Phosphorylation of eIF2α in the Ovarian Cells of *Spodoptera fruigiperda (Sf9):* Mechanisms Affecting Cell Death

A Thesis

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By

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DECLARATION

I, S. Aarti, hereby declare that this thesis entitled "Phosphorylation of eIF2α in the ovarian cells of *Spodoptera frugiperda (Sf9)*: Mechanisms affecting cell death" submitted by me under the guidance and supervision of Professor K. V. A. Ramaiah, is an original and independent research work. I also declare that it has not been submitted previously in part or in full to this University or any other University or Institution for the award of any degree or diploma.

Date:

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CERTIFICATE

This is to certify that this thesis entitled "Phosphorylation of eIF2α in the ovarian cells of *Spodoptera frugiperda (Sf9)*: Mechanisms affecting cell death" is a record of bonafide work done by S. Aarti, a research scholar for Ph.D. programme in Department of Biochemistry, School of Life Sciences, University of Hyderabad under my guidance and supervision.

The thesis has not been submitted previously in part or in full to this or any other University or Institution for the award of any degree or diploma.

Signature of the Supervisor

Head of the Department **Dean of the School**

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Aarti..

Contents

Abbreviations

ABC50	: ATP binding cassette protein-50
Ac-DEVD-AFC	: N-acetyl-Asp-Glu-Val-Asp-amino-4-triflouromethyl coumarin
Ac-DEVD-CHO	: N-acetyl-Asp-Glu-Val-Asp-aldehyde
AcMPV	: Autographa californica multiple nuclear polyhedrosis virus
ADP	: Adenosine 5' diphosphate
AIF	: Apoptosis inducing factor
AP	: Alkaline phosphatase
Apaf-1	: Apoptosis protease activating factor 1
APS	: Ammonium per sulphate
ATF	: Activated transcription factor
ATP	: Adenosine 5' triphosphate
BCIP	: 5-bromo-4-chloro-3-indolyl phosphate
Bcl 2	: B cell leukemia/lymphoma 2
BiP	: Immunoglobin heavy chain binding protein
bZIP	: Basic leucine zipper
CACH	: Childhood ataxia with central hypomyelination
CAT-1	: Cationic amino acid transporter
Cdc123	: Cell division cyclin protein 123
Chfr	: Checkpoint fork head associated with RING proteins
CEBP	: CCAAT-enhancer binding protein
CHOP	: C/EBP-homologous protein
CKII	: Caesin kinase II
CPK	: Creatine phospho kinase
CREB	: Cyclic AMP response element binding protein
CReP	: Constitutive repressor of eIF2α phosphorylation
CTD	: Carboxy terminal domain
DIABLO	: Direct IAP binding protein with low pI
DNA	: Deoxy ribonucleic acid
DNA-PK	: DNA dependent protein kinase
Ds	: double stranded
dsRBD	: double stranded RNA binding domain
DTT	: Dithiothreitol
DYRK	: Dual specificity tyrosine phosphorylated and regulated kinase
eIF	: Eukaryotic initiation factor
eIF2α	: alpha subunit of eIF2
eIF2α(P)	: Phosphorylated alpha subunit of eIF2
ER	: Endoplasmic reticulum
ERK	: Extracellular regulated kinase
FADD	: Fas-associated death domain
FANCC	: Fanconi's anaemia complementation group C
Fig	: Figure
GADD	: Growth arrest and DNA damage inducible protein
GCN	: General control non-derepressible
GDP	: Guanosine 5' diphosphate

GEF : Guanine nucleotide exchange factor
GNE : Guanine nucleotide exchange activity
GRP78 : Glucose regulated protein 78
GSH : Reduced form of glutathione
GSK3 : Glycogen synthase kinase 3
GSSG : Oxidized form of glutathione
GTP : Guanosine 5’ triphosphate
hDUS2 : Human dihydrouridine synthetase 2
HIV : Human immunodeficiency virus
HRI : Heme regulated inhibitor
HSP : Heat shock protein
HtrA2 : High temperature requirement protein A2
IAP : Inhibitor of apoptosis
IFN : Interferon
IRE1 : Inositol requiring enzyme 1
IRES : Internal ribosomal entry sites
IRF1 : Interferon regulatory factor 1
ISR : Integrated stress response
JNK : c-jun N-terminal kinase
KD : Kinase domain
kDa : Kilo dalton
LPS : Lipopolysaccharide
MAPK : Mitogen activated protein kinase
MetAP2 : Methionine aminopeptidase
Mcl-1 : Myeloid cell leukemia sequence 1
Mda7 : Melanoma differentiation associated gene 7
MEK : Mitogen activated ERK kinase
Min : Minute
Mr : Molecular weight marker
NBT : Nitro blue tetrazolium
NFκB : Nuclear factor κB
NTD : Amino terminal domain
OB : Oligonucleotide binding domain
ORF : Open reading frame
PACT : PKR associated activator
PAGE : Polyacrylamide gel electrophoresis
PDGF : Platelet derived growth factor
PERK : PKR like ER resident kinase
PI3K : Phosphatidyl inositol 3 kinase
PKA : cAMP dependent protein kinase
PKC : Calcium dependent protein kinase
PKR : Double stranded RNA dependent protein kinase
PIC : Pre-initiation complex
PPI : Protein phosphatase I
RBC : Red blood corpuscles
RNA : Ribomucleic acid

S	: Svedberg
S51D	: eIF2α mutation of ser51 eIF2α to Asp
SDS	: Sodium dodecyl sulphate
Sf9	: *Spodoptera frugiperda*
Smac	: Second mitochondria-derived activator of caspase
STAT	: Signal transducer and activator of transcription
TAR	: Tat responsive element
TEMED	: N'N'N'N'-tetra ethyl methyl ethyl diamine
TIF32	: Transcriptional intermediary factor 32
TNF	: Tumor necrosis factor
TRADD	: TNFR1 associated death domain
TRAIL	: TNF-related apoptosis inducing ligand
Tris	: Tris (hydroxyl methyl) amino methane
tRNA	: Transfer RNA
Tyk2	: Tyrosine kinase 2
UPR	: Unfolded protein response
UTR	: Untranslated region
VEGF	: Vascular endothelial growth factor
XIAP	: X-linked inhibitor of apoptosis
z-VAD-fmk	: Benzyoxycarbonyl-Val-Ala-Asp (o-methyl)-flouromethyl ketone

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The thesis work carried out here analyzes the importance of stress-induced eIF2α phosphorylation and activation of other signaling pathways in cell survival and death, in addition to changes in the inter-protein interactions and gene expression. Hence, a brief overview of the subunit composition, structure and function of eIF2 complex, phosphorylation of eIF2α and its physiological consequences, regulation and effects on gene expression is presented.

In brief, eukaryotic initiation factor 2 (eIF2), a heterotrimer, delivers initiator tRNA (Met-tRNAi) to the 40S ribosomal subunit in a GTP dependent manner, during the first step of initiation of protein synthesis in eukaryotes. Of the three subunits, the β- and γ-subunits of eIF2 are involved in various functions such as its ability to bind Met-tRNAi, GDP/GTP, and in the hydrolysis of GTP. β-subunit is implicated in mRNA binding and also serves as a hub for various protein-protein interactions. Both α- and β-subunits are substrates for several kinases. Although, the physiological significance of phosphorylation of β-subunit is not yet understood, phosphorylation of conserved Ser-51 residue in eIF2α is implicated in the regulation of initiation of protein synthesis and gene expression. Phosphorylation of a small portion of eIF2α inhibits protein synthesis in general and up-regulates translation of certain gene specific mRNAs such as ATF4 and GCN4, which are involved in regulating the genes affecting redox metabolism and amino acid biosynthesis. Phosphorylation of eIF2α is an integrated stress response (ISR) as it is phosphorylated by a variety of stress activated eIF2α kinases. It is a stress, survival and suicidal signal depending on the cellular location, changes in gene expression, inter-protein interactions and the activation of co-incident signaling pathways. In addition to its role in cell survival and death, phosphorylation of eIF2α plays a role in virus infection, unfolded protein response (UPR), amino acid starvation, and in memory. It couples protein synthesis with glucose sensing and glucose metabolism and thus, is also found to play an important role in cell metabolism and homeostasis.

1.1, A detailed overview on eIF2 subunit composition and interaction among the subunits;

Eukaryotic initiation factor 2 is a heterotrimeric guanine nucleotide binding protein comprising α, β and γ subunits (Barrieux and Rosenfeld, 1977; Picciano et al., 1973; Lloyd et al., 1980). Its primary role involves selection and recruitment of initiator tRNA to the 40 S ribosomal subunit in a GTP dependent manner, and also plays an important role in start site selection (Marintchev and Wagner, 2004)). Structurally, there exists a considerable sequence homology among different species for each of the subunits of eIF2, and this conserved nature of eIF2 implicates its importance in cell physiology (Kimball, 1999; Hershey and Merrick, 2000). For instance, the mammalian α, β and γ subunits are 58%, 47% and 72% identical to their respective counterparts in *Sacharomyces cerivisae*. The cDNA's of the subunits have been cloned and sequenced from a variety of species including *Drosophila* (Qu and Cavener, 1994; Ye and Cavener, 1994); wheat germ (Metz and Browning, 1997); and bovine systems (Green et al., 1991). The relative molecular mass for the human α, β and γ subunits as determined from their cDNA's are 36.2, 38 and 52 kDa respectively (Ernst et al., 1987; Pathak et al., 1988a; Schmitt et al., 2002; Hershey and Merrick, 2000), and appear to be very similar to that of their yeast counterparts, which correspond to 33.7, 35.4 and 49.2 kDa respectively (Cigan et al., 1989; Donahue et al., 1988; Hannig et al., 1993); Each of these individual subunits associate in a 1:1:1 ratio to form the functional protein, where the γ subunit forms the core of the protein and interacts with both α- and β-subunits, but the α- and β-subunits in turn do not interact with each other (Marintchev and Wagner, 2004). Unlike in archaeal and yeast systems, human α and β subunits interact with each other and with the γ subunit (Suragani et al. 2006; Rajesh et al., 2008; Schmitt et al., 2010). The structures of the individual subunits of archaeal, mammalian and yeast eIF2α, the N-terminal half of archaeal β, and the archaeal γ subunit have been determined and elucidated partially (Schmitt et al., 2002; Cho and Hoffman, 2002; Nonato et al., 2002; Dhaliwal and Hoffman, 2003; Ito et al., 2004; Stolboushkina et al., 2008).

1.2, Structure and Function of eIF2α ;

The α-subunit is the smallest subunit of eIF2 and does not exhibit any special functional feature other than the conserved phosphorylation site at Ser-51 residue, which serves as a substrate for the eIF2α kinases (Hershey, 1991; Dever et al., 1992). This serine residue is conserved in all eukaryotic eIF2α sequences (Ernst et al., 1987; Cigan et al., 1989; Qu and Cavener, 1994) but not in archaeal IF2α (Bult et al., 1996). However, a report indicates that archaeal IF2 (aIF2) is phosphorylated at its Ser-48 residue, but the kinase or the consequence of phosphorylation is not characterized (Tahara et al., 2004). In addition to the Ser-51 residue, yeast and wheat germ eIF2α contain casein kinase II sites, which are not conserved in mammalian system (Proud, 1992; Feng et al., 1994; Vanden Heuvel et al., 1995; Janaki et al., 1995). However, CKII phosphorylation is shown to reduce the eIF2 activity significantly only when combined with defective recycling of eIF2. GDP (Feng et al., 1994).

The structure of eIF2α as indicated by the N-terminal crystal structure (Nonato et al., 2002; Dhaliwal and Hoffman, 2003) and the complete NMR structure (Ito et al., 2004), indicate the presence of three domains: an N-terminal β barrel, a helical domain and a C-terminal α–β domain. The NTD consists of S1 and α-helical sub-domains and the S1 domain in turn consists of five stranded β- barrel, which adopts the oligonucleotide binding (OB) fold. The conserved Ser-51 residue is located in the β3/β4 loop (Ito et al., 2004). The amino terminal region of the α-subunit is implicated in AUG recognition (Donahue et al., 1988; Yoon and Donahue 1992). The C-terminus adopts the αβ conformation and consists of five β strands (β6-β10), two α helices (α6 and α7), and one loosely associated C-terminal helix α8 that has no specific or defined orientation with respect to the CTD or the NTD. Although there exists no sequence similarity between the CTD of eIF2α and CTD of eEF1Bα, which serves as a GEF involved in recycling of eEF1A, both of them possess a striking similarity in their tertiary structure (Ito et al., 2004; Monika et al., 2005; Schmitt et al., 2010). Mutational analyses reveal that the CTD of eIF2α is necessary for the interaction with the γ subunit and also plays an important role in stabilizing the Met-tRNA$_i^{Met}$ (Yatime et al., 2004; Ito et al., 2004). Consistent with the above observation, the CTD of eIF2α interacts with the γ-subunit of eIF2 and

resembles the eEF1A-eEF1Bα-CTD complex (Andersen et al., 2001). Physiologically, deletion of the α-subunit of eIF2 is found to be lethal in yeast and is associated with defective eIF2B associated GNE activity and Met-tRNA$_i^{Met}$ binding (Nika et al., 2001). This lethality was found to be reversed by the over expression of the β- and γ-subunits and Met-tRNA$_i^{Met}$, implicating the importance of α-subunit in Met-tRNA$_i^{Met}$ binding, and in regulating the eIF2B mediated GNE activity through physical interactions (Nika et al., 2001). Interestingly, studies in our laboratory have shown that the α-subunit which lacks G-binding domains can also bind GDP (Unpublished Results). This is consistent with the idea that the α-subunit resembles eEF1Bα, and probably the former has lost its GEF activity during evolution. The NTD and CTD do not interact with each other, but they are also not independent of each other. Although most of the features are highly conserved among eukaryotic and archaeal α-subunits, there exists a C-terminal acidic extension in the eukaryotic counterpart, which apparently has no known function but consists of a caspase cleavage site (Schmitt et al., 2010). Conserved negatively charged residues are present in the CTD of eIF2α which are predicted to interact with the poly lysine blocks present in the β-subunit of eIF2, and also with other initiation factors rich in basic amino acids (Ito et al., 2004).

1.3, Structure and Function of eIF2β;

The β-subunit of eIF2 shows multiple features and has been found to contain three different regions: the N-terminal, the central and the C-terminal regions (Pathak et al., 1988a; Donahue et al., 1988; Ye and Cavener, 1994; Gutierrez et al., 2004). The crystal structure of eukaryotic β subunit is not yet available. However, the archeal β-structure reveals an N-terminal helix connected to the central α–β domain through a linker (Schmitt et al., 2010). The N-terminal region has a number of distinct features such as phosphorylation sites for kinases like PKC and CKII; three poly lysine blocks containing 6-8 lysine residues each, which are involved in binding to eIF5, eIF2Bε and mRNA (Laurino et al., 1999; Asano et al., 1999), and two of the three guanine nucleotide binding domains (Dever et al., 1987), which are present in the human subunit but not in its yeast counterpart (Pathak et al., 1988a). Although mutations in the G-binding domains of the β-

subunit do not alter the GDP binding ability of eIF2, absence of β-subunit reduces partially the efficiency of γ-subunit in its gunanine nucleotide binding ability, thereby suggesting that the β-subunit may assist the γ subunit in binding GDP. The central region contains the binding site for eIF2γ (Hashimoto et al., 2002), whereas the C-terminal region contains a zinc binding domain which is required for mRNA binding and start site selection (Donahue et al., 1988). Mutation analyses reveal that the zinc finger motif is crucial for ternary complex formation and 40S binding (Donahue et al., 1988; Castilho-Valavicius et al., 1992), while the poly lysine blocks are necessary for the tight interaction between eIF2 and eIF2B (Asano et al., 1999). The C-terminal half of β subunit shows significant sequence homology with that of eIF5 (Das et al., 1997), suggesting that eIF5 and eIF2β compete with each other, and thereby influence the GTP hydrolysis. The β-subunit is also shown to contain certain sites which bind Met-tRNAi, and studies indicate that α–γ complex is severely defective in Met-tRNAi binding, thereby suggesting the involvement of β subunit in Met-tRNAi binding (Flynn et al., 1993; Huang et al., 1997). Further, the β subunit of eIF2 serves as a hub for interaction with various proteins such as eIF5 (Asano et al., 1999; Das et al., 1997), eIF2Bε (Kimball et al., 1998; Asano et al., 1999), eIF1A, eIF1, 40S ribosomal subunits, TIF32 (transcriptional intermediary factor 32)/eIF3a (Valasek et al., 2002), cellular phosphatase co-factor like Nck1 (Kebache et al., 2002), DNA-PK (Ting et al., 1998) and ATP binding cassette protein-50 (ABC50) (Tyzack et al., 2000), suggesting that it plays an important role in mediating the interactions necessary for the formation of 43S and 48S complexes and regulation of translation. In addition, the sequence of the β-subunit contains many ATP binding sites, and the binding of ATP causes the dissociation of Met-tRNAi and favours mRNA binding (Gonsky et al., 1990). Recent studies implicate that deletion of the β-subunit of eIF2 suppresses testicular cancer incidence by decreasing the total available eIF2 required for efficient translation and cell proliferation (Heaney et al., 2009).

