A GCN2-Like Eukaryotic Initiation Factor 2 Kinase Increases the Viability of Extracellular *Toxoplasma gondii* Parasites

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Toxoplasmosis is a significant opportunistic infection caused by the protozoan parasite *Toxoplasma gondii*, an obligate intracellular pathogen that relies on host cell nutrients for parasite proliferation. *Toxoplasma* parasites divide until they rupture the host cell, at which point the extracellular parasites must survive until they find a new host cell. Recent studies have indicated that phosphorylation of *Toxoplasma* eukaryotic translation initiation factor 2-alpha (TgIF2α) plays a key role in promoting parasite viability during times of extracellular stress. Here we report the cloning and characterization of a TgIF2kinase designated TgIF2K-D that is related to GCN2, a eukaryotic initiation factor 2e (eIF2e) kinase known to respond to nutrient starvation in other organisms. TgIF2K-D is present in the cytosol of both intra- and extracellular *Toxoplasma* parasites and facilitates translational control through TgIF2 phosphorylation in extracellular parasites. We generated a TgIF2K-D knockout parasite and demonstrated that loss of this eIF2 kinase leads to a significant fitness defect that stems from an inability of the parasite to adequately adapt to the environment outside host cells. This phenotype is consistent with that reported for our nonphosphorylatable TgIF2α mutant (S71A substitution), establishing that TgIF2K-D is the primary eIF2α kinase responsible for promoting extracellular viability of *Toxoplasma*. These studies suggest that eIF2α phosphorylation and translational control are an important mechanism by which vulnerable extracellular parasites protect themselves while searching for a new host cell. Additionally, TgIF2α is phosphorylated when intracellular parasites are deprived of nutrients, but this can occur independently of TgIF2K-D, indicating that this activity can be mediated by a different TgIF2K.

The ability to rapidly respond to stress is essential for cellular survival. Translational control through the phosphorylation of the alpha subunit of eukaryotic initiation factor 2 (eIF2α) is a well-conserved mechanism used by cells to repress global protein synthesis during times of nutrient scarcity (14, 48). In a GTP-driven process, eIF2 delivers the initiator tRNA to the translational machinery. When GCN2 phosphorylates eIF2α at its regulatory serine residue, this translation initiation factor becomes an inhibitor of its guanine nucleotide exchange factor, eIF2B, which results in reduced levels of the eIF2-GTP-Met-tRNAi ternary complex that are accessible to the translational machinery. Consequently, general protein synthesis is diminished, which conserves cellular resources and provides time for the cell to reprogram its genome to adapt to the stress. In addition to the GCN2 protein kinase that is activated during nutritional starvation, other eIF2α kinases respond to different stress conditions (41, 48). These protein kinases include PERK/PEK, activated by endoplasmic reticulum (ER) stress, and HRI and PKR, which respond to heme depletion and viral infection, respectively.

We have previously shown that the obligate intracellular parasite *Toxoplasma gondii* (phylum Apicomplexa) relies on phosphorylation of eIF2α (designated TgIF2α) and translational control to remain viable during the times it must persist without host cells (18). *Toxoplasma* can cause congenital birth defects, ocular disease, and life-threatening opportunistic infection (46). Current treatments consist of antifolates, which are problematic due to toxicity issues; therefore, there is an urgent need to develop novel therapies to treat this parasitic infection (6). Phosphorylation of eIF2α has recently been shown to be critical during multiple phases of the life cycle of apicomplexan parasites (8, 17, 18, 27, 51). We generated a TgIF2K-D mutant that no longer phosphorylates TgIF2α by mutating the regulatory serine (Ser71) to alanine (18). The TgIF2α-S71A mutant suffered a significant fitness defect in *vivo* and in *vitro* because the mutants were more susceptible to extracellular exposure. *Toxoplasma* expresses four putative eIF2α kinases designated TgIF2K-A to -D, and the underlying protein mediating this translational control in response to extracellular stress has not yet been identified (18, 27).

