Intersubunit and Interprotein Interactions of Bacterially Expressed Human eIF2 Subunits and the Importance of eIF2α Phosphorylation in Apoptosis

Thesis submitted for the degree of

Doctor of Philosophy

By

Rajesh Kamindla

(Regd No. 02LBPH08)

Department of Biochemistry

School of Life Sciences

University of Hyderabad

Hyderabad -500 046, Andhra Pradesh

INDIA

Department of Biochemistry
School of Life Sciences
University of Hyderabad
Hyderabad 500 046, Andhra Pradesh, India

DECLARATION

I hereby declare that the work presented in this thesis entitled **"Intersubunit and Interprotein Interactions of Bacterially Expressed Human eIF2 Subunits and the Importance of eIF2α Phosphorylation in Apoptosis"** has been carried out by me under the supervision of Prof. K. V. A. Ramaiah and this work has not been submitted for any degree or diploma of any university.

Supervisor	**Candidate**
Prof. K. V. A. Ramaiah	**Rajesh Kamindla**

Department of Biochemistry
School of Life Sciences
University of Hyderabad
Hyderabad 500 046, Andhra Pradesh, India

CERTIFICATE

This is to certify that Mr. **Rajesh Kamindla** has carried out the work in the present thesis under my supervision for a full period prescribed under the PhD ordinance of the university. I recommend his thesis entitled **"Intersubunit and Interprotein Interactions of Bacterially Expressed Human eIF2 Subunits and the Importance of eIF2α Phosphorylation in Apoptosis"** for submission for the award of the degree of Doctor of Philosophy of this university.

Supervisor **Head**

Prof. K. V. A. Ramaiah **Department of Biochemistry**

Dean

School of Life Sciences

Acknowledgements

Thanks to my supervisor **Prof K. V. A. Ramaiah** for his unfailing support, encouragement, insight and expertise that contributed to my education and training in my graduate career. His critical review of my research and thesis throughout my tenure in his laboratory allowed me to produce a higher quality thesis. In addition, his patience and enthusiasm helped foster my growth as a scientist.

I would like to thank **Prof A. S. Raghavendra**, Dean, School of Life Sciences, and Prof. **M. Ramanadham**, Head, Dept. of Biochemistry for providing all the necessary infrastructure and facilities to carry out my research work.

I extend my sincere thanks to **Prof Abani K. Bhuyan** for his guidance and support with the studies relating to the interprotein interactions. I owe my thanks to **Prof. N. Siva Kumar** and **Prof. Anand Kumar** for their suggestions and support during the course of this work and **Dr. Naresh** for his encouragement and support. I take this opportunity to express my gratitude to all the faculty members of the School of Life Sciences for their support.

I also want to thank all the non-teaching staff and animal house staff for their support.

I am thankful to my friendly lab members Rajasekhar, Hussain, Aparna, Pushpa, Aarti, Murthanna, Satyam and Yadi for creating a peaceful and amiable work atmosphere. I particularly thank Rajasekhar for helping me during the initial stages of my work and Aarti for all her support during the final stages of the work and the compilation of this thesis. I also want to thank all the project students associated with me.

I would like to thank all my friends; Gopi, Dinakar, Nagender, Gautham, Paul, Elisha, Manohar, Vyjayanthi, Yadaiah, etc. for their love and affection and making my stay at the University a cheerful and memorable one.

I owe my profound and immense sense of gratitude to my **beloved Parents** for their unconditional love, support, encouragement and patience without which it would have not been possible for me to complete this thesis. Thanks to my brother **Rohit** (**chotu**) and all my cousins for always being there for me and believing in my ability to achieve my goals.

Financial Support from **CSIR** is greatly acknowledged.

Above all, I owe my deep sense of gratitude to the **'Almighty'** for everything.

Rajesh Kamindla

Abbreviations

Ac-DEVD-AFC	N-acetyl-Asp-Glu-Val-Asp-amino-4-trifluoromethyl coumarin
Ac-DEVD-CHO	N-acetyl-Asp-Glu-Val-Asp-aldehyde
AcMNPV	Autographica californica nuclear polyhedrosis virus
AIF	Apoptosis inducing factor
AP	Alkaline phosphatase
Apaf-1	Apoptosis protein activating factor
APH	Acetyl phenyl hydrazine
ATF	Activated transcription factor
ATP	Adenosine 5' triphosphate
BCIP	5-bromo-4-chloro-3-indolyl phosphate
BiP	Immunoglobin heavy chain binding protein
Bis-acrylamide	N, N'-methylene-bis-acrylamide
BSA	Bovine serum albumin
cDNA	Complementary DNA
CHOP	C/EBP homology protein
Ci	Curie
CKII	Caesin kinase II
CP	Creatine phosphate
CPK	Creatine phosphor kinase
Cpm	Counts per minute
dATP	deoxy adenine triphosphate
dCTP	deoxy cytosine triphosphate
DMSO	Dimethyl Sulphoxide
DNA	deoxy ribonucleic acid
DNA-PK	DNA dependent protein kinase
DTT	Dithiothreitol
EDTA	Ethylene diamine tetraacetic acid
eEF	Eukaryotic elongation factor
eIF2	Eukaryotic initiation factor2
eIF2α	alpha subunit of eukaryotic initiation factor 2

eIF2α(P)	Phosphorylated alpha subunit of eukaryotic initiation factor 2
eIF4EBP	eIF4E binding protein
ER	Endoplasmic reticulum
eRF	Eukaryotic release factor
EtBr	Ethidium bromide
FBS	Foetal bovine serum
Fig	Figure
GADD	Growth arrest and DNA damage
GCN2	General nonderepressible kinase
GDP	Guanosine 5’ diphosphate
GSH	Reduced glutathione
GST	Glutathione-S-transferase
GTP	Guanosine 5’ triphosphate
Hrs	Hours
HRI	Heme regulated inhibitor
Hepes	N-[2-hydroxyl ethyl] piperazine-N’-[2-ethane sulphonic acid]
Hsp	Heat shock protein
IRE1	Inositol reqiring enzyme 1
IRES	Internal ribosomal entry sites
Kbp	Kilobase pairs
KDa	Kilo daltons
LB	Luria Bertani
Lt	Litre
Met	Methionine
Mr	Marker
mg	milligram
min	Minutes
ml	millilitre
mM	milli molar
MOI	Multiplicity of infection
NBT	Nitro blue tetrazolium

ng	Nano gram
PAGE	Polyacrylamide gel electrophoresis
PDI	Protein disulfide isomerase
PERK	PKR like ER resident kinase
pfu	Plaque forming units
p.i.	Post infection
PKR	Double stranded RNA dependent protein kinase
pmol	Pico mole
PMSF	Phenyl methyl sulphonyl chloride
Met. tRNAi	Initiator tRNA
mRNA	messenger RNA
tRNA	transfer RNA
RNAse	Ribonuclease
S	Svedberg unit
S48A	eIF2α mutation of Ser 48 to Ala
S51A	eIF2α mutation of Ser 51 to Ala
S51D	eIF2α mutation for Ser 51 to Asp
Ser	Serine
SDS	Sodium dodecyl sulphate
Sf9	Spodoptera frugiperda cell line
TCA	Trichloro acetic acid
TEMED	N',N',N',N' tetramethyl ethylene diamine
Tris	Tris (hydroxyl methyl) amino methane
UPR	Unfolded protein response
UV	Ultra violet
wt	Wild type
Δpk2	mutant pk2 deleted baculovirus
μg	microgram
μl	microlitre

List of Figures

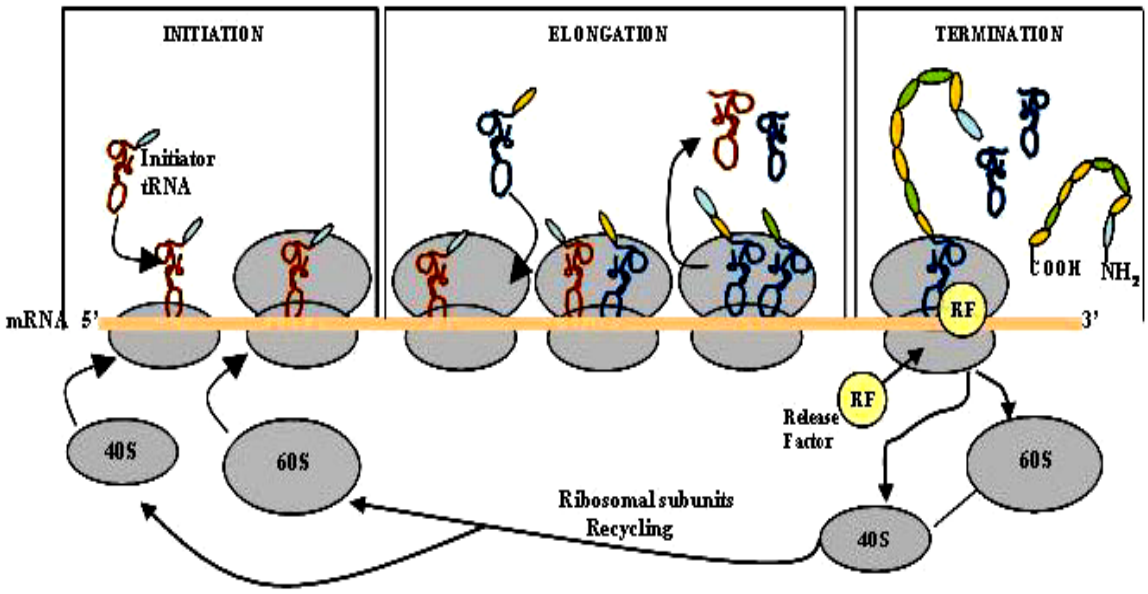
General Introduction

Introduction:

Protein synthesis is a process in which the genetic information encoded on the mRNA is translated into proteins. Most of the cellular processes like the enzymatic reactions on which the life depends, numerous structural, transport and regulatory functions are mediated mainly by proteins. Hence large proportions of cell's resources are devoted to translation. This energy expendable process therefore is closely monitored and regulated. Translation is a sophisticated process requiring extensive biological machinery such as the ribosomes, tRNAs, numerous protein factors and enzymes. The process of protein synthesis occurs generally in the cytoplasm or on the surface of endoplasmic reticulum. In eukaryotes, protein translation is uncoupled both temporally and spatially from RNA synthesis that occurs in the nucleus. The primary transcript of mRNA undergoes post-transcriptional modifications such as splicing (removal of introns and splicing of exons), 5' capping (addition of m^7 GTP) and 3' polyadenylation (addition of polyA tail) before being transported into the cytoplasm and translated thereafter. The sequence of amino acids to be incorporated into the protein is specified by the codon information on the mRNA. Amino acyl tRNA synthetase enzymes couple individual amino acids to the corresponding tRNAs in an ATP-dependent manner, and these tRNA coupled amino acids are transferred on to the template that is bound by ribosomes and translational factors.

This complex and dynamic process can be divided into four different phases: initiation, elongation, termination and ribosome recycling (**Fig A**). Although protein synthesis is the last step in gene expression pathway (DNA-RNA-Protein), translation regulation is critical for the survival and death of a cell. Cells apply translational controls/breaks because they are more focused, fast, flexible and reversible affecting the translation of selective messages (specific control) or whole class of mRNAs (global control). Regulation of translation can be exerted at different levels, however it is of the highest order at the initiation level. Keeping in view of the studies presented here in this thesis, the introduction is focused on the role of eukaryotic initiation factor 2 (eIF2) in the initiation step of eukaryotic protein synthesis and its intersubunit and interprotein interactions. This is followed by a detailed description of the structure, function of the individual subunits and the importance of the phosphorylation of the α-subunit of eIF2 in regulating protein synthesis.

Fig A:



Various steps in Translation: The process of protein synthesis, a complex pathway represents the last step of the gene expression pathway (DNA-RNA-Protein). It has been sub-divided into 4 major steps: 1, Initiation; 2, Elongation; 3. Termination and 4, Ribosome recycling. Each step is brought about by their respective specialized protein factors as described in the introduction. Translational regulation of gene expression is observed at each of these levels, however, it is of the highest order at the initiation level

Initiation: The initiation of protein synthesis is a complex and multistep pathway mediated by several proteins, accordingly called as initiation factors (IFs). It involves all the steps between the ribosomal subunit dissociation upon termination in the previous translation cycle to the assembly of elongation competent 80S ribosomal complex at the start codon on the mRNA (**Fig B**). In eukaryotes, the initiation is mediated by at least eleven different initiation factors and several significant interactions among them. Conceptually, this process can be divided into four steps:

a) Formation of 43S (Svedberg) preinitiation complex from Met-tRNAi, initiation factors and 40S ribosomal subunit.

b) Recruitment of the 43S complex to the 5' capped end of the mRNA to form 48S complex.

c) Scanning of the 5' untranslated region (UTR) of the mRNA and recognition of the initiator AUG codon, and

d) Assembly of the elongation competent 80S ribosomal complex.

Formation of 43S preinitiation complex: At the end of protein synthesis, 80S ribosomes are dissociated into their individual subunits and are bound by anti association factors to prevent their reassociation. This dissociation and anti association activities in eukaryotes are mainly accomplished by eIF3; its affinity towards the smaller ribosomal subunit increases in the presence of other factors like eIF1, eIF2.GTP.Met-tRNAi, mRNA and small U-rich RNAs (Marintchev and Wagner, 2004). Another protein, eIF6 has been shown to bind the 60S subunit and prevent its association with the 40S ribosomal subunit (Russell and Spremuli. 1979; Raychaudhuri et al., 1984). The first step in translation initiation appears to be the formation of a ternary complex (TC) containing eIF2, GTP and Met-tRNAi that subsequently joins the 40S subunits. This involves the selection of the initiator Met-tRNA, which in eukaryotes interacts specifically with eIF2.GTP complex. This interaction can be explained by the sequence divergence between the initiator and elongator forms of Met-tRNAs. The A1:U72 base pair is critical for the initiator function and is universally conserved amongst cytoplasmic eukaryotic initiator tRNAs and not in elongator tRNAs. In addition, the Met-tRNAi loop IV contains A60 in place of pyrimidine 60, and A54 instead of T54 present in the TψC sequence found in elongator tRNAs. Finally, the Met-tRNAi contains three consecutive G:C base pairs in the anticodon stem.

All these features enable the binding of the Met-tRNAi to eIF2, which delivers it in a GTP dependent manner to the 40S ribosomal subunit already bound by other initiation factors like eIFs 1, 1A, and 3 to form the 43S initiation complex. GTP bound form of eIF2 binds the Met-tRNAi more tightly recognizing determinants from both the tRNA and the Met moiety.

Binding of the ternary complex to the 40S subunit is also aided by eIF1, eIF1A, and the multi-subunit factor eIF3. In yeast, a multifactor complex (MFC) consisting of eIF1, eIF2, eIF3, eIF5, and Met-tRNAi assembles independently of the ribosome and its integrity depends on the AA-box at the C-terminal of eIF5 (Asano et al., 2000). The eukaryotic initiation factor 3 (eIF3), which acts as a central hub for interactions involving not only translational initiation factors but also a wide variety of cellular proteins that are components of cytoskeleton or proteasome (Pincheira et al., 2001; Hasek et al., 2000; Hou et al., 2000; Palecek et al., 2001; Lin et al., 2001; von Arnim and Chamovitz, 2003). It consists of five non-identical subunits (eIF3a, eIF3b, eIF3c, eIF3i and eIF3g) in yeast (Phan et al., 1998) and 11 subunits in mammals (eIF3A-3K) (Browning et al., 2001). The main function of eIF3 seems to prevent the 60S subunits from disrupting the 43S preinitiation complex. eIF5 is a GTPase activating protein (GAP) that stimulates the intrinsic GTPase activity of eIF2 complex (Das et al., 2001) and its C-terminus interacts with eIF2β and also associates with the p93 (Nip1) subunit of eIF3. eIF1 is the smallest of the initiation factors (12.7 and 12.3 KD in human and yeast respectively) and prevents eIF5-catalyzed 60S subunit joining in the absence of mRNA (Trachsel et al., 1977), implying a role in coordinating mRNA and tRNA binding to initiation complex. In concert with eIF1A, eIF1 promotes 48S complex formation with the ribosome positioned at the initiator AUG codon (Pestova et al., 1998). It also contacts the MFC through interactions with the eIF3a-CTD and eIF3c-NTD. eIF1A is also a small, stable protein of 17-22 KD (Wei et al., 1995) and is one of the most highly conserved of the initiation factors. Depletion of eIF1A in yeast cells results in polysome runoff and thus an inhibition of translation initiation (Kainuma and Hershey, 2001). The role of eIF1A in initiation is pleiotropic, as has been implicated in ribosome dissociation, ternary complex binding and mRNA binding to the ribosome (Wei et al., 1995). The carboxy terminal domain (CTD) of eIF1A interacts with the CTD of eIF5B, a homologue of bacterial IF2 (Choi et al., 2000)

and the NTD binds to eIF2 and eIF3 (Olsen et al., 2003).

Recruitment of the 43S complex to the 5' end of the mRNA to form 48S complex:

Eubacterial and archaeal mRNAs possess a Shine-Dalgarno sequence that facilitates ribosome recruitment by interacting directly with the complementary sequence at the 3' end of the 16S ribosomal RNA. Despite having a very similar 3' end, the eukaryotic 18S rRNA lacks such an element. So, to identify the 5' end of mRNAs, eukaryotic cells have opted for a specific structure- m^7Gppp cap (m^7G). Most of the mRNAs in eukaryotes are capped on their 5' ends and polyadenylated (poly A) at their 3' ends. The 5' m^7G cap on the mRNAs is recognized and bound by eIF4E which together with eIF4G and eIF4A forms the eIF4F complex. The binding of heterotrimeric eIF4F complex (consisting of 4E, 4G and 4A) to the m^7G cap commits the translational apparatus to the translation of that mRNA. The binding of the 43S preinitiation complex to the 5' terminal region of mRNA necessitates an unstructured mRNA region. To accomplish this, eIF4F, together with eIF4B, possesses ATP-dependent RNA helicase activity that presumably melts out secondary structure in the 5' proximal region of mRNA (Rozen et al., 1990). The eIF4A subunit is responsible for the RNA helicase activity and can catalyze RNA unwinding in the absence of eIF4E and eIF4G. It belongs to DEAD box family of proteases. However, the eIF4F complex possesses even stronger helicase activity and is likely the physiologically relevant form of activity. The ATPase and helicase activity of eIF4A are enhanced by eIF4B and eIF4H, which cause a switch in the eIF4F activity from nonprocessive to processive (Rogers et al., 1999). The factor, eIF4H, has homology to the RRM domain of eIF4B (Richter-Cook et al., 1998). The factor, eIF4E, is conserved across all eukaryotic kingdoms. The structures of yeast and mouse eIF4E bound to m^7G were solved (Marcotrigiano et al., 1997; Matsuo et al., 1997). Studies demonstrate the conservation of the cap binding mode and suggest that m7GDP binding occurs in a pocket on concave side of the protein. Specific recognition of the G in the m^7G cap by eIF4E occurs through hydrogen bonds to the side chain oxygens of Glu-103 and the main chain nitrogen of Trp-102. Indeed, all the amino acids involved in the cap recognition are conserved from yeast to humans, which strongly suggest a common ancestral origin (Aravind and Koonin, 2000). It is phosphorylated on one major site, serine 209 and this phosphorylation site lies on the same concave face and is in the vicinity of Lys 159. It has

been suggested that phosphorylation of this serine enables formation of a salt bridge, thereby clamping the protein to the m7G cap. Phosphorylation of eIF4E is correlated to increased translation rates and growth status of the cell (Kleijn et al., 1998), but it is still not clear whether phosphorylation increases (Minich et al., 1994) or decreases (Scheper et al., 2002) its cap binding affinity. The association of eIF4E with eIF4G and subsequent assembly of eIF4F complex is prevented by the binding of 4E binding proteins (BPs) to eIF4E. In turn, phosphorylation of 4E-BPs modulates their affinity for eIF4E; hypophosphorylated 4E-BPs bind efficiently to eIF4E, hyperphosphorylation abrogate their interaction with eIF4E (Lin et al., 1994; Pause et al., 1994; Fadden et al., 1997).

The factor, eIF4G, serves as a scaffolding protein mainly to bring together other components of the initiation pathway. Human eIF4G may be divided into three distinct domains of roughly similar size. The amino terminal (residues 1-634) region binds the poly A binding protein (PABP) and eIF4E, and is required for cap-dependent translation (Lamphear et al., 1995; Mader et al., 1995; Imataka et al., 1998). The interaction between eIF4G and PABP facilitates pseudo-circularization of the mRNA (Sachs and Varani, 2000) and may facilitate the reinitiation. The central domain (residues 635-1039) binds eIF3 and eIF4A (Imataka and Sonenberg, 1997) and possesses an RNA binding site (Pestova et al., 1996). The carboxy terminal region contains a second eIF4A binding site and binds to the kinase Mnk1. eIF4B (~69 KD) is a RNA binding protein that promotes the recruitment of ribosomes to mRNA and stimulates the RNA helicase activities of eIF4A and eIF4F.

Recognition of 'start site': Following the binding of the 43S complex to the m^7G cap at the proximal end of the mRNA, the ribosome seeks the initiation codon and binds there. The ribosome and the associated factors migrate or scans along the mRNA from the 5' terminus in an energy dependent process, until the anticodon (CAU) of Met-tRNAi can form a productive base pairing with AUG, the initiation codon. This mechanism which involves the location of the AUG codon by the ribosome is called as "scanning model" (Kozak, 1989 and 1999). This model suggests that the 5' proximal AUG is utilized in about 90% of mRNAs that employ scanning mechanism and presence of RNA secondary structures in the 5' UTR block scanning and prevent the 43S subunit from binding at the AUG. The efficiency of recognition of the 5' proximal AUG triplet may be influenced by its local sequence context and, in addition, a hairpin stem loop motif inserted some 18

nucleotides downstream from the initiator AUG can improve the efficiency with which it is recognized as an initiation codon. The consensus sequence (GCC(A/G)CCAUGG) around the start AUG codon provides an optimal context and is critical for the recognition at the initiation step. Initiation factors such as eIF1, eIF2 and eIF5 also play an important role in AUG recognition. Mutations in the yeast genes encoding the above factors or any of the subunits of eIF2 allow ribosomes to initiate at UUG instead of AUG. The rate of the eIF5 promoted GTPase reaction on eIF2 also plays an important role in the fidelity of initiation codon recognition. If the GTP hydrolysis proceeds more rapidly than normal, it allows the initiation at sites that normally would be bypassed or if the process is delayed than normal, the ribosome would stall at a non AUG codon such as UUG. eIF1 is required for the formation of 48S complexes and weakly promotes the correct positioning of 43S complex at the initiation codon. This activity is strongly enhanced by eIF1A (Pestova et al., 1998). eIF1A interacts with eIF5B (Choi et al., 2000) and structural studies suggest that eIF1A occupies the A site of the small ribosomal subunit and in conjunction with eIF5B directs the initiator tRNA to the P site (Roll-Mecak et al., 2001).

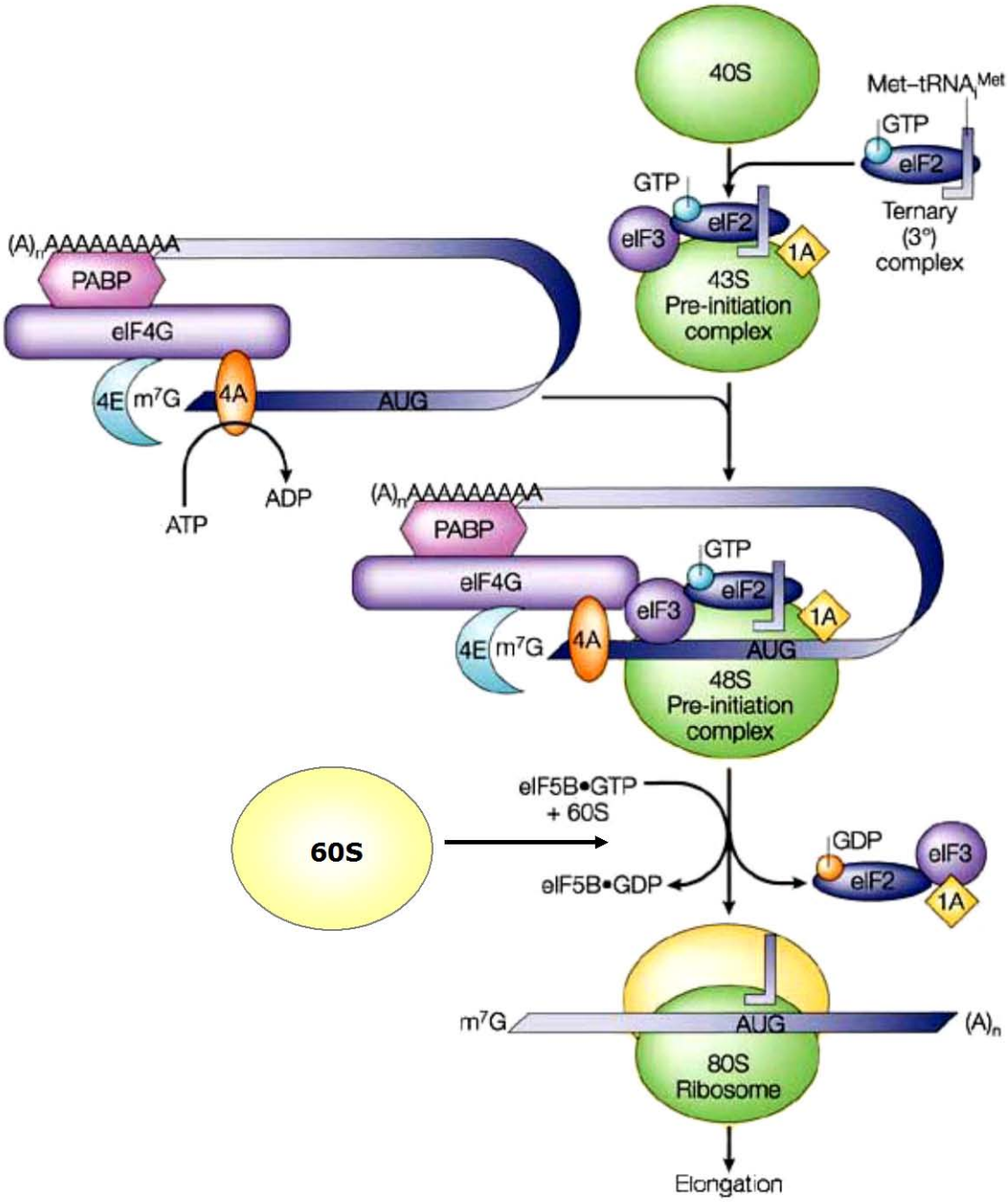
Joining of the 60S ribosomal subunit: Once the preinitiation complex has reached an initiator codon, base pairing between the AUG and the Met-tRNAi anticodon elicits a series of events that culminate in the joining of the 60S ribosomal subunit to form an active ribosome that is competent for elongation. This involves the release of the initiation factors bound to the 43S complex, which requires GTP hydrolysis. Recruitment of the 60S ribosomal subunit is not a passive reaction but necessitates activity of eIF5B-GTP. After the 48S complex is correctly assembled at the initiation codon, the bound initiation factors must be displaced to allow the joining of the 60S subunit and as mentioned above needs the hydrolysis of the GTP molecule bound to eIF2. Activation of the GTPase function of eIF2 requires its association with eIF5 and the 40S ribosomal subunit. Mutational studies have suggested that eIF2 and eIF5 act in concert to maintain the accuracy of the initiation codon recognition in eukaryotes. Intriguingly, eIF5 has not been found to interact with the GTP bound eIF2γ, but rather with eIF2β at the same site at which eIF2Bε also interacts (Das et al., 1997; Asano et al., 1999). This suggests that GTP hydrolysis is triggered by a conformational change in eIF2 when the initiator tRNA is correctly paired with the AUG codon. Further the amino terminus of eIF5 is similar to the carboxy terminus of eIF2β,

including the Cys_2-Cys_2 zinc finger motif, and is expected to adopt a similar fold (Cho and Hoffman, 2002). Recent observations suggest that eIF5 acts a bridge between eIF3 and eIF2, and therefore plays a role in the recruitment of the ternary complex to the 40S subunit (Asano et al., 2000). It has also been demonstrated that yeast eIF5 can interact simultaneously with eIF3 and eIF4G and could thus take part in 43S complex recruitment to the mRNA. These interactions are all mediated by the C- terminal domain of eIF5, while the GTPase activating region apparently resides in the N-terminus (Asano et al., 2001).

The joining of the 40S and 60S ribosomal subunits to form the 80S initiation complexes is stimulated by a recently discovered factor called eIF5B whose GTPase activity is stimulated probably because of the ribosomal subunit interaction. The GTP hydrolysis by eIF5B is not required for the 80S complex formation, but may be needed to promote dissociation of eIF5B and its efficient recycling in the junction reaction. In this respect, eIF5B resembles its prokaryotic homolog, IF2, which also hydrolyzes GTP to effect rapid dissociation from 70S ribosomes. In effect, two GTP hydrolysis reactions are required for initiation in eukaryotes: one with GTP bound to eIF2 and the other with GTP bound to eIF5B. Like bacterial IF2, eIF5B binds GTP and is a ribosome dependent GTPase, as its sequence contains the three consensus motifs found in GTP-binding proteins. By analogy with IF2, eIF5B might bind to the A site of the 40S and 60S ribosomal subunits, helping proper alignment of the 60S ribosomal subunit on the 48S initiation complex and ensuring placement of the Met-$tRNA_i$ in the P site. The unstructured C-terminus of eIF1A interacts with the C-terminus of eIF5B and this association is instrumental in releasing eIF1A from the ribosome after the subunit joining. It, in the absence of eIF5 also can stimulate the GTPase activity of eIF2, as eIF5B added to 40S initiation complexes causes the rapid dissociation of Met-$tRNA_i$ (Peterson et al., 1979). It is also reported that mammalian eIF5B is phosphorylated (Traugh et al., 1976), but the effect of this modification on the function has not been yet elucidated.

Fig B: Translation Initiation: The initiation step of translation is a multistep and multifactorial pathway mediated by at least 11 different initiation factors in eukaryotes (however, participation of all the initiation factors is not shown here). It is a rate-limiting limiting step in translation and is mainly regulated as it is more effective to control the onset of a given biological process than to interrupt it later. The first step of this complex pathway is the transfer of Met-tRNAi to the 40S ribosome by eIF2 in a GTP-dependent process to form the 43S pre-initiation complex. The mRNA is bound on its 5' end by trimeric eIF4F complex (eIF4A, 4E and 4G), and the interaction of poly A binding protein (PABP) with eIF4G, pseudo-circularizes the mRNA which aids in the reinitiation. The 43S complex now joins the mRNA to form the 48S initiation complex. 60S ribosomal subunit now joins the 48S complex assembled at the initiator AUG codon to form the elongation competent 80S ribosomal complex. The figure is derived from Klann and Dever, 2004.

Fig B:


40S
Met–tRNA$_i^{Met}$
GTP
eIF2
Ternary (3°) complex
GTP
eIF3
eIF2
1A
43S Pre-initiation complex
(A)$_n$AAAAAAAAA
PABP
eIF4G
4E
m^{7}G
4A
AUG
ATP
ADP
(A)$_n$AAAAAAAAA
PABP
eIF4G
GTP
eIF2
eIF3
1A
4E
m^{7}G
4A
AUG
48S Pre-initiation complex
60S
eIF5B•GTP + 60S
eIF5B•GDP
GDP
eIF2
eIF3
1A
m^{7}G
AUG
(A)$_n$
80S Ribosome
Elongation

Translation Elongation: Elongation process has three steps; amino acids as per the sequence information in the mRNA are brought one after another onto the template mRNA and positioned in the vacant 'A' site of ribosome. Peptide bond formation occurs between the adjacent amino acids (located in the 'P' and 'A' site). Afterwards, the mRNA is coordinately translocated by one codon and this movement facilitates movement of deacylated tRNA from the 'P' (peptidyl) site to the 'E' (exit) site of the ribosome and the peptidyl tRNA in the 'A'site to the 'P'site thus leaving again the 'A' site empty for the incoming amino acid. The process continues until a stop codon is encountered.

Eukaryotic elongation factors: The general eukaryotic protein biosynthesis elongation cycle is catalyzed by three polypeptide elongation factors: eEF1A, a heterotrimeric eEF1B and eEF2, which are homologous to their prokaryotic counterparts EF.TU, EF.TS and EF2 respectively. However in fungi, an additional factor eEF3 is required, which has no homolog in the bacterial system (Chakraburtty, 1999). Of the above mentioned elongation factors, eEF1A and eEF2 are GDP/GTP-binding proteins (G proteins), which are active when complexed to GTP and inactive in their GDP bound form, and eEF1B is a guanine nucleotide exchange factor (GEF). The guanine nucleotide exchange factor, eEF1B, serves exactly the same function as bacterial EF1B, to facilitate the off-rate for eEF1A bound GDP. It has three subunits in most species (αβγ), but only two in yeast (αγ). The α and β subunits contain nucleotide exchange activity, whereas no particular function is attributed to the γ subunit.

Mammalian eEF1A has evolved from prokaryotic EF1A by insertion of approximately 70 amino acids into 16 different sites, mostly at the loop regions based on the crystal structure for bacterial EF1A and serves the exact same function as EF1A, namely the formation of a ternary complex (eEF1A-GTP-aa-tRNA) which is then bound to the ribosomal A site in a codon dependent manner. Mammalian eEF1A is subjected to seven post translational modifications: five lysines that are methylated and two glutamic acid residues (301, 374) that form a peptide linkage with glycerylphosphorylethanolamine (Dever et al., 1989). The activity of eEF1A (and eEF1B) is regulated by phosphorylation (Janssen et al., 1988; Venema et al., 1991; Chang and Traugh, 1998; Sheu and Traugh, 1999) and the levels of mRNAs encoding eEF1A (and eEF2) are regulated.

The factor, eEF2, also catalyzes the exact same reaction as its bacterial counterpart EF2, the GTP dependent translocation of the peptidyl tRNA from the A site to the P site. Unlike EF2, eEF2 is subject to two post-translational modifications. The first is the multistep conversion of His-715 into diphthamide and second, phosphorylation. The exact physiological circumstances of these modifications are not known, although the phosphorylation of eEF2 has been shown to inhibit protein synthesis. The factor, eEF3 is unique to fungal protein synthesis and has no homologs in bacteria, archeabacteria, or higher eukaryotes. In contrast to the other two elongation factors, eEF3 interacts with both the ribosomal subunits, competes with eEF2 for binding to ribosomes, and stimulates eEF1A dependent binding of the cognate aminoacyl-tRNA- GTP complex to the A site of ribosome (Kovalchuke et al., 1998; Triana-Alanso, 1995). It has been suggested that eEF3 also facilitates the clearance of deacyl-tRNA from the E-site, which is required in every elongation cycle (Triana-Alanso, 1995; Kamath and Chakraburthy, 1989). eEF3 possesses both intrinsic and ribosome dependent ATPase and GTPase activities by virtue of repeated bipartite nucleotide binding domain characteristic of ABC transporters (Chakraburtty, 2001).

Elongation Cycle:

The initial step of elongation that is the binding of cognate amino acyl tRNA to the ribosome in presence of GTP is mediated by eEF1A subunit which is homologous to prokaryotic EF-Tu and GTP. The binding of GTP to the eEF1A is energetically favoured by the subsequent attachment of amino acyl tRNA followed by binding of the ternary complex to the ribosome. The ternary complex, aa-tRNA-eEF1A-GTP enters the ribosome, where the anticodon of the tRNA attempts to make a codon/anticodon interaction with the A site codon of the mRNA. Once the cognate recognition is made, the eEF1A-GTP is brought into the GTPase activation center of the ribosome, GTP is hydrolyzed and eEF1A-GDP is released from the ribosome. The recycling of eEF1A-GDP to eEF1.GTP requires eEF1B, a nucleotide exchange factor that is comparable to EF.TS in prokaryotes or to the initiation factor eIF2B of eukaryotes.

When the 3` CCA end of aa-tRNA enters the A site on the 50S subunit, the peptidyl transferase center of the ribosome quickly catalyzes the formation of a peptide bond

between the incoming amino acid and the peptide found in the peptidyl-tRNA binding site (P site). The tRNA is now placed in the mixed hybrid states with the newly formed peptidyl-tRNA in the A/P site, where the anticodon is still in contact with the codon of the A site of the 30S subunit, but the CCA end is at the P site of the 50S subunit. The newly deacetylated tRNA is left in a similar P/E site, with its CCA end in an exit site (E site) on the 50S subunit. At this stage, eEF2-GTP forces the peptidyl-tRNA out of the A site on the 30S subunit and prevents it to rebind the A site. Thus, the peptidyl-tRNA is brought fully into the P site, and the dacetylated tRNA fully into the E site. During this process, GTP bound to eEF2 will be hydrolyzed at the GTPase center and eEF2-GDP leaves the ribosome (Rodnina et al., 1997). The action of eEF2 that forces the peptidyl-tRNA into the P site accounts for the precise movement of the mRNA by 3 nucleotides. At this stage the empty A site in the ribosome is ready to receive the incoming aminoacylated tRNA and thus the elongation cycle continues until a stop codon is encountered.

Translation Termination:

The process of termination sets in when the 80S ribosome encounters/ recognizes one of the in-frame stop codons, UAA, UAG or UGA in the mRNA and results in the hydrolysis and release of the nascent polypeptide. Both prokaryotes and eukaryotes have two classes of specialized factors, called the release factors (RFs) that are required to mediate the termination reaction. The class-1 RFs mediate the recognition of a stop codon and promote the hydrolysis of the ester bond linking the polypeptide chain with the peptidyl (P) site tRNA by the peptidyl transferase center of the ribosome. The class-2 RFs are GTPases and stimulate the class 1 release factor activity (Mikuni et al., 1994; Stansfield et al., 1995; Zhouravleva et al., 1995; Frolova et al., 1996).

In eukaryotes, translation termination is catalyzed by eRF1, which recognizes all three termination codons. The presence of a GGQ sequence motif in class 1 release factors is universal to all the species and any substitutions in this sequence abolished the ability of human eRF1 to trigger peptidyl-tRNA hydrolysis (Frolova et al., 1999; Song et al., 2000). The crystal structure of human eRF1 protein has recently been determined (Song et al., 2000) and suggests that the polypeptide chain is organized into three domains in a structure reminiscent of the letter ‘Y’. The domains 1, 2, and 3 of eRF1 resemble the anticodon

loop, aminoacyl acceptor stem and T stem of the tRNA molecule respectively. The second release factor, eRF3, stimulates eRF1 in a GTP dependent manner and the GTPase activity of eRF3 requires eRF1 and ribosome. The universally conserved GGQ motif is similar to the aminoacyl group attached to the CCA-3` sequence of tRNA molecule (Song et al., 2000). The eRF1 protein binds to the A site of the ribosome so that the Gln-185 residue of the GGQ motif is in coordination with a water molecule and can mediate a nucleophilic attack on the ester bond of the peptidyl tRNA molecule in the P site, resulting in the hydrolysis of the nascent polypeptide chain (Song et al., 2000).