1.4, Structure and Function of eIF2γ;

The largest subunit of eIF2 is the γ-subunit and is found to be homologous to the bacterial SelB, EF1A and EF-Tu along with other G-proteins (Gaspar et al., 1994; Hannig et al., 1993; Erickson et al., 1997; Keeling et al., 1998). It contains all the three consensus domains (GXXXXGK, DXXG, NKXD) required for guanine nucleotide binding, and mutations in these domains decrease the binding of GDP to eIF2γ in yeast and mammals, implying that the γ-subunit serves as the primary guanine nucleotide binding site (Naranda et al., 1995). The γ-subunit is also thought to possess a latent GTPase activity, which is stimulated by its interaction with eIF2β and eIF5. In addition to guanine nucleotide binding function, γ-subunit is also implicated in binding Met-tRNAi through its N-terminus (Gaspar et al., 1994; Naranda et al., 1995; Erickson et al., 1997; Hannig et al., 1993). Recent reports indicate that the levels of the γ-subunit is regulated by cell division control 123 (cdc123) and checkpoint fork head associated with RING proteins (Chfr), which in turn promote cell cycle arrest at the G1 phase under conditions of nutrient starvation or in response to pheromones, thereby suggesting that the levels of the γ-subunit can in turn regulate cell cycle progression (Bieganwski et al., 2004). The crystal structure of the archaeal γ-subunit reveals an N-terminal G-domain with a β sheet flanked by 5 α helices, which are characteristic of other G binding proteins and GTPases. However, it also contains an extended zinc ribbon motif with two pairs of cysteines which may be important in mediating the α–γ or β–γ interaction (Roll-Mecak et al., 2004; Schmitt et al., 2010). In the physiological context, deletion of a segment of Y-chromosome encoding the γ-subunit of eIF2 is shown to be associated with failure in spermatogenesis and as a consequence causes sterility in mice and humans, implicating the importance of the availability of the trimeric eIF2 in germ cell development (Heaney et al., 2009).

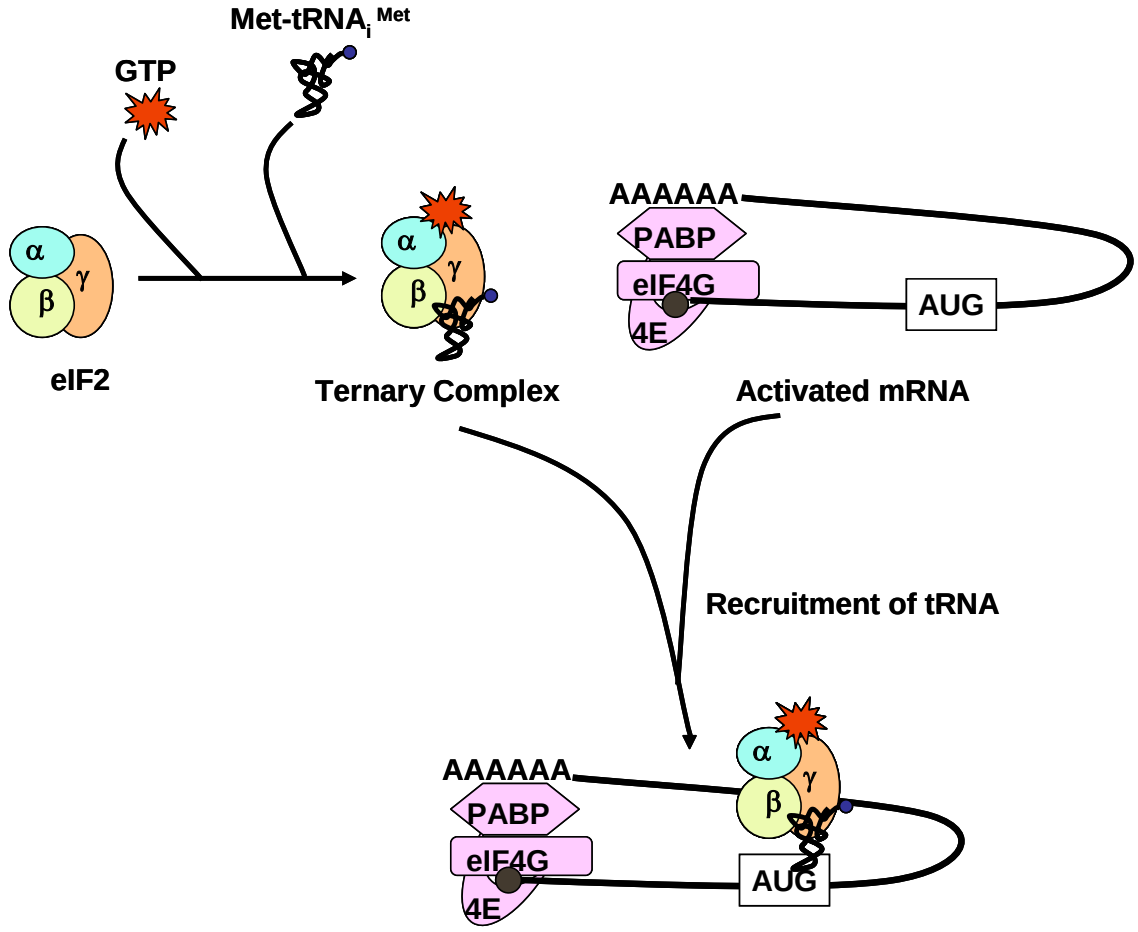
In conclusion, there exists a clear functional demarcation between each of these subunits. For instance, the α-subunit is primarily involved in regulating the function of eIF2, while the β-subunit serves as a hub for protein interaction and the γ-subunit is required for GTP/GDP and Met-tRNAi binding and has an intrinsic GTPase activity. Therefore, the

function and regulation of eIF2 is dependent on the co-ordinated interaction among these subunits and their interaction with other proteins.

1.5, Function of eIF2 in translation initiation;

Eukaryotic initiation factor 2 mediates a key step in translation initiation, which involves the selection and transfer of the initiator methionyl tRNA to the 40S ribosomal subunit in the form of a ternary complex comprising eIF2, GTP and Met-tRNA$_i^{Met}$, to form the 43S pre-initiation complex (PIC) (Lloyd et al., 1980; Pain, 1996; Kimball, 1999; Kapp and Lorsch, 2004; Schmitt et al., 2010). This reaction is promoted by other factors such as eIF3, eIF1A and eIF5B (Kolitz and Lorsch, 2010), and appears to be a critical step in the translation initiation process and therefore is highly regulated. The 43S pre-initiation complex binds mRNA and forms the 48S initiation complex, which then scans the mRNA for the initiator AUG codon (Hershey and Merrick, 2000; Kozak, 1999 and 2000). The fidelity of translation is dictated by the correct recognition of the start codon, and eIF2 is also shown to play an important role in start site selection along with other factors such as eIF1 and eIF5 (Donahue et al., 1988; Cigan et al., 1989; Castilho-Valavicius et al., 1990; Cui et al., 1998; Yoon and Donahue, 1992; Huang et al., 1997; Hashimoto et al., 2002; Preiss and Hentze, 2003). Upon recognition of the start codon, the 43S complex stops and establishes base pairing between the anti-codon of Met-tRNA$_i^{Met}$ and the start codon, forming a stable 48S initiation complex. At this point, eIF5 which binds to eIF2 in the 43S pre-initiation complex, stimulates the GTPase activity of the γ-subunit (Chaudhuri et al., 1994; Das et al., 1997; Asano et al., 1999; Das and Maithra, 2000; Das et al., 2001), thereby promoting hydrolysis of eIF2-GTP. As a consequence, eIF2-GDP and inorganic phosphate are formed and they remain associated with the PIC. eIF1 bound at the P-site detects the correct codon-anticodon base pairing because of its ability to alter the equilibrium of the PIC which is thought to exist in two forms, the 'open' (scanning competent) and 'closed' (scanning incompetent) conformations. Upon start codon recognition, eIF1 and 1A bound to the PIC, move farther away causing the dissociation of eIF1 (Pestova and Kolupaeva, 2002). At this step, the inorganic phosphate is released and enables the release of eIF2-GDP in complex with eIF5. The importance of eIF2 in start site selection is revealed by the mutational

Fig. A



Role of eIF2 in translation initiation

eIF2 plays an important role in delivering the initiator tRNA to the 40S ribosomal subunit. This is achieved by forming a ternary complex of eIF2, GTP and initiator tRNA (Met-tRNA$_i^{met}$). The ternary complex is then loaded on to the mRNA, which is activated by the binding of eIF4F complex to form the 43S pre-initiation complex. eIF2 along with eIF1 and 5, also plays an important role in start site selection, and places the anticodon of the tRNA at the correct AUG codon.

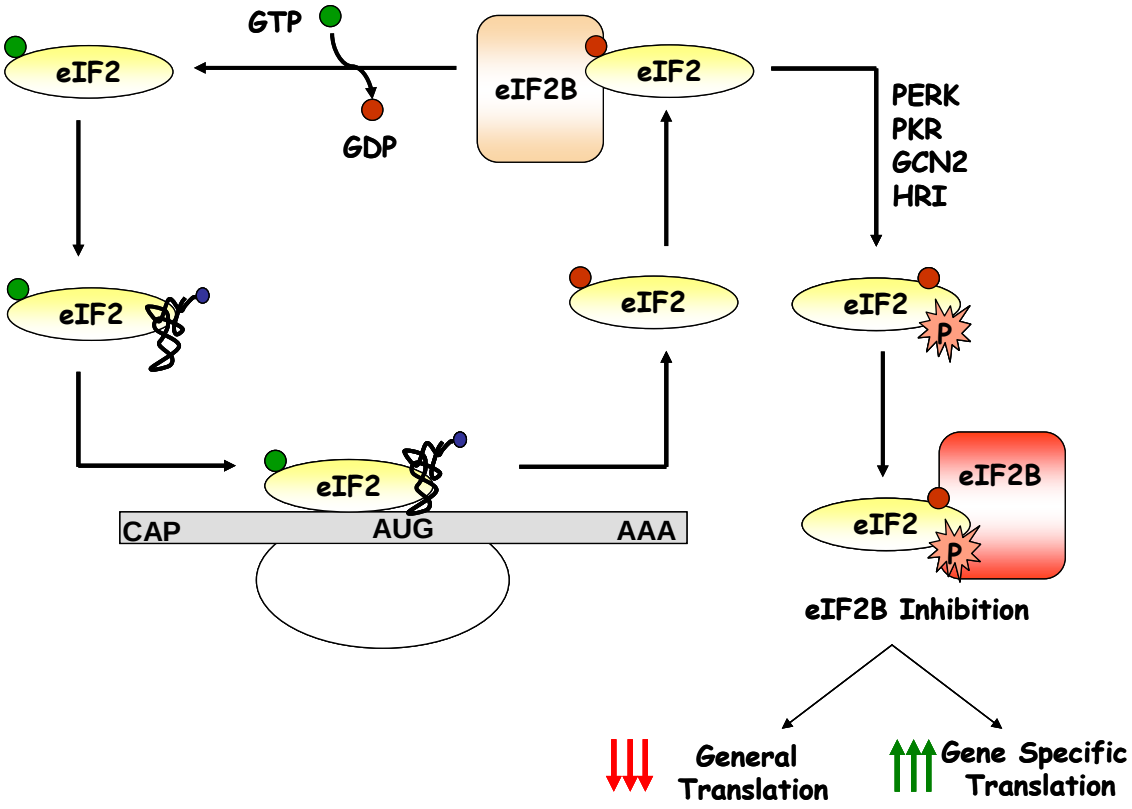
analyses, where in the mutants isolated for the three subunits of eIF2 show an error prone initiation site selection. For instance, mutations in the GTP-binding site of the γ-subunit of eIF2, affect initiation codon selection fidelity, and appear to increase both the eIF5-indepedent GTPase activity of eIF2, and the rate of dissociation of Met-tRNAi from ternary complex (Huang et al. 1997). The constitutive activation of the GTPase activity of eIF2 would lead to premature release of Met-tRNAi. Hence the GTPase stimulatory activity of eIF2 (Das and Maitra, 2001), should be precisely regulated to synchronize with the AUG codon selection. Similar to mutations in eIF2, mutations in eIF5 also reduce the fidelity of initiation site selection *in-vivo* and result in the hyper stimulation of GTPase activity of eIF2 (Huang et al. 1997).

1.6, Recycling and release of eIF2.GDP by eIF2B;

At the end of initiation, eIF2-GTP is hydrolyzed to eIF2-GDP, and is released from the 48S initiation complexes. Unless the GDP bound to eIF2 is exchanged with GTP, eIF2 cannot enter into another round of productive initiation. Since eIF2 has a higher affinity for GDP than for GTP at physiological concentrations of Mg^{2+} (Panniers and Henshaw, 1983; Panniers et al., 1988; Proud, 1992; Kapp and Lorsch, 2004), the GDP/GTP exchange on eIF2 is catalyzed by a rate-limiting heteropentameric protein called eIF2B (Konieczny and Safer, 1983; Matts et al., 1983). Although eIF2B exists in a 1:1 complex with eIF2 under physiological conditions (Cigan et al., 1991; Proud, 1992), the levels of eIF2B are approximately 20-30% of total eIF2α. Phosphorylation of a limited portion of eIF2α (~20-30%) is sufficient to inhibit all the available eIF2B activity (Leroux and London, 1982; Pain and Clemens, 1991; Price and Proud, 1994). Subsequent studies have shown that, this is because the trimeric eIF2 in which the α-subunit is phosphorylated, interacts with the five subunit eIF2B to form a 15 S complex {eIF2α(P). eIF2B}, in which the eIF2B becomes non-functional (Rowlands et al., 1988; Thomas et al., 1984). A type 1 protein phosphatase that dephosphorylates eIF2α in the 15 S complex, releases eIF2B and restores its function (Babu and Ramaiah, 1996; Brush et al., 2003).

