# Recombinant Human Translational Initiation Factor 2: Expression of Subunits, Purification and Characterization.

Thesis submitted to the University of Hyderabad for the degree of
**Doctor of Philosophy**

by

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## *DECLARATION*

I hereby declare that the work presented in this thesis entitled "**Recombinant Human Translational Initiation Factor 2: Expression of Subunits, Purification and Characterization**" has been carried out by me under the supervision of Prof. K. V. A. Ramaiah in Department of Biochemistry, School of Life Sciences, University of Hyderabad and this work has not been submitted for any degree or diploma of any other University or Institute.

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# CERTIFICATE

This is to certify that Mr. **S. N. V. S. Rajasekhar** has carried out the research work in the present thesis entitled "**Recombinant Human Translational Initiation Factor 2: Expression of Subunits, Purification and Characterization**" for the degree of **Doctor of philosophy** under my supervision at the Department of Biochemistry, School of Life Sciences, University of Hyderabad, Hyderabad, India.

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***Rajasekhar Suragani***

*Dedicated to my Parents*

# ABBREVIATIONS

| | |
|---|---|
| AcNPV | : Autographa californica nuclear poly hedrosis virus |
| AP | : Alkaline Phosphotase |
| APH | : Acetyl phenyl hydrazine |
| Asp | : Aspartic acid |
| ATF4 | : Activated transcription factor |
| ATP | : Adenosine 5' triphosphate |
| BCIP | : 5- bromo – 4 – chloro – 3 indoyl phosphate |
| BEVS | : Baculovirus expression vector system |
| Bis-acrylamide | : N,N'- methylene –bis –acrylamide |
| BSA | : Bovine serum albumin |
| BV | : Budded virion |
| Ci | : Curie |
| c-AMP | : Cyclic – Adenosine mono phosphate |
| CK2 | : Casein kinase II |
| CP | : Creatine phosphate |
| CPK | : Creatine phospho kinase |
| Cpm | : counts per minute |
| dATP | : deoxy adenine tri phosophate |
| dCTP | : deoxy cytosine tri phosphate |
| DEAE | : Diethyl amino ethyl |
| DMSO | : Di methyl sulphoxide |
| DNA | : de oxy ribonucleic acid |
| cDNA | : complementary DNA |
| DTT | : Dithiothreitol |
| EDTA | : Ethylene di amine tetra acetic acid |
| eEF | : Eukaryotic elongation factor |
| EGTA | : Ethylene –bis(β- amino ethyl ether) N',N''-tetraacetic acid |
| eIFs | : Eukaryotic initiation factors |
| eIF2 | : Eukaryotic initiation factor 2 |
| eIF2α | : alpha subunit of eukaryotic initiation factor 2 |
| eIF2(αP) | : Phosphorylated alpha subunit of eukaryotic initiation factor 2 |
| eIF2B/GEF/RF | : Guanine nucleotide exchange factor of eIF2 or reversing factor |
| eIF4E bp | : eIF4E binding protein |
| eRF | : eukaryotic releasing factor or termination factor |
| EtBr | : Ethidium bromide |
| FCS | : Fetal calf serum |
| Fig | : Figure |
| GDP | : Guanosine 5' di phosphate |
| GSH | : Reduced glutathione |
| GST | : Glutathione S-transferase |
| GTP | : Guanosine 5' tri phosphate |
| hrs | : Hours |
| HRI | : Haeme regulated inhibitor |
| HEPES | : N-[2-hydroxy ethyl] piperizine- N'- [2-ethane-sulphonic acid] |
| Hsp | : Heat shock protein |
| Kbp | : kilo base pairs |
| Kda | : Kilo daltons |
| LB | : Luria- Bertani |
| lt | : litre |
| Met | : Methionine |
| Mr | : Marker |
| mg | : milligram(s) |
| ml | : millilitre |

| | |
|---|---|
| min | : minutes |
| MOI | : Multiplicity of Infection |
| NBT | : Nitro blue tetrazolium |
| ng | : nanogram |
| PAGE | : Poly acrylamide gel electrophoresis |
| PERK | : Pancreatic endopolasmic – resident eIF2α kinase |
| pfu | : plaque forming units |
| p.i. | : post infection |
| PKR | : Double- stranded RNA- dependent eIF2α kinase |
| pmol | : pico moles |
| PMSF | : Phenyl methyl sulphonyl fluoride |
| RNA | : Ribonucleic acid |
| Ds RNA | : Double stranded Ribonucleic acid |
| Met.tRNAi | : initiator transfer RNA |
| mRNA | : messenger RNA |
| tRNA | : transfer RNA |
| RNAse | : Ribonuclease |
| rpm | : rotations per minute |
| S | : Svedberg |
| Ser | : serine |
| SDS | : sodium dodecyl sulohate |
| Sf9 | : Spodoptera frugiperda (fall army worm) cell line |
| S51A | : mutation of Ser51 of eIF2α to Ala |
| S51D | : mutation of Ser51 of eIF2α to Asp |
| TCA | : Tri chloro acetic acid |
| TEMED | : N',N',N',N'- tetra methyl ethylene diamine |
| TNM-FH | : Insect cell culture medium |
| TRIS | : Tris (hydroxy methyl) amino methane |
| UV | : ultraviolet |
| wt | : wildtype |
| µg | : microgram |
| µl | : microlitre |
| µM | : micro molar |

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# INTRODUCTION

Translation Initiation factor 2 in eukaryotes (eIF2) is a multifunctional heterotrimeric G-protein that delivers the initiator tRNA (Met-$tRNA_i^{Met}$) to the P-site of the small ribosomal subunit in a GTP dependent manner. It is a GTPase, a substrate for several kinases and caspase(s). The factor plays a crucial role in the mechanism and regulation of protein synthesis. The current understanding of eIF2 structure and function is based mostly on the results obtained from yeast because the ease with which this system can be manipulated genetically. However both yeast and mammalian systems have been well studied to understand the regulation of eIF2α phosphorylation by several kinases and the importance of eIF2α phosphorylation in the regulation of general and specific protein synthesis. Overall, the present understanding is that the α-subunit plays a crucial role in the regulation of protein synthesis, whereas the β and γ subunits are required for various functions of eIF2, such as, their ability to bind mRNA, GDP or GTP, and initiator tRNA (Met-$tRNA_i^{Met}$). Keeping in view of these studies presented here in this thesis, the introduction is focused on general protein synthesis in eukaryotes and the role of eIF2 in the intitiation step. This is followed by a detailed description of the subunit structure, function, interaction and regulation of eIF2.

**Protein synthesis:**

Translation is defined as a process in which information present in the messenger RNA template is decoded to the corresponding amino acid sequences of a protein by a special machinery that contains ribosomes, RNAs such as ribosomal and transfer RNAs, enzymes such as aminoacyl synthetases, and, several protein factors. The process of translation, or protein synthesis that occurs generally in cytoplasm or on the surface endoplasmic reticulum, is uncoupled in eukaryotes both temporally and spatially from RNA synthesis that occurs in the nucleus by a process called transcription. The synthesis of a mRNA transcript is a prerequisite for translation process. The primary transcript of mRNA however undergoes posttranscriptional modifications such as splicing (removal of introns and splicing of exons), 5' capping (addition of $m^7$ GTP at the 5' end) and 3' polyadenylation (addition of polyA tail at the 3'end), and, is transported into the cytoplasm before it is translated.

The complex and dynamic biochemical process of mRNA translation is divided for convenience into three phases: initiation, elongation and termination. The assembly of 80S ribosome marks the initiation phase with initiator-methionyl-transfer-RNA (Met-$tRNA_i^{Met}$) in its peptidyl (P-) site at the start codon of the mRNA. The actual polypeptide synthesis takes place during the elongation phase. Peptide bond formation between adjacent amino acids occurs on catalytic centers that are fundamentally formed by the ribosomal RNA (rRNA) of the 60S subunit. When ribosome reaches the stop codon, the signal for termination, the nascent polypeptide is released. The various events in initiation, elongation, and, the release of newly made polypeptide that occurs during termination step of protein synthesis are aided by specific protein factors called eIFs (eukaryotic initiation factors), EFs (Elongation factors) and RFs (releasing factors) respectively. Translation factors play a role both in the synthesis of proteins and also in their regulation.

The sequence of the open reading frame of a mRNA specifies the amino acids to be incorporated into the protein. The amino acids are brought on to the template that is bound by ribosomes and translational factors, one after another by the corresponding tRNAs where these are finally polymerized. Amino acyl synthetase enzymes couple the amino acids to the corresponding tRNAs and the process requires ATP. The anticodon of the amino acylated tRNAs recognizes the cognate codon in the translating mRNA. While the 'start codon' in mRNA is recognized by the initiator tRNA carrying methionine ($tRNAi^{Met}$ or methionylated initiator tRNA) generally, the amino acids specified by all other codons are recognized by elongator tRNAs.

**1. Initiation**: The initiation step in protein synthesis can be further subdivided into four to five steps depending on the convenience to explain the sequence of events that facilitate the formation of 80S initiation complex. These sub steps are:

1. formation of a 43 Svedberg (S) preinitiation complex from the small (40S) ribosomal subunit, initiation factors, and Met $tRNA_i^{Met}$,
2. recruitment of the 43S complex to the (capped) 5' end of the mRNA to form 48S preinitiation complex,

3. 'scanning' of the 5' untranslated region (UTR) of the mRNA and start codon recognition, and,
4. joining of the large ribosomal subunit (60S) to 48S complex to form 80S initiation complex.

**1.1 Formation of the 43S pre-initiation complex:**

At the end of translation, the 80S ribosome is dissociated into 40S and 60S subunits. Their association is initially prevented by specific anti-association factors like eIF6 (bound to 60S subunit), eIF1A and eIF3 (bound to 40S subunit). They are released after the formation of 48S initiation complex. The first and foremost step in the initiation appears to be the formation of a ternary complex containing eIF2, GTP and Met-tRNAi that subsequently joins the 40S subunits. The trimeric eIF2 complex recognizes and joins only the initiator tRNA carrying the methionine (Kapp and Lorsch 2004), whereas trimeric elongation factor 1 (EF1) recognizes all other elongator aminoacylated tRNAs (Miller and Weissbach, 1977; Kaziro, 1978). Both factors require GTP for their association with the respective amino acylated tRNAs. Initiator tRNA differs structurally from elongator aminoacyl tRNAs. The most important feature of initiator tRNA is that it contains the weak A1: U72 base pair at the end of acceptor stem (Basavappa and Sigler, 1991; Astrom et al., 1993; Farruggio et al., 1996, von Pawel-Rammingen et al., 1992). It promotes initiator function and also discriminates against its activity in elongation (Drabkin et al., 1998). Another feature of initiator tRNAs is the presence of three consecutive GC base pairs in the anticodon stem (G29: C41, G30: C40, G31: C39) that contribute to the initiator function. The lack of the characteristic TψC sequence present in the T loop of elongator tRNAs discriminates the elongator function (Hinnebusch, 2000; Kapp and Lorsch, 2004R). The initiator tRNAs contain Adenine at 54 and 60 positions instead of Thymine and characteristic pyrimidine at these positions. In fungi and plants, modification of phosphoribosyl group at position 64 in T loop in the initiator tRNA can probably cause a stearic hindrance so that the elongation factor EF1A cannot bind and thus blocks or prevents the elongator function of tRNA (Forster et al., 1993). In addition to the structural differences, initiator tRNA bound to

methionine is found to be translationally active where as yeast initiator tRNA bound by isoleucine cannot bind well with the heterotrimeric eIF2 (Wagner et al., 1984). Recent study suggests that eIF2 in its GTP bound form has a positive contact with the methionine on the initiator tRNA and this contact is lost when GTP is hydrolyzed or exchanged for GDP whereas contacts with the body of tRNA are not altered. The GTP dependent recognition of methionine moiety may prevent the joining of unacylated tRNAi and block eIF2.GDP entering the initiation pathway (Kapp and Lorsch 2004). In addition to its ability to bind Met-tRNAi, the eIF2 complex is also implicated in the selection of start AUG codon in mRNA since mutations in the subunits of yeast eIF2 allow the recognition of UUG codon by the anticodon of initiator tRNA (Donahue et al., et al., 1988).

Binding of the ternary complex to the 40S subunit is aided by eIF1, eIF1A, and the multi-subunit factor eIF3 (**Fig. 1.1**). In yeast, a multifactor complex (MFC) consisting of eIF1, eIF2, eIF3, eIF5, and Met-tRNA$_i^{Met}$ assembles independently of the ribosome and is an important functional unit during several stages of translation initiation (Asano et al., 2000). The multimeric protein complex eIF3, consists of five non-identical subunits (eIF3a, eIF3b, eIF3c, eIF3i and eIF3g in yeast) (Phan et al., 1998) and 11 subunits in mammals (Browning et al., 2001). It 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). eIF5 is a GTPase activating protein (GAP) that stimulates the intrinsic GTPase activity of eIF2 complex. eIF1s are monomeric proteins that are intimately linked to the positioning of the 40S ribosomal subunit at the translation initiation codon (Pestova et al., 1998). eIF1A is a small protein of 17 to 22 kDa (Wei et al., 1995) and promotes the binding of the ternary complex to the 40S subunit (Chaudhuri et al., 1999). 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). The interaction between eIF2 and the eIF1A NTD probably contributes to a step in the pathway following ternary complex recruitment. The β-subunit

**Figure 1.1 The translation initiation pathway.** eIF2 mediates the joining of Met-tRNA$_i^{Met}$ to the 40S ribosomal subunit in a GTP dependent manner. In yeast, a multifactor complex (MFC) associates at this stage and resulting 43S pre-initiation complex is recruited to the mRNA via interactions with the eIF4 factors bound at or near the cap structure of the mRNA and then scans the 5'UTR to identify the initiator codon. Recognition of the start AUG, involving base-pairing with the anti-codon loop of Met-tRNA$_i^{Met}$, triggers the release of the bound factors accompanied by two distinct GTP hydrolysis steps. The 40S ribosomal subunit joins the 60S subunit to form an elongation competent 80S ribosome, which enters into the elongation cycle. The figure is taken from Preiss and Hentze 2003.

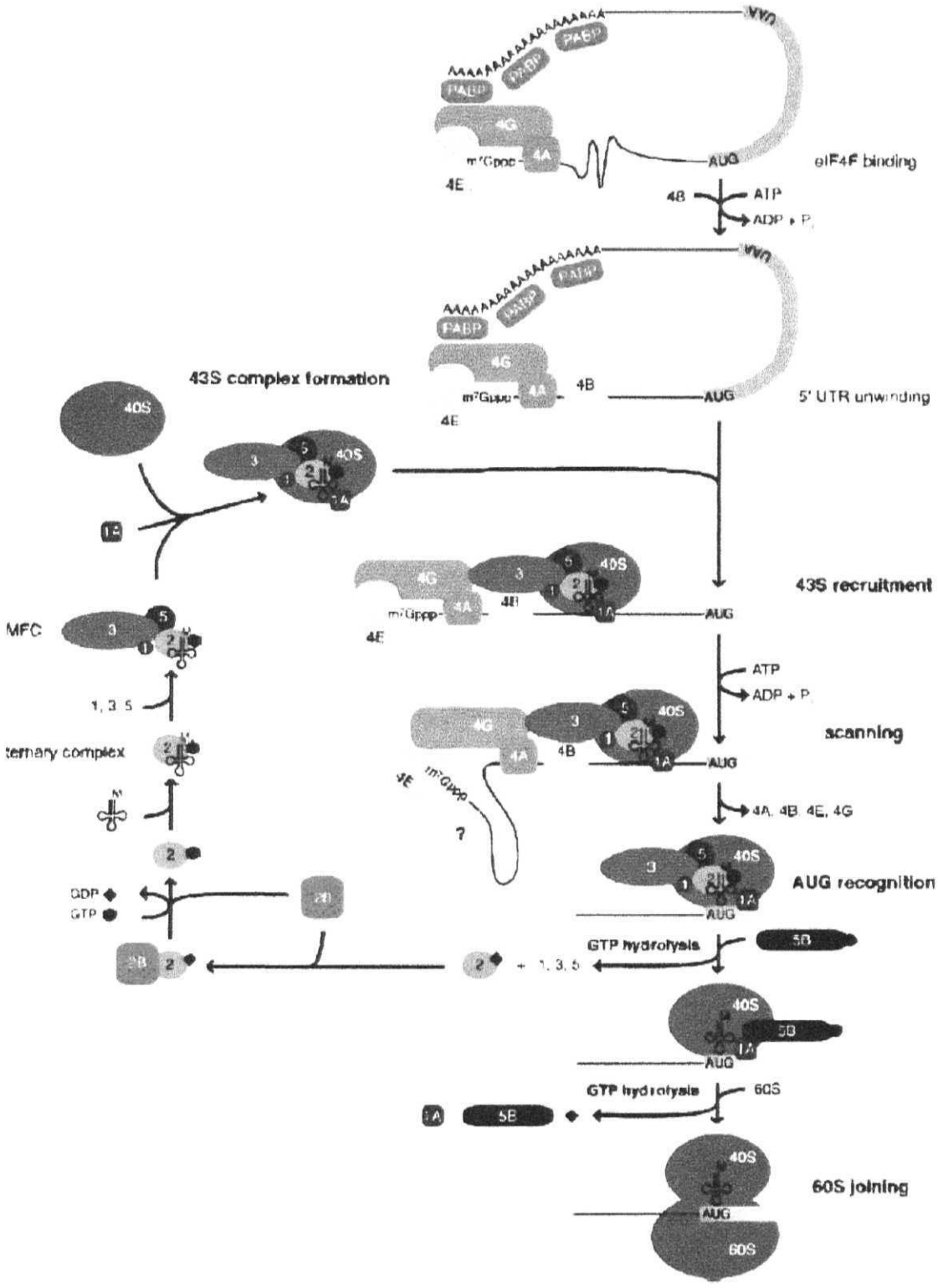


**Fig.1.1 Preiss and He ntze (2003)**

of eIF2 interacts with the C-terminal domain (CTD) of eIF3a and eIF5-CTD. eIF1 contacts the MFC through interactions with the eIF3a-CTD and eIF3c-NTD. The eIF3c-NTD also binds the CTD of eIF5. Further, eIF3a-NTD and eIF3c can interact with the ribosomal protein RPS0A located on the solvent side of the 40S subunit. The eIF3a-CTD can specifically bind to a short segment from domain I of 18S rRNA.

**1.2 Recruitment of the 43S complex (eIF3-eIF1A-40S-eIF2.GTP.Met-tRNA$_i^{Met}$) to the 5' end of the mRNA to form 48S complex:** Most of the eukaryotic mRNAs have a 5'methylated Guanosine (5'm$^7$G) and 3' polyA tail. The 43S pre-initiation complex binds at or near the 5'-end of capped mRNAs and scans the mRNA in a 5' to 3' direction until an AUG start codon in a proper context is encountered. Apparently before joining the 43S complex, the 5' m$^7$G cap of mRNA is bound by a heterotrimeric eIF4F complex and 3' end is bound by polyA binding protein (PABP). The heterotrimeric eIF4F complex consists of eIF4E, 4G and 4A. The factor eIF4E recognizes the 5'cap of the mRNAs. The shape of eIF4E resembles a cupped hand and its concave side provides a small hydrophobic slot for insertion of the cap structure and a contiguous region for mRNA binding. The opposite convex face of the eIF4E protein is in contact with eIF4G (Marcotrigiano et al., 1997, Matsuo et al., 1997, Tomoo, 2002). eIF4G is a multivalent adapter molecule and its primary function in translation is to enhance the binding of the eIF4F complex to the 5' end of the mRNA (Hentze 1997). The N-terminus of eIF4G has binding sites for eIF4E and poly (A)-binding protein (PABP). The interaction between eIF4G-PABP facilitates to pseudo-circularize the mRNA (Sachs and Varani, 2000). In addition, eIF4G central region interacts with mammalian eIF3 (Lamphear et al., 1995). This interaction between eIF3 and eIF4G helps the mRNA to join the 43S complex. The binding to eIF4F complex to 5'm$^7$G cap commits the translational apparatus to the translation of mRNA. The formation of eIF4F complex in mammalian systems is regulated by a family of translation repressor proteins (~12kDa) called eIF4E-BPs (eIF4E binding proteins). Binding of the 4E-BPs to eIF4E occurs through a 'core" sequence contained in eIF4E (YXXXXL Φ) in which X is any amino acid and Φ is a residue possessing an aliphatic

portion, most often L, but some times M and F [Mader et al., 1995]. Phosphorylation of specific serine and threonine residues of 4EBPs (4E-binding proteins) modulates the affinity of 4EPBPs to eIF4E. While hypo-phosphorylation leads to efficient binding of 4E-BPs to eIF4E, hyper-phosphorylation abrogates this interaction [Lin et al., 1994; Pause et al., 1994; Fadden et al., 1997].

The affinity of eIF4F complex for capped mRNAs is decreased due to the presence of stable secondary structure near the 5'cap structure. eIF4A is a DEAD box helicase, which unwinds the RNA secondary structure in the cap-proximal region of the mRNA aided by eIF4B, although these two proteins do not interact directly. eIF4A has a dumbbell structure consisting of two domains connected by a flexible linker [Caruthers et al., 2000]. It undergoes a cycle of changes in its conformation and RNA affinity as it binds ATP, hydrolyses and releases ADP and products [Lorsch and Herschlag, 1998a, 1998b]. The ATPase and helicase activities of eIF4A are stimulated by a factor called eIF4H that has homology to the RRM domain of eIF4B [Richter-Cook et al., 1998]. In summary, the assembly of the 43S preinitiation complex at the 5' cap structure of the mRNA is principally directed by the cap-binding protein eIF4E and co-ordinated by eIF3 and eIF4G, which provide multiple contact points to other initiation factors, the small ribosomal subunit, and finally to pseudo circularize of the mRNA.

**1. 3 Scanning of the 5' untranslated region in mRNA and AUG recognition:** Once assembled near the 5' end of the mRNA, the 43S complex scans along the mRNA from 5' UTR towards the 3'end in search of a first AUG triplet start codon (**Fig. 1.1**). 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 [Kozak 2002, 1999,1986 and 1987]. The minimal 40S-eIF3-eIF2-Met $tRNA_i^{met}$ complex can bind to the single stranded 5' un-translated region (UTR). In the presence of eIF1, 43S complex can reach and identify the initiation codon. However, the attachment of the 43S complex requires eIF4A, 4B and 4F. In the absence of these factors and ATP, 43S complexes cannot move through the weak internal secondary structures. Thus eIF4F complex is not only required

for ribosome attachment to mRNA but also in the scanning process. eIF1, at this step not only helps to identify the start AUG and discriminate from the non-cognate initiation codon but also in positioning of pre-initiation complexes at the appropriate context. Apart from initiator tRNA, the key point of contact with the mRNA in the decoding process, eIFs1, 2 and 5 appear to be the central players in maintaining the fidelity of start site selection (Kapp and Lorsch 2004). It is thought that probably eIF1 provides some kind of poof reading mechanism. It scrutinizes the anticodon-mRNA pairings and when the correct three base pairs are formed, eIF1 interacts with the resulting duplex and reduces the energy of the complex enough to stop scanning. The codon–anticodon interaction triggers a conformational change in 40S-subunit-bound eIF2, which leads to GTP hydrolysis. The GTP hydrolysis is aided by the GAP protein eIF5 that is bound to the β-subunit of eIF2. Thus the function of eIF2 in decoding process is to respond to identification of initiation codon by hydrolyzing GTP and then, at appropriate time, release the Met-tRNAi into the P-site. The GTP hydrolysis by eIF2 serves as a checkpoint for proper identification of the mRNA start codon. This is followed by the release of eIF2 and possibly other initiation factors (Sonenberg et al., 2000).

**1.4 Assembly of the 80S ribosome:** Once the 48S pre-initiation complex recognizes the initiator codon and base pairing between the AUG and the anticodon loop of the initiator tRNA has occurred, further series of events take place which results in the joining of the 60S subunit to form the complete 80S initiation complex, the final step of eukaryotic translational initiation. 60S subunit joining necessitates the release of initiation factors and also the anti-association factors from the 40S subunit and also requires a second GTP hydrolysis (**Fig. 1.1**) Until recently, hydrolysis of one molecule of GTP by eIF2 was thought to suffice for 60S joining. But the discovery of a yeast homologue of bacterial IF2, eIF5B, led to the idea that a second GTP hydrolysis stimulates the joining of 60S subunits to 48S initiation complexes. The *in vitro* reconstitution experiments also confirmed the role of eIF5B for the 80S ribosome assembly (Pestova et al., 2000, Lee et al., 2002). Thus, formation of an elongation competent 80S ribosome requires two distinct GTP hydrolysis steps one aided by the GAP protein, eIF5 bound to β-subunit of eIF2 and other

by eIF5B which predominantly serve as checkpoints for proper AUG codon identification and 80S assembly. Recent studies in mammalian systems suggest that the joining of 60S subunit to 48S initiation complex is a rate-limiting step. It requires external stimuli, and, the release of eIF6, an anti association phosphoprotein factor that is bound to 60S subunits (Valenzuela et al., 1982, Gingras et al., 1999, Rhoads, 1999, Ceci et al., 2003).

**1.5. Recycling and Release of eIF2.GDP by eIF2B:** The recycling of inactive eIF2.GDP, released at the end of initiation to active eIF2.GTP is catalyzed by a heteropentameric protein called eIF2B (Amesz et al., 1979; Siekierka et al., 1982; Panniers and Henshaw., 1983; Konieczny and Safer., 1983; Siekierka et al., 1981; Matts et al., 1983; Grace et al., 1982) (**Fig 1.2**). Since this factor is discovered simultaneously from many laboratories, it has enjoyed several names in the literature such as GEF (guanine nucleotide exchange factor), RF (reversing factor) and CoeIF2 etc. In physiological conditions, eIF2 complex has a higher affinity (~100 fold) for GDP than for GTP, although the values for the Kd GDP in the literature varies considerably (Walton and Gill, 1975; Proud, 1992; Kapp and Lorsch 2004, Kimball et al., 1987, Panniers et al., 1988). In the absence of $Mg^{2+}$, eIF2 affinity for GDP and GTP appears to be the same *in vitro*. The heteropentameric eIF2B is implicated not only in the recycling of eIF2.GDP to eIF2.GTP but also implicated in the release of eIF2.GDP. This is because many investigators found that eIF2.GDP is associated with 60S ribosomes of 80S initiation complexes and eIF2-eIF2B complex migrate together during purification schemes (deBenedetti and Baglioni 1983; Thomas et al., 1985; Gross et al., 1987; Matts et al., 1983). Hence the 60S bound eIF2.GDP appears to be a physiologically relevant intermediate. Interestingly, addition of purified eIF2B releases the eIF2. GDP bound to 60S subunits in quasiphysiological conditions (Thomas et al., 1985). Since the amount of eIF2.GDP bound to 60S subunits is significantly enhanced when the α-subunit in eIF2 is phosphorylated in translating reticulocyte lysates (Ramaiah et al., 1992), it is likely the eIF2 associated with 60S subunits of 80S initiation complexes is phosphorylated and is not released due to lack of functional eIF2B. Consistent with these results, 48S initiation complexes containing eIF2 are observed in the yeast eIF2B mutants (gcd1 and gcd2 that represent the γ and δ

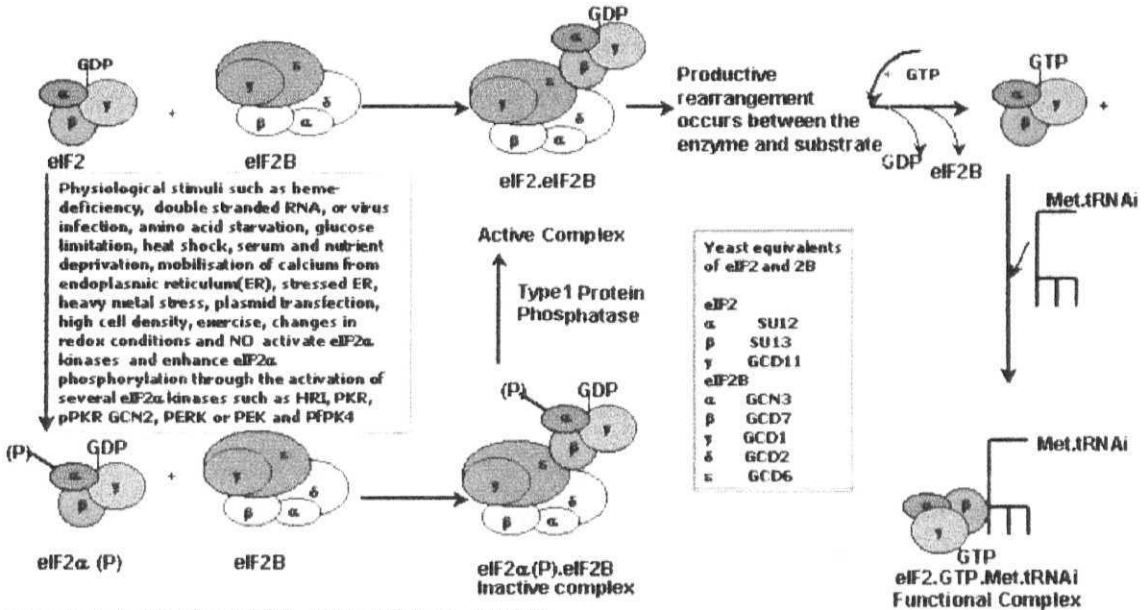


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**Figure 1.2: Guaninine Nucleotide Exchange on eIF2 by eIF2B: Effect of phosphorylation of serine 51 in eIF2α.**

subunits of eIF2B respectively). (Cigan et al., 1991 Foiani et al., 1991). Further, yeast GCD1/GCD6 (γ/ ε-subcomplex) catalytic subcomplex has high level of guanine nucleotide exchange activity in cell extracts but is not sufficient to provide the essential function of eIF2B *in vivo* (Pavitt et al., 1998). These findings therefore raise the possibility that the release of eIF2.GDP from ribosomes require eIF2B. In addition to eIF2α phosphorylation, the GNE activity of eIF2B is also altered by other means.