In this study, we hypothesized that extracellular parasites endure nutrient deprivation through the activity of a GCN2 orthologue. We show that TgIF2K-D is a GCN2-like eIF2α kinase in *Toxoplasma*. Through the generation of TgIF2K-D mutants, TgIF2K-D was shown to enhance the viability of parasites during times when they are deprived of their host cells. Parasites with a knockout of TgIF2K-D are unable to phosphorylate TgIF2α and initiate translational control in response to extracellular stress, phenocopying the TgIF2α-S71A mutant (18). Intracellular parasites also phosphorylate TgIF2α during nutrient deprivation, but this can occur independently of TgIF2K-D, suggesting the involvement of another TgIF2K. This study indicates that TgIF2K-D is the eIF2α kinase facil-

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Comparative fitness assay. The comparative fitness assay was carried out as described previously by Joyce et al. (18), with the exception that SYBR green-based quantitative real-time PCR (qPCR) was performed using primers that specifically delineated between parental ΔKu80 parasites, referred to as wild type (WT), and Δif2k-d parasites. In brief, equal numbers of filter-purified parental and Δif2k-d parasites (5 × 10^7) were cocultured in the same flask of HFF host cells. At 72 h postinfection, 10^7 parasites of the mixed population were isolated from the lysed culture and then transferred to a fresh HFF monolayer for an additional 72 h. This resulted in a total of 6 days of HFF infection by using two serial passages. Genomic DNA (gDNA) from the parasite samples was isolated using the DNeasy kit (Qiagen) and used in qPCR assays. Primers used to distinguish WT from Δif2k-d parasites included primers 15 and 16 and primers 17 and 18, as indicated. qPCR measurements were normalized by amplifying the 5'-UTR of TgIF2K-D, which is present in both WT and Δif2k-d parasites (primers 19 and 20). Twenty-five nanograms of gDNA was used in the qPCR assays, which were performed in triplicate using the 7500 real-time PCR system (Applied Biosystems). Relative quantification software (SDS software, version 1.2.1) was used for the analysis. As a specificity control, SYBR green assays employing gDNA purified from either WT or Δif2k-d parasites were carried out to verify the specificity of primers in the qPCR assay (data not shown).

Parasite proliferation assays. Toxoplasma recovery from extracellular stress was achieved using standard plating and plaque assays (35). Parental ΔKu80 (WT), Δif2k-d, and TgIF2e-S71A (18) parasites were physically released from host cells by syringe passage and then filtered to remove host cell debris. A total of 10^6 parasites were subjected to an extracellular stress assay for 0, 8, or 10 h in culture medium at 37°C and 5% CO_2 without host cells prior to infecting HFF host cells, as described previously (18). Parasites were quantitated using a standard plaque assay, where total number of plaques performed every hour. Replicate assays were carried out in triplicate using separate biological samples, and results of a representative experiment are shown. In the plaque assays, 500 WT, Δif2k-d, TgIF2e-S71A, or TgIF2k-D::DHFR* parasites were used to infect HFF monolayers in 12-well plates following extracellular incubation for up to 10 h, as indicated. The degree of host cell lysis at 7 days postinfection was determined by crystal violet staining of methanol-fixed cells. Measurements of the lysed areas were done using an Alpha Innotech imaging system and results of a representative experiment of three independent experiments are presented.

Analysis of nutrient starvation of intracellular Toxoplasma. To deprive intracellular Toxoplasma of nutrients, we employed a method recently developed by Anthony Sinai (University of Kentucky, unpublished). For these experiments, HFF cells and parasites were maintained in alpha minimal essential medium (αMEM)–7% fetal bovine serum (FBS)–2 mM glutamine (complete medium [CM]) (Gibco). Tachyzoites (10^6) were allowed to infect HFF monolayers in complete medium lacking methionine and cysteine. Culture medium lacking methionine and cysteine. Culture medium lacking methionine and cysteine. Tachyzoites were cultured in complete medium for 10 h in culture medium at 37°C and 5% CO_2 without host cells prior to infecting HFF host cells, as described previously (18). Parasites were quantitated using a standard plaque assay, where total number of plaques performed every hour. Replicate assays were carried out in triplicate using separate biological samples, and results of a representative experiment are shown. In the plaque assays, 500 WT, Δif2k-d, TgIF2e-S71A, or TgIF2k-D::DHFR* parasites were used to infect HFF monolayers in 12-well plates following extracellular incubation for up to 10 h, as indicated. The degree of host cell lysis at 7 days postinfection was determined by crystal violet staining of methanol-fixed cells. Measurements of the lysed areas were done using an Alpha Innotech imaging system and results of a representative experiment of three independent experiments are presented.
(PBS) and then lysed in 100 μl RIPA buffer (44). Uptake of the 35S during the 1-h pulse radiolabeling was similar for the WT and mutant parasites. For each sample, equal amounts of proteins were precipitated by adding trichloroacetic acid (TCA) to a final concentration of 10%. After incubation on ice for 30 min, samples were collected by centrifugation at 10,000 × g for 30 min at 4°C. The TCA precipitates were washed twice with acetone and resuspended in an equal volume of PBS. Incorporation of the radiolabeled amino acids was determined using a scintillation counter. Results of all radiolabeling experiments are presented as averages for three independent samples, with P values and standard errors determined using analysis of variance (ANOVA).