Ribosome recycling and Reinitiation:

After the completion of termination step and the release of polypeptide chain, the mRNA and tRNA which are still bound to the ribosome as post-termination complex have to be released and the ribosome has to be dissociated into its constituent subunits and then recycled in order to bind free new mRNA. In prokaryotes, a ribosome recycling factor, RRF, together with Elongation factor, EF-G.GTP and IF3 assist RRF in disassembling the post termination complex (Karimi et al., 1999). Eukaryotes do not have a RRF, instead eRF3 fulfills the role of RRF (Ramakrishnan, 2002; Kisselev et al., 2003). The synergistic action of the m^7G cap and the poly (A) tail, apparently through an interaction between the eIF4G and the poly (A)-binding protein (PABP), may contribute to the phenomenon of ribosome recycling. The terminating ribosome might initiate protein synthesis at an initiation codon near the site of termination, leading to the synthesis of a different protein from the same mRNA in an event called “reinitiation”.

Eukaryotic Initiation Factor 2 (eIF2): It is mainly involved in the selection and recruitment of Met-tRNAi to the 40S ribosomal subunit in a GTP dependent manner and also in the control of start site recognition. eIF2-GTP binds specifically to Met-tRNAi, whereas GTP hydrolysis or loss of the methionine moiety weakens the interaction of eIF2 with the initiator tRNA. The ternary complex (eIF2-GTP-Met-tRNAi) binds stably to the 40S ribosomal subunit and the interaction is further stabilized by other factors. Upon start site selection, eIF2 hydrolyzes GTP and is released as an inactive eIF2-GDP from the Met-tRNAi. In eukaryotes, the inactive eIF2-GDP is recycled to the active eIF2-GTP capable to

rebind the initiator tRNA, by a heteropentameric guanine nucleotide exchange factor (GEF) eIF2B, whereas archaea do not have an eIF2 GEF (Dever, 2002). Recent biochemical experiments showed that the affinity of eIF2.GDP for Met-tRNAi is only ~20 fold lower than that of eIF2.GTP. Furthermore, the affinities of both eIF2.GTP and eIF2.GDP for deacylated tRNAi were approximately equal to that of eIF2.GDP for Met-tRNAi. Therefore, the discrimination between GDP- and GTP-bound eIF2 and between Met-tRNAi and deacylated tRNAi is mainly through interactions with the methionine, whereas the rest of the binding interface appears unperturbed, at least in solution (Kapp & Lorsch, 2004). Structurally, eIF2 is a heterotrimeric protein with α, β and γ subunits. The α, β and γ subunits of eIF2 show a considerable amount of sequence similarity among different species. For instance, the human α, β and γ subunits are 58%, 47% and 72% identical to the corresponding subunits of Saccharomyces cerevisiae. The conservation among species underscores the importance of the role of eIF2 in cell viability. The trimeric native eIF2 results from the 1:1:1 association of the three subunits (Pathak et al., 1988; Schmitt et al., 2002). cDNAs for each of the subunits have been cloned and sequenced from a variety of species. The molecular weights of human eIF2 α-, β-, and γ-subunits are 36, 38 and 52 kDa respectively as calculated from their cDNA values (Ernst et al., 1987; Pathak et al., 1988; Gaspar et al., 1994). Based on interaction studies and structural features of archeal β and γ-subunits, the γ-subunit is proposed to be a central core in the structure of eIF2 complex interacting with the α and β-subunits on either side but little or no direct interaction between the α and β-subunits (Marintchev and Wagner, 2004).

eIF2*α*: It is the smallest subunit of the trimeric complex and is primarily implicated in the regulation of eIF2. Cloning, sequencing, structural, genetic, biochemical and mutational studies of genes coding for eIF2α, from various organisms indicate that the molecular mass of α-subunit is approximately 36.2, 34.7 or 41.6 kDa in mammals, yeast and in plants respectively (Hershey and Merrick, 2000; Browning, 1996). It is the key target in translational regulation as the phosphorylation of the Ser51 on eIF2α by different kinases results in general translation shutdown. This shutdown is caused by an increased affinity of phosphorylated eIF2α for eIF2B, which results in a decreased GDP/GTP exchange rate on eIF2, and thus a decreased population of the activated GTP bound form of eIF2 (Pavitt et

al., 1998). Phosphorylated yeast eIF2α was shown to bind tightly the regulatory subcomplex (consisting of α, β and δ subunits) of eIF2B (Krishnamoorthy et al., 2001). However, phosphorylation of eIF2α stimulates translation of certain mRNAs having upstream open reading frames (uORFs) such as ATF4 or GCN4. The solution structure of eIF2α determined by NMR spectroscopy suggests that it consists of two domains that are mobile to each other. The N-terminus domain consists of two subdomains, S1 subdomain (residues 15-85) and α-helical subdomain (residues 91-183) and matches with the NTD crystal structure of heIF2α (Ito et al., 2004; Dhaliwal and Hoffman, 2003; Nonato et al., 2002). The S1 domain consists of five stranded β-barrel and adopts the oligonucleotide binding (OB) domain. The conserved phosphorylation site Serine 51 is located on one of the loops of these β-strands (β3/β4) which is flexible in nature. The β barrel is followed by α-helical domain. The C-terminal domain adopts a αβ-fold and consists of five strands, two α helices and one loosely associated C-terminal helix that have no well defined direction. Although lacking sequence homology, the eIF2α-CTD is structurally homologous to CTD of the elongation factor eEF1Bα which is the GEF for eEF1A. Further, eIF2α interacts with eIF2γ-subunit and the binding surface corresponds to the eEF1Bα binding surface on eEF1A that resembles structurally the γ-subunit of eIF2 and suggests the association of eIF2α, eIF2γ and Met-tRNAi in the ternary complex. The GEF function of the α–subunit of eIF2 however, is lost probably in evolution and is catalyzed by heteropentameric eIF2B protein. It was recently reported, that archaeal eIF2γ binds specifically Met-tRNAi. The binding was not affected by the presence or absence of the β subunit. The α subunit or its CTD stabilized Met-tRNAi binding by ~50-fold (Yatime et al., 2004). In contrast, yeast eIF2α only stabilized Met-tRNAi binding to eIF2βγ by 5- to 10-fold (Nika et al., 2001). Interestingly, in addition to the conserved phosphorylation site at Ser-51, yeast eIF2α possess multiple phosphorylation sites for CKII at the C-terminal end and the phosphorylation by CKII may promote the productive interaction between eIF2-GDP and eIF2B.

eIF2β: The β-subunit of eIF2 ranges from 250 to 333 amino acids. It has several important features which include a stretch of polylysine residues or K boxes at the N-terminus and a Zinc-finger motif at its carboxy terminus that are conserved both in yeast, fruitfly and in

mammals (Donahue et al., 1988; Pathak et al., 1988; Ye and Cavenar, 1994). The molecular mass of the human β-subunit, calculated from its cDNA sequence, is 38 kDa (Pathak et al., 1988). However, it migrates as 50 kDa on 10% SDS-PAGE and this anomalous migration is attributed to the high percentage of basic residues present at the N-terminus (Pathak et al., 1988; Price et al., 1989). The wheat germ eIF2β is 38 kDa and is smaller than its α-subunit (42 kDa) (Janaki et al., 1995; Laxminarayana et al., 2002; Metz and Browning, 1997). eIF2β contains two domains conserved in all species: an NTD and a ZBD (Cho & Hoffman, 2002), as well as an eIF2γ-binding segment immediately preceding the NTD (Thompson et al., 2000). Eukaryotic eIF2β also contains a positively charged N-terminal tail, which binds to the GAP and GEF, eIF5 and eIF2B respectively (Dever, 2002). The structures of the two domains of archaeal eIF2β were determined (Cho and Hoffman, 2002; Gutierrez et al., 2004). The two domains are connected by a relatively flexible helical region. No interactions were reported between the eIF2β-NTD and eIF2β-ZBD (Cho & Hoffman, 2002; Gutierrez et al., 2004).

In addition to the above features, the N-terminal half of human but not yeast eIF2β-subunit has two of the three consensus guanine nucleotide binding domains (DXXG, NKXD) and they are about 125 residues apart, whereas, these are separated by 40-80 residues apart in other G-binding proteins (Dever et al., 1987). Biochemical and mutational analyses with mammalian and yeast eIF2β suggest that the zinc finger motif plays a role in the recognition of the initiation codon and the lysine rich regions in the β-subunit are important for eIF2 interaction with mRNA (Donahue et al., 1988, Asano et al., 1999; Laurino et al., 1999). The β-subunit is also implicated in the formation of ternary complex (eIF2.GTP.Met-tRNAi) that is essentially carried out by the γ-subunit of eIF2. Interestingly, it has been shown that the β-subunit of mammalian eIF2 can be proteolysed during purification or purposefully and suggests that eIF2 devoid of β-subunit can still function similarly to the wild type eIF2 in promoting ternary complex and 40S initiation complex (Mitsui et al., 1981). Subsequently, another study has shown that the absence of intact eIF2β-subunit has no effect on the binding of guanine nucleotides but the αγ-complex is severely defective in Met-tRNAi binding, thereby suggesting the involvement of β-subunit in Met-tRNAi binding (Flynn et al., 1993). This discrepancy may be due to

the fact that fragments of proteolysed β-subunit may still interact with the eIF2 complex (Proud, 1992). Cross-linking studies have implicated the β-subunit of mammalian eIF2 in nucleotide and Met-tRNAi binding indicating proximity of this subunit to binding sites present in the γ-subunit (Anthony et al., 1990; Gaspar et al., 1994). It may be likely that both the β and γ subunits of eIF2 are essential for binding of Met-tRNAi. This is supported further by biochemical analysis of important suppressor mutations in the yeast eIF2β and γ subunits and also of eIF5 that alter the rates of GTP hydrolysis and increase the dissociation of Met-tRNAi. In addition, the suppressor mutants of yeast eIF2β that map near or within the zinc finger domain in the carboxy terminus (S264Yand L254P mutants), have shown increased dissociation of Met-tRNAi from the ternary complex and increased intrinsic rate of GTP hydrolysis in the absence of eIF5 (Huang et al., 1997). These results suggest a role for β–subunit in Met-tRNAi binding, and also in GTP hydrolysis. Interestingly, the carboxy–terminal region in eIF2β has significant similarity with the amino terminal region in eIF5, a GTPase activating protein, including the zinc finger motif and raises the possibility that these homologous regions may be competing or interacting with common components of the translational machinery (Das et al., 1997).

The β-subunit is also a hub for protein interactions. It interacts with initiation factors such as eIF5, eIF2B, and indirectly with eIF3 a and c (Das et al., 1997; Kimball et al., 1998; Singh et al., 2004). The K-boxes at the N-terminus of eIF2β are required for interaction between eIF2 and eIF5 in addition to mRNA binding by eIF2. Recombinant rat eIF5 bound specifically to the β-subunit of the purified rabbit eIF2, and *in-vitro* binding assays using rat eIF5 and human eIF2β suggested that the second K-box was necessary and sufficient for their strong interaction. Further the K-boxes also mediate tight interaction between eIF2 and eIF2B in-vivo, promoting efficient recycling of eIF2 (Asano et al., 1999). The binding domain for eIF2β was mapped *in-vitro* to the carboxy terminal 40% of yeast eIF5. This region is highly conserved between yeast and mammalian eIF5 and has at the extreme carboxyl terminus a bipartite motif containing aromatic, hydrophobic and acidic (AA-boxes) residues in each of its parts. Interestingly, this motif is also found at the carboxyl termini of ε-subunit, the principal catalytic subunit of eIF2B (Koonin, 1995; Fabian et al., 1997; Pavitt et al., 1998). Further, the interactions of initiation factors in the multifactor complex (MFC) in yeast revealed that the β-subunit of eIF2 interacts directly

with the extreme CTD of eIF3a subunit, indirectly with the N-terminus domain of eIF3c subunit via the eIF5 CTD (Valasek et al., 2002), and also with eIF1 (Singh et al., 2004). These interactions play a role in the formation of 43S and 48S complexes. The β-subunit of mammalian eIF2 also interacts with other proteins such as PP1, a type 1 protein phosphatase, Nck1, a tyrosine kinase adaptor protein, and also with casein kinase II (CKII) (Wakula et al., 2006; Kebache et al., 2002; Llorens et al., 2006). Interestingly the mammalian β-subunit of eIF2 is found to interacts with DNA dependent protein kinase (DNA-PK), a nuclear enzyme and also serves as a substrate for DNA-PK which raises questions regarding its role if any in DNA repair (Ting et al., 1998). Further, the human eIF2β- subunit, unlike its yeast or archeal counter part, is phosphorylated by CKII (Ser 2 and 67 residues), PKC (Ser 13) and PKA (Ser 218) (Welsh et al., 1994). Phosphorylation of the β-subunit by CKII appears to be constitutive in HeLa cells and mutations at these sites alter the functions of the β-subunit (Llorens et al., 2006). The physiological significance of the β-subunit phosphorylation is not yet known.

The structure of the mammalian eIF2β is not available but the NMR structure of the aIF2β, the archaeal initiation factor from *Methanococcus jannaschii* and *Methanobacterium thermoautotrophicum* has been solved. These studies suggest that it contains three regions: an unfolded N-terminus domain, a core domain and a C-terminus Zn-finger domain, which is mobile with respect to the core domain and appears that both the N- and C-terminal domains are connected with a flexible linker (Cho and Hoffman, 2002; Gutierrez et al., 2004). Eukaryotic eIF2β also contains a positively charged N-terminal tail which binds to the eIF5 (the GTPase activating protein) and eIF2B respectively (Dever, 2002).

eIF2γ: It is the largest subunit of the eIF2 trimer and its molecular weight ranges from 51-58 KDa in different organisms. Unlike β-subunit of mammalian eIF2, the N-terminus region of γ-subunit contains all the three conserved consensus guanine nucleotide binding domains (GXXXXGK, DXXG, NKXD) and separated by 40-80 residues as reported in other GTP binding proteins suggesting that it is mainly involved in the binding GTP directly. Mutations in the NKXD consensus elements found in both the subunits indicate that GTP binds to the γ-subunit of eIF2 (Naranda et al., 1995). The N-terminus of eIF2γ is implicated in the binding of GTP and also Met-tRNAi (Gasper et al., 1994; Erickson et al.,

1997; Naranda et al., 1995). Further observations with two subunit form of eIF2 lacking the β-subunit could bind GDP but was unable to form a stable ternary complex with Met-tRNAi (Flynn et al., 1993). Thus, it is likely that eIF2γ binds GTP directly, and that the β and γ subunits each make important contributions to binding Met-tRNAi. It is homologous to elongation factors EF1A, eEF1A and the selenocycteine specific factor SelB/ eEFSec (Leibundgut et al., 2005). Upon the start codon recognition, eIF2 hydrolyzes GTP and this hydrolysis step is required for the release of the initiation complex. In analogy with proteins of the GTPase super family, the γ-subunit that contains the GTP binding domains has a GTPase activity that hydrolyses the GTP bound to eIF2. However in eukaryotes, GTP hydrolysis requires the presence of eIF5, which serves as a GTPase activating protein (GAP) for eIF2. A comparative study between archaea and eukaryotes suggests that eIF2 GTP hydrolysis and nucleotide exchange are more stringently controlled in eukaryotes, hence the need for a GAP (eIF5) and a GEF (eIF2B). The high degree of homology between eukaryotic and archaeal eIF2, suggests that the eukaryotic factor probably has retained the intrinsic ability to hydrolyze GTP, but this activity is efficiently repressed. According to this scenario, an alternative role of eIF5 could be to help derepress the GTPase activity of eIF2, rather than (or in addition to) acting as a classical GAP factor, stabilizing a transition state in GTP hydrolysis. In support of such an interpretation, eIF5 is homologous to the two core domains of eIF2β, covering almost the entire length of archaeal eIF2β, except for a short eIF2γ-binding segment that is absent in eIF5. Furthermore, mutations in, or deletion of the second of the eIF2β domains shared with eIF5 cause increased rate of spontaneous GTP hydrolysis by eIF2 (Hashimoto et al., 2002). Further the GTP hydrolysis of eIF2.GTP-Met-tRNAi stimulated by eIF5 requires 40S ribosomes (Chaudhuri et al., 1994). Thus it is likely the GTPase activity associated with the γ-subunit is stimulated by eIF2β and eIF5 interaction on the ribosome. This is consistent with the fact that the N-terminal polylysine residues of the eIF2β-subunit interact with the bipartite (AA) motifs in the C-terminus of eIF5 (Das et al., 1997; Asano et al., 1999). Although, the GTP-binding (G)/ GTPase domain resides in the γ subunit of the heterotrimeric eIF2, only eIF2β, and not eIF2γ, has been reported to directly bind to eIF5. However, a recent report with yeast proteins suggested that the γ-subunit interacts directly with its GAP, eIF5 (Alone and Dever, 2006).

The structure of archaeal eIF2γ was determined in the apo-form (Schmitt et al., 2002; Roll-Mecak et al., 2004) and GTP- and GDP-bound forms (Schmitt et al., 2002). It was found to be very similar to those of the elongation factors (EFs). In addition to the three-domain core as in EFs, archaeal eIF2γ has a small Zn-binding domain (ZBD) formed by four cysteines, inserted in the G domain The corresponding inserted segment in eukaryotic eIF2γ is even longer and essential for viability in yeast (Erickson et al., 1997), but may not bind zinc. Yeast eIF2γ, but not of humans also has a long N-terminal tail, whose deletion is tolerated *in vivo,* but a point mutation in it causes a growth defect (Erickson et al., 1997). Although the crystal structures appear similar, archaeal eIF2γ indicate a major difference to EF1A. Both apo- and GDP-bound forms of eIF2γ have a closed active orientation as the GTP-bound form of EF1A (and eIF2γ), instead of the open conformation found in apo-EF1A and EF1A.GDP. The orientation is unlikely to be due to crystal packing, because it is found in the structures from two different organisms, in different crystal forms (Schmitt et al., 2002; Roll-Mecak et al., 2004). Consistent with the structural data, recent biochemical experiments showed that the affinity of eIF2.GDP for Met-tRNAi is only ~20-fold lower than that of eIF2.GTP. Furthermore, the affinities of both eIF2.GTP and eIF2.GDP for deacylated tRNAi were approximately equal to that of eIF2.GDP for Met-tRNAi. Therefore, the discrimination between GDP- and GTP-bound eIF2 and between Met-tRNAi and deacylated tRNAi is mainly through interactions with the methionine, whereas the rest of the binding interface appears unchanged (Kapp and Lorsch, 2004). The Switch 1 and Switch 2 regions of the G-proteins, including those of the EF1A family, respond to ligand and nucleotide binding. It appears that the cooperativity between Met-tRNAi and GTP binding to eIF2 is mediated by their direct interactions with, and induction of conformational changes in, the switch regions.

Regulation of Protein Synthesis:

Although "gene expression" is often used synonymously with "transcription", the steady-state levels of proteins in eukaryotic cells are also strongly dependent on translational regulatory mechanisms. The regulation of gene expression at the level of translation is an important, but still not completely understood, control mechanism. However, it is becoming increasingly evident that the regulation of translation provides the cell with the plasticity that is needed to respond to rapid changes in the environment. Translation is the

final step in the flow of the genetic information, and regulation at this level allows for an immediate and rapid response to changes in physiological conditions. Such regulation is of increased importance under certain conditions, such as cellular stresses heat shock, hypoxia, nutrient deprivation and endoplasmic reticulum (ER) stress or apoptosis (Clemens et al., 2000, Harding et al., 2000) that require immediate changes in protein levels.

The estimated fraction of genes in a cell devoted to translation may be as high as 35 to 45% (Sonenberg et al., 2000), hence this costly process has to be strictly regulated. Two different modes of translational control occur: a) global control in which the translation of most mRNAs is affected; and b) mRNA-specific control where the translation of a defined group of mRNAs is modulated without affecting general protein biosynthesis. Global regulation chiefly occurs by the modification of translation factors but, mRNA specific regulation may be mediated by regulatory protein complexes that recognize particular elements present in the 5′ and/or 3′ untranslated regions (UTRs) of the target mRNA and also by the local changes in the activity of general translation factors (Sonenberg et al., 2000; Holcik et al., 2000). Translation is divided into three distinct phases: initiation, elongation and termination and all three phases are subject to regulatory mechanisms. The proteins eIF2, eIF2B, eIF4, S6, eEF1 and eEF2 are the major players involved in the regulation of translation through their post translational modifications such as phosphorylation, etc (Rhoads, 1999). But, under most circumstances initiation is the rate-limiting step in translation and is regulated. This mechanism presumably evolved because it is more effective to control the onset of a given biological process than to interrupt it later and, in the case of translation, to have to deal with the consequences of aberrant protein synthesis.

Phosphorylation of eIF2α and translation regulation: eIF2α phosphorylation on the conserved Ser51 residue by different kinases is an immediate response of a cell exposed to stress. Consistently, it is conceived to be a stress signal. The eIF2α kinases contain unique regulatory elements that sense different stress conditions and contribute to stress adaptation. Though kinases are stimulated by distinct stress stimuli because of their unique regulatory regions, they eventually converge upon eIF2α phosphorylation in mammalian cells (Wek et al., 2006) (**Fig C**). The catalytic domains of the eIF2α kinases show the 12 conserved subdomains of all Ser/Thr protein kinases and reveals similarities that make

them distinguishable from other eukaryotic kinases (Hanks and Hunter, 1995). It is notable that the eIF2α kinases possess relatively large insert sequences between catalytic domains IV and V (Samuel, 1993; Hanks and Hunter, 1995). Although the subdomain V is less conserved among the eukaryotic kinases, it shows a significant homology among the known eIF2α kinases, which suggests that this motif might contain the putative substrate specificity domain. The serine/threonine eIF2α kinases are characterized by two distinct kinase activities: trans-auto-phosphorylation, and the phosphorylation of their substrate, eIF2α. Four different eIF2α kinases are well characterized: (i) General control non-derepressible-2 (GCN2), (ii) Double stranded RNA dependent eIF2α kinase (PKR), (iii) PKR like ER-resident eIF2α kinase (PERK) and (iv) Heme regulated inhibitor (HRI). Although, these kinases share extensive homology in the kinase catalytic domains, the regulatory mechanisms of each of these kinases are very different.

1. **GCN2:** This eIF2α kinase is identified first in yeast. It is constitutively expressed, and gets activated in response to amino acid, glucose or purine starvation (Wek et al., 1990; Hinnebusch, 1997). Yeast GCN2 is a 1590 amino acid protein with a molecular mass of about 180 kDa. The catalytic domain of this kinase possesses a large insert domain of 110 amino acids between subdomain IV and V. Adjacent to the kinase domain, a 530 amino acid sequence related to the histidyl-tRNA synthetases (HisRS) is present, which is required for positive regulatory function of GCN2. Uncharged tRNAs that accumulate during amino acid starvation, binds to the regulatory domain and activates GCN2, which involves the dimerization and trans auto-phosphorylation of the kinase. Binding of uncharged tRNAs to His-RS like domain induces conformational change that disrupts the interaction between C-terminal and kinase domain. Auto-phosphorylation ensures further structural alteration of the kinase domain facilitating substrate binding and phosphorylation (Dong et al., 2000). In addition to the uncharged tRNAs, a GCN1/GCN20 complex is also required for the kinase activation. Apart from global shut down of protein synthesis, GCN2 kinase mediates a gene specific translational control of GCN4 mRNA in yeast. The GCN4 gene encodes a transcriptional activator of a large number of genes involved in amino acid and purine biosynthesis. The specific induction of GCN4 translation in response to eIF2α phosphorylation is mediated by four short open reading frames (uORFs) present in the GCN4 mRNA leader sequence. Mammalian cells also possesses an orthologue of GCN2,

activated by amino acid starvation and leads to the translation of ATF4 mRNA, a transcription factor of the same family as that of GCN4 (Wilson and Roach, 2002; Zhang et al., 2002; Vattem and Wek, 2004).

2. **PKR:** The interferon induced double stranded RNA-dependent protein kinase (PKR) phosphorylates eIF2α, in response to viral infections, which results in arrest of translation of both cellular and viral mRNAs, an efficient way to inhibit virus replication. Translation block observed in interferon treated vaccinia virus infected cells and their extracts showed restricted level of translation of cellular and viral mRNAs, led to the discovery of PKR (Friedman et al., 1972). In addition to the kinase domain (KD) shared by the other eIF2α kinases, PKR also has a dsRNA-binding domain (dsRBD) that regulates its activity. As a consequence of dsRNA accumulation in virus infected cells, PKR-triggered eIF2α phosphorylation inhibits translation of viral mRNAs and this constitutes the basic mechanism by which PKR exerts its antiviral activity on a wide spectrum of DNA and RNA viruses. In fact regulation of eIF2α phosphorylation is one of the central mechanisms in the fight between host and virus for their respective survival (see below). The dsRBD wrap around the dsRNA molecule for optimal protein-RNA interactions, and hence the optimal length of dsRNA (~80 bp or longer) is required for an effective activation (Manche et al., 1992; Garcia et al., 2006). In nonstressed cells, PKR is in a monomeric latent state due to the auto-inhibitory effect of its dsRBDs, which occlude the kinase domain. After binding dsRNA, PKR undergoes a number of conformational changes that relieve the auto-inhibitory interactions of the enzyme and allow subsequent substrate recognition. Replacement of the dsRBD with an unrelated domain such as glutathione S-transferase, that is able to dimerize, constitutively activates PKR, both *in-vitro* and *in-vivo*, underscoring the importance of dimerization prior to activation (Ung et al., 2001). After homodimerization, PKR undergoes rapid autophosphorylation in a stretch of amino acids termed the activation segment. Among these, amino acids Thr^{446} and Thr^{451} are consistently phosphorylated during activation. Autophosphorylation further stabilizes PKR dimerization, and increases the catalytic activity of the kinase (Zhang et al., 2001). Biochemical and genetic evidence have underscored the importance of homodimerization in PKR activation (Dey et al., 2005). In addition to its translational regulatory function, PKR has a role in signal transduction and transcriptional control through the IκB/NFκB

pathway (Kumar et al., 1994). PKR, which is expressed constitutively in mammalian cells, has also been implicated in the control of cell growth and proliferation with tumour suppressor function (Koromilas et al., 1992; Meurs et al., 1993). Apart from sensing viral infections, PKR is activated by other activators, such as pro-inflammatory stimuli, growth factors, cytokines, and oxidative stress. It also affects the activity of various factors involved in host defense, stress response and apoptosis, such as STAT, interferon regulatory factor 1 (IRF-1), p53, Jun N-terminal protein kinase (JNK), p38, and NF-κB (Garcia et al., 2006).

3. **PERK:** The PKR like ER resident kinase (PERK) is a transmembrane protein implicated in translational control in response to stresses that impair protein folding in the endoplasmic reticulum (Shi et al., 1998). The accumulation of unfolded proteins in the lumen of ER activates PERK, which is primarily an adaptive measure and a part of coordinated stress response triggered by ER, known as the unfolded protein response (UPR). Activation of PERK attenuates translation and halts the inflow of folding clients into the over burdened ER. PERK contains two domains: a kinase domain with similarity to the other eIF2α kinases and an N terminal domain similar to the IRE1, a protein involved in the unfolded protein response. Under normal conditions, the IRE1 domain binds the ER resident chaperone BiP/ GRP78, but in case of ER stress, BiP gets released from PERK to interact with client proteins in the ER lumen, leading to the oligomerization and subsequent trans auto-phosphorylation. Phosphorylation of serine and threonine residues in the activation loop of the kinase domain activates PERK and promotes eIF2α phosphorylation (Schroder and Kaufman, 2005). In addition to global translation shutdown, PERK mediated eIF2α phosphorylation also upregulates the translation of transcripts with multiple short uORF, like ATF4. ATF4 a bZIP transcription factor inturn induces ER chaperone genes like BiP, and those involved in amino acid transport, glutathione biosynthesis, and redox regulation (Harding et al., 2003).

4. **HRI:** This kinase was first discovered in reticulocytes, the precursors for erythroid cells which primarily manufacture hemoglobin. Heme deprived reticulocytes and their lysates showed rapid translation shut off, an event associated with phosphorylation of eIF2α. This inhibition of translation was rectified by supplementing with heme/iron (Waxman and Rabinovitz, 1966; Hunt et al., 1972). Subsequent studies led to the identification of HRI as

the kinase responsible for this and showed that the activity was repressed by heme (Chen and London, 1995). HRI is a polypeptide of 70 kDa with three regions: the N-terminus domain, the kinase insert domain which is a unique insertion sequence of ~140 amino acids between the subdomain IV and V (Chen et al., 1991; Mellor et al., 1994) and a C terminus domain. Both the N terminus and the kinase insert domain can bind heme, whereas the catalytic domains (kinase I and kinase II) and the C terminus cannot (Rafie-Kolpin et al., 2000). The N terminus is essential for the highly sensitive heme regulation of HRI. Both rabbit and rat HRI show two heme regulatory motifs (HRM), ACPYVM and RCPAQA respectively that are located in the kinase domain (Wek, 1994; Chen and London, 1995). However, they are unlikely to participate in the heme regulation as mutations in HRMs were unresponsive to heme (Zhu et al., 2006). HRI is activated in heme deficiency by multiple autophosphorylation (Bauer et al., 2001). Autophosphorylation is also important in the formation of active, stable HRI that is regulated by heme and, therefore, senses intracellular heme concentration. In low concentrations of heme, the non-covalent homodimer undergoes multiple auto-phosphorylations and phosphorylates eIF2α.

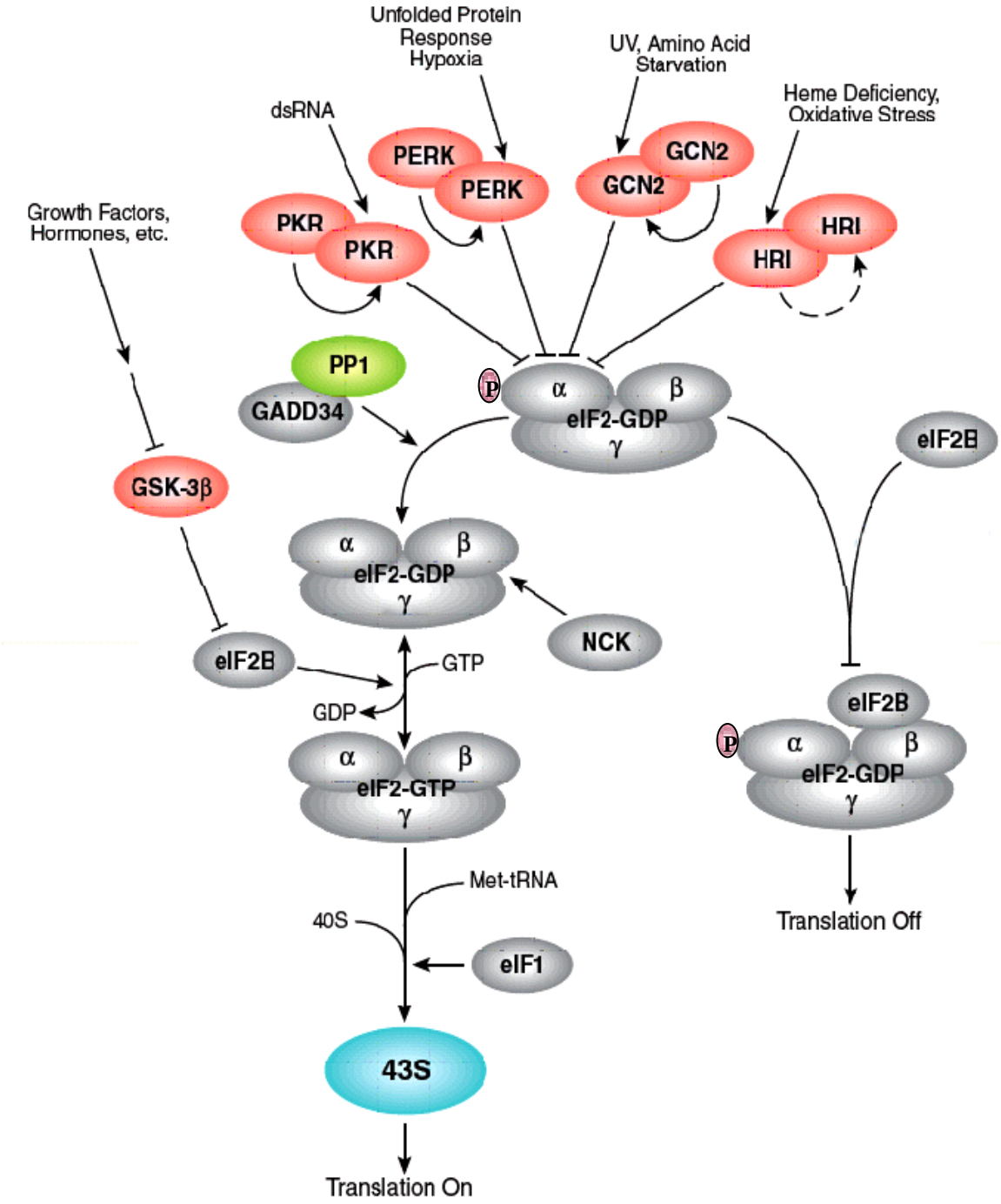
While the mammalian eIF2α is phosphorylated by different kinases on the conserved Ser51 only as described above, yeast eIF2α contains three additional phosphorylation sites at the C-terminus and are constitutively phosphorylated *in-vitro* and *in-vivo* by casein kinase II (Feng et al., 1994) and remain phosphorylated during heat shock, nitrogen starvation, or growth in poor carbon sources (Romero and Dahlberg, 1986).

Translation Initiation and Viral Tricks:

Viruses rely on the host cell for propagation, utilizing cellular machinery for the replication and assembly of viral components and the release of progeny virions. After the virion adsorbs and internalizes into the host cell, uncoating exposes the viral genome and associated proteins to the host milieu, whereupon genome replication and transcription take place. The translation of viral RNA is followed by the assembly of structural proteins, packaging of the viral genome, and eventual release of progeny virions. While some viruses encode or carry the enzymatic machinery required for autonomous genome replication and/or transcription, others recruit host polymerases to carryout this task.

Fig C: Phosphorylation of eIF2α and Translation Initiation Regulation: Phosphorylation of eukaryotic initiation factor 2 (eIF2) is a stress signal and is one of the important pathways to regulate translation initiation. In response to different stress conditions, four different well characterized kinases activated by dimerization and auto-phosphorylation, converge upon phosphorylation of the Ser51 residue in eIF2α resulting in either global control in which the translation of most mRNAs is affected; or mRNA-specific control where the translation of a defined group of mRNAs is modulated without affecting general protein biosynthesis. Phosphorylation of the α-subunit of eIF2 stabilizes the eIF2-GDP-eIF2B complex, inhibiting the GDP/GTP exchange activity of eIF2B. eIF2B is also inhibited by GSK-3β phosphorylation. These events result in a shutdown of cellular protein synthesis and can lead to apoptosis.The figure is derived from cell signaling pathways from Cell Signalling Technologies, USA.

Fig C:


Unfolded Protein
Response
Hypoxia
UV, Amino Acid
Starvation
Heme Deficiency,
Oxidative Stress
dsRNA
PERK
PERK
GCN2
GCN2
HRI
HRI
PKR
PKR
Growth Factors,
Hormones, etc.
PP1
GADD34
P
α
β
eIF2-GDP
γ
eIF2B
GSK-3β
α
β
eIF2-GDP
γ
NCK
eIF2B
GTP
GDP
α
β
eIF2-GTP
γ
Met-tRNA
40S
eIF1
43S
Translation On
eIF2B
P
α
β
eIF2-GDP
γ
Translation Off

In contrast, viruses do not encode or carry the machinery for mRNA translation. Thus, the ensuing stage of viral protein synthesis is completely dependent on the translational machinery of the host cell. Not surprisingly, viruses have devoted much attention to this dependency and have evolved strategies that reduce the impact of translational dependence on viral replication. Many viruses exploit regulated steps in the initiation of host protein synthesis to their own advantage. In addition, many viruses have evolved sophisticated mechanisms to ensure translation of their mRNAs while simultaneously inhibiting cellular mRNA translation (Gale et al., 2000). Many viruses have evolved various strategies for antagonizing the activation of PKR or its ability to inactivate eIF2–GTP recycling. Many viruses upon infection produces RNA or proteins that inhibit the activation of PKR, mimic eIF2 so that they act as pesudosubstrates of PKR and or dephosphorylate eIF2α. Hence the regulation of host cell eIF2α phosphorylation plays a critical role in the outcome of infection of many viruses.

Adenoviruses encode VA RNA_I, a small (160 nucleotide), abundant and highly structured RNA that competitively binds the dsRNA-binding domain of PKR and provides resistance to interferon. Because VA RNA_I possesses only one dsRNA end, its interaction with PKR in the dsRNA-binding site blocks PKR dimerization and activation by viral dsRNA. Epstein–Barr virus (EBV) synthesizes two RNAs, called as EBER RNAs which function similarly as adenovirus VA RNA_I. It is less certain whether human immunodeficiency virus (HIV) utilizes a structured RNA element known as TAR (trans-activating response RNA) to inhibit PKR. Another effective mechanism exploited by several unrelated viruses to undermine the attempts of a host to inactivate eIF2 by α-subunit phosphorylation involves the production of dsRNA-binding proteins that mask or sequester dsRNA and prevent activation of PKR. Proteins such as herpes simplex virus-1 (HSV-1) Us11 (Mulvey et al., 1999; Khoo et al., 2002), vaccinia virus E3L (a poxvirus) (Beattie et al., 1995), reovirus s3 (a dsRNA virus) (Lloyd and Shatkin, 1992) and influenza virus NS1 (Salvatore et al., 2002) all bind dsRNA, inhibit PKR activation and maintain protein synthesis during virus infection. Baculovirus infection of insect cells produces PK2, a truncated eIF2α kinase, a viral protein that is shown to inhibit the activation eIF2α kinases *in-vitro* (Dever, 1998). We have shown recently a mutant baculovirus devoid of its antiapoptotic gene p35 readily stimulates eIF2α phosphorylation and apoptosis in insect cells and inhibition of

caspase activation is found associated with inhibition of eIF2α phosphorylation. Since insect cell caspase can process PKR kinase in-vitro, it is likely the caspase processed eIF2α kinase is more active than unprocessed kinase (Aparna et al., 2003).

In addition of Us11, HSV-1 also encodes the viral γ34.5 protein, which complexes with the cellular protein phosphatase 1a (PP1a) and directs dephosphorylation of eIF2α preventing the accumulation of phosphorylated eIF2α. Salubrinal, a thio urea compound inhibits HSV infection since it inhibits the activity of eIF2α phosphatase produced by produced by host cells in response to HSV infection (Boyce et al., 2005). Vaccinia virus K3L protein and myxoma virus M156R proteins function as PKR pseudo-substrates that bind and sequester the kinase (Carroll et al., 1993; Ramelot et al., 2002). Semliki Forest virus (SFV) seems to tolerate eIF2α phosphorylation due to the presence of the enhancer element that functions well under these conditions. Genetic and biochemical data showed a highly stable RNA hairpin loop located downstream of the AUG initiator codon that is necessary to provide translational resistance to eIF2α phosphorylation. This structure can stall the ribosomes on the correct site to initiate translation of SV 26S mRNA, thus bypassing the requirement for a functional eIF2 (Schneider and Mohr, 2003).

