EUKARYOTIC CELL, Nov. 2007, p. 1979–1991
Vol. 6, No. 11
1535-9778/07/$8.00+0
doi:10.1128/EC.00249-07
Copyright © 2007, American Society for Microbiology. All Rights Reserved.

Novel Membrane-Bound eIF2α Kinase in the Flagellar Pocket of Trypanosoma brucei

Maria Carolina S. Moraes, 1 Teresa C. L. Jesus, 1 Nilce N. Hashimoto, 1 Madhusudan Dey, 2 Kevin J. Schwartz, 3 Viviane S. Alves, 1 Carla C. Avila, 1 James D. Bangs, 3 Thomas E. Dever, 2 Sergio Schenkman, 1 and Beatriz A. Castilho 1* 4

Departamento de Microbiologia, Imunologia e Parasitolgia, Universidade Federal de São Paulo, São Paulo, Brazil 1; Laboratory of Gene Regulation and Development, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, Maryland 2; and Department of Medical Microbiology and Immunology, University of Wisconsin School of Medicine and Public Health, Madison, Wisconsin 3

Received 11 July 2007/Accepted 3 September 2007

Translational regulation plays a pivotal role in stress adaptation programs in eukaryotes, from yeast to mammals (10). Little is known about translational regulation in trypanosomatids, an early diverging group of organisms in the eukaryotic lineage. Trypanosomas encompass important human pathogens, display differentiation processes elicited by contact with the distinct physiological milieu found in their insect vectors and mammalian hosts, likely representing stress situations. Trypanosoma brucei, the agent of African trypanosomiasis, encodes three potential eIF2α kinases (TbeIF2K1 to -K3). We show here that TbeIF2K2 is a transmembrane glycoprotein expressed both in procyclic and in bloodstream forms. The catalytic domain of TbeIF2K2 phosphorylates yeast and mammalian eIF2α at Ser51. It also phosphorylates the highly unusual form of eIF2α found in trypanosomatids specifically at residue Thr169 that corresponds to Ser51 in other eukaryotes. T. brucei eIF2α, however, is not a substrate for GCN2 or PKR in vitro. The putative regulatory domain of TbeIF2K2 does not share any sequence similarity with known eIF2α kinases. In both procyclic and bloodstream forms TbeIF2K2 is mainly localized in the membrane of the flagellar pocket, an organelle that is the exclusive site of exo- and endocytosis in these parasites. It can also be detected in endocytic compartments but not in lysosomes, suggesting that it is recycled between endosomes and the flagellar pocket. TbeIF2K2 location suggests a relevance in sensing protein or nutrient transport in T. brucei, an organism that relies heavily on posttranscriptional regulatory mechanisms to control gene expression in different environmental conditions. This is the first membrane-associated eIF2α kinase described in unicellular eukaryotes.

Translational control mediated by phosphorylation of the alpha subunit of the eukaryotic initiation factor 2 (eIF2α) is central to stress-induced programs of gene expression. Trypanosomatids, important human pathogens, display differentiation processes elicited by contact with the distinct physiological milieu found in their insect vectors and mammalian hosts, likely representing stress situations. Trypanosoma brucei, the agent of African trypanosomiasis, encodes three potential eIF2α kinases (TbeIF2K1 to -K3). We show here that TbeIF2K2 is a transmembrane glycoprotein expressed both in procyclic and in bloodstream forms. The catalytic domain of TbeIF2K2 phosphorylates yeast and mammalian eIF2α at Ser51. It also phosphorylates the highly unusual form of eIF2α found in trypanosomatids specifically at residue Thr169 that corresponds to Ser51 in other eukaryotes. T. brucei eIF2α, however, is not a substrate for GCN2 or PKR in vitro. The putative regulatory domain of TbeIF2K2 does not share any sequence similarity with known eIF2α kinases. In both procyclic and bloodstream forms TbeIF2K2 is mainly localized in the membrane of the flagellar pocket, an organelle that is the exclusive site of exo- and endocytosis in these parasites. It can also be detected in endocytic compartments but not in lysosomes, suggesting that it is recycled between endosomes and the flagellar pocket. TbeIF2K2 location suggests a relevance in sensing protein or nutrient transport in T. brucei, an organism that relies heavily on posttranscriptional regulatory mechanisms to control gene expression in different environmental conditions. This is the first membrane-associated eIF2α kinase described in unicellular eukaryotes.

Translated region sequences (16, 34, 48). General translation may be regulated during the differentiation process in T. brucei, as indicated by drastic alterations in polysome profiles (5).

One of the most conserved stress-activated regulatory pathways in eukaryotes involves the inhibition of protein synthesis through the phosphorylation of the alpha subunit of the translation initiation factor 2 (eIF2α) by a family of protein kinases that are activated by specific signals (10). eIF2, bound to GTP, delivers the initiator methionyl tRNA to the 40S ribosomal subunit in each round of translation initiation. For the formation of the 80S complex at the AUG initiator codon, GTP is hydrolyzed to GDP, with the release of eIF2-GDP. For a new cycle of initiation, GDP must be exchanged to GTP, in a process requiring the guanine exchange factor eIF2B. The phosphorylated form of eIF2 (eIF2α-P) is a poor substrate for eIF2B but has a much higher affinity for eIF2B than the unphosphorylated eIF2-GDP, acting as a competitive inhibitor.

Whereas high levels of eIF2α-P block translation, moderate levels of eIF2α-P, while still allowing protein synthesis to take place, lead to translational activation of specific messages, such as GCN4 in Saccharomyces cerevisiae (25) and ATF4 in mammals (50). These messages contain upstream open reading frames (uORFs) in their leader sequences that inhibit the scanning ribosome from reaching the main ORF. Upon a reduction in the amounts of eIF2-GTP, as a result of the phosphorylation of eIF2α, the ribosomes bypass the uORFs, and translate the main ORF. GCN4 and ATF4 are both bZIP transcriptional activators that regulate downstream responses
aimed at alleviating the initial stress condition. GCN4 activates hundreds of genes involved in amino acid biosynthesis and intermediary metabolism (37). ATF4, besides regulating amino acid metabolism, also participates in other stress remedial programs (22). Thus, eIF2α phosphorylation can provide a means for both general repression of protein synthesis, as well as gene-specific translational activation.