**Immunofluorescence assays.** HFF monolayers were grown on coverslips, infected for 24 h, and then fixed in 3% paraformaldehyde. Immunofluorescence analyses using a rat monoclonal antibody that recognizes the HA tag (Roche) followed by goat anti-rat Alexa Fluor 488 as the secondary antibody (Invitrogen) was performed as previously described (29).

**RESULTS**

**Characterization of the GCN2-like kinase TgIF2K-D.** The predicted gene TgME49_119610 (ToxoDB.org) was previously designated TgIF2K-D and is suggested to encode an orthologue of GCN2 (27), the eIF2α kinase that is well-documented as a responder to nutrient starvation stress in other species (14, 48). We used RT-PCR to identify and characterize the full-length TgIF2K-D cDNA. Our analysis revealed a predicted TgIF2K-D product consisting of 2,729 amino acid residues (GenBank accession number JF827031), which modifies the predicted sequence for TgME49_119610 due to a discrepancy at the exon 3/intron 3 boundary. The predicted start codon for the TgIF2K-D ORF matches the consensus sequence for translation initiation in Toxoplasma (39) and is preceded by an in-frame stop codon. RACE analyses indicated a 5′ untranslated region (5′-UTR) of 2,151 bp, which is consistent with the transcriptional start site (TSS) derived from the Full-parasites database (49) and chromatin immunoprecipitation-on chip (ChIP-Chip) data available in the ToxoDB, and a 3′-UTR of ~1,000 bp (see Fig. S1 in the supplemental material). The 5′-UTR was further validated by RT-PCR using primers flanking the TSS (see Fig. S2 in the supplemental material).

An alignment between TgIF2K-D and the eIF2α kinases from multiple species was compiled using BLAST and CLUSTALW (see Fig. S3 in the supplemental material). TgIF2K-D (residues 1,318 to 1,630) has the central features characteristic of eIF2α kinases, including an insert between subdomains IV and V (Fig. 1; see Fig. S3 in the supplemental material). As judged by BLAST analyses, this portion of TgIF2K-D is most closely related to putative eIF2α kinases from the parasites *Plasmidium falciparum* (AAAN37036; 4e−14) and *Trypanosoma brucei* (XP_828792.1; 6e−10) (25), followed by characterized GCN2 orthologues from *Arabidopsis thaliana* (CAD30860; 6e−32) (52), *Drosophila melanogaster* (AAC13490; 8e−27) (28), *Schizosaccharomyces pombe* (AAU11313; 2e−25) (50), and *Saccharomyces cerevisiae* (AAA34636; 1e−22) (47). Another hallmark feature of GCN2 is an RWD domain, which is present between residues 800 and ~1000 of TgIF2K-D, with a significance of 4e−6 as determined by the motif search program Pfam (9, 26) (see Fig. S4 in the supplemental material). The RWD in GCN2 from *S. cerevisiae* was reported to directly bind to the activator protein GCN1 (14, 26), and residue changes in GCN2 that blocked this binding, or abolition of the GCN2/GCN1 association by GCN1 binding with another RWD-containing protein, YIH1, blocked GCN2 phosphorylation of eIF2α in yeast depleted of amino acids (13, 14, 36, 37). *Toxoplasma* also has a predicted GCN1 orthologue (TGME49_031480) and a YIH1-related protein (TGME49_112350), supporting the idea that this network functions to regulate a GCN2-related eIF2α kinase in this parasite.

