**Toxoplasma gondii** Activates Hypoxia-inducible Factor (HIF) by Stabilizing the HIF-1α Subunit via Type I Activin-like Receptor Kinase Receptor Signaling

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**Toxoplasma gondii** is an intracellular protozoan parasite that can cause devastating disease in fetuses and immune-compromised individuals. We previously reported that the α subunit of the host cell transcription factor, hypoxia-inducible factor-1 (HIF-1), is up-regulated by infection and necessary for *Toxoplasma* growth. Under basal conditions, HIF-1α is constitutively expressed but rapidly targeted for proteasomal degradation after two proline residues are hydroxylated by a family of prolyl hydroxylases (PHDs). The PHDs are α-ketoglutarate-dependent dioxygenases that have low $K_m$ values for oxygen, making them important cellular oxygen sensors. Thus, when oxygen levels decrease, HIF-1α is not hydroxylated, and HIF-1 is activated. How *Toxoplasma* activates HIF-1 under normoxic conditions remains unknown. Here, we report that *Toxoplasma* infection increases HIF-1α stability by preventing HIF-1α prolyl hydroxylation. Infection significantly decreases PHD2 abundance, which is the key prolyl hydroxylase for regulating HIF-1α. The effects of *Toxoplasma* on HIF-1α abundance and prolyl hydroxylase activity require activin-like receptor kinase signaling. Finally, parasite growth is severely diminished when signaling from this family of receptors is inhibited. Together, these data indicate that PHD2 is a key host cell factor for *T. gondii* growth and represent a novel mechanism by which a microbial pathogen subverts host cell signaling and transcription to establish its replicative niche.

**Toxoplasma gondii** is an obligate intracellular protozoan parasite that can cause devastating disease in immune-compromised people and fetuses (1, 2). The ability for the parasite to cause disease is directly linked to its replication inside a parasitophorous vacuole within its host cell. From this vacuole, parasites scavenge nutrients from its host cell while causing reorganization of host organelles and cytoskeletal elements, preventing host cell apoptosis, and altering host gene expression (3–5). Therefore, *Toxoplasma* has developed mechanisms to manipulate host cell processes that permit it to grow. Identifying the parasite factors that cause these changes and the specific host pathways modulated by these factors is critical for developing new drugs to treat and prevent *Toxoplasma* infections and disease.

Previously, our laboratory demonstrated that *Toxoplasma* activates the host cell transcription factor hypoxia-inducible factor-1 (HIF-1)$^3$ (6). HIF-1 is activated by a parasite-derived, secreted factor independent of direct contact between the parasite and host cell. This is in contrast to other *Toxoplasma* factors that control host cell signaling by virtue of their secretion into the host cell cytoplasm (7–10). Significantly, HIF-1 is important for parasite growth at both normoxic and physiological O$_2$ levels but dispensable for host cell replication at either O$_2$ levels (6).

HIF-1 is a heterodimer composed of α and β subunits and is the master regulator of the cellular response to decreased O$_2$ availability (11). Although both subunits are constitutively expressed, the α subunit has a very short half-life (~2 min) because it is targeted in an O$_2$-dependent fashion to the proteasome by the von Hippel-Lindau E3 ubiquitin ligase (12–14). HIF-1α recognition by von Hippel-Lindau is dependent on the hydroxylation of HIF-1α on two proline residues (Pro$^{562}$ and Pro$^{564}$) in a domain termed the oxygen-dependent degradation domain (ODD) (15–19). Three HIF-1α prolyl hydroxylases (PHD1–3) have been identified. Gene knockout and siRNA-based studies indicate that PHD2 is the primary isoform responsible for regulating HIF-1α (20, 21). HIF-1-dependent transcription is regulated by an asparaginyl hydroxylase named factor inhibiting HIF (FIH) that hydroxylates Asn$^{803}$ in the C terminus of HIF-1α (22–24). The PHDs and FIH are oxygen and α-ketoglutarate-dependent dioxygenases that use ascorbic acid and Fe$^{2+}$ as cofactors. The affinity of these enzymes for O$_2$ is low ($K_m = 90–250$ μM), which renders them important cellular O$_2$ sensors (25, 26). Thus, when O$_2$ levels decrease, PHD and FIH activities are reduced, and two independent events...
take place: HIF-1α protein increases, and HIF-1α binds p300/CBP.

