that iron may have a much broader impact on the cell physiology of the parasite than observed in this study.2

Acknowledgments—We thank Dr. Fu-An Li at the proteomics core of IBMS for technical support, the metabolomics core of Academia Sinica for the use of facilities, and Buford C. Pruitt, Jr., for English editing of the manuscript.

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