The sequences of the histidyl-tRNA synthetase (HisRS) domain, which stimulates eIF2α kinase activity by binding to uncharged tRNAs accumulating during nutrient deprivation (14), appears to be less well conserved in the protozoan GCN2-like kinases. Analysis of the sequences flanking the C-terminal end of the protein kinase domain (residues 1750 to 2360) identified the PRGGRVY2299 sequence as the closest match to the histidine B sequence (AAGGRYD), which is characteristic for the HisRS-related domains (42). This weaker conservation of the HisRS-related sequences is a feature shared with other GCN2-related protein kinases from apicomplexans, including *P. falciparum* (8). TgIF2K-D also lacks the pseudokinase domain found in mammalian and yeast GCN2s, which is thought to contribute to the eIF2α kinase activity (33). The C terminus of GCN2 is important for dimerization and ribosome association (14, 48), and this region in TgIF2K-D (residues 2436 to 2499) is rich in hydrophobic and basic residues, which are suggested to contribute to these regulatory processes in this eIF2α kinase. Interestingly, this region shares sequence identity with GCN2-like kinases encoded in the apicomplexans *Neospora caninum* (NCLIV_010550; 3e−30), *Cryptosporidium muris* (CMU_027700; 0.011), *Plasmidium falciparum* (PF14_0264; 9e−08), *Plasmidium berghei* (PBANKA_101620, 4.8e−08), *Plasmidium knowlesi* (PKH_113740, 1.1e−07), and *Plasmidium vivax* (PVX_085120; 2e−07) (see Fig. S5 in the supplemental material). We designated this conserved region the C-terminal homology (CTH) region (Fig. 1).

Based on the presence of sequences related to the eIF2 kinases juxtaposed to the signature RWD domain, a putative histidine B-like sequence, and a C terminus rich in hydrophobic and basic residues, TgIF2K-D is suggested to be a parasite orthologue of GCN2 (Fig. 1). We therefore hypothesize that...
TgIF2K-D plays a critical role during nutrient deprivation experienced by extracellular Toxoplasma.

TgIF2K-D is expressed in intra- and extracellular parasites. Using RHΔku80 parasites engineered to have greater frequencies of homologous recombination (10, 15), we endogenously tagged TgIF2K-D with three HA epitopes (3×HA) at the C terminus. Western blot analyses of total protein lysate using antibodies specific for Toxoplasma tubulin. The faster-migrating TgIF2K-D3×HA variants (lower arrow) diminished when parasites were subjected to extracellular stress for 4 or 8 h. Samples were normalized in the immunoblot analysis using antibody specific for Toxo-kinase in other species (14, 48).

To identify the cellular location of TgIF2K-D, we also carried out immunofluorescence microscopy. The HA-tagged TgIF2K-D localized to the parasite cytosol in both intra- and extracellular parasites (Fig. 2C). A cytosolic localization is consistent with reports on GCN2 in other species (14, 48).

TgIF2K-D facilitates TgIF2α phosphorylation and translational control in extracellular parasites. Extracellular stress is a potent inducer of TgIF2α phosphorylation, and loss of translational control in the TgIF2α-S71A mutant reduced parasite viability (18). To address whether TgIF2K-D is required to manage extracellular stress, we generated knockdown and knockout parasite clones in the RHΔku80 background. The knockdown of TgIF2K-D involved an in-frame fusion of two HA tags and a 12-kDa destabilization domain (DD) at the C terminus of the endogenous TgIF2K-D in the RHΔku80 strain (see Fig. S6 in the supplemental material). The parasite clone, designated TgIF2K-D2xDD, allowed tunable expression of the TgIF2K-D protein. In the absence of the stabilizing ligand Shield-1, DD-tagged proteins are rapidly degraded (1, 2); TgIF2K-D2xDD parasites cultured without Shield-1 had no detectable levels of TgIF2K-D protein as assayed by Western blot analysis (see Fig. S6 in the supplemental material). The knockout of TgIF2K-D eliminated the entire genomic locus through homologous recombination and allelic replacement with a modified dihydrofolate reductase-thymidylate synthase (DHFR-TS) minigene, which confers resistance to pyrimethamine (Fig. 3A) (5). Δif2k-d was verified by PCR analyses of genomic DNA purified from pyrimethamine-resistant clones (Fig. 3B). In addition, total RNAs from the parental strain and a Δif2k-d knockout clone were isolated for RT-PCR analysis of the TgIF2K-D transcript. While TgIF2K-D mRNA was amplified from parental parasites, the corresponding transcript was not detected in Δif2k-d parasites (Fig. 3C). This parasite clone represents the first knockout of an eIF2α kinase in Toxoplasma.

