Parasite-specific eIF2 (eukaryotic initiation factor-2) kinase required for stress-induced translation control

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The ubiquitous intracellular parasite *Toxoplasma gondii* (phylum Apicomplexa) differentiates into an encysted form (bradyzoite) that can repeatedly re-emerge as a life-threatening acute infection (tachyzoite) upon impairment of immunity. Since the switch from tachyzoite to bradyzoite is a stress-induced response, we sought to identify components related to the phosphorylation of the α subunit of eIF2 (eukaryotic initiation factor-2), a well-characterized event associated with stress remediation in other eukaryotic systems. In addition to characterizing *Toxoplasma* eIF2α (TgIF2α), we have discovered a novel eIF2 protein kinase, designated TgIF2K-A (*Toxoplasma gondii* initiation factor-2 kinase). Although the catalytic domain of TgIF2K-A contains sequence and structural features that are conserved among members of the eIF2 kinase family, TgIF2K-A has an extended N-terminal region that is highly divergent from other eIF2 kinases. TgIF2K-A specifically phosphorylates the regulatory serine residue of yeast eIF2α *in vitro* and *in vivo*, and can modulate translation when expressed in the yeast model system. We also demonstrate that TgIF2K-A phosphorylates the analogous regulatory serine residue of recombinant TgIF2α *in vitro*. Finally, we demonstrate that TgIF2α phosphorylation in tachyzoites is enhanced in response to heat shock or alkaline stress, conditions known to induce parasite differentiation *in vitro*. Collectively, this study suggests that eIF2 kinase-mediated stress responses are conserved in Apicomplexa, and a novel family member exists that may control parasite-specific events, including the clinically relevant conversion into bradyzoite cysts.

Key words: Apicomplexa, eukaryotic initiation factor-2 (eIF2), stress, *Toxoplasma*, transcription, translation.

INTRODUCTION

The obligate intracellular parasite *Toxoplasma gondii* causes congenital birth defects and is a significant opportunistic pathogen. *Toxoplasma* infection is normally subdued by a healthy immune system, which induces the rapidly growing form of the parasite (tachyzoite) to differentiate into a virtually quiescent cyst form (bradyzoite). However, neonates and immunocompromised individuals lack the defence necessary to control *Toxoplasma* infection. A pressing need exists to develop new therapies to combat *Toxoplasma*, since the current treatment of pyrimethamine and sulphonamides has dangerous side effects and does not eliminate bradyzoite cysts, which are responsible for the continuous re-emergence of infection upon immune deficiency [1]. *Toxoplasma* is easily cultured *in vitro* and is genetically tractable, making it a useful model for the study of other parasites in its phylum, Apicomplexa [2,3]. The Apicomplexa consist of approx. 5000 parasitic protozoan species, distinguished by the presence of an elaborate group of specialized organelles at the apical end that facilitate invasion into host cells. Among the most notorious of apicomplexans is the malarial parasite *Plasmodium* spp., which threatens up to 40 % of the world's population and kills up to 2.7 million people a year [4].

As differentiation from tachyzoites to bradyzoites appears to be a stress-induced response [5], we addressed the molecular mechanisms controlling stress pathways in *Toxoplasma*. In other eukaryotes, cellular stress is ameliorated by a family of protein kinases that phosphorylate the α subunit of eIF2 (eukaryotic initiation factor-2). For example, activation of the eIF2 kinase GCN2 (general control non-derepressible 2) occurs in response to amino acid starvation [6]. The eIF2 combines with GTP and initiator Met-tRNA$_i^{Met}$, and is integral to ribosomal selection of initiation codons [7]. During this translation process, eIF2–GTP is hydrolysed to eIF2–GDP, and this initiation factor is released from the ribosomal machinery. A guanine nucleotide exchange factor, eIF2B, facilitates exchange of eIF2–GDP with the active form, eIF2–GTP [7]. Phosphorylation of eIF2 by GCN2 converts this initiation factor from a substrate into an inhibitor of the exchange factor, reducing the levels of eIF2–GTP available for translation initiation. In addition to reducing total protein synthesis, lowered levels of eIF2–GTP can enhance translation of specific mRNAs. In the well-characterized yeast *Saccharomyces cerevisiae*, phosphorylation of eIF2 enhances the translational expression of GCN4, a bZIP (basic-zipper) transcriptional activator of many genes that are involved in amino acid synthesis and related intermediary metabolism [6,8]. In nutrient-deprived mammalian cells, synthesis of a related bZIP regulator, ATF4 (activating transcription factor 4), is enhanced by phosphorylation of eIF2 [9]. ATF4 regulates genes involved in amino acid metabolism, relief of oxidative stress and apoptosis [10]. ATF4 also induces expression of the additional bZIP transcriptional regulators CHOP/GADD153 (growth-arrest and DNA-damage-inducible protein 153) and ATF3, leading to an amplified pattern of stress gene expression [10–12].

In addition to GCN2, three other eIF2 kinases have been identified in mammals, each associated with a distinct stress condition. PKR (double-stranded-RNA-dependent protein kinase) and HRI (haem-regulated inhibitor) are associated with viral infections

Abbreviations used: ATF(4), activating transcription factor (4); bZIP, basic zipper; eIF2, eukaryotic initiation factor-2; GCN(2), general control of non-derepressible (2); GST, glutathione S-transferase; HRI, haem-regulated inhibitor; ORF, open reading frame; PEK, pancreatic eIF-2α kinase; PKR, double-stranded-RNA-dependent protein kinase; RACE, rapid amplification of cDNA ends; RT, reverse transcriptase; TgIF2α, *Toxoplasma gendii* eIF2α; UTR, untranslated region.

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The nucleotide sequence data reported will appear in DDBJ, EMBL, GenBank® and GSDB Nucleotide Sequence Databases under the accession numbers AY518936 and AY518935 for TgIF2K-A and TgIF2α respectively.

and haemin deficiency respectively [13,14]. Homologues of HRI have also been identified in *Schizosaccharomyces pombe*, illustrating further the importance of eIF2 kinase in stress response pathways in 'lower' eukaryotic organisms [15]. The fourth eIF2 kinase is PEK (pancreatic eIF2α kinase; also known as Perk), which is activated by impaired protein secretory function that can occur during elevated protein misfolding in the lumen of the endoplasmic reticulum [9,16,17]. Homologues of PEK/Perk as well as GCN2 have also been found in the invertebrates *Caenorhabditis elegans* and *Drosophila melanogaster* [18].