Phosphorylation of eIF2α and regulation of eIF2B activity: At the end of the initiation step of protein synthesis, eIF2 released from the ribosome is bound to GDP, which must be replaced by GTP for it to join Met-tRNAi and form the ternary complex. This guanine nucleotide exchange (GNE) activity, a rate-limiting step, is mainly mediated by a heteropentameric protein eIF2B. Since eIF2 has a higher affinity for GDP (Erickson and Hannig, 1996), eIF2B functions to promote the nucleotide exchange. Joining Met-tRNAi stabilizes eIF2-GTP (Rowlands et al., 1988). eIF2B is a target of translational control by different mechanisms in response to cellular stresses. The well studied and evolutionary conserved mechanism is via phosphorylation of the α-subunit of eIF2, mediated by different kinases in response to various cellular stresses as described above. Due to the limiting amounts of eIF2B, low levels of phosphorylation of eIF2α (20-25%) are sufficient to attenuate protein synthesis as observed in heme-deficient reticulocyte lysates (Leroux and London, 1982) and in other mammalian cells (Rowlands et al., 1988). Further, addition of purified eIF2B (or reversing factor or RF) restores protein synthesis catalytically and

cells containing higher amounts of eIF2B are able to withstand higher levels of eIF2α phosphorylation (Matts et al., 1983; Rowlands et al., 1988; Clemens et al., 1982). eIF2B is a complex of five non-identical α–, β–, γ–, δ– and ε– subunits. Genetic and mutational analyses differentiated the yeast eIF2B into two sub-complexes: a regulatory sub-complex constituted of the α–, β– and δ– subunits and a catalytic sub-complex, constituted by the γ- and ε- subunits (Pavitt et al., 1997 and 98). Phosphorylation of Ser51 in eIF2α, leads to a non-productive interaction between α-subunit of eIF2 and the regulatory sub-complex of eIF2B (Krishnamoorthy et al., 2001; Pavitt et al., 1998), resulting in a tight complex formation between eIF2 and eIF2B holoproteins and the inhibition of the GNE activity of eIF2B (Sudhakar et al., 2000; Ramaiah et al., 1994) (**Fig D**). The distinction as observed in yeast eIF2B is not yet identified in mammalian eIF2B (Pavitt, 2005). In contrast to yeast, both the productive and non-productive interactions between mammalian eIF2 and eIF2B are mediated by the β-subunit rather than the α-subunit of eIF2 (Kimball et al., 1998; Suragani et al., 2005). In addition to phosphorylated eIF2α, mammalian eIF2B can also be regulated by its own phosphorylation. Four protein kinases are identified to phosphorylate eIF2B: CKI, CKII, DYRK (dual specificity tyrosine phosphorylated and regulated kinase) and GSK3 (glycogen synthase kinase 3) (Wang et al., 2001; Woods et al., 2001). DYRK phosphorylates eIF2Bε at Ser539 and acts as a priming kinase that allows phosphorylation at Ser535 by GSK3. Phosphorylation by GSK3, which is regulated by insulin signaling plays an important role in the regulation of eIF2B activity and also has a role in apoptosis (Pap and Cooper, 2002).

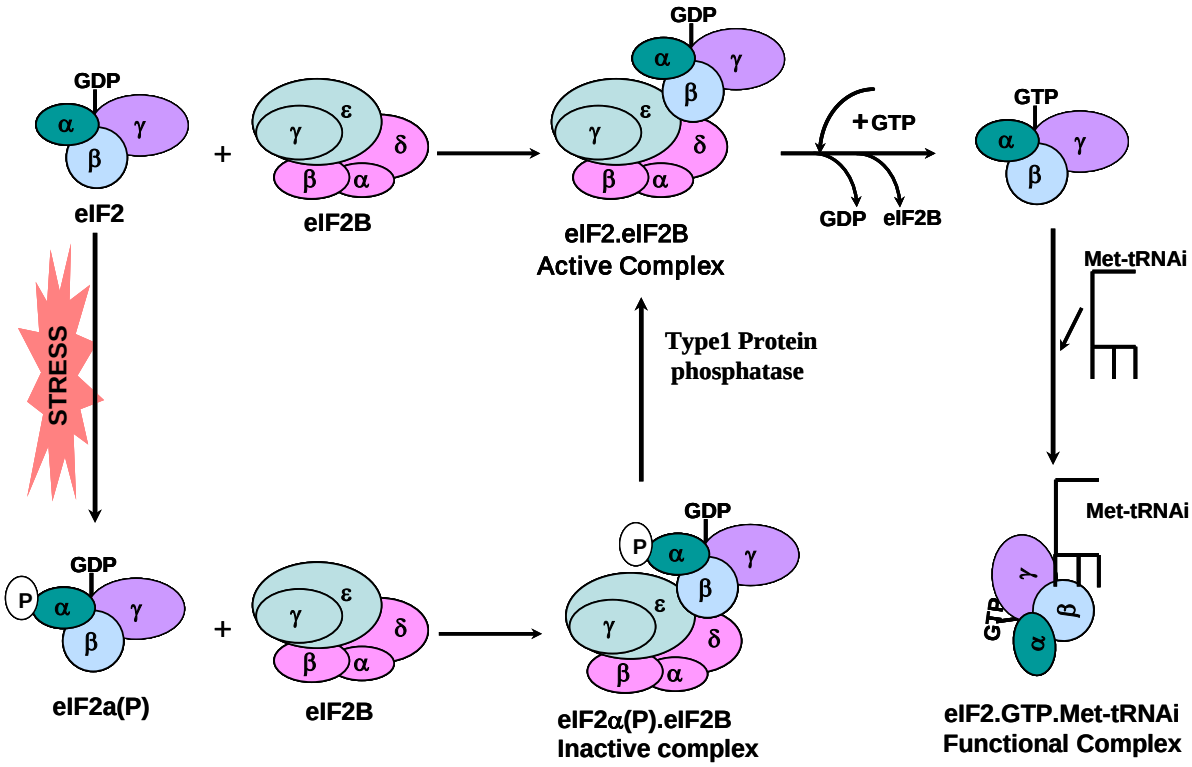
Regulation of translation initiation by dephosphorylation of eIF2α: Phosphorylation of the α-subunit of eIF2 on Ser51, leads to the inhibition of general translation with the concomitant promotion of the translation of specific mRNAs, which is a well-documented mechanism used by cells to respond to physiological stresses (Proud, 2005).

This is well illustrated by the increased translation of the activating transcription factor 4 (ATF4), a transcription factor that initiates a transcriptional program increasing the expression of specific products involved in stress responses. In addition to its regulation by eIF2α kinases, the levels of phosphorylated eIF2α are also controlled by eIF2α-phosphatases that specifically dephosphorylate the α-subunit. This is proposed as a feedback mechanism, allowing translational recovery on cellular stress insults. The eIF2α-

phosphatase activities identified essentially engage PP1 (**Fig D**) (Babu and Ramaiah, 1996) in molecular complexes with various regulatory proteins, such as CReP (Jousse et al., 2003), GADD34 (Novoa et al., 2001) or the virulence factor ICP34.5 (He et al., 1998). A recent report has shown that a SH2SH3 domain -containing adaptor protein Nck1 plays an important role in regulating the levels of phosphorylated eIF2α in ER stress conditions by being part of an eIF2α-holophosphatase complex containing PP1c and it interacts with the β-subunit of eIF2 complex (Latreille and Larose, 2006; Kebache et al., 2002). The exact mechanism, by which Nck1 modulates eIF2α phosphorylation, as well as its role in the holophosphatase complex, still remains to be defined. In contrast to the above observations, salubrinal, a thio urea compound selectively inhibits the dephosphorylation of eIF2α and protects the cells from ER stress induced apoptosis (Boyce et al., 2005).

Physiological significance of eIF2α phosphorylation: Phosphorylation of eIF2α is an immediate response of a cell in response to various kinds of stresses, and as described above, four known eIF2α kinases, PKR, PERK, HRI and GCN2 converge upon the phosphorylation of Ser51 residue in eIF2α in response to varied stress stimuli and help the cell to sense and adapt to various stress conditions. This elicits a stress-responsive gene-expression programme designed to alleviate cellular damage or alternatively induce apoptosis. The eIF2α kinases, mentioned above are activated under varied stress conditions such as suboptimal levels of amino acids, glucose or serum, heat shock, heavy metal stress, redox imbalance, oxidative stress, hypoxia, heme-deficiency, virus infection, UV irradiation and accumulation of unfolded proteins. In addition to inhibiting general protein synthesis, elevated levels of phosphorylated eIF2α mediates gene-specific translational upregulation of certain mRNAs that code for transcription factors that initiate stress-responsive signaling cascades such as redox regulation, amino acid metabolism, and in some cases also initiate apoptosis (Wek et al., 2006; Hinnebusch, 1994; Clemens, 2001; Holcik and Sonenberg, 2005; Harding et al., 2000; Srivastava et al., 1998; Schuener et al., 2006). Transcripts, which are upregulated in this manner, are ATF4, ATF3, CAT1, etc in mammalian cells and GCN4 in yeast (Wek et al., 2006; Harding et al., 2000; Hinnebusch, 1994). The mechanism by which these transcripts are regulated is similar as can be seen in the case of GCN4 in yeast and ATF4 in mammals as described below. The GCN4 mRNA

Fig D:



eIF2α Phosphorylation and eIF2B Regulation: In normal physiological situations, eIF2-GDP, released at the end of the initiation step, is recycled to eIF2-GTP by a heteropentameric guanine nucleotide exchange factor eIF2B, which now can bind Met-tRNAi and initiate another round of translation. In conditions where the cell is exposed to stress, phosphorylation of the Ser51 residue in the α-subunit of eIF2 results in a tight interaction between eIF2 and eIF2B holoproteins thereby inhibiting the GDP/GTP exchange activity, recycling of eIF2 and results in translation shutoff. This translation block can be released by the dephosphorylation of eIF2α by a type 1 phosphatase.

The figure is derived from Ramaiah et al., 1997; Babu and Ramaiah., 1996.

in yeast has four short upstream open reading frames (uORFs 1-4). In normal physiological conditions, when eIF2B is active, the small ribosomal subunits are recharged rapidly with ternary complex after translation of uORF1 making it possible for them to efficiently reinitiate at uORF4. Translation of uORF4 results in dissociation and release of ribosomes because of GC-rich sequence surrounding the uORF4 stop codon. Therefore only few recharged small ribosomal subunits reach the main ORF, representing GCN4 resulting in its low expression. Under conditions of amino acid/serum depletion, GCN2 kinase phosphorylates eIF2α that inhibits eIF2B resulting in low availability of active ternary complex. This physiological condition decreases the efficiency of the small ribosomal subunits in reinitiating at the uORFs and increases the number of ribosomes that continue to scan to the main ORF of GCN4 resulting in an increased induction of GCN4 translation. GCN4, a transcription factor can in turn induce genes involved in amino acid biosynthesis and related metabolic pathways (Hinnebusch, 2005). Similarly, in mammalian cells an identical mechanism involving multiple short uORFs upregulates the translation of ATF4 mRNA when eIF2α is phosphorylated. ATF4, a bZIP transcription factor is a part of the integrated stress response (ISR) and induces genes involved in amino acids transport, glutathione biosynthesis and oxidative stress response (Harding et al., 2003). The mechanism mediated by uORFs appears to upregulate the translation of genes with critical biological functions specifically when general translation is inhibited. The rapidly increasing list of genes containing uORFs that are probably upregulated by eIF2α phosphorylation includes the CCAAT/enhancer binding proteins α and β, human epidermal growth factor receptor-2, S-adenosylmethionine decarboxylase, and β2-adrenergic, retinoic acid, glucocorticoid, and estrogen receptors (Jefferson and Kimball, 2004). In addition, some mRNAs with IRES (internal ribosome entry sequences) elements in their 5'-UTR are also efficiently translated when eIF2α is phosphorylated (Gerlitz et al., 2002; Gebauer and Hentze, 2004). A number of cellular mRNAs such as vascular endothelial growth factor, hypoxia-inducible factor-1α, protein kinase C-δ, basic fibroblast growth factor, platelet derived growth factor (PDGF), c-myc, X-linked inhibitor of apoptosis (XIAP), cationic amino acid transporter 1 (CAT1) and ornithine decarboxylase contain IRES elements. Accumulating evidence suggests that, eIF2α phosphorylation mediated transcript-specific-translational upregulation is important for normal

physiological development, differentiation, stress response, adaptation, memory and in cell death (Gerlitz et al., 2002; Scheuner et al., 2001; Jefferson and Kimball, 2004; Brewer et al., 1999; Harding et al., 2000).

Several studies projected both prosurvival (Harding et al., 2001; Boyce et al., 2005) and proapoptotic (Scheuner et al., 2006; Aparna et al., 2003) nature of eIF2α phosphorylation. For example, expression of a non-phosphorylatable mutant S51A, of eIF2α can suppress apoptosis (Srivastava et al., 1998) whereas activation of PKR through FADD or TNFα pathway can induce or enhance apoptosis (Der et al., 1997; Balchandran et al., 1998; Scheuner et al., 2006). In fact, it was also shown that over-expression of a phosphomimetic mutant form S51D, of eIF2α is sufficient to activate caspase-3 and induce apoptosis (Scheuner et al., 2006). Infection of *Sf9* cells with a mutant baculovirus lacking P35, an anti-apoptotic protein, leads to increased levels of phosphorylated eIF2α, caspase activation and apoptosis (Aparna et al., 2003). Proapoptotic transcription factor CHOP (C/EBP homologous protein) induced via the eIF2α-ATF4 pathway can also probably induce apoptosis when eIF2α is phosphorylated (Marciniak et al., 2004). It is also pertinent to mention that translational attenuation rapidly brings down the levels of short lived anti-apoptotic proteins like IAPs (inhibitors of apoptosis), which allows an increase function of the proapoptotic proteins (Scheuner et al., 2006).

Paradoxically, eIF2α phosphorylation is also shown to protect cells from ER-stress induced apoptosis and viral infection. The protective effects of eIF2α phosphorylation in ER stress may be mediated by decline in client protein load on the overburdened ER and/or by reprogramming the induction of eIF2α–specific stress responsive genes that ensure restoration of ER homeostasis (Boyce et al., 2005; Harding et al., 2001). Repression of a constitutive eIF2α–specific phosphatase (CReP) or ER-stress induced eIF2α phosphatase offered cytoprotection against ER stress, viral infection and heat shock (Jousse et al., 2003; Boyce et al., 2005). Pre-emptive conditional phosphorylation of eIF2α also protected cells from lethal effects of oxidants, peroxynitrite donors and ER stress (Lu et al., 2004). Interestingly severe or prolonged ER-stress can also lead to apoptosis and CHOP is shown to play a significant role in this process (Schroder and Kaufman, 2005). The paradoxical effects of PERK or PKR mediated eIF2α phosphorylation can be visualized because of the

other potential targets of these kinases and/or by the spatial distribution of the targeted eIF2α in the cell. However, to understand the discrepancies involved in these ambiguous outcomes several clarifications still remain to be elucidated. Phosphorylation of eIF2α is also implicated in NF-κB activation, autophagy, hypoxic tumor survival and memory. Although the exact mechanism is unclear, NFκB activation, which plays a predominant role in inflammatory responses and immune development mediated by eIF2α phosphorylation primarily involves the repression of IκBα translation (Deng et al., 2005). ER stress stimuli such as nutrient deprivation and viral infection converge on eIF2α phosphorylation and also stimulate the eIF2α-kinase-dependent autophagy, an important conserved mechanism that involves bulk degradation of cellular contents by autophagolysosomes as an adaptation to environmental stress (Tallóczy et al., 2002). Nrf2, a substrate of the eIF2α kinase, PERK and ATF4, a product of eIF2α phosphorylation are shown to offer protection to tumors in hypoxic microenvironment by inducing an antioxidant gene expression programme (Cullinan and Diehl, 2006). From the above studies it is quite evident that the eIF2α phosphorylation and global translational control play a central role in several important cellular physiological processes.

Phosphorylation of eIF2β and regulation: The mammalian, but not yeast eIF2β-subunit is phosphorylated at least by four known kinases *in-vitro* as mentioned above. Studies have shown that purified and dephosphorylated mammalian eIF2 causes modest stimulation in the ability of eIF2 trimeric complex to bind GDP and this effect is reversed *in-vitro* by phosphorylation of CKII (Singh et al., 1994). A subsequent study has demonstrated that phosphorylation of the recombinant mammalian eIF2β by PKA, but not by CKII or PKC resulted in the stimulation of guanine nucleotide exchange of eIF2.GDP by eIF2B. This is consistent with the observation that PKA phosphorylation site is located in the C-terminus of the protein and this region interacts with the δ and ε-subunits of eIF2B (Kimball et al., 1998). Early studies have shown that the β-subunit undergoes phosphorylation in intact cells and may affect translation (Duncan and Hershey, 1984 and 85). Interestingly, enhanced phosphorylation of β-subunit is correlated to increased protein synthesis (Le et al., 1998). A recent report suggests that the phosphorylation of eIF2β by CKII is constitutive and mutations in these phosphorylation sites affect the functions of eIF2

(Llorens et al., 2006). Although, the β-subunit of eIF2 has multiple phosphorylation sites and is a substrate for four different kinases *in-vitro*, the functional or physiological effect of these phosphorylations have not been analysed in detail so far.

Objectives of the present work: Recent studies (Suragani et al., 2005 and 2006) from this laboratory have shown that the purified recombinant human eIF2 subunits expressed in insect cells using baculovirus expression system can interact with each other in ELISA and dot blot studies in addition to the ability of the α-subunit especially to interact with eIF2α kinases like recombinant purified PKR and PERK, and β-subunit to interact with purified eIF2B. However we also observed that both the α and β-subunits of human eIF2 are phosphorylated in insect cells during expression. Some of these observations (Suragani et al., 2005 and 2006), particularly, showing the interaction between the α and β-subunits of human eIF2, and the lack of interaction between human eIF2 α and eIF2B differ from the observations shown in yeast. The current model of eIF2 indicating that γ-subunit is central in the eIF2 trimer interacting with the α and β-subunits on either side and without any interaction between the α and β-subunits (Martinchev and Wagner, 2004) fits well with the current findings of yeast eIF2 where the β and γ-subunits are required for various functions of eIF2. Inhibition in the catalytic GDP/GTP exchange activity of eIF2B due to phosphorylation of the α-subunit of eIF2 is found to be mediated by a non-productive interaction between the regulatory subcomplex of eIF2B (α, β and δ) and the α-subunit of eIF2 in yeast. To demonstrate further and confirm that the subunits of human eIF2, particularly the α and β-subunits differ from their yeast counterparts and that the effects of phosphorylation of α-subunit on the regulation of mammalian eIF2B activity is mediated by the β-subunit of eIF2, I have under taken the present studies to express all the three subunits of human eIF2 in bacteria to obtain them in an unphosphorylated form and study the intersubunit and interprotein interactions of phosphorylated and unphosphorylated eIF2α and β-subunits.

The second part of the thesis is taken up to study the importance of eIF2α phosphorylation on apoptosis in insect cells. The ovarian cells of *Spodoptera frugiperda* (*Sf9),* a lepidopteran insect, are natural hosts for baculovirus infection and are used for the

expression of heterologous recombinant proteins. In addition, they are found to be good model systems for studying the process of apoptosis. An earlier study from this laboratory has shown that wildtype (wt) baculovirus, that produces p35, an anti apoptotic protein or synthetic caspase inhibitor like z-VADFMK, inhibits UV-mediated eIF2α phosphorylation and apoptosis in insect cells (Aparna et al., 2003). In contrast a mutant p35 baculovirus readily stimulates eIF2α phosphorylation and apoptosis. However some of the agents like tunicamycin, thapsigargin etc., that typically promote stress in the endoplasmic reticulum (ER) due to impaired protein glycosylation or calcium fluxes promote eIF2α phosphorylation without apoptosis. Unlike in mammals, *Sf9* insect cells may not carry an ER-stress induced apoptotic mechanisms as has been postulated recently (Hussain and Ramaiah, 2007). An earlier report suggested the baculovirus PK2 protein that resembles truncated eIF2α kinase, inhibits the eIF2α kinase activity (Dever et al., 1998). Further a recent report indicates that PKR-induced eIF2α phosphorylation stimulates caspase activity and apoptosis in mammalian cells (Scheuner et al., 2006). Based on these studies, I have evaluated the changes in phosphorylation status of eIF2α, caspase activity and apoptosis in uninfected, and wt and mutant pk2 baculovirus–infected *Sf9* cells in the presence and absence of UV-irradiation. As virus infection is known to promote ER stress and eIF2α phosphorylation is known to upregulate the expression of ATF4, a b-Zip transcription factor, the levels of Bip, an ER chaperone and ATF4 were monitored, while apoptosis was correlated to caspase activity.

Materials and Methods

Materials:

Most of the biochemicals, unless otherwise indicated, used in this study were obtained from Sigma, USA. The vector, pET32a and Enterokinase cleavage kit were obtained from Novagen, USA. Restriction endonucleases Nco1, Hind III, Xho-1 were from New England Biolabs. PCR and gel elution kits were procured from Eppendorf, USA. Ni-NTA agarose beads were obtained from Qiagen. Radioactive [γ-^{32}P]ATP was obtained from BRIT, India. Caspases 3 and 6 were obtained from Calbiochem, USA. Ac-DEVD-AFC and Ac-DEVD-CHO were procured from BD Pharmingen. TNM-FH insect cell culture medium was obtained from Sigma, USA. Fetal bovine serum was obtained from Gibco BRL. Sterilized filters (0.22 and 0.45 micron) were purchased from Millipore. Nitrocellulose membrane was purchased from Pall Biosciences, USA. Whatman I and III filter papers were obtained from Whatman, UK. Tissue culture flasks and Petri dishes were obtained from TPP plastic ware, Switzerland.

Monoclonal anti-his-tag, anti-eIF2β, anti-Nck1 and polyclonal antibodies against eIF2α, eIF5 and GADD 34 were obtained from Santacruz, USA. Polyclonal phosphospecific anti-eIF2α and anti-eIF2Bε antibodies were obtained from Cell Signalling, USA. Polyclonal anti- ATF4 and BiP antibodies were purchased from Abcam, UK. Secondary antibodies conjugated with alkaline phosphatase were obtained from Promega, USA.

Methodology:

Purification of recombinant human eIF2 subunits: Purification of the recombinant subunits from *E. coli* lysates was carried out using Ni-NTA agarose beads as described previously (Suragani et al., 2006), with the following modifications. BL-21 cells expressing human eIF2α, β or γ- were harvested and lysed at 4^0C in a buffer containing 20 mM Tris-HCl, pH 7.8, 300 mM NaCl and 1 μg /ml protease inhibitors as described above. Lysates were then clarified by centrifugation at 12, 000 rpm for 20 min and the supernatant was loaded onto a 1 ml pre-equilibrated Ni-NTA agarose resin at 4^0C. The column was washed with 10 volumes each of buffer 1 containing 20 mM Tris-HCl, pH 7.8, 500 mM NaCl and 30 mM imidazole and buffer II containing 100 mM NaCl and 50 mM imidazole.

Bound proteins were then eluted with Tris-HCl buffer containing 100 mM NaCl and 250 mM imidazole and separated by 10% SDS-PAGE.

His-tag cleavage of the subunits: Purified his-tagged subunits were subjected to his-tag cleavage by using enterokinase cleavage kit from Novagen. In this assay, ~20-30 µg of the protein was incubated with 0.2 U of enterokinase in the cleavage buffer, supplied by the manufacturer for 8 hrs-overnight at 20^{o}C. Recombinant enterokinase was then removed by passing the reaction mixture through EKapture agarose as per the manufacturer's instructions. His-tag cleaved proteins were analyzed on 10% SDS-PAGE.

Sodium Dodecyl Sulphate-Polyacrylamide gel electrophoresis (SDS-PAGE): Proteins were separated by a modified Laemmli method (1970). 8 ml of the 10% separation gel mixture contained the following: 1.875 ml of 2M Tris-HCl, pH 8.8, 2.5 ml of 30:0.8 acrylamide: bis-acrylamide, 75 µl of 10% SDS, 50 µl of 10% ammonium per sulphate (APS), 6 µl of TEMED and 3.75 ml of water. The 5% stacking gel mix in total volume of 2.5 ml contained: 1.875 ml of water, 0.375 ml of 2M Tris-HCl, pH 6.8, 0.325 ml of acrylamide: bis-acrylamide solution, 25 µl of 10% SDS, 50 µl of APS and 6 µl of TEMED. Proteins were prepared in a sample buffer containing 0.25M Tris-HCl, pH 6.8, 10% SDS, 40% glycerol, 5% β-mercaptoethanol and 0.05% bromophenol blue. Gel electrophoresis was carried out at 120 volts with Tris-SDS-Glycine buffer (0.3% Tris-HCl, 1.5% Glycine, 0.1% SDS) until the dye front ran into the lower buffer. Proteins in the gel were visualized by coomassie staining.

Western Transfer: After separation of proteins by SDS-PAGE, the proteins were transferred on to nitrocellulose membrane electrophoretically. Transfer of proteins was carried at 70 volts for 4 hr at 4^{o} C in transfer buffer (25 mM Tris and 195 mM Glycine in 40% methanol). Afterwards, the membrane was removed and stained with Ponceau S red solution. Marker proteins were marked and the excess stain was removed with double distilled water. Protein free regions of nitrocellulose were blocked with TBST (Tris-HCl, pH 8.0, 150 mM NaCl and 0.05% Tween-20) containing 3% milk powder for 1 hr at room temperature. The blocking solution was decanted and the membrane was rinsed once with TBST solution. The membrane was then incubated with a monoclonal primary antibody

(diluted 1:300) or a polyclonal antibody (1:1000) overnight at 4^{o}C with gentle shaking. The nitrocellulose membrane was washed with TBST for three times (x 10 min) to remove unbound antibody. The membrane was then incubated with alkaline phosphatase conjugated anti-mouse IgG or alkaline phosphatase conjugated anti-rabbit IgG (1:5000) for monoclonal or polyclonal antibodies respectively. Incubation was carried out for 1 hr at room temperature. Primary and secondary antibodies were stored at –20^{o} C for reuse. The membrane was washed with TBST three times (x 10 min), to remove unbound secondary antibody. Membrane was then treated with the developing solution (66 µl of NBT, 33 µl of BCIP in 10 ml of AP buffer containing 100 mM Tris-HCl, pH 9.5, 100 mM NaCl and 5 mM $MgCl_2$). The solution was removed soon after the development of bands and the blot was thoroughly washed with water and stored.

***In-vitro* phosphorylation of purified recombinant human eIF2 α (wt and mutants):** Phosphorylation of purified his-tagged eIF2α (wt and mutants) subunits were carried out in 25 µl reaction volume containing 20 mM Tris-HCl, pH 7.8, 80 mM KCl, 2 mM $Mg(oAc)_2$ and 100 µM cold ATP (30 µM of cold ATP was used in the reactions performed in the presence of labeled [γ^{32}P] ATP) in the presence of purified recombinant mouse PERK or PKR-GST for 10 min. The reactions were terminated by addition of SDS-sample buffer and boiled briefly. The proteins were separated by 10% SDS-PAGE and later transferred to a nitrocellulose membrane. The phosphorylation status of the recombinant proteins was analyzed by a phosphospecific anti-eIF2α antibody or phosphorimaging or by autoradiography.

***In- vitro* phosphorylation of purified recombinant human eIF2β:** Phosphorylation of he β-subunit was carried out in the presence of 10 µCi [γ^{32} P]-ATP (3000 Ci/ mmol) in 25 µl reaction volume and by the addition of 20 units of each of the kinases viz., casein kinase II (CK II), protein kinase A (PKA), protein kinase C (PKC) and DNA-dependent protein kinase (DNA-PK) as per manufacturer's instructions. Reaction mixtures contained 30 µM unlabelled ATP. Samples were separated by 10% SDS-PAGE and transferred to nitrocellulose membrane. The membrane was than monitored by a phosphorimage and then probed with an anti-eIF2β antibody.

Autoradiography/Phosphor-imaging: The dried gels or immunoblots containing labeled proteins were exposed to a phosphor imager screen for appropriate time depending on the radioactivity label used and the amount of radioactivity incorporated as determined by the GM-counter. The exposed screens were scanned in Typhoon phosphor imager (Amersham) according to manufacturer's instructions. Alternatively, the dried gels/immunoblots were exposed to X- ray film (Kodak) at -70^{o} C for the required time and developed by a set of photographic solutions obtained commercially as per conventional autoradiography.

Densitometric analysis: Densitometric analysis of the immunoblots was performed using 'ImageJ' software as per the instructions. The relative intensities obtained from three different individuals experiments were plotted as vertical bars.

Pull Down Assay: 50 µl of Ni-NTA resin was incubated with ~8 µg of one of the purified his-tagged recombinant subunits for 60 min at 4^{o}C. Unbound proteins were collected by centrifugation at 1500g. The resin bound subunit was incubated with one of enterokinase treated, his-tag cleaved, purified subunits to determine the interactions among the purified subunits. Alternatively, the resin bound subunits was also incubated with either reticulocyte or HeLa cell extracts to determine the interaction of the recombinant subunits with the subunits of the reticulocyte lysate eIF2 or with other proteins such as eIF2B, eIF5 or Nck1 of the extracts. These incubations were carried out at 4^{o}C. Unbound fractions were collected by centrifugation and the resin was extensively washed with TBS containing 0.1% Tween-20. The protein complex bound to the resin was then eluted with 500 mM imidazole. The unbound, wash and bound/eluted fractions were collected and separated by 10% SDS-PAGE, transferred to a nitrocellulose membrane and the membranes were treated with anti his-tag antibody for the γ-subunit, anti-eIF2α or β-antibodies for the detection of the specific subunits. Anti- eIF2Bε, eIF5 or Nck1 antibodies were used to detect the specific proteins as described in the figures.

Pull down assays were also carried out by incubating Ni-NTA resin with the phosphorylated α- or β- subunits to determine the effect of phosphorylation of these subunits on their intersubunit or interprotein interactions.

Far Western Analysis: In these studies, 5 μg of purified his-tagged recombinant α-, β- and γ- subunits were separated by 10% SDS-PAGE and transferred to a nitrocellulose membrane. The membranes were incubated with non-fat milk powder in TBS containing 0.1% Tween 20 (TBST) for one hr at room temperature to block the protein free regions of the membrane. Membranes were then incubated with 10 ml of diluted rabbit reticulocyte lysate for studying the interaction of proteins such as Nck1, eIF5, and the subunits of endogenous eIF2, or with the purified recombinant subunits of eIF2 in phosphate buffered-saline containing 0.1 % tween-20 for one hr at 4^0C. The membranes were then washed in TBST and treated with a rabbit polyclonal anti-eIF2α antibody or by a monoclonal anti-eIF2β antibody as described in the legends to figs to determine the interactions among the subunits or with anti-Nck1 or anti-eIF5 to determine the subunit interactions with the respective proteins of the lysate. The blots were washed and then treated with the respective alkaline-phosphatase conjugated secondary antibodies.

Cleavage of eIF2α and β-subunits by caspase 3: Caspase 3 mediated cleavage of the eIF2α- or β-subunits was carried out by incubating ~5 μg of the purified subunit with 300 ng of purified recombinant caspase 3 in a buffer containing 20 mM Hepes, pH 7.5, 250 mM sucrose, 50 mM KCl, 2.5 mM $MgCl_2$ and 1 mM DTT at 37^0 C. The recombinant α-subunit was incubated for 8 hrs while the β-subunit was incubated for 6 hrs. The samples were then separated by 10% SDS-PAGE and proteins were transferred to a nitrocellulose membrane. Cleavage of the proteins was analyzed by SDS-PAGE and western blotting.
To determine the effect of phosphorylation of α- and β-subunits on their cleavage by caspase 3, phosphorylation of the subunits was carried out with respective kinases as described above before and after the caspase 3 cleavage steps.

Preparation of rabbit reticulocyte lysates: White male Newzealand rabbits were used for the preparation of heme-deficient rabbit reticulocyte lysates. The lysates were prepared as described previously (Hunt et al., 1972; Ernst et al., 1978). Rabbits were injected with 1% acetyl phenyl hydrazine (APH) daily for four days and left for 3-4 days for anemia to set in. The rabbits were bled on the 7^{th} or 8^{th} day, through the ear or optic vein. Blood was collected in 30 ml pre-cooled corex tubes coated on the inner walls with heparin (300 IU for 30-50 ml of blood) and subsequent steps were carried out at 4^oC. The red blood cells

(RBC) were isolated by centrifugation at 3000 rpm for 5 minutes at 4^{o}C. The supernatant was carefully removed with a Pasteur pipette and the cell pellet was suspended in buffered saline (5 mM Hepes, pH 7.2, 130 mM NaCl, 5 mM $MgO(Ac)_2$ and 5 mM glucose). The suspension was then centrifuged at 3000 rpm for 5 min at 4^{o}C and the buffy coat layer present above the cell pellet was carefully removed. This step was repeated three times to ensure the complete removal of buffy coat. The cells were then lysed by adding equal volume of ice cold sterile water and incubated on ice for about 2-5 minutes. The suspension was centrifuged at 10000 rpm for 20 min at 4^{o}C and the supernatant was collected and stored as 250 µl aliquots in liquid nitrogen.

Immunoprecipitation: Interaction between recombinant eIF2α and cytochrome c of HeLa cell extracts was analyzed by immunoprecipitation. In this assay, recombinant eIF2α was mixed with purified cytochrome c and was immunoprecipitated by anti- eIF2α or cytochrome c antibodies. The immunoprecipitated samples were separated by SDS-PAGE and transferred to a nitrocellulose membrane. The proteins, eIF2α and cytochrome c were recognized by their respective antibodies. Further, immunoprecipitation was also carried out to demonstrate the interaction between the recombinant α-subunit of eIF2 and cytochrome c of HeLa cell extracts. In this experiment, recombinant purified eIF2α was added to cycloheximide (CHX) treated HeLa cell extracts (CHX treatment induces cytochrome c release from mitochondria and stimulates apoptosis). The reaction mixture was then immunoprecipitated with the addition of anti-cytochrome c antibody. The immunoprecipitated samples were separated by 15% SDS-PAGE and transferred to a nitrocellulose membrane. The western blot was probed with anti-eIF2α antibody to determine the interaction between eIF2α and cytochrome c.

Determination of equilibrium constant for the binding of eIF2α to cytochrome c: Titration experiments were carried out holding cytochrome c constant at one of the concentrations in the 1–4 µM range. A set of samples containing a fixed concentration of cytochrome c and variable concentration of eIF2α (0–5 µM) was prepared in a buffer composed of 20 mM Tris-HCl, pH 7.8, 100 mM NaCl, and 1 mM DTT. This procedure of equilibrium titration annulled dilution effects. Samples were incubated for ~2 hrs at 22 °C

before recording baseline-corrected optical absorption spectra in the 600–400 nm wavelength region using a Cary 100 (Varian) spectrophotometer. Absorbance at 415 nm, which is the Soret maximum for DTT-reduced cytochrome c, was used for binding analysis. The concentration of free eIF2α in the titration mixture is given by

$$[eIF2\alpha]free = \frac{[cytc]\times \Delta A}{\Delta A\infty}$$

in which, ΔA=Ax−Ao and ΔA∞=A∞−Ao, where Ao, Ax, and A∞ are 415-nm absorbances of solutions containing cytochrome c alone, cytochrome c in the presence of x concentration of eIF2α, and cytochrome c in the presence of infinite or saturating concentration of eIF2α, respectively. The association constant, K*ass*, was extracted from the equation

$$\log\left[\frac{\Delta A}{A\infty - Ax}\right] = \log Kass + \log[eIF2\alpha]free$$

The *x*-intercept of the plot of log [ΔA/ (A∞−Ax)] vs. log [eIF2α] gives the value of pKa for cytochrome c–eIF2α interaction.

Insect Cell Culture: *Spodoptera frugiperda* (*Sf9*) cells were grown in TNM-FH medium supplemented with 10% fetal bovine serum and 1% antibiotics at 27^{o}C in an incubator. After the cells are grown to confluence, the cells were dislodged from the flasks into fresh medium by gentle pipetting and transferred into new flasks for sub-culturing. Cells with >95% viability were used in all experiments.

Preparation of TNM-FH medium: TNM-FH medium is a modified Grace's basal insect cell culture medium (Grace, 1962) which is supplemented with lactalbumin hydrolysate and yeastolate ((Hink, 1970). This medium contains all the basic nutrients required for the growth of insect cells and it is buffered with sodium phosphate. In order to prepare 1 litre of TNM-FH medium, 46.3 g of TNM-FH powder medium was dissolved in 700 ml of distilled water and 0.35 g of NaHCO3 was added and the pH was adjusted to 6.2 using 10N NaOH. The medium was then made up to 1 litre and filter sterilized (0.22 microns) in the laminar hood. 10% fetal bovine serum and antibiotics were added later to make the complete medium.

Freezing and reviving of *Sf9* cells: *Sf9* cell stocks were prepared from >90% healthy log-phase cultures. The cells were harvested from the confluent cultures and suspended in a medium containing 10% DMSO. The cell suspension was aliquoted into screw-capped tubes at a final cell density of 4 X 10^6 cells/ml and later frozen gradually. The cells were incubated at 4^{o}C initially for 2 hrs, at –20^{o}C for 2 hrs and at -70^{o}C for 12 hrs before placing them finally in liquid nitrogen. For reviving, the frozen stocks were thawed by placing them in 37^{o}C water bath and later the contents were transferred into a culture flask. The cells were then washed in complete medium devoid of DMSO and incubated at 27^o C.

Preparation of cell extracts: *Sf9* cells were dislodged from the flasks or dishes and harvested by centrifugation at 4000 rpm for 5 min at 4^{o}C. Cell pellet was washed with ice cold PBS twice, and suspended in lysis buffer containing 20 mM Tris-HCl, pH 7.8, 1 mM $MgCl_2$, 1 mM DTT, 5 μg/ml each of pepstatin, aprotinin and leupeptin, and 250 μM PMSF. Cells were incubated in lysis buffer for 10 min on ice and lysed by pipetting. The suspension was clarified at 12000 rpm for 20 minutes at 4^{o}C and the supernatant was collected in a fresh eppendorf tube and stored at -70^{o}C.

Virus infection and UV irradiation of *Sf9* cells: For virus infection, cells with 80-90% viability were selected. The spent medium in the flask was replaced with 500 μl of fresh complete medium containing either wild type (wt) or a mutant pk2 deleted (Δpk2) baculovirus with a multiplicity of infection (MOI) of 10. The infection was given for one hour with intermittent rocking of the petridishes or culture flasks once in 15 minutes. After 1 hour, 1-4 ml of complete medium was added to the petridish or flask and incubated in a dark and humid environment for 30-35 hours at 27^{o}C in an incubator.
Uninfected or virus infected *Sf9* cells were irradiated with UV-B light (312 nm) for 60 seconds and incubated in dark at 27^{o}C for different time periods as described previously (Aparna et al., 2003).

Assays for apoptosis: 2-4 X 10^6 cells were used for each experiment and apoptosis was scored by monitoring plasma membrane blebbing under an inverted microscope equipped with a digital camera and the software MV500 DEMO. Trypan blue exclusion test was also

carried out to assess the viability of the cells by adding 0.4% Trypan blue to 50 μl cell suspension to a final concentration of 0.04% and then the cells were counted using a hemocytometer.

Caspase Activity: Cell extracts were prepared from the cells exposed to different treatments as described above. Caspase activity in these extracts was monitored as a measure of Ac-DEVD-AFC, a fluorogenic caspase substrate, hydrolysis. Cell extract containing ~150 μg of protein was incubated with 10 μM Ac-DEVD-AFC in a buffer containing 20 mM Tris-HCl, pH 7.2, 1 mM Mg^{2+}, 80 mM KCl and 1 mM DTT. Hydrolysis of Ac-DEVD-AFC was monitored from 450-550 nm in a Jobin Yvon, Horiba spectrofluorimeter.

Protein estimation: Cell lysate proteins and purified proteins were estimated by a Bio-rad protein estimation kit as per the manufacturer's instructions.

Part I

Chapter 1

Expression and Purification of the Human Eukaryotic Translation Initiation Factor 2 (eIF2) subunits in Bacteria: Characterization of the subunits.