**2. Elongation:** In the elongation phase of protein biosynthesis, the three most important steps are: i) joining of amino acylated tRNAs to A-site of ribosome based on the sequence information in mRNA; ii) peptide bond formation between adjacent amino acids and iii) movement or translocation of the ribosome. These steps are aided by different elongation factors. Step 1 in elongation requires eEF1A and eEF1B. They are comparable in function to the eIF2 and 2B proteins of initiation cycle. The tRNAs carrying amino acids are recognized by eEF1A.GTP. The ternary complex (eEF1A.GTP.aatRNA) then joins the 80S initiation complex in a codon-dependent manner. After the release of aminoacylated tRNA into the A-site of ribosome, the eEF1A.GTP is hydrolyzed to inactive eEF1A.GDP.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. The eEF1B is a heterotrimeric factor in which the catalytic activity is associated with the α and β-subunits. So far the function of the γ-subunit is unknown (Kinzy et al., 1994; Pape et al., 1998). The eukaryotic eEF1A is found associated with the endoplasmic reticulum (ER) membrane (Sanders et al., 1996). The activity of eEF1A and eEF1B are phosphoproteins and are regulated through phosphorylation mechanism (Venema et al., 1991; Chang and Traugh, 1998; Redpath et al., 1996; Belle et al., 1989; Mulner-Lorillon et al., 1992, 1994; Sheu and Traugh, 1999). The peptidyl transferase, the enzyme that catalyzes the peptide bond formation is thought to be located in the large subunit. Although no such protein is identified, the recent understanding is that the peptide bond formation appears to be catalyzed by a ribozyme (Moore and Steitz, 2003). After the formation of the peptide bond, GTP bound eEF2 factor facilitates the entry of peptidyl tRNA into

the P-site and the deacylated tRNA goes into the exit site (E). The GTPase center in ribosome then hydrolyzes the GTP bound to eEF2 so that eEF2 is released. Thus eEF2 is essentially implicated in the translocation where it will force the movement of peptidyl tRNA to P site. Once the translocation is completed, the elongation cycle will again resume by bringing the cognate amino acylated tRNA to the A site by the eEF1A factor. eEF2 is also a phosphoprotein with multiple sites and its phosphorylation status can regulate the elongation rates. The eEF2 kinase appears to be a dependent on $Ca^{2+}$ and calmodulin (Ryazanov et al., 1988; 1999, Carlberg, 1990; Hovland et al., 1999; Redpath and Proud, 1993). The elongation factors function in translation by mimicking the anticodon stem loop of tRNA molecules (Nissen et al., 1995; 2000). In addition to phosphorylation, the activity of eEF2 is also modified through ADP ribosylation (Fendrick et al., 1992). Yet another elongation factor, eEF3 is reported to be present exclusively in fungi which appears to be important in the release of nonacylated tRNA from the entry site of the ribosome (Triana-Alonso et al., 1995). The protein has 1044 aa and uses ATP as a substrate *in vivo*. The protein has sequence similarity to ribosomal protein S5 (Gontarek et al., 1998). It is an essential protein in yeast but surprisingly no homologs of this factor are discovered in higher eukaryotes or in archea.

**3. Termination:** Termination of newly made polypeptide chain occurs when the 80S complex reaches the termination codon, like UAA, UAG or UGA. The general framework of the translation termination process has been elucidated in (Nakamura et al. 1996). The presence of one of the three termination codons in the A site of the ribosome signals the polypeptide chain release factors to bind and recognize the termination signal. The end result of this process is the subsequent hydrolysis of the ester bond between the 3' nucleotide of the tRNA located in the P site of 60S ribosomal subunit and the nascent polypeptide chain. Ultimately, the polypeptide is released as a completed protein from the tRNA.

In eukaryotes, translation termination is catalyzed by eRF1, which recognizes all three-termination codons. The eRF1 protein functionally mimics tRNA molecules not only by interacting at the peptidyl transferase center of the large subunit, but also by

recognizing mRNA codon within the small subunit and by association with a GTPase. The molecular mechanisms, by which eRF1 decodes stop codons and promotes ribosome-catalyzed peptidyl tRNA hydrolysis, have been proposed based on the structure of eRF1 (Song et al. 2000). eRF1 has a conserved GGQ sequence motif which is equivalent to the aminoacyl group attached to the CCA-3' sequence found in the aminoacyl stem of a tRNA molecule (Song et al., 2000). eRF1 protein binds to the A-site of 60S ribosomal subunit in such a way that the Gln-185 residue of the GGQ motif is in coordination with a water molecule can mediate a nucleophilic attack on the ester bond of the peptidyl-tRNA molecule in the P-site, resulting in hydrolysis of the nascent polypeptide chain (Song et al., 2000).

A second release factor, eRF3, stimulates eRF1 activity in a GTP-dependent fashion (Frolova et al., 1996). The GTPase activity of eRF3 requires eRF1 and the ribosome. The GTPase function of eRF3 is thought to be involved in dissociation of the eRF1/eRF3 complex from the ribosome (Frolova et al. 1996). This is followed by the recycling of ribosomal subunits for a fresh round of initiation. The events that occur after peptide hydrolysis in eukaryotes and archea are not well understood as in bacteria. In bacteria, at the end of the termination stage the ribosome is left on the mRNA with a deacylated tRNA. The acceptor stem of the tRNA moves from the P-site to the E-site of the 50S subunit while the anticodon end remains in the P-site of the 30 S subunit (Moazed et al., 1989). This complex is recognized by ribosome release factor, RRF. Elongation factor, EF-G.GTP and IF3 then assist RRF in disassembling the post termination complex (Karimi et al., 1999). Homologs of ribosome release factors are not identified in eukaryotes and archaea and the mechanism of ribosomal recycling is mysterious.

**3.1. Recycling and reinitiation:** The synergistic action of the $m^7$Gcap 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. Further, the recently described interaction between eRF3 and PABP might bring the terminating ribosome close to the poly (A) tail and the $m^7$G cap (Hoshino et al., 1999). It is possible that a

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. This type of initiation event is called "reinitiation." The phenomenon of reinitiation has been shown to occur after the translation of short open reading frames (such as yeast GCN4 mRNA). However, reinitiation following the translation of a long open reading frame occurs extremely rarely.

**4. Structure and function of eIF2:** Translational initiation factor 2 in eukaryotes (eIF2) is a heterotrimeric complex consisting of α, β and γ-subunits. The molecular mass of trimeric eIF2 is approximately 127, 124 or 119 kDa in mammals, yeast and in plants respectively (Hershey and Merrick, 2000). The subunit structure, possible functions and phosphorylation sites of human eIF2 complex are mentioned in **Fig.1.3**. For example, the human eIF2 α, β, and γ sequences are respectively, 58%, 47%, and 72%, identical to the corresponding subunits from yeast. The trimeric native eIF2 results from the 1:1:1 association of the three subunits (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)

The complex eIF2 is a GTPase, a phosphoprotein and also a substrate for caspase(s) and GTP/GDP exchange factor called eIF2B. It is required for the joining of initiator tRNA to 40S subunits and in the recognition of start site on mRNA. The importance of eIF2 in the initiation cycle of protein synthesis is shown in **Fig.1.1** Phosphorylation of the conserved $ser^{51}$ residue in eIF2α that occurs by a family of eIF2α kinases in response to diverse stress signals inhibits the GNE activity of eIF2B (Hinnebusch, 2000; Clemens, 1995; Webb and Proud, 1997; Ramaiah et al., 1994; Pavitt et al., 1998; Sudhakar et al., 1999, 2000), impairs recycling of eIF2.GDP, inhibits protein synthesis in general, stimulates the translation of specific mRNAs like GCN4 and ATF4 that encode transcriptional factors, couples protein synthesis with glucose metabolism, and, also protects the cells from toxic effects of unfolded or denatured proteins, heat stress and virus infection (Rhoads, 1999,

Hinnebusch, 2000; Shi et al., 2003; Poulin and Sonenberg, 2003; Dever, 2002; Ron, 2002; Kaufman, 2002; Proud, 2005, Patil and Walter, 2001; Gale et al., 2000). Since α and β-subunits from yeast and archaea cannot form a dimer, and, phosphorylation of α-subunit alone is sufficient to inhibit the GNE activity of eIF2B, it is suggested that βγ-complex is sufficient for providing eIF2 function and the α-subunit of eIF2 and eIF2B serve primarily to regulate eIF2 activity (Erickson et al., 2001; Krishnamoorthy et al., 2001; Schmitt et al., 2002). Further, the amino acid sequences of the β-subunit facilitate the interaction of eIF2 complex with RNAs (Laurino et al., 1999; Rosen et al., 1982; Bommer and Kurzchalia, 1989; Flynn et al., 1993) and virtually with all factors in translational initiation that are required for 43S complex formation (**Fig. 1.1**). In mammalian systems, the β-subunit is a substrate for four different kinases and also mediates the inhibition in GNE activity of eIF2B that occurs due to eIF2α phosphorylation through its enhanced interaction with eIF2B (Clark et al., 1988, Welsh et al., 1994; Ting et al., 1998; Kimball, 1998)

**4.1 Structure of small or the α-subunit of eIF2:**

Cloning, sequencing, structural, genetic, biochemical and mutational studies of eIF2 subunit genes 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).Generally on a 10% SDS-PAGE, the rabbit reticulocyte eIF2α-subunit migrates at 38 kDa position (in our gels) lower to the β-and γ-subunits. The important functions of eIF2 such as its ability to bind GDP/GTP or Met-tRNAi are primarily ascribed to β- and γ-subunits (Anthony et al., 1987; 1990; Bommer et al., 1991; Bommer and Kurzchalia, 1989; Dholakia et al., 1989; Gaspar et al., 1994, Naranda et al., 1995). Genetic and biochemical analysis in yeast however suggest that α-subunit plays a role in Met-tRNAi binding. Deletion of α subunit of yeast is lethal and causes a defect in eIF2B catalyzed nucleotide exchange and Met-tRNAi binding (Nika et al., 2001). The lethality induced by lack of α subunit is suppressed by over expression of β- and γ subunits and Met-tRNAi. This is consistent with the interaction between α and γ-subunits, or, formation of stable αγ dimers (Schmitt et al., 2002; Ito et

al., 2004; Tahara et al., 2004). In yeast, steady state kinetic analysis of eIF2B-catalyzed GDP/GTP exchange using eIF2βγ.GDP as a substrate reveals a 16 fold increase in Km and an 8 fold increase in Vmax when compared with wt eIF2.GDP complex that includes the α-subunit. These findings therefore suggest that α-subunit plays a crucial role in Met-tRNAi binding and also in the structural interactions with eIF2B that determines wild type rates of nucleotide exchange *in vivo* (Nika et al., 2001). Very early studies have pointed out that eIF2α can be cross-linked to 18S rRNA (Westermann et al., 1980) and also to GTP (Barrieux and Rosenfeld, 1977; Bommer et al., 1988). These findings suggest the possible involvement of eIF2α in 43S complex formation and Met-tRNA$_i^{Met}$ binding.

The N-terminal crystal structure of human and yeast eIF2α (Nonato et al., 2002; Dhaliwal et al., 2003) and the complete NMR structure of human eIF2α (Ito et al., 2004) show that the N-terminal domain contains two subdomains: S1-type oligonucleotide/ oligosaccharide binding-fold (OB-fold) and an α-helical subdomain. OB-fold consists of the first 15-85 residues of the N-terminus and has a five-stranded β-barrel that is similar to the one originally identified in the S1 ribosomal protein (Arcus, 2002). However, this region in eIF2α does not have any of the clustered positive charges that are observed in other members of the nucleic acid binding super family. This is also consistent with the experimental results that there is limited binding of RNA to the α-subunit of eIF2. The β3 and β4 strands are connected by a conserved loop of 14 amino acids (47-61) that contains two $3_{10}$ helices. The phosphorylation site $51^{ser}$ is located between the two $3_{10}$ helices in the N-terminal OB-fold domain. The α-helical subdomain of NTD stretches between 91-183 aa residues and has five α-helices and one $3_{10}$ helix. The two subdomains of NTD are connected by a) hydrophobic core of amino acids and a disulfide bridge between Cys 69 and Cys 97. In the crystal structure of N–termimal two thirds of human eIF2α studied by Nonato et al., (2002), a negatively charged cavity is formed where the OB and helical subdomains come together. This groove reported to be present in human eIF2α, but not in the yeast subunit. Also, there are no reports suggesting the presence of disulfide bridge in yeast eIF2α (Dhaliwal et al., 2003). Further, it has been

observed that the conserved phosphorylation site in yeast eIF2α is more solvent exposed and visible in the electron density map than the human eIF2α. In addition to these differences in the crystal structure, there are other differences between yeast and mammalian eIF2α-subunits that are described below.

The C-terminal domain of eIF2α that has been recently characterized by solution NMR structure contains five β strands (β6-β10) and three α-helices (α6-α8). One of the α-helices present at the C-terminus (α-8) is loosely associated and has no defined orientation relative to CTD (Ito et al., 2004). Further, conserved negatively charged residues that are present in the CTD domain of eIF2α (after Pro274) are predicted to be in contact with the polylysine blocks in eIF2β and probably with other initiation factors that are rich in Arg/Lys residues. eIF2 α and γ-subunits can interact to form a heterodimer with yeast and archaeal eIF2 subunits (Thompson et al., 2000; Schmitt et al., 2002; Tahara et al., 2004). However, interactions between α and β -subunits, or the formation of αβ-dimers have not been reported. Structural, biochemical and mutational analysis of archaeal initiation factor 2α (eIF2α) suggest that the CTD of eIF2α is necessary and sufficient for the interaction with the γ-subunit and for stabilization of GTP bound Met-tRNAi (Yatime, et al., 2004; Ito et al., 2004). The recent crystal structure of archaeal γ-subunit suggests that the binding of eIF2α is localized to the side of eIF2γ adjacent to the Met-tRNAi$^{Met}$ binding (Roll-Mecak et al., 2003).

The most striking feature of the structure of human eIF2α is that the C-terminal αβ fold displays a very similar tertiary structure of the CTD of translational elongation factor eEF1Bα, despite lack of any significant sequence homology (Ito et al., 2004). The eEF1Bα is a guanine nucleotide exchange factor in the elongation cycle of protein synthesis and catalyzes the GDP/GTP exchange of eEF1A, which is functionally and structurally similar to the γ-subunit of eIF2. Lys $^{205}$ in eEF1Bα is a critical amino acid residue involved in the GNE activity of eEF1Bα. Consistent with the formation of αγ dimers, and for the ability of α-subunit to bind GTP in the cross-linking studies, the solution structure of human eIF2α suggests that the CTD of eIF2α may come close to $Mg^{2+}$ and GDP/GTP binding pocket of eIF2γ (Ito et al., 2004). This pocket contains basic

residues such as Arg $^{243}$ and Lys $^{275}$ at a similar site as Lys $^{205}$ in eEF1Bα. These findings raise a possibility that the α-subunit may be involved in the exchange of GTP for GDP to the γ-subunit of eIF2. The function of eIF2α however in the guanine nucleotide exchange of eIF2 is lost probably in evolution and is catalyzed by heteropentameric eIF2B protein. The possibility of archeal alpha-subunit involvement in nucleotide exchange activity however may exist because it contains the conserved basic residues at human positions 244 and 272 and the archael genome analysis suggests that it may not have comparable eIF2B like protein (Asano et al., 1999). Interestingly, a recent study describes aIF2 has similar affinities for GDP and GTP (Pedulla et al., 2005). The most important functional aspect of eIF2α, however, is that it regulates initiation of protein synthesis (discussed below).

**4.2 eIF2β-subunit:** The β-subunit of eIF2 ranges from 250 to 333 amino acids and has several interesting features relative to the other subunits. This subunit interacts with mRNA, eIF5, eIF2B, eIF3, Met-tRNAi and other proteins (**Fig.1.3**). Like eIF2α, the mammalian β-subunit of eIF2 is a substrate for several kinases *in vitro* (Ting et al., 1998; Clark et al., 1989; Welsh et al., 1994; Kimball et al., 1998). 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. This anamolous behaviour is attributed to the high percentage of basic residues (Pathak et al., 1988; Price et al., 1989). The wheat germ eIF2β is 38 kDa and is smaller than its α-subunit (42kDA) (Janaki et al., 1995; Laxminarayana et al., 2003; Metz and Browning, 1997). The reduced size of the β-subunit in part may be because of the lack of one of three polylysine motifs present in the mammalian β-subunit. The amino acid sequence of eIF2β indicates two most important features: three stretches of polylysine residues (K- boxes ranging 6-8 lysines) in the N- terminal half and a Cys-4-type zinc finger motif (with out $Zn^{2+}$) at C-terminus (Pathak et al., 1988). They are conserved in yeast, human and *Drosophila* (Donahue et al., 1988; Pathak et al., 1988; Ye and Cavenar, 1994). N-terminal half of the human β-subunit has only two of the three consensus guanine nucleotide binding domains (DXXG, NKXD) and they are about 125 residues apart, whereas, these are

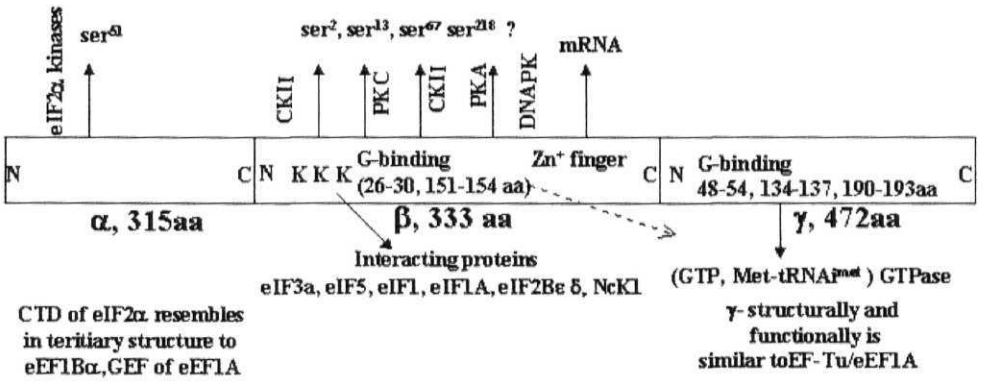



**Figure 1.3: Important features of Mammalian eIF2 subunits.**

separated by 40-80 residues apart in other G-binding proteins [Dever et al., 1987]. These two GDP or GTP binding domains are not found in yeast eIF2β [Pathak et al., 1988].

The β-subunit of eIF2 is primarily implicated in the binding of eIF2 complex to mRNA [Laurino et al., 1999]. However, it 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, β-subunit of mammalian eIF2 can be proteolysed during purification or purposefully [Mitsui et al., 1981]. An early report suggests that eIF2 devoid of β-subunit can still function similarly 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; Bommer and Kurzchalia 1989]. Based on genetic and biochemical studies in yeast cells, it has also been suggested that the C-terminal zinc finger structure in eIF2β is important in Met-tRNAi interaction [Castilho-Valavicius et al., 1992; Donahue et al., 1988]. It may be likely that this essential function of binding Met-tRNAi is carried out by both the β and γ subunits of eIF2. 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$^{Met}$ 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$^{Met}$ binding, and, also in GTP hydrolysis. Subsequent studies have shown that the C-terminal of eIF2β has considerable sequence homology and can also interact with eIF5

(Das et al., 1997a). Characterization of yeast eIF2γ suppressor mutant N135K (in the G2 motif) complex for the Met-tRNAi binding in the presence of GTP or GppNp (a non-hydrolyzable GTP), suggest that the wild type γ has some amount of eIF5 independent intrinsic GTPase activity and promotes the ternary complex formation in the absence of GTP hydrolysis. All the suppressor mutations in eIF2 β and γ- subunits result in aberrant start site selection (Huang et al., 1997) and is probably related to the binding strength of the β-subunit to the γ-subunit of eIF2 as has been suggested recently (Hashimoto et al., 2002).

The eIF2αγ subunit preparations that are devoid of the β-subunit also showed a reduction in the guanine nucleotide exchange activity of eIF2B (Flynn et al., 1993). This is consistent with the ability of C-terminus of β-subunit to interact with the δ- and ε-subunits of mammalian eIF2B (Kimball et al., 1998) as well as between the N-terminus of eIF2β and the ε-subunit of eIF2B in yeast. (Asano et al., 1999). Very early results have pointed out that eIF2 also binds ATP. 8-Azido ATP labels eIF2β (Dholakia etal., 1989). The sequence in eIF2β contains many ATP binding sites. ATP bound eIF2 complex can bind GTP but lowers the formation of ternary complex, eIF2.GTP.Met-tRNAi. It is therefore suggested that ATP binding to eIF2 may cause dissociation of Met-tRNAi and induces a change in the conformation of eIF2 that favors mRNA binding (Proud et al., 1991a).

Very early studies also showed that binding affinity of mRNA for eIF2 is related to translational efficiency (Rosen et al., 1982). Preparations of eIF2 depleted of β-subunit are defective in mRNA binding and that 4-thio-UTP-substituted encephalomyocarditis virus (EMCV) RNA can be cross-linked to the carboxy terminal of eIF2β that contains a conserved a Cys-4-type zinc finger motif (with out $Zn^{2+}$). *In vitro* experiments indicate that deletion of all the three lysine repeats or K-boxes from yeast eIF2β impair mRNA binding by purified eIF2 complex, but has no effect on the formation of eIF2.GTP.Met-tRNAi ternary complex. These findings therefore suggest that K-boxes are not essential for ternary complex formation or for the interaction between ternary complexes and 40S ribosomes (Laurino et al., 1999). These studies have further shown that one run of lysine residues, or argenines replacing lysine residues and, a region encompassing the zinc

finger motif are sufficient for mRNA-eIF2β interaction *in vivo* (Laurino et al., 1999).

Further, the N-terminal lysine repeats in eIF2β interact with the conserved bipartite motifs rich in aromatic and acidic residues in the C-terminal region of the catalytic subunit of eIF2Bε and eIF5 (Asano et al., 1999, Das et al., 1997, Das and Maitra, 2000). This interaction plays a role in the regulation of GDP/GTP exchange activity of eIF2B when eIF2α subunit is phosphorylated (Kimball et al., 1998). eIF5 can bind directly to the ternary complex via the β-subunit of eIF2 and this interaction is critical for the function of eIF5 (Asano et al., 2001; Das and Maitra, 2000). The interactions are found to be mutually exclusive because eIF2B and eIF5 carry opposing activities of eIF2 such as GDP/GTP exchange or promoting GTP hydrolysis respectively. Further, when the interactions of initiation factors in the multifactor complex (MFC) in yeast are analyzed, it has been observed 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.

A recent study describes that ATP –binding cassette protein-50 (ABC50) whose mRNA is increased rapidly following TNF-α stimulated synoviocytes and that plays a key role in the regulation of mRNA translation has been found to interact with eIF2 complex and stimulates the binding of Met-tRNAi to eIF2 (Tyzack et al., 2000). The fact that it stimulates the binding of Met-tRNAi to eIF2, interacts with initiating ribosomes, and, its interaction with purified eIF2 varies depending on the type of proteinase inhibitor used in the purification scheme, it is likely that ABC50 may be interacting with the β-subunit of eIF2. This idea is consistent with the fact that many researchers found that purified preparations of mammalian eIF2 lack the β-subunit and is accessible for protease degradation.

In addition to the above interactions with initiation factors, a recent study has pointed out that the carboxy terminal of β-subunit of eIF2 interacts with the third SH3 domain of non-catalytic region of tyrosine kinase adaptor protein (Nck1) that stimulates translation of both cap-dependent and cap independent translation. Since Nck1 binds to

insulin receptor and also to kinases such as CK1, these molecules can also regulate the activity of the effector molecules associated with the SH3 domain of Nck1 (Kebache et al., 2002). The β-subunit of eIF2 also interacts with DNA-PK, a nuclear enzyme and also serves as a substrate for DNA-PK raises questions regarding its role if any in DNA repair (Ting et al., 1998). Further, the human eIF2β- subunit is found earlier phosphorylated by CKII ($2^{ser}$ and $67^{ser}$ residues), PKC (at $13^{ser}$) and PKA ($218^{ser}$) (Issinger et al., 1976, Tuazon et al., 1980; Depali Roach et al 1981, Jagus et al., 1982, Clark et al., 1988, 89, Schatzmann et al., 1983, Welsh et al., 1994; Alcazar 1988). The physiological significance of the β-subunit phosphorylation is not studied in detail. However, it is shown that phosphorylation can alter certain biochemical reactions *in vitro* (discussed below).

Although a crystal structure of yeast or mammalian eIF2β- is not yet available, solution structure of the archaeal β-subunit has been recently solved (Cho and Hoffman 2002; Guttirrez et al., 2004). The N-terminal domain of *Methanococcus jannaschii* contains 4-stranded anti-parallel β-sheet and two α-helices. It is structurally, but not at the level of primary sequence, similar to the DNA-binding domain of the yeast heat shock transcription factor and a domain within ribosomal protein S4. This region corresponds to the central domain of yeast eIF2β. The C-terminal domain contains a zinc binding sequence of three antiparallel β-strands with four conserved cysteines arranged as two CXXC units separated by 17 amino acids. Both N and C-terminal domains are connected by a helical linker which plays an important role in maintaining the relative orientation of the two domains and the conserved hydrophobic residues in the linker may serve the contact regions for the α- or γ-subunits of eIF2 (Cho and Hoffman 2002). The mutations implicated in the selection of start codon in yeast eIF2β are conserved in the archaeal eIF2β-subunit. Some of them like the $Lys^{122}$, Arg $^{125}$, Val $^{126}$ and Ile $^{140}$ are located in close proximity in the structure of the C-terminal domain and may serve a site for the interaction of the other translational components implicated in the start site recognition. The β-subunit of aIF2 has ~50% similarity and ~30% identity with eukaryotic β-subunit (Cho and Hoffman 2002). It lacks the variable length of N-terminus that contains the polylysine

residues as in eukaryotes. This is consistent with the suggestion that archaea do not appear to contain eIF2B or eIF5 like proteins based on its genome analysis [Asano et al., 1999; Kyrpides and Woese, 1998]. The conserved sequences of eIF2β in archaea and eukaryotes, which are absent from eIF5, may mediate the interaction of β-subunit with the γ-subunit of eIF2 [Thompson et al., 2000].