To date, the only eIF2 kinase-related enzyme in apicomplexan protozoa is PfPK4, which was identified in *Plasmodium falciparum* [19]. Although PfPK4 is capable of phosphorylating itself as well as a synthetic peptide representing a portion of eIF2α, the specificity of PfPK4 for the eIF2α phosphorylation site Ser^{51} and its impact on translational control have not yet been established. In addition to characterizing *Toxoplasma* eIF2α, we demonstrate here that its phosphorylation occurs in response to heat shock or pH stress, treatments known to induce differentiation to bradyzoites *in vitro* [20]. We have identified an unusual eIF2 kinase in *Toxoplasma*, designated TgIF2K-A (*Toxoplasma gondii* initiation factor-2 kinase), which is distinct from the known family members of eIF2 kinases and appears to be restricted to parasites. This new eIF2 kinase family member is proposed to participate in the adaptive response of parasites to stress, as well as contributing to the clinically relevant process of parasite differentiation to bradyzoites.

EXPERIMENTAL

Plasmid constructs

Full-length cDNAs for TgIF2K-A and TgIF2α were obtained by 5′- and 3′-RACE (rapid amplification of cDNA ends) using GeneRacer™ (Invitrogen) with *Toxoplasma* total RNA and gene-specific primers based on ToxoDB sequences (http://ToxoDB.org). Multiple cDNAs were independently obtained and characterized by nucleotide sequencing. Plasmid pYES-GST-TgIF2K-A (where 'GST' is glutathione S-transferase) was created by installing a PCR-amplified product encoding amino acid residues 3974–5072 of TgIF2K-A into the restriction enzyme sites *Bam*HI and *Xba*I of a modified pYES (Invitrogen), a high-copy *URA3*-marked plasmid containing a galactose-inducible yeast promoter. The resulting plasmid, pYES-GST-TgIF2K-A, encodes the TgIF2K-A catalytic region inserted in-frame to an N-terminal GST tag. To make pET-TgIF2α, the cDNA encoding TgIF2α was inserted into pET-15 (Novagen) between *Nde*I and *Bam*HI restriction sites. The resulting plasmid, pET-TgIF2α, contains the TgIF2α coding region fused in-frame with an N-terminal polyhistidine tag. Galactose-inducible yeast expression plasmids encoding GST fusions to the kinase domain of PKR, PEK and the kinase-deficient PEK-K621M have been similarly constructed [21]. Plasmid constructs involved PCR using *PfuUltra*™ proofreading polymerase (Stratagene), and all DNA sequencing was performed by the IU Biochemistry Biotechnology Facility (Indianapolis, IN, U.S.A.). To generate the kinase-deficient version of TgIF2K-A [containing a K4038M ($Lys^{4038} \rightarrow$ Met) mutation], site-directed mutagenesis was performed using the QuikChange® II XL kit (Stratagene), according to the manufacturer's instructions.

Parasite culture and methods

Toxoplasma strains were cultured in human foreskin fibroblasts, as described previously [22]. To examine the effects of pH stress on TgIF2α phosphorylation, equal numbers of filter-purified tachyzoites were incubated in media of pH 7.1 (unstressed) and pH 8.1 (stressed) at 37 °C for 1 h [23]. Similarly, heat shock stress was examined by subjecting equivalent numbers of tachyzoites to either 37 °C or 43 °C for 1 h [20]. Post-incubation, parasites were collected by centrifugation and lysed in RIPA solution [50 mM Tris/HCl (pH 7.9)/150 mM NaCl/1.0 % Nonidet P-40/0.5 % (w/v) sodium deoxycholate/0.1 % (w/v) SDS] supplemented with phosphatase inhibitors (37 mM β-glycerophosphate and 47 mM NaF) and protease inhibitors (100 μM PMSF, 0.15 μM aprotinin, 1 μM pepstatin and 1 μM leupeptin). Proteins were quantified using the Bradford assay. Equal amounts of protein lysates were resolved by SDS/PAGE and then transferred to nitrocellulose filters. Immunoblot analyses were performed using a TBS-T solution containing 20 mM Tris/HCl, pH 7.9, 150 mM NaCl and 0.2 % (v/v) Tween 20 supplemented with 4 % non-fat milk. Filters were incubated with polyclonal antibody that specifically recognizes phosphorylated eIF2α at Ser^{51} (Research Genetics, Huntsville, AL, U.S.A.). Total eIF2α was measured using rabbit polyclonal antibody prepared against the peptide KGYIDLSK-RRVS, which recognizes both phosphorylated and non-phosphorylated forms of eIF2α. Antibody against constitutively expressed *Toxoplasma* tubulin (anti-TgβTub) was kindly given by Dr Morrissette and Dr Sibley (Washington University School of Medicine, St Louis, MO, U.S.A.) [24]. Following incubation of the filters with antibody, the blots were washed three times in TBS-T and incubated with TBS-T containing secondary antibody conjugated to horseradish peroxidase (Bio-Rad). The protein–antibody complexes were visualized using a chemiluminescent substrate.

RNA isolation and Northern blotting

Total RNA and mRNA were harvested from filter-purified parasites using TRIzol® (Invitrogen) or Poly(A)Pure™ (Ambion) respectively, each followed by treatment with DNase. Northern blotting was performed according to standard methods [25], with the exception that ExpressHyb™ (BD Biosciences) was used in place of conventional hybridization solution. Probes were radiolabelled using the Prime-A-Gene® system (Promega).

Sequence data and analysis tools

Preliminary genomic sequence data were accessed via http://toxoDB.org, Releases 1.0–2.2 [26]. Genomic data were provided by The Institute for Genomic Research (supported by the NIH grant AI05093), and by the Sanger Center (Wellcome Trust). DNA and protein sequences were analysed for homologues using BLAST programs at http://www.ncbi.nlm.nih.gov. Protein mapping and motif searches were performed using the Pfam HMM database (http://pfam.wustl.edu/hmmsearch.shtml). Alignments of multiple eIF2α and eIF2α kinase sequences were performed using Vector NTI 9.0 (Informax, Bethesda, MD, U.S.A.).

In vivo analysis of eIF2 kinase function in yeast

Plasmids encoding GST-tagged eIF2 kinase domains were introduced into isogenic yeast strains H1894 (*MATa ura3–52 leu2–3 leu2–112 gcn2Δ trp1Δ-63*) and J82 (*MATa ura3–52 leu2–3 leu2–112 gcn2Δ sui2Δ trp1Δ-63*, *p1098* [*SUI2-S51A LEU2*]), each deleted for the sole endogenous eIF2 kinase GCN2 [21]. The eIF2-kinase-expressing strains were grown overnight on the surface of agar plates containing synthetic medium with 2 % glucose (SD medium) supplemented with leucine, isoleucine, valine and tryptophan [27]. Cell patches were then replica-plated on to agar plates containing either SD or synthetic medium supplemented

with 10 % galactose and 2 % raffinose (S-Gal medium), supplemented with leucine, isoleucine, valine and tryptophan. Following incubation at 30 °C for 3 days, the agar dishes were electronically imaged. H1894-expressing strains were also cultured in S-Gal liquid culture for 6 h, and Western blots were performed as described above using antibody specific for phosphorylated eIF2α. Alternatively, rabbit polyclonal antibody prepared against recombinant yeast eIF2α was used to detect total levels of eIF2.