The five subunits of eIF2B can form two sub-complexes: catalytic and regulatory. The catalytic sub-complex comprises of γ- and ε-subunits and the regulatory sub-complex consists of α-, β- and δ-subunits. The ε-subunit of catalytic sub-complex is primarily

Fig. B



Recycling of eIF2.GDP by eIF2B

eIF2.GDP, released at the end of translation initiation has to be recycled back to active eIF2.GTP, to enter into another cycle of productive initiation. This is mediated by a heteropentameric protein called eIF2B, which by means of productive molecular rearrangement replaces GDP with GTP. Phosphorylation of eIF2α occurs in response to stress, and is mediated by four well characterized eIF2α kinases. Phosphorylated eIF2α forms a tight complex with eIF2B, and inhibits its guanine nucleotide exchange activity. This inhibition results in global shut down of translation. However, cetain gene specific mRNAs like ATF4, ATF5 and GCN4 are efficiently translated in response to eIF2α phosphorylation.

involved in the exchange of GDP on eIF2 with GTP (Fabian et al., 1997; Pavitt et al., 1998). The regulatory sub-complex of eIF2B is capable of differentiating the phosphorylated form of eIF2α from the unphosphorylated form (Pavitt et al., 1998), and binds more efficiently with the phosphorylated eIF2α (Rowlands et al., 1988; Pavitt et al., 1998; Sudhakar et al., 1999 and 2000; Krishnamoorthy et al., 2001). The mechanism of eIF2 recycling by eIF2B is uncertain, and various models have been proposed to explain this process (Manchester, 1987; Hinnebusch, 2000). The sequential mechanism proposed based on kinetic analyses appears to be consistent (Dholakia and Wahba, 1989), and suggests the formation of an intermediate complex comprising of GTP-eIF2B-eIF2-GTP. This model implicates the presence of two gunanine nucleotide binding sites, one each in eIF2 and eIF2B. Consistently, potential nucleotide binding site is present in the γ- and ε-subunits of eIF2B (Koonin, 1995). Although, photoaffinity labeling studies suggest the presence of a GTP binding site in the β-subunit of eIF2B (Dholakia and Wahba, 1989), this subunit is dispensible for GEF activity in vitro (Fabian et al., 1997; Pavitt et al., 1998).

In physiological conditions, eIF2 and eIF2B co-migrate together. Interestingly, eIF2 is also found on the 60S subunits of 80S initiation complexes, and is released by the addition of purified eIF2B to lysates, where protein synthesis is inhibited due to phosphorylation of eIF2α (Thomas et al., 1985; Ramaiah et al., 1992). Although the mechanism governing the translocation of eIF2 from 40S to 60S subunits is not understood, addition of purified eIF2B that restores inhibition of protein synthesis mediated by eIF2α phosphorylation also reduces the levels of eIF2 bound to the 60S subunits. These findings thereby suggest that eIF2B also participates in the recycling of ribosome bound eIF2.GDP.

The GNE activity of eIF2B is also regulated by its own phosphorylation. Atleast four kinases are known to phosphorylate the ε-subunit of eIF2B *in-vitro*. These include casein kinases (CK) I and II, glycogen synthase kinase 3 (GSK3), and dual specificity tyrosine phosphorylated and regulated kinase (DYRK) (Singh et al., 1996). Phosphorylation by casein kinases are shown to stimulate the activity of eIF2B (Singh et al., 1994 and 1996). In contrast, phosphorylation of Ser-535 in rat eIF2Bε or Ser-540 in the human

counterpart by GSK3 is required for inhibition of eIF2B activity in response to insulin (Wang et al., 2002; Pap and Cooper, 2002).

1.7, eIF2 phosphorylation sites;

Phosphorylation of eIF2α occurs in response to diverse stresses and is considered as a highly conserved adaptive mechanism in eukaryotes. It provides the cell with an efficient, rapid and reversible means to respond to a variety of stimuli. Phosphorylation of eIF2α is implicated in normal physiological development, cell differentiation, stress adaptation, apoptosis, learning and memory (Gerlitz et al., 2002; Scheuner et al., 2001; Kimball and Jefferson, 2004; Brewer et al., 1999; Harding et al., 2000; Costa-Mattioli et al., 2007). Although two phosphorylation sites have been identified at positions 48 and 51 (Wettenhall et al., 1986; Kudlicki et al., 1987), mutational analyses suggest that the conserved Ser-51 residue is the only phoshorylation site (Colthrust et al., 1987; Price and Proud, 1990; Pathak et al., 1988b). In addition to the phosphorylation site at the conserved Ser-51 residue, yeast eIF2α contains three casein kinase II phosphorylation sites at positions 292, 294 and 301 in the C- terminal region. Although non-phosphorylatable mutants at these positions did not confer any specific phenotype in wild type cells, they were found to exacerbate the growth defects of mutants lacking the eIF2α phosphorylation mediated regulation of eIF2B. These observations thereby suggest that CKII phosphorylation can affect eIF2 function only when there is a defect in eIF2 recycling, by promoting a non-productive interaction between eIF2-GDP and eIF2B (Feng et al., 1994). Similar CKII phosphorylation sites are also observed in plant eIF2α (Feng et al., 1994; Mehta et al., 1986; Janaki et al., 1995; Laxminarayana et al., 2002).

The β-subunit of human eIF2 is also shown to be phosphorylated by four kinases *in-vitro*. These include Protein kinase A (PKA), Protein kinase C (PKC), Caesin kinase II (CKII) and DNA dependent protein kinase (DNA-PK). The phosphorylation sites have been mapped at Ser-218 for PKA, Ser-13 for PKC, and Ser-2 and Ser-67 for CKII. However, the phosphorylation sites for DNA-PK have not yet been identified till date. Phosphorylation of the β-subunit of eIF2 is shown to occur under various conditions such as heat shock (Duncan and Hershey, 1984), serum deprivation (Duncan and Hershey, 1985), diabetes (Garcia et al., 1996) and during birth (Luis et al., 1993). Recent evidences

indicate that almost all of the cellular eIF2β is phosphorylated at Ser-2 by CKII under physiological conditions (Llorens et al., 2006). Interestingly, phosphorylation of the β-subunit does not affect the function of eIF2 in forming the ternary complex (Benne et al., 1976), but affects its interaction with GDP (Singh et al., 1994). Although there are multiple phosphorylation sites and kinases, the physiological significance of phosphorylation of the β-subunit remains to be elucidated.

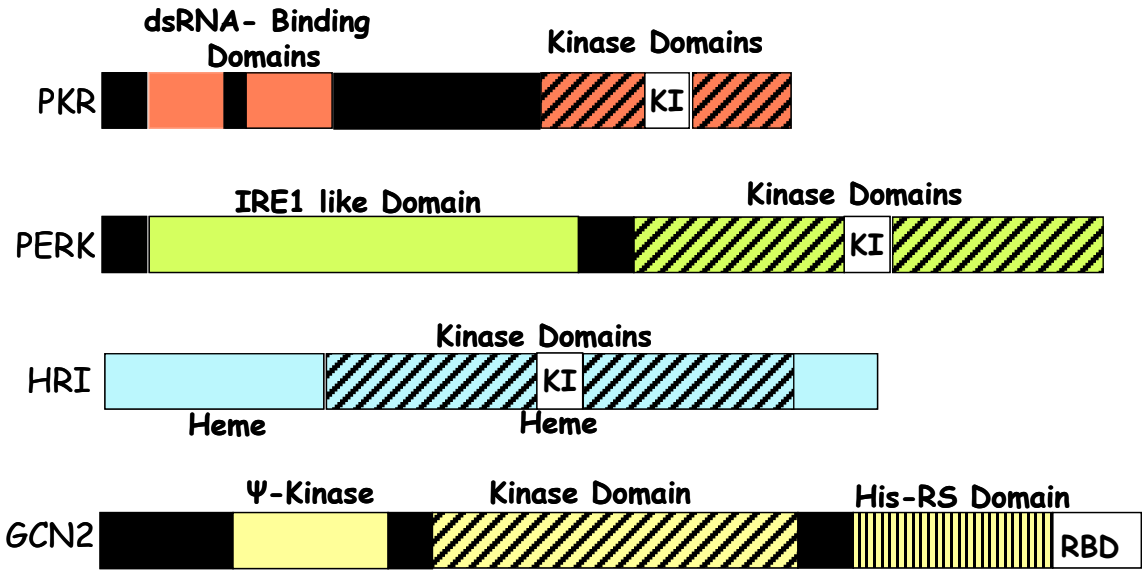
1.8, eIF2α kinases;

Multiple eIF2α kinases that phosphorylate the conserved Ser-51 residue have been identified and characterized in many species. For instance, four different kinases are identified in mammals, and each of them share a conserved kinase domain but differ in their regulatory domains, and therefore are activated in response to different stimuli. The four kinases include: GCN2 (General control non derepressible kinase 2); HRI (Heme regulated inhibitor); PKR (Double stranded RNA dependent protein kinase); PERK (PKR like endoplasmic reticulum resident kinase);

GCN2: It is highly expressed in liver and brain (Berlanga et al., 1999; Sood et al., 2000), and is activated in response to amino acid starvation by a mechanism that involves the binding of uncharged tRNAs to the regulatory domain, which is homologous to histidyl-tRNA synthetase (His-RS) (Wek et al., 1990; Rolfes and Hinnebusch, 1993; Harding et al., 2000; Yang et al., 2000; Zhang et al., 2002). Interestingly, GCN2 is also activated by other stresses such as UV irradiation and proteosomal inhibition (Wek et al., 2006). In a hypothetical model, the inactive form of GCN2 is speculated to occur as a dimer, where the C-terminal domain inhibits auto-phosphorylation and activation by interacting with the kinase domain. Binding of uncharged tRNAs to His-RS like domain, induces conformational change that disrupts the interaction between C-terminal and kinase domain. Auto-phosphorylation ensures further structural alteration of the kinase domain and facilitates substrate binding and phosphorylation (Hinnebusch, 2000).

HRI: It is predominantly expressed in erythroid cells and is activated in response to heme-deficiency, oxidative and heat stress, and exposure to certain diffusible gases in erythrocytes (Chen et al., 1991; Chen, 2000; Han et al., 2001; Uma et al., 2001; Zhang et al., 2002). Heme binds reversibly to the kinase insert region and promotes inter-subunit

Fig. C



eIF2a kinases

The figure depicts the homology among four different eIF2α kinases: PKR, PERK, GCN2 and HRI. The eIF2α kinases share a conserved kinase domain but differ in their regulatory domains. Although, different stresses are shown to activate different eIF2α kinases, all of them converge upon phosphorylation of eIF2α at Ser-51 position (Proud 2005).

disulphide bond formation of the homodimer, and inhibits its autokinase and eIF2α kinase activity (Chen et al., 1989). However, a decline in levels of heme, results in multiple auto-phosphorylation of the HRI homodimer, which in turn phosphorylates eIF2α.

PKR: It is ubiquitously expressed and is activated in response to dsRNA of cellular, viral and synthetic origin (Meurs et al., 1990; Williams, 1999; Kaufman, 2000). In addition, PKR is stimulated by a variety of signals such as pro-inflammatory stimuli, growth factors, cytokines and oxidative stress. Interestingly, a wide range of cellular stresses can activate PKR through PACT (PKR associated activator), and this activation is independent of dsRNA (Patel and Sen, 1998). PKR has two dsRNA binding domains (dsRBD) in the N-terminus, and a kinase domain (KD) near the C-terminus. In non-stressed cells, PKR is in a monomeric latent state due to the autoinhibitory effect of its dsRBDs, which interact with the kinase domain. After binding dsRNA, PKR undergoes a number of conformational changes that relieve the autoinhibitory interactions of the enzyme, and allow subsequent substrate recognition (Ung et al., 2001).

PERK: It is expressed in secretory tissues, particularly the pancreas, and is activated in response to accumulation of unfolded or malfolded proteins in the ER (Shi et al., 1998; Harding et al, 1999; Ron and Harding, 2000; Kaufman, 2004). PERK is a type 1 ER transmembrane protein with a regulatory luminal domain and catalytic cytoplasmic domain. In the non-stressed state, PERK is bound with BiP, an ER chaperone and also known as GRP78 (glucose regulated protein 78). In response to accumulation of misfolded proteins during ER stress, BiP is released from PERK to counter the increased load of unfolded proteins. Release of BiP from PERK, unmasks the oligomerization motif in the luminal domain, and facilitates its oligomerization and subsequent trans-auto-phosphorylation. Phosphorylation of serine and threonine residues in the activation loop of the kinase domain activates PERK, and promotes eIF2α phosphorylation (Schroder and Kaufman, 2005).

In addition to the above mentioned kinases, various eIF2α kinase homologues have been identified in a number of species and these include: TgIF2KA from *Toxoplasma,* which is activated in response to heat and alkaline stress, and promotes parasite differentiation. It appears to be distinct from other members of the eIF2α kinase family, and is restricted to

parasites (Sullivan et al., 2004); *Plasmodium* is shown to possess two eIF2α kinases, Pfpk4 and PfeIK1. Pfpk4 is activated in response to heme deficiency (Mohrle et al., 1997) while PfeIK1 is shown to be activated under conditions of nutrient starvation (Fennel et al., 2009); homologues of PERK and GCN2 have been identified in invertebrates like *Caenorhabditis elegans* (Shen et al., 2001) and *Drosophila melanogaster* (Pomar et al., 2003). In addition, a novel eIF2α kinase called BeK has been identified in *Bombyx mori,* which is activated in response to heat and osmotic stress. Interestingly, BeK does not share any homology with other eIF2α kinases in its regulatory domain, and therefore is thought to have a novel regulatory mechanism (Prasad et al., 2003).

1.9, Cellular regulators of eIF2α phosphorylation;

Phosphorylation of eIF2α is highly dynamic and regulated. In addition to the above mentioned kinases, there exist a wide number of cellular regulators which affect eIF2α phosphorylation. Primarily these regulators facilitate translational recovery upon stressful cellular insults. The eIF2α phosphatase activity is mainly attributed to PP1 (Babu and Ramaiah, 1996), which forms a complex with many regulatory proteins such as GADD34 (growth arrest and DNA damage inducible protein) (Novoa et al., 2001) and CReP (constitutive repressor of eIF2α phosphorylation) (Jousse et al., 2003). CReP is a constitutive regulatory subunit of PP1c, which contributes to the high eIF2α phosphatase activity in mammalian cells (Jousse et al., 2003). GADD34 is induced in response to stress by means of ATF4 dependent gene expression program, and serves as a feed back mechanism in regulating eIF2α phosphorylation (Novoa et al., 2001; Brush et al., 2003). Nck1, an SH2/SH3 domain containing adaptor protein, also forms a complex with PP1c, and regulates the levels of ER stress-induced eIF2α phosphorylation (Latrielle and Larose, 2006; Kebache et al., 2002). Although, many regulators affect eIF2α dephosphorylation, multiple cellular proteins are found to interact with eIF2α kinases and modulate their activity. $P58^{IPK}$ was first identified as an inhibitor of PKR, and belongs to the tetratricopeptide (TPR) family. Under normal conditions, $P58^{IPK}$ remains latent by forming a complex with Hsc (heat shock protein) 40. However, in response to stress, it

dissociates from Hsc40 and interacts directly with PKR, thereby disrupting PKR activity (Lee et al., 1994; Gale et al., 1996; Tan et al., 1998; Melville et al., 1999). Reports also indicate that $P58^{IPK}$ can interact with and inactivate PERK (Yan et al., 2002). Likewise, chaperones Hsp70 and 90 bind to and inhibit PKR phosphorylation and apoptosis. Inhibition of PKR activity by Hsp70 requires Fanconi's anaemia complementation group C (FANCC) protein (Pang et al., 2002). Human dihydrouridine synthetase 2 (hDUS2), which is a tRNA modifying enzyme is expressed in HT1080 cells, and serves as a potential PKR inhibitor (Mittelstadt et al., 2008). Nucleophosmin, a nucleolar phosphoprotein, implicated in ribosome biogenesis is also shown to interact with PKR and prevent PKR-mediated eIF2α phosphorylation and apoptosis (Pang et al., 2003). Similarly, glycoprotein p67, a methionine aminopeptidase 2 (MetAP2), inhibits eIF2α phosphorylation mediated by HRI, GCN2, PERK and PKR. However, the molecular mechanism of inhibition remains unclear (Gil et al., 2000; Datta et al., 2004). Although most of the cellular regulators of eIF2α kinases are found to inhibit eIF2α phosphorylation, RAX, a mouse protein and its human orthologue PACT, are found to activate PKR. RAX/PACT, which is phosphorylated in response to diverse stress conditions, is found to interact with PKR and activate it (Bennett et al., 2004; Patel et al., 2000). In addition, tumour suppressor melanoma differentiation associated gene 7 (Mda 7) and E2F1, are shown to induce PKR expression and activation in carcinoma cells (Pataer et al., 2005; Vorburger et al., 2005). The other less characterized inhibitors of PKR include: L18, a 60S ribosomal subunit protein (Kumar et al., 1999); Alu RNAs, which are RNA polymerase III transcripts (Chu et al., 1998); 15 kD inhibitor of PKR (dRF) from 3T3-F442A differentiated cells (Judware and Petryshyn, 1991 and 1992).