**4.3 eIF2γ-subunit:** The molecular mass of γ-subunit ranges from 51 to 58 KDa approximately in different organisms. It has a high degree of amino acid sequence similarity to the bacterial elongation factor EF-Tu or eukaryotic factor eEF1A and SELB proteins [Gaspar et al., 1994; Hannig et al.,1993; Erickson et al., 1997; Keeling et al., 1998]. Unlike β-subunit of eIF2, the N-terminus region of γ-subunit contains all three conserved consensus guanine nucleotide binding domains (GXXXXGK, DXXG, NKXD) and separated by 40-80 residues as reported in other GTP binding proteins (**Fig. 1.3**). Earlier biochemical studies with cross-linking agents implicated both the β-and γ-subunits in GTP binding [Anthony et al., 1990, Dholakia et al., 1989 and Bommer etal., 1989]. However, 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]. This result is further supported by the fact that β-subunit yeast eIF2 lacks both GTP binding domains found in mammalian eIF2. Hence the N-terminus of eIF2γ is implicated in the binding of GTP and Met-tRNAi [Gasper et al., 1994; Erickson et al., 1997; Naranda et al., 1995]. The GTP hydrolysis step is important for ribosomal recognition of 'start' AUG. eIF2 and eIF5 maintains the fidelity of translational initiation [Donahue et al., 1988]. In analogy with proteins of the GTPase super family, the γ-subunit that contains the GTP binding domains may have a latent GTPase activity that hydrolyses the GTP bound to eIF2. While the biochemical studies have shown that eIF5 can stimulate the GTP hydrolysis, no reports are available to indicate that GTPase activity is intrinsically associated with the γ-subunit of eIF2 [Das and Maitra 2000; Das et al., 2001]. However point mutations in yeast eIF2β in the C-terminus zinc finger motif that reduces the accuracy in start site selection can increase the GTP hydrolysis independent of eIF5 [Donahue et al., 1988;

Huang et al., 1997). 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 presumably associated with the γ-subunit is stimulated by eIF2β and eIF5 interaction on the ribosome. This is consistent with the fact that α and γ-subunits interact to form a dimer and the N-terminal polylysine residues of the β-subunit of eIF2 interact with the bipartite motifs (aromatic and acidic residues) in the C-terminal of eIF5 (Das et al., 1993 and Asano et al., 1999). Interestingly, the initiation step in eukaryotes requires the hydrolysis of two molecules of GTP. One of them is catalyzed by the γ-subunit of eIF2 that is stimulated by eIF5 and eIF2β. This step facilitates the hydrolysis of GTP bound eIF2 and occurs after the recognition of the 'start' site in mRNA by 48S initiation complexes. The second GTP hydrolysis occurs by eIF5B (Pestova et al., 2000) that promotes the joining of 48S initiation complexes to 60S subunits to form 80S initiation complexes. Interestingly recent studies indicate that eIF5B is homologous to bacterial IF2 that is equivalent to eIF2 in function in facilitating the binding of initiator tRNA to 30S ribosomal subunit (Gualerzi and Pon, 1990; Luchin et al., 1999; Grunberg-Manago, 1975). It is a single subunit protein with GTPase activity and thus IF2 homologs are conserved from bacteria to eukaryotes. In bacteria, however a single IF2 GTPase is sufficient to catalyze translational initiation (Lee et al., 2002). Archaea that lack eIF5, the GTP hydrolysis may be accomplished by an auxillary GTPase like IF2 that is homologous to eIF5B (Kyrpides and Woese, 1998).

Very early studies have indicated that ribosome-associated eIF2 trimeric complex is detected in cytoskeletal fraction of He La cells (Howe and Heshey, 1984, Gavrilova et al., 1987; Nielsen et al., 1983). This association may be important in the regulation of protein synthesis. However no reports are available subsequently implicating any of the subunits in the interaction with cytoskeletal material. A recent study suggests that eIF2γ levels are controlled by Cdc123 and Chfr (check-point fork head associated with RING proteins) proteins which in turn control the arrest of G1 cell cycle that occurs due to nutrient limitation, and, or the arrest of late G1 in cell cycle in haploids due to pheromone treatment. These studies suggest an interaction between eIF2γ and Cdc123 proteins. Too

much or too little Cdc123 function depresses the eIF2γ levels. Over-expression of them is also growth inhibitory or toxic to cells. These findings therefore suggest that Cdc123 and Chfr proteins control the cell cycle by controlling the eIF2γ levels (Bieganowski et al., 2004).

The crystal structure of eIF2γ without bound nucleotide (GDP or GTP) from two different organisms, *Methanococcus jannaschi* (Roll Mecak et al., 2004) and *Pyrococcus abyssi* (Schmitt et al., 2002) of archaea reveal that it consists of three domains and is similar to EF-Tu or eEF1A. Structure based sequence alignments of various eukaryotic and archaeal homologs indicate that the hydrophobic cores of each of these three domains map to the conserved residues, whereas random coiled portions in the structure map to the insertions and deletions. Domain I (1-226 in *M. jannaschii* and 1-205 in *P. abyssi*) is also called G domain that contains conserved sequences characteristic of other GTP binding proteins and shows significant structural homology with other GTPases. It consists of β-sheet of mixed polarity that is flanked by 5 α-helices. Domain I has an extended zinc ribbon motif that is not found in other GTPases. It projects out from the domain I and forms an overhang between I and II domains. This motif forms a metal binding knuckle and has two β-hairpin turns consisting of two pairs of cysteines. The metal ion in the knuckle probably stabilizes the relative positions of β-strands that do not make extensive hydrophobic contacts with one another. In mammalian γ-subunit, only one of the four cysteines is conserved and lacks zinc. Although the zinc ion is lost in evolution, the integrity of the motif may be preserved as has been observed in the zinc-containing motifs of mammalian transcription factors. This zinc containing specific extension may play a role in the binding of γ-subunit to α or β-subunits in eIF2. Domains II and III span between residues 236-344 and 347-437 in *M. jannaschii* and 206-308 and 309-410 in *P. abyssi* residues respectively. Both of them are β-barrels. Domain II is structurally similar to domain II of EF1A and domain IV of IF2 and eIF5B that are probably involved in the recognition of the 3' ends of tRNA. While domain II β-barrel contains ten anti-parallel β-strands and two $3_{10}$ helices, domain III contains six β-strands and is similar to domain III of EF1A. Ligand-induced conformational changes in eIF2γ are induced by two polypeptide segments called, switch 1 and 2. Switch 1 may contain an effector region that

probably interacts with Met-tRNAi, ribosome, and, or eIF5, and lies between domains I and II. Switch II lies in the middle of the structure and interacts with domain II and III. The eIF2γ without nucleotide binding shows a closed configuration with domain II packed against the G domain in the vicinity of the switch regions. The conformational changes undergone by eIF2γ on nucleotide binding and hydrolysis differ from those seen in EF1A despite high sequence identity. A positively charged/ hydrophobic surface that could support phosphate and or base recognition in eIF2γ is predicted to be involved in the binding of Met-tRNAi $^{Met}$ (Roll Mecak et al., 2004 and Schmitt et al., 2002). Structure based site-directed mutagenesis in archaea and the corresponding residues in yeast eIF2γ- reveals that domain II plays a critical role in the binding of Met-tRNAi $^{Met}$ to eIF2 and that the binding of the α-subunit of eIF2 is localized to the side of eIF2γ adjacent to the Met-tRNAi$^{Met}$ binding (Roll Mecak et al., 2004).

**5.0 Regulation:**

**5.1 eIF2α phosphorylation sites and kinases:** Regulation of translational initiation occurs chiefly through the phosphorylation of two factors eIF2 and eIF4E. Phosphorylation of eIF2α is considered to be a stress signal and regulates protein synthesis in response to physiological conditions such as heme-deficiency, viral infection, amino acid, nutrient or serum starvation, heat and oxidative stress, accumulation of unfolded proteins and stress in the endoplasmic reticulum (ER) (London et al., 1987; Jackson, 1991; Hershey, 1991; Rhoads, 1999, Hinnebusch, 2000; Poulin and Sonenberg, 2003; Clemens 1995; Dever, 2002; Ron, 2002; Kaufman, 2002; Proud, 1994, 2005, Patil and Walter, 2001). Phosphorylation of eIF2α is conserved and occurs in mammals (London et al., 1987; Jackson, 1991), yeast (Hinnebusch, 2000), insects (Aparna et al., 2003; Williams et al, 2001; Santoyo et al.,1997; Olsen et al., 1998; Pomar et al., 2003), plants (Benne et al., 1980; Shaikin et al., 1992; Mehta et al., 1986; Seal, 1983; Chang et al., 2000; Gil et al., 2000; Le et al., 1998; Laxminarayana et al., 2003; Janaki et al., 1996, Shaikin et al., 1992; Bilgin et al., 2003), parasites like *Plasmodium* (Surolia and Padmanaban et al., 1991; Mohrle et al., 1997) and *Taxoplasma* (Sullivan et al., 2004), and also in archaea (Tahara et al., 2004). Previous biochemical studies implicated that

human eIF2α may have two putative phosphorylation sites: $48^{ser}$ and $51^{ser}$ (Wettenhall et al., 1986; Kudlicki et al., 1987). However subsequent biochemical analysis and evaluation of site-specific mutants suggest that the conserved $51^{ser}$ is the only phosphorylation site *in vitro* and in lysates (Colthrust et al., 1987; Price and Proud, 1990; Pathak et al., 1988). The sequence around $51^{ser}$ is Ile-Leu-Leu-Ser-Glu-Leu-Ser-Arg-Arg-Arg-Ile-Arg-. The basic residues surrounding the phosphorylation site may be important in the substrate specificity of eIF2α kinases, as is the case for several other protein kinases (Proud et al., 1991). Target site in archaeal eIF2α is however found to be $48^{ser}$ (Tahara et al., 2004). Subsequent studies in eukaryotes have shown that phosphorylation of $51^{ser}$ residue is the cause for inhibition of protein synthesis (Kaufman et al., 1989; Price et al., 1991).

Several eIF2α kinases that phosphorylate eIF2α on the conserved $51^{ser}$ residue have been identified and characterized. These kinases require distinct stress signals and activate downstream response pathways by regulating translation. These include: heme-regulated eIF2α kinase (HRI) that links protein synthesis to heme availability in erythroid cells, and, is also activated by oxidative and heat stress, and, exposure to certain diffusible gases (Chen et al., 1991; Han et al., 2001; Lu et al., 2001; Uma et al., 2001; Zhan et al., 2002); GCN2 which is activated by nutritional stress such as amino acid or purine deprivation (Wek, 1990, 1995; Rolfes and Hinnebusch, 1993; Harding et al., 2000; Yang et al., 2000; Zhang et al., 2002); double-stranded RNA-dependent protein kinase (PKR), which controls an antiviral defense pathway that is induced by interferon (Meurs et al., 1990; Kaufman 2000; Williams, 1999), pancreatic eIF2α-kinase, PEK or PERK that resembles to PKR and is activated in response to the accumulation of unfolded/malfolded proteins (Shi et al., 1998; Harding et al., 1999); TgIF2K from Taxoplasma that is activated in response to heat and alkaline stress that are known to induce parasite differentiation *in vitro* from an acute infection form (tachyzoite) to encysted form (bradyzoite) (Sullivian et al., 2004), and, PfPK4 from plasmodium which is activated in response to heme- deficiency (Mohrle et al., 1997). In archaea, the gene product PH0512 has been found to serve as the kinase for archaeal eIF2α (Tahara et al., 2004). Despite reports that plants and insects contain PKR-like protein that is antigenically similar to

mammalian PKR [Crum et al., 1988; Langland et al., 1995], PKR-encoding sequences have not been detected or reported in these systems [Browning 2004; Aparna et al., 2003]. A recent study suggests that plants may lack homologs of PKR but contain genes with significant similarity to the double stranded RNA binding domains of PKR as well as ser/thr kinases that show significant similarity with the kinase domain of PKR. It is likely that the products of these two genes act together to perform the same function as in mammalian PKR [Bilgin et al., 2003]. GCN2 homologs have been isolated from plants and insects [Zhang et al., 2003; Santoyo et al., 1997; Olsen et al., 1998]. Although unfolded protein response is observed in yeast [Gething and Sambrook, 1992; Mori et al., 1992, 93; Cox et al., 1993; Patil and Walter 2001; Kaufman, 1999], insects [Williams et al., 2001] and in plants [Martinez and Chrispeels, 2003], PERK homologs are detected in insects [Pomar et al., 2003; Sood et al., 2000], but not in yeast and plants [Martinez and Chrispeels, 2003]. In addition to the stress signals mentioned above, eIF2α kinases like PKR and PERK also appear to be activated by caspases [Saleans et al., 2001; Aparna et al., 2003]. The four eukaryotic eIF2α kinases consist of a conserved eIF2α kinase domain linked to different regulatory domains [Dever, 1999]. While their regulation is different, they all phosphorylate eIF2α on its conserved $ser^{51}$ residue. Earlier studies with PKR have reported a region of PKR (aa 362-370) that is adjacent to the conserved insert domain in eIF2α kinases is required for kinase and for substrate binding activity [Cai and Williams, 1998]. Hydrophobicity in this region is found essential for kinase activity, while Glu-367 is required for binding to the substrate. Further, the GYID sequence is found to be important in the substrate for binding to the kinase [Sharp et al., 1997]. Interestingly, a recent paper describes that aIF2α does not contain the typical phosphorylation motif as in eIF2α but has two conserved serine residues at 44 and 48 positions corresponding to the phosphorylation motif in eIF2α. In addition, the conserved 81 $^{tyr}$ in the $K_{79}$GYID sequence in eIF2α is changed to histidine in aIF2α ($K_{75}$GHID). Mutational analysis of $77^{his}$ and $44^{ser}$ of aIF2α suggests that aIF2α may be recognized by human and archaeal eIF2α kinases in a manner different from $51^{ser}$ in eIF2α [Tahara et al., 2004].

Yeast eIF2α that contains three additional phosphorylation sites at the C-terminal

residue is 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 et al., 1986]. Mutational studies in yeast eIF2α suggest that the C–terminal phosphorylation of eIF2α may be necessary for optimal activity of eIF2 [Feng et al., 1994]. Artemia and plant eIF2α also contains CKII phosphorylation sites [Feng et al., 1994; Mehta et al., 1986; Janaki et al., 1995; Le et al., 1998 Laxminarayana et al., 2003]

**5.2 Phosphorylation of eIF2α and translation of mRNAs:** Phosphorylation of 20-30% of eIF2α inhibits protein synthesis in general in reticulocytes and also in other translating lysates prepared from mammalian cells [Leroux and London, 1982; Scorsone et al., 1987]. This phenomenon is conserved in mammals. The importance of phosphorylation of eIF2α in translational control during a wide variety of stress conditions has been elucidated by the expression of wt eIF2α and serine-to alanine mutants at residues 48 and 51(S48A and S51A). Although S48A mutant of eIF2α is a substrate for phosphorylation, over-expression of this mutant protein in stressed cells rescues protein synthesis inhibition that occurs due to endogenous eIF2α phosphorylation. [Choi et al., 1992, Kaufman et al., 1989, Murtha-Riel et al., 1993]. Subsequent studies with yeast and mammalian systems discovered that phosphorylation of eIF2α does not always lead to inhibition in protein synthesis but it is also found associated with the translational up regulation of transcription factors like GCN4 mRNA in yeast [Dever et al., 1992] and ATF4 (Activated Transcription factor 4) in mammalian systems which in turn stimulate the synthesis of several genes in amino acid biosynthesis or induced in stress [Harding et al., 2000; Scheuner et al., 2001]. A recent study further demonstrates a connection between eIF2 phosphorylation and activation of IRES (internal ribosome entry sites) elements, and, suggests that eIF2 phosphorylation has the potential to regulate expression of IRES-containing mRNAs under a range of non-physiological conditions [Gerlitz et al., 2002]. Interestingly, eIF2α phosphorylation that decreases the abundance of ternary complexes can stimulate the joining of RNA-binding proteins like TIA-1 (T-cell internal antigen-1) and TIAR (TIA-related) to the 48S

preinitiation complexes (Kimball et al., 2003; Kedersha and Anderson, 2002). Subsequent self-aggregation of these TIA proteins lead to the formation of stress granules. The aggregation of TIA proteins appears to be related to the reduced levels of HSP70. Hence it is suggested that the denaturation of proteins that signals eIF2α kinase activation and eIF2α phosphorylation, may also play an important role in stress granule formation (Kedersha and Anderson, 2002). Since eIF2α phosphorylation occurs in response to diverse stress conditions, it is suggested that the downstream response coordinated by eIF2α phosphorylation can be called as Integrated Stress Response (ISR) (Harding et al., 2001; Ron et al., 2002). Activation of ISR response by blocking the dephosphorylation of eIF2α by a constitutive phosphatase protects the cells from oxidative, nitrosative and ER stress (Jousse et al., 2003). This is consistent with the results that eIF2α phosphorylation, or inactivation of eIF2 activity that mimics the effect of eIF2α phosphorylation, induces a gene expression program with a special role in promoting resistance to oxidative stress (Tan et al., 2001; Harding et al., 2003). Given the diverse stress conditions activating both eIF2α kinases and NF-kB, a recent study demonstrates that phosphorylation of eIF2α is a fundamental to the process by which diverse physiological stresses are monitored and relayed to the activation of NF-kB (Jiang et al., 2003).

**5.3 Phosphorylation of eIF2α in malignancy, apoptosis and in glucose homeostasis:** Abrogation of eIF2α phosphorylation leads to malignancy in NIH 3T3 cells (Donze et al., 1995). In contrast cells, undergoing apoptosis have shown enhanced eIF2α phosphorylation (Srivastava et al., 1998; Aparna et al., 2003), there by suggesting that phosphorylation of eIF2α plays a critical role in cell growth. In cells transformed with the c-myc, v-src or v-abl oncogenes, elevated levels, but not phosphorylation of eIF2, are observed (Rosenwald, 1996; Rosenwald et al., 1993, Lobo et al., 2000). In some tumor cells, eIF2α phosphorylation is found higher than their normal counterparts (Lobo et al., 2000). This may be due to increased levels of eIF2B in these cells. Very early studies have indeed pointed

out that Ehrlich cell extracts can tolerate higher level of eIF2α phosphorylation than reticulocyte extracts and this is due to the fact that the former has higher amount of eIF2B relative to eIF2 than in reticulocyte lysates [Rowlands et al., 1988]. Depletion of intracellular calcium stores that activates eIF2α kinases and stimulate eIF2α phosphorylation as has been shown by Brostrom et al (1989) is used by Halperin's group to identify ant-cancer drugs such as clotrimazole, n-3 polyunsaturated fatty acid eicosopentaenonic acid (EPA), triglitazone and 3,3 –diphenyloxindole 1 that affect the intracellular $Ca^{2+}$ homeostasis. These compounds inhibit the expression of oncogenes such ras and c myc and growth promoting proteins cyclin A, E and D1 [Aktas et al., 1998; Palakurthi et al., 2000; 2001; Natarajan et al., 2004] and enhance eIF2α phosphorylation. Recent studies with knock-in mice harboring nonphosphorylatable form of eIF2α i.e S51A, in which the $51^{ser}$ residue is replaced by alanine, have shown that such genetically engineered mice suffer from acute hepatic failure and profound hypoglycemia. The fact that these mice survive through pregnancy without any defect at birth suggests that phosphorylation of eIF2α may not be important in embryonic development. However the neonates die soon after birth and can be rescued with a glucose injection. Consistent with the hypoglycemia observed in these mutant mice, the activity of phosphoenol pyruvate carboxy kinase (PEPCK) is significantly reduced. These findings suggest that eIF2α phosphorylation couples protein synthesis with glucose sensing, glucose metabolism and insulin production [Scheuner et al., 2001].

**5.4 Phosphorylation of eIF2α and regulation eIF2B activity:** The ability of eIF2 to participate in successive rounds of initiation is controlled by the availability of the guanine nucleotide exchange activity (GNE) associated with the heteropentameric eIF2B protein. This is due to limiting amounts of eIF2B that catalyzes the recycling of eIF2.GDP to eIF2.GTP. Consistent with this suggestion it has been observed a) low levels of phosphorylation of eIF2α (20-25%) are found sufficient to attenuate protein synthesis completely in heme-deficient reticulocyte lysates [Leroux and London, 1982] and in other mammalian cells [Rowlands et al., 1988], b) addition of purified eIF2B (or reversing factor or RF) restores protein synthesis catalytically and c) cells containing higher

amounts of eIF2B are able to withstand higher levels of eIF2α phosphorylation. These findings are also consistent with the reports that eIF2α phosphorylation inhibits the GDP/GTP exchange activity of eIF2B in mammalian cells (Matts et al., 1983; Panniers and Henshaw, 1983; Rowlands et al., 1988; Clemens et al., 1982). Mutants of eIF2α like S48A (which is a substrate for phosphorylation) or the nonphosphorylatable form like S51A that can mitigate protein synthesis inhibition caused by wt eIF2α phosphorylation are found to reduce the inhibition in the GDP/GTP exchange activity of eIF2B (Ramaiah et al., 1994). The expression of S51D mutant of eIF2α, which is shown to inhibit protein synthesis or promote apoptosis (Kaufman et al., 1989; Choi et al., 1992; Srivastava et al., 1998), results in the inhibition of the nucleotide exchange activity of eIF2B, thereby suggesting that S51D mutant of eIF2α is a phosphomimetic form (Ramaiah et al., 1994). These findings therefore lead to the hypothesis that phosphorylation of conserved $51^{ser}$ residue sequesters eIF2B into a complex in which the GNE activity of eIF2B becomes non-functional. Subsequently it has been demonstrated that phosphorylation of $51^{ser}$ in eIF2α forms a complex with eIF2B that can be separated on a sucrose gradient (Sudhakar et al., 1999, 2000). Since S48A mutant of eIF2α that is phosphorylated on its $51^{ser}$ is unable to interact with eIF2B as efficiently as phosphorylated wt eIF2α(P), the findings also suggested that $48^{ser}$ residue in human eIF2α is required for a high affinity interaction between eIF2 and eIF2B proteins (Sudhakar et al., 1999). Consistent with these results, a recent study (Dey et al., 2005) with yeast eIF2α has shown that mutations flanking $51^{ser}$ or away in the conserved K79GYID83 motif in eIF2α have been found to block translational regulation. While mutation at $49^{glu}$ does not affect the phosphorylation of eIF2α at its $51^{ser}$ residue, it however impairs the binding of eIF2B to eIF2α and is found similar to the results obtained with S48A mutations in human eIF2α. In contrast, mutation of $83^{asp}$ to alanine eliminated eIF2α phosphorylation both *in vivo* and *in vitro*. These findings therefore suggest that eIF2α kinases and eIF2B recognize overlapping surfaces on eIF2α. Subunit analysis of yeast eIF2B by immunoprecipitation and biochemical studies has shown that it contains two sub-complexes: a regulatory complex containing α, β and δ-subunits, and,

a catalytic complex consisting of γ and ε-subunits [Yang and Hinnebusch, 1996; Pavitt et al., 1997; Pavitt et al., 1998]. Both complexes can interact with eIF2 [Pavitt et al., 1998]. The interaction of eIF2 to the regulatory complex, but not with the catalytic complex is enhanced upon phosphorylation of the conserved $ser^{51}$ residue in eIF2α [Pavitt et al., 1998]. Hence it is suggested that binding of eIF2α (P) to the regulatory subcomplex prevents a productive interaction with the catalytic subcomplex thereby inhibiting the nucleotide exchange [Pavitt et al., 1998, Krishnamoorthy et al., 2001]. In mammalian eIF2 (rat), however, it has been observed that β-subunit of eIF2, mediates the inhibition in the GNE activity of eIF2B when the α-subunit is phosphorylated. This is consistent with the ability of mammalian eIF2β-subunit to interact with the δ and ε-subunits of eIF2B [Kimball et al., 1998]. In addition to eIF2α phosphorylation, the GNE activity of eIF2B is also altered by other means. Phosphorylation of the ε-subunit of eIF2B by casein kinases I and II enhances its activity, whereas it is decreased by phosphorylation by glycogen synthase kinase3 [Dholakia and Wabha, 1988; Akkaraju et al., 1991; Singh et al., 1996; Aroor et al., 1994, Wang et al., 2001]. It is also regulated oxidizing conditions such as GSSG [Kan et al., 1988], by redox state of pyridine dinucleotides, or pyrolloquinoline quinone, PQQ [Dholakia et al., 1986; Akkaraju et al., 1991; Oldfield et al., 1992; Ramaiah et al., 1997], and, by changes in adenylate energy charge [Kimball and Jefferson, 1995] and polyamines [Gross et al., 1988; Aroor et al., 1995].

**5.5 Dephosphorylation of eIF2α**: Both type1 and type 2 phosphatases and also physiological inhibitors of eIF2α kinase can regulate the phosphorylation of eIF2α either by dephosphorylating eIF2α or inactivating eIF2α kinases [Chen et al., 1989; Ernst et al., 1982; Petryshyn, et al., 1982; Tan et al., 2002; Wek et al., 1992]. Subsequently, using type1 phosphatase (PP1) inhibitors like okadaic acid and heat stable inhibitor-2, this laboratory has shown that the dephosphorylation of eIF2α(P) and the release of GNE activity of eIF2B activity are inhibited in heme-deficient reticulocyte lysates treated with the delayed addition of hemin. Based on these findings, it has been suggested that eIF2α kinase-phosphatase equilibrium regulates the GNE activity of eIF2B which is critical for the recycling of

eIF2.GDP to eIF2.GTP and a type 1 phosphatase may be physiologically relevant in the dephosphorylation of eIF2 α(P) (Babu and Ramaiah 1996;). The importance of a PP1 phosphatase activity in the dephosphorylation of eIF2 α(P) has been subsequently shown in herpes simplex virus-1-infected cells and also in ischemia-induced eIF2(alphaP) accumulation in PC12 cells (He et al., 1997; Munoz et al., 2000). In HSV-1- infected cells, the host cell invokes eIF2α phosphorylation to fight against the virus infection. However, the C-terminus of virus virulence factor encoded by $\gamma_1$34.5 gene (cellular catalytic subunit, PP1c) overrides the host cell defense mechanism, binds to host cell PP1α and targets it to dephosphorylate eIF2α (He et al., 1997). Recent studies suggest that stressful conditions that promote eIF2α phosphorylation have been found to increase the expression of GADD34 gene (Growth Arrest and DNA Damage) that contains a C-terminus domain homologous to γ34.5 protein (He et al., 1996; Novoa et al., 2001; Ma and Hendershot, 2003; Connor et al., 2001). This gene product is not found in unstressed cells. Expression of GADD34 reversed eIF2α phosphorylation induced by phosphatase inhibitors like okadaic acid but not calyculinA suggesting that PP1 being a component of GADD34 assembled eIF2α phosphatase (Brush et al., 2003). In contrast, Jousse et al (2003) have identified a novel regulatory subunit of an active holo-phosphatase PP1c called constitutive repressor of eIF2α phosphorylation (CreP or PPP1R15B) that specifically dephosphorylates eIF2α. CreP is unlike GADD 34 and its expression is not altered by stressful conditions. It is a short lived protein. Blocking the dephosphorylation, by interfering with the translation of the CreP gene protected mammalian cells against oxidative stress. In a screen for small molecules that protect cells from endoplasmic reticulum (ER) stress, Bruce et al (2005) recently identified salubrinal, a selective inhibitor of cellular complexes that dephosphorylates eIF2 alpha subunit. Salubrinal also blocks eIF2alpha dephosphorylation mediated by a herpes simplex virus protein and inhibits viral replication. These results suggest that selective chemical inhibitors of eIF2alpha dephosphorylation may be useful in diseases involving ER stress or viral infection.