Protein expression and purification

Yeast strain J82 expressing eIF2 kinase domains fused to GST were grown overnight to saturation in SD medium. Cells were inoculated into S-Gal medium at an D_{600} of 0.1, and grown for 20 h at 30 °C for induction of protein expression. Cells were collected by centrifugation and resuspended in solution A, containing 20 mM Tris/HCl, pH 7.9, 150 mM NaCl, 10 % glycerol, 1 mM 2-mercaptoethanol and protease inhibitors (100 μM PMSF, 0.15 μM aprotinin, 1 μM leupeptin and 1 μM pepstatin). Cells were lysed by using glass beads and vortex-mixed for 2 min at 15 s intervals, interspersed with incubation of samples on ice. After centrifugation at 2000 ***g*** to remove the glass beads and cell debris, the lysate was subjected to centrifugation at 16 000 ***g*** for 30 min. The protein content of the clarified cell lysate was measured using the Bradford method, with BSA as the standard. The GST-tagged fusion proteins in the lysate were partially purified by overnight incubation with glutathione–agarose beads and washing thrice with solution A. The washed beads were resuspended in solution A, and used directly in the *in vitro* kinase assay.

Escherichia coli BL21-CodonPlus® (Stratagene) were transformed with pET-TgIF2α. Recombinant protein was induced by adding 0.5 mM IPTG (isopropyl β-D-thiogalactoside) for 3 h at 37 °C, and purified by nickel-affinity chromatography (Qiagen).

In vitro eIF2 kinase assay

Kinase reactions were allowed to proceed with affinity-purified GST-tagged protein as indicated, 10 μCi of [γ-^{32}P]ATP in a final concentration of 50 μM and kinase buffer solution [20 mM Tris/HCl (pH 7.9)/50 mM KCl/10 mM $MgCl_2$/2 mM $MnCl_2$/5 mM 2-mercaptoethanol/protease inhibitors (100 μM PMSF, 0.15 μM aprotinin, 1 μM leupeptin and 1 μM pepstatin)] in a volume of 20 μl. An aliquot (1.3 μM) of recombinant yeast eIF2α was included in the reaction mixture, and the kinase assay was terminated by the addition of 2 % SDS after incubation at 30 °C for 10 min. Radiolabelled proteins were analysed by SDS/PAGE (12 % gels), fixed by Coomassie staining, dried and then visualized by autoradiography.

RESULTS

Characterization of *Toxoplasma* eIF2 kinases

Phosphorylation of eIF2α is a central mechanism for regulating stress gene expression in eukaryotic cells. To discern whether eIF2α phosphorylation participates in *Toxoplasma* stress responses, we searched for putative eIF2 kinase homologues encoded in the *Toxoplasma* genomic sequence (http://ToxoDB.org, Release 2.2) using the BLAST program and a query containing conserved hallmark sequences of the eIF2 kinase family [16]. Genome sequence entry Tgg_9402 encodes a polypeptide that possesses similarity to the catalytic sequences conserved among eIF2 kinases. We named the locus TgIF2K-A, and discerned the intron–exon boundaries by sequencing RT (reverse transcriptase)-PCR products spanning the genomic sequence data (Figure 1). The ORF (open reading frame) of the TgIF2K-A cDNA consists of

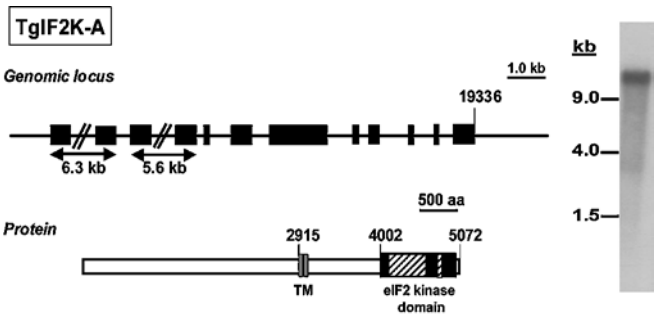

Figure 1 Structure and Northern analysis of TgIF2K-A

The open reading frame of the *Toxoplasma* eIF2 kinase homologue was mapped by using overlapping RT-PCR with primers based on sequences found in the *Toxoplasma* database. The predicted TgIF2K-A mRNA length is consistent with a Northern hybridization using a TgIF2K-A-specific probe, as shown in the right panel. In the genomic locus diagram, black boxes represent the exons and parallel lines denote regions too large to represent to scale. In the predicted protein diagram, 'TM' represents transmembrane domains, and the stippled boxes within the black box denote the two insert sequences within the kinase domain, between subdomains IV–V and VII–VIII respectively. aa, amino acids.

15 219 bp, and is predicted to encode a protein of 5072 amino acids. This coding region includes an initiating ATG codon that conforms to Kozak rules, and is preceded by an in-frame stop codon at -60 bp [28]. At the 3′-end of the TgIF2K-A cDNA is an approx. 600 bp UTR (untranslated region). Northern analysis revealed a single large transcript, in agreement with the $\approx$ 15 kb size of TgIF2K-A cDNA (Figure 1).

The catalytic region of TgIF2K-A is proximal to the C-terminus, and is closely related to the eIF2 kinase family (Figure 2), with the sum probability of the pairwise alignments ranging from $2\,e^{-18}$ with human PEK, $2\,e^{-12}$ with rabbit HRI, $2\,e^{-09}$ with mouse GCN2 and $4\,e^{-10}$ with human PKR. Notable features conserved in TgIF2K-A include the LFIQME(Y/F)(D/E) sequence in subdomain V that is important for catalytic activity, and an insert between kinase subdomains IV and V. Such inserts in the eIF2 kinases are quite variable in sequence composition and length, ranging from 32 amino acid residues in PKR to 223 residues in human PEK. The kinase insert in TgIF2K-A is longer than any previously reported (616 amino acids), and shares no significant similarity either with other members of the eIF2 kinase family or with any other known protein sequence. Similarly, the lengthy N-terminal domain upstream of the kinase catalytic domain of TgIF2K-A bears no resemblance to proteins entered in GenBank®. Protein motif searches SMART and TMHMM identified two transmembrane domains at residue positions 2915–2937 and 2942–2964, separated by only four amino acid residues.