GCN2, the sole eIF2α kinase in *S. cerevisiae*, is activated by amino acid deprivation and low levels of glucose or purine (24, 51). Mammals have three other eIF2α kinases in addition to GCN2: HRI, activated by the lack of heme; PKR, activated by double-stranded RNA and cytopotoxic stresses; and PEK/PERK, an ER transmembrane protein, the regulatory synthetase (HisRS domain) promotes its activation (36, 40). In each type of eIF2α kinase, the regulatory domain is located in the lumen of the ER which is normally bound to BiP; when unfolded proteins accumulate in the ER, BiP is released from the regulatory domain, which then dimerizes the protein, activating the cytoplasmic kinase domain (4, 32).

Activation of these kinases is mediated by dimerization and autophosphorylation of specific residues in the catalytic region. This phosphorylation event allows the binding of the substrate (15). Activation is controlled by regulatory domains specific to each type of eIF2α kinase. For GCN2, the binding of uncharged tRNAs to a region with similarity to histidinyl tRNA synthetase (HisRS domain) promotes its activation (36, 40). In PEK/PERK, an ER transmembrane protein, the regulatory domain located in the lumen of the ER is normally bound to BiP; when unfolded proteins accumulate in the ER, BiP is released from the regulatory domain, which then dimerizes the protein, activating the cytoplasmic kinase domain (4, 32).

Given the relevance of eIF2 signaling in stress remedial programs in all eukaryotes, and the obvious relevance of post-transcriptional regulation in trypanosomatids, we approached this regulatory pathway in *T. brucei*. Here we describe the characterization of an eIF2α kinase of *T. brucei* that is a membrane-associated protein localized at the flagellar pocket. The data presented here suggest that this kinase has an important role in the yeast strain described by the manufacturer (Invitrogen). The putative regulatory domains of the kinase were cloned by using the oligonucleotides BC215F (GGGGGATCCTTTTATGAGGAAAAGTTACCA) and BC437 (CTCCGCATCTTTCTTCTCCG) for *TbeIF2K*1065-1236, BC438 (GAATTCATGCCACAACTCTTTC) and BC441 (GAGCCTTTATATGGATAGATG) for *TbeIF2K*235-449, and BC446 (GGATCCATGCCACAACTCTTTC) and BC450 (CCGAGTGATATGGATAGATG) for *TbeIF2K*31-476. The amplified products were transferred to the plasmid pET28a (+) plasmid linearized with SacI-NotI, generating plasmid pBE520. The Scl-AbN-NotI fragment of pBE505 was cloned into the psiT28a (+) plasmid linearized with Scl-AbN-I, generating plasmid pBE587.

The kinase domain of *TbeIF2K* (residues 618 to 1000) was amplified with oligonucleotides BC442 (GGATCCTAGCCAAAACCTTTC) and BC445 (GAATTCAATGTGCTACGCG) and PCR products were cloned by using a ClaI site common to both fragments and introducing it into the yeast strain described by the manufacturer (Invitrogen). The putative regulatory domains of the kinase were cloned by using the oligonucleotides BC436 (AAGCTTTTTTATGAGGAAAAGTTACCA) and BC437 (CTCCGCATCTTTCTTCTCCG) for *TbeIF2K*1065-1236, BC438 (GAATTCATGCCACAACTCTTTC) and BC441 (GAGCCTTTATATGGATAGATG) for *TbeIF2K*235-449, and BC446 (GGATCCATGCCACAACTCTTTC) and BC450 (CCGAGTGATATGGATAGATG) for *TbeIF2K*31-476. The amplified products were transferred to the plasmid pET28a (+), generating plasmid pBE527.

Total RNA from *T. brucei* was obtained by TRizol extraction as described by the manufacturer (Invitrogen) and further purified by precipitation with 2 M LiCl. RNA was treated with DNase (1 U/µg), and 0.3 µg was used in the reaction with SuperScript One-Step RT-PCR using Platinum Taq (Invitrogen). The oligonucleotides used for the amplification of the sequences corresponding to the *TbeIF2* mRNA were BC338 (AACCCTATATTAGAACGATATTCC) (spliced leader sequence) and BC334 (GGGTTGATGACCTTACCGATG). The amplified products were transferred to the plasmid pET28a (+), generating plasmid pBE587.

Expression and purification of recombinant proteins. The soluble His*-TbeIF2* wild-type and His*-TbeIF2* T205A proteins expressed in *E. coli* BL21(DE3) were purified from cultures induced with 100 µM IPTG (isopropyl-β-D-thiogalactopyranoside) at 23°C overnight on nickel chelating resin as described by the manufacturer (QIAGEN). The putative regulatory domains of the kinases *TbeIF2*K1, *TbeIF2*K2, and *TbeIF2*K3 fused with a N-terminal His tag were expressed in *E. coli* BL21(DE3). The insoluble His*-TbeIF2*K1065-1236 protein was purified from cultures induced with 1 mM IPTG at 37°C for 2 h by preparative sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The insoluble His*-TbeIF2*K235-449 protein was purified from cultures induced with 1 mM IPTG at 23°C overnight on nickel chelating resin as described by the resin manufacturer. The soluble His*-TbeIF2*K1065-1236 protein was purified from cultures induced with 1 mM IPTG at 23°C overnight on nickel chelating resin. The soluble His*-TbeIF2*K235-449 protein was purified from cultures induced with 100 µM IPTG at 37°C for 2 h by preparative sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The insoluble His*-TbeIF2*K235-449 protein was purified from cultures induced with 1 mM IPTG at 23°C overnight on nickel chelating resin. The soluble His*-TbeIF2* was purified from cultures induced with 400 µM IPTG at 23°C overnight on glutathione-Sepharose 4B as described by the resin’s manufacturer (Amersham).
aprotinin/ml–10 μg of pepstatin A/ml–1 μg of antipain/ml–1 mM phenylmethylsulfonyl fluoride–10 mM sodium pyrophosphate–1 mM NaF. After freezing and thawing, the suspension was centrifuged at 10,000 × g for 15 min. The supernatant was kept as soluble material. The pellet was resuspended in 150 μl of the same buffer supplemented with 1% Triton X-100. After centrifugation at 10,000 × g for 15 min, the supernatant was kept as the membrane-enriched fraction. Final insoluble material was solubilized in buffer containing 8 M urea.