Increases in HIF-1α protein levels and activity are not restricted to hypoxic stress. Besides Toxoplasma, other bacterial, viral, and protozoan pathogens activate HIF-1 (27–33). HIF-1 is also activated by cytokines, growth factors, and other secreted factors (34–36). Although some of these agents increase HIF-1α protein by increasing its rate of translation, others act by stabilizing the protein. How HIF-1α is stabilized at normoxic O2 levels is largely unknown. Given its key role in regulating HIF-1α, dysregulation of PHD2 may be one way to stabilize HIF-1α under normoxic conditions. For example, HIF-1α can be activated by decreased availability of PHD2 cofactors and substrates (37). In addition, PHD2 activity can also be impacted by its abundance. Recent studies have highlighted two post-transcriptional mechanisms that control PHD2 protein levels. First, PHD2 binds the peptidyl prolyl cis/trans-isomerase FKBP38, and siRNA-mediated depletion of FKBP38 leads to increases in PHD2 protein abundance (38). TGFβ signaling through the Type I TGFβ receptor is a second mechanism to decrease PHD2 protein levels (39). The Type I TGFβ receptor is a member of the activin-like receptor kinase (ALK) family of receptors that bind several distinct ligand families including TGFβs, activins, and bone morphogenic proteins (40, 41). The cytoplasmic domains of the ALK receptors are serine/threonine kinases that are activated when the ligand induces the Type I receptor to dimerize with a Type II receptor. Seven Type I receptors (ALK1–7) have been identified and can be grouped based upon the homology of their cytoplasmic domains. The high degree of homology between ALK5 (Type I TGFβ receptor), ALK4, and ALK7 renders them similarly sensitive to a class of highly selective small molecule inhibitors that have very limited affects on other kinases including p38 MAPKs (42).

The goal of this work is to determine the mechanism by which Toxoplasma increases HIF-1α protein levels. We report that Toxoplasma infection increases HIF-1α stability by decreasing levels of HIF-1α hydroxylation. Alterations in HIF-1α stability are due to decreased PHD2 expression. Decreased PHD2 protein and HIF-1 activation was dependent on ALK signaling. Finally, we demonstrate that Toxoplasma growth was dependent on ALK signaling.

**EXPERIMENTAL PROCEDURES**

**Cells and Parasites**—The Toxoplasma strain RH was used for all experiments and maintained by serial passage in human foreskin fibroblast (HFF) cells in DMEM supplemented with 10% fetal bovine serum, glutamine, and penicillin/streptomycin as described (6). All other cells (HIF-1α WT, HIF-1α KO, and HeLa cells) were also grown in this medium. For all assays, the parasites were harvested from infected but nonlysed HFFs. The parasites were released from their host cells by passing them three times through a 27-gauge syringe needle. All of the cells and parasites were routinely tested with a mycoplasma detection kit (Lonzera, Basel, Switzerland) and found to be negative for mycoplasma contamination.

**Plasmids**—The HIF-1 luciferase reporter pGRE-luc and pTK-Rel were previously described (6). The GAL4 luciferase reporter (p5xGRE-luc) and the GAL4 DNA-binding domain fused to amino acids 737–826 of HIF-1α (pGBD-HIF-1α737–826 or pGBD as a empty vector control) were from Dr. Dan Peet (University of Adelaide) (22). FLAG-tagged PHD2 expression plasmid (p3XFLAG-PHD2) was from Dr. Richard Bruck (University of Texas Southwestern Medical Center). The ODD Renilla luciferase reporter (pTK-ODD-Rel) was generated by replacing the Renilla luciferase gene from pTK-Rel with the ODD-Rel fusion from pCMV-ODD-Rel kindly provided by Dr. Joan Conaway (Stowers Institute). pCDF1-SMAD7 was generated by amplifying full-length SMAD7 from MGC clone gi50960790 (Open Biosystems, Huntsville, AL) using primers containing SCA1/NOT1 restriction sites. The amplified fragment was cloned into pCDF1 (Systems Bioscience, Mountain View, CA) digested with SCA1 and NOT1.