The previously identified eIF2 kinase-related enzyme called PfPK4 exhibits the most similarity to TgIF2K-A, with the sum probability of $2\,e^{-62}$ in the pairwise alignment. Interestingly, PfPK4 also contains a lengthy insert, 511 amino acid residues in length, between subdomains IV and V, but it bears little resemblance to the analogous insert in TgIF2K-A. Similarly, the insert sequence comprising the activation loop between subdomains VII and VIII is notably longer than any reported previously (66 amino acids in TgIF2K-A and 42 amino acids in PfPK4) and, furthermore, lacks the second threonine residue 10 amino acids upstream of the S/APE motif, which is otherwise conserved in all known eIF2 kinases [29]. While high concentrations of haem were found to inhibit PfPK4 *in vitro*, the mechanism of action and physiological relevance of this eIF2 kinase remain unresolved [19]. The disparity in the non-catalytic domains between the novel parasite-specific eIF2 kinases TgIF2K-A and PfPK4 suggests that

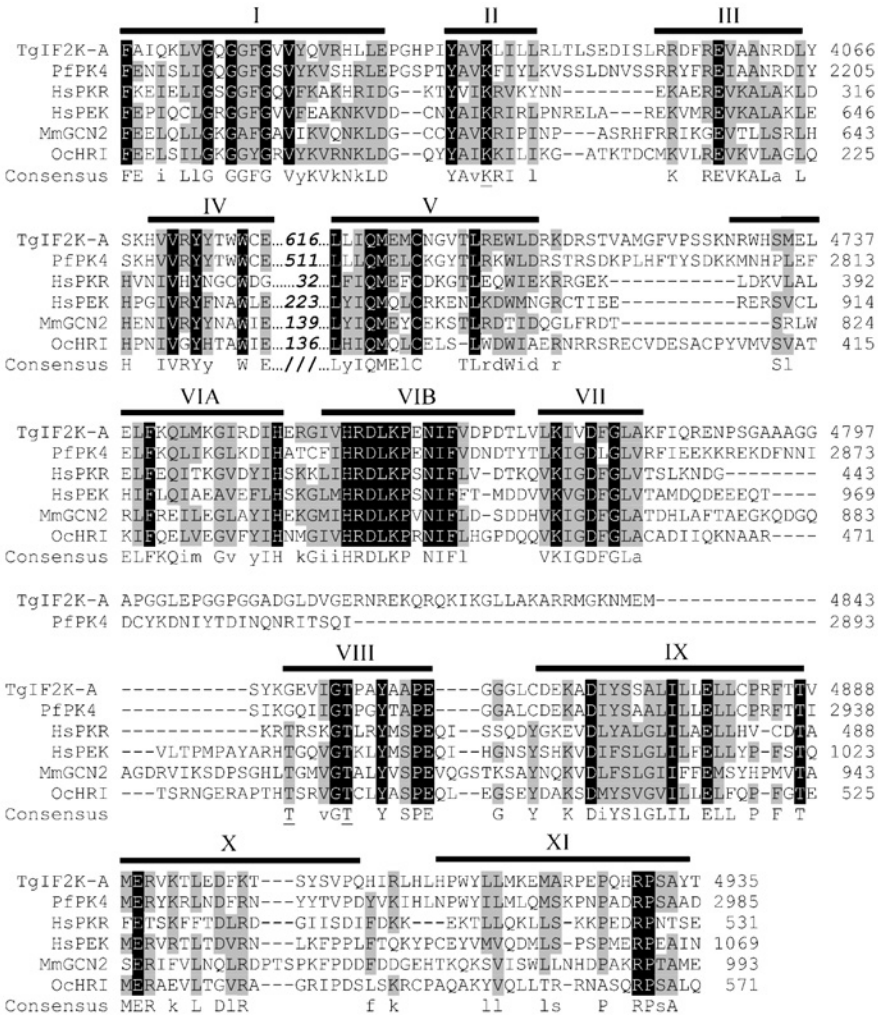

Figure 2 Comparative alignment of the catalytic kinase subdomains of representative eIF2 kinase family members

Residues shown in black boxes are identical in every species, whereas those that are chemically similar are shown in grey-shaded boxes. Upper-case letters in the consensus line represent identity, and lower-case letters denote conserved residues in at least four of the six species aligned. Dashes indicate gaps in the sequences that were used to maximize the alignment. The invariant lysine residue used to make kinase-deficient eIF2α kinases is underscored in the consensus line (K). The kinase inserts between subdomains IV and V are represented by dots and the number of amino acid residues comprising the insert sequence. Threonine residues in subdomain VIII known to be involved in autophosphorylation and kinase activation are underscored in the consensus line (T). Tg, *T. gondii* (accession no. AY518936); Pf, *P. falciparum* (accession no. NP_703939); Hs, *Homo sapiens* [PKR (accession no. XM_002661) and PEK (accession no. AF193339)]; Mm, *Mus musculus* (accession no. AF193343); Oc, *Oryctolagus cuniculus* (rabbit; accession no. P33279).

they have different modes of stress activation. The unusual kinase domain inserts and the lack of analogous regulatory regions found in other eIF2 kinases indicates that TgIF2K-A and PfPK4 are novel members of this family.

In our bioinformatics survey, we found no evidence for PKR, PEK/Perk or HRI in *Plasmodium* or *Toxoplasma*. However, *P. falciparum* appears to contain a GCN2 orthologue (chr14.phat_416). As noted above, this eIF2 kinase is associated with nutritional stress, and the predicted *P. falciparum* GCN2 has the hallmark feature of a regulatory domain similar to that of histidyl-tRNA synthetases, that is important for binding uncharged tRNA, which accumulates in response to amino acid starvation [6]. To date, we find no evidence of a GCN2 orthologue in the *Toxoplasma* genome sequence, despite $>10\times$ sequencing coverage.

TgIF2K-A regulates translation in a yeast model system

To determine whether TgIF2K-A is indeed an eIF2 kinase, we employed the well-characterized yeast model system [21,30]. In this system, a galactose-inducible promoter is used to express a heterologous eIF2 kinase in a yeast strain, such as H1894, that is deleted for its sole endogenous eIF2 kinase GCN2. Hyperphosphorylation of eIF2α that results from such expression of an eIF2 kinase leads to significant reduction in translation initiation and reduced growth. The catalytic portion of TgIF2K-A was cloned in-frame to an N-terminal GST tag in a yeast expression vector (pYES-GST-TgIF2K-A), and introduced into the H1894 strain. For controls, we introduced similar expression plasmids encoding GST fused to the catalytic domains of mammalian PKR or PEK into this yeast strain. Expression of TgIF2K-A or the mammalian eIF2 kinases elicited a severe slow growth in galactose-inducing medium (Figure 3A). By comparison, no growth defect was observed in the H1894-derived cells cultured in glucose medium, where the eIF2 kinases were not expressed.