**5.6 Viral and cellular inhibitors of eIF2α phosphorylation:** Also in the fight between

viruses and host cells, many times the viral proteins are made which inhibit host cell eIF2α phosphorylation, thereby facilitating the virus infection [Mathews 2000; Thompson and Sarnow, 2000; Gale et al., 2000; Hasnain et al., 2003]. While some of these protein products act by inhibiting the activation of double- stranded RNA dependent protein kinase, PKR, of the host cells, some of these viral products mimic structurally to eIF2α of the host cells. These pesudosubstrates or structural homologs of eIF2α such as vaccinia virus K3L [Davies et al., 1993; Carroll et al., 1993; Qian et al., 1996; Sharp et al., 1997], swine pox C8L [Kawagishi-Kobayashi et al., 2000] and myxoma virus M156R [Ramelot et al., 2002] proteins are found to compete with the eIF2α of the host cell to bind to eIF2α kinases and thereby decrease the phosphorylation of eIF2α.. Unlike K3L or C8L, M156R can be phosphorylated by PKR *in vitro* on its $67^{tyr}$ residue that aligns with $51^{ser}$ in eIF2α and can compete with eIF2α for phosphorylation. These pesudosubstrates have been used by several laboratories to investigate the molecular determinants that specify substrate recognition by PKR or eIF2α kinases. Comparisions of the structures of the viral homologs K3L, M156R and eIF2α indicated that residues important for eIF2α kinase binding are located at the conserved positions on the surface of β-barrel in the N-terminus of eIF2α [Ramelot et al., 2002]. In addition, many cellular proteins and RNAs also can regulate the eIF2α kinase activation and thereby eIF2α phosphorylation. Among them the very first described one is a small molecular weight protein, dRF that is induced upon differentiation of 3T3-F442A cells into adipocytes and is an inhibitor of double stranded RNA-dependent protein kinase, PKR [Judaware and Petryshyn, 1991, 1992]. The others include heat shock proteins such as hsp 70 that prevents the activation of heme-regulated eIF2α kinase [Matts and Hurst et al., 1992; Gross et al., 1994, Tulasiraman et al., 1998, Uma et al., 1999], PKR inhibitor proteins such as $P58^{IPK}$ which is activated by influenza virus infection [Tan et al., 1998; Barber et al., 1994], HIV-TAR RNA binding protein, TRBP [Cosentino et al., 1995; Benkirane et al, 1997] and the glycosylated p67protein, originally found associated with purified eIF2 [Wu et al., 1998; Gupta et al., 1997]; PKR-activating proteins, PACT, RAX (a murine homolog of human PACT)

and NF90 (Patel and Sen, 1998, Ito et al., 1999, Parker et al., 2001). Among the cellular RNAs, L18 RNA of the 60S ribosomes and high concentrations of Alu RNAs that are transcribed by RNA polymerase III and are induced upon stress conditions can regulate eIF2α phosphorylation through the inhibition of PKR (Kumar et al., 1999; Chu et al., 1998). Low concentrations of Alu RNAs however can activate PKR (Williams, 1999).

**5.7 eIF2α regulation and phosphorylation by caspases:** Both eIF2α and eIF2α kinases are substrates for caspases. Caspase 3 cleaves efficiently unphosphorylated than phosphorylated eIF2α and releases 11 or 14 amino acid peptides (Satoh et al., 1999; Marissen et al., 2000). Further, the *in vivo* target of a caspase appears to be eIF2 complex bound to eIF2B than eIF2.GDP released at the end of initiation. Caspase-cleaved eIF2.GDP binary complexes can exchange their bound GDP for GTP in the absence of eIF2B. *In vitro* experiments demonstrate that caspase-3 cleaved eIF2α fails to stimulate upstream AUG selection on a mRNA containing a viral internal ribosome entry site and is no longer capable of translating overall translation (Marissen et al., 2000). Further the conclusions of two different studies (Saleans et al., 2001; Aparna et al., 2003) have recently suggested that caspases can cleave eIF2α kinase (s) and the processed kinase is more efficient in phosphorylating the substrate eIF2α. One of these studies suggests that baculovirus p35, an antiapototic protein, mitigates eIF2α phosphorylation (Aparna et al., 2003). This appears to be again a viral strategy for infecting the host cells as mentioned above. These findings are also consistent with the idea that apoptosis is characterized by increased eIF2α phosphorylation (Srivastava et al., 1998 Balachandran et al., 2000; Aparna et al., 2003). Interestingly, cellular proteins like p58 IPK and p67 are also reported to be present in plants and may play a role in protecting the plants from viral induced pathogenesis or in plant growth and development (Bilgin et al., 2003; Langland et al., 1997).

**5.8 Phosphorylation of eIF2β and regulation:** The mammalian β-subunit of eIF2 is phosphorylated at least by four known kinases *in vitro* as mentioned above. There are no

reports as of now that yeast eIF2β can be phosphorylated like its mammalian counterpart. Very early 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 evaluated the ability of eIF2.GDP binary complex as a substrate for eIF2B-mediated guanine nucleotide exchange and has observed 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 by Duncan and Hershey (1984, 85) have shown that the β-subunit undergoes phosphorylation in intact cells and may affect translation. In plant seeds, eIF2β is multipily phosphorylated (Gallie et al., 1997). Phosphorylation of β-subunit is decreased after flowering. Interestingly, enhanced phosphorylation of β-subunit is correlated to increased protein synthesis (Le et al., 1998). Unlike eIF2α phosphorylation, eIF2β-phosphorylation could not be labeled with radioactive phosphate in translating reticulocyte lysates thereby suggesting that the phosphate groups are stable and do not turn over (Jagus et al, 1982). Since it has multiple phosphorylation sites and is a substrate for four different kinases, the functional or physiological effect of this phosphorylation has not been analysed in detail so far.

**6.0 Objectives:** Most of the recent work on eIF2 function has come from yeast genetics and mutations in yeast eIF2. Further yeast eIF2 complex is shown to interact with the catalytic and regulatory regions in eIF2B (Pavitt et al., 2000) and that phosphorylation of eIF2 alpha alone is sufficient to interact with the regulatory complex of eIF2B (Krishnammorthy et al., 2001). However a previous study with rat eIF2 has shown that the complex formation that occurs between eIF2 and 2B is mediated by the beta-subunit of eIF2 and phosphorylation of eIF2 alpha enhances this interaction (Kimball et al., 1998). Also studies with yeast eIF2 suggest that β and γ-subunits are important for function (Erickson et al., 2001) and the γ-subunit is central core in the eIF2 trimeric

complex (Thompson et al., 2000). Consistent with this proposition, yeast eIF2 γ –subunit interacts with the α and β-subunits to form the respective heterodimers but not the α and β-subunits. Our laboratory is working on the regulation of mammalian and plant eIF2. A previous thesis from this laboratory has documented the expression of wt and mutants of human eIF2α subunit in which the conserved 51 and 48 serine residues (the putative phosphorylation site) are replaced by alanine (S51A or S48A) or aspartic acid (S51D) using baculovirus expression system and studied the effect of phosphorylation of wt and mutants of eIF2α for their ability to interact with eIF2B. The interactions have been analyzed by sucrose gradient analysis (Sudhakar et al., 1999, 2000). The present work is envisaged to obtain the three subunit wt and mutant human eIF2 complexes in order to understand the intersubunit and inter-protein interactions. In this pursuit, we have constructed baculovirus vectors harboring the cDNAs of human wt eIF2α and mutants that have been previously expressed, and also the β and γ-subunits with a N-terminal 6x histidine tag. The recombinant constructs are expressed in the ovarian cells of lepidopteran insect, *Spodoptera frugiperda (Sf9)*. Expressed proteins are purified, characterized for their ability to serve as substrates for different kinases, and reconstituted *in vitro* to study the interaction between any two of the subunits and all three of them. Further the individual subunits, and also the recombinant trimeric complexes have been used to study the interaction between eIF2 and 2B, particularly when eIF2 alpha is phosphorylated. In addition, the interactions between eIF2 subunits and eIF2α kinases (PERK and PKR) have also been studied.

# METHODOLOGY

## MATERIALS

The various biochemicals used in the study were purchased from the following companies or obtained as kind gifts from other laboratories as mentioned below:

Baculovirus vectors and competent *E.coli* strains, Grace's insect cell culture media, fetal calf serum and cell-fectin were obtained from Invitrogen. Biochemicals used for the preparation of cell extracts and solutions were purchased from Sigma, Amersham and Calbiochem. 0.22 and 0.45 μM nitrocellulose membranes were obtained from Millipore. Whatman I and III were obtained from Whatmann. Restriction endonucleases, ligase and plasmid isolation and gel-extraction kits were purchased from Promega and New England Biolabs. [γ-32 P] ATP, inorganic $^{32}P$ and [$\alpha^{32}P$] GTP were obtained from BRIT and JONAKI. Phosphospecific anti-eIF2α antibody was purchased from Research Genetics, Inc, USA. Anti-his tag, anti-eIF2α, anti-eIF2β, anti-eIF2Bβ antibodies were obtained from Santacruz Biotechnology Inc. HRP conjugated secondary antibodies were purchased from Bangalore Genei.

**Gifts:** eIF2α-, mutants of eIF2α-(S48A, S51A and S51D), β- and γ-subunit cDNA's were generously provided by Prof. Randall J. Kaufman, University of Michigan Medical Center, Michigan. PERK-GST was a gift from Prof.David Ron, Skirball Institute of Molecular Medicine, New York, USA. PKR-GST was given as a kind gift by Bryan R.G. Williams, Lerner Research Institute, Cleveland Clinic Foundation, Ohio, USA.

### 1. Preparation of recombinant bacmid DNA harboring cDNA of human eIF2 subunits.

The preparation recombinant baculoviruses harboring recombinant human eIF2 α/β/γ subunits is depicted in **Fig. 2.0**

### 1.1. PCR amplification and cloning of amplified fragments into pGEMT vectors:

The ORF regions of human eIF2-subunit cDNAs were amplified from the parent vectors using the designed primers with the restriction sites at the 5' and 3' ends. The

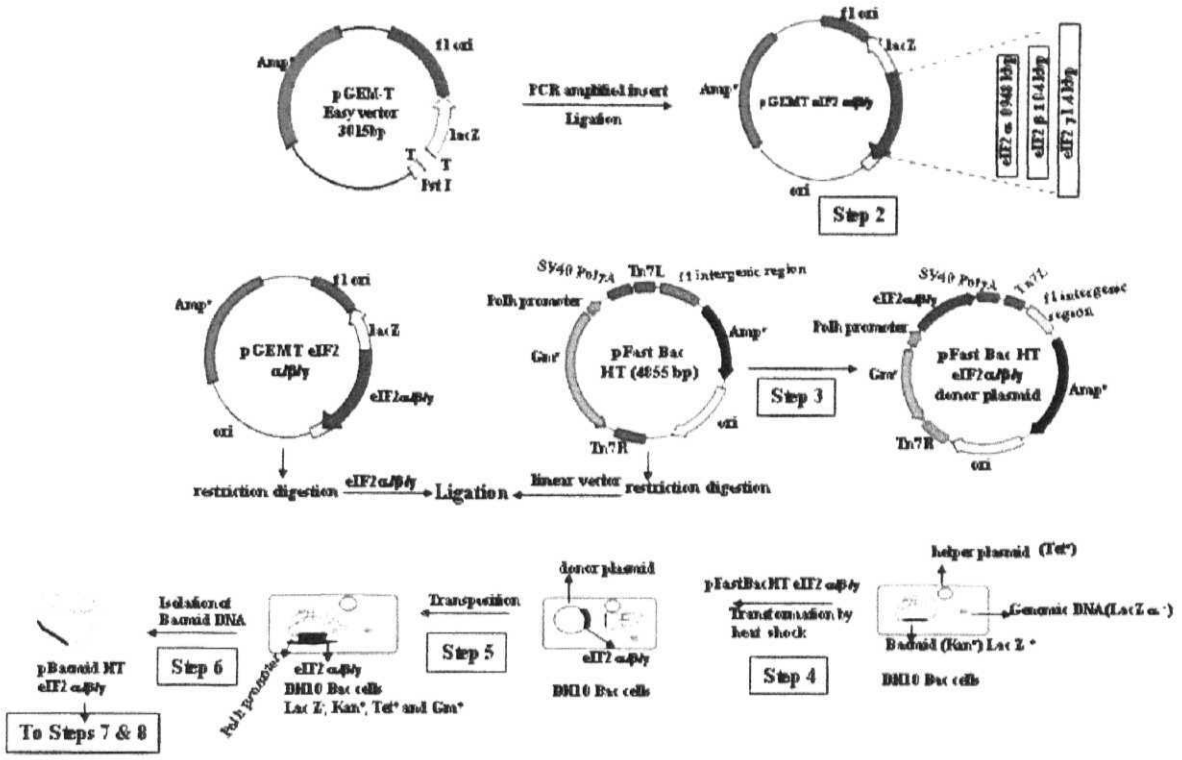



**Figure 2.0. Preparation of recombinant baculovirus harboring his-tagged eIF2 α/β/γ subunits and their expression in *Sf9* insect cells:**

Step 1: Human eIF2 α/β/γ cDNA is PCR amplified using gene-specific primer sequences.

Step 2: PCR amplified inserts are cloned into pGEMT vector.

Step 3: Human eIF2α/β/γ from pGEMT vector is isolated and sub cloned into pFast Bac HT vector.

Step 4: DH10 Bac cells are transformed with the pFast Bac HT vector.

Step 5: Transposition of the expression cassette from the donor plasmid to the Bacmid DNA.

Step 6: Isolation of Recombinant Bacmid DNA.

Step 7: Transfection of *Sf9* insect cells.

Step 8: Expression and purification of recombinant his-tagged human eIF2α/β/γ protein.

oligonucleotides used to amplify the α, β and γ cDNAs are mentioned below:

eIF2α forward: 5'TG**GGATCC**ATGCCGGGTCTAAGTTGT3' (Bam HI);

reverse: 5'GG**AAGCTT**TTAATCTTCAGCTTTGGCTT 3' (Hind III);

eIF2β forward: 5'GG**GGATCC**ATGTCTGGGGACGAGATG...3'(BamHI);

reverse: 5'AG**AAGCTT**TTAGTTAGCTTTGGCACGGAG...3'(HindIII) ;

eIF2 γ forward: 5' AG**GAATTC**ATGGCGGGCGGAGAAG.....3' (EcoRI);

reverse: 'TT**CTGCAG**TCAGTCATCATCTACTGTTGG.....3' (PstI).

The following PCR settings were employed in the reactions to amplify the cDNA's. eIF2α amplification: $94^0$C 30 sec, $50^0$C 30 sec, $72^0$C 1 min for 30 cycles; 2, eIF2β and γ-cDNA amplification: $94^0$C 30sec, $54^0$C 30 sec, $72^0$C 2 mins for 5 cycles and further $94^0$C 30 sec, $60^0$C 30 sec, $72^0$C 2 mins for 25 cycles.

The amplified cDNAs were analyzed by 1% agarose gel electrophoresis and the inserts were gel purified and then cloned into pGEMT vectors, which are used specifically to clone PCR amplified fragments. The ligation mixtures were then transformed into competent *E. coli.* (DH5α) cells prepared by using $CaCl_2$ by procedures as described (Sambrook etal., 1989). Heat shock method was used for transforming the *E.coli* cells. The transformation mixtures were then plated on Luria Bertani medium containing 1.5% agar, 100 μg/ml ampicillin, X-gal and IPTG and incubated at 37° C overnight. The recombinant pGEMT colonies were identified by blue white selection and ampicillin resistance. The recombinant colonies were inoculated into Luria Bertani broth containing 100 μg/ml ampicillin. Plasmid DNA was isolated from cultures by using plasmid isolation kit. The recombinant pGEMT plasmids harboring the eIF2α/β/γ inserts were digested using respective restriction enzymes and the samples were analyzed by 1% agarose gel electrophoresis to confirm the presence of insert.

**1.2. Sub-cloning of eIF2 α/β/γ cDNA from pGEMT vectors into Fast Bac HT vectors:** Fast Bac-HT donor vectors were used to generate viruses, which will express poly histidine-tagged proteins that can be readily purified on metal affinity resins. The first major component of the System is a pFastBac HT vector into which the gene(s) of interest will be cloned. They have a series of donor plasmids (HT a,b,c). To achieve high

level expression pf proteins, genes to be expressed were inserted into the multiple cloning site of a pFast Bac donor plasmid down stream from the polyhedron promoter (p10) of *Autographa californica* multiple nuclear polyhedrosis virus (AcMNPV). This expression cassette is flanked by the left and right arms of Tn7, and also contains a gentamicin resistance gene and an SV40 polyadenylation signal to form a mini Tn7.

1. The first step was cloning of the PCR amplified inserts from the pGEMT vectors into the Fast Bac donor vector at the required restriction sites. The recombinant colonies were ampicillin and gentamicin resistant. The recombinant plasmids were isolated and checked for the presence of the insert by restriction digestion analysis.
2. *E.coli* (DH10 Bac) cells were then transformed with the recombinant plasmid DNA. These cells have a parent plasmid that contains kanamycin resistance marker, and a segment of DNA encoding the lacZ alpha peptide. Inserted into the N-terminus of the lac Z alpha gene, is a short segment containing the attachment site for the bacterial transposon Tn7 (mini-attTn7) that does not disrupt the reading frame of the lacZ alpha peptide. The bacmid propagates in *E.coli* DH10Bac as a large plasmid that confers resistance to kanamycin and can complement a lacZ deletion present on the chromosome to form colonies that were blue (lac+) in the presence of a chromogenic substrate X-gal and the inducer IPTG. Recombinant bacmids of eIF-2 α/β/γ cDNA were constructed by transposing a mini-Tn7 element from a pFastBac donor plasmid to the mini-attTn7 attachment site on the bacmid when the Tn7 transposition functions were provided in trans by a helper plasmid (pMON7124). The helper plasmid confers resistance to tetracycline and encodes the transposase. The mini-Tn7 in the Fast-Bac donor plasmid contains an expression cassette consisting of gentamicin resistance gene, a baculovirus-specific promoter, a multiple cloning site, and an SV40 poly (A) signal inserted between the left and right arms of Tn7. The recombinant colonies were white in color in a background of blue colonies that harbour the unaltered bacmid on L.Bagar plate containing gentamicin, kanamycin and tetracycline.

3. The recombinant white colonies were checked for the presence of the eIF2 gamma cDNA by doing a colony PCR. pUC/ M13 amplification primers were directed at sequences on either side of the mini-attTn7 site within the lacZ alpha-complementation region of the bacmid. If the transposition had occurred, the PCR product by the primers will be 2430bp plus the size of the insert.
4. The recombinant positive clones were inoculated into L.B broth and incubated overnight at 37° C at 200 rpm. Later the recombinant bacmid DNA was isolated with the help of a plasmid DNA isolation kit, as described here previously.

**2. Maintenance, transfection of insect cells and generation of recombinant baculoviruses harboring human eIF2 α/β/γ- subunits:**

**2.1 Insect cell culture:** *Spodoptera frugiperda* (*Sf9*) cell lines (Vaughn etal., 1977), which serve as hosts for AcNPV were used for the expression of recombinant proteins. *Sf9* cells were maintained in complete Grace's insect cell culture medium (SF-900, Invitrogen USA) with 10% fetal calf serum and 1x antibiotic and antimycotic solution at 27° C as described by Summer and Smith, 1987. The doubling time of *Sf9* cells is 24 hours; cells were dislodged carefully and then distributed to four flasks for further growth. Confluent flasks containing >95% viable cells were used in all the experiments. Trypan blue exclusion test was carried out to assess the viability of cells.

**2.2 Freezing and reviving of *Sf9* cells:** *Sf9* cell line 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 million cell/ml and later frozen slowly. The cells were placed at 4° C initially then transferred to –20° C for 2 hours, -70° C for 12 hours before placing them finally in liquid nitrogen. The frozen stocks for reviving were thawed by placing them in 37° C water bath and later the contents were transferred into a culture flask. The cells were washed in complete medium devoid of DMSO and incubated at 27° C.

**2.3 Transfection:** Approximately 0.5 million cells/well were seeded in a six-well plate. The cells were washed once by replacing old complete medium with incomplete medium without serum and antibiotics. 5 μg of recombinant bacmid DNA was mixed with 5 μl of transfection reagent in 100 μl. The final volume was made up with medium without antibiotics and serum. The mixture was incubated for 45 mins at room temperature for formation of lipid DNA complexes. Later the medium from the cells was aspirated and overlaid with the lipid-DNA complexes gently and then added with 1 ml of incomplete medium and incubated for 5 hours at 27° C. A control transfection was done taking only cell-fectin reagent. The supernatant medium was later gently aspirated after incubation and saved as parent virus at 4° C. Fresh complete medium was then added to the cells and then allowed to infect for 72 hours. The cells were dislodged carefully in aseptic conditions and the cells were spun at 3K rpm for 10 mins. Then, the supernatant medium was collected and saved as 1st passage virus. The passage virus was reinfected to insect cells to increase the viral titers.

**2.4 Plaque assay:** The titre of the virus was determined by plaque assay. The procedure is as follows.

1. 2 x $10^6$ cells were seeded in each well of a six-well plate. Several dilutions of recombinant baculoviruses obtained form the transfections were prepared ranging from $1x10^{-1}$ to $10^{-7}$ in 100 μl volume.
2. The medium from the cells was removed and 100 μl of viral dilutions were added drop by drop into each well. The plates were then incubated at 27° C for one hour with gentle rocking for every 15 mins.
3. The viral inoculums were removed and 2 ml of low melting agarose prepared in insect cell medium was added to the monolayer cells in the wells of the six well plates. After the agarose is set, 1ml of complete medium was added to each well and the plates were incubated at 27° C for about 5 days. Later, the liquid medium was removed form the top of the agarose overlay and the plates were dried. 2 ml of neutral solution prepared in PBS pH 7.4 was added to each well.
4. The plates were incubated for about 2 hours at room temperature. The stain was

then removed and the plates were observed under microscope for the formation of clear plaques after 5-10 hours.

5. The plaques were counted for each dilution and the titre of the virus was determined by using the formula. Viral titre (pfu/ml)= average no. of plaques x innoculum plate$^{-1}$ x dilution factor$^{-1}$.

The low titre viruses were amplified by serial infection from 96 well plate to T-25cm$^2$ flask. After each infection, the cells in the flasks were incubated for one week for complete lysis of cells. A high titre of virus (6-8 x10$^7$ pfu/ml) was used for all the subsequent infections. Cells were infected at multiplicity of infection (MOI) 10 to express the recombinant proteins.

**3.0 Analysis of recombinant human his-tagged eIF2 α/β/γ- subunits expression in *Sf-9* cells.**

**3.1 Determination of human his tag eIF2α/β/γ expression by SDS-PAGE:** 2x10$^6$ *Sf9* cells were infected with recombinant baculoviruses harboring eIF2α (wt and mutant)/β/γ-subunits of eIF2 at a MOI of 10 and allowed to infect for 72 hours. After post infection the cells were harvested and spun at 3K rpm for 10 mins to collect the cell pellet. The cells were then washed twice with ice-cold PBS pH 7.4. Later the cells were suspended in 100 µl of 2X SDS-sample buffer and boiled for 5 mins before loading into the lanes of 10-12% SDS-Acrylamide gels. Uninfected *Sf9* cells were loaded in one lane as a control. The proteins in the gel were then transferred to a nitrocellulose membrane. The membrane was probed with monoclonal anti-histag antibody to detect the expression of recombinant proteins.

**3.2 Co-expression of recombinant eIF2 subunits by multiple infections:** Recombinant baculoviruses harboring eIF2 α/β/γ subunits were infected singly, or in combinations of two or three viruses to co-express the subunits of eIF2. The total MOI was fixed to 10 in all the infections. The cells or cell extracts were then analyzed by SDS-PAGE and western blotting for the co-expression of recombinant proteins.

**3.3 Preparation of cell extract:** Cell extract were prepared in lysis buffer containing 20 mM Tris-HCl, pH 7.8, 300 mM KCl, and 1% Nonidet P-40 containing protease inhibitors (10 μg/ml of leupeptin, pepstatin, aprotenin and 1 mM PMSF). The cells were incubated for 10 mins on ice and then the extracts were clarified by centrifugation at 10K for 20 mins. However, such a centrifugation resulted in the loss of gamma subunit but not the α and β-subunits from the supernatant. The cells expressing the gamma subunit were lysed in a buffer containing 8M urea in addition to the above components or the 10K pellet was treated with 1% Triton X-100 and vortexed for 15 minutes at $4^0$ C to release the association of gamma subunit with the cell membrane. The proteins in the extract were then resolved on 10% SDS-PAGE and visualized by coomassie staining and western blot analysis with the help of monoclonal anti-histag antibodies and or monoclonal anti-eIF2β antibodies. The supernatant of the cell extracts was stored in liquid nitrogen.

**3.4 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.

**3.5 Western Transfer:** After separation of proteins on SDS-PAGE, the proteins were transferred eletrophoretically on to nitrocellulose membrane. Transfer of proteins was carried at 70 volts for 4 hr at $4^\circ$ 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 with a ballpoint pen and the stain was removed with excess double distilled water. Regions of nitrocellulose free of proteins were blocked with TBST (Tris-HCl, pH 8.0, 150 mM NaCl and 0.05% Tween-20) containing 5% 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°C with gentle shaking. The nitrocellulose membrane was washed with TBST for three times (x 10 min) to remove unbound antibody. The nitrocellulose 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 done for 1 hr at room temperature. Primary and secondary antibodies were stored at –20° C for reuse. The nitrocellulose membrane was washed with TBST for three times (x 10 min) to remove unbound secondary antibody. Then membrane was treated with the color 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.

**4.0 Purification of recombinant his-tagged protein by Ni-NTA column chromatography.**

Ni-NTA-agarose beads (GIBCO BRL) were washed with 20 mM Tris-HCl, pH 7.8, 300 mM KCl, 10% glycerol and 0.1% Nonidet P-40 containing protease inhibitors (10 µg/ml of leupeptin, pepstatin, aprotenin and 1 mM PMSF). Washed beads were then incubated with the extracts for 30 minutes at $4^0$ C and were loaded in 1 ml plastic column. The column was washed with 10 column volumes of the above wash buffer to remove the unbound proteins. A second wash was carried out with 10 column volumes of wash buffer devoid of NP-40 but supplemented with 10 mM imidazole and 500 mM KCl to remove the non-specific proteins bound to the beads. Elution was carried out in 20 mM Tris-HCl, pH 7.8, 50 mM KCl, 1mM DTT, 10% glycerol and 200 mM imidazole. Peak

fractions of the protein were pooled, dialyzed in the ice-cold wash buffer devoid of imidazole. The γ-subunit was purified under denaturing conditions by the addition of 8M urea to the buffers. Proteins were later renatured gradually by dialyzing in ice-cold buffer consisting of Tris-HCl pH 7.8 (50-20mM), 100-50 mM KCl and 10% glycerol with decreasing concentrations of urea. Fractions were analyzed by 10% SDS-PAGE and the protein was identified by coomassie staining and also by immunoblot analysis. The proteins were aliquoted and stored at –70°C.

**5.0 Phosphorylation assays.**

**5.1 *Invitro* 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, 7.8, 80mM 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 [$\gamma^{32}$P] ATP) in the presence of purified recombinant mouse PERK or PKR-GST. 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 by phosphorimaging.

**5.2 *In vitro* phosphorylation of purified recombinant human eIF2β:** Phosphorylation of the β-subunit was carried out in the presence of 10 µCi [$\gamma^{32}$ 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.