Introduction:

The human eukaryotic translation initiation factor 2 (eIF2) is a trimer and plays a critical role in the initiation of translation by transferring the Met-tRNAi to the 40S ribosomes in a GTP dependent manner. The native eIF2 consists of α, β and γ subunits in 1:1:1 ratio. Phosphorylation of the conserved Ser51 residue in eIF2α is one of the key mechanisms in the regulation of translation. Although, this mechanism is conserved both in yeast and mammals, the mechanistic details involved in the regulation of protein synthesis through phosphorylation of eIF2α appear to differ. Recent studies from this lab have expressed the human eIF2 subunits in insect cells using the baculoviral expression system to study the intersubunit interactions and observed that the recombinant α and β subunits are phosphorylated in the insect cells during expression and that all three subunits interact with each other in a dot blot and ELISA assays (Suragani et al., 2006 and 2005).

One of the objectives of the present thesis is to express the subunits of human eIF2 in bacteria which are not phosphorylated during expression in order to further understand the intersubunit and interprotein interactions and to evaluate the effect of phosphorylation of the α and β-subunits on these interactions. Accordingly, the first part of the chapter deals with the cloning of the subunits of human eIF2 into bacteria, time-course of their expression, the ability of the α and β-subunits to serve as substrates for bacterial kinases during expression, purification of the subunits by affinity matrix and the phosphorylation of the purified subunits by purified kinases *in-vitro*. Since eIF2 has a high affinity for GDP in the presence of physiological concentrations of magnesium (Mg^{2+}), and the GDP bound eIF2 cannot participate in initiation unless the bound GDP is exchanged for GTP, we have also evaluated the abilities of the subunits alone and the combinations of different subunits to bind and retain labeled GDP.

Results & Discussion:

Cloning of cDNAs of the subunits of human eIF2 into pET32a vector: The cDNAs representing the subunits of human eIF2 and mutants of eIF2α harbored in the baculovirus transfer vector, pFast Bac HT (Suragani et al., 2006), were amplified in the DH5α cells of *E. coli*. The mutants of eIF2α are as follows: S51A represents a non-phosphorylatable form where the 51 serine residue is replaced by an alanine; S48A, a mutant of eIF2α in

which serine 48 is replaced by alanine and contains the conserved 51 phosphorylaton site, and S51D, a phosphomimetic form of eIF2α in which the 51 serine residue is replaced by aspartic acid. Plasmid DNA was isolated and the cDNAs of α-, β-, and mutants of eIF2α were then excised from the parent plasmid using Nco1 and Hind III. However, restriction endonucleases Nco1 and Xho1 were used to release the γ-cDNA. The excised and released cDNAs were then ligated into the pET32a vector linearized by the restriction endonucleases as mentioned above. The flow chart depicting the release of cDNAs from parent vector to cloning them into pET32a vector with a 6x his-tag has been shown in **Fig 1.1.** The recombinant clones of pET32a vector harboring the subunits of human eIF2 were confirmed by restriction digestion analysis as shown in **Fig 1.1A** and also by PCR using gene-specific primers as shown in **Fig.1.1B.** The clones were then transformed into BL21(DE3)pLysS cells and the protein expression was induced in the presence of 1 mM IPTG. Expressed proteins were then purified using Ni-NTA agarose affinity matrix.

Expression and purification of recombinant subunits of human eIF2:

BL21(DE3)pLysS cells, transformed with recombinant clones were grown in LB broth containing ampicillin and chloramphenicol. 1 mM IPTG was then added to the cultures and incubated for 3-4 hrs to induce the expression of recombinant proteins. Cells were then harvested and the cell pellet was resuspended in SDS sample buffer. The expression of the recombinant α-, β- and γ- subunits of human eIF2, in response to IPTG was analyzed by a western blot and probed by an anti his-tag antibody. There was negligible or no expression without the addition of IPTG (**Fig 1.2**).

Time based expression of the recombinant eIF2 subunits indicates that the expression is time-dependent (**Figs 1.3 A-C**). The expression of the α and β subunits starts at 30 min post induction (**Figs 1.3 A** and **B**), and that of the γ subunit starts around 1 hr (**1.3C**). Optimum expression of all the subunits is observed around 4 hrs post induction. After 4 hrs, the expression of the proteins remained more or less constant. The expression of the subunits in extracts prepared from IPTG-treated cells was detected in coomassie stained gels (**Fig 1.3. A, B** and **C**).

Fig 1.1:

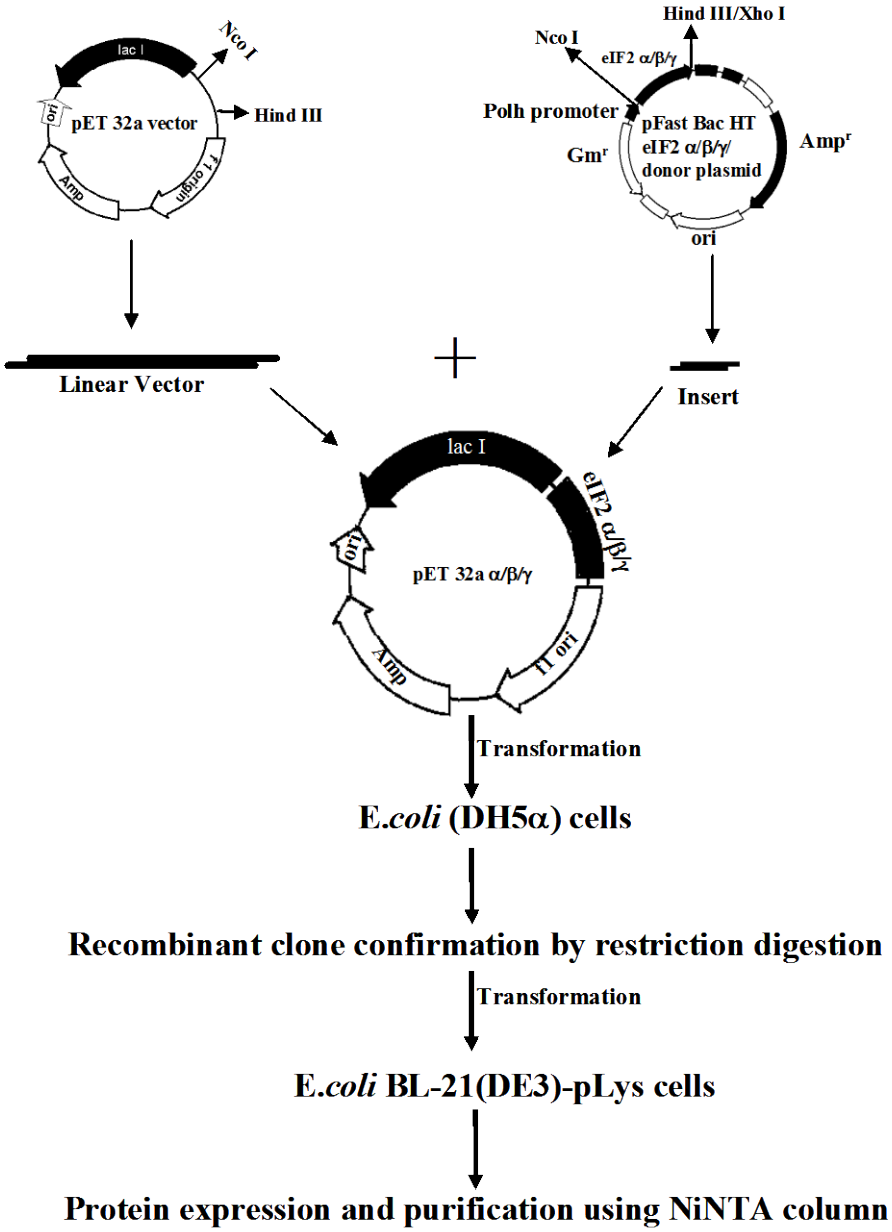


Fig 1.1:

The figure depicts the flowchart describing the cloning of the subunits of human eIF2 α-, β- and γ- and the muntants of eIF2α into pET 32a vector. The inserts representing the cDNAs of the eIF2 subunits harbored in the pFAST Bac vector were excised and ligated into the linearised pET32a vector as described in the text.

Fig 1.1A: Restriction analysis of recombinant clones: The excised cDNAs of eIF2α-, β-, γ- and mutants of eIF2α were ligated into pET32a vector and the ligation mixture was transformed into DH5α cells. Antibiotic resistant colonies were selected and recombinant plasmids were isolated as described in 'Materials and Methods'. The plasmids were subjected to restriction digestion with Nco I and Hind III for recombinant pET32a vector harboring wt and mutants of eIF2α and β- subunits, while Nco I and Xho I were used for pET32a harboring the γ-subunit for confirmation of the recombinant clones. The samples were analyzed by 1% agarose gel electrophoresis. The lanes are as mentioned in the figure.

Fig 1.1B: Recombinant clones confirmation by PCR: The recombinant clones were further confirmed by PCR performed using gene specific primers for eIF2α, β and γ subunits as described earlier (Suragani et al., 2006). The samples were analyzed by 1% agarose gel electrophoresis. Various lanes are as mentioned in the figure.

Fig 1.1A:

Fig 1.1B:

Fig 1.2:

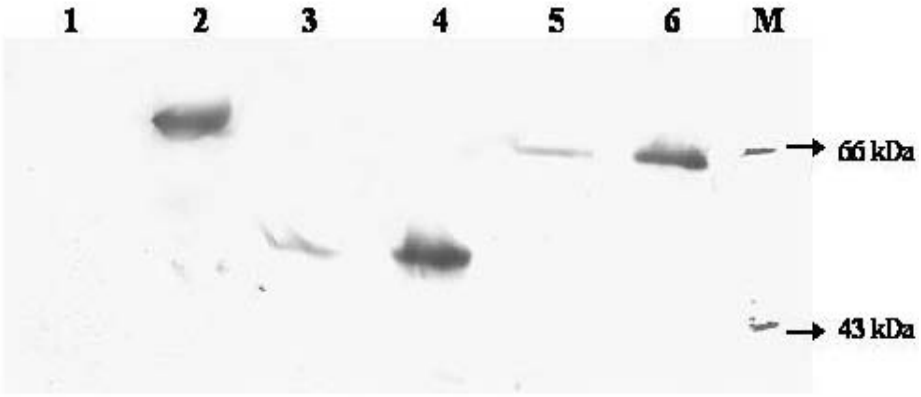
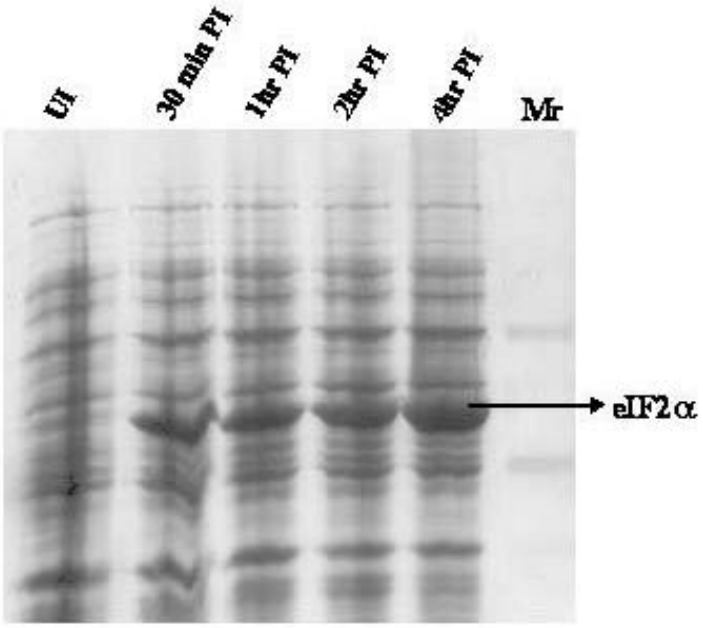


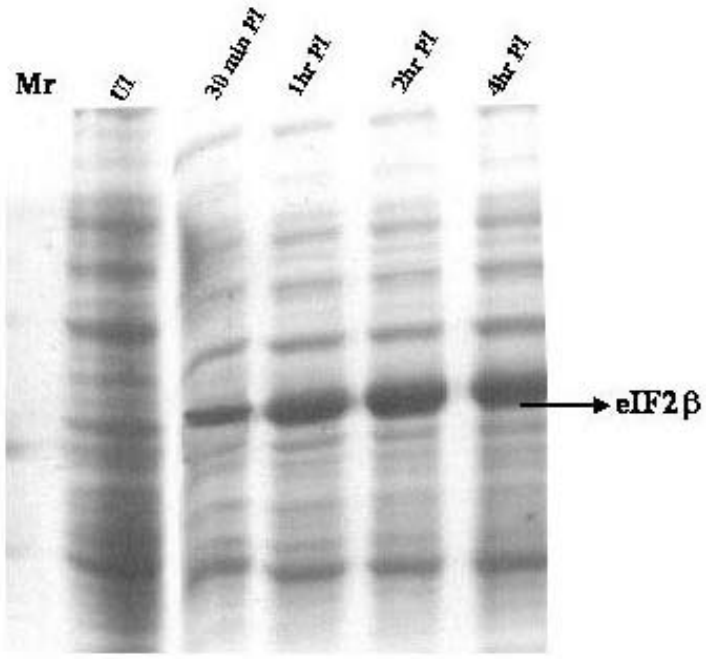
Fig 1.2: Expression of the subunits of human eIF2 in bacteria: The cell pellets were suspended in SDS-PAGE sample buffer and the samples analyzed on 10% SDS-PAGE. The proteins were transferred onto nitrocellulose membrane. The figure represents a western blot probed with anti-histag antibody. Lanes 1 and 2 represent the expression of the γ-subunit, whereas lanes 3, 4 and 5, 6 represent the expression of α- and β-subunits respectively in the absence and presence of 1 mM IPTG. Lane M represents molecular weight marker.

Fig. 1.3A:



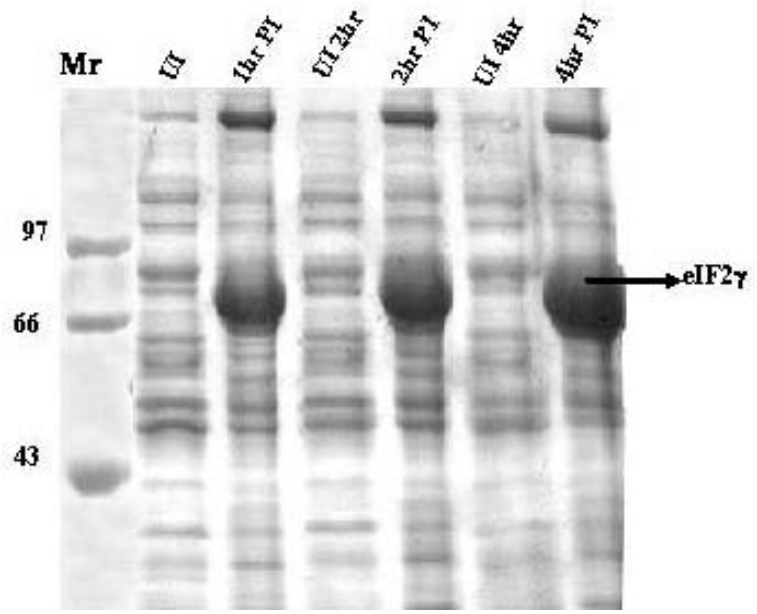
Expression of the α-subunit of human eIF2.

Fig. 1.3B:



Expression of the β-subunit of human eIF2.

Fig. 1.3C:



Expression of the γ-subunit of human eIF2.

Fig. 1.3A, B and C: Time-course of expression of recombinant eIF2*α*, β or γ: Transformed BL-21(DE3)-pLysS E.coli cells were cultured at 37^0 C till an O.D of 0.8 at 600 nm was attained. 1 mM IPTG was then added to cultures to induce protein expression for different time periods ranging from 30 min-4 hrs. Cell pellets from different time points were briefly boiled in SDS-sample buffer and separated by 10% SDS-PAGE. The figures are coomassie stained gels indicating the time-course of expression of α (**1.3A**), β (**1.3B**) and γ (**1.3C**). Various lanes are as follows: UI represents uninduced culture and the other lanes represent the induced cultures for different time periods as depicted in the figure.

Recombinant subunits were purified by Ni-NTA affinity chromatography. The columns were washed with low concentrations (30 and 50 mM) of imidazole and the bound fractions were then eluted by 250 mM imidazole as described in the 'Materials and Methods'. The purification profile of the recombinant subunits was analyzed by coomassie stained gels (**Fig 1.4 A, B** and **C**).

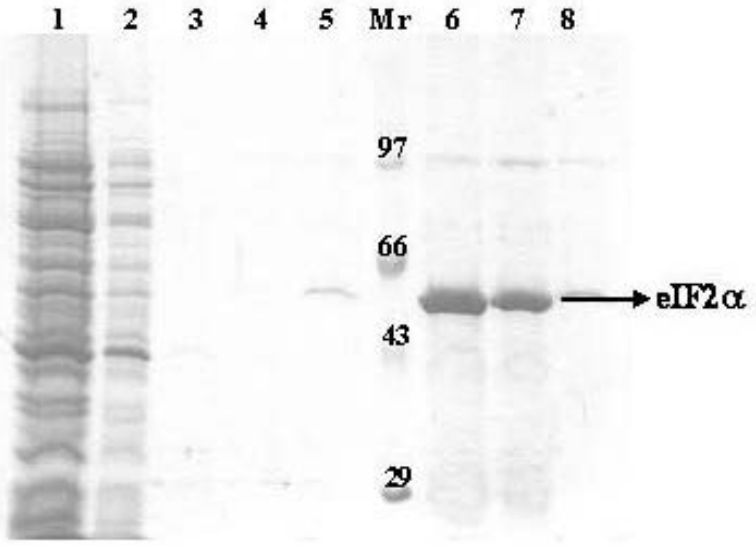
Phosphorylation of the recombinant α- and β- subunits:

Since the α- and β- subunits of human eIF2 are phosphorylated *in-vitro* by various purified kinases and are also found phosphorylated during their expression in insect cells (Suragani et al., 2006), I evaluated the ability of bacterially expressed α- and β- subunits of human eIF2 to serve as substrates for different kinases *in-vitro* and also by bacterial extracts (**Figs 1.5 A-C**). Our observations here indicate that bacterially expressed recombinant human eIF2 α- (wt and S48A mutant) and β- subunits were phosphorylated *in-vitro* by purified kinases as shown in **Fig 1.5 A** and **B** respectively. Unlike the baculovirus expressed subunits which are phosphorylated in insect cells, the bacterially expressed subunits are not phosphorylated by any of the bacterial kinases (**Fig 1.5C**).

[H3] GDP binding by the recombinant subunits: The ability of the recombinant α-, β- and γ- subunits of human eIF2, to bind [H^3] GDP was analyzed as described in 'Materials and Methods'. Although, the γ-subunit is implicated in GDP binding and the β-subunit is suggested to aid the γ-subunit in the above function (Nika et al., 2001), it was observed here that all the three subunits bind to labeled GDP. However, the GDP dissociation analysis which is inverse to the retention efficiency in the presence of excess unlabeled GDP suggests that the trimeric complex binds and retains the GDP most efficiently (**Fig 1.6**).

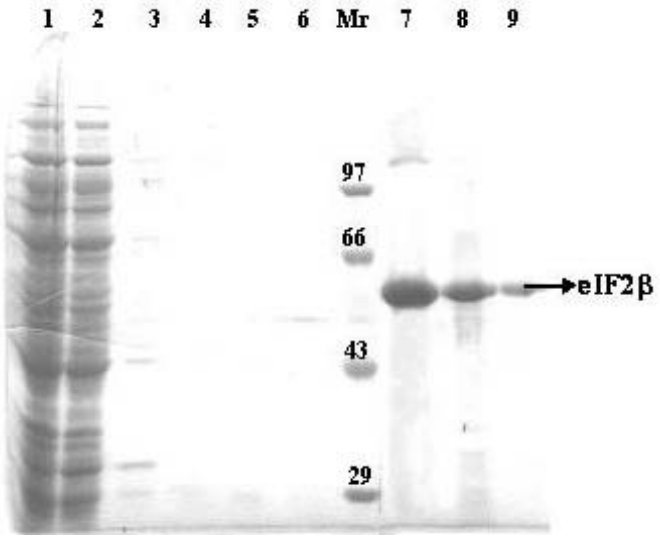
Polyclonal antibodies against the recombinant eIF2α- subunit: The bacterially expressed and purified eIF2α- subunit was used to raise polyclonal antibodies in rabbit according to the standard protocols described in the book 'Molecular Cloning' by Sambrook et al. The ability of the antibodies to detect and immunoprecipitate the α-subunit of reticulocyte and HeLa cell lysates indicates that the antibody recognizes specifically the α-subunit of eIF2 (**Fig 1.7**).

Fig. 1.4A:



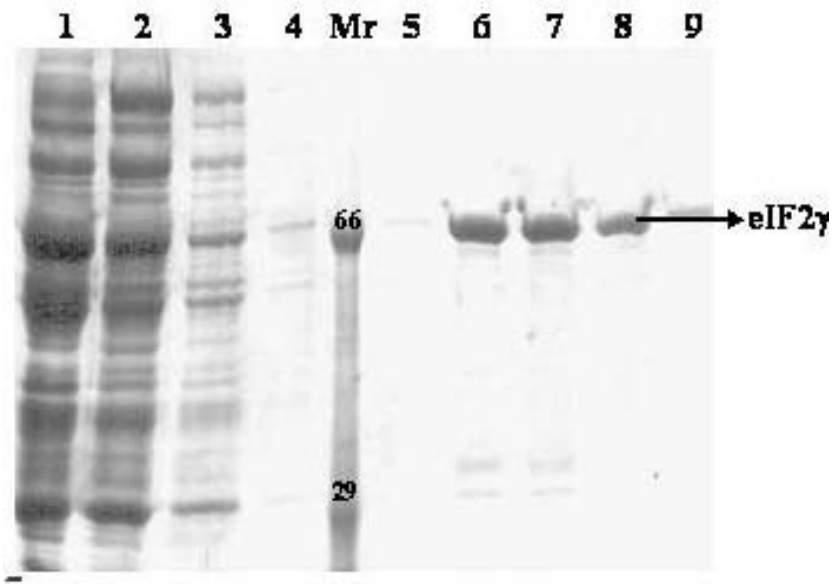
Purification profile of recombinant **eIF2α** by Ni-NTA chromatography: The figure represents a coomassie stained gel. Various lanes are as follows: lane 1, flowthrough or unbound fraction; lanes 2-5, wash fractions; Mr depicts the molecular weight marker and lanes 6-8, represent the elution fractions.

Fig. 1.4B:



Purification profile of recombinant **eIF2β** by Ni-NTA chromatography: Various lanes are as follows: lane 1, flowthrough or unbound fraction; lanes 2-6, wash fractions; Mr depicts the molecular weight marker and lanes 7-9, represent the eluted fractions.

Fig. 1.4C:



Purification profile of recombinant **eIF2γ** by Ni-NTA chromatography: Various lanes are as follows: lane 1, flowthrough or unbound fraction; lanes 2-4, wash fractions; Mr depicts the molecular weight marker and lanes 5-9, represent elution fractions.

Fig. 1.4 A, B and C: Purification of the recombinant subunits by Ni-NTA agarose: The cultures expressing recombinant his-tagged subunits of human eIF2 were harvested and the cell pellet was processed as described in 'Materials and Methods'. The cell lysate was passed through pre-equilibrated Ni-NTA resin. The column was processed as described in 'Materials and Methods' and the bound proteins were eluted with 250 mM Imidazole. The figures represent coomassie stained gels indicating purification of recombinant human **eIF2 α**, (**1.4A**), **β** (**1.4B**) and **γ** (**1.4C**).

Fig 1.5A: Phosphorylation of recombinant eIF2α (wt and mutants): The bacterially expressed and purified human eIF2α and its mutants were phosphorylated by purified recombinant PERK *in-vitro* in the presence of [γ-^{32}P] ATP as described in Materials and Methods. The samples were separated by 10% SDS-PAGE and transferred to a nitrocellulose membrane and the membrane was exposed to an X-ray film. **Panel (a)** represents an autoradiogram indicating the phosphorylation status of the proteins in the presence and absence of PERK. **Panel (b)** represents a western blot indicating the levels of the eIF2α-subunit and its mutants as detected by an anti-eIF2α antibody. Various lanes are as follows: 1) eIF2α (wt), 2) eIF2α (wt) + PERK, 3) S51A, 4) S51A+ PERK, 5) S48A, 6) S48A+ PERK, 7) S51D, 8) S51D+ PERK.

The wt and S48A, but not S51D or S51A mutants of human eIF2 α-subunit were phosphorylated in the presence of PERK.

Fig 1.5B: Phosphorylation of the recombinant β-subunit: The purified recombinant β-subunit expressed in bacteria was phosphorylated in the presence of [γ-^{32}P] ATP by purified kinases like PKA, PKC, CK II and DNA-PK *in-vitro* as per the manufacturer's instructions and as described in Materials and Methods.

Panel **(a)** represents the phosphorylation status of recombinant eIF2β as judged by a phosphorimage, and panel **(b)** is a western blot indicating the levels of the β-subunit in the reactions as judged by a monoclonal anti-eIF2β antibody. Various lanes are as follows: lanes 1, 3, 5, and 7, represent control lanes containing the β-subunit alone; lane 2, eIF2β + CK II; lane 4, eIF2β + PKC; lane 6, eIF2β + DNA-PK; lane 8, eIF2β + PKA.

Fig. 1.5A

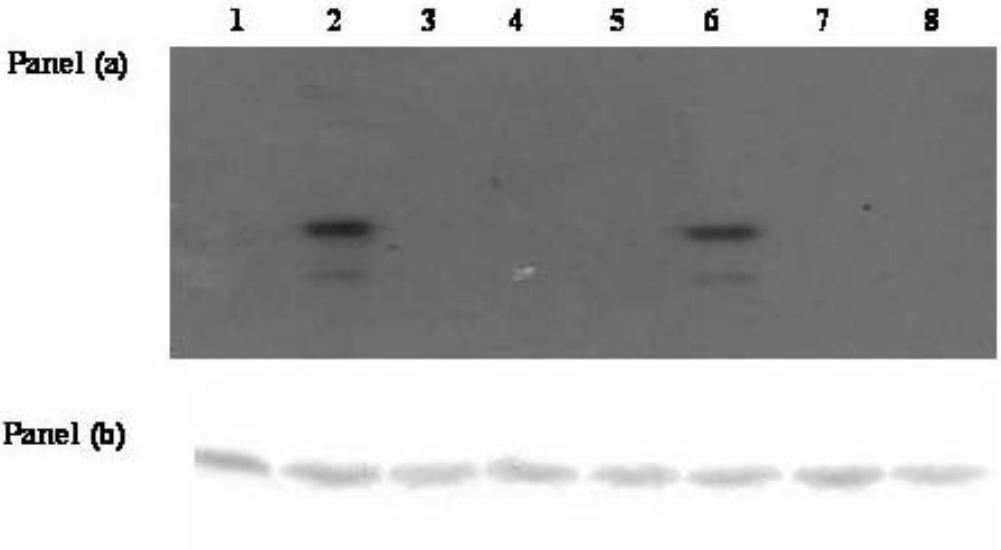


Fig. 1.5B

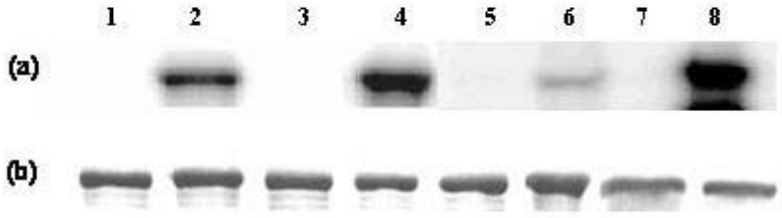


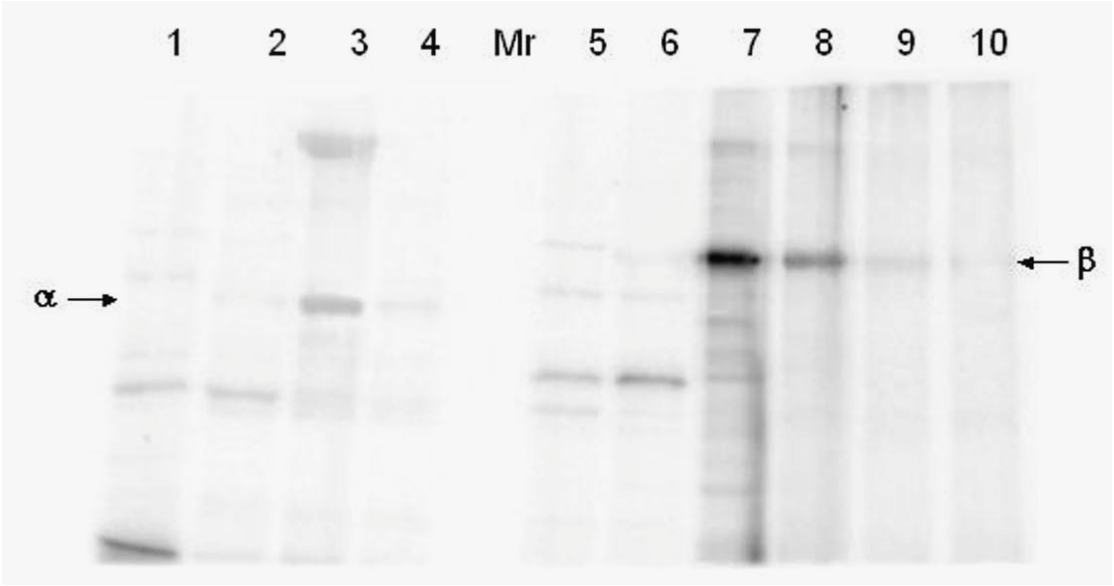
Fig. 1.5C: Phosphorylation status of recombinant α and β-subunits in bacterial extracts and by purified kinases *in-vitro*:

Extracts of BL21(DE3)pLysS, *E. coli* cells was prepared as described in 'Materials and Methods'. 5 μg of purified recombinant α or β subunits was incubated with bacterial cell extracts containing ~25 μg of total protein in the presence of a phosphorylation buffer and 10μ Ci of [γ-^{32}P]ATP in a total volume of 25 μl at 30^0 C for 10 min. Purified subunits were also incubated separately without extracts in the presence of a phosphorylation buffer labelled γ-^{32}[P]ATP with 0.5 μg of PERK, an eIF2 α kinase or with purified eIF2β-subunit specific kinases like PKA, PKC and CKII that are adjusted to the same specific activity at 30^0C for 10 min as described in 'Materials and Methods'. Samples were separated by10% SDS-PAGE and transferred to a nitrocellulose membrane. The membranes were then analyzed by phosphorimage (**Panel I**) and western blot (**Panel II**).

Various lanes in both the panels are as follows: 1, Bacterial extract; 2, extract + eIF2α; 3, eIF2α + PERK; 4, α + BSA; Mr, Molecular weight Markers; 5, extract; 6, extract + eIF2β; 7, eIF2β + PKA; 8, eIF2β + PKC; 9, eIF2β+ CKII; 10, eIF2β + BSA.

Fig 1.5C

Panel I



Panel II

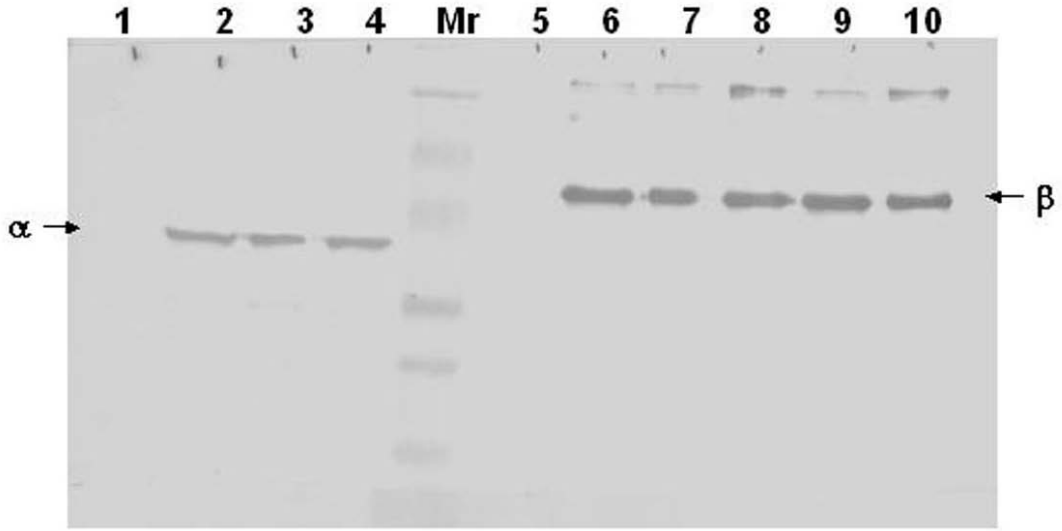


Fig. 1.6:

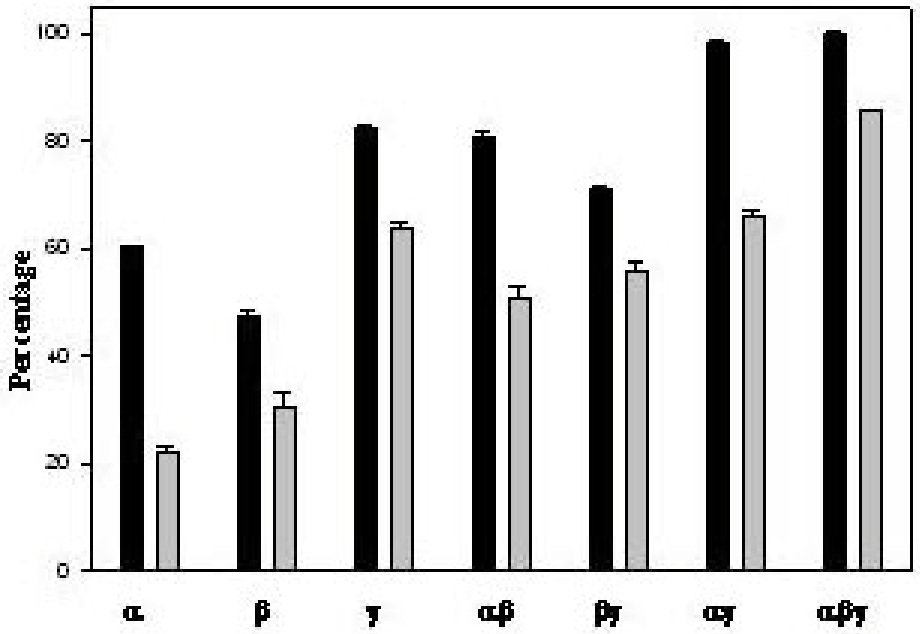


Fig 1.6: [H^3] GDP binding by the recombinant eIF2 subunits: The binding of [H^3] GDP and the retention efficiency in the presence of excess unlabeled GDP of the individual subunits, complexes containing two of the subunits and the trimeric complex was analyzed as described in 'Materials and Methods'. The dark bars represent the binding efficiency and the grey bars represent the retaining efficiency of the bound GDP in the presence of unlabelled GDP. The maximum binding and retention efficiency was found for the trimeric eIF2 and was taken as the 100% count (binding cpm = 1498; retention of bound [H^3] GDP in the presence of excess unlabelled GDP cpm = 934).

Fig 1.7:

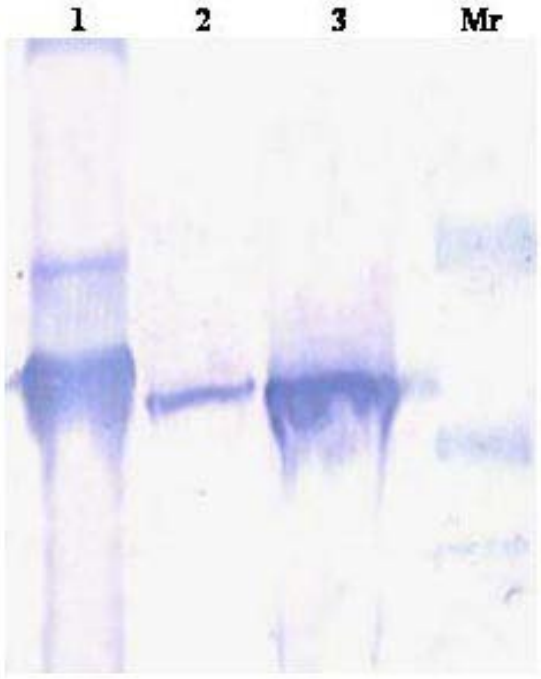


Fig 1.7: Immunoprecipitation of α-subunit of eIF2 by antibodies raised against recombinant human eIF2α: Antibodies raised against recombinant α-subunit of human eIF2 were used to immunoprecipitate the eIF2α-subunit from HeLa and rabbit reticulocyte extracts. The immunoprecipitated samples were separated on 10% SDS-PAGE and transferred to a nitrocellulose membrane. The figure is a western blot probed with a commercial anti-eIF2α antibody. Various lanes are: lane 1, recombinant eIF2α; lane 2, eIF2α from HeLa cell extracts; lane 3, eIF2α from rabbit reticulocyte extracts; Mr depicts molecular weight marker.

Chapter 2

Intersubunit and Interprotein Interactions of α- and β-Subunits of Human eIF2: Effect of Phosphorylation.

Introduction:

The initial step of the translation initiation is the transfer of the Met-tRNAi to the 40S ribosomes, which is mediated by the eukaryotic initiation factor 2 (eIF2). It also plays an important role in the recognition of the initiator AUG codon on the mRNA, GDP/GTP binding and hydrolysis etc., These multifaceted actions of eIF2 are accomplished by the coordinated interactions within the subunits and also with other proteins. Much effort has gone into determining the interactions among the components of the initiation machinery and a lot of them are yet to be deciphered. Most of the information now available on the intersubunit and interprotein interactions of eIF2 has been derived from studies on yeast and based on these studies it was proposed that the γ-subunit forms the central core in the structure of eIF2 interacting with α- and β-subunits on either side, and that there is no interaction between the α- and β-subunits (Marintchev and Wagner, 2004). In contrast to these above observations, our recent studies with the baculovirus expressed α- and β-subunits of human eIF2, indicate that they interact with each other in addition to interacting with the γ-subunit in ELISA and dot blot assays (Suragani et al., 2005).

Further, eIF2 also interacts with other initiation factors like eIF2B, eIF5, etc., and also with other proteins such as PP1, Nck1, CK II, etc,. Studies from yeast have indicated that the non-productive interaction between eIF2 and eIF2B, that occurs as a consequence of eIF2α phosphorylation inhibits the GDP/GTP exchange activity of eIF2B and is mainly mediated by the α-subunit of eIF2 and the regulatory subcomplex comprising the α, β and δ subunits (Pavitt et al., 1998; Krishnamoorthy et al., 2001), but not the catalytic subcomplex constituted of γ- and ε-subunits of eIF2B. But, an earlier observation (Kimball et al., 1998) and also recent observations from our lab (Suragani et al., 2005) have shown that the β- rather than α-subunit of mammalian eIF2 interacts with eIF2B. These observations suggest that the interaction patterns of mammalian eIF2 subunits are different from that of the yeast. Although, earlier studies have pointed that α subunit of eIF2 acts as a substrate for caspases (Satoh et al., 1999, Marissen et al., 2000), no such reports exist for the β-subunit or the effect of phosphorylation of the α- or β-subunits on their cleavage by caspases. Persistent eIF2α phosphorylation is implicated in promoting apoptosis under certain conditions that is mediated by cytochrome c (Aparna et al., 2003; Sahdev et al., 2003).