Antiseras and immunoblots. Antibodies were obtained in rabbits by immunization with the recombinant proteins purified from E. coli. Monospecific antibodies to His-TbeIF2K235–449 were obtained by adsorption to the purified recombinant His-TbeIF2K235–449 protein immobilized on a nitrocellulose filter, as described previously (39). For immunoblots, membranes were blocked with 5% nonfat milk in double-distilled H2O for 1 h at 23°C, followed by incubation with primary antibodies for 1 h at 23°C or overnight at 4°C. The following conditions were used: (i) anti-TbeIF2 θrolled 1:500 in PBS, (ii) purified anti-TbeIF2K235–449 dilitated 1:500 in PBS, (iii) anti-(mammalian)IF2A (Cell Signaling) diluted 1:100 in Tris-buffered saline (TBS)–0.1% Tween 20–5% bovine serum albumin (BSA), (iv) anti-(yeast)IF2A (23) diluted 1:500 in PBS, (v) anti-IF2α–P (Bio-source) diluted 1:1,000 in TBS–0.1% Tween 20–5% BSA, (vi) anti-GST (laboratory reagent, 1:5,000 in PBS), and (vii) anti-ThiB (2) diluted 1:60,000 in PBS–0.1% Tween 20–5% BSA. After washes with 0.1% Tween 20 in PBS or TBS, bound antibodies were detected by using horseradish peroxidase (HRP), protein A (Amersham Biosciences) diluted 1:4,000 in PBS for anti-TbeIF2α, anti-TbeIF2K235–449, anti-GST, anti-(yeast)IF2A, anti-IF2α–P, and anti-BP and by using HRP-conjugated goat anti-mouse immunoglobulin G (IgG; Santa Cruz Biotechnology, Inc.) diluted 1:4,000 in PBS–0.1% Tween 20–5% BSA for anti-(m)IF2A. The bound antibodies were detected by using enhanced chemiluminescence (Amersham Biosciences). When necessary, the antibodies were stripped off the membranes by incubation with 100 mM β-mercaptoethanol, 2% SDS, and 62.5 mM Tris-HCl (pH 6.7) at 50°C for 30 min.

Immunoprecipitation. Extract (700 μg) of induced J82 yeast strain carrying the plasmid pBE553 (GST-TbeIF2K2547) or pEGST (GST) was preclarified by incubation with 20 μl of protein A-Sepharose CL-4B beads (Amersham Biosciences) and preimmune serum in buffer containing 10 mM Tris-HCl (pH 7.5), 100 mM NaCl, 70 mM glucose [pH 7.5], 1 mM MgCl2, and 1 mM NaF. The supernatant was incubated for 2 h at 4°C with 20 μl of protein A-Sepharose beads preincubated with anti-GST antibodies. The beads were washed three times with the same buffer, and the material bound to the beads was used in the in vitro kinase assays.

In vitro phosphorylation assays. Kinase assays using purified PKR or GCN2 were performed as described previously (13). Phosphorylation reactions using the immunoprecipitated GST-TbeIF2K2547 contained 30 μCi of [γ-32P]ATP in buffer containing 4 mM Tris-HCl (pH 7.5), 100 mM NaCl, 70 mM glucose [pH 7.5], 2 mM magnesium acetate in a volume of 30 μl. Reactions were allowed to proceed for 1 h at 23°C. Proteins were denatured in the absence of β-mercaptoethanol (to avoid the comigration of the IgG heavy chain and TbeIF2α), resolved by SDS-PAGE, and stained with Coomassie blue; the incorporation of radioactive phosphate into TbeIF2α was detected on a Typhoon phosphorimager.

Tomato lectin uptake and localization studies. Cultured bloodcell cultures were washed with HEPES buffered-saline (50 mM HEPES, 50 mM NaCl, 5 mM KCl, 70 mM glucose [pH 7.5]) and resuspended at 104/ml in HEM9-BSA (serum-free HM9 medium supplemented with 0.5 μg of bovine serum albumin/ml) containing tomato lectin-conjugate (TL; Vector Laboratories, Burlingame, CA) at 20 μg/ml in PBS. Cells were incubated at 5°C for 15°C for 30 min, followed by extensive washing with PBS. Washed cells were lightly prefixed with 0.1% formaldehyde and then fixed onto slides with methanol-acetone as described previously (1). Cells were then stained with Alexa 633-streptavidin (Molecular Probes, Eugene, OR) to detect both internalized TL at the same time cells were also stained with rabbit anti-TbeIF2K2 and/or monoclonal anti-p67 (1) to detect the lysosome. Appropriate Alexa 488- and Alexa 633-conjugated goat anti-IgG (Molecular Probes) were used as secondary antibodies. Cells were also stained with 500 ng of DAPI (4′,6′-diamidino-2-phenylindole/ml) Serial image stacks (0.2-μm Z-increment) were collected at 100× (PlanApo oil immersion 1.4 NA) on a motorized Zeiss Axioskop II equipped with a rear-mounted excitation filter wheel, a triple-pass (DAPI-fluorescein isothiocyanate-Texas Red) emission cube, differential interference contrast optics, and a Orca AG charge-coupled device camera (Hamamatsu, Bridgewater, NJ). All images were collected with OpenLab 5.0 software (Improvision, Inc., Lexington, MA), and fluorescence images were deconvolved by using a constrained iterative algorithm, pseudocolored, and merged by using Velocity 4.0 software (Improvision).

Dephyosphorylation and deglycosylation assays. A total of 5 μg of total protein from the membrane-enriched fraction was incubated with 20 U of calf intestinal alkaline phosphatase (Promega) in buffer containing 50 mM Tris-HCl (pH 9.3 at 25°C), 1 mM MgCl2, 100 μM ZnCl2, and 1 mM spermidine in a volume of 10 μl at 37°C for 3 h. Deglycosylation was performed with the membrane-enriched protein fraction (20 μg) of procyelic parasites, denatured at 100°C for 5 min in buffer containing 50 mM sodium phosphate (pH 5.5), 0.1% SDS, and 50 mM β-mercaptoethanol. After incubation on ice for 10 min, endoglycosidase H (Sigma) was added to the mixture, and the reaction was allowed to proceed for 18 h at 37°C.