1.10, eIF2α phosphorylation regulates global and specific translation;

Phosphorylation of eIF2α converts the protein from substrate into a competitive inhibitor of eIF2B, and thereby interferes with recycling of eIF2 to its active GTP bound form, and limits the ternary complex formation (Dever et al., 1992; Gebauer and Hentze, 2004; Holcik and Sonenberg, 2005; Ron and Harding, 2007). As a result, afflicted cells show a global shutdown of translation which enables them to prevent the synthesis of unwanted proteins that could interfere with cellular stress response. The translational attenuation

mediated by eIF2α phosphorylation is exemplified by the fact that the translation of cyclin D1, a key protein involved in cell cycle regulation, is tightly regulated by phosphorylated eIF2α. PERK and GCN2 mediated eIF2α phosphorylation leads to a reduction in cyclin D1 levels by translational attenuation, and promotes G1 cell cycle arrest (Aktas et al. 1998; Brewer et al., 1999; Hamanaka et al., 2005;). As cell division places extreme energy demands on the cell, G1 arrest through eIF2α phosphorylation provides the cell with an opportunity to divert the cellular resources towards mechanisms contributing to restore cellular homeostasis (Brewer and Diehl, 2000). Although translation of most mRNAs is down-regulated by eIF2α phosphorylation, the latter also stimulates the translation of specific mRNAs such as ATF4 and ATF5 in mammals and GCN4 in yeast (Hinnebusch, 1994; Harding et al., 2000; Wek et al., 2006; Zhou et al., 2008), which are required to alleviate stress. The mechanism of specific induction of certain mRNAs in response to eIF2α phosphorylation is well illustrated by the general amino acid control response in *S. cerevisae*. Amino acid starvation leads to the activation of GCN2 kinase in yeast and results in the inhibition of translation. However, the 5' UTR of GCN4 mRNA contains four short upstream open reading frames (uORFs). Under normal conditions, where there is no limitation for the ternary complex, the ribosomes scan and translate these uORFs and dissociate from the mRNA prior to the GCN4 start codon. In contrast, under conditions of nutrient starvation, eIF2α is phosphorylated by GCN2, thereby decreasing the levels of ternary complex. As a consequence, a fraction of the scanning ribosome form the active translational complex only after the uORFs have been bypassed, thereby allowing initiation at the authentic GCN4 start codon (Hinnebusch and Natarajan, 2002). Similarly, translation of ATF4 in higher eukaryotes is also mediated by two uORFs, where in the uORF2 overlaps with ATF4 ORF and inhibits its translation under conditions of low levels of eIF2α phosphorylation. However, in cases where there is a high level of eIF2α phosphorylation, the ribosomes scan past the uORF2, thereby allowing the translation of ATF4 (Harding et al., 2000; Scheuner et al., 2001; Vattem and Wek, 2004). CCAAT/enhancer binding proteins α and β (C/EBP α and β), contain one uORF in a reading frame and has multiple initiation sites, thereby facilitating the synthesis of multiple isoforms. The isoform ratio is modulated by

phosphorylation of eIF2α, as the phosphorylated form of eIF2 favors the synthesis of full length isoforms, which promote proliferation arrest and contact inhibition in cells. In contrast, limited phosphorylation of eIF2α results in truncated isoforms of C/EBP α and β (Calkhoven et al., 2000). Recent report also indicates that an isoform of protein kinase c, PKC ν, contains two uORFs and like ATF4, its translation is dependent on GCN2 mediated eIF2α phosphorylation (Raveh-Amit et al., 2009). There are numerous other examples of mRNAs such as ATF3, oncoprotein mouse double-minute 2, human epidermal growth factor receptor-2, S-adenosylmethionine decarboxylase, and β2-adrenergic, retinoic acid, glucocorticoid, and estrogen receptors in mammals, whose translation is regulated by uORFs. However, their dependence on eIF2α phosphorylation is not clear. In addition, phosphorylation of eIF2α also promotes cap-independent translation of certain mRNAs that contain internal ribosomal entry sites (IRES) in their 5' UTR (Holcik and Sonenberg, 2005). The best characterized IRES-containing mRNA that is regulated by eIF2α phosphorylation is cationic amino acid transporter (CAT1) mRNA (Fernandez et al., 2002). Other mRNAs containing IRES elements whose translation is up-regulated in reponse to stress include: platelet derived growth factor 2 (PDGF2), protein kinase C-δ, hypoxia-inducible factor-1α, X-linked inhibitor of apoptosis (XIAP), ornithine decarboxylase, vascular endothelial growth factor (VEGF) and c-Myc (Gerlitz et al., 2002; Gebauer and Hentze, 2004; Holcik and Sonenberg, 2005). However, it remains elusive whether mere shut down of cap dependent translation induces efficient translation of such IRES containing mRNAs, which remain usually latent under normal conditions, or if there exists a molecular switch like eIF2, which allows the shift from cap-dependent translation to IRES mediated translation.

1.11, Importance of eIF2α phosphorylation in normal cellular physiology;

Apart from being a stress adaptive pathway, eIF2α phosphorylation is also essential for normal cell physiology, differentiation and cell death. The first clue in implicating the importance of eIF2α phosphorylation in regulating intermediary metabolism came from studies on homozygous mice harboring a non-phosphorylatable form of eIF2α. The embryos died shortly after birth due to their inability to maintain blood glucose levels as

a result of defective pancreatic islet development (Scheuner et al., 2001; Harding et al., 2002; Scheuner and Kaufman, 2008). Heterozygous mice too showed subtle metabolic defects such as enhanced body weight and hyperlipidemia. Interestingly, when the heterozygous mice were placed on a high fat diet, they became obese and glucose intolerant (Scheuner et al., 2005; Marciniak et al., 2006). Similar to the mice harboring non-phosphorylatable form of eIF2α, *Perk* $^{-/-}$ mice show stunted growth and develop neonatal insulin dependent diabetes (Zhang et al., 2002). While *Gcn2* $^{-/-}$ mice grow and reproduce normally when fed freely, they lose body mass heavily when fed on leucine free diet when compared to wild type mice (Anthony et al., 2004). Further, *Gcn 2*$^{-/-}$ mice also show reduced viability upon amino acid starvation (Zhang et al., 2002). *Hri* $^{-/-}$ mice demonstrate abnormal erythroid response to iron and heme deficiency, and show a reduced survival rate in response to erythroid stress, thereby establishing its role as a physiological regulator for the synthesis of the α and β globins in erythrocytes (Han et al., 2001; Han et al., 2005). Although, homozygous PKR knockout mice are fertile and develop normally, they show defects in IFN- and dsRNA mediated signaling (Kumar et al., 1997). In addition, *Pkr* $^{-/-}$ mice are compromised in their ability to respond to viral challenges (Yang et al., 1995; Abraham et al., 1999) These observations readily explain the profound metabolic consequences as a result of deprivation of eIF2α phosphorylation mediated translational control.

1.12, Role of eIF2α phosphorylation in virus sustenance;

Viruses, for their productive infection, are dependent on the host translational apparatus for the synthesis of their own proteins, as they do not encode components of the translational machinery. Therefore, a critical part of the cellular anti-viral response involves the shut down of global translation to dampen virus production (Schneider and Mohr, 2003). On the other hand, viruses employ intricate strategies to effectively manipulate the elaborate cellular regulatory network, in order to gain access to the host cell translation machinery, and to counteract host defense mechanisms that act at the translational level. Phosphorylation of eIF2α is a major player in inhibiting viral translation and sustenance. For instance, accumulation of double stranded RNA intermediates within the virus infected cells, signal the viral intrusion by triggering the

Table 1

Viral Regulators of eIF2α phosphorylation				
i) RNA and proteins which directly modulate PKR				
1	Adenovirus	VAI RNA	It's a highly structured dsRNA which interacts with PKR and inactivates it.	Schneider, 2000
2	Epstein-Barr virus (EBV)	EBER-1 and 2	Interacts with PKR and inhibits its activation.	Sharp et al., 1993
3	Human immunodeficiency virus (HIV1)	Tat responsive region RNA (TAR)	It is a highly structured RNA element which interacts with PKR and can activate or blocks its kinase function in-vitro.	Gunnery et al., 1990
4	HIV1	Tat	Interacts with PKR directly and prevents its activation. Tat is phosphorylated by PKR and phosphorylated Tat facilitates tight binding of TAR to PKR.	McMillan et al., 1995; Brand et al., 1997; Endo-Munoz et al., 2005
5	Hepatitis C virus	NS5A	Interferon sensitive determining region (ISDR) of NS5A interacts with PKR and inhibits its activity.	Gale et al., 1997
6	Influenza virus	$P58^{IPK}$	$P58^{IPK}$, an inhibitor of PKR, is activated upon influenza virus infection	Lee et al., 1994
7	Herpes simplex virus	Us11	Interacts with N-terminal 170 residues of PKR and inhibit its activation	Mohr and Gluzman, 1996; Cassady et al., 1998
8	Baculovirus	PK2	Resembles the C-terminus of the kinase domain and inhibits it.	Dever et al., 1998
9	Human Herpes Virus-8	v-IRF2	Physically interacts with PKR and prevents its auto-phosphorylation.	Burysek and Pitha, 2001
10	Human cytomegalovirus (hCMV)	IRS1, TRS1	Directly interact with both PKR and dsRNA.	Cassady, 2005

11	Murine cytomegalovirus (mCMV)	m142 and m143	m142 and 143 bind to each other and interact with PKR and inhibit its activity.	Valchanova et al., 2006; Child et al., 2006 and 2009;
12	EBV	SM	RXP domain of the protein interacts with both PKR and dsRNA and inhibits PKR activation	Poppers et al., 2003
13	Ebola virus	VP35	Interacts with PKR and reverses its activation	Schumann et al., 2009
14	Adeno virus	E1B-55K/E4orf6	Blocks PKR activation and eIF2α phosphorylation	Spurgeon and Ornelles, 2009
ii) Proteins affecting PKR stability				
15	Poliovirus	Cellular proteinase	Degrades PKR	Black et al., 1989, 1993
16	Rift valley fever virus	NSs	Promotes post-transcriptional down regulation of PKR and also promotes its degradation.	Ikegami et al., 2009
iii) Proteins masking or sequestering dsRNA				
17	Vaccinia virus	E3L	Sequesters dsRNA and prevents PKR activation and also prevents the activation of 2'-5' oligo adenylate synthetase	Chang et al., 1992; Rivas et al., 1998
18	Cytomegalovirus	TRS1	Sequesters RNA and prevents PKR activation	Cassady, 2005
19	Influenza virus	NS1	Blocks the dsRNA mediated activation of PKR.	Lu et al., 1995; Bergmann, 2000
20	Porcine rotavirus group C	NSP3	Homologous to dsRNA binding proteins	Langland et al., 1994
21	Rotavirus group A	NSP5	Sequence independent RNA binding protein.	Vende et al., 2002
22	Reovirus	σ3, σ4	Binds to dsRNA and blocks PKR activation.	Beattie et al., 1995; Yue and Shatkin, 1997; Jacobs and Langland, 1998
23	EBV	SM	RXP domain of the protein interacts with both PKR and dsRNA and inhibits PKR activation.	Poppers et al., 2003
24	Ebola virus	VP35	Binds dsRNA through its C-	Feng et al., 2007;

			terminal cluster of basic amino acids and blocks PKR activation.	Schumann et al., 2009
25	Betanodavirus	B2	Binds dsRNA independent of its sequence and blocks PKR.	Fenner et al., 2006
ii) Proteins mimicking eIF2 (Pseudosubstrates)				
26	Hepatitis C virus	E2	Identical to the phosphorylation sites on PKR and eIF2α	Taylor et al., 1999
27	Myxoma virus	M156R	Structurally mimics eIF2α and competes with PKR	Ramelot et al., 2002
28	Vaccinia virus	K3L	Homologous to eIF2α and serves as a pseudo substrate for PKR	Davies et al., 1992, 1993; Carroll et al., 1993
29	Swine Pox virus	C8L	Structurally similar to the N-terminal third of eIF2α	Kobayashi et al., 2000
30	Amblystoma tigrinum virus	ReIF2H	Homologous to eIF2α and serves as a pseudo substrate for PKR	Essbaur et al., 2001
31	HIV-1	TAT	Homologous to eIF2 and competes with PKR	Gunnery et al., 1990
iii) Proteins limiting dsRNA				
32	Sendai virus	SeV C	Limits dsRNA production by regulating viral genome replication and transcription	Takeuchi et al., 2008
33	Measles virus	MeV C	Limits production of dsRNA.	Nakatsu et al., 2006
34	Simian virus 5	P, V	Limits the synthesis of aberrant viral mRNA and dsRNAs.	Ong et al., 2005; Gainey et al., 2008
vi) Proteins causing relocation of PKR to nucleus or other insoluble cytoplasmic compartments				
35	Human papilloma virus	E6 and E7	Causes relocalization of PKR into dense clumps in cytoplasm.	Hebner et al., 2006
36	mCMV	m142 and m143	Promotes nuclear recolalization.	Child et al., 2009

37	hCMV	TRS1	Causes nuclear relocation.	Hakki et al., 2006
vii)Proteins modulating PERK				
38	Herpes simplex virus 1 (HSV1)	γ_B glycoprotein	Regulates viral protein accumulation by interacting with the ER luminal domain of PERK	Mulvey et al., 2007
39	EBV	Latent membrane protein 1 (LMP1)	Activates PERK to induce phosphorylation of eIF2 and subsequent ATF4 translation. ATF4 in turn trans-activates LMP promoter.	Lee and Sugden, 2008
viii)Viral proteins affecting cellular inhibitors/activators of eIF2α phosphorylation				
40	HIV1	Adenosine deaminase acting on RNA 1 (ADAR1)	Activates cellular p58IPK	Clerzius et al., 2009
41	HIV1	TRBP	TRBP interacts with PACT through dsRBD and modulates PKR activity.	Daher et al., 2009
42	HSV1	γ_2Us11	Inhibits cellular PACT	Peters et al., 2002
43	HSV1	$\gamma_1$34.5	Co-activates protein phosphatase 1	He et al., 1997 and 1998
44	HPV 18	E6	Physically interacts with PP1/GADD34 complex and mediates translational recovery	Kazemi et al., 2004