Consistent with these observations, a recent report has shown the localization of phosphorylated eIF2α with cytochrome c in ischemic neuronal cells (Page et al., 2003).
Since baculovirus expressed human eIF2α and β- subunits are phosphorylated during expression, as shown in our earlier studies (Suragani et al., 2006), I expressed the human eIF2 subunits in bacteria to primarily obtain them in an unphosphorylated status and to evaluate the effect of phosphorylation of the α- and β-subunits on the intersubunit interaction and also on their interaction with other proteins using pull down and far western analyses. Our findings here indicate that the interaction between α- and β- subunits is enhanced by the phosphorylation of the α- but not β-subunit of eIF2. The non-productive interaction that occurs between phosphorylated α- subunit in the eIF2 complex and eIF2B holoprotein is mediated by the β-subunit. Interestingly, it was observed here, that the phosphorylation of eIF2α or β- subunits resist caspase 3 action. Recombinant bacterially expressed eIF2β subunit is found to interact with Nck1, a co-factor of eIF2α phosphatase and eIF5, a GTPase activating protein of reticulocyte and HeLa cell extracts and the interaction with Nck1, but not with eIF5 is modified by the phosphorylation of the β-subunit. Interestingly, we also observed that phosphorylated eIF2α interacts efficiently with cytochrome c than the unphosphorylated form.

Results:

Phosphorylation of eIF2α enhances its interaction with eIF2β and eIF2B: Previously, we have shown that baculovirus-expressed human eIF2 subunits interact with each other in ELISA and dot blot assays (Suragani et al., 2005). Bacterially expressed subunits of eIF2 are also found to interact with each other to form αβ, βγ and αγ complexes both in far western analysis (**Fig 2.1A**) and pull down experiments (**2.1B**).

In a far western analysis, antibodies against the subunits of eIF2 have been used to detect the interaction between the recombinant subunits separated on a membrane and the subunits of reticulocyte lysate eIF2 (**Fig. 2.1A**). In panel (**a**), the recombinant α-, β- and γ-subunits of eIF2, separated on a membrane were detected by an anti his-tag antibody to indicate the levels of these subunits used in the interaction studies. Lanes 2 and 3 of panel (**b**) indicate the interaction between the recombinant β and γ-subunits with the α-subunit of

lysate eIF2 as detected by an anti-eIF2α antibody which also recognizes the uninteracting recombinant α-subunit as shown in lane 1. Panel (**c**) indicates the interactions between the recombinant α- and γ- subunits with the β-subunit of lysate eIF2 as detected by an anti β-antibody (lanes 1 and 3). The antibody also recognizes the uninteracting β-subunit (lane 2). Panels (**d**) and (**e**), represent the controls where the membrane containing different recombinant subunits were incubated with anti eIF2α- or β- antibodies respectively to determine the specificity of the antibodies.

Complementing these observations, we observed purified subunits interact with each other to form similar complexes in a pull down assay as described in the legend to **Fig 2. 1B.** In panel (i), his-tagged γ-subunit is incubated with the Ni-NTA-agarose resin, and the resin was then washed before incubating it with his-tag cleaved recombinant α-subunit to determine the interaction between γ- and α-subunits. Unbound or wash fractions showed very little of the γ- or α-subunits (lanes 1-4 of **Fig. 2.1B**). The α-subunit co-elutes with the γ-subunit (lanes, 5 and 6; upper portion containing γ and the lower portion indicating the α-subunit). Similarly, we observed that β-subunit co-elutes with the γ-subunit and the α-subunit co-elutes with the β-subunit (Fig. 2.1 B panels ii and iii). These findings reiterate our earlier conclusions (Suragani et al., 2005) that unlike yeast, human eIF2α and β-subunits interact with each other in addition to their interactions with the γ-subunit.

Phosphorylation of the α-subunit enhances its interaction with the β-subunit: Unlike baculovirus expressed eIF2 subunits (Suragani et al., 2006), bacterially expressed subunits of human eIF2 are not phosphorylated by any of the bacterial kinases (**Fig. 1. 5C**). However, they are phosphorylated *in-vitro* by their respective kinases (**Fig. 1.5 A&B**). Hence we could evaluate here the effect of phosphorylation on α-β interaction (**Fig. 2. 2A**). To determine the importance of phosphorylation of the α-subunit on the interaction between α and β-subunits, recombinant eIF2α was phosphorylated *in-vitro* by a purified recombinant ER-resident eIF2α kinase and bound to Ni-NTA resin prior to incubating with the his-tag cleaved β-subunit (**Fig. 2.2A**). Immunoblot analysis of the

Fig 2.1A:

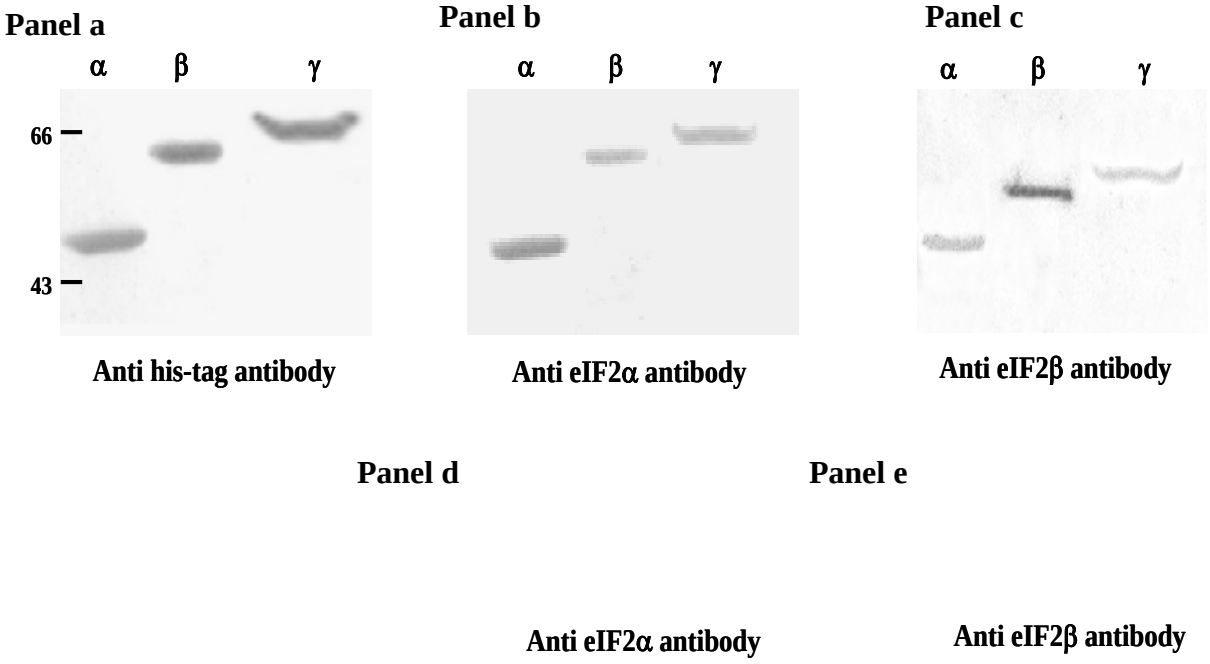
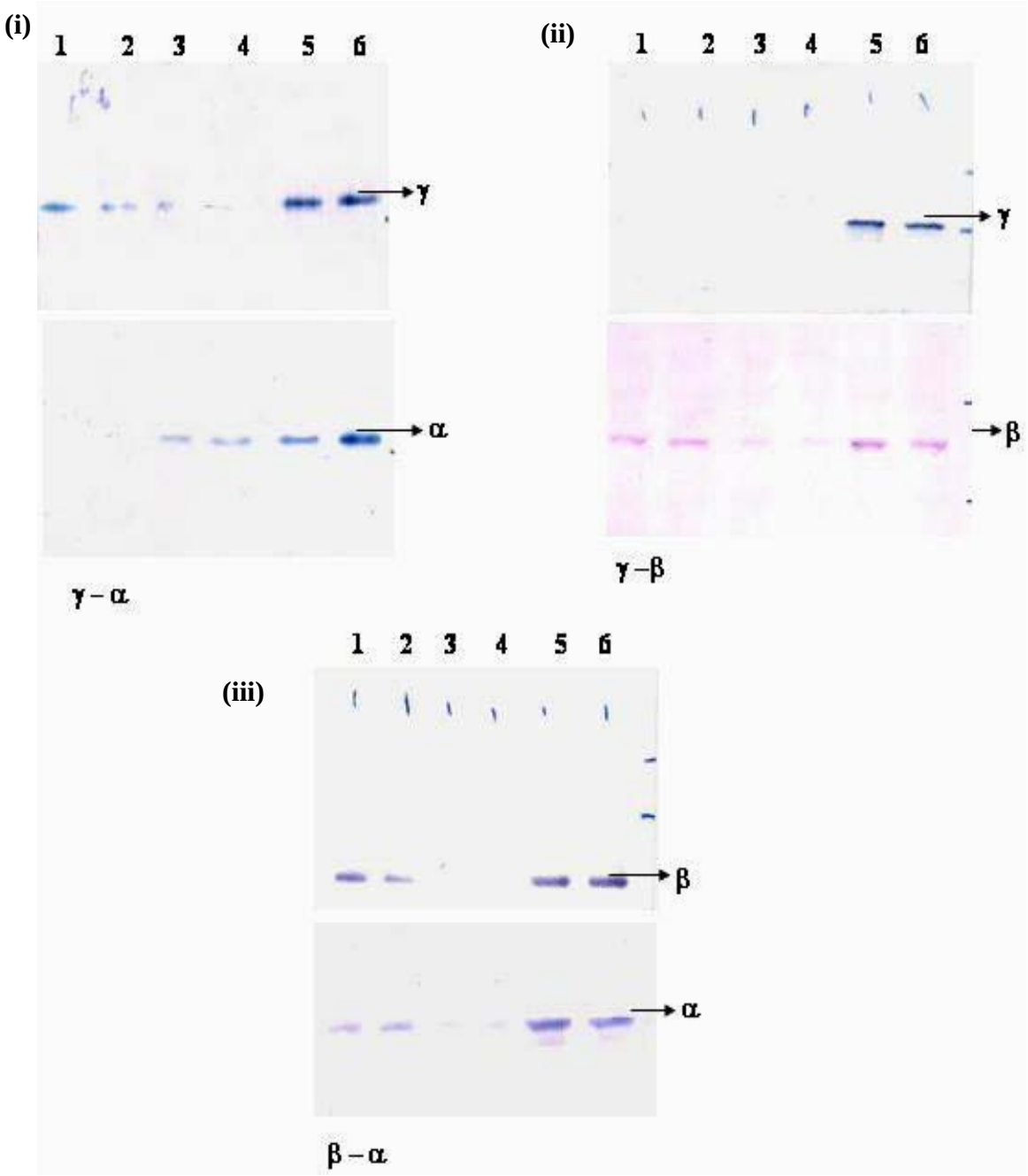


Fig 2.1A: Interaction between subunits; Far western analysis: Interaction between purified recombinant eIF2 subunits with the subunits of rabbit reticulocyte lysate eIF2, were determined by far western analysis. In this assay, 5 μg of purified his-tagged recombinant α, β and γ- subunits were separated by 10% SDS-PAGE, transferred to a nitrocellulose membrane and then incubated with recombinant subunits or with rabbit reticulocyte lysates. The membranes were processed as described in 'Materials and Methods'. The lanes of panel **(a)** represent the levels of recombinant eIF2α, β and γ loaded in the respective lanes as determined by anti his-tag antibody. Panel **(b)** represents the interaction between recombinant β and reticulocyte lysate α (in lane 2) or between recombinant γ and lysate α (lane 3) as judged by an anti-eIF2α antibody. Lane 1 represents recombinant eIF2α. The lanes of panel **(c)** represent the interaction between recombinant α and reticulocyte lysate β (in lane 1) or between recombinant γ and lysate β (lane 3) as judged by anti-eIF2β antibody. Lane 2 represents recombinant eIF2β. Panels **(d)** and **(e)** represent the controls, where the different subunits on the membrane are incubated with anti eIF2α- or β-antibodies respectively. The antibodies recognize the specific subunits only.

Fig 2.1B: Intersubunit interactions by pull down assays: Pull down assays as described in Materials and Methods, were carried out to study the interactions among the purified recombinant eIF2 subunits. In panels (**i**) and (**ii**), recombinant purified γ –subunit or in panel (**iii**), purified β-subunit were bound to the Ni-NTA resin. The resin bound proteins were incubated with enterokinase treated, his-tag cleaved recombinant α- or β-subunit in panels (**i**) and (**ii**) respectively, or with α- subunit in panel (**iii**) to monitor the interactions between the recombinant **γ- α**, **γ-β** and **β-α** respectively. The unbound, wash and bound/eluted fractions were collected and separated by 10% SDS-PAGE, transferred to a nitrocellulose membrane. The membranes were probed with anti his-tag antibody for the γ-subunit and anti eIF2α- or β- antibodies for the specific subunits respectively as shown in the figure. The figure is a western blot. Various lanes are as follows: In all panels, lanes 1 and 2 represent unbound fractions, lanes 3 and 4 represent wash fractions, and lanes 5 and 6 are eluted fractions.

Fig 2.1B:



unbound, wash and eluted fractions of this pull down assay indicate that β-subunit interacts and co-elutes with the α-subunit (lane 7, panels **i** and **ii**) and this interaction is enhanced (lane 8, panel i) when eIF2α is phosphorylated (lane 8, panel iii, and iv, the bar diagram). However, we have not observed any enhanced interaction between the α- and β-subunits when β-subunit is phosphorylated (panel v).

The above enhanced interaction between α and β-subunits upon phosphorylation of eIF2α was further confirmed by mutants of eIF2α such as S51A (the nonphosphorylatable form), S51D (a phosphomimetic form) and S48A (a mutant eIF2α that is phosphorylated on its serine 51 residue) (**Fig. 2. 2B**). The observations indicate that the phosphorylated wt eIF2α and phosphomimetic form S51D interacts more efficiently with the recombinant his-tag cleaved β-subunit of eIF2 than the nonphosphorylatable S51A (lanes 14 and 16 versus lane 15). Also we observed the mutant phosphorylated or unphosphorylated S48A eIF2α cannot interact so efficiently with the β-subunit as wt phosphorylated eIF2α (lanes 17 and 18 versus lane 14).

Phosphorylated eIF2α enhances its interaction with eIF2B and eIF2β of reticulocyte lysates: To understand the physiological significance of the above interaction, we studied the interaction of the recombinant α -subunit with the eIF2B and the β-subunit of eIF2 of the lysate. An earlier study in yeast has shown that phosphorylated eIF2α interacts with the regulatory complex of eIF2B in a non-productive manner that inhibits the GDP/GTP exchange activity of eIF2B (Krishnamoorthy et al., 2001; Pavitt et al., 1998). In contrast to this observation, a previous study from this laboratory has shown that baculovirus expressed and phosphorylated human eIF2α- subunit interacts with purified eIF2B only in the presence of β-subunit of eIF2 in an ELISA study (Suragani et al., 2005). Here we have evaluated the interaction of bacterially expressed, purified and phosphorylated human eIF2α interaction with the eIF2B and eIF2β-proteins of the rabbit reticulocyte lysate in a pull down assay (**Fig. 3. 1**). Analysis of the various unbound and bound fractions using anti-eIF2Bε, anti-eIF2β and α-antibodies (panels I- III respectively) indicate that phosphorylated eIF2α-subunit interacts efficiently with the lysate eIF2B protein as

Fig.2.2A. Effect of phosphorylation of the α-subunit on its interaction with the recombinant β-subunit: Phosphorylation of the α-subunit was carried as described in 'Materials and Methods'. Pulldown assays with 5 μg of unphosphorylated and phosphorylated α-subunit of eIF2 and 5 μg of enterokinase treated, his-tag cleaved β-subunit were carried out as described in the legend to **Fig 2.1C**. Unbound and bound fractions were separated by 10% SDS-PAGE, transferred to nitrocellulose membrane. The western blots are probed with anti eIF2 α- or β-antibodies to detect the specific subunits as in Panels **(i)** and **(ii)** respectively. Phosphorylation status of the α-subunit of different fractions was assessed by an Amersham typhoon phosphorimager as in panel **(iii)**. Various lanes in all the panels are as follows: lanes 1and 2, unbound fractions, lanes 3-6 wash fractions and lanes 7 and 8 eluted fractions. Panel **(iv)** represents bar diagram indicating the relative levels of the β-subunit in unbound (bars 1 and 2) and in eluted fractions (bars 3 and 4) in the presence of unphosphorylated and phosphorylated α-subunit respectively. Panel **(v)** represents the interaction of recombinant β-subunit phosphorylated with different kinases with the his-tag cleaved α-subunit as detected by anti-eIF2α antibody in the upper portion and anti-eIF2β antibody in the lower portion. Various lanes are as follows: lanes 1-4, represent the unbound fractions; lanes 5-8, wash fractions and lanes 9-12, represent the elution fractions of a pull down assay.

Fig 2.2A

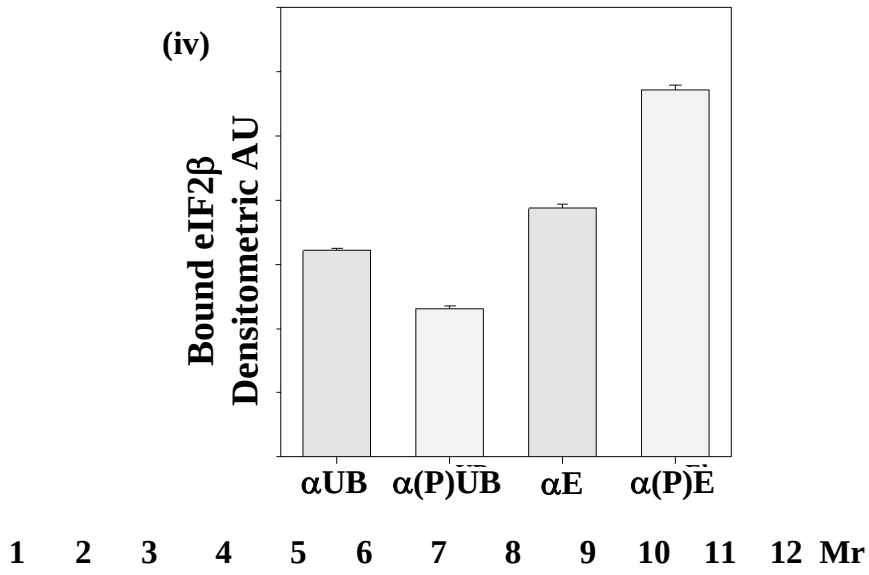

(iv)
Bound eIF2β
Densitometric AU
αUB
α(P)UB
αE
α(P)E
1 2 3 4 5 6 7 8 9 10 11 12 Mr

Fig 2.2B

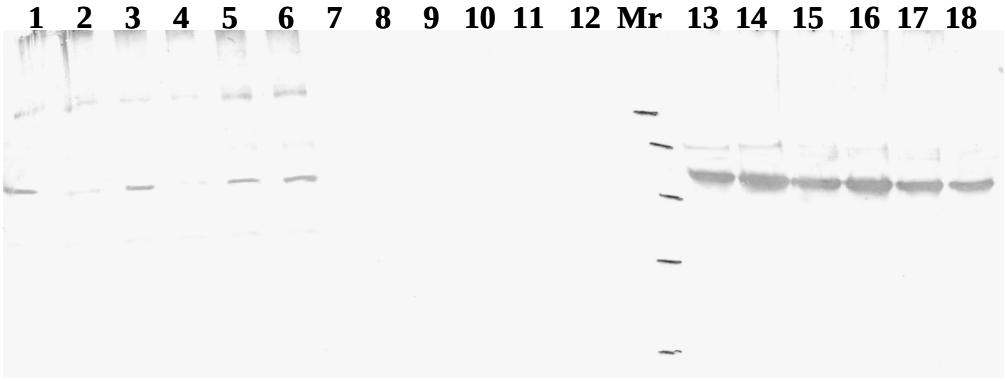


Fig 2.2B Interaction of unphosphorylated and phosphorylated recombinant eIF2α (wt and mutants) with the β-subunit: Interactions between the unphosphorylated and phosphorylated wt and mutants (S51A, S51D and S48A) of eIF2α with his-tag cleaved recombinant β-subunit was analyzed by a pull down assay. The figure is a western blot probed with an anti-eIF2β antibody. Panel **(i)** indicates the levels of β-subunit co-eluted with the wt and mutants of eIF2α (lanes 13-18), and panel **(ii)** indicates levels of eIF2α as detected by anti-eIF2α antibody. The lanes in the figure are as follows: Lanes 1 and 2, represent the unbound fractions of unphosphorylated and phosphorylated eIF2α; 3 and 4, unbound fractions of S51A and S51D; 5 and 6, unbound fractions of S48A in the presence and absence of kinase respectively. Lanes 7-12 represent the corresponding wash fractions and 13-18, the corresponding elution/bound fractions of the pull down assay.

measured by the intensity of the ε-subunit and also with the β-subunit of lysate eIF2 (**Fig 3.1**, Panel I). Bar diagram indicates the relative levels as indicated (Panel IV). The diminished interaction between the β-subunit of eIF2 and phosphorylated S48A mutant of eIF2α, and the enhanced interaction between the β-subunit and S51D, a phosphomimetic form of eIF2α support our previous and present observations that phosphorylation of Ser51 in eIF2α and the maintenance of the Ser48 residue in eIF2α aids in a complex formation between eIF2 and eIF2B holoproteins (Ramaiah et al., 1994; Sudhakar et al., 1999 and 2000) and that this interaction is mediated by the β-subunit of eIF2. Hence we suggest that the interaction between eIF2α- and β-subunits is physiologically relevant and regulates the productive and non-productive interaction between eIF2 and eIF2B holoproteins, and thereby the GDP/GTP exchange activity of eIF2B. However, the mechanism by which phosphorylation of α-subunit causes the β-subunit to switch from productive to non-productive interaction is not known.

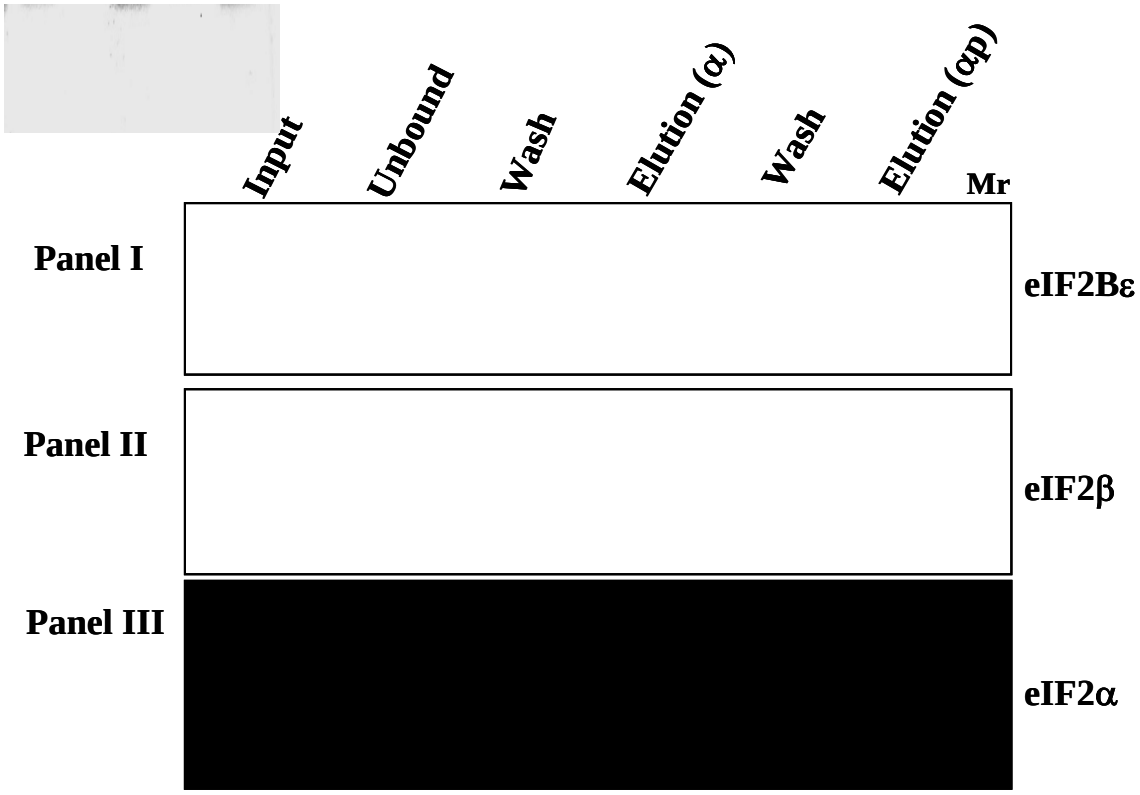
Based on the crystal structural data of yeast and human eIF2α, it has been suggested that the interaction between phosphorylated eIF2α and the regulatory sub-complex of eIF2B can occur due to better surface complementarity and/or more favorable electrostatic interactions (Marintchev and Wagner, 2004). As there is no direct interaction between eIF2α and eIF2B in mammals (Suragani et al., 2005; Kimball et al., 1998), it is likely that phosphorylation of eIF2α induces a conformational change in the eIF2 complex so that β-subunit of eIF2 comes in close proximity presumably to the regulatory sub-complex or more precisely to the δ-subunit of eIF2B. A more detailed study involving the mutations of the subunits is required to determine the possible regions involved in the interaction between α and β-subunits. However, it is likely that the N-terminal region of human β-subunit that is not homologous to eIF5 may be involved in the interaction with the α-subunit of mammalian eIF2.

β-subunit interacts with Nck1 and eIF5: The β-subunit in eIF2 aids the γ-subunit in all its functions (Hinnebusch, 2005) and is also reported to interact with several initiation factors, with mRNA, Nck1, a cofactor of an eIF2α phosphatase, CKII and type1 phosphatase (Kapp and Lorsch, 2004; Laurino et al., 1998; Gaspar et al., 1994; Llorens et -

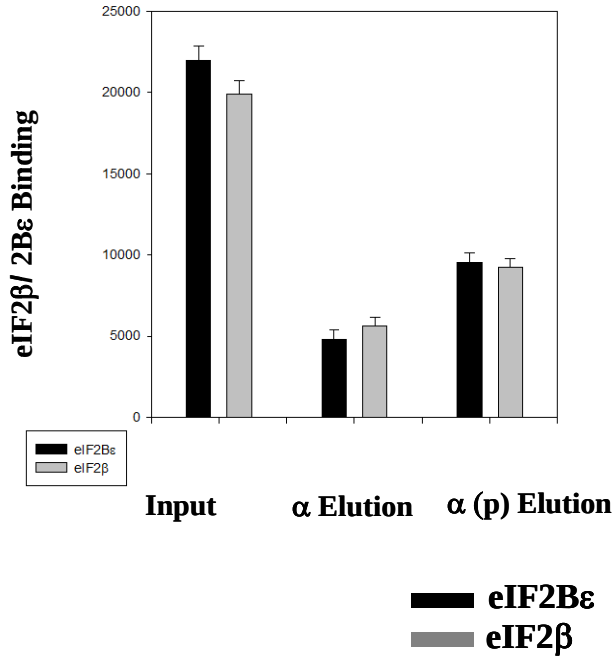
Fig 3.1: Interaction of recombinant eIF2α with eIF2β and eIF2Bε of reticulocyte lystes: Effect of α phosphorylation: Recombinant eIF2α was phosphorylated by PERK in the presence of 100 µM unlabelled ATP as described in Materials and Methods. Both unphosphorylated and phosphorylated eIF2α were bound to Ni-NTA resin. Pull down assay in the presence of lysate was carried out as described in Materials and Methods. Various unbound and bound fractions were separated on a 10% SDS-PAGE and transferred onto a nitrocellulose membrane. The figure is a western blot. Panels I and II, indicate the levels of eIF2B and the eIF2β of the lysates co-eluted with unphosphorylated and phosphorylated recombinant eIF2α as detected by an anti-eIF2Bε and anti-eIF2β antibodies. Panel III, indicates the levels of eIF2α eluted under similar conditions. Various lanes are as follows: lane 1, input; lane 2, unbound fraction; lanes 3 and 5, wash fractions and lanes 4 and 6, eluted fractions.

Panel IV, represents a bar diagram indicating the relative levels of eIF2Bε (dark bars) and eIF2β (grey bars) from reticulocyte lysate eluted in the presence of unphosphorylated and phosphorylated α-subunit.

Fig 3.1:



Panel IV



-al., 2006; Kebache et al., 2002; Wakula et al., 2006). Here we have evaluated the interaction of bacterially expressed recombinant β-subunit with eIF5, a GTPase activating protein, and Nck1 of reticulocyte and HeLa cell extracts by far western and pull down analysis (**Figs. 3.2A and B**). In far western analysis, the three subunits of human eIF2 are separated by 10% SDS-PAGE and transferred to a nitrocellulose membrane (**Fig. 3.2 A** panel **i**). The membranes were incubated with rabbit reticulocyte lysates (panels ii and iii) and probed with anti- Nck1 or eIF5 antibodies to detect the interaction of these subunits with Nck1 (ii) or eIF5 (iii) respectively. Our observations here, indicate that bacterially expressed human eIF2 β-, but not α and γ-subunits interacts with Nck1 and eIF5 (ii and iii) respectively. However, we also observed a faint interaction between the recombinant eIF2γ-subunit and eIF5 (panel iii).

Consistent with these above observations, the recombinant β-subunit was able to interact with Nck1 and eIF5 of the reticulocyte and HeLa cell extracts in a pull down assay (**Fig 3.2B**). Panel I indicates the interaction between eIF2β and Nck1, where the reticulocyte or HeLa cell lysate specific Nck1, coelutes with eIF2β (lanes 4 and 8 of the upper portion representing the reticulocyte and HeLa cell Nck1 respectively) as detected by an anti Nck1 antibody, while the lower portion indicates eIF2β as detected by an anti eIF2β antibody. Similarly, panel II indicates the interaction of the recombinant eIF2β-subunit with eIF5 of the extracts.

Further, the effect of phosphorylation of eIF2β on its interaction with Nck1 and eIF5 of reticulocyte lysate was also studied here. The recombinant eIF2β-subunit was able to interact with Nck1 of reticulocyte lysate in a pull down assay as analyzed by an anti-Nck1 antibody (**Fig.3. 2C, panel i**, lane 9) as also stated above (**Fig 3.2B**). This interaction was enhanced when the β-subunit was phosphorylated by PKA and PKC (lanes 10 and 11) and decreased when it was phosphorylated by CKII (lane 12). However, the amount of the β-subunit eluted under similar conditions was almost same (**Fig. 3.2C, panel ii**). Phosphorylation status of β-subunit for the corresponding lanes is shown in the phosphorimage (**panel iii**).

Fig 3.2A.

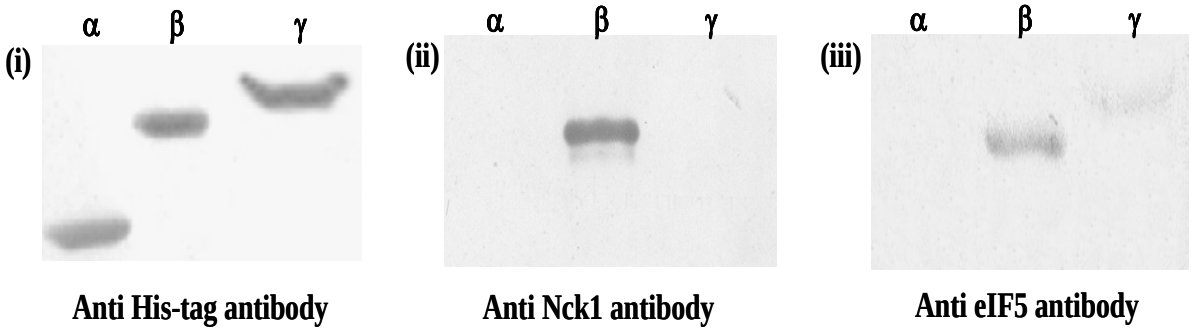


Fig 3.2A: Interaction of recombinant eIF2 subunits with Nck1 and eIF5: Far western assay was carried out as described in Materials and Methods to determine the interaction between different subunits of eIF2 and Nck1 or eIF5 of lysates. The figure is an immunoblot, where panel **(i)** indicate the levels of recombinant subunits used in the assay as detected by an anti his-tag antibody. Panels **(ii)** and **(iii)** indicate the interaction between the recombinant eIF2 subunits with Nck1 and eIF5 of reticulocyte lysate as detected by an anti Nck1 or eIF5 antibodies.

Fig 3.2B:

Fig 3.2B: Interaction of the β-subunit with Nck1 and eIF5- Pull down assay: Recombinant eIF2β-subunit was bound to Ni-NTA resin and pull down assay in the presence of lysate was carried out as described in 'Materials and Methods'. Various unbound and bound fractions were separated on a 10% SDS-PAGE and transferred onto a nitrocellulose membrane. The figure is a western blot. Panels I and II depicts the interaction of the β-subunit with Nck1 and eIF5 respectively of reticulocyte and HeLa cell lysates. The upper portion of both panels indicates Nck1 or eIF5 as detected by anti- Nck1 or eIF5 antibodies, co-eluted with the recombinant β-subunit as shown in the lower portion. Various lanes in both panels are as follows: lanes 1 and 5 unbound; lanes 2, 3, 6 and 7 represent wash fractions and lanes 4 and 8 are elution fractions.

Fig 3.2C: Phosphorylation of the β-subunit- Interaction with Nck1 and eIF5: The recombinant subunit was phosphorylated in the presence of [γ^{32}P] ATP with PKA, PKC and CKII as described in 'Materials and Methods'. Pull down assay with the unphosphorylated and phosphorylated subunits in the presence of lysates was carried out as described in 'Materials and Methods'. **Panel A** represents the interaction between unphosphorylated and phosphorylated eIF2β by different kinases with Nck1 of reticulocyte lysate. The upper portion (**i**) of the western blot represents the levels of Nck1, as analyzed by an anti-Nck1 antibody co-eluted with β-subunit indicated in the lower portion (**ii**) as judged by an anti-eIF2β antibody. The various lanes are as follows: lane 1, unbound β-subunit without any kinase ; lanes 2-4 represent unbound β-subunit phosphorylated by PKA, PKC and CKII respectively; lanes 5- 8 represent the wash fractions corresponding to lanes 1-4, and lanes 9-12 represent the eluted fractions of Nck1 or eIF2β corresponding to lanes 1-4. Panel (**iii**) corresponds to the phosphorimage indicating the phosphorylation status of the β-subunit by different kinases in different fractions as mentioned above.

Panel B represents the interaction between unphosphorylated and phosphorylated eIF2β with eIF5 of reticulocyte lysate. Top portion (**iii**) is a western blot representing the levels of reticulocyte lysate eIF5 co-eluted with unphosphorylated (lane 5) and phosphorylated β-subunit (lane 6) as analyzed by an anti-eIF5 antibody. Panel (**iv**) indicates the phosphorylation status of eIF2β by PKA as analyzed by phosphorimage. Various lanes are as follows: lanes 1 and 2 unbound; lanes 3 and 4, wash fractions and lanes 5 and 6 represent eluted/bound fractions of the unphosphorylated and phosphorylated β-subunit respectively.

Fig 3.2C:

However, the levels of eIF5 co-eluted with unphosphorylated (panel **iv**, lane 5) and PKA phosphorylated β-subunit (lane 6) was almost similar suggesting that the phosphorylation of the β-subunit (panel v) does not affect its interaction with eIF5. The fact that Nck1, a cofactor of eIF2α phosphatase, interacts with the β-subunit and phosphorylation of the β-subunit modifies its interaction with Nck1 also suggests that probably the phosphorylation of eIF2α is regulated through changes in the β-subunit phosphorylation and its interaction with Nck1.

Caspase 3 interacts and processes the recombinant α- and β-subunits of eIF2: Dot blot analysis indicates that caspase 3 interacts with recombinant eIF2α and β-subunits (**Fig 3.3**). Panel A, indicates the interaction between caspase 3 spotted on the membrane and recombinant eIF2α expressed both in insect cells and bacteria. CPK and BSA serve as controls. Similarly recombinant eIF2β also interacts with caspase 3 as indicated in panel II.

Phosphorylation of α-and β-subunits resists caspase-3 action: Previous studies have shown that caspase-3 processes readily unphosphorylated eIF2α (Satoh et al., 1999; Marissen et al., 2000). Phosphorylation of eIF2α is a cause and consequence of caspase activation. Phosphorylated eIF2α is required to maintain the adaptive response and to induce caspase activation and apoptosis as has been suggested (Scheuner et al., 2006; Aparna et al., 2003; Harding et al., 2000). However no such information is available on the β-subunit or on the effect of phosphorylation of these subunits on the caspase action. Hence we have studied the ability of the bacterially expressed, phosphorylated and unphosphorylated human eIF2α and β-subunits to serve as substrates for caspase-3 (**Fig 3.4A**). Bacterially expressed human eIF2α was cleaved by caspase-3 in a time dependent manner and the cleavage was inhibited by Ac-DEVD CHO, a caspase-3 inhibitor (**Fig 3.4A,** Panels I and II) as expected. Caspase-3 was also found to process the bacterially expressed β-subunit in a time-dependent manner and the caspase inhibitor mitigates caspase-3 action (**Fig 3.4A,** Panels III and IV).

Fig 3.3.

Panel A

Insect cell expressed eIF2α **Bacteria expressed eIF2α** **Control**

Panel B

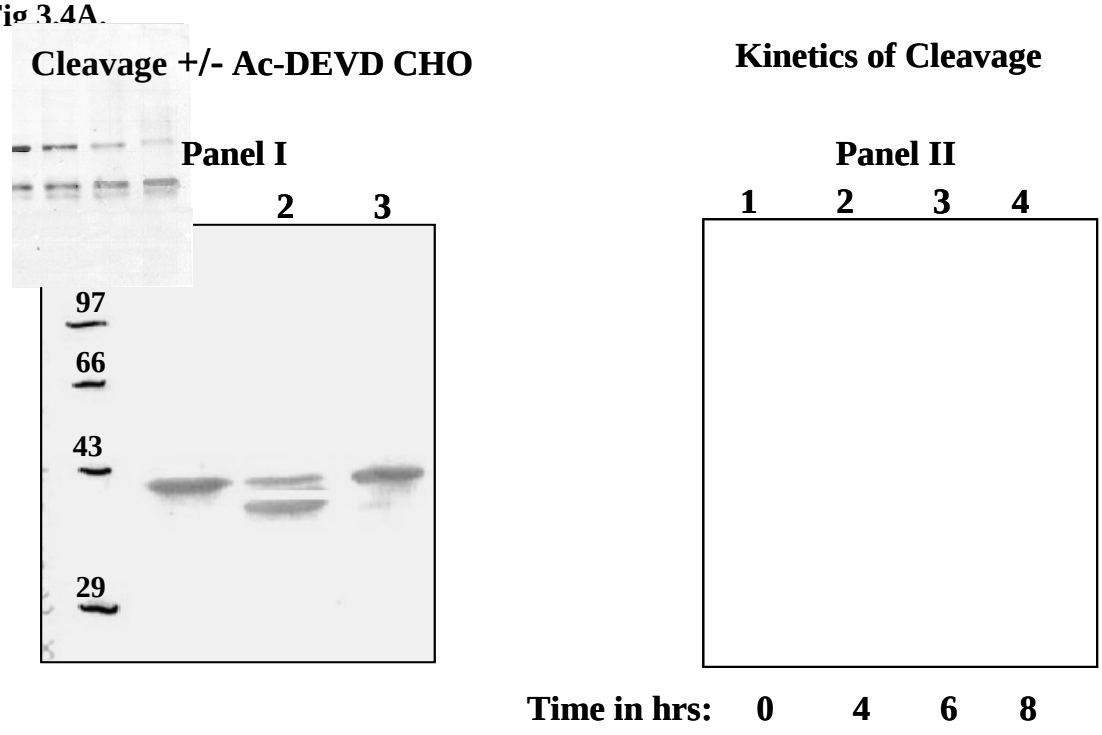
Insect cell expressed eIF2β **Bacteria expressed eIF2β** **Control**

Fig 3.3: Interaction of recombinant α- and β-subunits with caspase 3: The interaction of the recombinant α- and β-subunits with caspase 3 was analyzed by a dot blot assay. Caspase 3 was spotted on a nitrocellulose membrane, air dried and blocked with 3% non fat milk powder in TBST for 60 min. The membranes were then incubated with insect cell or bacterially expressed α-subunit (**Panel A**) or β-subunit (**Panel B**) as indicated in the figure. CPK and BSA were used as control proteins. Panels A and B, indicate the interaction between caspase 3 spotted on the membrane and eIF2α or eIF2β, as detected by an anti eIF2α- or β- antibodies.