RESULTS

Trypanosomatids encode three potential eIF2α kinases. Sequences for three potential eIF2α kinases are found in the genome of T. brucei, with similar counterparts in other two trypanosomatids, Trypanosoma cruzi and Leishmania major (3, 15, 26). We named these proteins TbeIF2K1 through TbeIF2K3. The alignment of the catalytic kinase domains (KD) of the T. brucei proteins and the known eIF2α kinases is shown in Fig. 1. The three proteins contain an insert between domains IV and V, typical of eIF2α kinases (21). The inserts range from 46 residues in TbeIF2K2 to 119 in TbeIF2K3. These sizes are within the range found for the eIF2α kinases, of which the extreme is the T. gondii TgKA, composed of 615 residues. TbeIF2K3 has another large insert located between domains VIB and VII, which is unusual for this class of kinases. An interesting feature of the T. brucei proteins is the site of putative autophosphorylation. As indicated by the boxed arrow in Fig. 1, TbeIF2K2 has a serine residue where all other kinases have a threonine residue. Phosphorylation of this residue in PKR promotes the recognition of the substrate (13). The presence of the serine residue in TbeIF2K2 was confirmed by DNA sequencing. Residue T487 of PKR, indicated with a boxed arrowhead, is an important determinant of specificity for recognition of the substrate. This residue is within an α-helical domain that in eIF2α kinases is longer than in other class of kinases. This extended configuration, along with its orientation, determines the substrate specificity. This threonine is found in TbeIF2K2 and TbeIF2K3 but not in TbeIF2K1. The remaining residues that are typical of this class of kinases are for the most part conserved in the T. brucei proteins, as indicated by the arrowheads in the alignment of Fig. 1. Figure 2 depicts the putative regulatory domains of the three T. brucei kinases relative to their catalytic regions. TbeIF2K1 is apparently a GCN2 ortholog, containing a ring finger, WD repeat domain (RWD) and a HisRS-like domain, which in yeast GCN2 are involved in GCN1 and uncharged tRNA binding, respectively. The HisRS-like region of TbeIF2K1 was detected by the Phyre prediction engine (www.sbg.bio.ic.ac.uk/phyre). The domain architecture is similar to GCN2, with the RWD and HisRS-like regions flanking the kinase domain. No pseudokinase region was detected in TbeIF2K1. TbeIF2K2 and TbeIF2K3 putative regulatory domains have no similar counterparts in any database, except the other trypanosomatids orthologs. TbeIF2K2 has a predicted N-terminal signal sequence and a potential transmembrane domain, suggesting association with membranes and with a topological similarity to PEK/PERK, i.e., a type I transmembrane protein with a C-terminal cytosolic kinase domain and a large N-terminal ectoplasmic domain.

Reverse transcription-PCR (RT-PCR) indicated that the mRNAs encoding for the three proteins are present in both bloodstream and procyclic forms of the parasite (Fig. 2B).
Antibodies were then raised against the putative regulatory regions of the three proteins. Although for TbeIF2K1 and TbeIF2K3 the antisera recognized the recombinant proteins, no clear signal was detected in immunoblots of whole-cell extracts of the parasites (data not shown). It is possible that these are very low abundance proteins or that they are only translated under specific conditions not tested here. For TbeIF2K2, the antiserum recognized a protein with an apparent molecular mass of approximately 130 to 140 kDa, slightly larger than expected (111 kDa), suggesting that it might be glycosylated (Fig. 2C). Consistent with this observation, the putative ectoplasmic domain (residues 1 to 464) contains four potential Asn-X-Ser/Thr N glycosylation sites (Asn63, -165, -217, and -444). Given the suggestive membrane localization of this protein, which would be unique among eIF2/H9251 kinases in unicellular eukaryotes, we focused this work on the characterization of TbeIF2K2.

TbeIF2K2 is localized to flagellar pocket and endosomal membranes. Indication of the membrane-bound nature of TbeIF2K2 was obtained by partial fractionation of cell extracts. Parasites were lysed in buffer lacking detergents, and the insoluble material was extracted in the same buffer containing 1% Triton X-100. As shown in Fig. 3A, virtually all TbeIF2K2 partitioned to the detergent-solubilized material. As a control, we probed the lower part of the same nitrocellulose membrane with antibodies directed against TbeIF2/H9251, a cytoplasmic protein (see below). Endoglycosidase H treatment of the membrane enriched fraction resulted in a decrease in size of TbeIF2K2, confirming the presence of N-glycans and thus its import and transit through the ER (Fig. 3B).

Immunofluorescence analysis of T. brucei cells using affinity-purified antibodies (Fig. 4A) showed that TbeIF2K2 is highly concentrated in a structure near the kinetoplast in both procyclic and bloodstream parasites, a finding suggestive of the flagellar pocket (Fig. 4B and Fig. 5). Preincubation of the antibodies with the purified recombinant protein abolished the signal, ascertaining the specificity of the reaction (Fig. 4B, bottom panels). We then performed a colocalization analysis with TL, which labels exclusively the flagellar pocket of bloodstream when intact cells are incubated at 5°C to block endocytosis (6, 41) (Fig. 5). The anti-TbeIF2K2 signal for the most part overlapped the TL spot, as shown in panels A to D, confirming the flagellar pocket localization, although weak internal labeling associated with the lysosomal marker p67 was found in some cells (panels E to H, small arrow). Seen more frequently, however, was a strong independent TbeIF2K2 signal.
in the region between the flagellar pocket and lysosome suggestive of partial endosomal localization (Fig. 5E to H, arrowhead). To investigate this possibility, we performed a TL uptake experiment by incubation of the cells at 15°C. At this temperature, TL is internalized into endosomes but subsequent delivery to the lysosome is minimized (6). Under these conditions TbeIF2K2 again colocalized prominently with TL in the flagellar pocket, but a strong colocalization was also apparent in an internal tubular endosome compartment characteristic of the low temperature block (Fig. 5I to L and M to P). This tubular compartment did not stain with anti-p67 consistent with a block in endosome-to-lysosome trafficking (Fig. 5Q to T). These localization data taken together indicate that TbeIF2K2 is in the membrane of the flagellar pocket and that it can be recycled through the endocytic pathway.