To address whether TgIF2K-A eIF2 kinase activity is required for the slow growth, we introduced a mutant version of TgIF2K-A containing a methionine in place of the kinase-invariant lysine residue Lys^{4038} into the H1894 strain, and found no growth defect

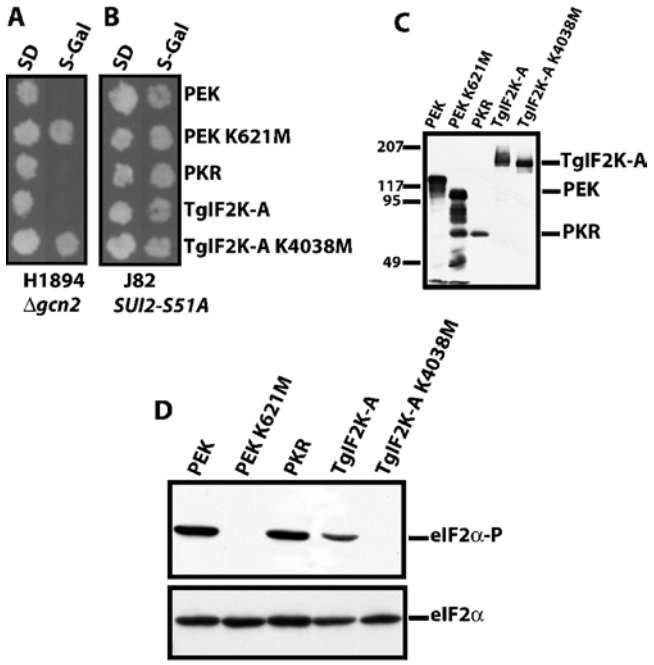

Figure 3 TgIF2K-A is an eIF2 kinase that mediates translational control in the yeast model system

To verify that TgIF2K-A can regulate translation through phosphorylation of eIF2α at Ser^{51}, we expressed the catalytic domains of TgIF2K-A or mammalian eIF2 kinases PEK or PKR fused to an N-terminal GST tag in the well-characterized yeast model system. (**A**) Plasmids encoding TgIF2K-A, the kinase-deficient TgIF2K-A K4038M, human PKR, human PEK or the kinase-defective PEK K621M were introduced into yeast strain H1894 that contains a deletion in its sole eIF2 kinase, GCN2. The H1894-derived strains were cultured on agar plates containing galactose (S-Gal), a condition that induces the expression of the eIF2 kinases via a galactose-inducible promoter. Alternatively, these strains were patched on to agar medium containing glucose (SD), which leads to low levels of eIF2 kinase expression. (**B**) The same experiment was repeated using the yeast strain J82, which also contains a non-phosphorylatable form of eIF2α-S51A (*SUI2-S51A*), and is isogenic to H1894. (**C**) The levels of the indicated eIF2 kinases were measured by immunoblotting using an antibody that specifically recognizes the GST tag. Locations of the eIF2 kinases in the immunoblot are indicated. (**D**) H1894 cells expressing the indicated eIF2 kinases were grown in galactose-inducible medium, and the levels of eIF2α phosphorylated at Ser^{51} were measured by immunoblotting using polyclonal antibodies specific for phosphorylated eIF2α. (bottom panel). Total eIF2α levels were measured in the H1894-derived strains by immunoblot analysis, by using antibodies that recognize both phosphorylated and non-phosphorylated versions of eIF2α.

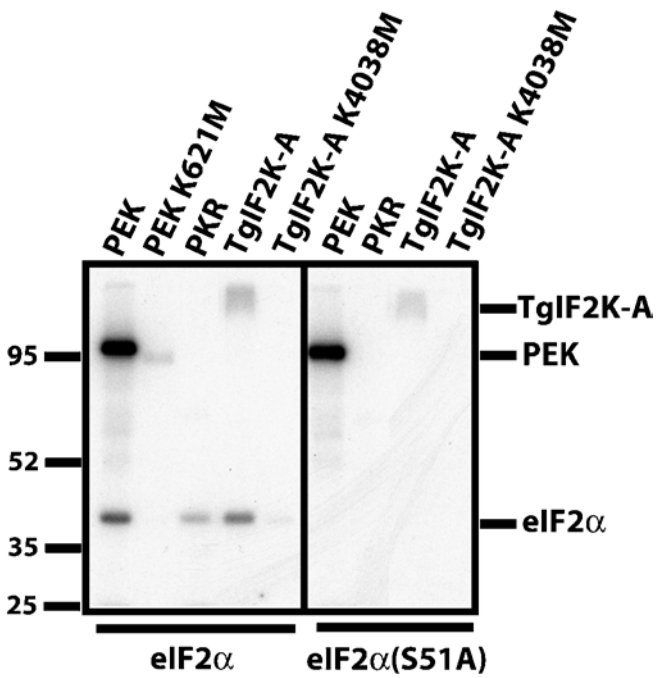

Figure 4 TgIF2K-A phosphorylates eIF2α *in vitro*

The eIF2α kinases expressed in the yeast strain J82 were purified by virtue of the GST tag and included in an *in vitro* kinase assay using recombinant yeast eIF2α (left panel) or a mutant version of eIF2α (S51A) (right panel) and [γ-^{32}P]ATP, as described in the Experimental section. Proteins in the kinase reactions were separated by SDS/PAGE and visualized by autoradiography. Radiolabelled eIF2α and eIF2 kinases are shown. Protein molecular-mass markers are indicated in kDa.

in the galactose medium (Figure 3A). Furthermore, we introduced the wild-type and mutant versions of TgIF2K-A into strain J82, which contains an alanine at the eIF2α phosphorylation site Ser^{51} (SUI2-S51A), and observed efficient growth in both galactose and glucose media (Figure 3B). Comparable levels of wild-type and mutant versions of TgIF2K-A were measured by immunoblotting (Figure 3C). Also evident in Figure 3(C) is that both PEK and TgIF2K-A exhibit a shift to a slower migrating form compared with their corresponding kinase mutant forms. This has been observed previously for PEK, and was shown to be due to autophosphorylation [17].

To determine directly whether eIF2α is phosphorylated in TgIF2K-A-expressing cells, protein lysates were prepared from the H1894 transformants grown in galactose medium, and phosphorylation of eIF2α at Ser^{51} was measured by Western blot analyses (Figure 3D). Phosphorylated eIF2α was observed in the H1894 strain expressing TgIF2K-A or the mammalian eIF2 kinases PKR or PEK. No eIF2α phosphorylation was detected in yeast cells expressing the kinase-defective TgIF2K-A or PEK. Levels of total eIF2α were similar among these H1894-derived cells, as judged by Western blotting using polyclonal antibody that recognizes both phosphorylated and non-phosphorylated versions of eIF2α (Figure 3D). Collectively, these experiments demonstrate that TgIF2K-A, like other eIF2 kinases, regulates translation *in vivo* by phosphorylating eIF2α at Ser^{51}. Kinase assays were also performed *in vitro* to confirm the specificity of TgIF2K-A for the Ser^{51} residue of eIF2α. We utilized the J82-derived transformants, because the eIF2α Ser^{51} $\rightarrow$ Ala (S51A) mutant alleviated the slow growth phenotype associated with expression of functional TgIF2K-A. The yeast cells were grown in galactose-inducing medium, and the GST-tagged eIF2 kinases were purified using glutathione–affinity chromatography. TgIF2K-A efficiently phosphorylated recombinant yeast eIF2α, whereas no phosphorylation was detected using the mutant eIF2α S51A substrate (Figure 4). Phosphorylation of eIF2α is blocked in the mutant version of TgIF2K-A (K4038M), consistent with its observed loss of *in vivo* function. This specificity for *in vitro* phosphorylation of eIF2α at Ser^{51} was conserved in the well-characterized mammalian eIF2 kinases PKR and PEK.