**Northern Blotting**—Host cells were infected with parasites at a multiplicity of infection of 10:1 (parasites:host cells), and 18 h later total RNA was purified with the Stratagene Absolutely RNA MicroPrep kit (La Jolla, CA) and Northern blotted as previously described (43). Murine β-actin and HIF-1α probes were generated by reverse transcriptase PCR from total RNA using the following primers: β-actin forward, 5′-GAGGCTTTTAC-GGATGTCAA-3′; and HIF-1α reverse, 5′-CCAGATCATGTTT-GAGACCT-3′; HIF-1α forward, 5′-GAGTTCGAAACTCGAAAAG-3′; and HIF-1α reverse, 5′-ACCTTAGTCTCATCG-TCCCTC-3′. The probes were labeled with [α-32P]dGTP with the random primed DNA labeling kit (Roche Applied Science) and hybridized with Express-Hyb (Clontech, Palo Alto, CA). The blots were exposed to film and analyzed using the Image-QuaNT program (Molecular Dynamics, Sunnyvale, CA).

**Western Blotting**—Parasites were added to host cells at a multiplicity of infection of 10:1 (parasites:host cells). The cells were washed with ice-cold PBS and lysed in ice-cold radioimmunoprecipitation assay lysis buffer supplemented with EDTA-free protease inhibitor mixture (Roche Applied Science). The lysates were centrifuged to remove any remaining cellular debris, and protein concentration was determined with the DC protein assay (Bio-Rad). The samples were separated by SDS-PAGE, transferred to a PVDF membrane, blocked with 5% BSA, and Western blotted with anti-HIF-1α (BD Biosciences, San Jose, CA), anti-PH1D1 (catalog no. NB100–310; Novus, Littleton, CO), anti-PHD2 (catalog no. NB100–113; Novus), anti-PHD3 (catalog no. NB100–303; Novus), anti-actin (Ambion, Austin, TX), anti-hydroxylated HIF-1α P402 and P564 (44), or anti-FKBP38 (Abcam, Cambridge, MA) antibodies. The proteins were detected using ECL Western blotting detection kit (GE Healthcare) and imaged using FluoroChemQ (Cell Biosciences, Santa Clara, CA). Densitometry was performed using the AlphaInnotech software, and protein amounts were determined by normalizing to β-actin levels.

**Luciferase Assays**—HIF-1α WT cells were transfected using Lipofectamine (Invitrogen) as previously described (45). When infected cells were exposed to 3% O2, the parasites were allowed to invade for 1 h before transferring the infected cells to a hypoxia work station (Ruskinn, Cincinnati, OH). Unless otherwise indicated, the cells were harvested 24 h post-infection, which preceded parasite egress. The cells were then washed with PBS, lysed, and assayed using the Dual-Glo luciferase assay
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kit (Promega, Madison, WI). For all of the assays, the appropriate empty vector controls were included.

Toxoplasma Growth and Invasion Assays—Toxoplasma growth was measured using β-galactosidase-expressing parasites from Dr. Gustavo Arrizabalaga (University of Idaho) (46, 47). Briefly, parasites were added to host cells, and after 72 h the medium was removed. Z-buffer (100 μl) containing 20 μM chloroformol-β-d-galactopyranoside was then added to each well for 15 min, and the A420 was measured (48). The numbers of parasites in each well were determined by linear regression analysis from a standard curve generated in each plate. To specifically measure parasite replication, parasites were added to confluent HFFs on 12-mm² glass coverslips at a multiplicity of infection of 1:4 (parasites:host cells). After 12 or 24 h, the cells were fixed and stained with a rabbit polyclonal antibody against the tachyzoite surface protein SAG1 (from Dr. John Boothroyd, Stanford University). Fifty vacuoles were counted for each time point. Synchronized parasite invasion assays were performed with GFP+ parasites using a high potassium buffer as described (49). Sixty minutes after adding invasion buffer, the cells were fixed by adding formaldehyde (final concentration, 3%) directly to the wells. The cells were stained with rabbit anti-SAG1 antiserum and detected with anti-rabbit AlexaFluor 594 (Invitrogen) without permeabilizing the cells. Intracellular parasites were scored as GFP+/SAG1+, and extracellular parasites were scored as GFP+/SAG1+. A total of 200 parasites were counted in each experiment.