45	Respiratory syncitial virus (RSV)	RSV N	Sequesters PKR away from eIF2 by binding the kinase domain and also aids in the association of eIF2 with PP2A.	Groskreutz et al., 2010
46	African swine fever virus	DP71L	Recruites PP1 catalytic subunit and promotes dephosphorylation of eIF2α	Zhang et al., 2010
ix)Viral proteins circumventing eIF2 dependent translational arrest				
47	Rotavirus	NSP3	Sequesters poly-A binding proteins thereby preventing eIF2 dependent stress granule assembly.	Montero et al., 2008
48	Sindbis virus (SV) and Semliki forest virus (SFV)	DLP	SV26 mRNA translation is resistant to eIF2α phosphorylation. This requires a downstream RNA structure which acts as a translation enhancer and facilitates translation initiation in the absence of functional eIF2.	Ventoso et al., 2006
49	Cow pox virus	CP77	Suppresses PKR activation and phosphorylation of eIF2α. May rescue viral translation by functioning as a component of translation initiation complex.	Hsiao et al., 2004

interferon response (Pe'ery and Mathews, 2000). PKR, a cellular eIF2α kinase is up-regulated and activated by interferon α/β, in response to double stranded RNA in virus infected cells (Kaufman, 2000). Unopposed, activated PKR effectively phosphorylates eIF2α, and therefore would deplete active eIF2 and intervene with viral and cellular protein synthesis. Therefore, inhibition of translation at the initial stage of viral infection would enable to effectively curtail viral replication. However, numerous viruses encode multiple factors which counter this cellular response, by affecting eIF2α phosphorylation directly or indirectly. The viral factors have been classified based on their mechanism of action (Table 1). These include RNA and proteins which directly interact with PKR and inactivate it. Non-coding RNAs such as AdVARNA (Schneider, 2000) or the EBERs produced in cells latently infected with Epstein-Barr virus (Sharp et al., 1993), bind to PKR and prevent it from dimerization and activation. In contrast, ssRNA genome of hepatitis delta agent activates PKR (Robertson et al., 1996; Circle et al., 1997). Likewise, the HIV Tat responsive element TAR, has been reported to both activate and inhibit PKR (Gunnery et al., 1990; Maitra et al., 1994). Another prominent class of inhibitors are dsRNA binding proteins, which function by binding and sequestering dsRNA, and thereby prevent PKR activation (Hatada et al., 1999; Bergmann et al., 2000; Salvatore et al., 2002; Khoo et al., 2002). The members of this class include E3L (Fierro-Monti and Mathews, 2000), HCMV- TRS1 and IRS2 (Child et al., 2004), influenza virus- NS1, EBV-SM, and HSV1-Us11 (Romano et al 1998; Tan and Katze, 1998; Poppers et al., 2003). Viruses also encode for certain proteins like K3L, C8L, E2 etc., which resemble eIF2 and serve as pseudo-substrates and overcome translational shutdown. Interestingly, some viruses like Vaccinia and HSV-1 encode factors that prevent accumulation of phosphorylated eIF2α, taking advantage of targets both upstream and downstream to PKR activation. For instance, $_{\gamma 1}$34.5, a HSV-1 encoded gene product prevents the accumulation of phosphorylated eIF2α by recruiting the cellular protein phosphatase 1α (PP1α) (He et al., 1997). This is achieved through the action of the C-terminus of the $_{\gamma 1}$34.5 protein, which is homologous to a domain in the GADD34 protein, a co-factor of protein phosphatase 1α (Chou and Roizman, 1994). Baculovirus which specifically infects the insect cells such as *Spodoptera frugiperda*, encodes two anti-apoptotic

proteins P35 and Pk2, which affect eIF2α phosphorylation. P35 is shown to inhibit caspases and prevents caspase mediated eIF2α kinase activation (Aparna et al., 2003), whereas, Pk2 is an inhibitor of eIF2α kinase and blocks eIF2α phosphorylation (Dever et al., 1998). In conclusion, irrespective of the mechanism involved, the ensuing contest between virus and host cell to determine the abundance of unphosphorylated and active eIF2, is a key feature of viral pathogenesis (Leib et al., 1999, 2000; Brandt and Jacobs, 2001).

1.13, Role of eIF2α phosphorylation in signal transduction;

Phosphorylation of eIF2α has been shown to have an interdependent relationship with various signaling pathways, and the connections are being uncovered to a greater extent. The various signaling cascades showing interdependency with phosphorylation of eIF2α include MEK/ERK, stress activated protein kinases such as JNK and p38 MAP kinases, PI3K pathway etc. Phosphorylation of eIF2α by the nutrient sensing kinase, GCN2, is dependent on MEK activity; as recent studies indicate that inhibition of MEK activation in HepG2 human hepatoma cells, blocked eIF2α phosphorylation and ATF4 synthesis under conditions of amino acid starvation (Thiaville et al, 2008). Further, knockdown of *GADD34* did not prevent the dependence of eIF2α phosphorylation on MEK, indicating that MEK functions through eIF2α kinase activation rather than suppression of an eIF2α phosphatase (Thiaville et al, 2008). Similarly, PKR has also been implicated to be activated by ERK2 and RSK2 (receptor signaling kinase) mediated phosphorylation of its threonine 451 residue, in response to UV-A irradiation in JB6 cells (Zykova et al, 2007). PKR also serves as a substrate for tyrosine kinases like Janus kinase 1 (Jak 1) and tyrosine kinase 2 (Tyk2) in response to interferons (Su et al., 2007), which can regulate the activity of PKR through tyrosine phosphorylation at residues 101 and 293. Apart from serving as substrates for various kinases, eIF2α kinases are also essential for activating other signaling cascades in response to cellular stresses. For instance, PKR has been shown to mediate the activation of important transcription factors such as nuclear factor kB (NF-kB), activating transcription factor 2 (ATF2), signal transducer and activator of transcription 1 (STAT1) and interferon regulatory factor 1 (IRF1) (Yang et al., 1995; Der

et al., 1997; Kumar et al., 1997; Bandyopadhyay et al., 2000; Ramana et al., 2000; Zamanian-Daryoush et al., 2000). PKR controls IFN and dsRNA signaling pathways by modulation of STAT1 and STAT3 transcription factors. *Pkr* $^{-/-}$ cells are defective in STAT1 phosphorylation at Ser-727 residue, which results in defective transactivation of STAT1 (Ramana et al., 2000). STAT1 is also a target for PKR mediated activation, in response to lipopolysaccharide (LPS) in glial cells (Lee et al., 2005). Association of PKR with STAT3 is required for the full activation of STAT3 in response to platelet derived growth factor (Deb et al., 2001). PKR is also involved in regulating p53 activity, by interacting with the C-terminus of p53 and phosphorylates it on the Ser-392 residue. Phosphorylation of p53 by PKR causes cell cycle arrest and regulates transcription of target genes (Cuddihy et al., 1999). Recent studies suggest that PKR activates p38 MAPK and JNK, which in turn serves as a link between PKR and its pro-inflammatory properties (Goh et al., 2000).

Studies indicate that eIF2α kinases are also required for the induction of Akt/PKB phosphorylation in response to either IFN-γ or dsRNA treatment, both of which induce the PI3K pathway (Platanias, 2005; Sen and Sarkar, 2005). Thus, the functional interplay between PI3K activation and translational control mediated by eIF2α phosphorylation, would possibly activate cytoprotective mechanisms in case of ER stress (Schroder and Kaufman, 2005). However, in case of viral infection, PI3K activation might enable efficient expression of genes required for mediating an antiviral response (Kazemi et al., 2007).

Caspases are also shown to process eIF2α kinases such as PKR and PERK, and enhance the efficiency of eIF2α kinases (Saleans et al., 2001; Pushpanjali and Ramaiah, 2010). The ability of caspases to activate several kinases also raises the question whether eIF2α phosphorylation mediated caspase activation is the cause for the activation of other kinases.

1.14, Importance of eIF2α phosphorylation in memory;

Local translation of mRNAs at synapses, play an important role in eliciting and maintaining the ability to learn and memorize. Strong genetic evidence implicating the role of eIF2α phosphorylation in learning and memory is provided by studies using

Gcn2 $^{-/-}$ mice and eIF2$\alpha^{ser51Ala/+}$ heterozygous knock in mice. These mutants exhibited enhanced memory using different training protocols (Costa-Mattioli et al., 2007). Activated GCN2 suppresses memory formation by phosphorylating eIF2α, which in turn results in enhanced translation of ATF4 mRNA in the brain. ATF4 acts as a memory suppressor, as it inhibits the transcription factor cAMP response element binding protein (CREB)-mediated gene expression that are critical for long term plasticity and memory (Kandel, 2001).

1.15, Aberrant eIF2α phosphorylation in pathophysiology;

Aberrations affecting eIF2α phosphorylation are associated with a variety of inherited disorders like diabetes, stroke, eating disorders, viral infections, anemia, neurological diseases etc. The Wolcott-Rallison syndrome has been linked with mutations in the catalytic domain of the eIF2α kinase, PERK (Delepine et al., 2000; Senee et al., 2004). Mutations in PERK abolish its catalytic activity, leading to destruction of pancreatic β cells and there by leads to permanent diabetes. The disease is also associated with multiple pathological conditions such as multiple systemic disorders, including epiphyseal dysplasia, osteoporosis and growth retardation (Wolcott and Rallison, 1972; Stoss et al., 1982). Likewise, loss of PERK function in mice recapitulates many of the defects of human syndrome including diabetes due to destruction of pancreatic β cells after birth and pancreatic failure (Harding et al, 1999 and 2001; Zhang et al., 2002). Similarly, mice harbouring a homozygous knock in mutation in eIF2α (Ser 51 Ala), were shown to have defects in embryonic β cell survival, liver glycogen storage, postnatal induction of gluconeogenesis (Scheuner et al., 2001). Pathology of a wide variety of heme deficiency disorders is associated with aberrant HRI induced eIF2α phosphorylation. These include iron deficiency anemia (Han et al., 2001), erythropoietic protoporphyria and β-thalassemia intermediates (Han et al., 2005). The hallmarks of deficient HRI induced eIF2α phosphorylation in these anemias are increased globin inclusions, decreased red blood cell number and aggravation of ineffective erythropoiesis (Chen, 2000; Han et al., 2001 and 2005). Therefore, HRI induced eIF2α phosphorylation is critical for safeguarding the proper cell size, number and hemoglobin content of RBC.

Moreover, HRI induced eIF2α phosphorylation can also modify other forms of red cell diseases involving heme and globin synthesis like sideroblastic anemia and unstable hemoglobin hemolytic anemia. Further, phosphorylation of eIF2α (Chang et al., 2002) and PKR activation (Onuki et al., 2004; Peel and Bredesen, 2003) are shown to be associated with neuronal degeneration in Alzheimer's disease and other neurodegenerative disorders (Peel, 2004). eIF2α phosphorylation dependent ATF4 expression regulates multiple factors and genes implicated in schizophrenia or bipolar disorder (Carter et al., 2007). Long term depression is also associated with enhanced eIF2α phosphorylation (Hou et al., 2006). Childhood ataxia with cerebral hypomyelination (CACH, also known as vanishing white matter disease), is a rare genetic syndrome caused by loss of function mutations in one of the subunit of eIF2B, the guanine nucleotide exchange factor for eIF2 (Leegwater et al., 2001; Richardson et al., 2004; Proud, 2005). These mutations in eIF2B causing CACH mimic the consequences of eIF2α phosphorylation.

1.16, Pro-survival effects of eIF2α phosphorylation;

The protective role of eIF2α phosphorylation is evident under ER stress conditions, where phosphorylated eIF2α mediates a decline in protein load and ensures proper folding and thereby brings about ER homeostasis. Further, activation of eIF2α kinases in response to DNA damage, virus infection and ER stress, induce and activate glycogen synthase kinase 3 beta (GSK3β), which compromises p53 mediated apoptosis by promoting nuclear export and degradation of p53 (Baltzis et al., 2007). The pro-survival properties of eIF2α phosphorylation are also associated with the activation of phosphatidyl inositide 3 (PI3) kinase signaling pathway and activation of NF-κB as a result of inhibited IκB translation. Both NF-κB and PI3K have been found to cause changes in gene expression that are cytoprotective in nature (Deng et al., 2004; Jiang et al, 2003). In addition, hibernation, one of the most stress resistant states of central nervous system, is associated with high levels of eIF2α phosphorylation and considered to be an adaptive mechanism (Frerichs et al., 1998).

1.17, Role of eIF2α phosphorylation in apoptosis;

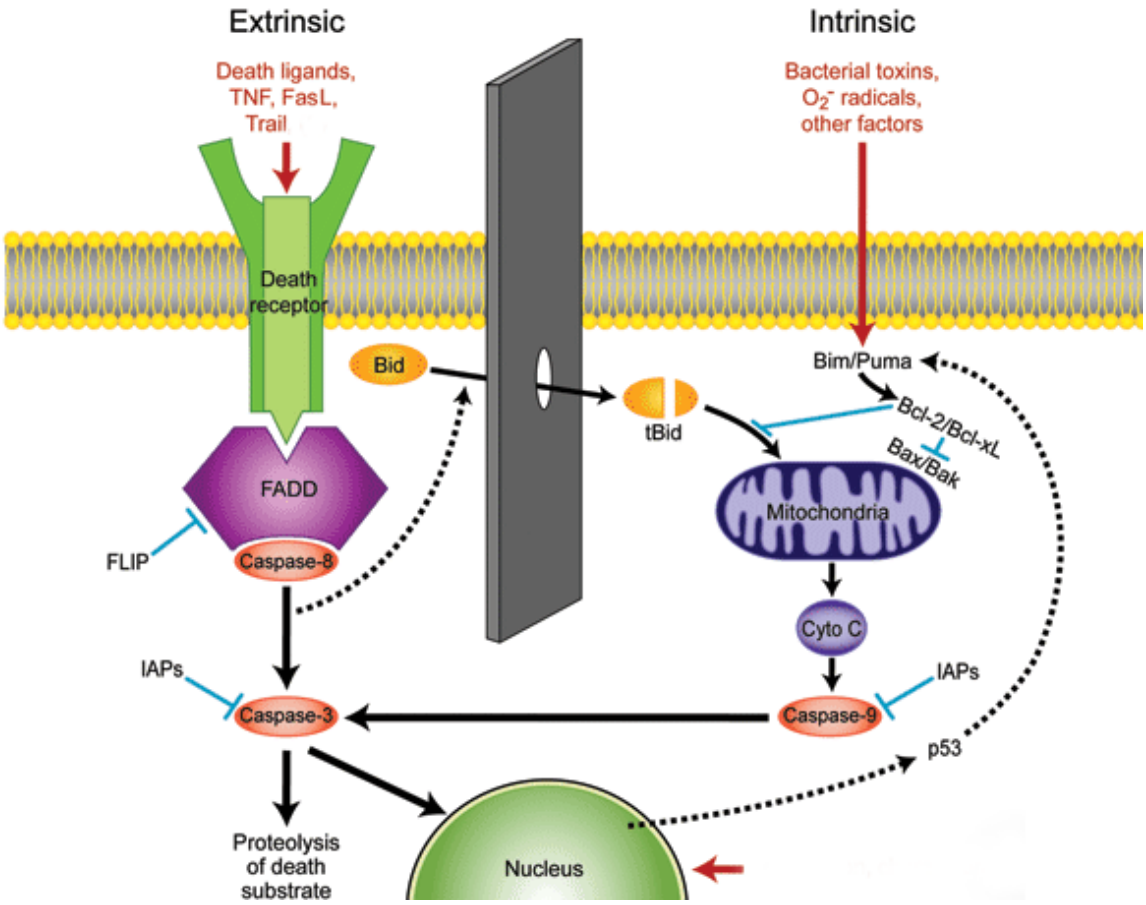
Induction of apoptosis coincides with a rapid, incomplete inhibition of the rate of protein synthesis (Deckwerth and Johnson 1993; Morley et al., 1998; Zhou et al., 1998, Scott and Adebodun, 1999), which is mediated by changes in the phosphorylation status of multiple translation initiation factors and targeted cleavage of these factors by caspases (Clemens et al., 2000; Morley et al., 2005). Double stranded RNA dependent protein kinase, PKR, plays an important role in regulating the apoptotic process (Barber, 2005). Studies with recombinant vaccinia viruses expressing PKR, suggest that active PKR can promote apoptosis in HeLa cells (Lee and Esteban, 1994), and like wise murine fibroblasts lacking PKR were resistant to TNFα induced apoptosis (Der et al., 1997). The most prominent and well studied substrate of PKR is the α subunit of eIF2. The extent of phosphorylation of eIF2α is often enhanced following exposure to a variety of pro-apoptotic stimuli, with PKR being the strongest candidate kinase in most of the cases (Morley et al., 1998, 2000; Saelens et al., 2001; Jeffrey et al., 2002; Aparna et al., 2003). In this context, enhanced levels of eIF2α was observed in certain tumors, and the level of expression correlated with the biological aggressiveness of the tumor (Clemens and Bommer, 1999), suggesting that regulation of eIF2 may be critical for cellular growth. This is consistent with the fact that constitutive expression of a non-phosphorylatable form of eIF2α (Ser 51 Ala), or expression of a catalytically inactive eIF2α kinase, PKR, was shown to transform NIH-3T3 cells (Donze et al., 1995, Koromilas et al. 1992; Meurs et al. 1993; Barber et al. 1995). In contrast, over-expression of a phosphomimetic form of eIF2α (Ser 51 Asp) in COS-1 monkey cells (Srivastava et al. 1998; Scheuner et al. 2006), or transient or inducible over expression of PKR, activates apoptosis in several cell systems (Lee and Esteban 1994; Balachandran et al. 1998, Srivastava et al. 1998, Donze et al. 1999, Scheuner et al. 2006). Apoptosis in these cells corresponds to caspase 3 activation. These studies point out that eIF2α phosphorylation has a direct role in stimulating caspase activation and apoptosis. Small fraction of eIF2α is also cleaved by caspases to give a carboxy terminal truncated product (Satoh et al., 1999; Marissen et al., 2000; Saelens et al 2001; Pushpanjali and Ramaiah, 2010). Significance of this cleavage is

unclear. However, it is likely that the cleaved form of eIF2α, unlike the intact eIF2α, does not require eIF2B for its guanine nucleotide exchange (Marissen et al., 2000). The caspase mediated cleavage of eIF2α may render a population of eIF2 to be constitutively active and independent of its phosphorylation status, thereby protecting protein synthesis from complete down regulation. This could be particularly relevant for proteins like CHOP and GADD34, which are induced via the eIF2α-ATF4 pathway and are known to promote apoptosis when eIF2α is phosphorylated (Marciniak et al., 2004). Apart from inducing the levels of pro-apoptotic proteins, phosphorylation of eIF2α is also shown to down regulate pro-survival proteins like IAPs (inhibitor of apoptosis proteins), which shift the balance towards cell death (Scheuner et al., 2006). Overall, eIF2α phosphorylation mediated specific translational regulation along with its ability to restrict global translation is important in cells undergoing division, or entering apoptosis in response to various stress stimuli.