**5.3 *Insitu* phosphorylation of recombinant human eIF2 α/β:** Insect cells (2 X $10^{6}$) were seeded in 35 mm dishes. The cells were infected with recombinant baculoviruses

expressing α (wt and mutants) or β or γ subunits of human eIF2. After 48 hrs of infection, 100 μCi of in-organic [$^{32}$P] was added and allowed for incorporation for 12 hours. After 12 hours, the cells were harvested by centrifugation. The cells were washed twice with 1xPBS and lysed with lysis buffer as described previously. The proteins in the cell extracts or whole cells were then analysed by 12% SDS-PAGE and then transferred to nitrocellulose membrane. The radiolabelled proteins were analysed by phosphorimaging and later with anti-phosphospecific eIF2α or anti-histag antibodies.

**6.0 Ternary complex formation (eIF2 .GTP. [$^{35}$S] Met-tRNA$_i^{Met}$).**

**6.1 Charging of bovine liver tRNA with [$^{35}$S] methionine:** Bulk charging of bovine liver tRNA was carried out in a 100 μl reactions containing 50 μg of tRNA, 2mM ATP, 100 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 10 μM [$^{35}$S] methionine (5 μM cold and 5 μM labeled) and 100 units of *E.coli* aminoacyl tRNA synthetase. Reaction was carried out at 37° C for 10 minutes and was terminated by addition of 100 μl of 1M NaOAc (pH 5.5). Charged tRNA was then deproteinized by mixing thoroughly with 0.2M Tris buffer (pH 8.0) saturated phenol and the mixture was centrifuged at 12K for 10 mins at 4° C. The aqueous layer was then carefully taken and dialyzed at 4° C initially against a high salt buffer containing 50 mM NaOAc (pH 5.0) and 0.5 M NaCl for 2 hours and then against 20 mM NaOAc (pH5.0) a low salt buffer. 5.0 μl of dialyzed charged tRNA was spotted on a Whattman paper and the paper was air-dried. Then the paper was put in boiling 10% TCA for 10 min and then in cold TCA for 5 min. The paper was then put in 70% ethanol for 5min to remove the TCA and then for 5 mins in acetone to remove ethanol. The filter paper was air-dried and the bound radioactivity of measured by scintillation counting. 5.0 μl of charged gave approximately 49,271.6 cpm on 10% TCA precipitation.

**6.2 [$^{35}$S] Met-tRNAi binding to recombinant human eIF2 subunits:** Over-expressed recombinant human eIF2 subunits were incubated at 37°C for 15 minutes with 10 μl of charged [$^{35}$S] met tRNAi (~ 100,000 cpm) in a 75 μl reaction mixture containing 20 mM Tris-HCl, 2 mM DTT, 1.5 mM$Mg^{+2}$, 75.2 mM KCl, 266 μM GTP. Reactions also

contained 130 μg of BSA as carrier protein. Reactions were terminated by the addition of cold wash buffer containing 20 mM Tris-HCl (pH7.8) 100 mM KCl and 5 mM $Mg2^{+}$. Wash buffer devoid of $Mg^{2+}$ was used to stop the reactions that lacked $Mg^{2+}$. Reaction mixture was filtered through 0.45 μM nitrocellulose membranes using a Millipore filter unit. The filters were washed twice with the cold wash buffer, dried and counted in a scintillation counter. Any other modifications are mentioned in the respective figure legends.

**6.3 Binding of purified recombinant α,β and γ- subunits of human eIF2 with [α–$^{32}$P]GTP by UV cross-linking:** Purified recombinant human eIF2 α, β and γ-subunit were incubated with 10 μCi of [α$^{32}$P] GTP in the presence of 20 mM Hepes (pH 7.9), 10 mM $MgCl_2$, 50mM NaCl, 50 μM cold GTP. The reaction mixtures were then placed under U.V. light for 15 mins at 120K μJ/cm$^2$. The samples were analyzed by 12% SDS-PAGE and transferred to nitrocellulose membrane. The western blot was then exposed to X-ray film.

**7.0 Inter subunit and inter protein interactions:**

**7.1 Dot blot assays:** In this method, the subunits of eIF2 were spotted on a nitrocellulose filter paper, air dried, UV-cross linked, and, then blocked with 3% BSA for one hour. Afterwards, the membrane was incubated with the interacting protein of interest for about 1 hour in1x phosphate buffered saline (PBST, pH, 7.4) and 0.05 % Tween 20 and then the membrane was washed thrice in the same buffer. Membranes were then incubated with the respective primary and alkaline phosphatase-conjugated secondary against the interacting protein to determine the interaction among the subunits of eIF2 and also with purified recombinant PERK. NBT and BCIP substrates were used to develop the membrane.

**7.2 Enzyme Linked Immuno-Sorbent Assay (ELISA):** Here, the recombinant subunits of eIF2 (~ 200 ng of the total protein with equimolar concentrations of the subunits) were coated into the wells of the microtitre plates for about 2 hrs at room temperature. Wells

were washed once with 1X PBST and blocked with 1% BSA for about another two hours. Afterwards, ~200 ng of the purified protein of interest, or rabbit reticulocyte lysate (10 µl) consisting the protein of interest, was added to the wells and incubated for 2 hrs. The wells were washed five times with 1x PBST, treated with the respective primary antibodies against the interacting protein for 2 hrs and then with the respective horseradish peroxidase-conjugated secondary antibodies. $H_2O_2$-TMB was used as a substrate for color development. Reactions were stopped finally by the addition 1N $H_2SO_4$. The microtitre plates were read in an ELISA reader at 450 nm. Two to four sets of control assays were carried out as shown in the respective figures to determine the specificity of the interaction between the protein coated in the wells and the interacting protein and also to determine the specificity or cross reactivity of the antibodies against the protein used in the wells or against the interacting proteins. One set of control wells were loaded with BSA instead of any of the subunits of eIF2 and then treated with the protein of interest or reticulocyte lysates before the addition of the respective primary and secondary antibodies against the interacting protein to determine the nonspecific interaction between the protein of interest and a control protein like BSA. The results of all ELISA assays were taken out from a mean of three experiments. Sigma Plot was used to analyze the data.

**7.3 Pull down assay:** Insect cell extracts expressing wt or mutants of eIF2α, or control extracts (20 µl) were incubated with purified PERK or PKR (~ 3 µg) in 1X PBST containing 10 µg/ml of protease inhibitors (leupeptin, pepstatin and aprotenin) for 10 min at room temperature. The mixtures were then added to 50 µl of pre-equilibrated Ni-NTA beads and incubated on ice for 45 mins. The beads were then centrifuged at 3.5K for 3 minutes and the unbound proteins in the supernatant were saved. The beads were washed thrice with 1X PBST and his tag proteins were eluted with PBST buffer containing 500 mM imidazole. All the fractions were separated by 10% SDS-PAGE and the proteins were transferred to a nitrocellulose membrane. The upper half of the membrane was probed with the anti-PERK or anti-PKR antibodies while the lower half with anti-eIF2α antibody.

**7.4 Interaction between human recombinant eIF2α and PERK by fluorescence spectroscopy:** All Fluorescence measurements were performed at a controlled temperature of 25° C in a 1-cm path length cuvette using a Jasco-Spectro fluorimeter as described (Raghu et al., 2002). The slit width was fixed at 5 nm for all the measurements. Initially the increase in intrinsic tryptophan fluorescence of PERK was followed with increasing additions of PERK to buffer. Similar measurements were also done with purified eIF2α in buffer containing 20 mM Tris-HCl, pH 7.8, 50 mM KCl, 10% v/v glycerol. Then eIF2α (0.01-1 μM) was incremented to PERK (5μM), after each addition of small aliquots of concentrated eIF2α, the mixture was thoroughly mixed and the emission spectrum (300-400 nm) was recorded by exciting at 295 nm. Similar measurements were also done with PERK (0.01-1 μM) incrementing to eIF2α (5μM) and the data points were fitted to the following equation,

$$F = a / 1+10^{n\,(Kd-c)}$$

Where c = concentration of ligand (eIF2α), F = fluorescence intensity,

a = intercept to y axis, n = number of binding sites and $K_d$ = dissociation constant.

# RESULTS

**1. Preparation of recombinant bacmid DNA harboring subunits of human eIF2:** Previously recombinant baculoviruses harboring human eIF2α wt and mutants (S51A, S51D and S48A) of eIF2α were generated using baculovirus transfer vector pBAKPAK8 and Bsu 361 digest of pBaKPAK6, baculovirus DNA. Recombinant baculoviruses were transfected into the ovarian cells of *Spodoptera frugiperda (Sf9)* for the expression of the corresponding proteins. (Sudhakar, 2000; Krishnamoorthy, 1998). In these studies, pFast Bac HT baculoviral vectors were prepared harboring the c-DNAs of human eIF2α, β, or γ and, also the mutants of eIF2α with his tag at the N-terminus end.

**1.1 Cloning of human eIF2 subunits (α/β/γ) in baculoviral HT-vectors:** The parent plasmids containing the cDNA of human eIF2α (wt or mutants)/β/γ subunits were amplified in *E.coli* (DH5α) cells and then the open reading frames (ORF) of these subunits were PCR amplified using the designed primers (**Fig. 3.0**) by procedures as described in 'Materials and Methods'. The amplified fragments were gel purified and then sub-cloned into pGEMT vectors. The positive recombinant colonies were confirmed by double antibiotic selection and were white in color. The plasmid DNA harboring the recombinant human eIF2 cDNA sequences was subjected to restriction digestion for the confirmation of clones and release of inserts (**Fig. 3.1**, panel A). These inserts were then subcloned into Fast Bac HT vectors. The recombinant clones were gentamicin and ampicillin resistant. The clones were confirmed by restriction digestion analysis (**Fig. 3.1**, panel B). Recombinant bacmid DNA harboring the cDNAs of eIF2 subunits were generated by procedures as described in 'Materials and Methods'. The presence of insert in the exact position in the expression cassette was determined by colony PCR (**Fig. 3.2**).

**2. Kinetics of expression of human eIF2 subunits in insect cells:** *Spodoptera frugiperda (Sf9)* cells were transfected with the recombinant bacmids harboring human eIF2 subunits by procedures described in 'Materials and Methods'. The insect cells expressed the corresponding subunit proteins very efficiently. Expression of recombinant proteins was analyzed at 72 hrs PI by 10% SDS-PAGE (**Fig. 3.3 A**) and by western blot analysis (**Fig. 3.3 B**). Uninfected *Sf9* insect cell extract (**Fig. 3.3**, lane 1) was used as a

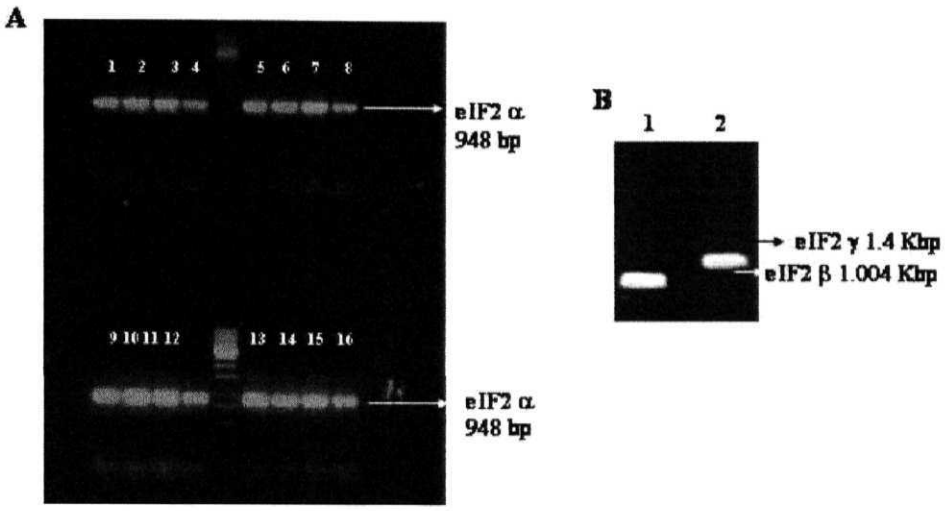



**Figure 3.0. PCR amplification of human eIF2 α/β/γ cDNA.** The open reading frames of human eIF2 α/β/γ were amplified from parent vector by procedures as described in 'Materials and Methods'. The reactions for eIF2α (wt and mutants) were performed at different concentrations of $Mg^{2+}$ (05, 1.0, 1.5, 2.0 mM) to achieve optimum amplification whereas eIF2β or γ-subunit was amplified at an optimum $Mg^{2+}$ concentration of 1.5 mM. The samples were then analyzed by 1% agarose gel electrophoresis. The various lanes are as follows: Panel A, lanes 1-4, eIF2α wt; and mutants of eIF2α, lanes 5-8, S51A; 9-12, S51D and 13-16 S48A respectively. Panel B, lanes 1 and 2 are eIF2β and eIF2γ respectively.

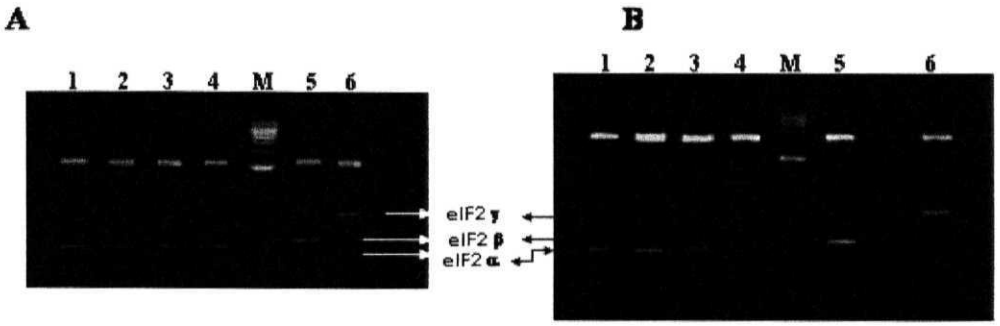



**Figure 3.1. Restriction Digestion analysis of recombinant pGEMT and pFast BacHT eIF2α/β/γ for confirmation of recombinant clones.** The PCR amplified cDNA of eIF2α (wt and mutants)/β/γ are ligated into pGEMT vectors and then sub cloned into pFast Bac HT vectors. The recombinant plasmids are isolated by procedures described in 'Materials and Methods' and then subjected to restriction digestion analysis for the clone confirmation. The samples are analyzed by 1-% agarose gel electrophoresis.

The various lanes in the figure are as follows: Panel A, lanes 1-4, pGEMT eIF2α wt and mutants (S51A, S51D and S48A) +BamHI + Hind III; 5, pGEMT eIF2β + BamHI + Hind III; 6, pGEMT eIF2γ + E.coRI + PstI. Panel B, lanes 1-4, pHTb eIF2α wt and mutants (S51A, S51D and S48A) +BamHI + Hind III; 5, pHTb eIF2β + BamHI + Hind III; 6, pHTa eIF2γ + E.coRI + PstI; Lane M in both the panels represents 1Kb Bangalore genei molecular weight ladder.

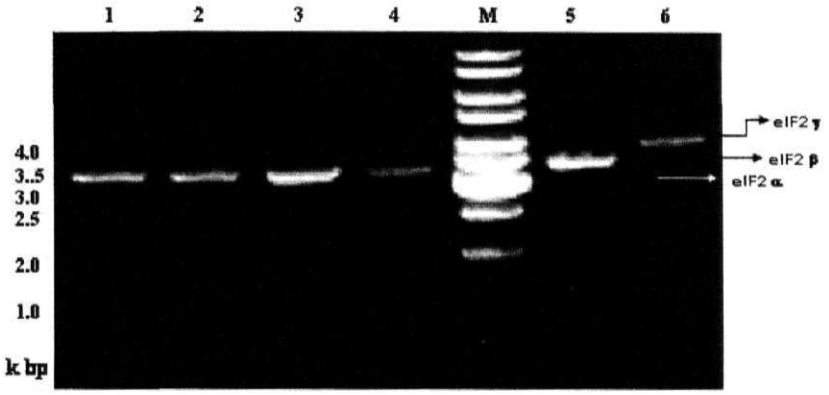



**Figure 3.2 Confirmation of recombinant bacmids harboring the eIF2α/β/γ subunits by colony PCR.** Recombinant white, gentamicin, kanamycin and tetracyclin resistant colonies were picked up and suspended in 10μl of sterile water and subjected to PCR using M13 primers. The M13 primers are were used to identify the transposition of the expression cassette harboring eIF2α/β/γ cDNA at the exact position in the bacmids DNA. The size of amplified fragments obtained were 2430 bp+ the size of the insert.

The various lanes are as follows: 1-4, eIF2 α wt and mutants (S51A, S51D and S48A) 3.378 kbp; M, NEB 1kb extension marker; 5, eIF2β, 3.434 kbp and 6, eIF2γ, 3.83 kbp respectively.

control. A kinetic analysis of β and γ-subunit expression in these studies showed that the expression of β-subunit starts at 24 hrs PI (**Fig. 3.4 A**) and was similar to the expression of eIF2α as had been observed in earlier studies [Sudhakar et al., 1999, 2000]. However, the expression of γ-subunit starts at 48 hrs PI (**Fig. 3.4 B**). Expression of recombinant β and γ- subunits was recognized by stain intensity (upper panels in **Fig. 3.4 A and B**), and also by immunoblot analysis using anti-eIF2β antibody or anti-histag antibody respectively (lower panels in **Fig. 3.4 A and B**). Optimum expression in both cases occurred at 72 hrs PI. Afterwards, the expression levels were reduced probably due to cell lysis caused by viral infection. Further, the expression of γ-subunit was lower compared to the expression of α- and β- subunits (**Fig. 3.3**). We suspect that the reduced and slow expression of γ-subunit relative to β and α-subunits of eIF2 may be because of the depletion of endogenous initiator tRNA. This interpretation is consistent with the fact that γ-subunit is implicated in the binding of Met-tRNAi and GDP and over expression may be toxic to cells [Schmitt et al., 2002]. A recent study in yeast implicates the importance of γ-subunit levels in cell growth and its interaction with cell division controlling proteins like Cdc 123. Expression of too much or too little γ-subunit is found growth inhibitory [Bieganowski et al., 2004]. The migration of the recombinant β-subunit protein (~54 kDa with his tag) in 12% SDS-PAGE was anomalous and did not correspond to the real molecular mass (expected to be ~39 kDa without his tag) as observed previously by others while analyzing the β-subunit of the purified trimeric eIF2 [Pathak et al., 1988; Price et al., 1989].

**2.1 Co-Expression and purification of the subunits.** To achieve the expression of all the three subunits individually, or in combination with other subunits, cells were infected individually or in combination with the respective recombinant viruses to produce αβγ, αβ, βγ or αγ as shown in **fig. 3.5.** Cells expressing the subunits of eIF2 were boiled and processed with the SDS-sample buffer. The expression of the recombinant subunits was analyzed by 12 % SDS-PAGE and the protein was detected with an anti-histag antibody as shown in **fig. 3.5 A**. Although, cells were able to express together any of the two subunits or all the three subunits, their expression levels are not stoichiometric and

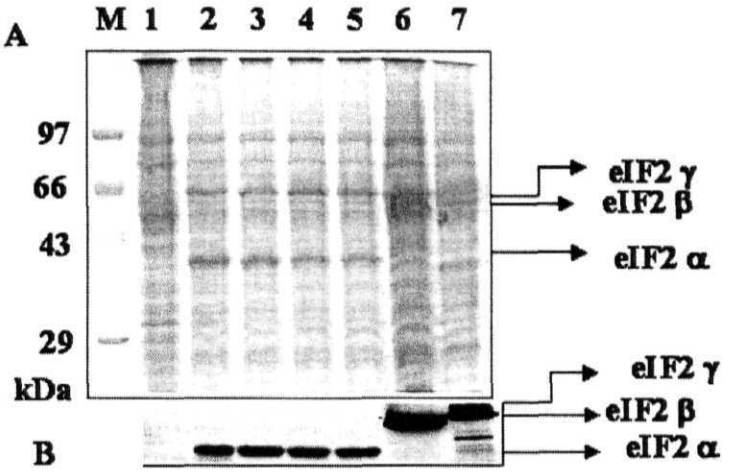



**FIGURE 3.3. Expression of human eIF2 subunits and mutants of eIF2α in *Sf9* cells.** N-terminal histidine-tagged recombinant subunits of human eIF2 cDNA, and, the mutants of eIF2α were prepared and expressed in *Sf9* cells as described in 'Materials and Methods'. Cells were boiled in SDS-sample buffer and the proteins were separated by 10% SDS-PAGE. Panel A represents coomassie blue-stained gel. Panel B represents the corresponding western blot probed by an anti-histag antibody. Different lanes are as follows: lane M, molecular weight markers; lane 1, control *Sf9* cells, lane 2, wt eIF2α; lane 3, S51A eIF2α; lane 4, S51D eIF2α; lane 4, S48A eIF2α; lane 5, eIF2β; lane 6, eIF2γ.

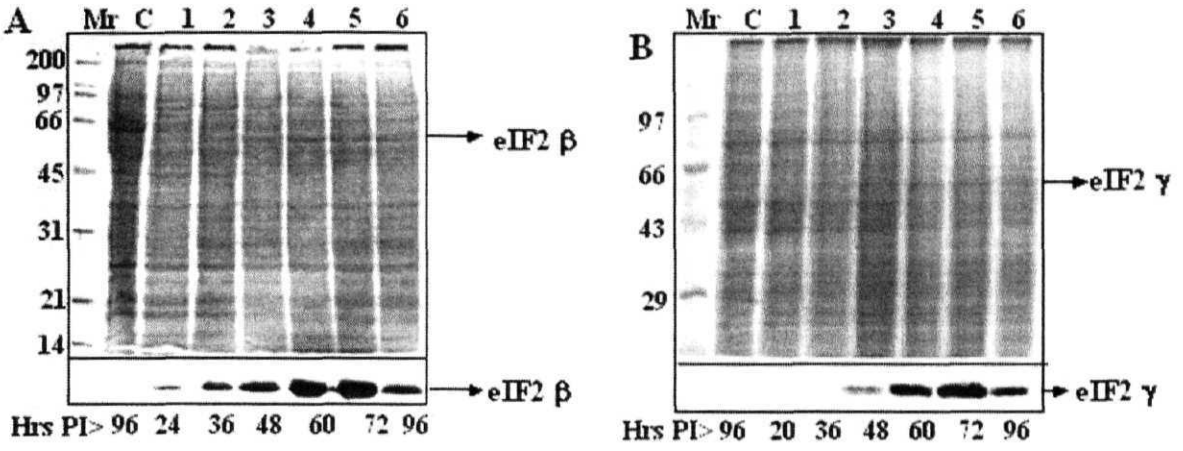



**FIGURE 3.4. Kinetics of expression of the recombinant human eIF2β and γ- subunits.** *Sf9* cells infected with recombinant baculoviruses were analyzed at different time points, 24-96 hrs post infection (PI). Uninfected *Sf9* cell extracts were prepared as described above and loaded in lane C. Each lane contains approximately 50 μg of extract protein and the top panels of the figure A and B are coomassie stained 12% SDS-polyacrylamide gels. Lower panels, corresponding to the stained gels in A and B, represent the expression of recombinant eIF2β and γ- subunits as analyzed by monoclonal anti-eIF2β or anti-his tag antibodies respectively. Mr represents molecular weight markers.

reflects probably to the efficiency of the viral titre carrying the respective subunit genes. Interestingly, expression of the γ-subunit of eIF2 could be detected in gels if the cells were boiled and processed with the SDS-sample buffer (**Fig. 3.5 A**), but not in the supernatants of the cell extracts that were processed or centrifuged at 10K to remove the cell pellet (**Fig. 3.5 B**). The absence of γ- subunit in the 10K supernatants was observed when it was expressed singly or in combination with other subunits as analyzed by 10 % SDS gels (**Fig. 3.5 B**). It was however detected in the 10K pellet as shown in **fig. 3C** (lane, 1vs 2). This may be either due to the formation of inclusion bodies in insect cells or the recombinant γ-subunit is associated with cytoskeletal fraction of the insect cells. This latter interpretation is consistent with early reports indicating the association of initiation factors including eIF2 with cytoskeletal framework in He La cells [(Howe and Hershey 1984)]. Since the γ-subunit of eIF2 has marked sequence [(Gaspar et al., 1994)] and structural [(Schmitt et al., 2002)] similarity with eEF1A that is known to bind actin [(Yang et al., 1990)], it may be possible that the interaction between eIF2 complex and cytoskeleton (if any) may be mediated by the γ-subunit. However no reports are available that indicate the association of γ-subunit of eIF2 with membrane or any cytoskeletal material.

**3. Purification of recombinant human histag eIF2 α (wt and mutants)/β/ γ-subunits by Ni-NTA chromatography**. The recombinant proteins expressed in insect cells were purified by using procedures and described in 'Materials and Methods'. All the three subunits of eIF2 and the mutants of eIF2α were purified using Ni-NTA agarose matrix to 90% homogeneity in a single step (**Fig. 3.6**). Since, γ-subunit was found associated to the 10K pellet, we employed denaturing conditions as mentioned in 'Materials and Methods' to purify the γ-subunit (**Fig. 3.6**).

**4. Phosphorylation of α and β-subunits.** Purified recombinant α and β- subunits were tested for their ability to serve as substrates for different kinases. Phosphorylation of the α-subunit was carried out in the presence of [γ-$^{32}$P] ATP and by purified recombinant double-stranded RNA-dependent eIF2α kinase (PKR), and, also by PERK (**Fig. 3.7A**).

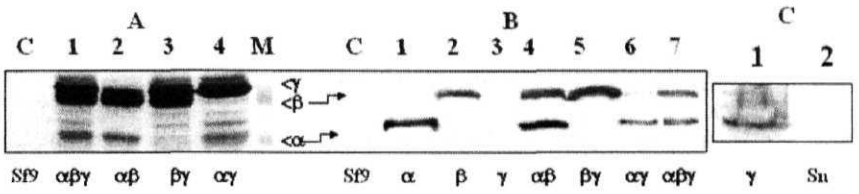



**FIGURE 3.5. Co-expression of human eIF2 subunits by multiple infections in *Sf9* cells and in cell extracts.** *Sf9* cells expressing single subunits of human recombinant eIF2 , or, in combination with other subunits, at 72 hrs PI, were boiled directly in SDS-sample buffer and separated by 12% SDS-PAGE (Panel A), or, centrifuged at 10 K. The 10K supernatants were resolved on 10% SDS-PAGE in panel B while the 10K pellet obtained from cells expressing the γ-subunit was separated by 10% SDS-PAGE in panel C. All gels were transferred to a nitrocellulose membrane and probed with a monoclonal anti histag-antibody.

Panel A represent the following: C, uninfected *Sf9* cells; 1, eIF2 α + β+ γ; 2, α + β; 3, β + γ; 4, α + γ; M, molecular weight markers. Panel B represent the following: C, uninfected *Sf9* cell extract; 1, eIF2α; 2, eIF2β; 3, eIF2γ; 4, eIF2α + β; 5, eIF2β + γ; 6, eIF2α + γ; 7, eIF2α + β + γ. Panel C represents the 10K pellet (lane 1) and supernatant (lane 2) obtained from cell extracts expressing the γ-subunit.

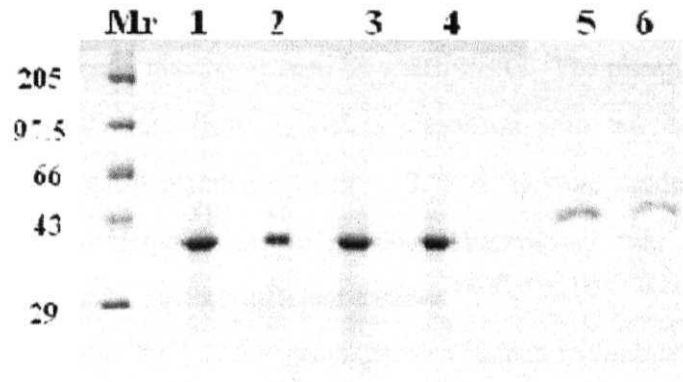



**FIGURE 3.6. Purification of recombinant eIF2α (wt and mutants), β and γ- subunits.** Baculovirus expressed recombinant human eIF2 subunits containing N-terminal 6x-histag were purified as described in 'Materials and Methods'. While eIF2α and β were purified from the cell extracts, the γ- subunit was purified under denaturing conditions as described in 'Materials and Methods'. Purified proteins were separated by 10% SDS-PAGE and analyzed by coomassie staining. The figure is a stained gel. Various lanes represent the following: Mr, molecular weight markers; 1, eIF2α wt; 2, eIF2α S51A; 3, eIF2α S51D; 4, eIF2α S48A; 5, eIF2β; 6, eIF2 γ.