Flow Cytometry—HIF-1α WT cells were mock infected or infected with tachyzoites at a multiplicity of infection of 1:1 in the absence or presence of SB505124. After 12 or 24 h, the cells were fixed and stained with a rabbit polyclonal antibody against the tachyzoite surface protein SAG1 (from Dr. John Boothroyd, Stanford University). Fifty vacuoles were counted for each time point. Synchronized parasite invasion assays were performed with GFP+ parasites using a high potassium buffer as described (49). Sixty minutes after adding invasion buffer, the cells were fixed by adding formaldehyde (final concentration, 3%) directly to the wells. The cells were stained with rabbit anti-SAG1 antiserum and detected with anti-rabbit AlexaFluor 594 (Invitrogen) without permeabilizing the cells. Intracellular parasites were scored as GFP+/SAG1+, and extracellular parasites were scored as GFP+/SAG1+. A total of 200 parasites were counted in each experiment.

Toxoplasma Infection Stabilizes HIF-1α Protein.—Toxoplasma up-regulates host HIF-1α protein levels and requires HIF-1α for growth at both normoxic and physiological oxygen tensions. Because increasing HIF-1α protein levels is a key step in activating the HIF-1 transcription factor complex, we sought to define the mechanism underlying how Toxoplasma up-regulates HIF-1α protein levels. These experiments were performed under normoxic conditions because examining parasite regulation of HIF-1 at decreased O2 tension is complicated because of O2-dependent HIF-1α stabilization. Thus, we examined whether infection leads to changes in HIF-1α transcription, translation, or stability. First, HIF-1α wild type or knock-out cells were mock or parasite-infected for 18 h, after which total RNA was isolated and probed by Northern blotting to detect HIF-1α or β-actin (as a normalization control) mRNAs. The data indicated that infection did not significantly alter HIF-1α transcript levels (Fig. 1A). As expected, full-length HIF-1α mRNA was undetectable in HIF-1α knockout cells, demonstrating the specificity of the HIF-1α probe. These data are also consistent with previous microarray studies showing that HIF-1α transcript abundance was not altered in Toxoplasma-infected HFFs (8, 43).

We next addressed how infection affected HIF-1α translation by comparing its rate of synthesis in Toxoplasma-infected cells and cells treated with the hypoxia mimetic CoCl2, which stabilizes HIF-1α protein by inhibiting its hydroxylation (51). Eighteen hours after they were either infected or treated with CoCl2, the cells were exposed for increasing times to the proteasome inhibitor MG132. Lysates were prepared, separated by SDS-PAGE, and then immunoblotted with anti-HIF-1α antibodies. Densitometric analysis indicated that relative to CoCl2-treated, or Toxoplasma-infected for 18 h and then treated with MG132 for the indicated times. Lysates were prepared and Western blotted to detect HIF-1α and actin as a loading control. Densitometry indicated no significant increase in HIF-1α levels in the Toxoplasma-infected cells. C. HFFs were mock infected, CoCl2-treated, or Toxoplasma-infected for 18 h and then treated with cycloheximide (CHX) for the indicated times. Lysates were prepared and Western blotted to detect anti-HIF-1α. Shown is a representative blot from three independent experiments. We next addressed how infection affected HIF-1α translation by comparing its rate of synthesis in Toxoplasma-infected cells and cells treated with the hypoxia mimetic CoCl2, which stabilizes HIF-1α protein by inhibiting its hydroxylation (51). Eighteen hours after they were either infected or treated with CoCl2, the cells were exposed for increasing times to the proteasome inhibitor MG132. Lysates were prepared, separated by SDS-PAGE, and then immunoblotted with anti-HIF-1α antibodies. Densitometric analysis indicated that relative to CoCl2-treated, or Toxoplasma-infected for 18 h and then treated with cycloheximide (CHX) for the indicated times. Lysates were prepared and Western blotted to detect anti-HIF-1α. Shown is a representative blot from three independent experiments.