Next we addressed whether TgIF2K-D is required for induced TgIF2α phosphorylation when the parasite is outside the host cell. As observed previously (18), parental WT parasites showed TgIF2α phosphorylation after 8 h of incubation in the extracellular environment (Fig. 4A). In comparison, there was minimal TgIF2α phosphorylation in the TgIF2K-D2xDD knockdown parasites following extracellular exposure (Fig. 4A). To test the specificity of TgIF2K-D in responding to extracellular stress, we subjected WT and Δif2k-d parasites to the calcium ionophore A23187, a known inducer of ER stress and TgIF2α phosphorylation (27). As shown in Fig. 4B, the Δif2k-d parasites were not defective for TgIF2α phosphorylation in response to ER stress. These results support the model that each TgIF2α kinase in Toxo-plasma recognizes distinct stress arrangements and TgIF2K-D is central for inducing TgIF2α phosphorylation when parasites are outside the host cell.

Under stress conditions, eIF2α phosphorylation represses general translation as part of the cellular stress response (41). To compare translational control in WT versus Δif2k-d parasites, we measured the incorporation of radiolabeled Cys/Met in parasites subjected to extracellular stress for 1 and 8 h. As expected, protein synthesis was repressed by greater than 90%
in WT parasites subjected to 8 h of extracellular stress; however, in the Δif2k-d and TgIF2α-S71A mutant parasites, protein synthesis was diminished by only about 40% (Fig. 4C). We conclude that TgIF2K-D is likely to be the primary eIF2α kinase that mediates translational control in response to extracellular stress.

Parasites lacking TgIF2K-D exhibit a fitness defect. Recently we reported that TgIF2α-S71A mutants are outcompeted by wild-type parasites when placed in a “head-to-head” competition assay, as the mutant struggles to cope with the extracellular environment experienced while finding a new host cell (18). Given that the Δif2k-d mutant failed to phosphorylate

FIG. 3. Generation of a TgIF2K-D knockout. (A) The TgIF2K-D genomic locus, depicted with 18 exons, was replaced by a minigene conferring resistance to pyrimethamine (DHFR*) using homologous recombination in Δku80 RH strain parasites. The numbered arrows indicate the positions of primers used to screen genomic DNA from transfected pyrimethamine-resistant clones and parental (WT) parasites. Primer sequences are listed in Table S1 in the supplemental material. (B) Genomic PCR assays used gDNA harvested from WT or Δif2k-d parasites and the indicated primers to validate replacement of the TgIF2K-D genomic locus. (C) The absence of TgIF2K-D mRNA in the Δif2k-d parasites was confirmed by RT-PCR analysis using primers upstream and downstream of the encoded protein kinase domain (primers 11 and 12). Toxoplasma actin mRNA was amplified as a positive control (primers 13 and 14). A no-template control (Ø) was included in all PCRs.