Cloning of the eIF2α in *Toxoplasma*

Data from ToxoDB Release 1.0 entries Tgg_5585, Tgg_3749 and Tgg_2375 resemble different portions of eIF2α proteins from other species. Primers designed on the basis of these data were used to generate an ORF, designated TgIF2α, which was subsequently verified by an independent RT-PCR analysis of *Toxoplasma* RNA. 5′-RACE yielded an $\approx$0.6 kb 5′-UTR containing an in-frame stop codon 87 nt upstream of the proposed start ATG codon, which conforms to Kozak consensus rules [28]. 3′-RACE revealed a 3′-UTR of $\approx$0.9 kb. Northern blot analysis of

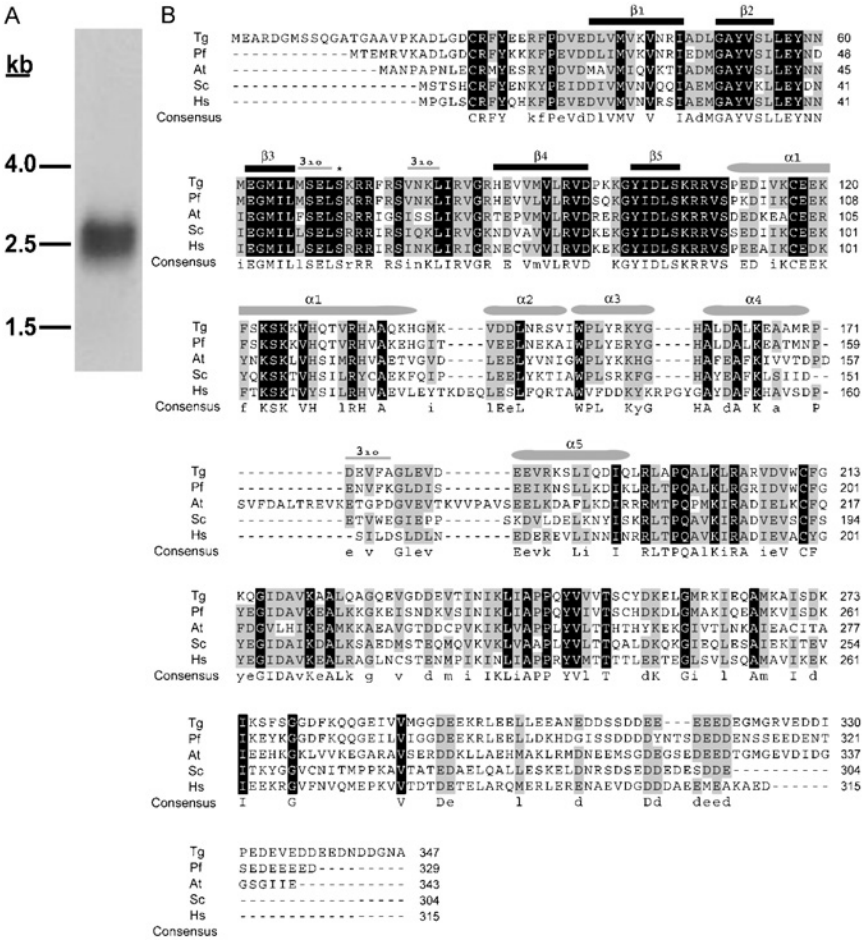

Figure 5 Characterization of TgIF2α

(**A**) A Northern blot probed with cDNA-derived sequence of TgIF2α is shown. (**B**) Comparison of the TgIF2α sequences with other eIF2α subunits. The deduced amino acid sequence of TgIF2α was aligned with other eIF2α polypeptides. Residues shown in black boxes are identical in every species, whereas those shown in grey-shaded boxes are residues that are chemically similar. Upper-case letters in the consensus line represent identity, and lower-case letters denote conserved residues in at least four of the five species aligned. The asterisk denotes the conserved serine residue phosphorylated by eIF2 kinases (residue Ser[71] in TgIF2α). Dashes indicate gaps in the sequences that were used to maximize the alignment. The β-strands, α-helices and 3_{10} helices are represented by bars above the alignments, based on the crystal structure of yeast eIF2α [31]. Tg, *T. gondii* (accession no. AY518935); Pf, *P. falciparum* (accession no. PF07_0117); At, *Arabidopsis thaliana* (accession no. AAK29673); Sc, *Sacch. cerevisiae* (accession no. P20459); Hs. *H. sapiens* (accession no. NP_004085).

Toxoplasma mRNA probed with the TgIF2α ORF showed hybridization to a transcript of the predicted ≈2.5 kb size (Figure 5A). TgIF2α is 347 amino acid residues in length, and most closely resembles eIF2α from *Plasmodium* spp. and yeast (Figure 5B). One feature of note in the two apicomplexan eIF2α proteins is the unique substitution of lysine for the arginine immediately C-terminal to the serine phosphorylation site. The arginine is well conserved among eukaryotes from plants, fungi and mammals. The predicted protein sequence of TgIF2α is consistent with structural features previously reported in the crystal structures for yeast and human eIF2α. As shown in Figure 5(B), a helical domain follows the β-barrel in the N-terminus, and the regulatory serine residue is located at the C-terminal end of the first conserved 3_{10} helix [31,32]. Additionally, Tyr[28], Asp[37], Glu[57], Tyr[58] and Asp[162], the five residues comprising the negatively charged groove predicted to interact with the β subunit of eIF2, are conserved in TgIF2α [31,32]. Also conserved in TgIF2α are Glu[69], Lys[99], Gly[100] and Arg[108], the residues mediating interaction with eIF2B [33].

Although we have shown that TgIF2K-A efficiently phosphorylates yeast eIF2α, we wanted to address whether this protein kinase could also phosphorylate the *Toxoplasma* version of the translation initiation factor. TgIF2α fused to an N-terminal polyhistidine tag was produced in *E. coli*, and purified by using nickel-chelation resin. Recombinant TgIF2α (Figure 6B, lanes 1–6) migrates with a predicted molecular mass of 42 kDa, including the 3 kDa polyhistidine tag, a size modestly larger than that measured for similarly tagged yeast eIF2α (Figure 6B, lanes 7 and 8). The TgIF2α was included in the kinase reaction mixture, and found to be phosphorylated by purified TgIF2K-A, but not by the kinase-deficient version of this eIF2 kinase (Figure 6A, lanes 5 and 6). Furthermore, mammalian eIF2 kinases PEK or PKR phosphorylated TgIF2α (Figure 6A, lanes 2 and 4). These results suggest that there are conserved regulatory features between eIF2α and eIF2 kinases among *Toxoplasma* and mammals.