RESULTS

Toxoplasma Infection Stabilizes HIF-1α Protein.—Toxoplasma up-regulates host HIF-1α protein levels and requires HIF-1α for growth at both normoxic and physiological oxygen tensions. Because increasing HIF-1α protein levels is a key step in activating the HIF-1 transcription factor complex, we sought to define the mechanism underlying how Toxoplasma up-regulates HIF-1α protein levels. These experiments were performed under normoxic conditions because examining parasite regulation of HIF-1 at decreased O2 tension is complicated because of O2-dependent HIF-1α stabilization. Thus, we examined whether infection leads to changes in HIF-1α transcription, translation, or stability. First, HIF-1α wild type or knock-out cells were mock or parasite-infected for 18 h, after which total RNA was isolated and probed by Northern blotting to detect HIF-1α or β-actin (as a normalization control) mRNAs. The data indicated that infection did not significantly alter HIF-1α transcript levels (Fig. 1A). As expected, full-length HIF-1α mRNA was undetectable in HIF-1α knockout cells, demonstrating the specificity of the HIF-1α probe. These data are also consistent with previous microarray studies showing that HIF-1α transcript abundance was not altered in Toxoplasma-infected HFFs (8, 43).

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the von Hippel-Lindau ubiquitin ligase protein. The finding that Toxoplasma infection increased HIF-1α stability led us to test the hypothesis that Toxoplasma impairs HIF-1α prolyl hydroxylation. To monitor this modification, the cells were transfected with pTK-ODD-Rel, which contains the luciferase gene fused downstream to the O2 domain of HIF-1α. Transfected host cells were then either mock or parasite-infected or treated with CoCl2, and luciferase activity was measured. Shown are the averages and standard deviations of six independent experiments. *, p < 0.05 Student’s t test. B, HFFs were mock infected, CoCl2-treated, Toxoplasma-infected (Toxo) for 18 h, or MG132-treated for 2 h. C, the lysates were prepared and Western blotted with antibodies against either total HIF-1α, Pro402-OH HIF-1α, or Pro564-OH HIF-1α or actin as a loading control. Shown are representative blots from three independent experiments. D, cells cotransfected with pG5XGRE-luciferase reporter and a Renilla luciferase reporter and a Renilla luciferase reporter were mock infected, Toxoplasma-infected, or shifted to 3% oxygen for 24 h. The lysates were collected, and luciferase activity was measured. Shown are the averages and standard deviations of three independent experiments. *, p < 0.05 Student’s t test.

It is possible that the effect of Toxoplasma on HIF-1α prolyl hydroxylation was due to a global defect in the activity of O2/α-ketoglutarate-dependent hydroxylases. To address this issue, we examined how infection affected the HIF-1α asparaginyl hydroxylase FIH that, like the PHDs, requires iron, ascorbate, O2, and α-ketoglutarate as substrate and cofactors. Thus, cells were transfected with the p5XGRE-luciferase reporter and a plasmid encoding either the GalDBD-HIF-1α737–826, which contains the asparagine residue targeted by FIH, or pGBD as a control. As expected, exposure of the transfected cells to hypoxia potently increased luciferase expression because of a reduction in FIH activity. In contrast, luciferase expression was unchanged in Toxoplasma-infected cells, indicating that infection had no apparent effect on FIH activity (Fig. 2D). Together, these data indicate that Toxoplasma specifically decreases the HIF-1α PHD.

Toxoplasma Infection Specifically Decreases PHD2 Protein Levels—We next hypothesized that Toxoplasma affected HIF-1α PHD activity by decreasing the expression of the prolyl hydroxylase enzymes. Thus, lysates from mock or parasite-infected cells were prepared 6 or 18 h post-infection and Western blotted with antibodies to detect the three HIF-1α PHDs: PHD1, PHD2, and PHD3. Densitometric analysis indicated that there was no detectable decrease in PHD1 protein levels in Toxoplasma-infected cells, and we failed to reproducibly detect PHD3 protein in either mock or parasite-infected cells (Fig. 3A and data not shown). In contrast, PHD2 protein levels were decreased as early as 6 h post-infection, and by 18 h the protein was reduced by 64%. As a second approach, the cells were transfected with a plasmid encoding FLAG-tagged PHD2 and then either mock or parasite-infected. Eighteen hours later, lysates were prepared and analyzed by Western blotting. The data showed that the levels of both the endogenous and epitope-tagged PHD2 were significantly reduced in parasite-infected cells by 67 and 55%, respectively (Fig. 3B). These data indicate that Toxoplasma specifically reduces expression of PHD2, which is the key prolyl hydroxylase in regulating HIF-1α levels.