Both these kinases phosphorylated the recombinant substrate efficiently (**Fig. 3.7A**, lanes 2 and 3 respectively). No phosphorylation was detected in the absence of kinases (lane 1). Further, the phosphorylation of the mutants of eIF2α by PERK and unlabelled ATP was carried out and the phosphorylation was analyzed by a phosphospecific anti-eIF2α-antibody (**Fig 3.7B**). As expected, S48A (lane 8), but not S51A or S51D (lanes 4 and 6), mutant of eIF2α, was efficiently phosphorylated by added PERK. The phosphorylation of S48A is comparable to wt eIF2α (lane 2) and is consistent with our earlier studies (Sudhakar et al., 1999, 2000). Control reactions (lanes 1, 3, 5 & 7) were carried out in the absence added kinases. Consistent with the previous observations that β-subunit of purified eIF2 is also a substrate for four different kinases (Welsh et al., 1994; Clark et al., 1989; Ting et al., 1998), we observed here that the baculovirus-expressed human recombinant β-subunit was also phosphorylated *in vitro* by these different kinases (**Fig. 3.9**) viz., CK-II, (**Fig. 7**, lane 2) PKC (lane 3), PKA (lane 4) and DNA-PK (lane 5). In the absence of any added kinase, no significant phosphorylation was observed (lane 1).

**5. Phosphorylation of eIF2 α (wt) in the presence of β, γ, and, β + γ subunits:** Phosphorylation of wt eIF2α was carried out alone, or in the presence of β, γ or β + γ-subunits by PERK in a reaction mixture as described in 'Materials and Methods'. Previously, we observed that phosphorylation of recombinant human eIF2α- purified from insect cells is not as efficient as the phosphorylation of the α-subunit of purified rabbit eIF2 complex (Sudhakar et al., 1999). Although this may be because of a viral protein, like PK2, which is shown to inhibit eIF2α kinases (Dever et al., 1998) and can co-migrate with the baculovirus-expressed recombinant eIF2α, we could not eliminate the possibility that the phosphorylation efficiency of the recombinant α-subunit is independent of the other two subunits. To rule out this possibility, the phosphorylation of recombinant eIF2α is evaluated independently and in the presence of the other two purified recombinant subunits. The phosphorylation of eIF2α alone (Fig 3.8, lane 1) was however not influenced by the presence of β-subunit (lane, 2), or γ-subunit (lane, 3) or β and γ-subunits (lane, 4).

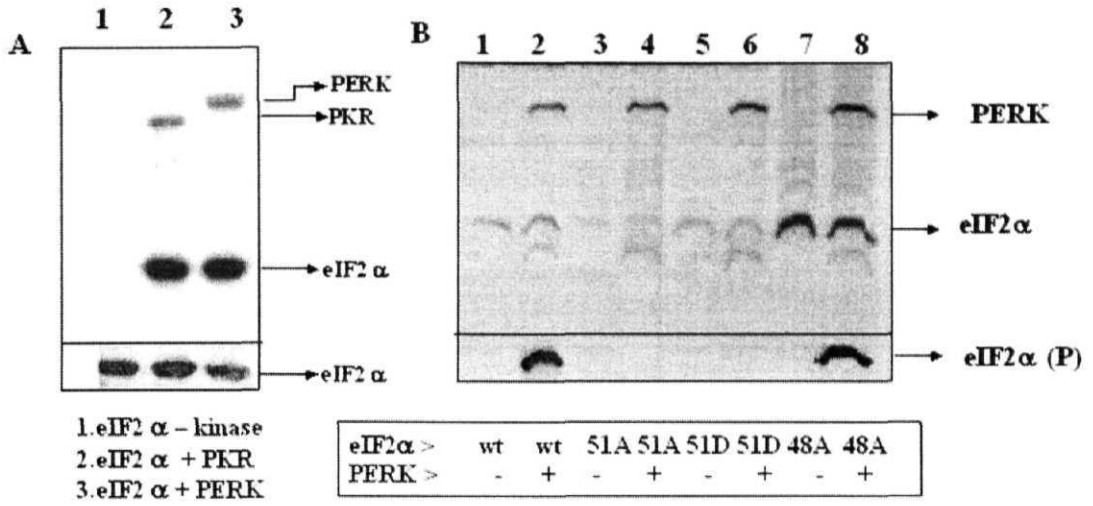



**FIGURE 3.7 Phosphorylation of recombinant wt and mutants of eIF2α.** Baculovirus-expressed his-tagged human eIF2α wt and mutants were purified using Ni-NTA agarose affinity matrix and were phosphorylated by a purified recombinant PKR or PERK *in vitro* in the presence of 20 mM Tris-HCl, pH 7.8, 100 mM KCl, 2.0 mM $Mg^{2+}$, 1 mM DTT, and 100 μM ATP at $30^0$ C for 10 minutes. Samples were separated by 10% SDS-PAGE and transferred to a nitrocellulose membrane. Panel A represents phosphorylation of wt eIF2α by PKR and PERK in the presence of [γ-$^{32}$P] ATP. The figure is a phosphorimage. Lanes represent the following: 1, eIF2α alone; 2. eIF2α + PKR; 3. eIF2α + PERK. The lower panel represents the amount of protein used in the reactions as analyzed by a western blot. Panel B represents the phosphorylation of eIF2α wt and mutants in the presence and absence of PERK. Upper panel represents the amounts of protein used in the phosphorylation reaction mixture as judged by coomassie stain. Lower panel represents the phosphorylation of eIF2α as determined by a polyclonal phosphospecific anti-eIF2α antibody. The various lanes are as follows: 1, wt eIF2α -PERK 2, wt eIF2α + PERK; 3, S51A eIF2α - PERK; 4, S51A eIF2α + PERK; 5, S51D eIF2α – PERK; 6, S51D eIF2α + PERK; 7, S48A eIF2α - PERK; 8, S48A eIF2α + PERK.

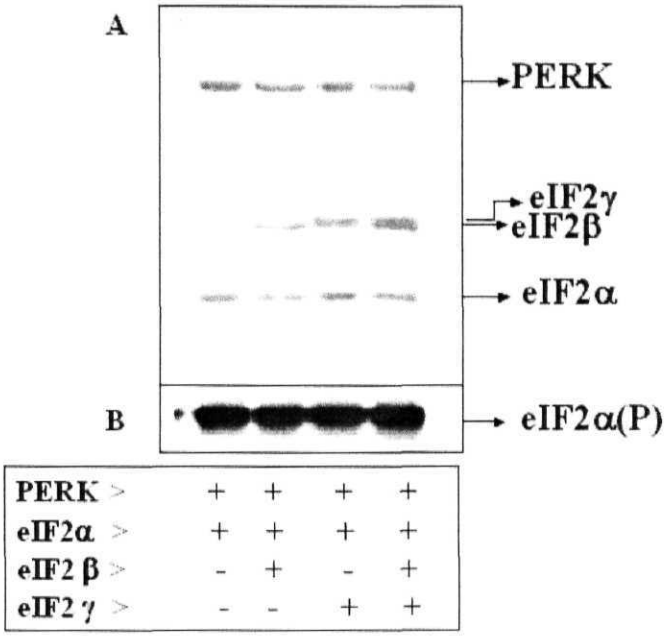



**FIGURE 3.8. Phosphorylation of recombinant wt eIF2α in the presence of β, γ or β and γ- subunits.** Phosphorylation of wt eIF2α was carried out alone, or in the presence of β, γ or β and γ-subunits by PERK in a reaction mixture as described above. Panel A represents ponceauS stained membrane indicating the levels of PERK and the recombinant his tag eIF2 subunits. Panel B represents the corresponding immunoblot analysis of eIF2α phosphorylation as judged by a phosphospecific anti-eIF2α antibody. The various lanes represent the following: 1, eIF2α + PERK; 2, eIF2α, β + PERK; 3, eIF2α, γ + PERK; 4, eIF2α, β, γ + PERK.

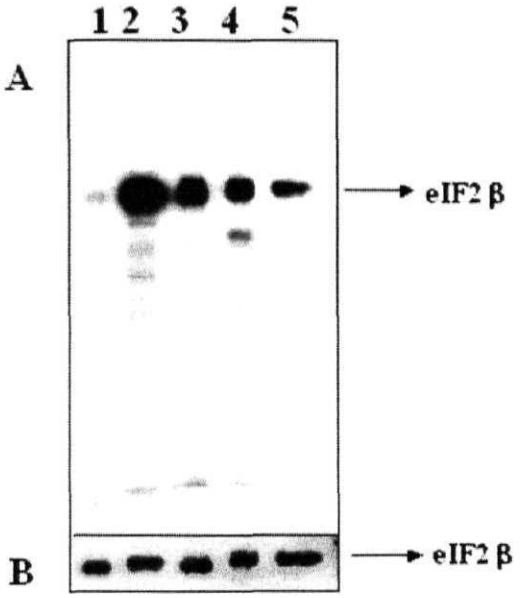



**FIGURE 3.9. Phosphorylation of recombinant human his-tagged eIF2β-subunit by different kinases.** Purified bacuolvirus-expressed recombinant human eIF2β was carried out in the presence of [γ-$^{32}$P] ATP by CK-II, PKC, PKA and DNA-PK *in vitro* and as described in 'Materials and Methods'. The lanes are as follows. 1, eIF2β - kinase; 2, eIF2β + CKII; 3, eIF2β + PKC; 4, eIF2β + PKA; 5, eIF2β + DNAPK. Panel A represents the phosphorylation status of eIF2β as judged by phosphorimage and panel B represents the eIF2β protein present in the reactions as judged by a monoclonal anti-eIF2β antibody.

**5.1. *In-situ* phosphorylation of eIF2 α and β- subunits:** To determine the phosphorylation ability of the recombinant proteins in situ, we labeled the cells with inorganic phosphate and analyzed their phosphorylation status by phosphorimager or by phosphospecific antibodies as mentioned in the Methods. We obtained evidence that wt eIF2α and a mutant of eIF2α, S48A are phosphorylated in cells (**Fig. 4.0 A** lanes 2, 4). S51A, the nonphosphorylatable form of eIF2α was not phosphorylated as expected (lane 3). Endogenous insect cell eIF2α whose migration was slightly below the recombinant his tagged eIF2α was found phosphorylated in uninfected and in recombinant virus-infected cell extracts (lane 1 versus lanes 2-4). Consistent with earlier results (Sudhakar et al., 1999; Aparna et al., 2003), phosphorylation of native insect cell eIF2α was decreased in virus-infected cells probably because of viral proteins like pk2 and p35 that serve as kinase or caspase inhibitors respectively (Dever et al., 1998 Aparna et al., 2003). We also observed the recombinant β-subunit of eIF2 was phosphorylated in cultured cells as shown in lane 6 of **Fig. 4.0 B**. No phosphorylation of the γ-subunit was observed under those conditions (lane 5). The expression levels of α-subunit was detected by a polyclonal anti-eIF2α antibody whereas the expression of β and γ-subunits subunits was detected by anti-histag antibodies as shown in the lower panels of **Fig. 4.0 A and B.**

**6.0 Inter-subunit interactions of recombinant human eIF2 subunits:** Recent studies with archaeal and yeast eIF2 suggest that γ-subunit is the core subunit of trimeric eIF2 based on its ability to interact with α and β-subunits to form αγ and βγ dimers and the inability of α-subunit to interact with β-subunit to form αβ dimer (Hashimoto et al., 2002; Schmitt et al., 2002; Tahara et al., 2004). Interestingly, a recent report indicates that no complex formation could be detected between purified human eIF2α and γ- subunits produced in bacteria (Ito et al., 2004). However, it is not clear if this recombinant protein is nonfunctional or requires some other components like initiator tRNA or eIF2β-subunit. Dot blot and ELISA methods were used here to determine the interaction among the subunits of eIF2, and of eIF2 and eIF2B complexes *in vitro*. In the dot blot method, purified α, β, γ or, βγ (**Fig. 4.1**, panel A), or, αγ (panel B) subunits of eIF2 were prepared and spotted on the four corners of a nitrocellulose membrane. A control blot was carried out with CPK

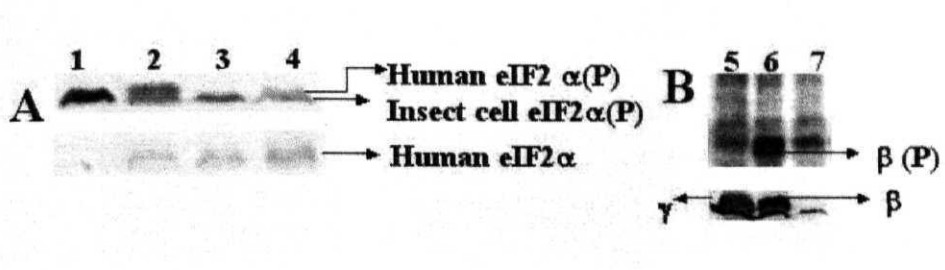



**Figure 4.0. *In-situ* phosphorylation of recombinant human eIF2 subunits.** *Sf9* insect cells were infected with recombinant baculoviruses expressing α (wt and mutants), β or γ subunits of human eIF2. After 48 hrs of infection, 100 μCi of in-organic [$^{32}$P] was added and allowed to incorporate for 12 hours. Later, the cells were harvested and the samples were separated by 10% SDS polyacrylamide gel and then the proteins were transferred to nitrocellulose membrane by western transfer. The radio labeled proteins were analyzed by phosphor imaging or with anti-phosphospecific eIF2α or anti-his tag antibodies. In panel A the top panel shows the phosphorylation of eIF2 α (wt and mutants) detected by anti-phosphospecific eIF2α antibodies and the lower panel represents the corresponding blot in the upper panel with anti-his tag antibodies. In Panel B, the upper panel represents the phosphor image showing the phosphorylation of eIF2β and γ and the lower panel is the corresponding blot detected with anti-his tag antibodies. The various lanes in the figure are as follow: 1, Control uninfected *Sf9* cells; 2, eIF2α (wt); 3, eIF2α (51A); 4, eIF2α (48A); 5, eIF2γ; 6, eIF2β; 7, Control uninfected *Sf9* cells.

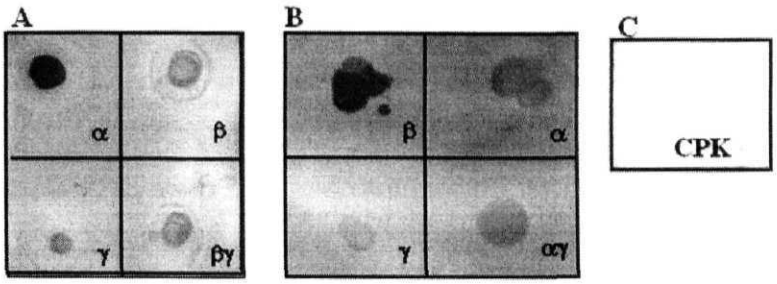



**FIGURE 4.1. Inter-subunit interactions of recombinant human eIF2.** Purified recombinant subunits of eIF2 (~ 500 ng), alone, or in combination with one of the other subunits, were spotted on the four corners of a nitrocellulose membrane as shown in panel A and panel B. Membranes were air dried and UV-cross linked for a minute. Afterwards, they were blocked with 3% BSA for 60 min at room temperature. Membrane of Panel A was then incubated with purified recombinant α-subunit protein, while membrane in panel B was incubated with β-subunit for 60 min at room temperature. Both the membranes were then incubated with the respective primary and alkaline phosphatase-conjugated secondary antibodies. Panel C represents a control membrane carrying ~2 μg creatine phosphokinase (CPK) that was treated with eIF2α-subunit for 60 min and the interaction between CPK and α-subunit was assessed by anti-eIF2α antibody.

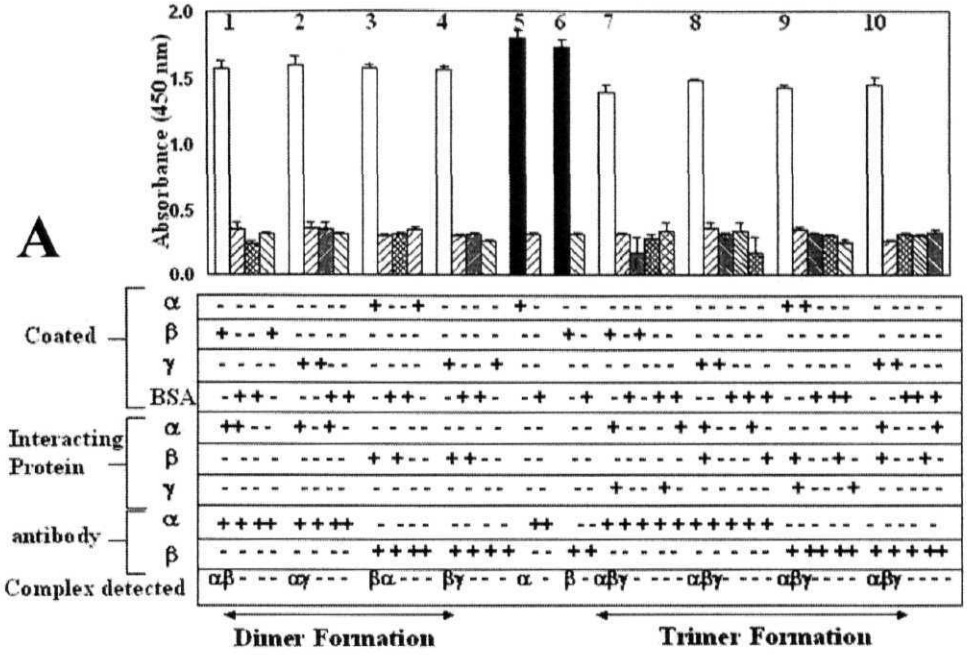
spotted on the filter instead of any of the eIF2 subunits (panel C). After blocking the membranes with BSA, membranes in panel A and C were treated with recombinant eIF2α protein, whereas, the membrane in panel B was treated with eIF2β- protein. After the treatments, as described in 'Materials and Methods', panels A and C were probed with polyclonal anti-eIF2α and panel B was probed with monoclonal anti-eIF2β antibodies. While CPK, spotted as the control, did not interact with eIF2α protein (panel C), the eIF2 β, γ or βγ-subunits (panel A) however were found to interact with eIF2α-subunit there by suggesting that these subunits can form heterodimers and heterotrimeric complexes. Similarly, eIF2β-subunit also interacted with α, γ or αγ-complex to yield the possible heterodimer and heterotrimeric complexes (panel B).

Complementing the dot blot results, ELISA assays were also performed as described in 'Materials and Methods' to show the interaction between two or the three subunits of eIF2 *in vitro*, or, the interaction between recombinant subunit (s) of human eIF2 with the subunits of eIF2 in rabbit reticulocyte lysate as shown in the respective panels A and B of **fig. 4.2**. The findings suggest that all the three possible heterodimers αβ, αγ and βγ and a heterotrimeric complex of human eIF2 can be formed *in vitro*. The formation of αβ dimer appears to be possible based on the crystal structure of the N-terminal segment of human eIF2α (Nonato et al., 2002) where in it is suggested that the highly charged polylysine motif present on the β-subunit can interact with the negatively charged groove with highly conserved residues formed in human eIF2α. The three polylysine rich boxes in the N-terminal segment of eIF2β that appear to be important in protein-protein interactions, have also been implicated in the binding of eIF2 to both eIF5 and eIF2B (Asano et al., 1999).

**6.1 Interaction between recombinant subunits and the subunits of rabbit reticulocyte lysate eIF2 complex:** In the previous studies (Sudhakar et al., 1999, 2000) the effect of eIF2α phosphorylation on the interaction between lysate eIF2 and eIF2B was determined by using sucrose gradient analysis. The addition of recombinant S48A and S51A mutants of human eIF2α reduced the 15S complex (eIF2.α(P).eIF2B) formation.

**FIGURE 4.2. Inter-subunit interactions of recombinant eIF2 and formation of chimeric trimeric eIF2.** In panel A, formation of heterodimeric and trimeric complexes were determined with the addition of any of the two subunits (bars 1-4) or any of the two subunits with the third subunit (bars 7-10). In bars 1-4, wells of the microtitre plates were coated with 50 µl (~200 ng) of the purified recombinant eIF2 β, γ, α and γ respectively. All samples were prepared in 1x PBST. After blocking with 1% BSA, the wells were incubated with purified α-(bars1-2), or, β-(bars 3-4) protein for 2 hrs at room temperature. Wells were washed as described in 'Materials and Methods' and probed with anti-eIF2α (bars 1-2), or, β-(bars 3-4) antibody to show the formation of αβ, αγ, βα and βγ- heterodimer complexes. The hatched bars, aligning bars 1-4, represent various controls as shown in the figure. In reactions 5 and 6 (solid black bars), the wells were coated with α or β-proteins and were probed with the respective antibodies to demonstrate the specificity of the antibodies against the respective proteins. The hatched bars aligning bars 5 and 6 represent the nonspecific interaction between the antibody and a negative control protein like BSA. Bars 7-10 represent formation of heterotrimeric complexes. Here the corresponding wells were coated with single subunits containing β, γ, α or γ respectively, blocked with BSA, and incubated with a precomplexed dimeric solution containing αγ, αβ, βγ, and αβ. Reactions were then probed with α (bars 7 and 8) or β- (bars 9 and 10) antibodies as described in 'Materials and Methods' to determine the formation of αβγ complexes. The unfilled bars represent specific interaction between or among the subunits of eIF2 where as the hatched bars of the figure represent the control reactions to indicate the nonspecific interaction.

In panel B, wells of the microtitre plate, corresponding to bars 1-6, were coated with α, β, α, β, γ and γ-subunits respectively and then blocked with 1% BSA for about 2hrs. Afterwards, reticulocyte lysate (10µl) was added to wells 1, 2, 5 and 6, but not in 3 and 4. The respective reactions were then probed with β, α, α, and β- antibodies to show that reticulocyte eIF2 subunits can interact with the coated recombinant subunit protein in the wells to form chimeric trimeric complexes as shown in bars 1, 2, 5 and 6. Wells corresponding to bars 3 and 4 (filled bars) were coated with α or β-subunit and probed with the respective antibodies to demonstrate the specificity of the eIF2α and β-antibodies. All hatched bars represent the nonspecific interactions.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1 2 3 4 5 6 7 8 9 10
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α
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Interacting Protein
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Complex detected
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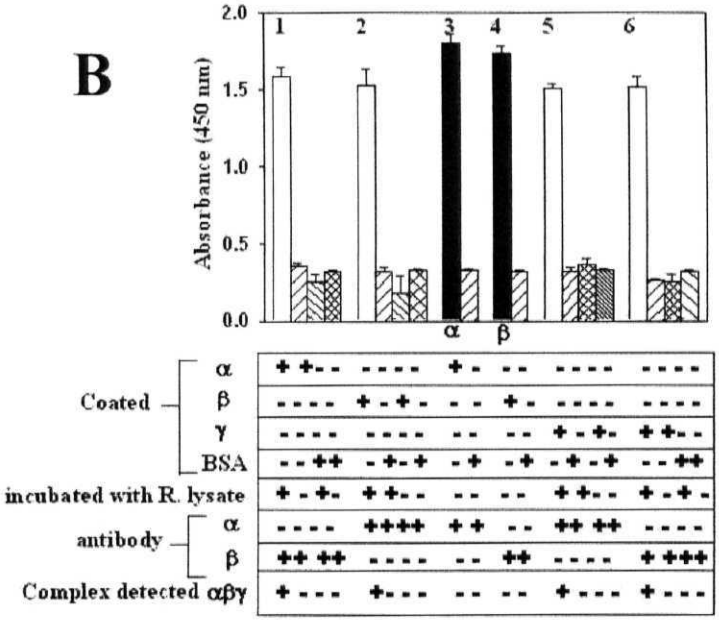
Dimer Formation
Trimer Formation


B
Absorbance (450 nm)
2.0
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α
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Coated
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β
γ
BSA
incubated with R. lysate
antibody
α
β
Complex detected αβγ

Further, addition of recombinant S51D mutant of eIF2α, to hemin-supplemented lysates, promoted the formation of 15S complex that is consistent with its behavior as a phosphomimetic form. Based on these studies it has been suggested that the added recombinant α-subunit can exchange out the α-subunit the native eIF2 complex in lysates and may interact presumably with the other two subunits of lysate eIF2 to form a chimeric heterotrimeric complex. The results presented here (**Fig. 4.2 B**) also support the above suggestion that one of the recombinant subunits of eIF2 can be used to pull down or determine its association with the other two subunits of the lysate eIF2. These findings also suggest that the α and β-subunits of human eIF2, unlike their yeast and archeal counterparts, can interact together to form αβ dimers.

**7.0 Ternary complex (eIF2. GTP. [$^{35}$S] Met-tRNA$_i^{Met}$) formation:** The complex eIF2 obtained from purification studies has been shown to interact with charged methionyl initiator tRNA in a GTP dependent manner forming a stable ternary complex (eIF2.GTP.Met-tRNAi$^{met}$) which is delivered then to 40S ribosomal subunits to form 43S complex in the initiation step of protein synthesis (Hinnebusch, 2000; Hershey, 1991; Kapp and Lorsch, 2004). Structural, mutational and biochemical studies of yeast eIF2γ-subunit suggest that it is primarily involved in Met-tRNAi and GTP binding (Roll-Mecak et al., 2004; Erickson and Hanning, 1996). A previous study that actually describes the cloning and expression of human eIF2γ-subunit has carried out cross linking studies with purified rabbit eIF2 complex and suggested that both the β and γ-subunits of eIF2 are in close proximity to methionyl tRNAi in ternary complexes (Gaspar et al., 1994). No such reports are however available with the recombinant γ-subunit of mammalian eIF2. In order to identify the subunit of human eIF2 that is involved in this interaction, purified recombinant individual subunits were incubated in the presence of [$^{35}$S] Met-tRNA$_i$, GTP and $Mg^{+2}$ (**Fig. 4.3**).