FIG. 4. TgIF2K-D phosphorylates TgIF2α and represses protein synthesis in response to extracellular stress. (A) Wild-type (WT), TgIF2K-DΔADD (DD), and Δif2k-d parasites were exposed for 0 or 8 h to the extracellular environment. TgIF2α phosphorylation was analyzed by separating cell lysates via denaturing SDS-PAGE, followed by Western blotting using antibodies to total TgIF2α or phosphorylated TgIF2α (TgIF2α-P). (B) WT and Δif2k-d tachyzoites were treated with 5 μM calcium ionophore A23187 for 30 min and then analyzed for TgIF2α-P by immunoblotting. (C) WT, TgIF2α-S71A, and Δif2k-d parasites were physically released from host cells and incubated for 1 or 8 h in DMEM culture medium. One hour prior to harvesting, the parasites were incubated in the presence of [35S]Cys/Met. Lysates were prepared, and equal amounts of protein were precipitated with TCA. Levels of incorporation of radiolabeled amino acids were determined via scintillation counting. Three experiments were performed, and incorporation of the radiolabel is represented as a percentage of that measured for parasites subject to 1 h of stress. Error bars indicate the standard error, and significance was determined using a two-tailed Student’s t test, with P < 0.05, as indicated by the asterisks.
TgIF2α in response to extracellular stress (Fig. 4A), we tested whether the ∆if2k-d parasites would be outcompeted by parental wild-type parasites using the head-to-head fitness assay. Equal numbers of WT and ∆if2k-d parasites were premixed and transferred into the same culture flask containing a confluent monolayer of HFF cells (Fig. 5A). Samples were taken prior to infection and after day 6 for genomic DNA isolation.

The relative amounts of WT and ∆if2k-d parasites were determined using a SYBR green-based quantitative PCR assay and primers specific for WT or ∆if2k-d parasites. Primers that amplify DNAs from both strains were used to ensure normalization between the samples (Fig. 5B). WT parasites outgrew the mutant parasites by day 6 (Fig. 5C), establishing that parasites lacking TgIF2K-D exhibit reduced fitness in the parasite lytic cycle.

**TgIF2K-D promotes the viability of extracellular tachyzoites.**

We evaluated whether the reduced fitness seen in the ∆if2k-d parasites involved impaired motility or host cell invasion using gliding assays and a red/green adhesion and invasion assay. As reported for the TgIF2α-S71A mutant, ∆if2k-d parasites exhibited no deficiencies in motility or the ability to attach and invade host cells (see Fig. S7 and S8 in the supplemental material).

As mentioned above, parasites deficient for TgIF2K-D suffer a loss in viability due to an inability to respond appropriately to the extracellular stress experienced while outside host cells. To further address the role of TgIF2K-D in the extracellular stress response, the WT and different mutant parasites (TgIF2α-S71A, ∆if2k-d, and TgIF2K-D2xDD, without Shield) were incubated outside host cells in DMEM for between 0 and 10 h prior to being applied to a fresh host cell monolayer. After 7 days, the infected host cells were fixed and stained to determine the degree of host cell lysis. With increased periods of extracellular stress, ∆if2k-d parasites showed sharply reduced infection and lysis of host cells that was similar to that measured for the TgIF2α-S71A mutants (Fig. 6A). This defect was more pronounced in the ∆if2k-d parasites than in the TgIF2K-D2xDD knockdown parasites, suggesting that there are residual levels of functional TgIF2K-D despite the absence of Shield.

To further characterize the role of translational control in the resistance to extracellular stress, we also analyzed the doubling rate of the ∆if2k-d parasites. ∆if2k-d parasites proliferated at a rate similar to that of the WT when allowed to infect a new host cell monolayer immediately upon release from their initial host cells (Fig. 6B, 0-h extracellular stress). However, consistent with the plaque assay, extracellular stress led to a significant reduction in the proliferation of ∆if2k-d parasites. WT parasites subjected to extracellular stress for 10 h grew to an average of ~17 parasites/vacuole, but ∆if2k-d parasites grew only to ~10 parasites/vacuole (Fig. 6B). This reduction in doubling time was also observed when the TgIF2α-S71A mutants were subjected to extracellular stress prior to infection of the HFF cells. Collectively, these studies establish that TgIF2K-D is critical for promoting survival of extracellular tachyzoites through translational control mediated by the phosphorylation of TgIF2α.