Toxoplasma Reduces Prolyl Hydroxylase Activity by Signaling through an Activin-like Receptor Kinase—The prolyl hydroxylase-3/3 iso-
results in decreased PHD2 protein levels (38). We therefore tested whether decreased PHD2 protein levels were due to the infection up-regulating FKBP38 protein levels. Thus, lysates from mock or parasite-infected cells were Western blotted with anti-FKBP38 antibodies. The data indicated that infection led to a reproducible decrease in FKBP38 expression (supplemental Fig. S1). This result strongly suggests that FKBP38-mediated degradation of PHD2, which is mediated by increases in FKBP38 protein, is likely not the mechanism controlling PHD2 expression in *Toxoplasma*-infected host cells.

TGFβ activates HIF-1 by decreasing PHD2 protein levels (39). This decrease in PHD2 protein levels is dependent on signaling from the ALK5 Type I TGFβ receptor because highly specific pharmacological inhibitors prevent TGFβ-stimulated decreases in PHD2. To test whether Type I ALK receptor signaling regulates HIF-1 activation in *Toxoplasma*-infected host cells, pHRE-luc-transfected cells were mock or parasite-infected in the presence of increasing concentrations of SB505124, which is a highly specific ALK4,5,7 inhibitor (42). Lysates were prepared 18 h later, and luciferase activity was measured. The data indicated that the inhibitor potently blocked *Toxoplasma* induced pHRE-luc activity (Fig. 4A). The effect of SB505124 on parasite stimulation of HIF-1 was not a general consequence of altering HIF-1 signaling because the drug did not significantly affect pHRE-luc activation in cells exposed to decreased O2 tension (Fig. 4B).

Next, we tested whether the effects of the drug on parasite activation of pHRE-luc activity corresponded to a concomitant decrease in HIF-1α protein levels in *Toxoplasma*-infected cells. Thus, vehicle- or SB505124-treated cells were mock or parasite-infected. The lysates were prepared 18 h later and Western blotted to examine HIF-1α protein levels. As we previously demonstrated, infection dramatically increased HIF-1α abundance (6). HIF-1α protein levels were, however, significantly reduced in *Toxoplasma*-infected cells treated with SB505124 (Fig. 4C).

It is possible that the drug impacts *Toxoplasma* induction of HIF-1 by having a general affect on the ability of parasites to signal to their host cell. We therefore tested whether SB505124 blocked *Toxoplasma* inhibition of IFNγ signaling (53–55). Hence, vehicle- or drug-treated HIF-1α wild type cells were first infected with GFP7 parasites and 6 h later were treated with IFNγ for an additional 18 h. Expression of the costimulatory molecule, PD-L1, was then examined by flow cytometry because in fibroblasts as well as macrophages its expression is critically dependent on IFNγ (56–59) and because it is up-regulated more rapidly and robustly than MHC Class II.4 Similar to the effect of *Toxoplasma* on other IFNγ-regulated genes, PD-L1 expression was significantly reduced on the surface of *Toxoplasma*-infected cells (>95% versus 35%). This inhibition of PD-L1 expression was not affected by the presence of SB505124, indicating that the drug does overtly interfere with *Toxoplasma* signaling to its host cell (supplemental Fig. S2).

Caveats associated with using pharmacological inhibitors are that they may target the parasite and not the host and may affect cellular targets besides the Type I ALK4,5,7 receptor family. We therefore compared *Toxoplasma* up-regulation of HRE-luc activity when either the host cells or parasites were pretreated with the SB505124. The data indicated that HRE-luc activity was reduced by pretreating the host cells, but not the parasites, indicating that the drug did not have an unexpected, irreversible effect on the parasite (Fig. 4D).

As an alternative approach to inhibiting ALK4,5,7 signaling, we examined the effect of SMAD7 overexpression on pHRE-luc activity in *Toxoplasma*-infected cells. SMAD7 is an endogenous inhibitor of ALK4,5,7 by preventing activation of downstream effectors and by recruiting E3 ubiquitin ligases to the activated receptors (60–62). Host cells cotransfected with SMAD7 and the HRE-luc reporter were mock or parasite-in-
The drug were infected with growth. Thus, cells treated with increasing concentrations of cation were abrogated by treating the cells with SB505124 (Fig. 5).