1.18, Apoptosis ;

Apoptosis is a widespread and morphologically distinct process of cell death necessary for the removal of unwanted, damaged, mutated or infected cells. It is the best characterized and highly regulated form of cell death which is conserved through out evolution (Samali et al., 1996; Lockshin and Zakeri, 2007). Diverse stress stimuli such as chemotherapeutic drugs, irradiation, redox imbalance, ER stress etc., are known to trigger apoptosis. However, the pathways triggering apoptosis varies from cell type to cell type. Caspases, a family of cysteine proteases, act as common death effector molecules in various forms of apoptosis (Degterev et al., 2003). Caspases are synthesized as inactive zymogens, which upon activation cleave various substrates, resulting in distinct morphological features such as DNA fragmentation, nuclear shrinking and blebbing (Lockshin and Zakeri, 2007; Degterev et al., 2003; Samali, 2007). Apoptosis can be initiated by both external and internal stimuli such as UV induced DNA damage, oncogenic transformation, drugs, virus infection and a variety of external signals. These various stimuli lead to the activation of either the intrinsic or extrinsic apoptotic pathway.

Fig. D



Apoptosis: Intrinsic and Extrinsic pathways

Programmed cell death, which is more commonly known as apoptosis, is an inter-related collection of pathways and mechanisms utilized to eliminate excess or unwanted cells. Cells also undergo apoptosis in response to detrimental stress or in the presence of appropriate stimuli. This figure depicts the intrinsic and extrinsic pathways of apoptosis. This figure is adapted from medscape.

The extrinsic pathway is triggered by the binding of external (death) ligands to their cognate death receptors as exemplified by the stimulation of receptors belonging to the tumour necrosis factor (TNF) superfamily such as CD95 (APO-1/Fas) or TNF-related apoptosis inducing ligand (TRAIL). Stimulation of receptors by ligand, is accompanied with receptor aggregation and recruitment of the adaptor molecules such as TNFR1 associated death domain (TRADD) and Fas-associated death domain (FADD) and pro-caspase-8 to form the death inducing signaling complex (DISC) leading to the self activation of pro-caspase 8 (Kischkel et al., 1995; Locksley et al., 2001; Ashkenazi, 2008). Active caspase 8 facilitates the activation of downstream caspases by processing them (Ashkenazi, 2008). The intrinsic pathway is initiated by non-receptor mediated signals originating within the cells from a series of death triggering genes, which in insect systems include the *hid, grim* and *reaper* genes of drosophila and in mammalian cells the *Smac/Diablo*, *GSPT1* and *Omi/HtrA2* genes. The intrinsic pathway is characterized by the permeabilization of the outer mitochondrial membrane and the release of several pro-apoptotic factors such as cytochrome c, second mitochondria-derived activator of caspase (Smac)/ direct IAP binding protein with low pI (DIABLO), apoptosis inducing factor (AIF), endoG, high temperature requirement protein A2 (HtrA2)/Omi etc., into the cytosol. The precise mechanism of cytochrome c release remains unclear, but is regulated by the antagonistic activities of the Bcl-2 family of proteins. Once released into the cytosol, cytochrome c binds to an adaptor protein, Apaf-1, which self oligomerizes and recruits pro-caspase 9 to form the apoptosome complex. This promotes the auto processing of pro-caspase 9, which in turn recruits and cleaves pro-caspase 3 that is then released into the cytosol to degrade target substrates proteolytically. The intrinsic pathway is controlled by a balance between the anti-apoptotic members such as Bcl-2, Bcl-Xl and Mcl-1, and pro-apoptotic members such as Bax, Bak and BH3 domain only molecules (Adams and Cory, 2007). The BH3 only proteins interact with the anti-apoptotic members of the Bcl-2 family of proteins and act on the mitochondrial membrane resulting in the release of cytochrome c (Letai et al., 2002). Activation of caspases is also regulated by a family of proteins which serve as cellular inhibitors of caspases and are termed as “Inhibitor of Apoptosis Proteins (IAPs)” (LaCasse et al., 2008).

1.19, Objectives ;

From the detailed overview presented above it is evident that phosphorylation of eIF2α is not only important in translational regulation but also plays a very important role in cellular homeostasis. Although there are several reports describing that phosphorylation of eIF2α in itself is a death signal (Srivastava et al., 1998; Scheuner et al., 2006), a recent paper (Boyce et al., 2005) describes that a cell permeable thiourea compound, salubrinal, acts as a selective inhibitor of eIF2α phosphatases and protects cells from endoplasmic-reticulum(ER) stress, which indicates that eIF2α phosphorylation is also a survival/ protective signal. Related to the above studies, this laboratory has shown previously that the ovarian cells of *Spodoptera frugiperda (Sf*9) can express a phosphomimetic form of eIF2α, using a baculovirus expression vector (Sudhakar et al., 2000; Suragani et al., 2006). Unlike mammalian cells, expression of the phosphomimetic form of eIF2α did not promote cell death in *Sf9* cells. An analysis of the above results, suggest that it may be because of the expression of P35, an anti-apoptotic protein produced by baculovirus. Consistently, a mutant baculovirus devoid of p35 gene readily stimulates eIF2α phosphorylation and cell death (Aparna et al., 2003). Complimenting these observations, UV-induced eIF2α phosphorylation and cell death were significantly mitigated in wt baculovirus infected cells. In addition, expression of a phosphomimetic form of eIF2α promotes UV–irradiation induced cell death, whereas, expression of a non phosphorylatable form of eIF2α mitigates UV irradiation induced cell death, suggesting that eIF2α phosphorylation may be a death signal.

Baculoviruses produce P35 and Pk2 proteins. P35, a known anti-apoptotic protein was found to mitigate eIF2α phosphorylation as mentioned above. Another study has shown that baculovirus Pk2, a truncated eIF2α kinase like protein was shown to inhibit eIF2α kinase activation *in-vitro* (Dever et al., 1998). Complementary to these results, it was also observed that infection of insect cells with a mutant baculovirus lacking the *Pk2* gene promotes eIF2α phosphorylation and offers less protection from stress induced apoptosis compared to the wild type virus (Aarti et al., 2010). These findings suggest a definite role for eIF2α phosphorylation in cell death, and also indicate that the mechanisms underlying eIF2α phosphorylation mediated cell survival and death are

different in different stresses. Keeping in view of these observations, the present study has been undertaken with the following objectives to further understand the importance of eIF2α phosphorylation on cell death and survival and on the co-incident signaling pathways.

a) Why and how eIF2α phosphorylation does not always lead to cell death in *Sf9* cells?

b) Is eIF2α phosphorylation a cause or a consequence of caspase activation?

c) Are there any other mechanisms affecting eIF2α phosphorylation mediated cell death?

2. Materials and Methods

- **2.1:** *Biochemicals and Antibodies*
- ***2.2:*** *Cell Culture and treatments*
- ***2.3:*** *Preparation of Cell Extracts*
- ***2.4:*** *Protein estimation*
- ***2.5:*** *In-vitro Caspase Activity*
- ***2.6:*** *Immunostaining*
- ***2.7:*** *Immunodepletion*
- ***2.8:*** *Purification of recombinant human eIF2α wt and phosphomimetic S51D forms*
- ***2.9:*** *In-vitro caspase activation in cell extracts*
- ***2.10:*** *Pull Down assay*
- ***2.11:*** *Far-western Analysis*
- ***2.12:*** *Immunoprecipitation*
- ***2.13:*** *SDS-Polyacrylamide gel electrophoresis*
- ***2.14:*** *Western Blotting*
- ***2.15:*** *Densitometry*
- ***2.16:*** *Statistics*

2.1, Biochemicals and Antibodies:

Biochemicals used in this study, unless and otherwise indicated, were obtained from Sigma, USA. Fetal Bovine Serum was from Biowest, USA. Protein A sepharose beads were obtained from Amersham, USA. Rabbit polyclonal and monoclonal phosphospecific anti-eIF2α antibody, rabbit polyclonal anti-BiP and phosphospecific anti p38 antibodies were purchased from Cell Signaling, USA. Mouse monoclonal anti-BiP antibody, z-VAD-fmk, Ac-DEVD-AFC were from BD-Biosciences, USA. Rabbit polyclonal anti-ATF4 antibody was from Abcam, UK. Mouse monoclonal anti-active caspase 3 and alkaline phosphatase conjugated goat anti-mouse and donkey anti-rabbit secondary antibodies were from Promega, USA. Mouse monoclonal anti-CHOP, anti-BiP, phospho-ERK and phospho-JNK, FITC and TRITC conjugated bovine anti-rabbit and goat anti-mouse secondary antibodies, were from Santa Cruz, USA. Specific inhibitors like U0126, SB203580 and SP60025 were kindly provided by Dr. Rajesh Kamindla.

2.2, Cell Culture and treatments:

Sf9 insect cells were maintained at 27^{o} C in TNM-FH medium supplemented with 10% fetal bovine serum (FBS), 50 U of penicillin G and 50 μg/ ml streptomycin as described previously (Aparna et al., 2003). Cells were grown to 90-95% confluence prior to exposure to UV-B irradiation (312 nm), cycloheximide, tunicamycin, thapsigargin, brefeldin A, DTT, GSH, GSSG and salubrinal at the indicated concentrations and time periods. To determine the importance of caspase activation on eIF2α phosphorylation, cells were treated with 50 μM caspase inhibitor, z-VAD-fmk prior to exposing the cells to different stressors or at other time points as mentioned in the legends to figures. MAPK inhibitors were added to the cells 2 hours prior to induction of stress wherever indicated. Cell viability was assessed by morphological examination of cells under VWR-AE31 inverted microscope equipped with a digital camera and the software motic and also by trypan blue analysis as mentioned previously (Aparna et al., 2003).

2.3, Preparation of cell extracts:

Sf9 cells treated with diverse agents were harvested by centrifugation at 2000 rpm for 10 min and lysed in buffer containing 20 mM Tris, 80 mM KCl, 1 mM EDTA, 1 mM DTT, 1 mM PMSF and 10 μg/ ml leupeptin, aprotinin and pepstatin and incubated on ice for 10 min. the lysate was centrifuged at 14000 rpm for 20 min and the supernatant was stored at -70^{o} C as whole cell extract. Cell extracts were separated by 10% SDS-PAGE and transferred onto a nitrocellulose membrane (Millipore). The membrane was probed with various antibodies as mentioned and then with alkaline phosphatase conjugated secondary antibodies and the blots were developed using NBT and BCIP.

2.4, *Protein estimation:*

Protein estimation was done using BioRad's protein estimation reagent as per manufacturer's instruction.

2.5, Caspase Activity:

Caspase 3 like activity was measured spectrofluorimetrically where in *Sf9* cells that had been treated with various agents were lysed as described earlier and an aliquot of the whole cell extract containing 50 μg of protein was incubated with 5 μM Ac-DEVD-AFC, caspase 3 substrate, for 1 hr at 37^{o} C. AFC hydrolysis was monitored by measuring fluorescence (excitation, 400 nm; emission, 500 nm) in a Flouromax-3, Jobin Yvon Horiba spectrofluorimeter.

2.6, Immunostaining:

Sf9 cells were plated on chamber slides and treated with diverse stressors. The cells were then fixed in PBS containing 0.1% Triton X-100 and 4% paraformaldehyde. After washing with ice cold PBS, cells were incubated with 10% FBS for 1 hr at room temperature and then with rabbit monoclonal phosphospecific anti-eIF2α, mouse monoclonal anti-BiP, mouse monoclonal anti-CHOP or mouse monoclonal anti-phosphoJNK antibodies, overnight at 4^{o} C. The unbound primary antibody was washed using PBS and the binding of the primary antibody was visualized after incubating with respective secondary antibodies conjugated with FITC or TRITC for 1 hr, under a Leica

TCS SP2 AOBS laser scanning confocal microscope equipped with Leica confocal software.

2.7, Immunodepletion:

Immunodepletion of BiP was carried out in 250 µl aliquots of extracts prepared from cells treated with 12 µM tunicamycin containing approximately 2.5 mg of protein. The extracts were incubated with 5 µg of anti-BiP antibody or 5 µg of control antibody (goat anti-mouse immunoglobulin G) in the presence of 50 µl (packed volume) of protein A sepharose resin for 4 hrs at 4° C with gentle rocking. The resin was collected by centrifugation at 4000 rpm, 10 min at 4° C and the supernatant was saved as depleted extracts. Control and BiP-depleted extracts were analysed for the levels of BiP using immunoblot analysis.

2.8, Purification of recombinant human eIF2 subunits

Purification of the recombinant subunits from *E. coli* lysates was carried out using Ni-NTA agarose beads as described previously (Rajesh et al., 2008), with the following modifications. BL-21 cells expressing human wt and mutant S51D eIF2α were harvested and lysed at 4^0C in a buffer containing 20 mM Tris-HCl, pH 7.8, 300 mM NaCl and 1 µg /ml protease inhibitors as described above. Lysates were then clarified by centrifugation at 12, 000 rpm for 20 min and the supernatant was loaded onto a 1 ml pre-equilibrated Ni-NTA agarose resin at 4^0C. The column was washed with 10 volumes each of buffer 1 containing 20 mM Tris-HCl (pH 7.8), 500 mM NaCl and 30 mM imidazole and buffer II containing 100 mM NaCl and 50 mM imidazole. Bound proteins were then eluted with Tris-HCl buffer containing 100 mM NaCl and 250 mM imidazole and separated by 10% SDS-PAGE.