Fig 3.4A. Caspase 3 cleaves recombinant α- and β-subunits of eIF2: Panels I and II represent the processing and time-course of the cleavage of recombinant eIF2α subunit by caspase 3 respectively. The caspase 3 cleavage of the α-subunits is mitigated in the presence of Ac-DEVD-CHO, a specific inhibitor of caspase 3 (lanes 3 vs 2 of Panel I). The time-course of the cleavage indicates that maximum cleavage of the α-subunit is observed at around 8 hrs (Panel II).

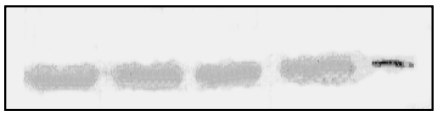
Panels III and IV represent the processing and time-course of the cleavage of recombinant β-subunit of eIF2 by caspase 3 respectively. Caspase 3 processes the β-subunit efficiently and the cleavage is inhibited in the presence of Ac-DEVD-CHO, a specific inhibitor of caspase 3 (lanes 6 vs 5 of Panel III). Maximum cleavage of the β-subunit is observed at around 6 hrs (Panel IV). The subunits are not accessible as substrates for caspase 6 (Panel V).

Fig 3.4A.



Time in hrs: 0 1 2 4 6

Panel V



eIF2β + Caspase 6

Phosphorylation of bacterially expressed eIF2α was carried out in the presence of recombinant mouse PERK and labeled ATP prior to caspase treatment (**Fig 3.4B**, Panel A, lane 4) and also after caspase treatment (lane 2). Both processed and unprocessed eIF2α subunit serve as substrates for kinase. However, phosphorylated eIF2α resists caspase-3 processing (**Fig 3.4B**, lane 4 vs. 2). Analysis of phosphorylation of bacterially expressed recombinant human β-subunit reveals that it is phosphorylated by PKA (**Fig 3.4B**, lower part of panel B, lane 5), PKC (lane 6) and CKII (lane 7). However, PKA is found to phosphorylate more efficiently than PKC or CKII *in-vitro* (5 vs. 6 and 7). As has been observed with the α-subunit, the β-subunit is efficiently processed by caspase-3 prior to its phosphorylation than after phosphorylation by any of the three kinases tested here i.e., PKA, PKC and CKII (**Fig. 3.4B**, upper portion, lanes 12, 13 and 14 vs. lanes 8, 9 and 10). Recombinant α- and β-subunits of human eIF2 serve as substrates for caspase 3 (**Fig 3.4A**) but phosphorylation of these subunits makes them resistant to caspase 3 action (**Fig 3.4B)**. However, these subunits are not accessible as substrates for caspase 6 (**Fig 3.4A,** panel V).

Since β-subunit interacts with eIF2α, eIF2B, eIF5 and Nck1, aids the γ-subunit in many of the eIF2 functions, regulates the productive and non-productive interactions between eIF2 and 2B complexes and is a substrate for kinases and caspase (s), these support the idea that the β-subunit of mammalian eIF2 plays a critical role in the function and regulation of eIF2 activity.

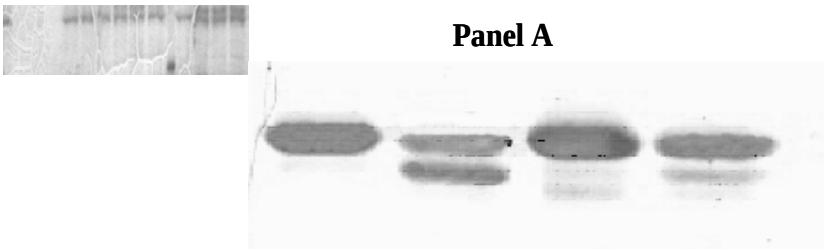
Cytochrome c interacts with eIF2*α*: Since eIF2α interacts with caspases (Marissen et al., 2000), its phosphorylation is implicated in apoptosis (Scheuner et al., 2006; Aparna et al., 2003) and a recent study has shown the that phosphorylated eIF2α and cytochrome c are co-localized in ischemic neuron\al cells (Page et al., 2003), we have studied here, the interaction between recombinant human eIF2α and purified horse cytochrome c using co-immunoprecipitation, pull down assays and the equilibrium constant for the binding of eIF2α and cytochtrome c was also calculated (**Figs 3.5 A-C**).

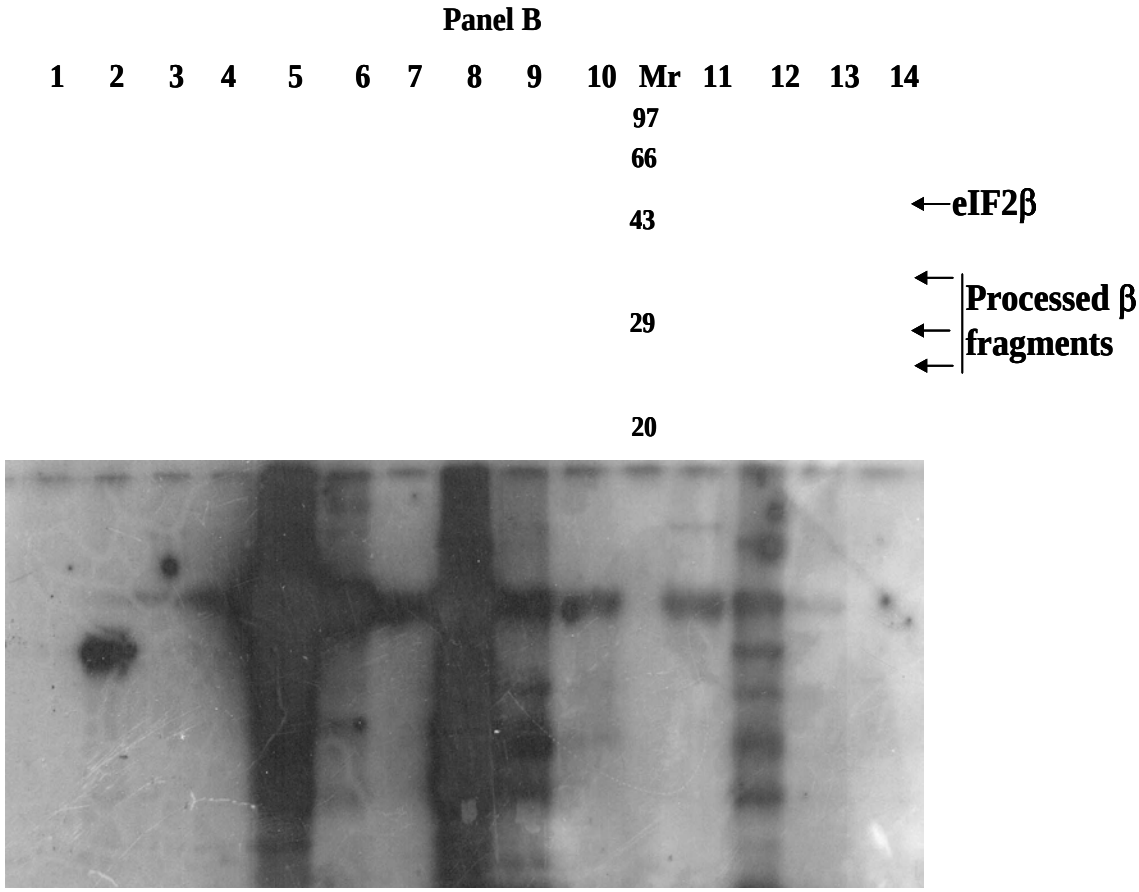
Immunoprecipitation assays were carried out as described in ‘Materials and Methods’ and the immunoprecipitated samples were separated by SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was probed with anti- eIF2α or cytochrome c

Fig 3.4B: Effect of phosphorylation of α- and β-subunits on their cleavage by caspase 3: As the recombinant α- and β-subunits are phosphorylated by their respective kinases (**Figs 1.5 A** and **B**) and also serve as substrates for caspase 3 (**Fig 4.2A**), the effect of phosphorylation of the subunits by respective kinases on their cleavage by caspase 3 was analyzed here. Panel A represents the effect of phosphorylation of the α-subunit on its cleavage by caspase 3. Phosphorylation of the α-subunit by PERK was carried out in the presence of [γ^{32} P] ATP before and after caspase 3 processing as described in 'Materials and Methods'. The figure represents a phosphorimage indicating the phosphorylation status of the α-subunt before and after caspase 3 treatment. Various lanes are as follows: lane1, eIF2α-subunit + PERK; lane 2, eIF2α + caspase 3 + PERK; lane 3, eIF2α + PERK; lane 4, eIF2α + PERK + caspase 3.

Panel B represents the cleavage of unphosphorylated and phosphorylated β-subunit by caspase 3. Phosphorylation of the β-subunit by PKA, PKC and CKII was carried out in the presence of [γ^{32} P] ATP before and after caspase 3 processing as described in 'Materials and Methods'. The upper portion is a coomassie stained gel indicating the caspase 3 cleavage of the phosphorylated (lanes 8, 9 and 10) and unphosphorylated β-subunit (lanes 12, 13 and 14) respectively. Lower portion represents the corresponding autoradiogram of the phosphorylation of β-subunit by different kinases before and after caspase 3 treatment. The various lanes are as follows: lane 1, + eIF2β; lane 2, + PKA; lane 3, + PKC; lane 4, + CKII; lane 5, eIF2β + PKA; lane 6, eIF2β + PKC; lane 7, eIF2β + CKII; lane 8, PKA phosphorylated eIF2β + caspase 3; lane 9, PKC phosphorylated eIF2β + caspase 3; lane, 10 CKII phosphorylated eIF2β + caspase 3; Mr represents marker lane; lane 11, eIF2β + CKII; lane 12, caspase 3 treated β + PKA; lane 13, caspase 3 treated β+ PKC; lane 14, caspase 3 treated β + CKII.

Fig 3.4B.


Panel A


Panel B
1 2 3 4 5 6 7 8 9 10 Mr 11 12 13 14
97
66
43
29
20
eIF2β
Processed β
fragments

antibodies to determine the interaction between these two proteins (**Fig 3.5A**, panels I and II). Complementing these observations with purified cytochrome c, we have also carried out another experiment as described in 'Materials and Materials' to demonstrate the interaction between the recombinant α-subunit of eIF2 and cytochrome c of HeLa cell extracts (**Fig 3.5A** panel III). Results of these experiments suggest that eIF2α interacts with cytochrome c. Determination of the equilibrium constant for the binding of eIF2α and cytochrome c at 415 nm resulted in the Kass ~2.7 μM^{-1}, which indicates a significant binding between the two proteins (**Fig 3.5B**). Further the effect of phosphorylation of eIF2α was also analyzed by a pull down assay (**Fig 3.5C**). The results indicate that phosphorylated eIF2α interacts efficiently with cytochrome c than the unphosphorylated form (**Fig 3.5C** Panel I and panel II representing a bar diagram). We have also analyzed the ability of cytochrome c bound eIF2 to serve as a substrate for caspase 3 (**Fig 3.6** Panel A). The results indicate that eIF2α interaction with cytochrome c does not alter the efficiency of eIF2α processing by caspase 3, although eIF2α can interact with both cytochrome c and caspase 3 at the same time (**3.6** Panel B). However, the physiological significance of this interaction is not clear and requires further studies.

Fig 3.5A.

Fig 3.5A: Interaction between eIF2α and Cytochrome c: Co-immunoprecipitation studies with either anti- eIF2α or cytochrome c antibodies were carried out as described in Materials and Methods. Panels I and II represent the interaction between the two purified proteins. Panel I shows the interaction where the complex was precipitated with anti-eIF2α antibody, fractions were separated on 10% SDS-PAGE and transferred onto a nitrocellulose membrane, which was probed with anti-cytochrome c antibody to detect the interacting protein. Likewise in panel II, the complex precipitated by anti-cytochrome c antibody and the membrane probed with anti-eIF2α antibody to detect the interacting proteins. Panel III represents the interaction of the recombinant α-subunit with the cytochrome c of HeLa cells. The complex precipitated with anti-cytochrome c antibody and the membrane was probed with anti-eIF2α antibody to detect the interaction. Various lanes are as follows: 1, Unbound; 2 and 3, Wash fractions; and 4, Eluted fractions.

Fig 3.5B: Interaction between eIF2α and Cytochrome c – Determination of the binding constant: The equilibrium constant for the binding of eIF2α with Cytochrome c was determined as described in 'Materials and Methods'. Panel **(i)** represents the changes in the visible absorption spectra of the cytochrome c in the presence of various concentrations of eIF2α. Panel **(ii)** represents the analysis of the absorbance values as a function of eIF2α concentration. The equilibrium association constant is calculated from the value of pK_{ass}, which is obtained from the X intercept of the straight line.

Equilibrium binding constant as calculated using equations as described in 'Materials and Methods' is found to be ~2.7 μM^{-1}.

Fig 3.5B:

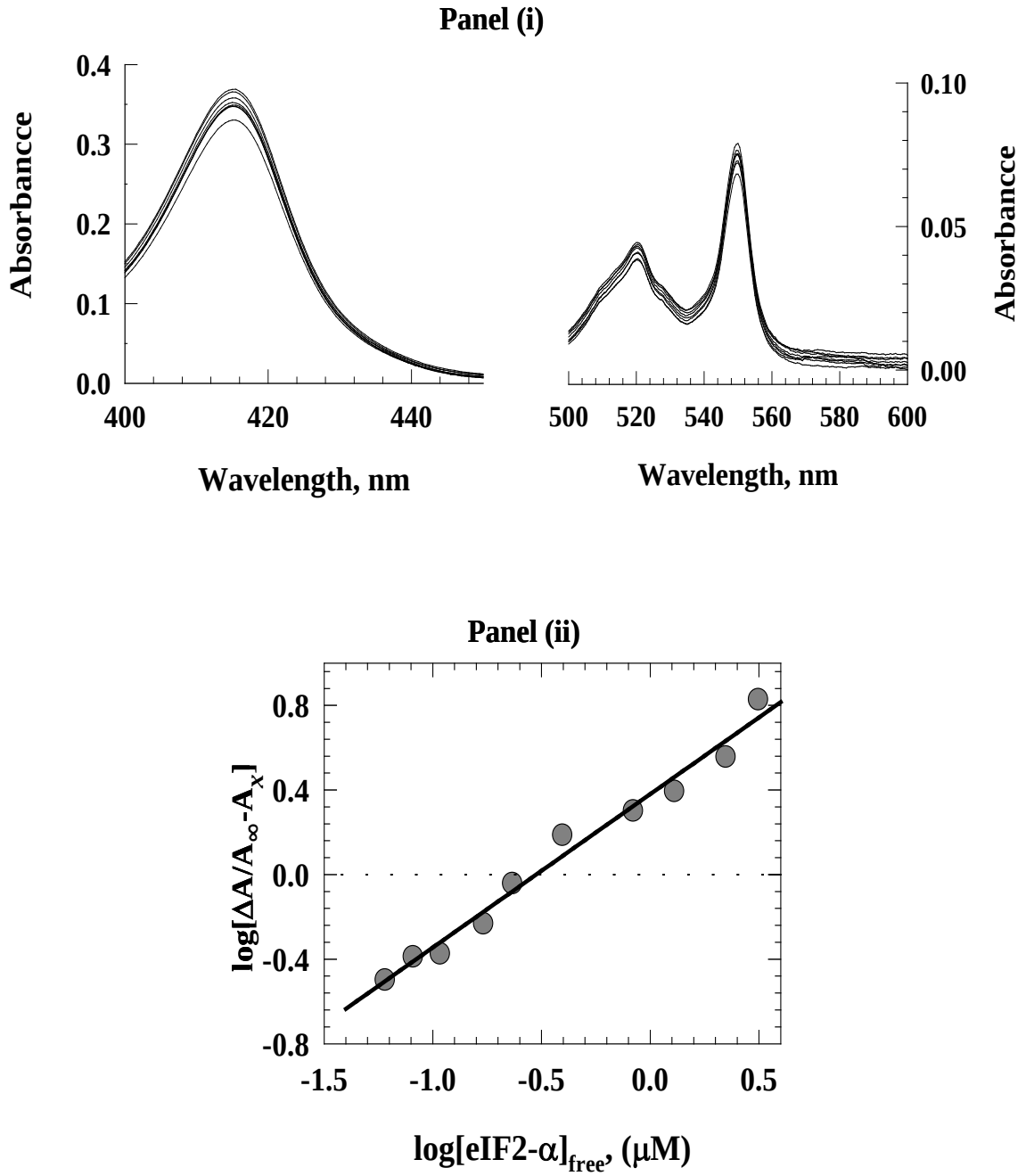

Panel (i)
Absorbancce
0.4
0.3
0.2
0.1
0.0
400
420
440
Wavelength, nm
0.10
0.05
0.00
500
520
540
560
580
600
Absorbancce
Wavelength, nm
Panel (ii)
log[ΔA/A∞-Aχ]
0.8
0.4
0.0
-0.4
-0.8
-1.5
-1.0
-0.5
0.0
0.5
log[eIF2-α]free, (μM)

Fig 3.5C.

Fig 3.5C: Phosphorylation of eIF2α- Effect on eIF2α and Cytochrome c interaction: Recombinant eIF2α was phosphorylated by PERK in the presence of 100 µM unlabelled ATP as described in 'Materials and Methods'. Pull down assay with unphosphorylated and phosphorylated eIF2α and cytochrome c was carried out as described in 'Materials and Methods'. Various unbound and bound fractions were separated on a 10% SDS-PAGE and transferred onto a nitrocellulose membrane. The figure is a western blot. The upper portion of panel I represent the levels of Cyt c co-eluted with unphosphorylated (lane 5) and phosphorylated eIF2α (lane 6). Bottom portion of panel I depicts the levels of eIF2α eluted under these conditions. Various lanes are lanes 1 and 2, unbound fractions of unphosphorylated and phosphorylated eIF2α respectively; lanes 3 and 4, corresponding wash fractions; lanes 5 and 6 are corresponding elution fractions. Panel II is a bar diagram representing the quantification of the corresponding fractions.

Fig 3.6.

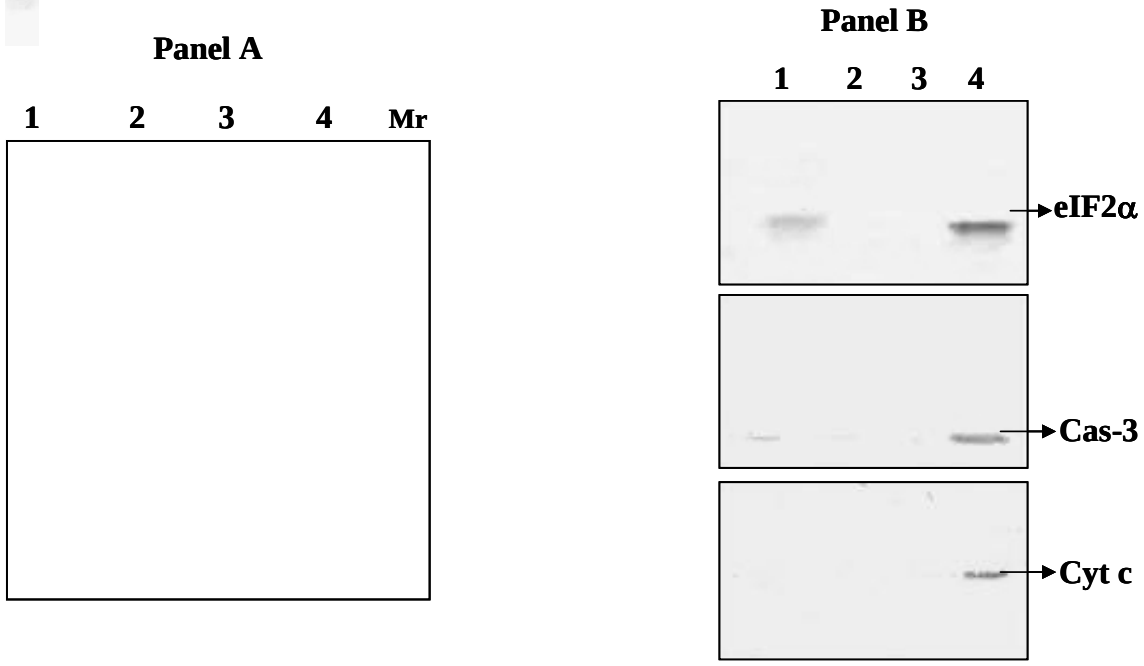


Fig 3.6: eIF2α - Cytochrome c interaction: Effect on caspase 3 cleavage of the α-subunit: The figure is a western blot. Panel A represents the cleavage of recombinant α-subunit of human eIF2 by caspase 3 in the absence (lane 3) and presence of cytochrome c (lane 4). Caspase 3 cleavage of recombinant α-subunit in the presence of cytochrome c was carried out as described in 'Materials and Methods'. Panel B represents the interaction between recombinant eIF2α, cytochrome c and caspase 3 by a pull down assay carried out with three proteins as described in 'Materials and Methods'. Various lanes in Panel A are as follows: 1, eIF2α; 2, eIF2α + Cyt c; 3, eIF2α + Caspase 3; 4, eIF2α + Cyt c + Caspase 3 and Mr represent Marker. Lanes in the top, middle and bottom portions of panel B are lane1, unbound fraction; lanes 2 and 3, wash fractions and lane 4 represent the elution fraction.

Part 2

Chapter 1

Phosphorylation of eIF2α : Adaptive and Apoptotic Functions

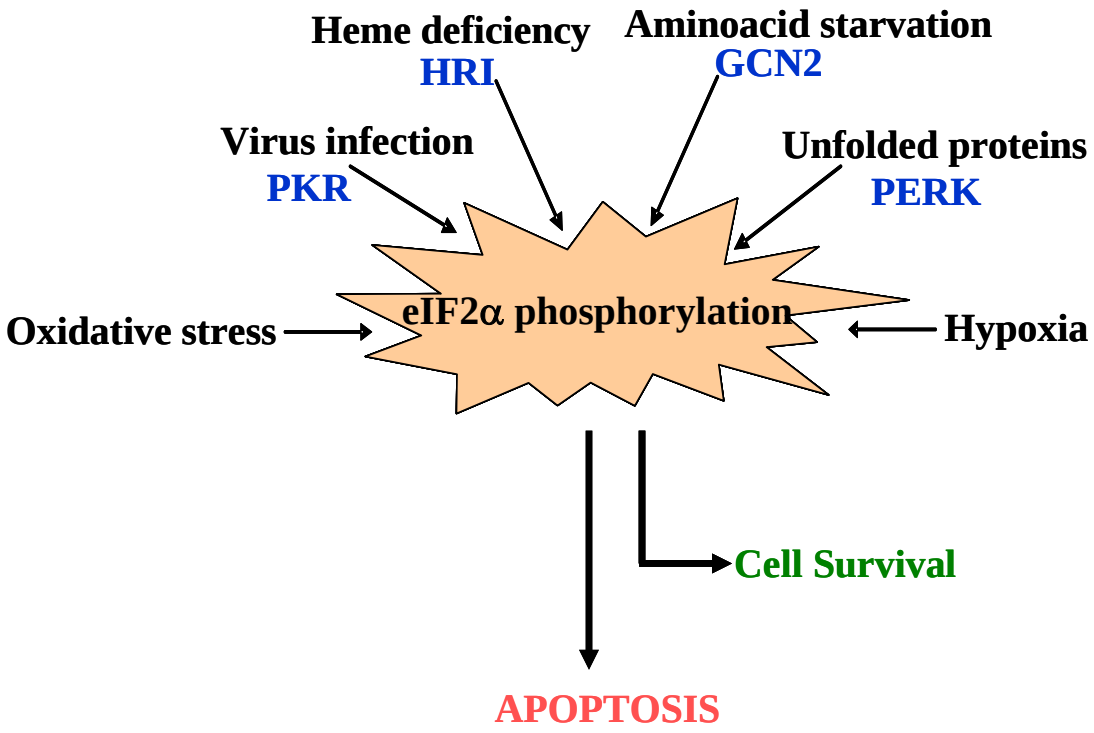
Introduction:

Phosphorylation of eIF2α is a stress signal: Phosphorylation of the conserved Ser51 residue in the α-subunit of eIF2 is a stress signal and it occurs in response to multiple stress conditions such as heme-deficiency, virus infection, amino acid starvation or nutrient deprivation, heavy metal stress, changes in cellular redox and calcium levels and accumulation of unfolded proteins that can occur in response to excessive protein synthesis or improper modifications on the proteins (**Fig E**). Further phosphorylation of eIF2α occurs both in the cytosol and also in the endoplasmic reticulum (ER) in response to the activation of respective eIF2α kinases. While PERK is an ER resident eIF2α kinase, the three other well characterized eIF2α kinases viz., GCN2, HRI and PKR are cytosolic kinases and they all require different activation signals (see General Introduction) although they all phosphorylate the Ser51 residue in eIF2α. In fact, phosphorylation of eIF2α affects only after birth in response to diverge stress conditions but not during development of embryo as substantiated by the knock-in experiments (Scheuner et al., 2001).

Recent studies with ER stress-induced adaptive signaling cascade have shown to contain two arms: translational attenuation by eIF2α phosphorylation followed by transcriptional induction of chaperones like BiP. Prolonged stress beyond the adaptive limit of a cell however can trigger apoptosis, the suicide of unhealthy cells in a programmed manner. Apoptosis that occurs in response to both cytosolic and ER stress is mediated by the mitochondria (intrinsic pathway) and/or through the activation of proapoptotic down stream kinases that are typically triggered in the death-induced receptor-mediated extrinsic apoptotic pathway.

Phosphorylation of eIF2α affects gene expression programme: The decline in general translation that occurs through eIF2α phosphorylation is clearly seen as an adaptive mechanism to protect cells from unfolded protein accumulation, relieve the burden of the folding chaperones and to save the amino acid pools (Harding et al., 2000). However, the translations of many mRNAs are not arrested but are upregulated in response to eIF2α phosphorylation induced gene expression programme. These include mRNAs with multiple upstream open reading frames (uORFs) like ATF4 in mammals and GCN4 in

Fig E:

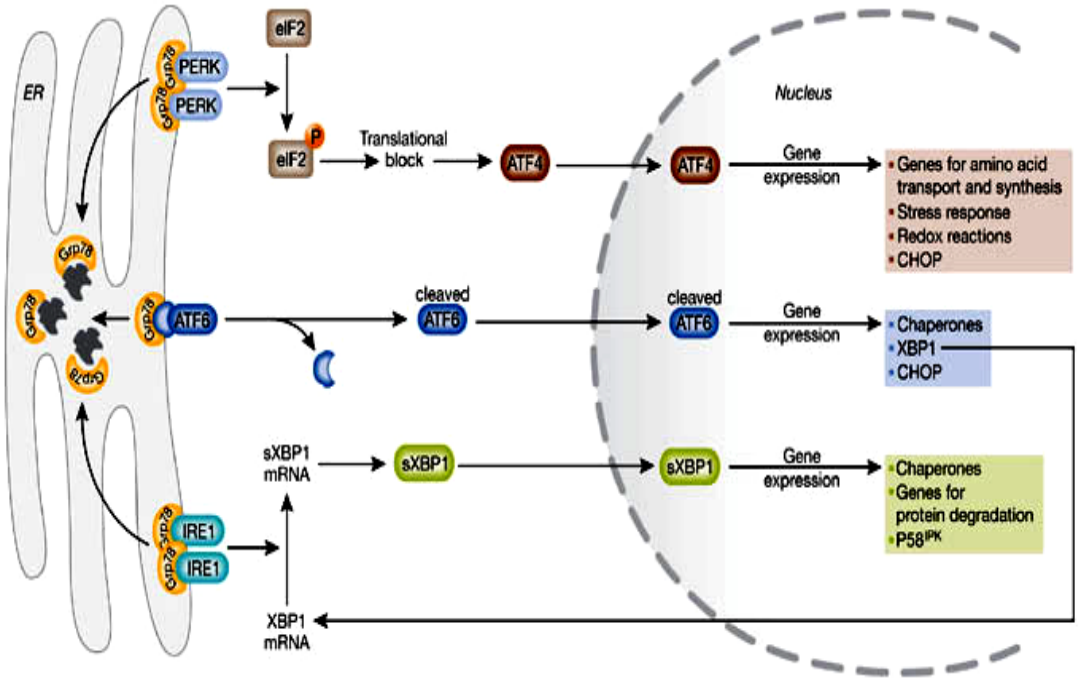


Phosphorylation of eIF2α is a stress signal: In response to various stresses, four different kinases phosphorylate Ser51 position on the α-subunit of eIF2. Generally, phosphorylation of eIF2α is considered to be a stress signal and may promote apoptosis. But, recent studies have suggested a cytoprotective role in response to ER stress induced cell death. These paradoxical effects of eIF2α phosphorylation may be because of co-incident signaling activities that promote activation of eIF2α kinases.

yeast which in turn facilitate the synthesis of several genes involved in amino acid synthesis, transport and redox chemistry (Vattem and Wek, 2004; Hinnebusch, 2005; Harding et al., 2003). Also many mRNAs that contain internal ribosome entry sequences (IRESs) like CAT-1 and growth factor-related genes such as PDGF, VEGF, c-Myc have been upregulated during eIF2α phosphorylation. Phosphorylation of eIF2α also regulates the mRNAs that code for cellular antiapoptotic proteins (IAPs) (Gerlitz et al., 2002; Scheuner et al., 2001; Jefferson and Kimball, 2004; Brewer et al., 1999; Harding et al., 2000; Holcik and Sonenberg, 2005). For example ER stress induces human IAP-2 at the translational level (Warnakulasuriyarachchi et al., 2004) where as the translation of certain IAPs decreases in response to PKR-induced eIF2α phosphorylation during TNFα-induced apoptosis (Scheuner et al., 2006). Growth arrest DNA-damage-inducible gene-153 (GADD-153), also called CHOP (CCAAT/enhancer binding protein or C/EBP), a proapoptotic transcription factor (Oyodomari and Mori, 2004) is induced in response to PERK- eIF2α phosphorylation and ATF4 expression in mammals (**Fig F**). Expression of CHOP leads to the induction of several proapoptotic proteins and one of them is GADD 34, a cofactor of eIF2α phosphatase. GADD34 appears to play both proapoptotic and prosurvival roles. Dephosphorylation of eIF2α by GADD-34 in the overburdened ER aggravates ER stress by enhancing the folding client load. Translational restoration may facilitate the synthesis of proapoptotic proteins. Alternatively, GADD-34 mediated translational restoration is required for the adaptive cell survival measures (Marciniak and Ron, 2006).

Phosphorylation of eIF2α mediates caspase activation and apoptosis: In an earlier study, we observed that many agents such as UV light, etoposide, cycloheximide, tunicamycin, A23187, EGTA promote phosphorylation of eIF2α in the ovarian cells of Spodoptera frugiperda (*Sf9*), a lepidopteran insect, that are natural hosts of baculovirus infection. However, only some of these stress conditions lead to cell death. Interestingly we observed that caspase inhibitors or wt baculovirus that produces an antiapoptotic p35 protein inhibit UV-induced eIF2α phosphorylation and apoptosis. However, the caspase inhibitors do not affect the eIF2α phosphorylation induced by a typical ER stress promoting agent like tunicamycin, thereby suggesting that eIF2α phosphorylation is a

Fig F:



Unfolded protein response (UPR): In mammalian cells IRE-1, PERK and ATF6 are the major players in ER stress signaling pathway. Under normal conditions these three ER-transmembrane proteins are held inactive by the binding of BiP/GRP78 to their ER luminal domains. Accumulation of unfolded proteins in the lumen of ER under the conditions of nutrient deprivation, calcium fluxes, redox imbalance, viral infection, etc, evokes a stress response pathway known as unfolded protein response (UPR). This induces the release of BiP bound to the above mentioned transducers to facilitate protein folding and is followed by the activation of IRE-1 and PERK by oligomerization and autophosphorylation. Active PERK phosphorylates eIF2α and attenuates general translation, reducing the ER client protein load. Phosphorylation of eIF2α upregulates the translation of ATF4, a potent bZIP transcription factor that induces CHOP and genes involved in redox regulation, and amino acid metabolism. Active IRE-1 splices XBP1 mRNA and the spliced XBP1 mRNA generates a potent bZIP transcription factor that translocates to nucleus and upregulates transcription of genes that encode chaperones. ATF6 is activated through its processing by golgi resident S1P and S2P proteases. Processed p50ATF6 also translocates to nucleus and upregulates ER chaperone and ERAD genes (Schroder and Kaufman, 2005).

The figure is derived from Szegezdi et al., 2006.

cause and consequence of caspase activation in cells treated with proapoptotic agents. Complementing these observations and the above suggestion, a mutant baculovirus devoid of its p35 gene is found to stimulate eIF2α and apoptosis (Aparna et al., 2003). Subsequent studies have shown that UV-induced apoptosis in *Sf9* cells is mediated by the intrinsic pathway (Sahdev et al., 2005). While UV is known to induce GCN2 kinase like activity, typical ER stressors like tunicamycin may be inducing PERK, an ER-resident eIF2α kinase. Although prolonged cytosolic and ER stressors are known to promote apoptosis in mammalian cells, we suspect that ER stress-induced apoptotic mechanisms are not operating in *Sf9* cells. A recent study in mammalian cells indicates that PKR-mediated eIF2α phosphorylation however can stimulate a caspase activity and apoptosis (Scheuner et al., 2006). Interestingly, baculovirus also contains a homolog of truncated eIF2α kinase called PK2 that is found to inhibit eIF2α phosphorylation in yeast and the activation of eIF2α kinases *in-vitro* (Dever et al., 1998)

To determine further the importance of eIF2α phosphorylation in promoting apoptosis in *Sf9* cells, we have evaluated here the changes in phosphorylation status of eIF2α, caspase activity and apoptosis in uninfected, and wt and mutant pk2 (Δpk2) baculovirus infected *Sf9* cells in the presence and absence of UV-irradiation. As virus infection is known to promote ER stress, we monitored eIF2α phosphorylation, ATF4, a bZIP transcription factor produced in response to eIF2α phosphorylation, and Bip, an ER chaperone. Our observations indicate that *Sf9* cells infected by pk2 deletion virus show relatively higher levels of eIF2α phosphorylation compared to the cells infected by wt virus. Unlike p35 deletion baculovirus that induces eIF2α phosphorylation and readily stimulates apoptosis (Aparna et al., 2003), pk2 deletion virus however does not promote apoptosis readily. Only prolonged infection with pk2 virus can lead to apoptosis. This is due to the fact that pk2 deletion virus also contains p35 gene as in wt virus. These findings therefore favor the idea that eIF2α phosphorylation promotes caspase activation and apoptosis. This is further supported by the fact that UV-induced eIF2α phosphorylation, caspase activity and apoptosis in *Sf9* cells is diminished more effectively by wt baculovirus infection compared to pk2 deletion virus. An analysis of BiP levels indicate that it increases in cells infected by wt and mutant baculovirus, but not in uninfected or UV-treated cells suggesting that virus

infection promotes ER stress. ATF4 levels are maintained high in pk2 deletion virus-infected cells but decline in wt baculovirus-infected cells and correlate to enhanced phosphorylation status of eIF2α observed in pk2 deletion virus infected cells. Since UV irradiation alone does not stimulate BiP or ATF4 significantly, these findings suggest that ER-stress-induced eIF2α phosphorylation pathway may be important for the expression of ATF4. Further the levels of Gadd-34, the cofactor of eIF2α phosphatase increases in cells infected by wt and mutant baculovirus but not by UV irradiation suggesting that ER-stress induced pathway plays an important role in the induction of GADD-34. Although eIF2α phosphorylation and ATF4 levels decline in wt baculovirus infected cells, significant decline in GADD-34 levels however are not observed. This finding suggests that the induction of GADD-34 may also occur through other ER stress-induced signaling mechanisms like IRE-1 or ATF6 pathways other than PERK-eIF2α phosphorylation of pathway as has been suggested (**Fig F**) (Oyadomari and Mori, 2004).

Results:

Mutant pk2 (Δpk2) baculovirus infection stimulates apoptosis and caspase activation at prolonged infection times: Previous studies from this lab have shown that *Sf9* cells infected with a mutant p35, but not wild-type baculovirus, readily induced eIF2α phosphorylation and apoptosis (Aparna et al, 2003). A recent study indicates that PKR induced phosphorylation of eIF2α induces apoptosis (Scheuner et al, 2006). Here we analyzed apoptosis, caspase activation and eIF2α phosphorylation in pk2 mutant baculovirus infected cells Sf9 cells and compared the results to cells infected by wt baculovirus (**Fig 4.1 A-C)**. The cells were infected with wildtype (wt) or mutant pk2 virus (Δpk2) as described in Materials and Methods, for time periods as mentioned in the legends to the figure 4.1. Apoptosis was scored by observing the changes in morphology of cells under an inverted microscope as mentioned in 'Methods'. Both wt and mutant virus infected cells appeared similar to the control cells during early periods of infection. (**Fig 4.1A**, **i** and **ii**) However mutant (Δpk2) but not wt baculovirus infected cells displayed 10-15% apoptosis at a prolonged infection times (40-48 hrs) as observed by membrane blebbing and formation of apoptotic bodies (**Fig 4.1A, v** vs **iv)**. Trypan blue exclusion test

was also carried out to score for apoptosis. Since one of the characteristic events in apoptosis is caspase activation, Ac-DEVD-AFC, a fluorogenic substrate of caspase 3, was used to measure the activation of *Sf9* caspases. A considerable caspase activity was observed in the extracts of the *Sf9* cells infected with the mutant virus (Δpk2) for prolonged time periods (**Fig 4.1B**), complementing the morphological observations of the cells (**Fig 4.1A**). These findings therefore suggest pk2 mutant virus stimulates small but significant amounts of caspase activation and apoptosis at late stages of infection compared to the cells infected by wt baculovirus.