The kinase domain of TbeIF2K2 phosphorylates yeast and mammalian eIF2α. Given the unusual localization of this protein and the sequence divergence relative to other known eIF2α kinases, we studied the substrate specificity of TbeIF2K2. The putative kinase domain comprising residues 618 to 1009 (TbeIF2K2KD) was expressed in yeast as a fusion to GST, under the control of the galactose-inducible GAL1 promoter. Dimerization of eIF2α kinases is a requirement for their activation, and fusion of the kinase domain to heterologous dimerization domains, such as GST, results in a constitutively active protein, as shown for PKR and PEK/PERK (4, 47). Expression of GST-TbeIF2K2KD in strain J80, which contains the wild-type yeast eIF2α protein and lacks the chromosomal copy of GCN2, resulted in the complete inhibition of...
growth on medium containing galactose (Fig. 6A). When the same protein was expressed in the isogenic strain J82, in which the serine 51 residue of eIF2α is replaced by alanine, not a target for the eIF2α kinases, the cells were capable of growing on galactose (Fig. 6A). The expression of GST-TbeIF2K2KD in the J82 cells is shown in a Western blot with anti-GST antibodies (Fig. 6B). Direct evidence for the phosphorylation of eIF2α by TbeIF2K2 in strain J80 was obtained by immunoblotting total cell extracts, prepared after induction with galactose, using antibodies that specifically recognize the phosphorylated form of eIF2α (eIF2α-P) (Fig. 6C). Thus, TbeIF2K2 phosphorylates yeast eIF2α specifically at S51, therefore inhibiting translation and growth. The efficiency of phosphorylation of eIF2α by TbeIF2K2 was compared to that of PKR in in vitro phosphorylation assays. GST-TbeIF2K2KD, immunoprecipitated from the yeast J82 cell extract, and GST-PKRKD, purified from E. coli, were incubated with mammalian eIF2α purified from E. coli, and the reactions were analyzed by immunoblots with anti-eIF2α-P antibodies and normalized with the levels of total eIF2α (Fig. 6D). The amounts of the two kinases were equivalent, as judged from probing the upper portion of the membrane with anti-GST antibodies. These experiments, together with the in vivo results in yeast, indicated that TbeIF2K2 has the same ability as PKR to recognize and phosphorylate yeast or mammalian eIF2α, even though there are marked sequence divergences in the regions of these kinases that have been implicated in autophosphorylation and substrate recognition.

**TbeIF2K2 phosphorylates TbeIF2α at T169.** Inspection of the trypanosomatid genomic database revealed an ortholog of eIF2α with conserved features with other known eIF2α sequences, as shown in the alignment in Fig. 7. Interestingly, however, in place of the residue corresponding to S51 that is phosphorylated in all eukaryotes, trypanosomatid eIF2α has a threonine residue. In addition, we noticed that the AUG codon assigned as the initiator for the three inspected trypanosomatid sequences (T. brucei, T. cruzi, and L. major) was not conserved. To ascertain the sequence of the transcript, we performed RT-PCR from total mRNA of T. brucei using as a forward primer an oligonucleotide corresponding to the spliced leader sequence and as a reverse primer an oligonucleotide complementary to a conserved region, located just after the putative threonine residue (T169) corresponding to S51 of other eukaryotes. Sequencing of this fragment confirmed the threonine residue and the extension in the N-terminal region in the T. brucei eIF2α (TbeIF2α), with no homology to any eukaryotic sequence, except the other trypanosomatids’ orthologs (Fig. 7A). Using the same experimental approach, we determined that these features were also found in the T. cruzi eIF2α protein (data not shown). To determine whether this extended protein was expressed in T. brucei, we raised antibodies against TbeIF2α. The only protein recognized by the antisera has the expected size of the extended eIF2α (Fig. 7B). The difference in size between the endogenous and the recombinant protein used as control is due to the presence of the His tag sequence in the latter.

Given the unusual features of TbeIF2α, we then addressed whether this protein could substitute for the yeast counterpart and be a substrate for the known eIF2α kinases. The complete TbeIF2α sequence was cloned under the control of the GAL1 promoter, in a LEU2 vector. This plasmid was used to transform yeast strain H1643, which has a deletion of the chromosomal copy of the SUI2 gene, encoding eIF2α, and is maintained viable by the presence of the SUI2 gene in a URA3 plasmid. This strain is unable to grow in the presence of 5-fluoroorotic acid (5-FOA), which selects for Ura− cells arising...
from the spontaneous loss of the URA3 plasmid and therefore of the SUI2 gene. As a control, we used a LEU2 plasmid expressing yeast eIF2α from the same promoter. The expression of TbeIF2α did not allow growth in 5-FOA, indicating that TbeIF2α cannot functionally substitute for the yeast eIF2α (Fig. 8A). Because the presence of the N-terminal extension could hinder the function of TbeIF2α in yeast, we performed the same assay using a truncated form of the protein.

**FIG. 5.** Localization of TbeIF2K2 in bloodstream cells. Bloodstream trypanosomes were incubated for 30 min with biotinyl TL at 5°C to allow flagellar pocket binding (A to D and E to H) or at 15°C to allow binding and uptake into the endosomal compartment (I to L, M to P, and Q to T) as described in Materials and Methods. Fixed and permeabilized cells were then stained with fluorescent streptavidin to detect bound or internalized TL (red), and as indicated with purified anti-TbeIF2K2 antibodies (αTbK2) and or anti-p67 (middle panels). (A to H) Cells stained with anti-TbeIF2K2 (green) and anti-p67 (red). The discrete positioning of the anterior lysosome and the posterior flagellar pocket allow simultaneous imaging of these organelles in the same channel. (I to P) Cells stained with anti-TbeIF2K2 (green). (Q to T) Cells stained with anti-p67 (green). Merged DAPI/differential interference contrast images are presented in the leftmost panels with kinetoplast (k) and nucleus (n) labeled, and merged three-channel fluorescent images are presented in rightmost panels. The positions of the lysosome (Lys), endosome (Endo), and flagellar pocket (FP) are indicated. The arrowhead in panel H indicates a region of discrete TbeIF2K2 signal, presumably the endosome, that does not colocalize with the nearby flagellar pocket. Bars, 5 µm.
This truncated protein was functional in yeast, allowing the growth of yeast cells on 5-FOA-containing medium (Fig. 8A). Immunoblots of total cell extracts prepared from independent isolates from the 5-FOA plates showed that TbeIF2α/H9251125-419 was the sole eIF2α present in these cells (Fig. 8B).