2.9, In-vitro caspase activation in cell extracts:

Wt eIF2α or mutant phosphomimetic S51D form of human recombinant eIF2α were purified from BL21-DE3 as described previously and 5 µg of either of the recombinant proteins were incubated for 1 hr at 37^o C with cell extracts prepared from untreated and 12 µM tunicamycin treated cells or BiP depleted extracts containing 100 µg protein. The

samples were then incubated for 1 hr at 37^{o} C with caspase assay buffer containing 5 μM Ac-DEVD-AFC and the caspase activity was monitored.

2.10, Pull Down Assay:

50 μl of Ni-NTA resin was incubated with ~10 μg of one of the purified his-tagged recombinant subunits for 60 min at 4^{o}C. Unbound proteins were collected by centrifugation at 1500g. The resin bound subunit was incubated with either HeLa cell extracts prepared from tunicamycin treated cells or from Sf9 cell extracts prepared from UV-irradiated or tunicamycin treated cells to determine the interaction of the recombinant subunits with BiP and caspase of the lysate. These incubations were carried out at 4^{o}C. Unbound fractions were collected by centrifugation and the resin was extensively washed with TBS containing 0.1% Tween-20. The protein complex bound to the resin was then eluted with 500 mM imidazole. The unbound, wash and bound/eluted fractions were collected and separated by 10% SDS-PAGE, transferred to a nitrocellulose membrane and the membranes were probed with respective antibodies for the detection of the specific proteins.

2.11, Far Western Analysis:

In these studies, 5 μg of purified his-tagged recombinant wt and mutant S51D eIF2α subunits were separated by 10% SDS-PAGE and transferred to a nitrocellulose membrane. The membranes were incubated with non-fat milk powder in TBS containing 0.1% tween 20 (TBST) for one hr at room temperature to block the protein free regions of the membrane. Membranes were then incubated with 10 ml of diluted Sf9 cell extracts prepared from UV irradiated or tunicamycin treated cells for studying the interaction of proteins such as BiP and caspase 3, in phosphate buffered-saline containing 0.1 % tween-20 for one hr at 4^{0}C. The membranes were then washed in TBST and probed with respective primary and secondary antibodies as described in the legends to figs to determine the interactions of the subunits with BiP and caspase 3.

2.12, Immunoprecipitation:

Interaction between unphosphorylated and phosphorylated eIF2α with BiP and caspase 3 of HeLa cell extracts prepared from tunicamycin treated cells or *Sf9* cell extracts prepared from UV-irradiated or tunicamycin treated cells were analyzed by immunoprecipitation. In this assay, BiP, unphosphorylated and phosphorylated eIF2α; caspase 3 were immunoprecipitated by respective antibodies. In case of *Sf9* cell extracts, the caspase 3, eIF2α and phosphospecific eIF2α antibodies were added to UV-irradiated extracts and the immunocomplex was then incubated with tunicamycin treated extracts to monitor BiP-caspase, BiP-eIF2α and BiP-eIF2α(P) interaction. The immunoprecipitated samples were separated by SDS-PAGE and transferred to a nitrocellulose membrane and the proteins were recognized by their respective antibodies and with the interacting protein specific antibodies.

2.13, Sodium dodecyl sulphate polyacrylamide gel electrphoresis (SDS-PAGE):
Proteins were separated by a modified Laemmli method (1970). 8 ml of the 10% resolving gel mixture contained the following: 1.875 ml of 2 M 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), 6µl 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 2 M 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. Protein were prepared in a sample buffer containing 0.25 M 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. The proteins in the gel were visualized by coomassie staining or transferred onto nitrocellulose membrane.

2.14, Western blotting*:*

After separation of proteins on SDS-PAGE, the proteins were transferred eletrophoretically by wet method on to nitrocellulose membrane. Transfer of proteins was carried at 25 volts/100 milli-amps overnight in cold room in standard Biorad transfer unit.

For a standard western blot, the transfer buffer contained 25 mM Tris buffer and 195 mM glycine in 20% methanol, however for efficient transfer of low molecular weight proteins the methanol concentration was increased to 40% and for transfer of high molecular weight proteins the methanol percentage was reduced to 10%. After the transfer the membrane is removed and stained with ponceau S stain to check for transfer of protein onto the membrane. The ponseau S stain is subsequently removed by washing with excess 1x TBST (10 mM Tris-HCl pH 8, 150 mM NaCl containing 0.05% Tween 20). Regions of nitrocellulose membrane, free of proteins were blocked 5% milk solution prepared in TBST for 1 hr at room temperature. After briefly rinsing the blocked membrane with TBST, the membrane is incubated with a primary antibody solution at 4^{o}C overnight, with gentle shaking. Later the membrane is washed with TBST three times for 15 min each. Then membrane is incubated with appropriate alkaline phosphatase (ALP) conjugated secondary antibody for 1 hr at room temperature followed by three washes with TBST for 15 min each. Finally the blot is developed by treating with ALP substrates NBT and BCIP (66µl and 33µl of 20 mg/ml stocks) in 10 ml of ALP buffer containing 100 mM Tris-HCl pH 9.5, 100 mM NaCl and 5 mM $MgCl_2$. The solution is removed soon after the appearance of bands and the blot washed with water, air-dried and scanned. Each immunoblot was repeated at least three times with different batches of extracts.

2.15, Densitometric analysis:

Densitometric analysis of the immunoblots was performed using Image-J software as per the protocol given in the software user manual. The relative intensities obtained from three different individuals experiments were plotted as vertical bars depicted with standard error.

2.16, Statistics:

All the results were expressed as mean ± SEM and they are representative of three independent experiments. Significance was tested using student t-test using sigma plot 9.0. A probability of $P < 0.05$ was considered statistically significant.

3. Results and Discussion

Phosphorylation of eIF2α in Sf9 Cells: A Stress, Survival and Suicidal Signal

Phosphorylation of eIF2a: A stress, survival and suicidal signal.

- *3.1, Stress induced phosphorylation of eIF2α elicits expression of prosurvival and pro-apoptotic proteins*
- *3.2, eIF2α phosphorylation is a cause and consequence of caspase activation*
- *3.3, BiP expression resists apoptosis and interferes with caspase activation*
- *3.4, Phosphorylation of eIF2α reduces its interaction with BiP and caspase3*
- *Fig. 1 Stress induced changes in eIF2α(P), ATF4, BiP, JNK(P) and CHOP.*
- *Fig. 2 Morphology of Sf9 cells in response to different stressors:*
- *Fig. 3 Analysis of Sf-caspase fragment and activity in stressed cells:*
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- *Fig.9 Morphology of UV-irradiated cells pre-treated with tunicamycin.*
- *Fig.10 Analysis of BiP and phosphorylated eIF2α in UV-irradiated cell pre-treated with tunicamycin:*
- *Fig.11 Sf-caspase activation and caspase-3 like fragment release in Sf9 cell extracts: Effect of recombinant phosphomimetic form (S51D)of eIF2α:*
- *Fig.12 Interaction among eIF2α, BiP and Sf-caspase: Coimmunoprecipitation studies.*
- *Fig.13 Interaction among eIF2α, BiP and Sf-caspase: Far-westernanalysis.*
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3.1, Phosphorylation of eIF2α: A stress, survival and suicidal signal

Phosphorylation of eIF2α is a stress signal and occurs both in cytosol and in the ER by respective eIF2α kinases. Although, both the compartments carry out protein synthesis and harbor their respective chaperones, they differ significantly in their chemical composition (relative levels of GSH/GSSG and calcium), topology and in protein folding machinery. eIF2α phosphorylation provides a fundamental mechanism to couple the rate of protein synthesis with the capacity to fold proteins under conditions of different physiological stress, such as nutrient deprivation or viral infection. Although phosphorylation of eIF2α is implicated in apoptosis, recent evidences support the idea that eIF2α phosphorylation evokes a survival signaling response under conditions of accumulation of unfolded proteins in the lumen of endoplasmic reticulum (ER).

BiP (immunoglobulin heavy chain binding protein), an ER chaperone belongs to the family of HSP70 chaperones. It is known to be a master regulator of three ER stress sensors: PERK (ER-resident eIF2α kinase), IRE-1 (Inositol requiring enzyme-1), and ATF6 (Activated transcriptional factor 6). It plays a key role in the unfolded protein response (UPR). Unfolded or malfolded proteins arise when protein synthesis exceeds protein folding capacity of the cell, because of improper post translational modifications of proteins or their degradation, and perturbations in ER calcium levels or in its oxidizing environment. In the absence of ER stress, the ER stress sensors are bound by BiP and are in an inactive state. Stress in ER leads to the release of BiP and consequent activation of ER stress sensors that result in translational attenuation mediated by PERK-activated phosphorylation of eIF2α, and transcriptional induction of chaperones mediated by IRE-1 and ATF-6 activation (Marciniak and Ron, 2006; Kaufman, 1999; Hussain and Ramaiah, 2007). Because of its ability to interact with various proteins, BiP is known to play a role in folding and assembly of proteins, export and degradation of proteins, regulation of ER stress sensors and the signaling pathways, calcium homeostasis, alleviation from ER-stress induced apoptosis, tumor cell proliferation, survival and angiogenesis (Dudek et al., 2009; Dong et al., 2008; Li and Lee, 2006; Luo et al., 2006; Ranganathan et al., 2006; Fu et al., 2007). In addition, BiP is also shown to inhibit caspases 3, 7 and ER-resident caspase-12 (Reddy et al., 2003; Rao et al., 2002). In ER–stressed conditions, BiP expression occurs down stream to PERK activation-mediated-eIF2α phosphorylation,

and as a result of the activation of ER stress sensors, IRE-1 and ATF6 in many mammalian cell types.

To determine the importance of stress-induced phosphorylation of the α-subunit of eukaryotic initiation factor 2 (eIF2α), a factor involved in translational initiation, in survival and suicide mechanisms, the present study analysed eIF2α phosphorylation, changes in the expression of certain genes such as ATF4 (activated transcription factor), BiP, phospho-JNK (c-Jun N-terminal kinase), CHOP (C/EBP homologous protein), caspase activation and cell death in the ovarian cells of *Spodoptera frugiperda (Sf9)* in response to diverse stressors. The analysis reveals that the expression of BiP, an ER (endoplasmic reticulum) chaperone, following eIF2α phosphorylation promotes cell survival whereas expression of CHOP and enhanced phospho-JNK are associated with cell death. However, expression of ATF4 (activated transcription factor), unlike the expression of BiP, occurs in response to eIF2α phosphorylation. A pan caspase inhibitor, z-VAD fmk, which mitigates the late stages, but not the initial stages of UV irradiation-induced eIF2α phosphorylation, does not affect brefeldin-induced eIF2α phosphorylation. Addition of a recombinant mutant phosphomimetic form, but not wt eIF2α, stimulates caspase activity and generates an active *Sf9* caspase in cell extracts prepared from untreated cells or in BiP-depleted tunicamycin-treated extracts. This is consistent with the observation that tunicamycin-treated cells expressing BiP resist partly UV-induced caspase activation and apoptosis. These findings therefore suggest that the stress-induced eIF2α phosphorylation in *Sf9* cells, the absence of BiP expression, as is the case with many non-ER stressors, is a cause and consequence of caspase activation. To further understand the importance of BiP and phosphorylation of eIF2α in regulating caspase activation, changes in inter-protein interactions were analyzed in *Sf9* cell extracts. The observations indicate that phosphorylation of eIF2α reduces its affinity for both active caspase and BiP. These findings together suggest that phosphorylation of eIF2α is primarily a stress signal and its ability to modulate caspase activation and evoke an adaptive or apoptotic response depends on its location, changes in gene expression the coincident signaling activities and inter-protein interactions.

3.2, Stress induced phosphorylation of eIF2α elicits expression of prosurvival and proapoptotic proteins

Previous observations from our laboratory indicate that phosphorylation of eIF2α is a stress signal and is associated with caspase activation and apoptosis. However, not all stressors which induce eIF2α phosphorylation were able to trigger caspase activation and apoptosis (Aparna et al., 2003). To further understand the importance of eIF2α phosphorylation in cell survival and suicide mechanisms, eIF2α phosphorylation, changes in the expression of ATF4 (activated transcription factor 4), BiP (immunoglobin binding protein), phosphorylation of JNK (c-Jun N-terminal kinase), CHOP (C/EBP homologous protein), caspase activation and cell death were analyzed in the presence of diverse stressors, to determine whether the enhanced phosphorylation is a result of stress in the cytosol or in the endoplasmic reticulum (ER), and if there exists any difference in the survival and suicide mechanisms between the ER and cytosol associated eIF2α phosphorylation. The stressors used in this study include: UV-B irradiation, a genotoxic stress; cycloheximide, a translation elongation inhibitor; tunicamycin, an N-glycosylation inhibitor; thapsigargin, a calcium ATPase inhibitor; brefeldin A, an ER to golgi transport inhibitor; salubrinal, a specific eIF2α phosphatase inhibitor; redox stressors such as dithiothreitol, reduced and oxidized forms of glutathione; Expression of BiP, an ER specific chaperone is used to determine the stress in the ER and for changes in eIF2α phosphorylation mediated gene expression. Expression of ATF4, like GCN4 in yeast, is used as marker to study the eIF2α phosphorylation mediated gene expression. Expression of CHOP and phosphorylation of JNK are used as pro-apoptotic markers here along with caspase activation.

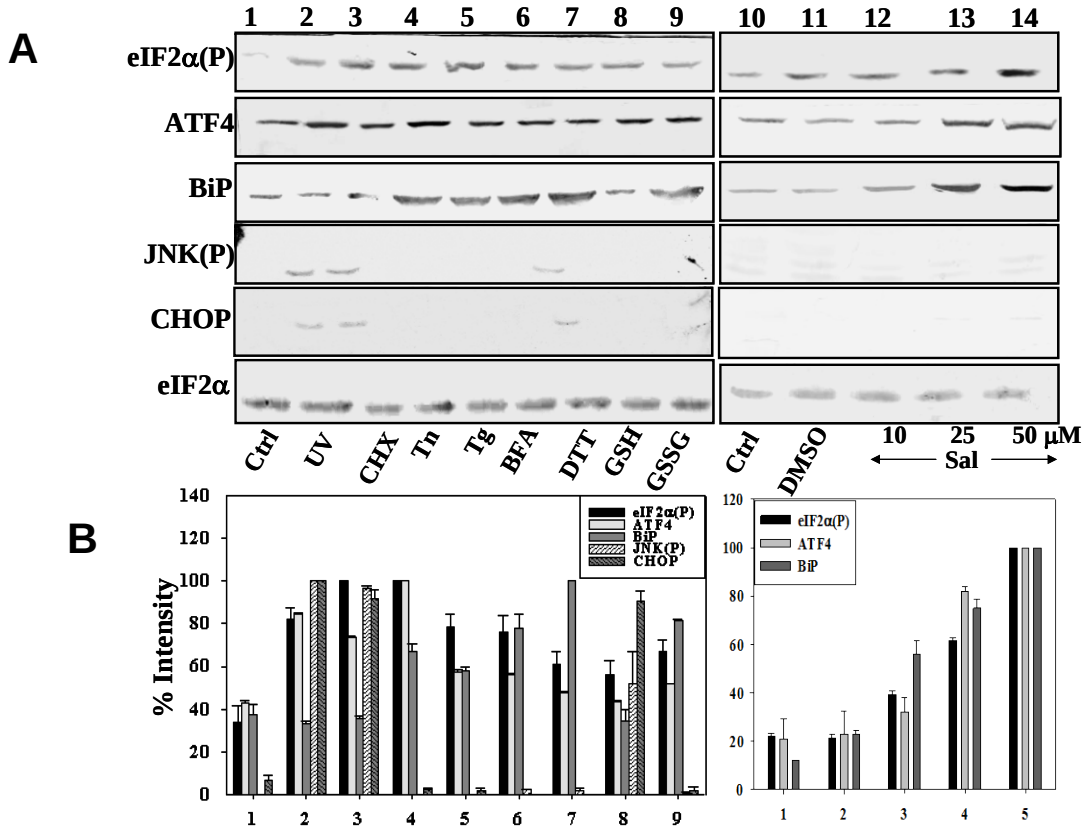
All stressors stimulated eIF2α phosphorylation **(Fig. 1)**. However, UV-irradiation, cycoheximide and GSH–treatments promoted caspase activation and cell death **(Figs. 2 and 3)**. *Sf9* cells treated with these apoptotic stressors expressed low levels of BiP **(Fig. 1A, lanes 2, 3 and 8)** when compared to other stressors such as tunicamycin, thapsigargin, brefeldin, DTT, GSSG and salubrinal **(lanes 4-7, 9 and 10)**. This is consistent with the notion that tunicamycin, thapsigargin and brefeldin are typical ER stressors. Unlike the expression of BiP, that was limited to cells treated with typical ER stressors, ATF4 expression **(Fig. 1A)** was found directly related to eIF2α