Infection-induced decreases in PHD2 levels that PHD2 protein levels decreased 60% in infected cells, but infection-induced decreases in PHD2 levels. Densitometric analysis indicated that PHD2 protein levels in cle- or SB505124-treated cells were mock or parasite-infected. Hence, pTK-ODD-Rel-transfected cells were pretreated with vehicle or SB505124 and then mock or parasite-infected for 18 h, at which time luciferase activity was measured. We found that consistent with the HIF-1 data, SB505124 blocked decrease in PHD2.

Next, we tested whether the drug also impacted decreased PHD2 protein levels in Toxoplasma-infected cells. Thus, vehicle- or SB505124-treated cells were mock or parasite-infected. The lysates were prepared 18 h later and Western blotted to examine PHD2 protein levels. Densitometric analysis indicated that PHD2 protein levels decreased 60% in Toxoplasma-infected cells, but infection-induced decreases in PHD2 levels were abrogated by treating the cells with SB505124 (Fig. 5A).

ALK4,5,7 Receptor Inhibition Reduces Toxoplasma Replication—We next examined the effect of SB505124 on parasite growth. Thus, cells treated with increasing concentrations of the drug were infected with β-galactosidase-expressing parasites, and 3 days later the parasite numbers were determined. The data indicated that the drug reduced parasite growth with an apparent IC_{50} of 2 μM (Fig. 6A). Decreases in Toxoplasma growth in the drug-treated cells were likely not a consequence of decreased cell viability because previous work demonstrated that cell viability is not affected by SB505124 at the concentrations we used (42). In addition, 5 μM SB505124 had no detectable impact on the growth of an unrelated intracellular pathogen, West Nile virus (not shown).

To determine how the drug reduced parasite growth, Toxoplasma invasion was assessed with GFP-expressing parasites using a potassium-based synchronized invasion assay (63). No apparent difference in parasite invasion between mock and drug-treated cells was observed, indicating that ALK4,5,7 signaling is not required for parasite invasion (Fig. 6B). We next tested whether the drug impacted parasite replication by counting numbers of parasites/vacuole in vehicle- or drug-treated cells 12 or 24 h post-infection. We found that relative to mock treated cells, parasite replication was severely reduced in SB505124-treated cells (Fig. 6C). Together, these data indicate that SB505124 reduces tachyzoite growth by reducing its rate of replication.

DISCUSSION

As all intracellular parasites must do, Toxoplasma modifies its host cell to create an environment permissive for its own growth. These changes include rearrangement of host cytoskeleton (64–66); relocalization of host mitochondria, endoplasmic reticulum, and lysosomes to the parasitophorous vacuole (67, 68); inhibition of host cell apoptosis (69–71); and modulation of host cell transcription (8, 43, 72, 73) by a variety of transcription factors including HIF-1, STAT3, EGR2, AP-1,
and NF-κB (6–8, 45, 74). In this report, we defined the mechanism by which Toxoplasma activates HIF-1: increasing the stability, but not synthesis, of the HIF-1α subunit. This was surprising because our experiments were performed under normoxic conditions and because Toxoplasma activates host phosphatidylinositol 3-kinases (75, 76), which regulates HIF-1α protein synthesis (34, 77). Moreover, we found that Toxoplasma infection significantly decreased prolyl hydroxylase activity and expression of PHD2, which is the key regulator of HIF-1α protein degradation. Finally, we showed that Toxoplasma-dependent increases in HIF-1α protein and activity and decreases in PHD2 protein and prolyl hydroxylase activity were dependent on Type I activin-like kinase receptor signaling (supplemental Fig. S3).

In many cases, neither the biological significance of the parasite-induced changes to its host cell nor the molecular mechanisms mediating these changes are known. HIF-1 is one of the few host factors modulated by infection that is established to be necessary for parasite growth (6). Thus, the data in this report are significant because they define PHD2 as a key host cell factor targeted by infection. Moreover, parasite regulation of PHD2 via Type I ALK4,5,7 receptor family signaling indicates that this signaling pathway is important for infection and represents a novel drug target to treat Toxoplasma infections.