**TgIF2α is phosphorylated in starved intracellular parasites in the absence of TgIF2K-D.**

In addition to nutrient deprivation experienced while outside a host cell, parasites can be deprived of host nutrients while intracellular as well. *Toxoplasma* is auxotrophic for several key metabolites and amino acids, including tryptophan. Tryptophan starvation has been shown to be a mechanism of gamma interferon (IFN-γ)-mediated parasite growth inhibition (31). We tested whether intracellular parasites phosphorylated TgIF2α in response to nutrient starvation and whether TgIF2K-D mediated this activity. We subjected infected HFFs to starvation medium for 8 h, which is composed of complete medium diluted to a 6% final concentration in HBSS (6% SM). TgIF2α phosphorylation was detected in parasites incubated in 6% SM for 8 h, indicating that translational control is initiated in response to nutrient deprivation in intracellular parasites (Fig. 7A). The ∆if2k-d mutants were still able to phosphorylate TgIF2α in response to nutrient deprivation in intracellular parasites (Fig. 7A), suggesting that a different TgIF2K is activated in starved intracellular parasites.

To further characterize the role of TgIF2α phosphorylation in intracellular parasites when nutrients become limiting, we analyzed the recovery of wild-type, ∆if2k-d, and TgIF2α-S71A mutants following exposure to 6% SM using a standard dou-
bling assay. Wild-type and Δif2k-d parasites exhibited no difference in their ability to recover following incubation for 4 or 8 h in 6%-SM (Fig. 7B). In contrast, TgIF2α-S71A parasites exhibited a greater defect in recovering from the intracellular nutrient starvation (Fig. 7B). These data demonstrate that phosphorylation of TgIF2α promotes the viability of intracellular tachyzoites that experience nutrient deprivation but that this response can be mediated by a TgIF2K other than TgIF2K-D.

**DISCUSSION**

In this study, we generated and characterized the first eIF2α kinase knockout in the obligate intracellular parasite Toxoplasma. The TgIF2K-D knockout showed reduced TgIF2α phosphorylation and translational control in response to extracellular stress, along with reduced viability when outside the host cell (Fig. 4 and 6). This phenotype was also observed for the TgIF2α-S71A mutant, supporting the idea that induced TgIF2K-D phosphorylation of TgIF2α is central for Toxoplasma to persist in the extracellular environment (Fig. 6) (18). Intracellular tachyzoites proliferate within a parasitophorous vacuole membrane that operates as a molecular sieve and regulates the acquisition of nutrients from the host cell (38, 40). Upon exit from their host cell, the tachyzoites must find a new host cell in order to survive and replicate. The extracellular environment is likely to be reduced in essential nutrients that are available to the parasite, and/or the tachyzoites may not be equipped with the uptake mechanisms needed to acquire them. Our data suggest that TgIF2α phosphorylation serves to protect the parasite during this period of vulnerability. Reductions in global translation would allow the tachyzoites to conserve energy and nutrients and may also
induce preferential translation of key proteins required for extracellular survival (e.g., membrane transporters or a new array of metabolic enzymes). Such preferential translation of transcripts, such as ATF4 in mammals and GCN4 in S. cerevisiae, during eIF2α phosphorylation is central for ameliorating nutrient stress (14, 41, 45, 48). The ability of the parasite to overcome extracellular stress is suggested to be important for pathogenesis, as demonstrated by our prior report that the TgIF2α-S71A mutant has reduced virulence when inoculated into mice (18). Toxoplasma strains differing in virulence are also suggested to differ in their ability to initiate translational control; hypervirulent strains are able to phosphorylate TgIF2α faster and more robustly than hypovirulent strains during extracellular stress (18).

Mechanisms by which Toxoplasma copes with the extracellular environment. The mechanisms employed by tachyzoites to overcome the dramatic changes in their extracellular environment are poorly understood but have recently emerged as a new area of intensive research. Microarray analyses have revealed significant changes in the transcriptome between intra- and extracellular tachyzoites (12, 21). Generally, intracellular parasites favor expression of genes involved in metabolism and DNA replication, while Toxoplasma cells in the extracellular environment activate genes focused on invasion, motility, and signal transduction.

Coincident with the reprogramming of the transcriptome, extracellular parasites form a novel plant-like vacuole/vacuolar compartment (PLV/VAC). The PLV/VAC may protect parasites from osmotic or ionic stresses encountered outside host cells or mediate the proteolytic maturation of proproteins targeted to micronemes, a cellular compartment important for the parasite invasion into host cells (11, 24, 30). Several studies have also shown that extracellular parasites undergo a metabolic shift from oxidative phosphorylation to glycolysis in order to generate the ATP required for gliding motility and invasion (22, 32). Collectively, these studies suggest that tachyzoites undergo extensive changes in their morphology, metabolism, and transcriptome when transitioning to the extracellular environment.