We next addressed whether TbeIF2α was a substrate for yeast GCN2 by assessing the growth of these isolates on media containing 3-aminotriazole. The isolates expressing either TbeIF2α/H9251125-419 or TbeIF2α/H9251125-419(T169A) were capable of growth on 3-aminotriazole (data not shown). This is probably due to the formation of a defective ternary complex. Thus, an in vivo assay for GCN2-mediated phosphorylation of TbeIF2α/H9251125-419 was not possible. In vitro phosphorylation assays were then performed with purified TbeIF2α, PKR, and GCN2. The complete TbeIF2α, purified from E. coli as a His tag fusion, was incubated with purified PKR or GCN2 in the presence of [33P]ATP. TbeIF2α was not phosphorylated by GCN2 and only weakly phosphorylated by PKR (Fig. 9). The truncated eIF2α/H9251125-419 protein was not phosphorylated by PKR or GCN2 either (data not shown). To address whether TbeIF2α was a substrate for TbeIF2K2, GST-TbeIF2K2KD immunoprecipitated from extracts of strain J82 grown on galactose, using anti-GST antibodies, and used in in vitro phosphorylation assays with purified TbeIF2α or TbeIF2α/T169A. TbeIF2K2 was capable of phosphorylating T. brucei eIF2α but not the mutant protein TbeIF2α/T169A (Fig. 9). These results clearly show that TbeIF2K2 phosphorylates TbeIF2α specifically at T169. Thus, TbeIF2K2 recognizes yeast, mammalian, and trypanosomatid...
eIF2α, but TbeIF2α is only efficiently recognized by the trypanosomatid kinase.

**Modifications of TbeIF2K2 in bloodstream forms.** Two bands corresponding to TbeIF2K2 can be detected on prolonged runs on SDS–7% PAGE of membrane-enriched fractions from bloodstream parasites obtained under normal growth conditions in well-established medium, whereas only one band is seen for procyclics (Fig. 10A). The procyclic protein migrates in a position intermediary between the two bands observed in bloodstream forms. In analogy to PEK/PERK, which shows a drastic change in migration due to phosphorylation when activated, we reasoned that in the bloodstream forms TbeIF2K2 could be partially activated. To determine whether the slower-migrating protein was phosphorylated, the membrane-enriched fractions were treated with alkaline phosphatase. As shown in Fig. 10, the phosphatase treatment caused a shift of the bloodstream protein to the faster mobility band. In contrast, in procyclics the phosphatase treatment did not alter the mobility of the protein. Thus, in bloodstream forms, a fraction of the TbeIF2K2 protein is phosphorylated under normal in vitro growth conditions. Whether the phosphorylated form of TbeIF2K2 found in bloodstream parasites represents the activated form of the kinase remains to be elucidated.

**Bloodstream forms are highly sensitive to high cell densities in culture.** While pleomorphic strains differentiate from slender to stumpy forms above a critical density, the monomorphic strain MITat 1.2 used in the present study rapidly die (49). During the present study we found that TbeIF2K2 is affected by the density of the culture of bloodstream forms. Membrane and soluble fractions prepared from parasites obtained from culture samples taken at 12-h intervals, starting from a density of 2.4 × 10^6 cells/ml were analyzed in immunoblots for TbeIF2K2. As shown in Fig. 10B, TbeIF2K2 is virtually absent in parasites obtained from the culture in which cell death was evident. As a control, BiP expression was not affected, as shown by probing the same filter with anti-BiP antibodies. These results suggest that TbeIF2K2 may be subject to either downregulation of expression and/or to proteolytic degradation under these conditions.

**DISCUSSION**

We described here two highly unusual components of translational regulation found in *T. brucei* that seem to be conserved in *T. cruzi* and *L. major*. Considering that these organisms represent a deeply rooted branch of the eukaryotic kingdom, it is interesting that trypanosomatids have evolved an eIF2α subunit that is divergent from all other eukaryotes, regarding both the threonine residue in place of Ser51 and the unique N-terminal extension. Perhaps most surprising was the discovery of an eIF2α kinase that is membrane associated and completely constrained to a subcellular structure that is typical of this group of protozoa. Importantly, the data presented here clearly indicated that TbeIF2K2 is a kinase that phosphorylates eIF2α in a highly specific manner. Its substrate range, however, differs from the known eIF2α kinases. Although TbeIF2K2 was capable of phosphorylating yeast, mammalian, and the *T. brucei* eIF2α, neither PKR nor GCN2 efficiently phosphorylated TbeIF2α in vitro. This difference is not due to the presence in TbeIF2α of a threonine in the position corresponding to serine 51 since both PKR and GCN2 can phosphorylate yeast or mammalian eIF2α at a threonine residue in place of Ser51 (31). The specificity of substrate recognition by PKR is given by a contiguous surface on one face of eIF2α comprising the S51 region; the kinase determinants G30, A31, and Met44; and the
invariant peripheral docking site comprised by the KGYID motif, located 28 residues C terminal to the S51 residue (9). Of these, only residues G30 and M44 differ in the trypanosomatids’ protein, being replaced by serine and isoleucine, respectively. Mutations in both G30 and M44 abolish phosphorylation of eIF2α by PKR (14). These substitutions alone could then account for the poor ability of TbeIF2α to function as a substrate for PKR. However, other divergent residues in TbeIF2α in positions that are invariant in other eIF2α in this critical region may also hinder its recognition by PKR. TbeIF2K2 phosphorylation of mammalian, yeast, and T. brucei eIF2α suggests that the substrate-binding sites on this kinase can accommodate a wider range of residues.

Phosphorylation of eIF2α appears to be relevant as a mechanism of regulation of protein synthesis in these parasites as suggested by the existence of three eIF2α kinases in these parasites as suggested by the existence of three eIF2α kinases, one of which we have clearly shown to be a bona fide eIF2α kinase, and by the presence of orthologs of the five subunits of the GTP exchange factor, eIF2B, of which the regulatory subunits α, β, and δ mediate the inhibitory effect caused by increased levels of eIF2α-P in the cells. It is not clear at the moment whether the phosphorylation of TbeIF2α would result in a downstream regulatory signaling cascade, since there is an apparent lack of transcriptional factors of the bZIP type in trypanosomatids that could function as GCN4 or ATF4 (26). It seems then that this signaling may only involve the downregulation of general translation during specific situations. It is possible that proteins with no transcriptional role might be translated from messages containing uORFs when TbeIF2α is phosphorylated.