TgIF2α phosphorylation accompanies parasite stress

Tachyzoites were subjected to heat shock (43 °C) or alkaline stress (pH 8.1) to address whether phosphorylation of TgIF2α occurs in these parasites in response to an environmental insult. The pH stress is commonly used to induce differentiation to bradyzoite cysts in culture [23], and heat shock stress has also been reported to

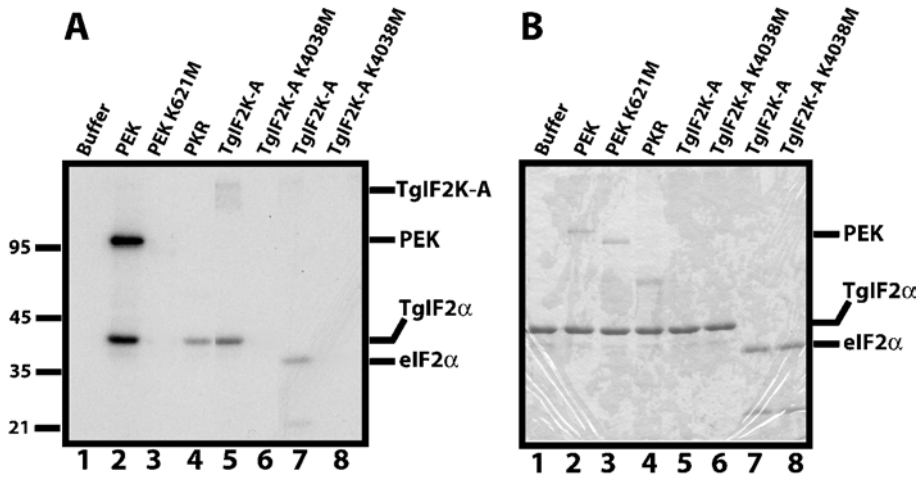

Figure 6 eIF2 kinases phosphorylate recombinant TgIF2α

(**A**) TgIF2α (lanes 1–6) or yeast eIF2α (lanes 7 and 8) were included in kinase reaction mixtures containing [γ-^{32}P]ATP and eIF2α kinases TgIF2K-A, PEK or PKR, as shown. Proteins in the *in vitro* kinase reactions were separated by SDS/PAGE and visualized by autoradiography. ^{32}P-labelled eIF2α and eIF2 kinases are shown. (**B**) Proteins separated by SDS/PAGE were visualized by Coomassie Blue staining. Protein molecular-mass markers are shown in kDa.

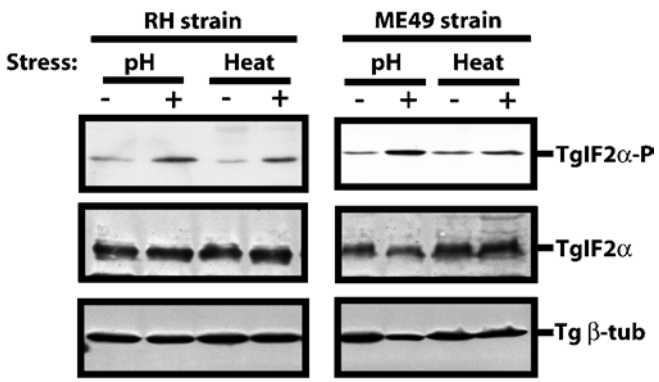

Figure 7 Stress conditions induce eIF2α phosphorylation in *Toxoplasma*

Tachyzoites from *Toxoplasma* strains RH and ME49 were incubated for 1 h in the presence of either pH or heat shock stresses, as indicated by the lanes labelled '+'. An equal number of tachyzoites was incubated under physiological conditions as controls (non-stressed, as shown by the lanes labelled '−'). Equivalent amounts of protein lysates prepared from these parasite samples were analysed by immunoblotting using polyclonal antibodies that specifically recognize phosphorylated TgIF2α (TgIF2α-P), total TgIF2α or β-tubulin (Tg βTub).

facilitate stage switching [20]. As shown in Figure 7, tachyzoites from two independent *Toxoplasma* strains subjected to high pH or heat shock stress exhibited enhanced phosphorylation of TgIF2α compared with tachyzoites incubated under unstressed conditions. Levels of total TgIF2α or the constitutively expressed tubulin were unchanged in response to these environmental insults. These results demonstrate a stress-induced phosphorylation of eIF2α at the conserved serine residue in Apicomplexa, and establish an intriguing link between eIF2α phosphorylation and conditions that induce parasite differentiation.

DISCUSSION

Novel eIF2 kinase present in an apicomplexan parasite

Four eIF2 protein kinases have been well characterized to date, each inducing stress gene expression in response to a unique subset of environmental stress conditions. Phosphorylation of eIF2α at a conserved serine residue (Ser^{51} in yeast and Ser^{71} in *Toxoplasma*) is a stress response conserved in *Toxoplasma*, and we have identified an eIF2 kinase, TgIF2K-A, that mediates this translational response. TgIF2K-A specifically phosphorylates yeast eIF2α at the regulatory serine residue *in vivo* and *in vitro*, and can facilitate translational control when expressed in the yeast model system. TgIF2K-A has sequence similarities unique to the catalytic domains of eIF2 kinases, and shares the placement of an insert sequence between kinase subdomains IV and V. Interestingly, TgIF2K-A contains an extended activation loop due to an additional kinase insert located between subdomains VII and VIII. In all other eIF2α kinases reported, two conserved threonine residues are located 5 and 10 amino acids N-terminal to the S/APE motif in subdomain VIII, and have been shown to be required for high-level kinase function in human PKR and yeast GCN2 [29]. TgIF2K-A contains only one threonine residue 5 positions upstream of its APE motif, and a serine residue 17 positions upstream that is also conserved in PfPK4. For MAP kinase (mitogen-activated protein kinase) and Cdk2 (cyclin-dependent kinase 2), it has been proposed that phosphorylation of residues in the activation loop changes its conformation, allowing substrate to bind. It is tempting to speculate that autophosphorylation of the residues in the activation loop of TgIF2K-A may contribute to a conformational change, leading to the activation of this eIF2 kinase in response to *Toxoplasma* stress.

TgIF2K-A is most closely related to PfPK4 in *Plasmodium* [19], although the N-terminal sequences appear to be quite divergent, suggesting distinct regulatory mechanisms. These novel eIF2 kinases appear to be restricted to apicomplexan parasites. Phosphorylation of TgIF2α in response to alkaline and heat stress, conditions known to induce parasite differentiation *in vitro*, suggests a role for TgIF2K-A in stress remediation and possibly in the induction of a gene expression program required for life cycle change.