Translational control through TgIF2α phosphorylation provides an additional mechanism that can modulate Toxoplasma gene expression that is designed to facilitate extracellular survival. In support of this model, our data showed that parasites lacking the GCN2-like TgIF2K-D are significantly impaired in their ability to survive outside host cells. In addition to TgIF2K-D, Toxoplasma is suggested to express three other eIF2α kinases that are each proposed to respond to unique stress arrangements or environmental cues. TgIF2K-A resides in the parasite endoplasmic reticulum and is suggested to function analogously to mammalian PEK/PERK (27, 44). TgIF2K-B is a parasite-specific eIF2α kinase likely to respond to a cytosolic stress (27). Finally, TgIF2K-C is another GCN2-like protein kinase present in the Toxoplasma genome (27). However, this putative eIF2α kinase appears to lack an RWD that was reported to be essential for GCN2 activity in the yeast model system (13, 19, 20). We do not yet understand the functional significance of two related GCN2 eIF2α kinases in Toxoplasma, although this study demonstrates that deletion of TgIF2K-D alone is sufficient to disrupt the translational control required for the parasite to cope with the extracellular environment. It is tempting to speculate that TgIF2K-C is activated to phosphorylate TgIF2α during nutrient deprivation experienced by intracellular parasites. Our data show that while TgIF2α is phosphorylated in starved intracellular parasites, TgIF2K-D is dispensable for this response. It will be important to identify the TgIF2K involved in phosphorylating TgIF2α under intracellular starvation conditions, since TgIF2α-S71A is deficient in recovering from this stress. Collectively, our data further highlight the eIF2α kinase stress response pathway as a potential therapeutic target.

GCN2-like protein kinases in parasites. The tandem arrangement of GCN2-related eIF2α kinases is also found in the related parasite Plasmodium falciparum. Conservation of multiple GCN2-related protein kinases may indicate that each phosphorylates eIF2α in response to distinct stress conditions. The P. falciparum PF14_0264 product is most closely related to TgIF2K-D and contains an RWD domain, while PefK1 appears to lack an RWD domain and has recently been reported to respond to amino acid starvation during the intraerythrocyte ring stage (8). This observation suggests that the RWD/GCN1
regulatory network may not be essential for invoking translational control during periods of certain nutritional deficiencies.

GCN2-like protein kinases lacking the RWD domain are not restricted to Apicomplexa. Three GCN2-related kinases (IFKA through -C) have been described in Dictyostelium, but only IFKC possesses an RWD domain (34). Dictyostelium is capable of developing a fruiting body, a process that is induced upon nutrient starvation. Although they are involved in regulating Dictyostelium development, neither IFKA nor IFKB appears to represent the initial sensor for this stress, supporting the idea that different GCN2 isoforms sense distinct stress conditions (3, 7). The role of IFKC in this process has not yet been studied.

In the case of mammalian GCN2, different mRNA isoforms have been identified, leading to the expression of one GCN2 variant lacking the RWD domain (42). The reason for the absence of this domain is still enigmatic but is likely to affect the regulation of eIF2α kinase activity. How the different versions of GCN2 protein kinases interplay and respond to stress will be an interesting topic for future investigation.

Regulation of translation through TgIF2K-independent mechanisms. Our radiolabeling experiments revealed that Δif2k-d and TgIF2α-S71A mutant parasites subjected to extracellular stress reduce protein synthesis by about 40% (Fig. 4C). While this is much different from the 90% reduction observed in cellular Toxoplasma gondii tachyzoites do not require carbon source uptake for ATP maintenance, gliding motility and invasion in the first hour of their differentiation mutants are also impaired with respect to switching into a novel extracellular morphology. Toxoplasma gondii: a selectable dihydrofolate reductase-thymidylate synthase marker based on drug-resistance mutations in malaria. Proc. Natl. Acad. Sci. U. S. A. 96:11703–11707.

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