The predicted domain structure of TbeIF2K2 suggests that it is an integral membrane protein with an N-terminal luminal or ectoplasmic domain and a C-terminal cytoplasmic kinase domain. Our findings that it requires detergent for solubilization and that it is N glycosylated are consistent with this interpretation. Surprisingly, however, given its topological similarity to the metazoan ER kinase PEK/PERK, TbeIF2K2 is prominently localized to the flagellar pocket and closely associated endosomal membranes. The flagellar pocket localization was also confirmed by immunofluorescence assays with different antibodies raised against another region of the protein, encom-
passing the kinase domain (data not shown). The flagellar pocket is the only site where endocytosis and exocytosis occur in these parasites, and all proteins destined to the plasma membrane are directed to the flagellar pocket before reaching the parasite surface (19). Trypanosomes have a dense extracellular surface coat composed of variant surface glycoprotein that restricts macromolecular access to the plasma membrane; thus, the flagellar pocket functions as the primary portal for cross talk with the host. In this regard the predicted topology of TbeIF2K2, with a cytoplasmic kinase domain and a potential extracellular regulatory domain, is very suggestive of a role in sensory signaling. A limited repertoire of transmembrane proteins have been characterized in the general endomembrane system of bloodstream trypanosomes, including the lysosomal marker p67 (1), the endosomal marker membrane-bound acid phosphatase (17), and the invariant surface glycoproteins ISG65/75, which recycle between the endosome and cell surface (7). However, none of these is likely to have a role in sensory signaling. Bloodstream trypanosomes do have a flagellar membrane-associated adenylate cyclase with an architecture analogous to that of TbeIF2K2 and that is primarily localized to the flagellum membrane, but ligands for this potential receptor have never been identified (38).

Just what process may regulate TbeIF2K2 activity is currently uncertain, but its localization in the flagellar pocket suggests as one possibility the endocytic cargo load, which is greatly enhanced in the bloodstream stage of the life cycle (18). TbeIF2K2 is constitutively expressed but has a higher basal phosphorylation state in bloodstream parasites, and perhaps this regulates kinase activity which could, in turn, control endocytosis indirectly through translational attenuation of proteins destined to the cell membrane. Another possibility is that TbeIF2K2 activity is regulated during life cycle differentiation. In natural infections pleomorphic bloodstream parasites differentiate in a density-dependent manner from dividing long slender forms to nondividing short stumpy forms that are preadapted for transmission to the tsetse fly (33). Concomitant

FIG. 9. In vitro phosphorylation of TbeIF2α. Purified His₆-TbeIF2α was used in in vitro reactions with purified GCN2 (left panels) and purified PKR (upper right panels) and with immunoprecipitated GST-TbeIF2K2⁰⁰ for the GCN2 and PKR assays and purified His₆-TbeIF2α-Thr1₆₉Ala for the TbeIF2K2 assay. Exposure times are indicated as “short” or “long.” The total protein was visualized on the gels by Coomassie R250 stain.

FIG. 10. Modifications of TbeIF2K2 in bloodstream parasites. (A) Phosphorylation of TbeIF2K2. Membrane-enriched (mb) and soluble (sol) fractions (5 μg of total protein) of bloodstream (B) and procyclic (P) parasites were subjected to immunoblot with anti-TbeIF2K2 antibodies and with anti-BiP serum (left panels) (BiP partitions to both soluble and membrane enriched fractions). Membrane-enriched fractions from bloodstream (B) and procyclic (P) parasites were treated with calf intestinal alkaline phosphatase (CIAP), and subjected to immunoblot with anti-TbeIF2K2 antibodies (right panel). (B) TbeIF2K2 in high-density cultures of bloodstream forms. Membrane (mb) and soluble (sol) fractions (10 μg of total protein) from bloodstream forms grown to late logarithmic phase (2.4 × 10⁶ cells/ml) and 12 h (2.0 × 10⁶ cells/ml) or 24 h (4.0 × 10⁵ cells/ml) later were subjected to immunoblotting with anti-TbeIF2K2 antibodies (TbK2) or anti-BiP (BiP) serum. The indicated number of cells corresponds to live parasites as determined by their motility at each time point.
with this growth arrest phenotype, levels of protein synthesis drop dramatically (5). This “quorum-sensing” process is stimulated by a parasite-derived small molecule called stumpy induction factor (SIF) (49). Neither SIF nor its receptor have been identified, but the location of TbeIF2K2 and its ability to phosphorylate endogenous eIF2 and thereby regulate translation make it an attractive candidate receptor. The loss of TbeIF2K2 in high-density cultures of monomorphic bloodstream trypanosomes, which are resistant to SIF, may be related as a cause or a consequence to this process.

Another issue raised by our study is how an eIF2α kinase present exclusively in a particularly small area of the cell could regulate general translation. Protein synthesis in T. brucei is not restricted to any region of the cell, since ribosomes are spread in the cytoplasm, as seen in many published electron microscopy analysis. TbeIF2α is also found distributed in the cytoplasm, as judged by immunofluorescence analysis using the antibodies described here (data not shown). We can foresee two possibilities: (i) TbeIF2K2 may regulate localized protein synthesis in the vicinity of the flagellar pocket, or (ii) activated TbeIF2K2 may change cellular localization, for example, by being internalized via endocytic vesicles, with the catalytic domain thus gaining access to a large pool of cytoplasmic eIF2α.

This latter mechanism could perhaps account for the fraction of TbeIF2K2 found in endosomes and that found phosphorylated in bloodstream cells.

The N-terminal ectoplasmic domain of TbeIF2K2 that may serve a regulatory function is conserved in the orthologs of other trypanosomatids. It shares 31 and 24% identity (45 and 38% similarity) with eIF2K2 from T. cruzi and L. major, respectively. The T. cruzi protein is very similar to TbeIF2K2, containing a signal sequence at its N terminus and a transmembrane region in the exact same position as in the T. brucei protein. For the Leishmania protein, although clearly similar to TbeIF2K2 within the regulatory region, there are several insertions relative to TbeIF2K2. In addition, the signal sequence starts at residue 40, which may not qualify it for directing the protein to the membrane. It will certainly be interesting to localize the eIF2K2 proteins in the cells of both T. cruzi and L. major since these parasites seem to present slightly distinct exo- and endocytic systems relative to the better characterized membrane network described for T. brucei. The putative regulatory N-terminal domain does not show any sequence similarity with Pek/Perk or with any other known or predicted protein, including those of other parasites such as Plasmodium, Toxoplasma, Trichomonas, and Giardia. These observations taken together indicate that this kinase evolved only in the trypanosomatid lineage, but within it, it has somewhat diverged, perhaps reflecting differences in life cycles and cell biology.