Mutant pk2 baculovirus infection enhances eIF2α phosphorylation: Phosphorylation of eIF2α on Ser51 is conceived to be a stress signal and occurs in response to various kinds of stress conditions as described earlier. The levels of phosphorylated eIF2α in the extracts of the uninfected and infected cells, determined by a phosphospecific anti-eIF2α antibody indicate an increase in the levels in the early phases of the viral infection (8 hrs). The levels of phosphorylated eIF2α declined with prolonged infection times (16, 24 and 36 hrs) in the case of wt baculovirus infection (**Fig 4.1C**, **i**, lanes 4, 6 and 8) but increased in the mutant (Δpk2) baculovirus infection (**Fig 4.1C**, **i**, lanes 5, 7 and 9). The initial increase in the levels of eIF2α phosphorylation, and its subsequent decline in wt baculovirus-infected cells correlate to the reports that p35 expression occurs six to eight hrs after infection. The gradual increase in eIF2α phosphorylation in pk2 deletion virus-infected cells relates the fact that PK2 protein is a late gene product (Dever et al., 1998).

Baculovirus infection enhances ATF4 and Bip expression: Virus infection in general is known to cause a stress in the endoplasmic reticulum (ER) and also phosphorylation of eIF2α by different mechanisms. Phosphorylation of eIF2α upregulates the expression of certain mRNAs with uORFs as a part of an adaptive response of the cell against the stress (Dever et al., 2002). Kinetics of expression indicates that the levels of ATF4, a bZIP transcription factor, increase both in wt and mutant (Δpk2) baculovirus infected *Sf9* cells at the initial hrs of infection (8 hrs). The levels of ATF4 however were maintained high in the mutant (Δpk2) virus infected cells but declined in wt virus infected cells with time (**Fig 4.1C**, **ii**) and correlate to the levels of eIF2α phosphorylation (**Fig 4.1C**, **i**). This is consistent with the reasoning that ATF4 expression is induced in response to eIF2α

phosphorylation (Vattem and Wek, 2004). Both wt and mutant (Δpk2) baculovirus infection lead to an increased expression of Bip, an ER resident chaperone (**Fig 4.1C**, **iii**) and GADD 34, a co-factor of eIF2α phosphatase (**Fig 4.1C**, **iv**). This is consistent with the idea that virus infection generally cause an ER stress and increase ER chaperone levels to maintain the ER client load (Harding et al., 2003) or to balance the protein folding to ongoing protein synthesis. Expression of GADD-34 down stream to eIF2α phosphorylation is considered to be a proapoptotic or prosurvival signal. The protein primarily dephosphorylates eIF2α and can restore translation of proapoptotic or prosurvival proteins whose synthesis is inhibited due to phosphorylation of eIF2α. But under prolonged ER stress and eIF2α phosphorylation, enhanced GADD-34 levels may enhance the unfolded protein response and thereby the stress in the ER.

***Sf9* cells infected with baculovirus resist UV induced Apoptosis:** UV-irradiation of *Sf9* cells for a brief period of 60 sec and incubation for 15 hrs at 27^{o}C, resulted in ~90% apoptosis (**Fig 4.2A**, **iv**). Infection of the cells with wt baculovirus that produces P35 antiapoptotic protein, inhibit UV irradiation-induced apoptosis (**Fig 4.2A**, **iii**). These observations were consistent with the previous reports from this lab (Aparna et al., 2003). Interestingly, infection of the cells with the mutant (Δpk2) baculovirus also resists the UV-induced apoptosis (**Fig 4.2A**, **vi**) to a large extent. However the resistance to apoptosis offered by the mutant (Δpk2) baculovirus in response to UV-irradiation induced apoptosis was lower compared to wt baculovirus-infection. While wt baculovirus abrogates UV-induced apoptosis almost by 95%, mutant pk2 bacuovirus-infected cells reduce apoptosis to an extent of 70- 75%. In other words, the protection offered to *Sf9* cells by wt baculovirus infection against UV induced apoptosis was more efficient than that offered by the mutant (Δpk2) baculovirus infection. The above observations were further complemented by the levels of phosphorylated eIF2α (**Fig 4.2B**, Panel I) and caspase activity (**Fig 4.2B**, Panel II), in uninfected and virus-infected cells exposed to UV irradiation. The eIF2α phosphorylation levels were high in uninfected and UV irradiation treated cells, which came down with wt baculovirus infection (**Fig 4.2B**, Panel I, lane 8 versus 1). However, mutant (Δpk2) baculovirus infection was unable to bring down the phosphorylation levels as efficiently as the wt baculovirus infection (**Fig 4.2B**, Panel I, lane 14 vs 8).

Fig 4.1A: Morphology of *Sf9* cells infected with wt and mutant (Δpk2) baculovirus: *Sf9* cells were infected with wildtype (wt) or a pk2 deleted mutant (Δpk2) Autographa californica nuclear polyhedrosis virus (AcNPV) baculovirus as described in 'Materials and Methods' and incubated at 27^{o}C for 36 and 48 hrs respectively as indicated in the figure. Uninfected cells were used as a control. The morphology of the cells was observed under an inverted microscope at 20X magnification and apoptosis was scored as described in 'Materials and Methods'. Various panels are as follows: **i**, uninfected control *Sf9* cells; **ii** and **iv**, cells infected with wt baculovirus for 36 and 48 hours respectively; and **iii** and **v**, cells infected with mutant (Δpk2) virus for 36 and 48 hours respectively.

Fig 4.1B: Caspase activity of Uninfected and virus infected *Sf9* cells: *Sf9* cells infected with wt or mutant (Δpk2) virus for 36 and 48 hrs, and uninfected cells, were harvested and lysed as described in 'Materials and Methods'. Caspase activity of the cell extracts containing ~150 µg of total protein was determined as a measure of Ac-DEVD-AFC (flurogenic caspase substrate) cleavage at 450-550 nm in a spectrofluorimeter as described in 'Materials and Methods'. Various curves representing the caspase activities are as follows: 1, Blank; 2, uninfected control; 3, cells infected with wild type virus for 36 hours; 3, cells infected with wild type virus for 48 hours; 5, cells infected with Δpk2 virus for 36 hours; and 6, cells infected with Δpk2 virus for 48 hours.

Fig 4.1A

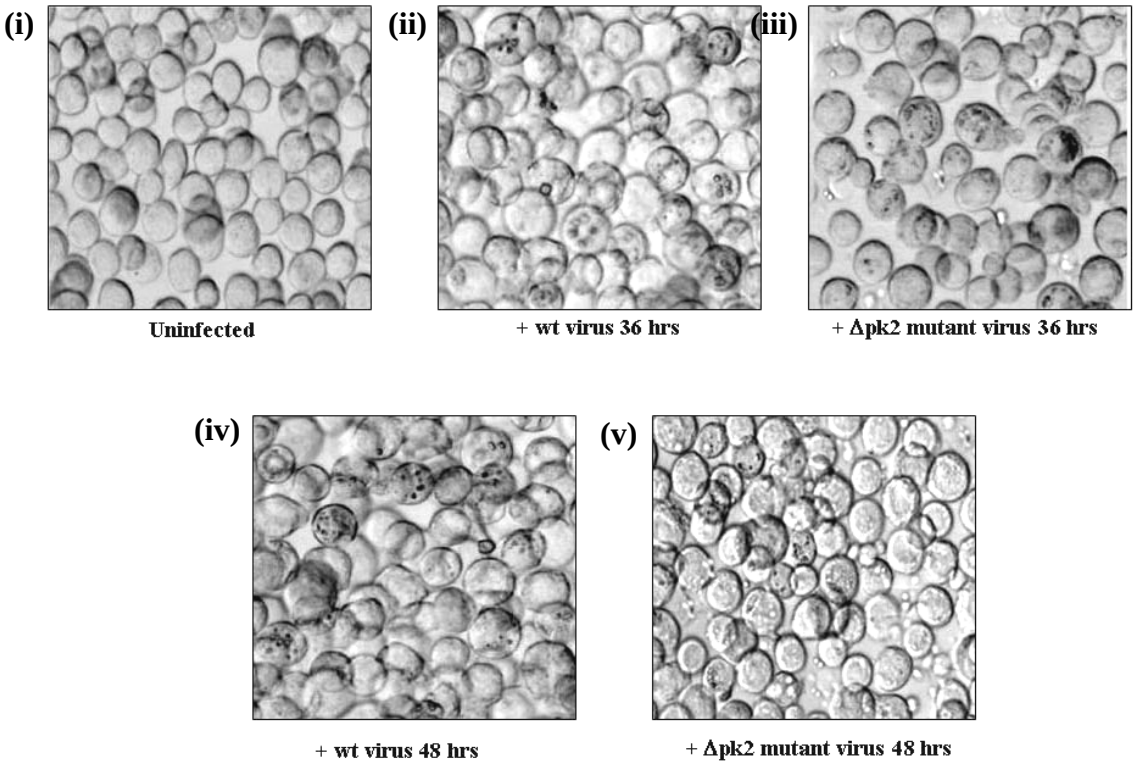


Fig 4.1B

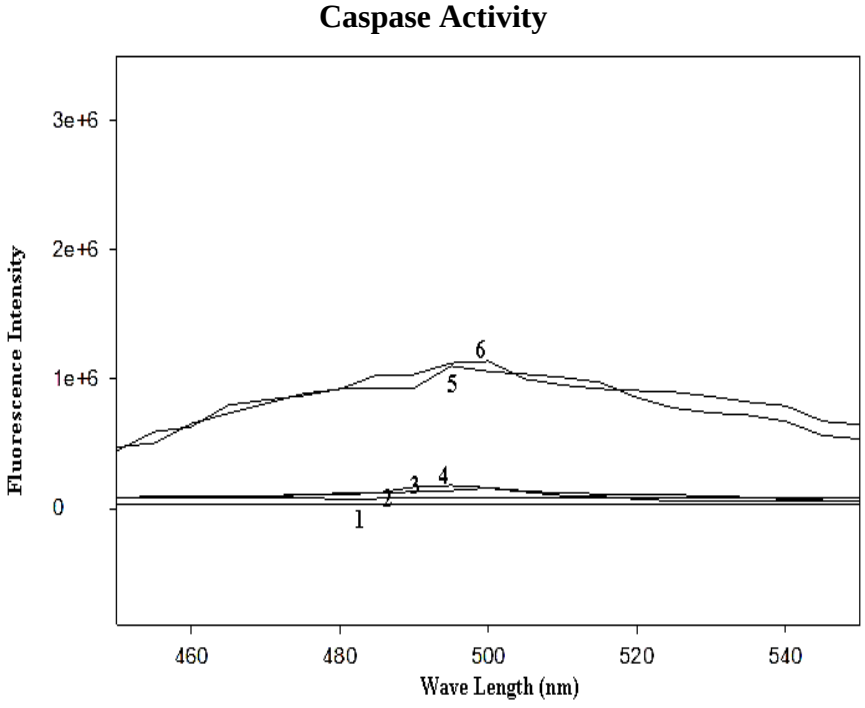
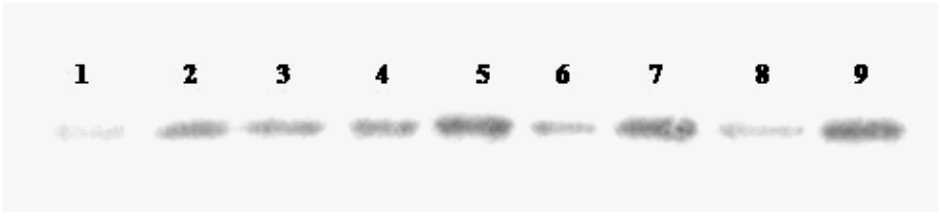


Fig 4.1C: Effect of virus infection on eIF2α phosphorylation and expression of various ER stress markers: *Sf9* cells were infected with wt or mutant (Δpk2) baculovirus for different time periods as indicated below. Cell extracts were prepared as described in 'Materials and Methods' and ~ 75 µg total proteins was separated by 10% SDS-PAGE. The proteins were transferred to a nitrocellulose membrane as described in Materials and Methods. The membrane was probed with a phosphospecific anti-eIF2α, antibody to determine the effect of wt and mutant virus infection on the levels of phosphorylated α-subunit of eIF2. To analyze the effect of virus infection to induce an ER stress, the membrane was also probed with anti- ATF4, Bip or GADD 34 antibodies, as indicated in the figure. Various lanes are as follows: 1, control/uninfected; 2, wt virus infected for 15 hrs; 3, Δpk2 virus infected for 15 hrs; 4, wt virus infected for 24 hrs; 5, Δpk2 virus infected for 24 hrs; 6, wt virus infected for 36 hrs; 7, Δpk2 virus infected for 36 hrs; 8, wt virus infected for 48 hrs; 9, Δpk2 virus infected for 48 hrs;

Fig 4.1C

(i)

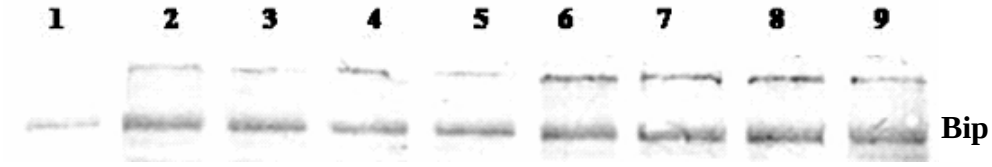


eIF2α Phopshorylation

(ii)

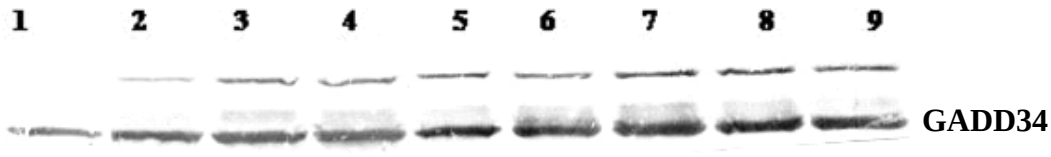
ATF4 expression

(iii)



Bip expression

(iv)



GADD34 expression

The enhanced levels in the caspase activity in uninfected and UV-treated *Sf9* cells as measured by the cleavage of Ac-DEVD-AFC, were significantly lowered by wt baculovirus but not by mutant (Δpk2) baculovirus infection (**Fig 4.2B**, Panel II). The ability of wt and mutant baculovirus infection to bring down the UV-induced caspase activation and apoptosis is attributed to the presence of an anti-apoptotic baculoviral protein P35. However, the enhanced levels of caspase activation and apoptosis induced by the mutant (Δpk2) baculovirus upon prolonged infection times may be due to the high levels of phosphorylation of eIF2α caused by the lack of PK2, a late gene baculoviral protein which serves as a specific eIF2α kinase inhibitor.

Discussion: We analyzed here specifically the importance of eIF2α phosphorylation in promoting apoptosis using a mutant baculovirus-infection. The mutant baculovirus lacks pk2 gene which codes for an eIF2α kinase inhibitor (Dever et al., 1998). Consistent with this notion, we observed here that cells infected by pk2 mutant baculovirus for prolonged time periods displayed enhanced phosphorylation of eIF2α and a small but significant amount of caspase activation and apoptosis (10-15%), compared to uninfected or wt virus-infected cells (**Fig. 4.1**). This result is further complimented by the fact that UV- induced apoptosis, is more efficiently abrogated by wt baculovirus infection but not by pk2 deletion virus infection (**Fig. 4.2**). It may seem that the protection offered by the pk2 mutant (Δpk2) virus-infection is due to the expression of P35, an anti apoptotic baculoviral protein by the infected cells. Although p35 mutant baculovirus-infected cells displayed higher level of eIF2α phosphorylation like pk2 mutant virus-infected cells, the p35 mutant virus infected cells undergo apoptosis more readily (Aparna et al., 2003). Earlier studies from this laboratory has also shown that caspase-inhibitors like zVAD-FMK can decrease eIF2α phosphorylation as observed with wt baculovirus infection that produces antiapoptotic P35 protein, hinting that caspase activation may also lead to further increase in eIF2α kinase activation. This suggestion is further corroborated with the evidence that caspase processed PKR is more active than unprocessed kinase in mammalian cells (Saelens et al., 2001). But subsequent unpublished observations from our lab have shown that eIF2α phosphorylation that occurs in the initial stages of UV-irradiated cells, unlike

Fig 4.2A

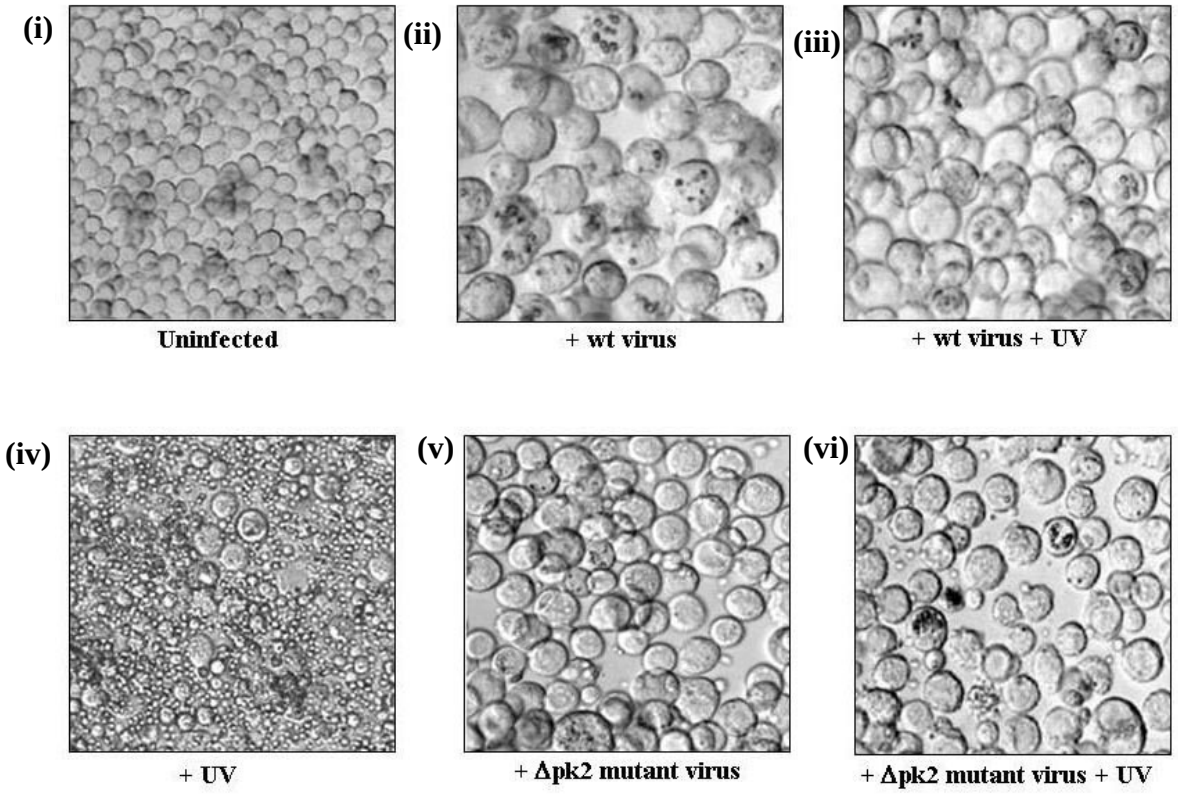


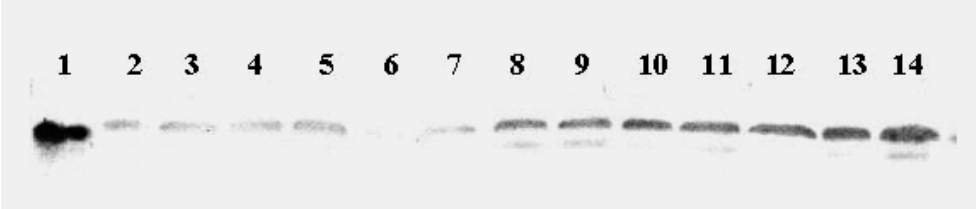
Fig 4.2A: Wildtype but not mutant (Δpk2) baculovirus infected *Sf9* cells efficiently resist UV-induced apoptosis: *Sf9* cells were infected with wt or mutant (Δpk2) baculovirus for ~35 hrs and then exposed to UV-B (312 nm) light for 60 sec. The cells were then incubated for 15 hrs at 27^{o}C. The changes in the morphology of cells and induction of apoptosis were observed under the microscope. Uninfected cells were also exposed to UV light as described above. Various panels in the figure are as follows: (i), uninfected and untreated cells; (ii), wt baculovirus infected cells; (iii), wt virus infected and UV treated; (iv), uninfected and UV treated; (v), mutant (Δpk2) baculovirus infected cells; (vi), mutant (Δpk2) baculovirus infected and UV treated.

Fig 4.2B: Effect of wt and mutant (Δpk2) virus infection on UV-induced eIF2α phosphorylation and Caspase activity: Uninfected and wt or mutant (Δpk2) baculovirus infected *Sf9* cells were harvested and cell extracts were prepared as described in 'Materials and Methods'. In panel I, extracts containing ~75 µg of protein was separated on a 10% SDS-PAGE and transferred to a nitrocellulose membrane as described in 'Materials and Methods'. The membrane was probed with anti- phosphospecific eIF2α antibody to analyze the effect of virus infection on UV light induced eIF2α phosphorylation. Various lanes are as follows: 1, +UV 15 hrs; 2, uninfected and untreated cells; 3, +wt virus - UV 5 hrs; 4, +wt virus - UV 10 hrs; 5, +wt virus - UV 15 hrs; 6, +wt virus + UV 5 hrs; 7, +wt virus + UV 10 hrs; 8, +wt virus + UV 15 hrs; 9, +Δpk2 virus - UV 5 hrs; 10, +Δpk2 virus - UV 10 hrs; 11, +Δpk2 virus - UV 15 hrs; 12, +Δpk2 virus + UV 5 hrs; 13, +Δpk2 virus + UV 10 hrs; 14, +Δpk2 virus + UV 15 hrs.

In panel II, the above extracts from 5 and 15 hrs time periods were used to determine the caspase activity by using Ac-DEVD-AFC as described in 'Materials and Methods'. Various panels are as follows: (**i**) represent caspase activity of uninfected and wt or mutant (Δpk2) baculovirus infected cells treated with or without UV and the corresponding bar diagram; and (**ii**) represent the caspase activities of uninfected and virus infected cells in comparison to that of uninfected and UV treated cells and the corresponding bar diagram. Various curves are as indicated in the figure.

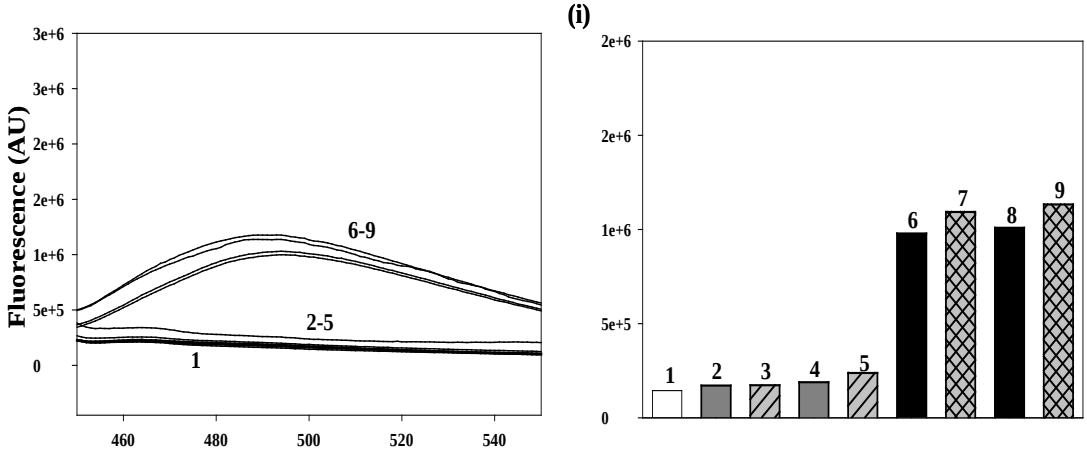
Fig 4.2B

Panel I



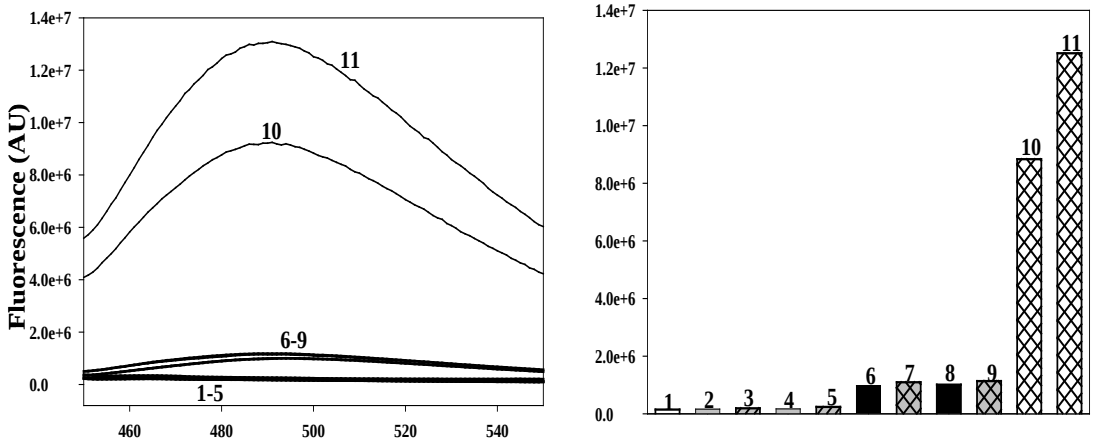
Panel II

(i)



Curve/Bar	1	2	3	4	5	6	7	8	9
Virus	-	wt	wt	wt	wt	Δpk2	Δpk2	Δpk2	Δpk2
UV	-	-	+	-	+	-	+	-	+
Time(h)	15	5	5	15	15	5	5	15	15

(ii)



Curve/Bar	1	2	3	4	5	6	7	8	9	10	11
Virus	-	wt	wt	wt	wt	Δpk2	Δpk2	Δpk2	Δpk2	-	-
UV	-	+	-	+	-	+	-	+	+	+	+
Time(h)	15	5	5	15	15	5	5	15	15	5	15

the phosphorylation that occurs at the late time points, is not significantly inhibited by caspase inhibitors (unpublished observations) thereby suggesting that eIF2α phosphorylation is a cause and consequence of caspase activation. This argument is further supported by the fact PKR-mediated eIF2α phosphorylation stimulates caspase activation in mammalian cells (Scheuner et al., 2006).

However earlier observations from this laboratory suggest that eIF2α phosphorylation does not always lead to apoptosis as obseved in cells treated with typical ER stress inducing agents like tunicamycin, thapsigargin, A23187 (Aparna et al., 2003). This is probably because of the absence of upstream caspases like caspase12 in *Sf9* cells with which phosphorylated eIF2α can interact and trigger its activation. The present information available suggests that baculovirus P35 protein blocks the cleavage of P25 protein to P19 thereby inhibiting the maturation of caspases(s). P25 and P12 are the cleavage products of *proSf1* caspase. Studies conducted *in-vitro* and *in vivo* (Hasnain et al., 2003) demonstrate that baculovirus P35 also functions directly as an anti-oxidant by removing the free radicals and consequently the activation of upstream caspases and cell death in H_2O_2 treated *Sf9* cells. Hence it is suggested that baculovirus P35 protein can act as an inhibitor of caspases and also as an anti-oxidant by removing free radicals (Sahdev et al., 2003; Sah et al., 1994).

It appears that both oxidative stress and persistent eIF2α phosphorylation play a role in apopotosis. Both of them can occur in response to different agents both in the cytosol as well as in ER. In the ER, disruption of oxidative environment induces accumulation of unfolded proteins due to the failure in the formation of of –S-S bridges in proteins and the consequent unfolded protein response can trigger eIF2α phosphorylation in the ER through an ER-resident eIF2α kinase pathway. In *Sf9* cells, however probably due to a lack of caspase in the ER in the vicinity of phosphorylated eIF2, ER-stress induced eIF2α phosphorylation does not lead to cell death. This idea is consistent with the fact that none of the typical ER-stressors like tunicamycin, Thapsigargin, A23187 that induce eIF2α phosphorylation cannot stimulate apoptosis (Aparna et al., 2003). However in baculovirus infection, we find here can increase ER stress because of the enhanced BiP levels in infected cells and the mutant baculovirus p35 but not pk2 can stimulate readily apoptosis.

In the absence of P35 protein, baculovirus infection must be promoting a tremendous amount of oxidative stress that is apparently not confined to ER but can also be felt by the cytosol where it can trigger caspase activation directly or through phosphorylation of eIF2α and cell death. In the case of non-ER stressors, or the agents that do not induce BiP like the UV-irradiation, activation of a cytosolic eIF2α kinase like PKR or GCN2 may lead to the phosphorylation of eIF2α and caspase activation.

Literature Cited

- Alone PV, Dever TE. (2006) Direct binding of translation initiation factor eIF2gamma-G domain to its GTPase-activating and GDP-GTP exchange factors eIF5 and eIF2B epsilon J Biol. Chem. 281: 12636-44.
- Anthony DD Jr, Kinzy TG, Merrick WC. (1990) Affinity labeling of eukaryotic initiation factor 2 and elongation factor 1 alpha beta gamma with GTP analogs. Arch. Biochem. Biophys. 281: 157-62.
- Aparna G, Bhuyan AK, Sahdev S, Hasnain SE, Kaufman RJ, Ramaiah KV. (2003) Stress-induced apoptosis in Spodoptera frugiperda (Sf9) cells: baculovirus p35 mitigates eIF2 alpha phosphorylation. Biochemistry, 42: 15352-60.
- Aravind L, Koonin EV. (2000) Eukaryote-specific domains in translation initiation factors: implications for translation regulation and evolution of the translation system. Genome Res. 10: 1172-84.
- Asano K, Phan L, Anderson J, Hinnebusch AG. (1998) Complex formation by all five homologues of mammalian translation initiation factor 3 subunits from yeast Saccharomyces cerevisiae. J Biol. Chem. 273: 18573-85.
- Asano K, Krishnamoorthy T, Phan L, Pavitt GD, Hinnebusch AG. (1999) Conserved bipartite motifs in yeast eIF5 and eIF2Bepsilon, GTPase-activating and GDP-GTP exchange factors in translation initiation, mediate binding to their common substrate eIF2. EMBO J. 18: 1673-88.
- Asano K, Clayton J, Shalev A, Hinnebusch AG. (2000) A multifactor complex of eukaryotic initiation factors, eIF1, eIF2, eIF3, eIF5, and initiator tRNA (Met) is an important translation initiation intermediate invivo. Genes Dev. 14: 2534-46.
- Asano K, Shalev A, Phan L, Nielsen K, Clayton J, Valásek L, Donahue TF, Hinnebusch AG. (2001) Multiple roles for the C-terminal domain of eIF5 in translation initiation complex assembly and GTPase activation. EMBO J. 20: 2326-37.
- Babu SV, Ramaiah KV. (1996) Type 1 phosphatase inhibitors reduce the restoration of guanine nucleotide exchange activity of eukaryotic initiation factor 2B inhibited reticulocyte lysates rescued by hemin. Arch. Biochem. Biophys. 327: 201-8.

- Balachandran S, Kim CN, Yeh WC, Mak TW, Bhalla K, Barber GN. (1998) Activation of the dsRNA-dependent protein kinase, PKR, induces apoptosis through FADD-mediated death signaling. EMBO J. 17: 6888-902.
- Beattie E, Paoletti E, Tartaglia J. (1995) Distinct patterns of IFN sensitivity observed in cells infected with vaccinia K3L- and E3L- mutant viruses. Virology, 210: 254-63.
- Boyce M, Bryant KF, Jousse C, Long K, Harding HP, Scheuner D, Kaufman RJ, Ma D, Coen DM, Ron D, Yuan J. (2005) A selective inhibitor of eIF2alpha dephosphorylation protects cells from ER stress. Science, 307: 935-9.
- Brewer JW, Hendershot LM, Sherr CJ, Diehl JA. (1999) Mammalian unfolded protein response inhibits cyclin D1 translation and cell-cycle progression. Proc. Natl. Acad. Sci. USA. 96: 8505-10.
- Browning KS. (1996) The plant translational apparatus. Plant Mol. Biol. 32: 107-44.
- Browning KS, Gallie DR, Hershey JW, Hinnebusch AG, Maitra U, Merrick WC, Norbury C. (2001) Unified nomenclature for the subunits of eukaryotic initiation factor 3. Trends. Biochem. Sci. 26: 284.
- Carroll K, Elroy-Stein O, Moss B, Jagus R. (1993) Recombinant vaccinia virus K3L gene product prevents activation of double-stranded RNA-dependent, initiation factor 2 alpha-specific protein kinase. J Biol. Chem. 268: 12837-42.
- Chakraburtty K. (1999) Functional interaction of yeast elongation factor 3 with yeast ribosomes. Int. J Biochem. Cell Biol. 31: 163-73.
- Chakraburtty K. (2001) Translational regulation by ABC systems. Res. Microbiol. 152: 391-9.
- Chang YW, Traugh JA. (1998) Insulin stimulation of phosphorylation of elongation factor 1 (eEF-1) enhances elongation activity. Eur. J Biochem. 251: 201-7.
- Chaudhuri J, Das K, Maitra U. (1994) Purification and characterization of bacterially expressed mammalian translation initiation factor 5 (eIF-5): demonstration that eIF-5 forms a specific complex with eIF-2. Biochemistry, 33: 4794-9.

- Chen JJ, London IM. (1995) Regulation of protein synthesis by heme-regulated eIF-2 alpha kinase. Trends Biochem. Sci. 20: 105-8.
- Chen JJ, Throop MS, Gehrke L, Kuo I, Pal JK, Brodsky M, London IM. (1991) Cloning of the cDNA of the heme-regulated eukaryotic initiation factor 2 alpha (eIF-2 alpha) kinase of rabbit reticulocytes: homology to yeast GCN2 protein kinase and human double-stranded-RNA-dependent eIF-2 alpha kinase. Proc. Natl. Acad. Sci. USA. 88: 7729-33.
- Cho S, Hoffman DW. (2002) Structure of the beta subunit of translation initiation factor 2 from the archaeon Methanococcus jannaschii: a representative of the eIF2beta/eIF5 family of proteins. Biochemistry, 41: 5730-42.
- Choi SK, Olsen DS, Roll-Mecak A, Martung A, Remo KL, Burley SK, Hinnebusch AG, Dever TE. (2000) Physical and functional interaction between the eukaryotic orthologs of prokaryotic translation initiation factors IF1 and IF2. Mol. Cell Biol. 20: 7183-91.
- Clemens MJ, Pain VM, Wong ST, Henshaw EC. (1982) Phosphorylation inhibits guanine nucleotide exchange on eukaryotic initiation factor 2. Nature, 296: 93-5.
- Clemens MJ, Bushell M, Jeffrey IW, Pain VM, Morley SJ. (2000) Translation initiation factor modifications and the regulation of protein synthesis in apoptotic cells. Cell Death Differ. 7: 603-15.
- Clemens MJ. (2001) Initiation factor eIF2 alpha phosphorylation in stress responses and apoptosis. Prog. Mol Subcell. Biol. 27: 57-89.
- Cullinan SB, Diehl JA. (2006) Coordination of ER and oxidative stress signaling: the PERK/Nrf2 signaling pathway. Int. J Biochem. Cell Biol. 38: 317-32.
- Das S, Maiti T, Das K, Maitra U. (1997) Specific interaction of eukaryotic translation initiation factor 5 (eIF5) with the beta-subunit of eIF2. J Biol. Chem. 272: 31712-8.
- Das S, Ghosh R, Maitra U. (2001) Eukaryotic translation initiation factor 5 functions as a GTPase-activating protein. J Biol. Chem. 276: 6720-6.

- Deng J, Lu PD, Zhang Y, Scheuner D, Kaufman RJ, Sonenberg N, Harding HP, Ron D. (2004) Translational repression mediates activation of nuclear factor kappa B by phosphorylated translation initiation factor 2. Mol. Cell Biol. 24: 10161-68.
- Der SD, Yang YL, Weissmann C, Williams BR. (1997) A double-stranded RNA-activated protein kinase-dependent pathway mediating stress-induced apoptosis. Proc. Natl. Acad. Sci USA. 94: 3279-83.
- Dever TE, Glynias MJ, Merrick WC. (1987) GTP-binding domain: three consensus sequence elements with distinct spacing. Proc. Natl. Acad. Sci. USA. 84: 1814-8.
- Dever TE, Costello CE, Owens CL, Rosenberry TL, Merrick WC. (1989) Location of seven post-translational modifications in rabbit elongation factor 1 alpha including dimethyllysine, trimethyllysine, and glycerylphosphorylethanolamine. J Biol. Chem. 264: 20518-25.
- Dever TE, Sripriya R, McLachlin JR, Lu J, Fabian JR, Kimball SR, Miller LK. (1998) Disruption of cellular translational control by a viral truncated eukaryotic translation initiation factor 2 alpha kinase homolog. Proc. Natl. Acad. Sci. USA. 95: 4164-9.
- Dever TE. (2002) Gene-specific regulation by general translation factors. Cell, 108: 545-56.
- Dey M, Cao C, Dar AC, Tamura T, Ozato K, Sicheri F, Dever TE. (2005) Mechanistic link between PKR dimerization, autophosphorylation, and eIF2alpha substrate recognition. Cell, 122(6): 901-13.
- Dhaliwal S, Hoffman DW. (2003) The crystal structure of the N-terminal region of the alpha subunit of translation initiation factor 2 (eIF2alpha) from Saccharomyces cerevisiae provides a view of the loop containing serine 51, the target of the eIF2alpha-specific kinases. J Mol. Biol. 334: 187-95.
- Donahue TF, Cigan AM, Pabich EK, Valavicius BC. (1988) Mutations at a Zn(II) finger motif in the yeast eIF-2 beta gene alter ribosomal start-site selection during the scanning process. Cell, 54: 621-32.

- Dong J, Qiu H, Garcia-Barrio M, Anderson J, Hinnebusch AG. (2000) Uncharged tRNA activates GCN2 by displacing the protein kinase moiety from a bipartite tRNA-binding domain. Mol. Cell, 6: 269-79.
- Duncan R, Hershey JW. (1984) Heat shock-induced translational alterations in HeLa cells. Initiation factor modifications and the inhibition of translation. J Biol. Chem. 259: 11882-9.
- Duncan R, Hershey JW. (1985) Regulation of initiation factors during translational repression caused by serum depletion. Covalent modification. J Biol. Chem. 260: 5493-7.
- Erickson FL, Hannig EM. (1996) Ligand interactions with eukaryotic translation initiation factor 2: role of the gamma-subunit. EMBO J. 15: 6311-20.
- Erickson FL, Harding LD, Dorris DR, Hannig EM. (1997) Functional analysis of homologs of translation initiation factor 2gamma in yeast. Mol. Gen. Genet. 253: 711-9.
- Ernst H, Duncan RF, Hershey JW. (1987) Cloning and sequencing of complementary DNAs encoding the alpha-subunit of translational initiation factor eIF-2. Characterization of the protein and its messenger RNA. J Biol. Chem. 262: 1206-12.
- Fabian JR, Kimball SR, Heinzinger NK, Jefferson LS. (1997) Subunit assembly and guanine nucleotide exchange activity of eukaryotic initiation factor-2B expressed in Sf9 cells. J Biol. Chem. 272: 12359-65.
- Fadden P, Haystead TA, Lawrence JC Jr. (1997) Identification of phosphorylation sites in the translational regulator, PHAS-I, that are controlled by insulin and rapamycin in rat adipocytes. J Biol. Chem. 272: 10240-7.
- Feng L, Yoon H, Donahue TF. (1994) Casein kinase II mediates multiple phosphorylation of Saccharomyces cerevisiae eIF-2 alpha (encoded by SUI2), which is required for optimal eIF-2 function in S. cerevisiae. Mol. Cell Biol. 14: 5139-53.
- Flynn A, Oldfield S, Proud CG. (1993) The role of the beta-subunit of initiation factor eIF-2 in initiation complex formation. Biochim. Biophys. Acta. 1174: 117-21.