The binding of [$^{35}$S] Met-tRNA$_i$ was significantly higher in the presence of γ-subunit compared to the presence of α or β-subunits (**Fig. 4.3**, bar 3 versus 1 and 2). The α-subunit showed least binding (**Fig. 4.3,** bar 1). These results while suggesting that γ-subunit is primarily involved in Met-tRNAi binding it does not exclude out that β and α-

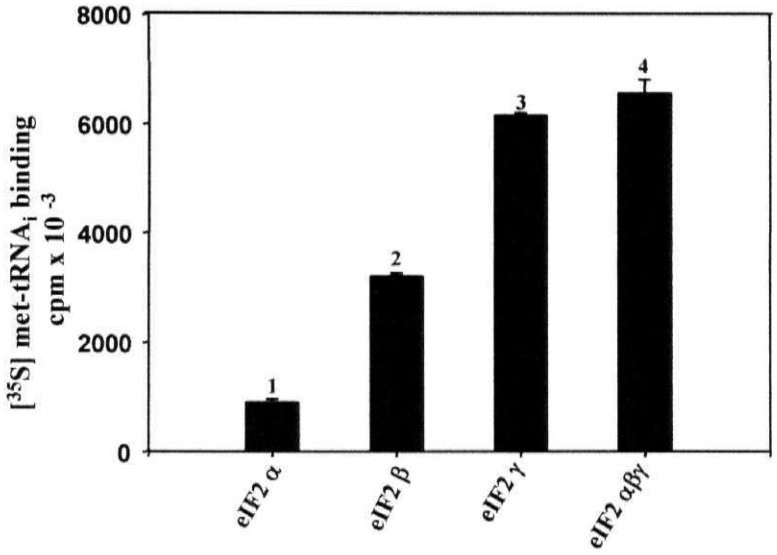


**Figure 4.3. Interaction of recombinant subunits of human eIF2 with [$^{35}$S] Met. tRNA$_i^{Met}$.**

Bovine liver tRNA was charged initially with [$^{35}$S] methionine in the presence of E.coli amino acyl tRNA synthetase by procedures as described in 'Materials and Methods'. In order to identify the subunit of eIF2 interacting with initiator tRNAi, recombinant human 2 μg of eIF2 subunits (α, β or γ) were incubated with methionylated tRNA$_i$ in the presence of GTP and $Mg^{2+}$ for the formation of ternary complex (eIF2.GTP. [$^{35}$S] Met.tRNAi$^{Met}$) in bars 1-3. In bar 4, Met-RNAi binding was studied for a mixture of 2μg of total protein consisting of α, β and γ-subunits combined in a 1:1:1 molar ratio. The reaction mixture was filtered through 0.22 μM nitrocellulose filters. The bound complex was analyzed by liquid scintillation counting as described in 'Materials and Methods'. The bars in the figure denote the amount of bound radioactivity, which corresponds to the amount of TC formed.

subunits of trimeric complex are not involved in this reaction. Consistent with this interpretation, the reconstituted trimeric eIF2 complex consisting of equimolar amounts of the three subunits (**Fig 4.3** bar 4) showed enhanced, stable TC formation than the individual subunits. This is observed in spite of the fact that the trimeric complex has lesser amounts of each of these subunits (for a total of 2 μg complex in equimolar ratio) compared to the amount of protein used in the single subunit reactions (2 μg each). The above interpretation is also consistent with the facts that the genetic and biochemical analyses in yeast indicate that deletion of α subunit is found lethal and causes a defect in eIF2B catalyzed nucleotide exchange and Met-tRNAi binding (Nika et al., 2001). Further the recent crystal structures of human α and yeast γ-subunits (Roll-Mecak et al., 2003, Ito et al., 2004) and archaeal IF2γ-subunit suggest that the binding of eIF2α is localized to the side of eIF2γ adjacent to the Met-tRNAi$^{Met}$ (Roll-Mecak et al., 2003, Ito et al., 2004 and Yatime et al., 2003). Also earlier biochemical and mutational studies implicated that both β and γ- subunits of mammalian eIF2 are important in GTP binding and thereby it has been reasoned out that perhaps the β-subunit is also required for Met-tRNAi binding (Flynn et al., 1993; Anthony et al., 1987; Bommer et al., 1988a; Bommer and Kurzchalia et al., 1989; Dholakia et al., 1989; Gaspar et al., 1994).

**7.1 Kinetics of Ternary complex formation: Effect of GDP and $Mg^{2+}$:** Earlier studies with purified eIF2 complex suggested that the ternary complex formation by purified eIF2 requires GTP. GDP was found inhibitory. In the presence of physiological concentrations of $Mg^{2+}$, GDP has higher affinity for eIF2 (Panniers et al., 1988). The GDP bound eIF2 is inactive and cannot join Met-tRNAi. This necessitates the requirement for a guanine nucleotide exchange factor, like eIF2B, to replace the GDP bound to eIF2 for GTP. To evaluate the importance of GTP in Met-tRNAi binding of the recombinant reconstituted human eIF2 trimeric complexes, the ternary complex (TC) formation was monitored in the presence of GTP or GDP in a time-dependent manner. The formation of TC was significantly higher at all time points in the presence of GTP than in the presence of GDP thereby suggesting that GDP inhibits the reaction (**Fig. 4.4**). These reactions were carried out in the presence of 2 mM $Mg^{2+}$. Since earlier studies suggested that $Mg^{2+}$

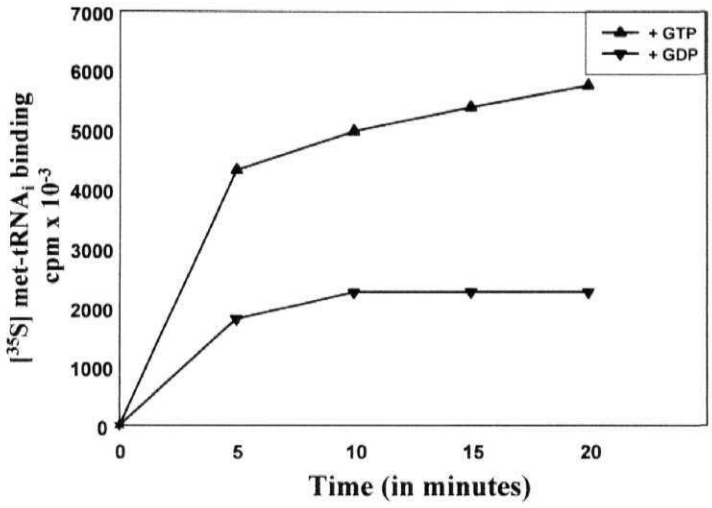


**Figure 4.4. Kinetics of ternary complex formation in the presence of GTP and GDP.** Recombinant subunits were mixed in solution in 1:1:1 molar ratio and the resultant complex of eIF2 was analyzed for its ability to form ternary complex in the presence of GTP and GDP. At five minutes time intervals, each sample was filtered through the nitrocellulose membranes and the amount of bound radioactivity was analyzed by liquid scintillation counting. The upright triangles (-Δ-Δ-) denote the reaction carried in the presence of GTP and inverted triangles (-∇-∇-) represent the reactions carried in the presence of GDP.

enhances the affinity of eIF2 for GDP, the formation of ternary complex was also evaluated in the presence and absence of $Mg^{2+}$. Maximum formation of ternary complexwas observed in the absence of $Mg^{2+}$ and in the presence of GTP (**Fig. 4.5** bar# 2). Presence of $Mg^{2+}$ inhibited the reaction significantly (bar # 5). Inclusion of GDP, instead of GTP, also inhibited the joining of Met-tRNAi (bar # 3 and 6) as has been shown in the previous result (**Fig. 4.4**). However presence or absence of $Mg^{2+}$ did not cause a significant difference in the formation of the ternary complex in the presence of GDP (bar # 3 vs 6). The inhibition in GTP-dependent ternary complex formation by $Mg^{2+}$ may be because of the formation of small amounts of GDP due to GTP hydrolysis. This suggestion is consistent with the following facts that most of the GTP preparations are hydrolyzed upon storage, eIF2 has higher affinity for GDP in the presence of $Mg^{2+}$, and, is inhibitory for the ternary complex formation as has been reported by others and has been observed here. These findings therefore suggest that the recombinant eIF2 complex is functionally similar to the native complex.

**8. Cross-linking studies with guanine nucleotides:** Mutational analysis of yeast and human eIF2-subunits suggest that the γ-subunit of eIF2 is primarily implicated in GTP binding (Erickson and Hannig, 1996; Naranda et al., 1995). This is supported by the fact that yeast γ-subunit but not the β-subunit, has the characteristic GTP binding domains (Pathak et al., 1988). However, the N-terminal half of the human β-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 including in the γ-subunit of eIF2. Earlier biochemical studies with cross-linking agents implicated both the β-and γ-subunits of mammalian eIF2 in GTP binding (Anthony et al., 1990, Dholakia et al., 1989; Gaspar et al., 1994; and Bommer etal., 1989). Except for a couple of old reports (Barrieux and Rosenfeld, 1977, Bommer et al., 1988). no other reports indicate that α-subunit is involved in GTP binding. Here when we assayed the ability of recombinant human eIF2 subunits to cross link GTP by UV, we observed that all subunits including the α-subunit can be cross linked to GTP (**Fig. 4.6**, lanes 1-4). Interestingly even BSA was also

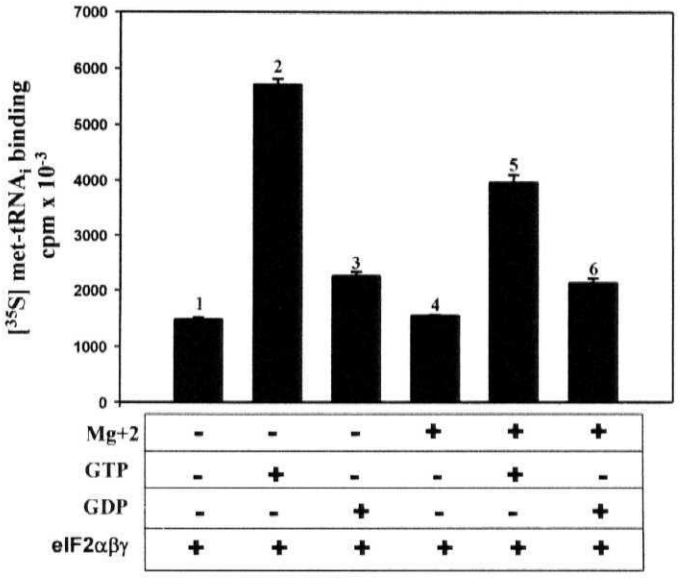


**Figure 4.5. $Mg^{2+}$ dependent formation of ternary complex.** Reconstituted recombinant eIF2 complexes were incubated with labeled [$^{35}$S] Met. $tRNA_i^{Met}$ in the presence of GTP or GDP and with or without $Mg^{2+}$. The reactions carried out in the absence of $Mg^{2+}$ were terminated by stop buffers devoid of $Mg^{2+}$. The bound radioactivity was analyzed by liquid scintillation counting. Bars in the graph represent the amount of ternary complex formed.

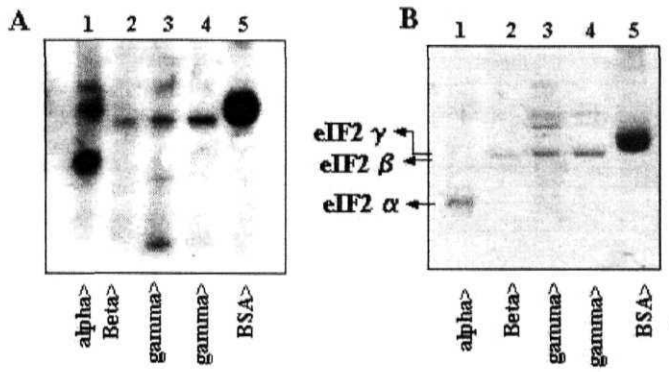


**Figure 4.6. Interaction of recombinant subunits of eIF2 with GTP.** Purified recombinant subunits of eIF2 were incubated in the presence of 10 μCi of labeled GTP and then U.V. cross-linked by the procedures as described in 'Materials and Methods'. The samples are then separated by 12% SDS-polyacrylamide gels and then transferred to a nitro-cellulose membrane. The amount of bound radioactivity was analyzed by phosphorimaging. Panel A in the figure is the phosphorimage and panel B is the Ponceau S stained blot corresponding to the lanes of the phosphorimage in panel A showing the amount of protein used in the reaction. The various lanes in the blot are as follows. 1, eIF2α: 2, eIF2β; 3, eIF2γ; 4, eIF2γ; 5, BSA.

cross- linked. To determine the specificity of the cross-linking, we used control proteins other than BSA such CPK (creatine phosphofructokinase) and two of the eIF2α kinases: PKR and PERK. The specificity of cross-linking is assessed in the presence of unlabelled ATP and GDP as competitors (**Fig. 4.7**). Consistent with the previous result, all the three subunits of eIF2 bound to GTP even when ATP was used as a competitor (**Fig. 4.7**, lanes 5-7). In the presence of unlabeled GDP as a competitor, the binding of labeled GTP decreased to a large extent (**Fig. 4.7**, lanes 12-14). However, β- and γ- subunits showed more significant decrease compared to the α-subunit (**Fig. 4.7**, lane 12 versus 13 and 14). These results indicating the binding of GTP to β-and γ- subunits of eIF2, and, also to the α-subunit are consistent with the earlier reports [Anthony et al., 1990; Dholakia et al., 1989; Bommer et al., 1988,1989; Barrieux and Rosenfeld 1977]. The binding of GTP to the α-subunit is somewhat unexpected keeping in view of the fact that it does not contain any of the G-binding motifs. However, the results of recent crystal structure suggest that human eIF2α at the C-terminus displays a very similar structure of the C-terminal domain of eEF1Bα, despite lack of any significant sequence homology [Ito et al., 2004]. The eEF1Bα is a guanine nucleotide exchange factor of eEF1A in elongation cycle and is comparable in its function to eIF2B in the initiation cycle. Although α-subunit of eIF2 is not reported to be involved in the exchange of guanine nucleotides, it is likely that it may be able to bind GTP like eEF1Bα or eIF2B. BSA and CPK also showed similar binding of labeled GTP in the presence of ATP and the binding decreased in the presence of GDP indicating the specificity of GTP binding to these proteins (**Fig. 4.7**, lanes 1, 2). Further, other control proteins like PKR and PERK in the reaction did not show any binding in the presence of ATP suggesting that these two proteins are not G-binding proteins (**Fig. 4.7**, lanes 3, 4). However, the GDP pretreated, but not ATP treated, PKR and PERK kinases were cross-linked to same extent with labeled GTP. This suggests that these kinases bind primarily to ATP. But in the absence of ATP, they may bind to GTP to the same extent.

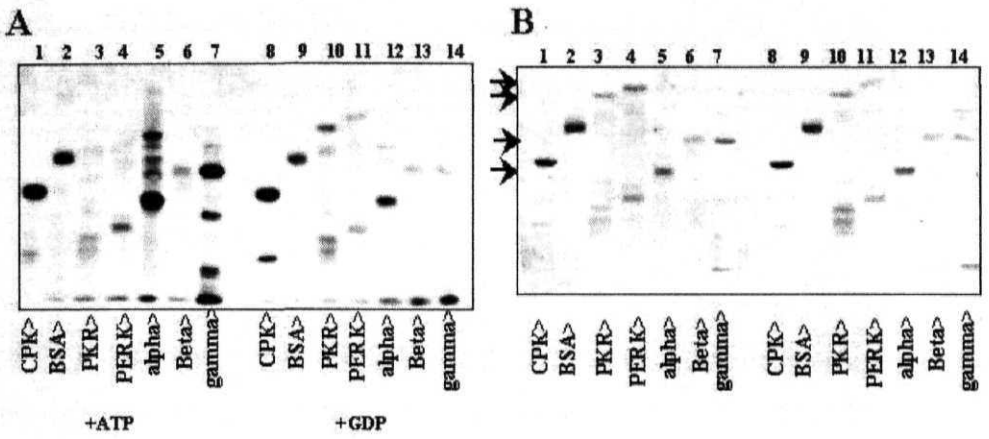


**Figure 4.7. Interaction of recombinant subunits of eIF2 with α-($^{32}$P)GTP in the presence of unlabeled ATP or GDP.** Purified recombinant human eIF2 subunits were incubated with 10 μCi of labeled GTP in the presence of 100 μM cold ATP or GDP as a competitor. The samples were then U.V. cross-linked by procedures as described in 'Materials and Methods'. PKR, PERK, CPK and BSA were used as the controls in the reactions. The samples were then separated by 12% SDS-polyacrylamide gels and then transferred to a nitrocellulose membrane. The amount of bound radioactivity was analyzed by phosphorimaging. Panel A in the figure is the phosphorimage and panel B is the Ponceau S stained blot corresponding to the phosphorimage in the panel A showing the amount of protein used in the reaction. The various lanes in the blot are as follows. 1, CPK+ [α-$^{32}$P] GTP+ cold ATP; 2, BSA+ [α-$^{32}$P] GTP+ cold ATP; 3, PKR+ [α$^{32}$P] GTP+ cold ATP; 4, PERK + [α-$^{32}$P] GTP+ cold ATP; 5, eIF2α + [α-$^{32}$P] GTP+ cold ATP; 6, eIF2β + [α-$^{32}$P] GTP+ cold ATP; 7, eIF2γ + [α-$^{32}$P] GTP+ cold ATP; 8, CPK+ [α-$^{32}$P] GTP+ cold GDP; 9, BSA + [α-$^{32}$P] GTP + cold GDP; 10, PKR+ [α-$^{32}$P] GTP+ cold GDP; 11, PERK + [α-$^{32}$P] GTP+ cold GDP; 12, eIF2α + [α-$^{32}$P] GTP+ cold GDP; 13, eIF2β + [α-$^{32}$P] GTP+ cold GDP; 14, PKR+ [α-$^{32}$P] GTP+ cold GDP.

**9. Inter Proteins interactions between subunits of eIF2 and eIF2B and eIF2α kinases.**

**9.1 β-subunit of human eIF2 interacts with eIF2B:** Previous reports on the interaction between the subunits of rat and yeast eIF2 with eIF2B proteins are different. Yeast eIF2 complex is shown to interact with the regulatory complex (consisting of α, β and δ-subunits) and catalytic complex (comprising the ε and γ-subunits) of yeast eIF2B and the interaction of eIF2 with the regulatory complex, but not with the catalytic complex, is enhanced specifically by the phosphorylation of eIF2α subunit alone [Pavitt et al., 1998]. Subsequent study has shown that recombinant yeast eIF2α alone can interact with the eIF2B holoprotein and also with the regulatory complex of eIF2B. The interaction with the regulatory complex is dependent upon phosphorylation of eIF2α [Krishnamoorthy et al., 2001]. However, studies with rat eIF2 has shown that phosphorylated or unphosphorylated recombinant eIF2α- subunit is unable to interact with eIF2B, where as the β-subunit is found to interact with the ε- and δ-subunits of eIF2B [Kimball et al., 1998]. This interaction is impaired when the correct folding of β-subunit of eIF2 is presumably impaired by deletion of certain residues in the C-terminus domain. In order to identify the subunit (s) of human eIF2 that can interact with eIF2B, we have carried out ELISA studies to determine the interaction between the subunits of eIF2 and the guanine nucleotide exchange factor, eIF2B, as shown in **fig. 4.8**. The plates were coated with the respective recombinant subunits, blocked with BSA and then incubated with purified rabbit reticulocyte eIF2B protein (**Fig. 4.8**, bars, 1-10) or without eIF2B (bars, 11-17). The plates were washed, treated with an anti-eIF2Bβ-antibody and respective HRP-conjugated secondary antibody as described in 'Materials and Methods'. We observed that purified rabbit reticulocyte eIF2B did not interact efficiently with the single subunits of human recombinant wt eIF2α (**Fig. 4.8** bar 1) or, mutants of eIF2α (S51A, S51D, S48A) that were not treated with PKR (bars 3, 5 and 7) or, phosphorylated by PKR (bars 2, 4, 6 and 8 respectively). Further, the interaction between recombinant eIF2β-subunit and purified eIF2B was found to be much higher and more significant compared to the interaction observed with the α, or γ-subunits (compare bar 9 vs 1-8 or 10 respectively).

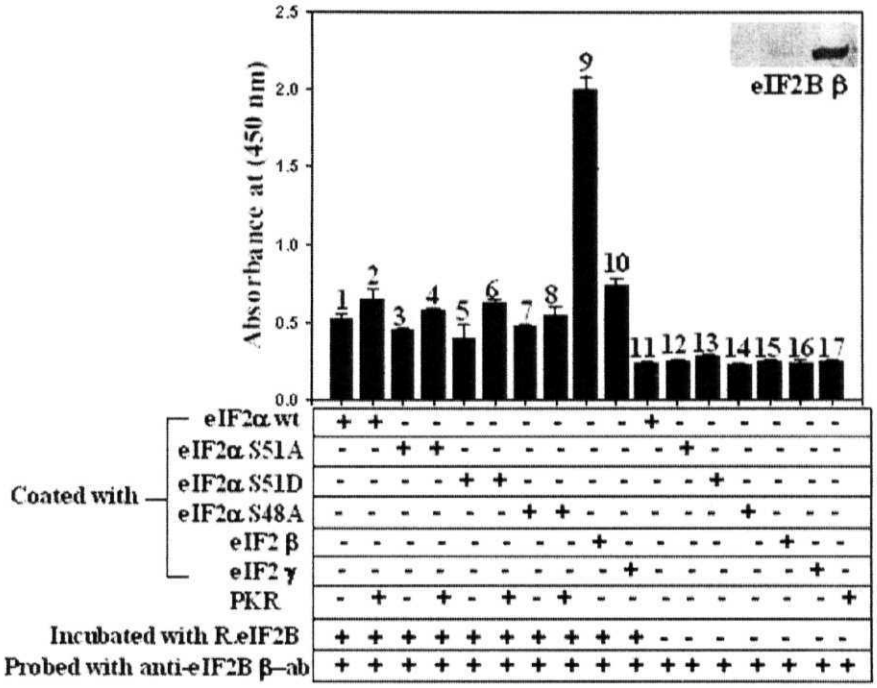


**FIGURE 4.8. Interaction of recombinant subunits of eIF2 with purified rabbit reticulocyte eIF2B.** The wells of microtitre plates were coated with wt eIF2α, mutants of eIF2α, β or γ-subunits. In order to determine the importance of phosphorylation of wt and mutants of eIF2α in the interaction with rabbit reticulocyte eIF2B protein, the wt and mutants of eIF2α were pretreated with PKR, wherever indicated in the figure. In step 2, eIF2B protein (~200 ng) that was purified from the 35K supernatant of rabbit reticulocyte lysate as described previously (Matts et al., 1983), was allowed to interact with the proteins coated in the wells for 2 hrs. Afterwards, wells were washed and treated with a monoclonal anti-eIF2Bβ-antibody. Control reactions carried out in bars 11-17 lack eIF2B protein. Insert shows the western blot analysis of β-subunit of purified eIF2B by monoclonal anti-eIF2Bβ antibody.

A small amount of interaction that occurred between α, or γ-subunits and 2B (bars 1, 2 and 10) may be due to minor contamination of eIF2 present in the purified eIF2B preparation. This result showing the interaction between the β-subunit of eIF2 and eIF2B is consistent with the earlier findings obtained by using rat eIF2 subunits (Kimball et al., 1998). From these results, it appears that eIF2β, but not α (unphosphorylated, phosphorylated or mutants) or γ-subunits of mammalian eIF2 interacts with purified eIF2B protein (**Fig. 4.8**, 11) or lysate eIF2B (**Fig. 5.0**).

**9.2. Recombinant trimeric complexes containing phosphorylated wt eIF2α, or S51D eIF2α, interact efficiently with eIF2B:** ELISA assays, as described above were carried out to determine the interaction between purified eIF2B and wt or mutants of eIF2 trimeric complexes where the wt α-subunit is replaced by S51A or S51D or S48A. Phosphorylation of serine 51 residue in wt eIF2α in the reconstituted trimeric complex of eIF2 by PKR enhanced its interaction with purified eIF2B (**Fig. 4.9**, bar 1 vs 5). This result is further supported by mutant eIF2 complexes that are prepared by replacing the wt eIF2α with mutants of eIF2α. The eIF2 trimeric complex composed of S51A, a nonphosphorylatable form, did not show enhanced interaction in the presence of PKR (bars 2 vs 6). In contrast, the eIF2 trimer containing S51D, a phosphomimetic form of eIF2α, showed enhanced interaction compared to the unphosphorylated wt eIF2α (bar 3 vs 1) or eIF2 trimer containing S51A (bar 3 vs 2). PKR addition to this mutant eIF2 trimer did not enhance the interaction with eIF2B any further as expected (bar 7 vs 3). Earlier by using recombinant human S48A mutant which can be phosphorylated on its 51 serine residue, it has been suggested that the serine 48 residue in eIF2α is required for a high affinity interaction between eIF2(αP) complex and eIF2B in rabbit reticulocyte lysates (Sudhakar et al., 1999). Consistent with this result, we obtained here that the mutant trimeric eIF2 complex containing S48Aα-subunit was unable to interact efficiently like the wt or S51D when the 51serine residue in S48A was phosphorylated by PKR (bars 4 vs 8). Control reactions lacking eIF2B were shown in bars, 9-13.

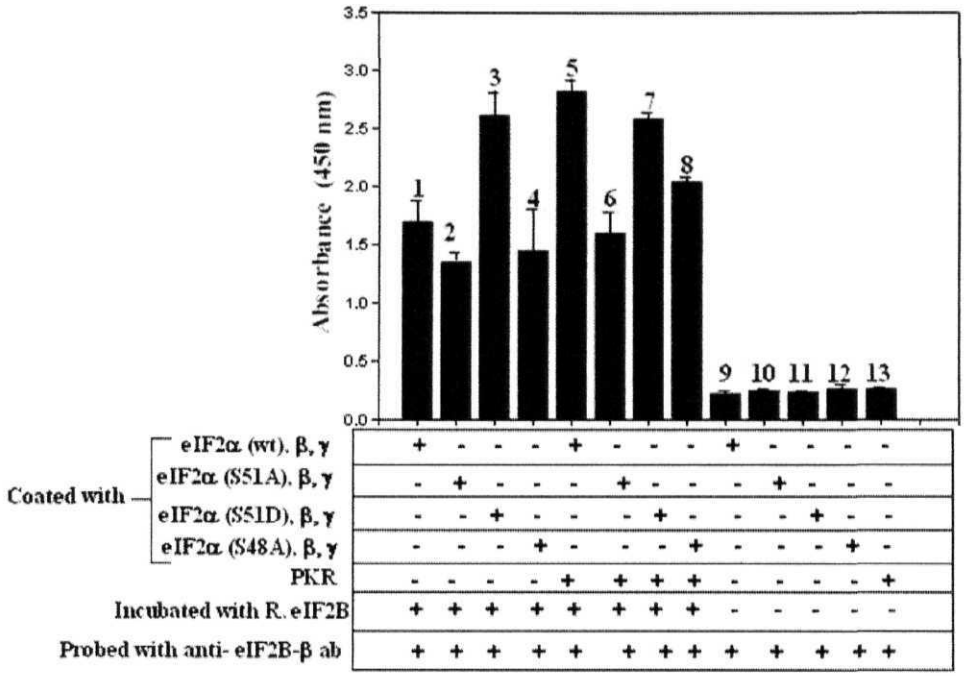


**FIGURE 4.9. Interaction between reconstituted eIF2 trimeric complex and purified eIF2B.** Heterotrimerc eIF2 complexes containing wt or mutants of eIF2α were prepared, supplemented with and with out PKR (in order to obtain unphosphorylated and phosphorylated eIF2α- subunit) as shown in the figure, and then coated to the wells of microtitre plates. Afterwards, the plates were incubated with purified rabbit reticulocyte eIF2B protein and probed with monoclonal anti-eIF2Bβ-antibodies and the respective HRP-conjugated secondary antibody. Controls carried out in bars 9-13 lack eIF2B protein. The plates were processed and analyzed as described in the legend to figure 9.

**9.3 Interaction of reticulocyte lysate eIF2B with reconstituted chimeric trimeric eIF2 complexes:** Heme-deficiency and double-stranded RNA treatment leads to the phosphorylation of endogenous eIF2α in reticulocyte lysates due to the activation of heme-regulated kinase (HRI) and RNA-dependent Protein Kinase, PKR. Phosphorylation of eIF2α inhibits the lysate eIF2B activity, presumably due to formation of eIF2α(P).eIF2B complex in which eIF2B is inactive. Further, we observed here that purified eIF2β- subunit coated to the wells of microtitre plates can interact with lysate α and β- subunits to form a possible chimeric trimeric eIF2 complex and this trimeric complex interacts efficiently with purified eIF2B (**Fig. 5.0 B**). Hence, we studied the interaction between such a chimeric trimeric complex of eIF2 formed in microtitre plates in the presence of eIF2β-subunit with native eIF2B present in heme-deficient, hemin-supplemented and PKR-treated lysates (**Fig. 5.0**). Phosphorylation of eIF2α was significantly enhanced in heme-deficient lysates or, in hemin and PKR-treated lysates compared to hemin-supplemented lysates (data not shown). Consistent with increased eIF2α phosphorylation in heme-deficient and PKR-treated lysates (bars 1 and 3), enhanced interaction between chimeric trimeric eIF2 and eIF2B was also observed (bars 1 and 3 vs 2). Similar results were obtained in lysates with the addition of reconstituted trimeric complexes of eIF2 consisting wt eIF2α or S51D (data not shown).