Components of the yeast nutrient stress pathway are conserved in *Toxoplasma*

In our bioinformatics analyses, we found no evidence for PKR, PEK/Perk or HRI in *Plasmodium* or *Toxoplasma*. However,

P. falciparum appears to express a GCN2 orthologue (chr14.phat_416). This eIF2 kinase is associated with nutritional stress, and the predicted *P. falciparum* GCN2 has the hallmark feature of a regulatory domain similar to the histidyl-tRNA synthetases, which is important for binding uncharged tRNA that accumulates in response to amino acid starvation. To date, we find no evidence of such a homologue in the *Toxoplasma* sequence databases. However, GCN1 and GCN20 appear to be present in *Toxoplasma*, as evidenced by ToxoDB Release 2.2 entries Tgg_4107 and Tgg_4586 respectively. Essential for the activation of GCN2 in yeast, GCN1 and GCN20 are proposed to deliver uncharged tRNA to GCN2 in the context of the A site of the ribosome [34–36]. The presence of GCN1 and GCN20 in *Toxoplasma* supports conserved regulation of GCN2, and perhaps it has simply not yet been identified, despite 10× sequencing coverage of the parasite genome. Alternatively, GCN1 and GCN20 may carry out translation functions independently of GCN2 in *Toxoplasma*, or a unique eIF2 kinase such as TgIF2K-A may carry out some functions in this parasite analogous to GCN2.

Stress and *Toxoplasma* translational control

This study demonstrates for the first time that stress conditions in parasites can induce eIF2α phosphorylation. In addition to eIF2α, analysis of the *Toxoplasma* genome sequence indicates that this parasite has probable orthologues for eIF2β (Tgg_3970) and eIF2γ (Tgg_4227). The presence of each of the three subunits of eIF2 indicates that this translation factor has a conserved role in the delivery of initiator tRNA to the *Toxoplasma* translation apparatus. Phosphorylation of eIF2α has been shown to reduce the guanine nucleotide exchange activity of mammalian and yeast eIF2B, contributing to global and gene-specific translational control. The eIF2B in yeast and mammals is a five-subunit complex that provides for the catalytic and regulatory features of the exchange factor [37–39]. Encoded within the *Toxoplasma* genome sequence are predicted orthologues of eIF2B subunits, including ε (GCD6) that is critical for exchange activity, δ (GCD2) and α (GCN3). The α subunit is noteworthy, given that it is the only subunit of yeast eIF2B that is dispensable for *in vivo* exchange activity, but is essential for inhibition of eIF2B by phosphorylated eIF2 [37]. These sequence relationships between *Toxoplasma* and other well-characterized mammalian and yeast model systems support the conservation of the translational control mechanism in stressed *Toxoplasma*.

Phosphorylation of eIF2α induces translational expression of bZIP proteins GCN4 and ATF4 in yeast and mammalian cells respectively. However, in *Toxoplasma* and other Apicomplexa genomes, we have not identified any *bona fide* bZIP proteins. Interestingly, apicomplexans do harbour the histone acetyltransferase GCN5 [40,41] and an SWI/SNF homologue [42], components of complexes that are recruited by GCN4 in yeast [43,44]. We propose that apicomplexan parasites possess a transcriptional activator analogous to the master regulator, yeast GCN4, which bridges the communication between stress-induced translational regulation and alterations in the expressed genome, as depicted in the model shown in Figure 8. Expression of genes required for stress remediation could also function in bradyzoite differentiation. It is interesting to note that in *Aspergillus nidulans* the GCN2 orthologue and the related GCN4 transcription factor, CPCA, function in a stress response pathway that blocks formation of cleistothecia, or fruit bodies, when nutrients are limiting [45]. Formation of these complex reproductive structures consumes macromolecules and energy, and induction of CPCA expression signals that this fungi lacks adequate nutrients to carry out this complex process. A GCN2 orthologue is also involved in triggering a developmental programme involving formation of fruiting bodies in response to nutrient starvation in *Dictyostelium* [46]. Considered with the data we present here, these studies highlight the intimate relationship between stress induction of eIF2 kinases and differentiation.

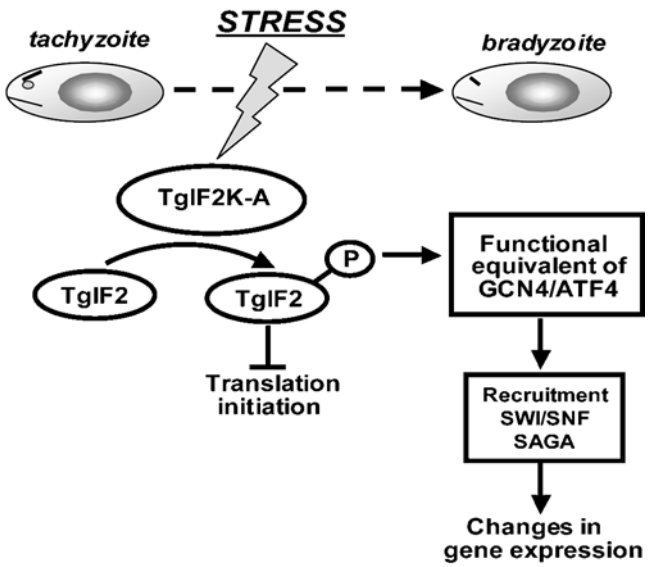

Figure 8 Model for stress-induced phosphorylation of eIF2 in *Toxoplasma*

The stress conditions that drive the differentiation of tachyzoites to bradyzoites induce phosphorylation of TgIF2α. Phosphorylation of TgIF2α is proposed to trigger changes in gene expression both at the translational and transcriptional levels. Although no bZIP transcriptional activators such as GCN4 exist in *Toxoplasma*, we propose an analogous transcriptional regulator functions to recruit co-activators such as SWI/SNF and GCN5 in response to cellular stress.

Stress in *Toxoplasma* induces TgIF2α phosphorylation

In addition to eIF2α phosphorylation, exposing tachyzoites to high temperatures or alkaline pH promotes differentiation to bradyzoites *in vitro* [20,23]. Our observation that parasites phosphorylate TgIF2α under such conditions is an important example of eIF2α being associated with pH stress. In addition to these stress events, there are other treatments that induce parasite differentiation, including chemical stress (sodium arsenate), γ-interferon and mitochondrial inhibitors [5]. Whether TgIF2α is phosphorylated under these conditions remains to be determined. Since the maintenance and reactivation of bradyzoites is critical to pathogenesis, the identification of factors modulating stage conversion remains a primary focus of research. The correlation of TgIF2α phosphorylation with parasite stress, and the identification of a candidate eIF2 kinase representing a divergent novel family member, opens the door to a new facet of parasite biology that may be exploited in drug design.

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