- Friedman RM, Metz DH, Esteban RM, Tovell DR, Ball LA, Kerr IM. (1972) Mechanism of interferon action: inhibition of viral messenger ribonucleic acid translation in L-cell extracts. J Virol. 10: 1184-98.
- Frolova L, Le Goff X, Zhouravleva G, Davydova E, Philippe M, Kisselev L. (1996) Eukaryotic polypeptide chain release factor eRF3 is an eRF1- and ribosome-dependent guanosine triphosphatase. RNA. 2: 334-41.
- Frolova LY, Tsivkovskii RY, Sivolobova GF, Oparina NY, Serpinsky OI, Blinov VM, Tatkov SI, Kisselev LL. (1999) Mutations in the highly conserved GGQ motif of class 1 polypeptide release factors abolish ability of human eRF1 to trigger peptidyl-tRNA hydrolysis. RNA. 5: 1014-20.
- Gale M Jr, Tan SL, Katze MG. (2000) Translational control of viral gene expression in eukaryotes. Microbiol. Mol. Biol. Rev. 64: 239-80.
- García MA, Gil J, Ventoso I, Guerra S, Domingo E, Rivas C, Esteban M. (2006) Impact of protein kinase PKR in cell biology: from antiviral to antiproliferative action. Microbiol Mol. Biol. Rev. 70: 1032-60.
- Gaspar NJ, Kinzy TG, Scherer BJ, Hümbelin M, Hershey JW, Merrick WC. (1994) Translation initiation factor eIF-2. Cloning and expression of the human cDNA encoding the gamma-subunit. J Biol. Chem. 269: 3415-22.
- Gebauer F, Hentze MW. (2004) Molecular mechanisms of translational control. Nat. Rev. Mol. Cell Biol. 5: 827-35.
- Gerlitz G, Jagus R, Elroy-Stein O. (2002) Phosphorylation of initiation factor-2 alpha is required for activation of internal translation initiation during cell differentiation. Eur. J Biochem. 269: 2810-9.
- Gutiérrez P, Osborne MJ, Siddiqui N, Trempe JF, Arrowsmith C, Gehring K. (2004) Structure of the archaeal translation initiation factor aIF2 beta from Methanobacterium thermoautotrophicum: implications for translation initiation. Protein Sci. 13: 659-67.
- Hanks SK, Hunter T. (1995) Protein kinases 6. The eukaryotic protein kinase superfamily: kinase (catalytic) domain structure and classification. FASEB J. 9: 576-96.

- Harding HP, Novoa I, Zhang Y, Zeng H, Wek R, Schapira M, Ron D. (2000) Regulated translation initiation controls stress-induced gene expression in mammalian cells. Mol. Cell, 6: 1099-108.
- Harding HP, Zhang Y, Bertolotti A, Zeng H, Ron D. (2000) Perk is essential for translational regulation and cell survival during the unfolded protein response. Mol. Cell, 5: 897-904.
- Harding HP, Zeng H, Zhang Y, Jungries R, Chung P, Plesken H, Sabatini DD, Ron D. (2001) Diabetes mellitus and exocrine pancreatic dysfunction in perk-/- mice reveals a role for translational control in secretory cell survival. Mol. Cell, 7: 1153-63.
- Harding HP, Zhang Y, Zeng H, Novoa I, Lu PD, Calfon M, Sadri N, Yun C, Popko B, Paules R, Stojdl DF, Bell JC, Hettmann T, Leiden JM, Ron D. (2003) An integrated stress response regulates amino acid metabolism and resistance to oxidative stress. Mol Cell, 11: 619-33.
- Hasek J, Kovarik P, Valásek L, Malínská K, Schneider J, Kohlwein SD, Ruis H. (2000) Rpg1p, the subunit of the Saccharomyces cerevisiae eIF3 core complex, is a microtubule-interacting protein. Cell Motil. Cytoskeleton, 45: 235-46.
- Hashimoto NN, Carnevalli LS, Castilho BA. (2002) Translation initiation at non-AUG codons mediated by weakened association of eukaryotic initiation factor (eIF) 2 subunits. Biochem. J. 367: 359-68.
- Hasnain SE, Begum R, Ramaiah KV, Sahdev S, Shajil EM, Taneja TK, Mohan M, Athar M, Sah NK, Krishnaveni M. (2003) Host-pathogen interactions during apoptosis. J Biosci. 28: 349-58.
- He B, Gross M, Roizman B. (1998) The gamma134.5 protein of herpes simplex virus 1 has the structural and functional attributes of a protein phosphatase 1 regulatory subunit and is present in a high molecular weight complex with the enzyme in infected cells. J Biol. Chem. 273: 20737-43.
- Hershey JWB, Merrick, M. B. The pathway and mechanism of initiation of protein synthesis. In Sonenberg N, Hershey, JWB and Mathews, MB. (2000) Translational

Control of Gene Expression, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York. pp 33–88.

- Hinnebusch AG. (1994) The eIF-2 alpha kinases: regulators of protein synthesis in starvation and stress. Semin. Cell Biol. 5: 417-26.
- Hinnebusch AG. (1994) Translational control of GCN4: an in vivo barometer of initiation-factor activity. Trends Biochem. Sci. 19: 409-14.
- Hinnebusch AG. (1997) Translational regulation of yeast GCN4. A window on factors that control initiator-tRNA binding to the ribosome. J Biol. Chem. 272: 21661-64.
- Hinnebusch AG. (2005) Translational regulation of GCN4 and the general amino acid control of yeast. Annu. Rev. Microbiol. 59: 407-50.
- Holcik M, Sonenberg N, Korneluk RG. (2000) Internal ribosome initiation of translation and the control of cell death. Trends Genet. 16: 469-73.
- Holcik M, Sonenberg N. (2005) Translational control in stress and apoptosis. Nat Rev Mol. Cell Biol. 6: 318-27.
- Hou CL, Tang C, Roffler SR, Tang TK. (2000) Protein 4.1R binding to eIF3-p44 suggests an interaction between the cytoskeletal network and the translation apparatus. Blood , 96: 747-53.
- Huang HK, Yoon H, Hannig EM, Donahue TF. (1997) GTP hydrolysis controls stringent selection of the AUG start codon during translation initiation in Saccharomyces cerevisiae. Genes Dev. 11: 2396-413.
- Hunt T, Vanderhoff G, London IM. (1972) Control of globin synthesis: the role of heme. J Mol. Biol. 66: 471-81.
- Hussain SG, Ramaiah KV. (2007) Reduced eIF2alpha phosphorylation and increased proapoptotic proteins in aging. Biochem. Biophys. Res. Commun. 355: 365-70.
- Imataka H, Gradi A, Sonenberg N. (1998) A newly identified N-terminal amino acid sequence of human eIF4G binds poly(A)-binding protein and functions in poly(A)-dependent translation. EMBO J. 17: 7480-9.

- Imataka H, Sonenberg N. (1997) Human eukaryotic translation initiation factor 4G (eIF4G) possesses two separate and independent binding sites for eIF4A. Mol. Cell Biol. 17: 6940-7.
- Ito T, Marintchev A, Wagner G. (2004) Solution structure of human initiation factor eIF2alpha reveals homology to the elongation factor eEF1B. Structure, 12: 1693-704.
- Janaki N, Krishna VM, Ramaiah KV. (1995) Phosphorylation of wheat germ initiation factor 2 (eIF-2) by N-ethylmaleimide-treated wheat germ lysates and by purified casein kinase II does not affect the guanine nucleotide exchange on eIF-2. Arch. Biochem. Biophys. 324: 1-8.
- Jefferson LS. Kimball SR. (2004) Amino acids as regulators of gene expression. Nutr. Metab (Lond). 1: 3.
- Jousse C, Oyadomari S, Novoa I, Lu P, Zhang Y, Harding HP, Ron D. (2003) Inhibition of a constitutive translation initiation factor 2alpha phosphatase, CReP, promotes survival of stressed cells. J Cell Biol. 163: 767-75.
- Kainuma M, Hershey JW. (2001) Depletion and deletion analyses of eucaryotic translation initiation factor 1A in Saccharomyces cerevisiae. Biochimie. 83: 505-14.
- Kamath A, Chakraburtty K. (1989) Role of yeast elongation factor 3 in the elongation cycle. J Biol. Chem. 264: 15423-8.
- Kapp LD, Lorsch JR. (2004) The molecular mechanics of eukaryotic translation. Annu. Rev. Biochem. 73: 657-704.
- Karimi R, Pavlov MY, Buckingham RH, Ehrenberg M. (1999) Novel roles for classical factors at the interface between translation termination and initiation. Mol. Cell. 601-9.
- Kebache S, Zuo D, Chevet E, Larose L. (2002) Modulation of protein translation by Nck-1. Proc. Natl. Acad. Sci USA. 99:5406-11.
- Khoo D, Perez C, Mohr I. (2002) Characterization of RNA determinants recognized by the arginine- and proline-rich region of Us11, a herpes simplex virus type 1-encoded double-stranded RNA binding protein that prevents PKR activation. J Virol. 76: 11971-81.

- Kimball SR, Heinzinger NK, Horetsky RL, Jefferson LS. (1998) Identification of interprotein interactions between the subunits of eukaryotic initiation factors eIF2 and eIF2B. J Biol. Chem. 273: 3039-44.
- Kimball SR, Jefferson LS. (2004) Amino acids as regulators of gene expression. Nutr Metab (Lond). 1(1):3.
- Kisselev L, Ehrenberg M, Frolova L. (2003) Termination of translation: interplay of mRNA, rRNAs and release factors? EMBO J. 22: 175-82.
- Kleijn M, Scheper GC, Voorma HO, Thomas AA. (1998) Regulation of translation initiation factors by signal transduction. Eur. J Biochem. 253: 531-44.
- Koonin EV. (1995) Multidomain organization of eukaryotic guanine nucleotide exchange translation initiation factor eIF-2B subunits revealed by analysis of conserved sequence motifs. Protein Sci. 4: 1608-17.
- Koromilas AE, Roy S, Barber GN, Katze MG, Sonenberg N. (1992) Malignant transformation by a mutant of the IFN-inducible dsRNA-dependent protein kinase. Science, 257: 1685-9.
- Kovalchuke O, Kambampati R, Pladies E, Chakraburtty K. (1998) Competition and cooperation amongst yeast elongation factors. Eur. J Biochem. 258: 986-93.
- Kozak M. (1989) The scanning model for translation: an update. J Cell Biol. 108: 229-41.
- Kozak M. (1999) Initiation of translation in prokaryotes and eukaryotes. Gene, 234: 187-208.
- Krishnamoorthy T, Pavitt GD, Zhang F, Dever TE, Hinnebusch AG. (2001) Tight binding of the phosphorylated alpha subunit of initiation factor 2 (eIF2alpha) to the regulatory subunits of guanine nucleotide exchange factor eIF2B is required for inhibition of translation initiation. Mol. Cell Biol. 21: 5018-30.
- Kruh J, Borsook H. (1956), Hemoglobin synthesis in rabbit reticulocytes in vitro. J Biol. Chem. 220: 905-15.

- Kumar A, Haque J, Lacoste J, Hiscott J, Williams BR. (1994) Double-stranded RNA-dependent protein kinase activates transcription factor NF-kappa B by phosphorylating I kappa B. Proc. Natl. Acad. Sci. USA. 91: 6288-92.
- Lamphear BJ, Kirchweger R, Skern T, Rhoads RE. (1995) Mapping of functional domains in eukaryotic protein synthesis initiation factor 4G (eIF4G) with picornaviral proteases. Implications for cap-dependent and cap-independent translational initiation. J Biol. Chem. 270: 21975-83.
- Latreille M, Larose L. (2006) Nck in a complex containing the catalytic subunit of protein phosphatase 1 regulates eukaryotic initiation factor 2alpha signaling and cell survival to endoplasmic reticulum stress. J Biol. Chem. 281: 26633-44.
- Laurino JP, Thompson GM, Pacheco E, Castilho BA. (1999) The beta subunit of eukaryotic translation initiation factor 2 binds mRNA through the lysine repeats and a region comprising the C2-C2 motif. Mol. Cell Biol. 19: 3224.
- Laxminarayana B, Krishna VM, Janaki N, Ramaiah KV. (2002) Translation and phosphorylation of wheat germ lysate: phosphorylation of wheat germ initiation factor 2 by casein kinase II and in N-ethylmaleimide-treated lysates. Arch. Biochem. Biophys. 400: 85-96.
- Le H, Browning KS, Gallie DR. (1998) The phosphorylation state of the wheat translation initiation factors eIF4B, eIF4A, and eIF2 is differentially regulated during seed development and germination. J Biol. Chem. 273: 20084-9.
- Leibundgut M, Frick C, Thanbichler M, Böck A, Ban N. (2005) Selenocysteine tRNA-specific elongation factor SelB is a structural chimaera of elongation and initiation factors. EMBO J. 24: 11-22.
- Leroux A, London IM. (1982) Regulation of protein synthesis by phosphorylation of eukaryotic initiation factor 2 alpha in intact reticulocytes and reticulocyte lysates. Proc. Natl. Acad. Sci. USA. 79: 2147-51.
- Lin PJ, Chang CH, Yao PC, Hsieh HC, Hsieh MJ, Kao CL, Tsai KT. (1994) Enhancement of endothelium-dependent contraction of the canine coronary artery by UW solution. Transplantation, 58: 1323-8.

- Lin L, Holbro T, Alonso G, Gerosa D, Burger MM. (2001) Molecular interaction between human tumor marker protein p150, the largest subunit of eIF3, and intermediate filament protein K7. J Cell Biochem. 80: 483-90.
- Llorens F, Duarri A, Sarró E, Roher N, Plana M, Itarte E. (2006) The N-terminal domain of the human eIF2beta subunit and the CK2 phosphorylation sites are required for its function. Biochem. J. 394: 227-36.
- Lloyd RM, Shatkin AJ. (1992) Translational stimulation by reovirus polypeptide sigma 3: substitution for VAI RNA and inhibition of phosphorylation of the alpha subunit of eukaryotic initiation factor 2. J Virol. 66: 6878-84.
- Mader S, Lee H, Pause A, Sonenberg N. (1995) The translation initiation factor eIF-4E binds to a common motif shared by the translation factor eIF-4 gamma and the translational repressors 4E-binding proteins. Mol. Cell Biol. 15: 4990-7.
- Manche L, Green SR, Schmedt C, Mathews MB. (1992) Interactions between double-stranded RNA regulators and the protein kinase DAI. Mol. Cell Biol. 12: 5238-48.
- Marcotrigiano J, Gingras AC, Sonenberg N, Burley SK. (1997) Cocrystal structure of the messenger RNA 5' cap-binding protein (eIF4E) bound to 7-methyl-GDP. Cell, 89: 951-61.
- Marciniak SJ, Yun CY, Oyadomari S, Novoa I, Zhang Y, Jungreis R, Nagata K,Harding HP, Ron D. (2004)CHOP induces death by promoting protein synthesis and oxidation in the stressed endoplasmic reticulum. Genes Dev. 18: 3066-77.
- Marciniak SJ, Ron D. (2006) Endoplasmic reticulum stress signaling in disease. Physiol. Rev. 86: 1133-49.
- Marintchev A, Wagner G. (2004) Translation initiation: structures, mechanisms and evolution. Q Rev. Biophys. 37: 197-284.
- Matsuo H, Li H, McGuire AM, Fletcher CM, Gingras AC, Sonenberg N, Wagner G. (1997) Structure of translation factor eIF4E bound to m7GDP and interaction with 4E-binding protein. Nat. Struct. Biol. 9: 717-24.

- Matts RL, Levin DH, London IM. (1983) Effect of phosphorylation of the alpha-subunit of eukaryotic initiation factor 2 on the function of reversing factor in the initiation of protein synthesis. Proc. Natl. Acad. Sci. USA. 80: 2559-63.
- Mellor H, Flowers KM, Kimball SR, Jefferson LS. (1994) Cloning and characterization of cDNA encoding rat hemin-sensitive initiation factor-2 alpha (eIF-2 alpha) kinase. Evidence for multitissue expression. J Biol. Chem. 269: 10201-4.
- Metz AM, Browning KS. (1997) Assignment of the beta-subunit of wheat eIF2 by protein and DNA sequence analysis and immunoanalysis. Arch. Biochem. Biophys. 342: 187-9.
- Meurs EF, Galabru J, Barber GN, Katze MG, Hovanessian AG. (1993) Tumor suppressor function of the interferon-induced double-stranded RNA-activated protein kinase. Proc. Natl. Acad. Sci. USA. 90: 232-6.
- Mikuni O, Ito K, Moffat J, Matsumura K, McCaughan K, Nobukuni T, Tate W, Nakamura Y. (1994) Identification of the prfC gene, which encodes peptide-chain-release factor 3 of Escherichia coli. Proc. Natl. Acad. Sci. USA. 91: 5798-802.
- Minich WB, Balasta ML, Goss DJ, Rhoads RE. (1994) Chromatographic resolution of in vivo phosphorylated and nonphosphorylated eukaryotic translation initiation factor eIF-4E: increased cap affinity of the phosphorylated form. Proc. Natl. Acad. Sci. USA. 91: 7668-72.
- Mitsui K, Datta A, Ochoa S. (1981) Removal of beta subunit of the eukaryotic polypeptide chain initiation factor 2 by limited proteolysis. Proc. Natl. Acad. Sci. USA. 78: 4128-32.
- Mulvey M, Poppers J, Ladd A, Mohr I. (1999) A herpesvirus ribosome-associated, RNA binding protein confers a growth advantage upon mutants deficient in a GADD34-related function. J Virol. 73: 3375-85.
- Naranda T, Sirangelo I, Fabbri BJ, Hershey JW. (1995) Mutations in the NKXD consensus element indicate that GTP binds to the gamma-subunit of translation initiation factor eIF2. FEBS Lett. 372: 249-52.

- Nika J, Rippel S, Hannig EM. (2001) Biochemical analysis of the eIF2beta gamma complex reveals a structural function for eIF2alpha in catalyzed nucleotide exchange. J Biol. Chem. 276: 1051-6.
- Nonato MC, Widom J, Clardy J. (2002) Crystal structure of the N-terminal segment of human eukaryotic translation initiation factor 2alpha. J Biol. Chem. 277: 17057-61.
- Novoa I, Zeng H, Harding HP, Ron D. (2001) Feedback inhibition of the unfolded protein response by GADD34-mediated dephosphorylation of eIF2alpha. J Cell Biol. 153: 1011-22.
- Olsen DS, Savner EM, Mathew A, Zhang F, Krishnamoorthy T, Phan L, Hinnebusch AG. (2003) Domains of eIF1A that mediate binding to eIF2, eIF3 and eIF5B and promote ternary complex recruitment in vivo. EMBO J. 22: 193-204.
- Oyadomari S, Mori M. (2004) Roles of CHOP/GADD153 in endoplasmic reticulum stress. Cell Death Differ. 11: 381-9.
- Palecek J, Hasek J, Ruis H. (2001) Rpg1p/Tif32p, a subunit of translation initiation factor 3, interacts with actin-associated protein Sla2p. Biochem. Biophys. Res. Commun. 282: 1244-50.
- Pap M, Cooper GM. (2002) Role of translation initiation factor 2B in control of cell survival by the phosphatidylinositol 3-kinase/Akt/glycogen synthase kinase 3beta signaling pathway. Mol. Cell Biol. 22: 578-86.
- Pathak VK, Nielsen PJ, Trachsel H, Hershey JW. (1988) Structure of the beta subunit of translational initiation factor eIF-2. Cell, 54: 633-9.
- Pathak VK, Schindler D, Hershey JW. (1988) Generation of a mutant form of protein synthesis initiation factor eIF-2 lacking the site of phosphorylation by eIF-2 kinases. Mol. Cell Biol. 8: 993-5.
- Pause A, Belsham GJ, Gingras AC, Donzé O, Lin TA, Lawrence JC Jr, Sonenberg N. (1994) Insulin-dependent stimulation of protein synthesis by phosphorylation of a regulator of 5'-cap function. Nature, 371: 762-7.

- Pavitt GD, Yang W, Hinnebusch AG. (1997) Homologous segments in three subunits of the guanine nucleotide exchange factor eIF2B mediate translational regulation by phosphorylation of eIF2. Mol. Cell Biol. 17: 1298-313.
- Pavitt GD, Ramaiah KV, Kimball SR, Hinnebusch AG. (1998) eIF2 independently binds two distinct eIF2B subcomplexes that catalyze and regulate guanine-nucleotide exchange. Genes Dev. 12: 514-26.
- Pavitt GD. (2005) eIF2B, a mediator of general and gene-specific translational control. Biochem. Soc. Trans. 33: 1487-92.
- Pestova TV, Shatsky IN, Hellen CU. (1996) Functional dissection of eukaryotic initiation factor 4F: the 4A subunit and the central domain of the 4G subunit are sufficient to mediate internal entry of 43S preinitiation complexes. Mol. Cell Biol. 16: 6870-8.
- Pestova TV, Borukhov SI, Hellen CU. (1998) Eukaryotic ribosomes require initiation factors 1 and 1A to locate initiation codons. Nature, 394: 854-9.
- Peterson DT, Safer B, Merrick WC. (1979) Role of eukaryotic initiation factor 5 in the formation of 80 S initiation complexes. J Biol. Chem. 254: 7730-5.
- Phan L, Zhang X, Asano K, Anderson J, Vornlocher HP, Greenberg JR, Qin J, Hinnebusch AG. (1998) Identification of a translation initiation factor 3 (eIF3) core complex, conserved in yeast and mammals, that interacts with eIF5. Mol. Cell Biol. 18: 4935–46.
- Pincheira R, Chen Q, Huang Z, Zhang JT. (2001) Two subcellular localizations of eIF3 p170 and its interaction with membrane-bound microfilaments: implications for alternative functions of p170. Eur. J Cell Biol. 80: 410-8.
- Price NT, Nakielny SF, Clark SJ, Proud CG. (1989) The two forms of the beta-subunit of initiation factor-2 from reticulocyte lysates arise from proteolytic degradation. Biochim. Biophys. Acta. 1008: 177-82.
- Proud CG. (1992) Protein phosphorylation in translational control. Curr. Top.Cell Regul. 32: 243-369.

- Proud CG. (2005) eIF2 and the control of cell physiology. Semin. Cell Dev. Biol. 16: 3-12.
- Rabinovitz M, Waxman HS. (1965) Dependence of polyribosome structure in reticulocytes on iron; implication on the tape theory of haemoglobin synthesis. Nature, 206: 897-900.
- Ramaiah KV, Davies MV, Chen JJ, Kaufman RJ. (1994) Expression of mutant eukaryotic initiation factor 2 alpha subunit (eIF-2 alpha) reduces inhibition of guanine nucleotide exchange activity of eIF-2B mediated by eIF-2 alpha phosphorylation. Mol. Cell Biol. 14: 4546-53.
- Ramakrishnan V. (2002) Ribosome structure and the mechanism of translation. Cell, 108: 557-72.
- Ramelot TA, Cort JR, Yee AA, Liu F, Goshe MB, Edwards AM, Smith RD, Arrowsmith CH, Dever TE, Kennedy MA. (2002) Myxoma virus immunomodulatory protein M156R is a structural mimic of eukaryotic translation initiation factor eIF2alpha. J Mol. Biol. 322: 943-54.
- Rafie-Kolpin M, Chefalo PJ, Hussain Z, Hahn J, Uma S, Matts RL, Chen JJ. (2000) Two heme-binding domains of heme-regulated eukaryotic initiation factor-2alpha kinase. N terminus and kinase insertion. J Biol. Chem. 275: 5171-8.
- Raychaudhuri P, Stringer EA, Valenzuela DM, Maitra U. (1984) Ribosomal subunit antiassociation activity in rabbit reticulocyte lysates. Evidence for a low molecular weight ribosomal subunit antiassociation protein factor (Mr = 25,000). J Biol. Chem. 259: 11930-5.
- Rhoads RE. (1999) Signal transduction pathways that regulate eukaryotic protein synthesis. J Biol. Chem. 274: 30337-40.
- Richter-Cook NJ, Dever TE, Hensold JO, Merrick WC. (1998) Purification and characterization of a new eukaryotic protein translation factor. Eukaryotic initiation factor 4H. J Biol. Chem. 273: 7579-87.
- Rodnina MV, Savelsbergh A, Katunin VI, Wintermeyer W. (1997) Hydrolysis of GTP by elongation factor G drives tRNA movement on the ribosome. Nature, 385: 37-41.

- Rogers GW Jr, Richter NJ, Merrick WC. (1999) Biochemical and kinetic characterization of the RNA helicase activity of eukaryotic initiation factor 4A. J Biol. Chem. 274: 12236-44.
- Roll-Mecak A, Cao C, Dever TE, Burley SK. (2000) X-Ray structures of the universal translation initiation factor IF2/eIF5B: conformational changes on GDP and GTP binding. Cell, 103: 781-92.
- Roll-Mecak A, Shin BS, Dever TE, Burley SK. (2001) Engaging the ribosome: universal IFs of translation. Trends Biochem. Sci. 26: 705-9.
- Roll-Mecak A, Alone P, Cao C, Dever TE, Burley SK. (2004) X-ray structure of translation initiation factor eIF2gamma: implications for tRNA and eIF2alpha binding. J Biol. Chem. 279: 10634-42.
- Romero DP, Dahlberg AE. (1986) The alpha subunit of initiation factor 2 is phosphorylated in vivo in the yeast Saccharomyces cerevisiae. Mol. Cell Biol. 6: 1044-9.
- Rowlands AG, Panniers R, Henshaw EC. (1988) The catalytic mechanism of guanine nucleotide exchange factor action and competitive inhibition by phosphorylated eukaryotic initiation factor 2. J Biol. Chem. 263: 5526-33.
- Rowlands AG, Montine KS, Henshaw EC, Panniers R. (1988) Physiological stresses inhibit guanine-nucleotide-exchange factor in Ehrlich cells. Eur. J Biochem. 175: 93-9.
- Rozen F, Edery I, Meerovitch K, Dever TE, Merrick WC, Sonenberg N. (1990) Bidirectional RNA helicase activity of eucaryotic translation initiation factors 4A and 4F. Mol. Cell Biol. 10: 1134-44.
- Russell DW, Spremulli LL. (1979) Purification and characterization of a ribosome dissociation factor (eukaryotic initiation factor 6) from wheat germ. J Biol. Chem. 254: 8796-800.
- Sachs AB, Varani G. (2000) Eukaryotic translation initiation: there are (at least) two sides to every story. Nat. Struct. Biol. 7: 356-61.

- Saelens X, Kalai M, Vandenabeele P. (2001) Translation inhibition in apoptosis: caspase-dependent PKR activation and eIF2-alpha phosphorylation. J Biol. Chem. 276: 41620-8.
- Sah NK, Taneja TK, Pathak N, Begum R, Athar M, Hasnain SE. (1999) The baculovirus antiapoptotic p35 gene also functions via an oxidant-dependent pathway. Proc. Natl. Acad. Sci USA. 96: 4838-43.
- Sahdev S, Taneja TK, Mohan M, Sah NK, Khar AK, Hasnain SE, Athar M. (2003) Baculoviral p35 inhibits oxidant-induced activation of mitochondrial apoptotic pathway. Biochem. Biophys. Res. Commun. 307: 483-90.
- Salvatore M, Basler CF, Parisien JP, Horvath CM, Bourmakina S, Zheng H, Muster T, Palese P, García-Sastre A. (2002) Effects of influenza A virus NS1 protein on protein expression: the NS1 protein enhances translation and is not required for shutoff of host protein synthesis. J Virol. 76: 1206-12.
- Samuel CE. (1993) The eIF-2 alpha protein kinases, regulators of translation in eukaryotes from yeasts to humans. J Biol. Chem. 268: 7603-6.
- Scheper GC, van Kollenburg B, Hu J, Luo Y, Goss DJ, Proud CG. 2002 Phosphorylation of eukaryotic initiation factor 4E markedly reduces its affinity for capped mRNA. J Biol. Chem. 277: 3303-9.
- Schmitt E, Blanquet S, Mechulam Y. (2002) The large subunit of initiation factor aIF2 is a close structural homologue of elongation factors. EMBO J. 21: 1821-32.
- Scheuner D, Song B, McEwen E, Liu C, Laybutt R, Gillespie P, Saunders T, Bonner-Weir S, Kaufman RJ. (2001) Translational control is required for the unfolded protein response and in vivo glucose homeostasis. Mol. Cell, 7: 1165-76.
- Scheuner D, Patel R, Wang F, Lee K, Kumar K, Wu J, Nilsson A, Karin M, Kaufman RJ. (2006) Double-stranded RNA-dependent protein kinase phosphorylation of the alpha-subunit of eukaryotic translation initiation factor 2 mediates apoptosis. J Biol. Chem. 281: 21458-68.
- Schneider RJ, Mohr I. (2003) Translation initiation and viral tricks. Trends Biochem. Sci. 28: 130-6.

- Sheu GT, Traugh JA. (1999) A structural model for elongation factor 1 (EF-1) and phosphorylation by protein kinase CKII. Mol. Cell Biochem. 191: 181-6.
- Schröder M, Kaufman RJ. (2005) The mammalian unfolded protein response. Annu. Rev. Biochem. 74:739-89.
- Singh LP, Arorr AR, Wahba AJ. (1994) Phosphorylation of the guanine nucleotide exchange factor and eukaryotic initiation factor 2 by casein kinase II regulates guanine nucleotide binding and GDP/GTP exchange. Biochemistry, 33: 9152-7.
- Shi Y, Vattem KM, Sood R, An J, Liang J, Stramm L, Wek RC. (1998) Identification and characterization of pancreatic eukaryotic initiation factor 2 α-subunit kinase, PEK, involved in translational control. Mol. Cell Biol. 18: 7499–7509.
- Singh CR, Yamamoto Y, Asano K. (2004) Physical association of eukaryotic initiation factor (eIF) 5 carboxyl-terminal domain with the lysine-rich eIF2beta segment strongly enhances its binding to eIF3. J Biol. Chem. 279: 49644-55.
- Singh CR, He H, Ii M, Yamamoto Y, Asano K. (2004) Efficient incorporation of eukaryotic initiation factor 1 into the multifactor complex is critical for formation of functional ribosomal preinitiation complexes in vivo. J Biol. Chem. 279: 31910-20.
- Sonenberg N, Hershey JWB, Mathews BM (eds). (2000) Translational Control of Gene Expression, Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press.
- Song H, Mugnier P, Das AK, Webb HM, Evans DR, Tuite MF, Hemmings BA, Barford D. (2000) The crystal structure of human eukaryotic release factor eRF1--mechanism of stop codon recognition and peptidyl-tRNA hydrolysis. Cell, 100: 311-21.
- Srivastava SP, Kumar KU, Kaufman RJ. (1998) Phosphorylation of eukaryotic translation initiation factor 2 mediates apoptosis in response to activation of the double-stranded RNA-dependent protein kinase. J Biol. Chem. 273: 2416-23.
- Stansfield I, Jones KM, Tuite MF. (1995) The end in sight: terminating translation in eukaryotes. Trends Biochem. Sci. 20: 489-91.

- Sudhakar A, Krishnamoorthy T, Jain A, Chatterjee U, Hasnain SE, Kaufman RJ, Ramaiah KV. (1999) Serine 48 in initiation factor 2 alpha (eIF2 alpha) is required for high-affinity interaction between eIF2 alpha(P) and eIF2B. Biochemistry, 38: 15398-405.
- Sudhakar A, Ramachandran A, Ghosh S, Hasnain SE, Kaufman RJ, Ramaiah KV. (2000) Phosphorylation of serine 51 in initiation factor 2 alpha (eIF2 alpha) promotes complex formation between eIF2 alpha(P) and eIF2B and causes inhibition in the guanine nucleotide exchange activity of eIF2B. Biochemistry, 39: 12929-38.
- Suragani RN, Kamindla R, Ehtesham NZ, Ramaiah KV. (2005) Interaction of recombinant human eIF2 subunits with eIF2B and eIF2alpha kinases. Biochem. Biophys. Res. Commun. 338: 1766-72.
- Suragani RN, Ghosh S, Ehtesham NZ, Ramaiah KV. (2006) Expression and purification of the subunits of human translational initiation factor 2 (eIF2): phosphorylation of eIF2 alpha and beta. Protein Expr. Purif. 47: 225-33.
- Tallóczy Z, Jiang W, Virgin IV HW, Leib DA, Scheuner D, Kaufman RJ, Eskelinen E, Levine B. (2002) Regulation of starvation- and virus-induced autophagy by the eIF2 kinase signaling pathway. Proc. Natl. Acad. Sci. USA. 99: 190-195.
- Thompson GM, Pacheco E, Melo EO, Castilho BA. (2000) Conserved sequences in the beta subunit of archaeal and eukaryal translation initiation factor 2 (eIF2), absent from eIF5, mediate interaction with eIF2gamma. Biochem. J. 347: 703-9.
- Ting NS, Kao PN, Chan DW, Lintott LG, Lees-Miller SP. (1998) DNA-dependent protein kinase interacts with antigen receptor response element binding proteins NF90 and NF45. J Biol. Chem. 273: 2136-45.
- Trachsel H, Erni B, Schreier MH, Staehelin T. (1977) Initiation of mammalian protein synthesis. II. The assembly of the initiation complex with purified initiation factors. J Mol. Biol. 116: 755-67.
- Traugh JA, Tahara SM, Sharp SB, Safer B, Merrick WC. (1976) Factors involved in initiation of haemoglobin synthesis can be phosphorylated invitro. Nature, 263: 163-5.

- Triana-Alonso FJ, Chakraburtty K, Nierhaus KH. (1995) The elongation factor 3 unique in higher fungi and essential for protein biosynthesis is an E site factor. J Biol. Chem. 270: 20473-8.
- Ung TL, Cao C, Lu J, Ozato K, Dever TE. (2001) Heterologous dimerization domains functionally substitute for the double-stranded RNA binding domains of the kinase PKR. EMBO J. 20: 3728-37.
- Valásek L, Nielsen KH, Hinnebusch AG. (2002) Direct eIF2-eIF3 contact in the multifactor complex is important for translation initiation in vivo. EMBO J. 21: 5886-98.
- Vattem KM, Wek RC. (2004) Reinitiation involving upstream ORFs regulates ATF4 mRNA translation in mammalian cells. Proc. Natl. Acad. Sci. USA. 101: 11269-74.
- Venema RC, Peters HI, Traugh JA. (1991) Phosphorylation of elongation factor 1 (EF-1) and valyl-tRNA synthetase by protein kinase C and stimulation of EF-1 activity. J Biol. Chem. 266: 12574-80.
- Venema RC, Peters HI, Traugh JA. (1991) Phosphorylation of valyl-tRNA synthetase and elongation factor 1 in response to phorbol esters is associated with stimulation of both activities. J Biol. Chem. 266: 11993-8.
- Von Arnim AG, Chamovitz DA. (2003) Protein homeostasis: a degrading role for Int6/eIF3e. Curr. Biol. 13: R323-5.
- Wakula P, Beullens M, van Eynde A, Ceulemans H, Stalmans W, Bollen M. (2006) The translation initiation factor eIF2beta is an interactor of protein phosphatase-1. Biochem. J. 400: 377-83.
- Wang X, Paulin FE, Campbell LE, Gomez E, O'Brien K, Morrice N, Proud CG. (2001) Eukaryotic initiation factor 2B: identification of multiple phosphorylation sites in the epsilon-subunit and their functions in vivo. EMBO J. 20: 4349-59.
- Warnakulasuriyarachchi D, Cerquozzi S, Cheung HH, Holcík M. (2004) Translational induction of the inhibitor of apoptosis protein HIAP2 during endoplasmic reticulum stress attenuates cell death and is mediated via an inducible internal ribosome entry site element. J Biol. Chem. 279: 17148-57.

- Waxman HS, Rabinovitz. (1966) M. Control of reticulocyte polyribosome content and haemoglobin synthesis by heme. Biochim. Biophys. Acta, 129: 369-379.
- Whalen SG, Gingras AC, Amankwa L, Mader S, Branton PE, Aebersold R, Sonenberg N. (1996) Phosphorylation of eIF-4E on serine 209 by protein kinase C is inhibited by the translational repressors, 4E-binding proteins. J Biol. Chem. 271: 11831-7.
- Wei CL, Kainuma M, Hershey JW. (1995) Characterization of yeast translation initiation factor 1A and cloning of its essential gene. J Biol. Chem. 270: 22788-94.
- Wek RC, Ramirez M, Jackson BM, Hinnebusch AG. (1990) Identification of positive-acting domains in GCN2 protein kinase required for translational activation of GCN4 expression. Mol. Cell Biol. 10: 2820-31.
- Wek RC. (1994) eIF-2 kinases: regulators of general and gene-specific translation initiation.Trends Biochem. Sci. 19: 491-6.
- Wek RC, Jiang HY, Anthony TG. (2006) Coping with stress: eIF2 kinases and translational control. Biochem. Soc. Trans. 34: 7-11.
- Welsh GI, Price NT, Bladergroen BA, Bloomberg G, Proud CG. (1994) Identification of novel phosphorylation sites in the beta-subunit of translation initiation factor eIF-2. Biochem. Biophys. Res. Commun. 201: 1279-88.
- Wilson WA, Roach PJ. (2002) Nutrient-regulated protein kinases in budding yeast. Cell, 111: 155-8.
- Woods YL, Cohen P, Becker W, Jakes R, Goedert M, Wang X, Proud CG. (2001) The kinase DYRK phosphorylates protein-synthesis initiation factor eIF2Bepsilon at Ser539 and the microtubule-associated protein tau at Thr212: potential role for DYRK as a glycogen synthase kinase 3-priming kinase. Biochem. J. 355: 609-15.
- Yatime L, Schmitt E, Blanquet S, Mechulam Y. (2004) Functional molecular mapping of archaeal translation initiation factor 2. J Biol. Chem. 279: 15984-93.
- Ye X, Cavener DR. (1994) Isolation and characterization of the Drosophila melanogaster gene encoding translation-initiation factor eIF-2 beta. Gene, 142: 271-4.

- Zhang F, Romano PR, Nagamura-Inoue T, Tian B, Dever TE, Mathews MB, Ozato K, Hinnebusch AG. (2001) Binding of double-stranded RNA to protein kinase PKR is required for dimerization and promotes critical autophosphorylation events in the activation loop. J Biol. Chem. 276: 24946-58.
- Zhang P, McGrath BC, Reinert J, Olsen DS, Lei L, Gill S, Wek SA, Vattem KM, Wek RC, Kimball SR, Jefferson LS, Cavener DR. (2002) The GCN2 eIF2alpha kinase is required for adaptation to amino acid deprivation in mice. Mol. Cell Biol. 22: 6681-8.
- Zhouravleva G, Frolova L, Le Goff X, Le Guellec R, Inge-Vechtomov S, Kisselev L, Philippe M. (1995) Termination of translation in eukaryotes is governed by two interacting polypeptide chain release factors, eRF1 and eRF3. EMBO J. 14: 4065-72.
- Zhu R, Zhang YB, Chen YD, Dong CW, Zhang FT, Zhang QY, Gui JF. (2006) Molecular cloning and stress-induced expression of paralichthys olivaceus heme-regulated initiation factor 2alpha kinase. Dev. Comp. Immunol. 30: 1047-59.

Publications