ACKNOWLEDGMENTS

We thank Ronald Wek (University of Indiana) for helpful discussions. This study was supported by grants from Fundação de Amparo à Pesquisa no Estado de São Paulo (FAPESP) to B.A.C. and to S.S. and by National Institutes of Health grants AI056866 and AI35739 to J.D.B. M.C.S.M and T.C.L.J. were supported by doctoral fellowships, and N.N.H. and V.S.A. were supported by postdoctoral fellowships from FAPESP.
transmembrane eIF2α kinase in T. brucei

17. Engstler, M., F. Weise, K. Bopp, G. C. Grunfelder, M. Gunzel, N. Hedd- gott, and P. Overath. 2005. The membrane-bound histidine phosphatase ThMBAP1 is essential for endocytosis and membrane recycling in Trypanosoma brucei. J. Cell Sci. 118:2105–2118.

18. Field, M. C., and M. Carrington. 2004. Intracellular membrane transport systems in Trypanosoma brucei. Traffic 5:905–913.

19. Gull, K. 2003. Host-parasite interactions and trypanosome morphogenesis: a flagellar pocketful of goodies. Curr. Opin. Microbiol. 6:365–370.

20. Han, A. P., C. Yu, L. Lu, Y. Fujitake, C. Brown, G. Chui, M. Flemming, P. Leboulich, S. H. Okin, and J. J. Chen. 2001. Heme-regulated eIF2α kinase (HRI) is required for translational regulation and survival of erythroid precursors in iron deficiency. EMBO J. 20:6999–6918.

21. Hanks, S. K., and T. Hunter. 1995. Protein kinases: the eukaryotic protein kinase superfamily: catalytic domain structure and classification. FASEB J. 9:576–596.

22. Harding, H. P., Y. Zhang, H. Zeng, I. Novoa, P. D. Lu, M. Calfon, N. Sadri, C. Schauer, J. Schein, R. Paules, D. F. Stojdl, J. C. Bell, T. Hettmann, J. M. Leiden, and D. Ron. 2003. An integrated stress response regulates amino acid metabolism and resistance to oxidative stress. Mol. Cell 11:619–633.

23. Hashimoto, N. N., L. S. Carnevalli, and B. A. Castilho. 2002. Translation initiation at non-AUG codons mediated by weakened association of eukaryotic initiation factor 2 (eIF2) subunits. Biochem. J. 367:359–368.

24. Hinnebusch, A. G. 1997. Translational regulation of yeast GCN4. A window on factors that control initiation-tRNA binding to the ribosome. J. Biol. Chem. 272:21661–21664.

25. Hinnebusch, A. G. 1993. Gene-specific translational control of the yeast GCN4 gene by phosphorylation of eukaryotic initiation factor 2. Mol. Microbiol. 10:215–223.

26. Ivens, A. C., C. C. Peacock, E. A. Worthington, L. Murphy, G. Aggarwal, M. Berriman, E. Sikela, M. A. Rajandream, E. Adlem, R. Aert, A. Anupama, Z. Apostolou, P. Attipoe, N. Bason, C. Bauser, A. Beck, S. M. Beverley, G. Bothe, C. V. Bruschi, M. Collins, E. Cadag, L. Ciaroni, C. Clayton, R. M. Coulson, A. C. Koo, R. R. Davies, J. De Gaudenzi, B. E. Dobson, A. Duesterhoft, G. Fazelina, N. Fosker, A. C. Frasch, A. Fraser, M. Fuchs, C. Gabel, A. Goble, A. Goaiffe, D. Harris, C. Hertz-Fowler, H. Hilbert, D. Horn, Y. Huang, S. Klages, A. Knights, M. Kube, N. Larke, L. Litvin, A. Lord, T. Louie, M. Marra, D. Masuy, K. Mottram, S. Muller-Auer, H. Munden, S. Nelson, H. Norbertczak, K. Oliver, S. O'Neill, M. Pentyon, T. Mohl, C. Price, B. Purnelle, M. A. Quail, E. Rabbinowitsch, R. Reinhardt, M. Rieger, J. Rinta, J. Robben, L. Robertson, J. C. Ruiz, S. Rutter, D. Saunders, M. Sherman, F. Sherman, C. M. Pereira, E. Sattlegger, H. Y. Jiang, B. M. Longo, C. A. Jaqueta, A. G. Vattem, B. N. Bauer, T. E. Dever, J. J. Chen, and R. C. Wek. 2004. An integrated stress response regulates amino acid starvation in mammalian cells. Biochem. J. 376:561–576.

27. Ivens, A. C., C. C. Peacock, E. A. Worthington, L. Murphy, G. Aggarwal, M. Berriman, E. Sikela, M. A. Rajandream, E. Adlem, R. Aert, A. Anupama, Z. Apostolou, P. Attipoe, N. Bason, C. Bauser, A. Beck, S. M. Beverley, G. Bothe, C. V. Bruschi, M. Collins, E. Cadag, L. Ciaroni, C. Clayton, R. M. Coulson, A. C. Koo, R. R. Davies, J. De Gaudenzi, B. E. Dobson, A. Duesterhoft, G. Fazelina, N. Fosker, A. C. Frasch, A. Fraser, M. Fuchs, C. Gabel, A. Goble, A. Goaiffe, D. Harris, C. Hertz-Fowler, H. Hilbert, D. Horn, Y. Huang, S. Klages, A. Knights, M. Kube, N. Larke, L. Litvin, A. Lord, T. Louie, M. Marra, D. Masuy, K. Mottram, S. Muller-Auer, H. Munden, S. Nelson, H. Norbertczak, K. Oliver, S. O'Neill, M. Pentyon, T. Mohl, C. Price, B. Purnelle, M. A. Quail, E. Rabbinowitsch, R. Reinhardt, M. Rieger, J. Rinta, J. Robben, L. Robertson, J. C. Ruiz, S. Rutter, D. Saunders, M. Sherman, F. Sherman, C. M. Pereira, E. Sattlegger, H. Y. Jiang, B. M. Longo, C. A. Jaqueta, A. G. Vattem, B. N. Bauer, T. E. Dever, J. J. Chen, and R. C. Wek. 2004. An integrated stress response regulates amino acid starvation in mammalian cells. Biochem. J. 376:561–576.