Previous work suggested that TGFβ decreases PHD2 protein levels by reducing PHD2 mRNA abundance (39). However, microarray (8, 43) and RT-PCR data have failed to detect decreased PHD2 mRNA levels in Toxoplasma-infected cells. Thus, Toxoplasma post-transcriptionally regulates PHD2 levels by affecting PHD2 protein synthesis and/or stability. We favor the latter mechanism for two reasons. First, PHD2 is a relatively long-lived enzyme with a half-life of >20 h (38), but a significant decrease in PHD2 protein levels was observed within 6 h post-infection. Hence, the effect that reduced PHD2 would have on total PHD2 protein levels would only be apparent at later time points. Second, the data in Fig. 3B were generated using an epitope-tagged PHD2 construct that contains the full-length PHD2 open reading frame without either endogenous 5′- or 3′-untranslated regions. This is significant because changes in untranslated region secondary structure are commonly associated with translational regulation (78). It is, however, possible that a microRNA targeting the PHD2 coding sequence may regulate PHD2 translation. Our future work will focus on defining the mechanism by which Toxoplasma affects PHD2 levels.

Signaling by the three Type I receptors (ALK4, ALK5, and ALK7) targeted by SB505124 is complex for a variety of reasons. First, the three receptors are expressed throughout the body and often are coexpressed by the same cell (79, 80). In addition, each of the receptors can bind and transduce signaling from numerous ligands e.g. TGFβ and GDF11 bind ALK5, activin A, and myostatin bind ALK4, and activin AB and activin B interact with ALK7 (81, 82). Moreover, some ligands interact with more than one receptor. Nodal, which functions in embryonic development, binds both ALK4 and ALK7 (83). Finally, some ALK4,5,7 ligands are multimeric complexes whose individual subunits can be part of different multimeric complexes that bind to similar as well as different receptors. As an example, activin A is a homodimer of Inhibin β (INHBA) that binds ALK4. INHBA is also a subunit of activin AB (the other subunit is Inhibin b) that binds ALK5. To add to this complexity, INHBA can also interact with Inhibin α to form a complex named Inhibin, which is an antagonist of activin signaling. The function of INHBA expression may be particularly relevant to Toxoplasma infections because INHBA mRNA is up-regulated in Toxoplasma-infected cells (8, 43). Interestingly, TGFβ is also up-regulated in Toxoplasma infections (84–87) and in some cases can increase host cell susceptibility to infection (88). Thus, it is quite likely that signaling by more than one ALK4,5,7 ligand can signal through one or more receptors to activate HIF-1 in Toxoplasma-infected cells. It is also possible that the parasite expresses and secretes its own ligand that binds to ALK4,5 or 7. Our future work will focus on determining which of the Type I receptors are important for Toxoplasma infections and whether host- and/or parasite-derived ligands activate this receptor(s).

HIF-1 was originally identified as a critical player in cell response to hypoxic stress (89). It is, however, becoming increasingly apparent that HIF-1 is activated by diverse stimuli under normoxic conditions. A key question resulting from these studies is whether HIF-1α functions similarly when it is activated at either O2-rich or -poor conditions. HIF-1α has two transactivation domains that regulate distinct and common sets of genes (90). The C-terminal transactivation domain is regulated by FIH, which in vitro has a higher Km for O2 than the PHDs. Thus, the N-terminal transactivation domain is most likely to be active under O2-replete conditions such as Toxoplasma-infected cells. This conclusion is consistent with our data that although Toxoplasma could stimulate a HIF-1-regulated luciferase reporter, it could not overcome FIH-mediated inhibition of the C-TAD. Thus, future work aimed at identifying HIF-1 target genes that promote parasite growth most likely can exclude those regulated by the C-TAD.

At 21% O2, parasite growth was decreased in HIF-1α KO cells by ~70% (6), which was in contrast to the dramatic effect that SB505124 had on parasite replication. This could be due to the fact that other host proteins regulated by ALK4,5,7 can compensate for the loss of HIF-1α at 21% O2. Alternatively, it is possible that Type I receptor signaling regulates several pathways that act independently to promote parasite growth. Thus, whereas HIF-1α/PHD2 may be one target of Type I receptor signaling, other pathways may also contribute to parasite growth (supplemental Fig. S3). Possible targets may include MAPK and phosphatidylinositol 3-kinase-dependent signaling, which are activated in Toxoplasma-infected cells and are also regulated by these receptors (40, 91, 92), but whether the loss of other individual pathways will impact parasite growth to the same extent as the loss of HIF-1α is unknown and will need to be tested as additional host factors that are regulated by Toxoplasma are identified.

5 M. Wiley and I. J. Blader, unpublished data.
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