**9.4. Wt and mutants of eIF2α subunits interact with PERK and PKR:** Earlier studies had shown that mutations in double-stranded RNA-dependent kinase that inhibited the kinase activation were not important for binding the substrate eIF2α (Cai and Williams 1998). No such studies on the interaction between PERK, the recently characterized pancreatic endoplasmic-resident eIF2α- kinase and its substrate eIF2α- have been carried out. Here, we studied the interaction between recombinant purified mouse PERK with recombinant eIF2 subunits in dot blot assays (**Fig. 5.1**) and also by pull down assays (**Fig. 5.2** A and B). In the dot blot assay (**Fig 5.1**), as described in 'Materials and Methods', the nitrocellulose membrane was spotted with recombinant subunits of eIF2α, β or γ separately in three different corners and purified PERK in one corner. The proteins on the

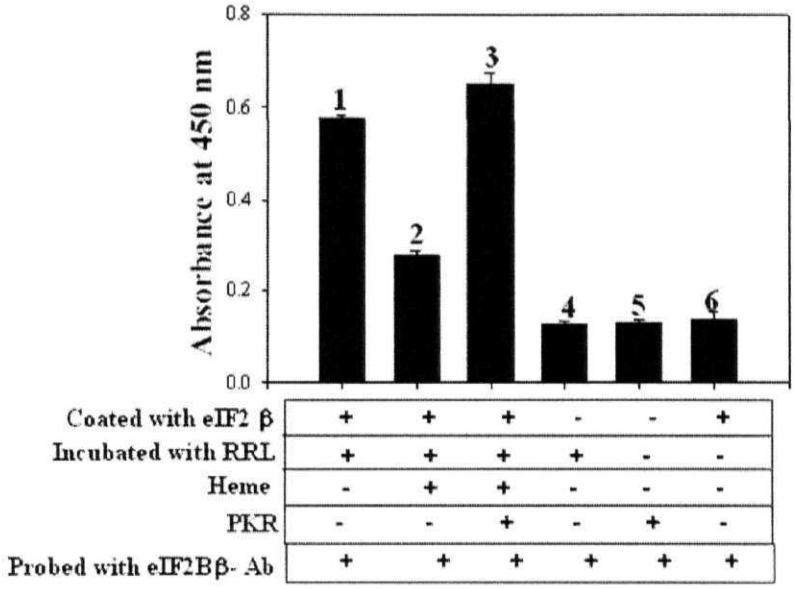


**FIGURE 5.0. Interaction between recombinant human eIF2β with eIF2B in rabbit reticulocyte lysate.** Purified recombinant human eIF2β-subunit was coated to the wells of microtitre plates. In the next step, the wells were incubated with heme-deficient, hemin-supplemented, and or heme and PKR–treated lysates (5 μl). Heme deficient and heme and PKR-treated lysates have shown enhanced phosphorylation of lysate eIF2α (data not shown). In the third step, the plates were probed with anti-eIF2Bβ antibodies and the respective secondary antibodies. The plates were processed and analyzed as described in the legend to figure 9.

membrane were UV-cross linked, treated with BSA, incubated with purified PERK - protein, washed, and, treated with primary and secondary antibodies as mentioned in the figure legend (**Fig. 5.1 A**). As can be seen from the color intensity that eIF2α-, but not β- and γ- subunits, interacts with PERK (**Fig. 5.1 A**). In panel B, the proteins were spotted as shown in the figure but the blot was not incubated with PERK protein as in panel A to determine the specificity of the PERK antibody. Panel C was treated with anti-histag antibody to determine the amounts of recombinant his-tagged eIF2 subunits used in the reactions. These studies indicate that only eIF2α- serves as the substrate for PERK. The dot blot assays (**Fig. 4.1 and 5.1**) are also found to be very reliable to assess specific interactions between proteins.

The binding of PERK and PKR to wt, or mutants of eIF2α was also evaluated by binding the his-tagged recombinant proteins of eIF2α to Ni-NTA agarose resin using pull down assays as described in 'Materials and Methods' (**Fig. 5.2 A and B**). The bound fractions (lanes 12-15), eluted with 500 mM imidazole, contained both the proteins (PERK or PKR in the top panels of A and B and eIF2α- in the corresponding bottom panels). Excess proteins that could not be bound to the Ni-NTA agarose matrix were found in the unbound fraction (lanes 1-5). Amount of proteins used in the reaction mixtures was shown in lanes 17- 21. Control insect cell extracts were used in the reaction. While the insect cell eIF2α- migrates below the recombinant human eIF2α-subunit, however its binding to PERK/PKR cannot be observed in the bound fractions as it does not carry the his tag. Since both wt and mutants of eIF2α- (S51A. S51D and S48A) can bind to PERK or PKR, the findings suggest that the PERK or PKR interacts with eIF2α- at a site other than the region surrounding the phosphorylation site.

Further, the binding of PERK and eIF2α was also monitored by fluorescence spectroscopy and analyzed by fluorescence resonance energy transfer (FRET) (**Fig. 5.3**) Increasing the concentration of α-subunit decreases PERK fluorescence, whereas, addition of PERK enhances the fluorescence of eIF2α. The most likely explanation of this behavior is that the conformation of eIF2α subunit is perturbed, as PERK is incremented. The perturbation, presumably, increases the fluorescence quantum yield of

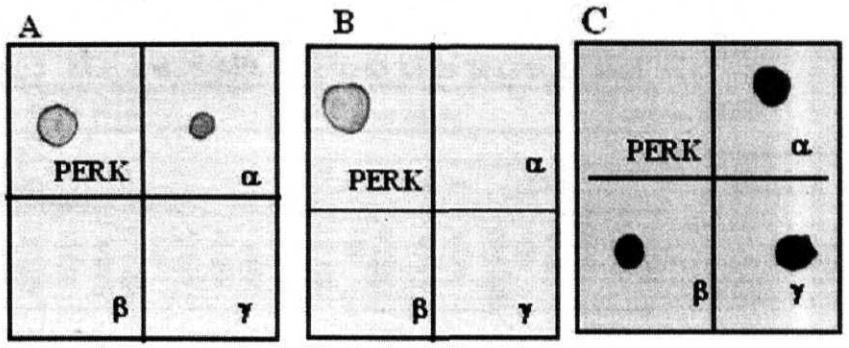


**FIGURE 5.1. Dot blot analysis of the interaction between PERK and recombinant eIF2 subunits:** Purified recombinant subunits of eIF2 (~ 500 ng), and or PERK were spotted on the four corners of a nitrocellulose membrane as shown in panels A, B and C. Membranes were air dried and UV-cross linked for one minute. Afterwards, they were blocked with 3% BSA for 60 min at room temperature. Membrane in panels A, but not in panel B and C, was then incubated with purified recombinant mouse PERK protein. Membranes in panel A and B were probed with a monoclonal anti PERK-antibody. The membrane in panel C was treated with a monoclonal anti-his tag antibody to detect the amount of eIF2 subunits loaded in each corner of the membrane. The membranes were processed as described in ' Materials and Methods'.

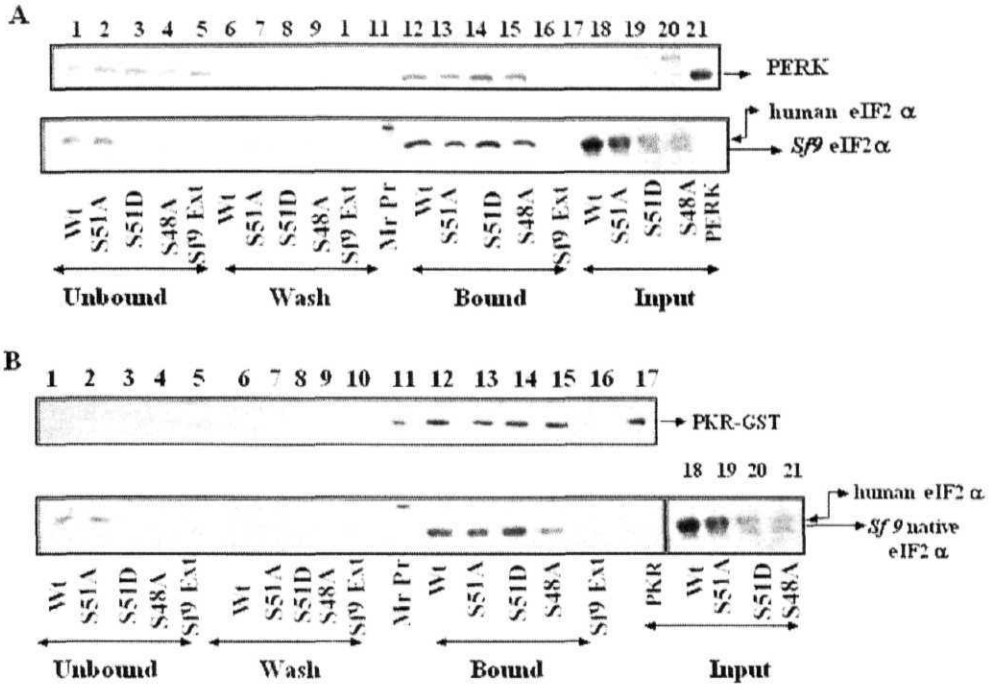


**FIGURE 5.2. Interaction between recombinant wt and mutants of human eIF2α with purified recombinant PERK and PKR.** Insect cell extracts expressing wt or mutants of eIF2α, or control extracts were incubated with purified PERK or PKR (~ 3 μg) in 1x-PBST for 10 min at room temperature. The mixtures were then added to 50 μl of pre-equilibrated Ni-NTA beads and incubated on ice for 45 mins in 1xPBST. The beads were then centrifuged and the unbound proteins in the supernatant were saved. The beads were washed thrice with PBST buffer and the histag proteins were eluted in the same buffer containing 500 mM imidazole. All fractions were separated by 10% SDS-PAGE and the proteins were transferred to a nitrocellulose membrane. The upper half of the membrane was probed with the anti-PERK (Panel A) or anti-PKR antibody (Panel B) while the lower half in both panels was treated with anti-eIF2α antibody. 50% of the protein used in the reaction was shown in the input lanes The figure is a western blot.

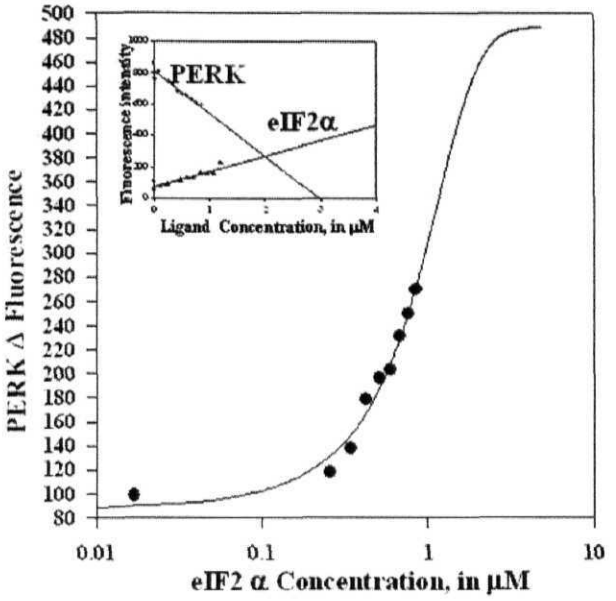


**FIGURE 5.3. Interaction between eIF2α and PERK by fluorescence spectroscopy.** Increasing concentrations of recombinant human eIF2α-subunit was added to 5 μM purified recombinant PERK protein. Samples were excited at 280 nm and the quenching in fluorescence was observed at 336 nm. Kd constants of eIF2α binding to PERK were calculated as described in 'Materials and Methods'. Figure represents the ΔF (fluorescence at a particular concentration of ligand) values plotted against increasing concentrations of eIF2α, the ligand. Inset represents the fluorescence resonance energy transfer from PERK to eIF2α. Quenching in net fluorescence (F) of PERK in the presence of eIF2α, or, the actual increase (Δ F) in fluorescence of eIF2α in the presence of PERK.

the eIF2α tryptophan. These findings, therefore, suggest that these two proteins interact with each other and thus complement the results obtained by dot blot analysis and pulldown assays (**Fig. 5.1 and 5.2**). FRET analysis suggests that the energy transfer is occurring from PERK to eIF2α (insert in **Fig. 5.3**). The dissociation constant of α-subunit-PERK complex ($K_d$ α-subunit) was calculated as described in 'Materials and Methods' and was found to be 0.74 μM. Stoichiometric binding was observed between eIF2α and PERK.

# DISCUSSION

This laboratory previously expressed the α-subunit of human eIF2 and the variants without his tag using baculovirus expression system (Sudhakar et al., 2000). Now, all the three subunits of human eIF2 (α, β and γ) and also three variants of eIF2α (S51A, S51D and S48A) were expressed with an N-terminal 6x histag using pFast Bac baculovirus transfer vectors. The protein profiles indicate that the visible synthesis of γ-subunit starts at 48hr PI, whereas the expression of α- and β-subunits starts at 24hr PI (**Fig. 3.4**). Further, the expression of γ-subunit is found to be lower compared to the expression of α- and β-subunits and also the γ-subunit is found associated with the 10K pellet fraction of the cell extracts rather than with the 10K supernatant (**Fig. 3.5**). In order to purify this subunit, we employed denaturing conditions using urea. Also, addition of 1% triton x-100 could release the γ-subunit from the cell pellet. Hence, it is likely that the γ-subunit may be associated with a membrane or cytoskeletal fraction. This interpretation is consistent with early reports indicating the association of initiation factors including eIF2 with cytoskeletal framework in HeLa cells (Howe and Hershey, 1984), marked sequence (Gaspar et al., 1994), and structural similarities (Schmitt et al., 2002) between eIF2γ and elongation factor, eEF1A which is also shown to interact with actin, a cytoskeletal material (Yang et al., 1990). However no reports are available that indicate the association of γ-subunit of eIF2 with membrane or any cytoskeletal material. Alternatively, the γ-subunit may be aggregated and forming inclusion bodies during the expression. Hence the protein is also purified under denaturing conditions. The purified, dialyzed and renatured protein is apparently in the right conformation because of its ability to bind GTP and Met-tRNAi (**Figs. 4.3 and 4.6**). We suspect that the reduced and slow expression of γ-subunit relative to β and α-subunits of eIF2 may be because of the depletion of endogenous initiator tRNA. This interpretation is consistent with the fact that γ-subunit is implicated in the binding of Met-tRNAi and GDP and over expression may be toxic to cells (Schmitt et al., 2002). A recent study also suggests that γ-subunit expression in yeast is controlled by proteins such as Cdc123 and Chfr which can arrest the G1 cell cycle, and, the levels of γ-expression (high or low) are found growth inhibitory (Bieganowski et al., 2004).

Further, studies with archaeal and yeast eIF2 suggested that γ-subunit is the core

subunit of trimeric eIF2 based on the formation of αγ, βγ, but not αβ dimers [Hashimoto et al., 2002; Schmitt et al., 2002; Thompson et al., 2000; Tahara et al., 2004]. Interestingly, a recent report indicates that no complex formation could be detected between purified human eIF2α and γ- subunits produced in bacteria [Ito et al., 2004]. However, it is not clear if this recombinant protein is nonfunctional or requires some other components like initiator tRNA or eIF2β-subunit. In our studies, we observed all possible interactions between the recombinant subunits: αγ, βγ and also αβ in dot blot and ELISA studies (**Figs. 4.1 and 4.2**).

Further, the recombinant subunits expressed and purified here are found functional. The purified eIF2α- and β-subunits are phosphorylated efficiently by several kinases (**Figs. 3.7 and 3.9**) as reported earlier [Dever, 2002; Welsh et al., 1994; Clark et al., 1989; Ting et al., 1998]. A Previous study from this laboratory has shown that phosphorylation of recombinant human eIF2α- purified from insect cells is not as efficient as the phosphorylation of the α-subunit of purified rabbit eIF2 complex [Sudhakar et al., 1999]. This reduction in eIF2α phosphorylation may be because of a contamination or comigration of a viral protein, like PK2 that inhibits eIF2α kinase activity [Dever et al., 1998], or, the lack of the other two subunits in the phosphorylation mixture. To rule out the second possibility, the phosphorylation of recombinant eIF2α was evaluated independently and also in the presence of the other two purified recombinant subunits. Addition of other subunits did not alter the phosphorylation status of eIF2α (**Fig. 3.8**).

Results of dot blot, pull down, and, fluorescence spectroscopic assays (**Figs. 5.1, 5.2 and 5.3**) suggest that wt and mutants of eIF2α subunits interact with purified recombinant PERK and PKR kinases. Previous studies with PKR and eIF2α interaction indicate that GYID amino acid residues (80-83) present in the N-terminal 123 region in eIF2α interact with PKR [Sharp et al., 1997]. Consistent with these results, mutations at 51 or 48 serine residues to alanine or aspartic acid in the recombinant human eIF2α have not altered the ability of these mutants of eIF2α subunit to interact with PKR or PERK as analysed by pull down assays here (**Fig. 5.2**). Further, the α-subunit, but not β and γ-

subunits, interacts with PERK, as has been observed here in the dot blot assays (**Fig. 5.1**) which is consistent with the fact that eIF2α is the substrate for these kinases. This experiment also highlights the specificity of interactions observed in dot blot assays between the recombinant subunits of human eIF2 (**Fig. 4.1**). Further fluorescence spectroscopic studies indicate that eIF2α interacts with PERK, as shown by the quenching of PERK intrinsic fluorescence (**Fig. 5.3**). The resonance energy is transferred from PERK to eIF2α and the binding is stoichiometric and Kd of α-subunit is 0.74 μM.

The recombinant γ-subunit is primarily implicated in GTP and Met-tRNAi binding. Consistent with this idea our results indicate that γ-subunit can join Met-tRNAi much more efficiently than β or α-subunits (**Fig. 4.3**). However the formation of ternary complex in the presence of all the three subunits of eIF2 or any one of the three subunits, suggests that the binding of Met-tRNAi to the γ-subunit requires the proximity of the other two subunits. This suggestion is consistent with earlier cross-linking studies (Gaspar et al., 1994) and also that the preparations devoid of β-subunit in eIF2 complex are defective in Met-tRNAi binding (Flynn et al., 1993). Structure based site-directed mutagenesis in archaea and the corresponding residues in yeast eIF2γ- reveals that domain II plays a critical role in the binding of Met-tRNAi $^{Met}$ to eIF2 and that the binding of the α-subunit of eIF2 is localized to the side of eIF2γ adjacent to the Met-tRNAi$^{Met}$ binding (Roll Mecak et al., 2004) and the CTD of eIF2α is necessary for the stabilization of GTP bound Met-tRNAi (Roll Mecak et al., 2003; Yatime et al., 2004; Ito et al., 2004). Consistent with these structural studies in eIF2α, we also observed here that all the three subunits of eIF2, including eIF2α, could be cross-linked to GTP (**Fig. 4.6**).

Using sucrose gradient analysis, previous studies from this laboratory have shown that phosphorylation of reticulocyte eIF2α promotes the formation of 15S complex, eIF2α(P).eIF2B (Sudhakar et al., 1999; 2000). The addition of recombinant S48A and S51A mutants of human eIF2α reduce the 15S complex formation. Further, addition of recombinant S51D mutant of eIF2α, to hemin-supplemented lysates, promoted the formation of 15S complex that is consistent with its behavior as a phosphomimetic form.

Based on these studies, it was suggested that the mutant proteins replace or exchange out the native eIF2α present in reticulocyte lysates to form a chimeric heterotrimeric complex. The results presented here on the interaction among the three subunits lend further support for the formation of chimeric trimeric complex (**Fig. 4.2B**) in lysates with the addition of recombinant α-subunit of eIF2. Further, results with the reconstituted human eIF2 subunits (**Figs. 4.9 and 5.0**) also support our earlier observations [Sudhakar et al., 1999] that phosphorylation of serine 51 residue in eIF2α promotes a tight binding between eIF2 and eIF2B and this interaction also requires serine 48 residue in eIF2α.

Previous reports on the interaction between the subunits of rat and yeast eIF2 with eIF2B proteins are different. Yeast eIF2 complex is shown to interact with the regulatory complex (consisting of α, β and δ-subunits) and catalytic complex (comprising the ε and γ-subunits) of yeast eIF2B and the interaction of eIF2 with the regulatory complex, but not with the catalytic complex, is enhanced specifically by the phosphorylation of eIF2α subunit in the trimeric complex [Pavitt et al., 1998]. A subsequent study has shown that recombinant yeast eIF2α alone can interact with the eIF2B holoprotein and also with the regulatory complex of eIF2B. The interaction with the regulatory complex is dependent upon phosphorylation of eIF2α [Krishnamoorthy et al., 2001]. However, studies with rat eIF2 has shown that phosphorylated or unphosphorylated recombinant eIF2α- subunit is unable to interact with eIF2B, whereas the β-subunit of eIF2 is found to interact with the ε- and δ-subunits of eIF2B [Kimball et al., 1998]. This interaction is impaired when correct folding of the β-subunit of eIF2 is presumably impaired by deletion of certain residues in the C-terminus domain. Consistent with this latter result in mammalian systems, it has been observed here that human eIF2β, but not α (unphosphorylated, phosphorylated or mutants) or γ-subunits, interacts with purified eIF2B protein (**Figs. 4.8 and 4.9**) or reticulocyte lysate eIF2B (**Fig. 5.0**) and this interaction is enhanced upon phosphorylation of eIF2α in the reconstituted trimeric complex (**Figs. 4.8 and 4.9**). Although no previous reports are available showing any interaction between eIF2 α and β either in mammalian or in archaeal systems, such an interaction is essential for the β-subunit in eIF2 to relay

the changes in the phosphorylation status of eIF2α to the regulatory complex in eIF2B. This suggestion is supported by our observations that α and β-subunits of human eIF2 indeed interact. Hence, it is likely that the interactions between eIF2 and eIF2B in yeast and mammalian systems may be somewhat different. Consistent with this notion, the recently solved crystal structures of yeast and human eIF2α pointed certain differences in the N-terminal domain [Dhaliwal et al., 2003]. These include the presence of disulfide bond that connects the residues between 69-97 in human protein, an additional 4-residue relative to the yeast protein in the α1 and α2 helices and between α3 and α4 helices, and, a conserved loop consisting of residues 51-65 is visible in the electron density maps of yeast eIF2α, but not in the human protein. However, it is not clear if any of these differences in the crystal structure between yeast and human eIF2α can explain the differences in their abilities to interact with the eIF2B or eIF2β- proteins. The crystal structure of the N-terminal segment of human eIF2α envisages a possibility of interaction between the highly conserved polylysine motif present in the β-subunit and the negatively charged groove with highly conserved residues formed in human eIF2α [Nonato et al., 2002]. The three polylysine rich boxes in the N-terminal segment of eIF2β appear to be important in protein-protein interactions, have also been implicated in the binding of eIF2 to both eIF5 and eIF2B [Asano et al., 1999]. Consistent with these suggestions, archaeal eIF2β that lacks the N-terminal polylysine motifs also lacks eIF5 and eIF2B proteins and cannot form a αβ dimer. Hence one possibility is that the polylysine region in the β-subunit may be important not only for the interaction with eIF2B but also with the α-subunit of eIF2. However, the polylysine regions are also present in the β-subunits of both human and yeast eIF2, and, the *in vivo* data suggest that yeast eIF2 cannot form αβ dimer [Thompson et al., 2000]. So it is likely that phosphorylation of human eIF2α induces a conformational change in the β-subunit of eIF2 that facilitates a nonproductive interaction with the δ-subunit of the regulatory complex in eIF2B. This is somewhat analogous to the interactions proposed between yeast eIF2α(P) and the regulatory complex to explain the inhibition in the GNE activity of eIF2B [Krishnamoorthy et al., 2001].

Based on mutational and genetic studies in yeast, it is suggested that eIF2βγ forms a central functional core whereas the α-subunit of eIF2 and the eIF2B form a regulatory core that modulates the level of eIF2 function by regulating the GNE activity of eIF2B and the formation of ternary complex in *vivo* (Erickson et al 2001). Further, it has been shown that the presence of α-subunit in yeast eIF2 complex retards the GNE activity of eIF2B, thereby suggesting a structural role for eIF2α in the nucleotide exchange (Nika et al., 2001). However we think that it is somewhat different from the subunits of mammalian eIF2. We suspect, based on our studies here and also from others that the β-subunit of mammalian eIF2 plays a central role because of its ability to interact with the other two subunits of eIF2 and to form ternary complexes through its interaction with the γ-subunit. Most importantly, the recombinant human β-subunit regulates the GNE activity of eIF2B indirectly through its interaction with phosphorylated eIF2α, and, directly through its interactions with the δ and/ or ε-subunits of eIF2B. In addition, the β-subunit interacts with eIF5 (the GTPase Activating Protein), ribosomes, and, has a role in start site recognition in mRNA. Further, the human eIF2β-subunit, unlike its yeast counter part, is a substrate for several kinases as has been shown here (**Fig. 3.9**), and, the phosphorylation of it by PKA increases the GNE activity of eIF2B as has been described earlier (Kimball et al., 1998). In addition to its interactions with translational factors, eIF2β is also shown to interact with Nck1, a tyrosine kinase adaptor protein (Kebache et al., 2002). Very early observations of mammalian eIF2 complex, that is devoid of the β-subunit, is found to be severely defective in the formation of ternary complexes and exhibited reduced rates of eIF2B catalyzed nucleotide exchange *in vitro* (Flynn et al., 1993). All these findings make us to hypothesize that the β-subunit of mammalian eIF2 is somewhat evolved compared to yeast or archaea and appears to be a key player in the function and regulation of eIF2.

# SUMMARY

1. We have over expressed α (wt and mutants), β and γ-subunits independently or in combination with other subunits with a 6x his tag at the N-terminus using baculovirus expression system and purified them using Ni-NTA agarose.
2. Using Elisa and dot blot analyses, we observed that any of the two recombinant subunits of eIF2 or all the three subunits can interact with each other *in vitro* and in lysates. Purification studies suggest that γ-subunit, but not α- and β-, may be aggregated or associated with a membrane or cytoskeletal fraction.
3. The purified recombinant α (wt and S48A mutant) and β- subunits are efficiently phosphorylated by different kinases.
4. Purified α, β and γ-subunits can bind GTP and Met-tRNAi, although the relative binding affinities appear to be different. Based on properly spaced GTP-binding sequences and its similarity with EF1, the γ-subunit is primarily involved in GTP and Met-tRNA$_i$ binding. However, these functions apparently require the coordination of other two subunits.
5. Phosphorylation of eIF2α can take place independently and is not affected by the presence of the other two subunits.
6. It has also been observed here that eIF2α (wt and mutants), but not β and γ-subunits, interact with recombinant PERK (pancreatic endoplasmic resident kinase) and PKR (double-stranded RNA-dependent kinase) irrespective of whether or not they serve as substrates for these kinases thereby suggesting that the amino acid sequence surrounding the phosphorylation site in the substrate may not be important for the interaction with the kinases.
7. Further, fluorescence spectroscopy and fluorescence resonance energy transfer (FRET) analysis reveal that the energy transfer occurs from PERK to eIF2α, the dissociation constant of α-subunit-PERK complex ($K_d$ α-subunit) is 0.74 μM and the interaction is stoichiometric.
8. The β-subunit, rather than the α and γ-subunits, of human eIF2 is observed to interact specifically with the purified, or rabbit reticulocyte lysate eIF2B in ELISA (Enzyme-linked immunosorbent assay) studies and this interaction is enhanced when wt eIF2α in the trimeric complex is phosphorylated. Consistent

with this result, the mutant phosphomimetic trimeric complex formed by eIF2α S51D has a higher affinity for eIF2B.

9. The findings that β subunit interacts with the α- subunit and also with purified eIF2B. The interaction between β-subunit of eIF2 and eIF2B is enhanced upon phosphorylation of eIF2α thereby suggesting that β-subunit in mammalian eIF2 complex plays a critical role in eIF2 function and regulation. This is also consistent with the suggestion that it is a substrate for several kinases and interacts with a host of proteins and RNA.

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