phosphorylation. This observation is consistent with the previous findings that eIF2α phosphorylation inhibits translation of general mRNAs but can up regulate gene-specific mRNAs like ATF4 (Vattem and Wek, 2004). Salubrinal, a selective inhibitor of eIF2α phosphatase, which was shown to protect mammalian cells from Herpes Simplex Virus-mediated ER stress (Boyce et al., 2005), also promoted eIF2α phosphorylation and BiP expression in *Sf9* cells in a concentration dependent manner **(Fig. 1A, lanes 12-14)** without promoting caspase activation or apoptosis **(Figs. 2 and 3)**. Expression of CHOP and phosphorylation of JNK are restricted to apoptotic stressors such as UV irradiated, cycloheximide and GSH treated cells **(Fig. 1A)**. Consistent with the western blot analyses, enhanced eIF2α phosphorylation was also detected by immuno-fluorescence in UV-irradiated cells and in brefeldin–treated cells compared to controls **(Fig. 4, column 1)**. However, BiP expression was enhanced in brefeldin-treated cells than in UV-irradiated cells **(Fig. 4, column 3)**. Immuno-localization experiments revealed that eIF2α phosphorylation and BiP were mainly localized outside and around the nucleus **(Fig. 4, columns 2 and 4 respectively)**. In addition to BiP and eIF2α phosphorylation, expression of pro-apoptotic proteins like CHOP **(Fig. 5, panel A)** and phospho-JNK **(Fig. 5, panel B)** were also analyzed here. The levels of both these proteins were significantly higher in UV-irradiated cells than in brefeldin-treated cells. This is consistent with the results that UV-irradiation, but not brefeldin treatment promoted cell death. These findings therefore suggest that eIF2α phosphorylation associated with BiP expression that occurs due to ER stress does not lead to apoptosis. In contrast, non-ER stress-induced eIF2α phosphorylation as has been observed in cells treated with UV-irradiation, cycloheximide and reduced glutathione, stimulate caspase activation and cell death **(Figs. 2 and 3)**.

Since ER stress induced eIF2α phosphorylation fails to stimulate caspase activation and apoptosis, a time course analysis for the aforementioned proteins was carried out to identify if there exists any difference in their induction with respect to time, in response to ER and non-ER stressors. The results from this analysis indicate that phosphorylation of eIF2α in response to a non-ER stress such as UV irradiation is biphasic, (i.e) it occurs immediately in response to stress at 1 hr, followed by a decline and then an increase at

the later stages **(Fig. 6A)**. However, ER stressor like brefeldin, promotes a gradual increase in phosphorylation of eIF2α, in a time dependent manner **(Fig. 6B)**. BiP expression is not significantly altered in UV irradiated cells but shows a time dependent increase in case of brefeldin treated cells **(Fig. 6A versus 6B)**. In contrast, caspase activation is stimulated in response to UV irradiation at 5 hrs of treatment and appears to be time dependent **(Fig. 6 C)**. These observations indicate that phosphorylation of eIF2α is an immediate stress response and promotes BiP expression in case of ER stress and caspase activation in case of non-ER stress.

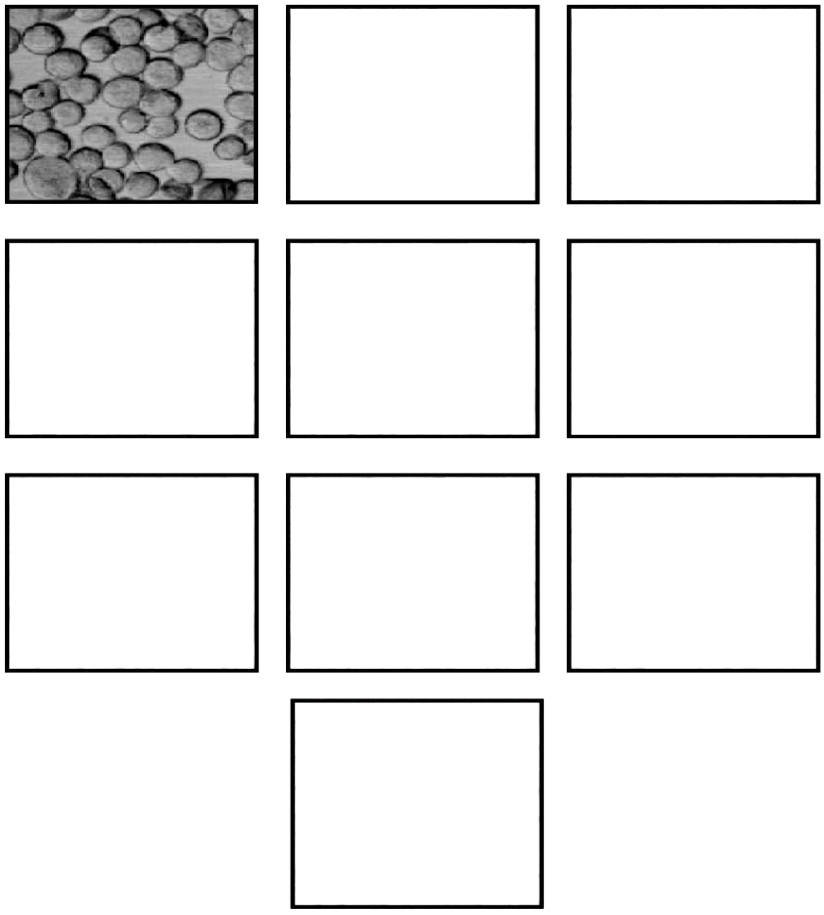
Fig. 1



Stress induced changes in eIF2α(P), ATF4, BiP, JNK(P) and CHOP.

Sf9 cells were treated with the following agents and incubated at 27^{o} C for 15 hrs. Untreated *Sf9* cells incubated for 15 hrs at 27^{o} C were used as controls. Cell extracts were prepared as described in the 'Materials and Methods'. Lysates containing 75 μg protein / lane were separated on a 10% SDS-PAGE and the levels of phosphorylated eIF2α, BiP ATF4, Phospho-JNK, CHOP and total eIF2α were determined by immunoblotting using respective antibodies. Panel a, represents western blots indicating the levels of different proteins as mentioned in the figure. The various lanes are as follows: 1) control, 2) 60 sec UV-B irradiation (312 nm), 3) 1 mM cycloheximide (CHX), 4) 12 μM tunicamycin (Tn), 5) 2 μM thapsigargin (Tg), 6) 36 μM brefeldin A(BF), 7) 5 mM DTT, 8) 10 mM GSH, 9) 5 mM GSSG 10) control 11) DMSO 12) 25 μM salubrinal (sal) 13) 50 μM sal 14) 100 μM sal. The bar diagrams in panel B, represent the quantification of the blots corresponding to panel A.

Fig. 2

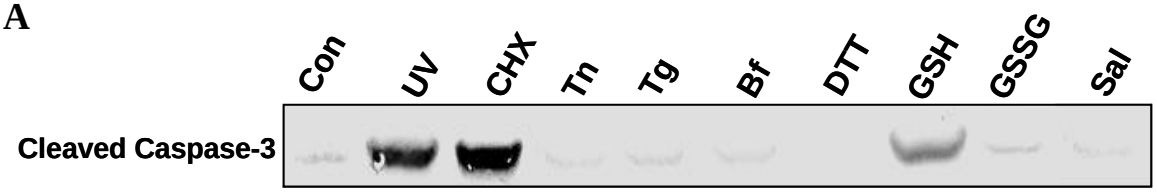


Morphology of Sf9 cells in response to different stressors.

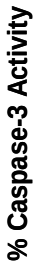
Sf9 cells were treated with the following agents and incubated at 27^{o} C for 15 hrs. Untreated *Sf9* cells incubated for 15 hrs at 27^{0} C were used as controls. The figure represents morphology of cells as viewed under inverted microscope, at 200X magnification. The percentage of apoptosis as calculated by trypan blue staining is mentioned below each treatment. The various treatments are as follows: 1) control, 2) 60 sec UV-B irradiation (312 nm), 3) 1 mM cycloheximide (CHX), 4) 12 μM tunicamycin (Tn), 5) 2 μM thapsigargin (Tg), 6) 36 μM brefeldin A(BF), 7) 5 mM DTT, 8) 10 mM GSH, 9) 5 mM GSSG and 10) 50 μM salubrinal (sal).

Fig. 3

A



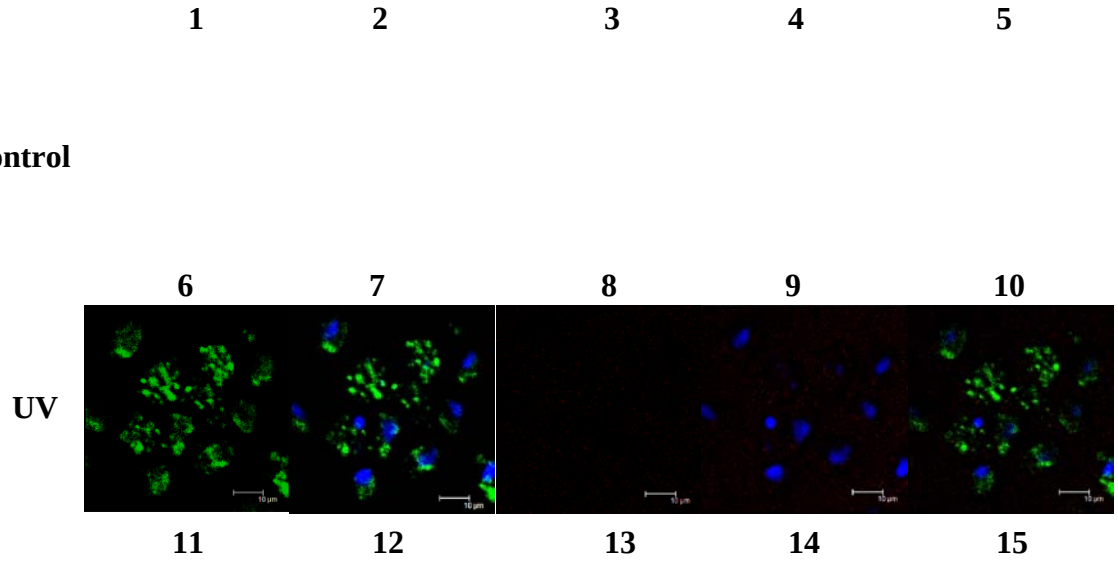
B



Analysis of Sf-caspase fragment and activity in stressed cells.

Sf9 cell extracts prepared from the cells treated with the agents as mentioned in the legend to Fig 3.2 were analyzed for the activation of caspase, using a specific antibody against cleaved caspase 3, which represents the active form of caspase. In addition, the caspase activity was also assayed spectrofluorimetrically, using Ac-DEVD-AFC as the substrate as mentioned in 'Methods'. Panel A, represents a western blot probed for cleaved caspase and panel B, represents the corresponding caspase activity. The various lanes in panel A which correspond to the bars in panel B are: 1) control, 2) 60 sec UV-B irradiation (312 nm), 3) 1 mM cycloheximide (CHX), 4) 12 μM tunicamycin (Tn), 5) 2 μM thapsigargin (Tg), 6) 36 μM brefeldin A(BF), 7) 5 mM DTT, 8) 10 mM GSH, 9) 5 mM GSSG and 10) 50 μM salubrinal (sal).

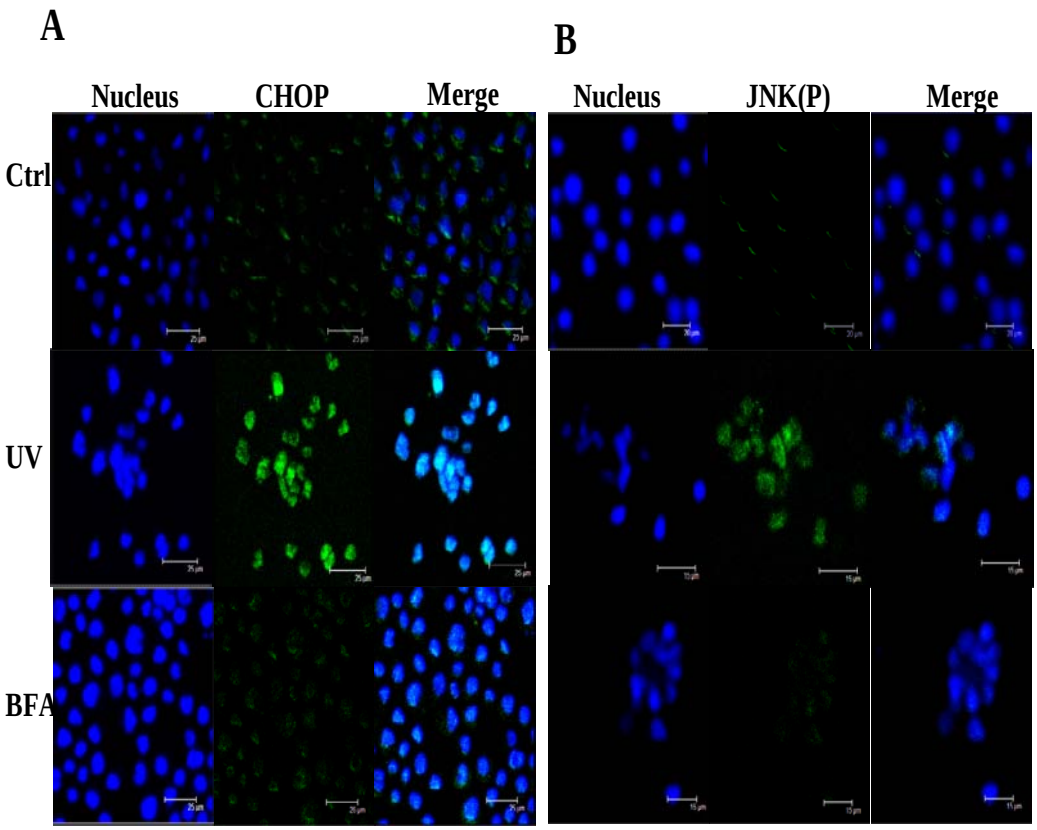
Fig. 4



Analysis of phosphorylated eIF2α and BiP in UV-irradiated and brefeldin-treated cells by confocal microscopy.

Sf9 cells exposed to UV-B light (312 nm) for 60 sec and then incubated at 27^{o} C for 15 hrs or treated with 36 μM brefeldin A for 15 hrs at 27^{o} C were then immunostained with mouse monoclonal anti-BiP and rabbit monoclonal phosphospecific anti-eIF2α antibodies along with the Hoechst stain and viewed by confocal microscopy. Green fluorescence indicates phosphorylated eIF2α, red indicates BiP and blue staining represents the nucleus. The various panels represent the following; Panels (or rows) 1-5 represent controls, 6-10 represent UV-irradiated cells and 11-15 represent 36 μM brefeldin A-treated cells. The columns represent eIF2α(P) in the non-nuclear region, eIF2α (P) in the nuclei, BiP, in the non-nuclear region, BiP in the nuclear region and the colocalization of eIF2 α(P) and BiP as indicated up and above the columns.

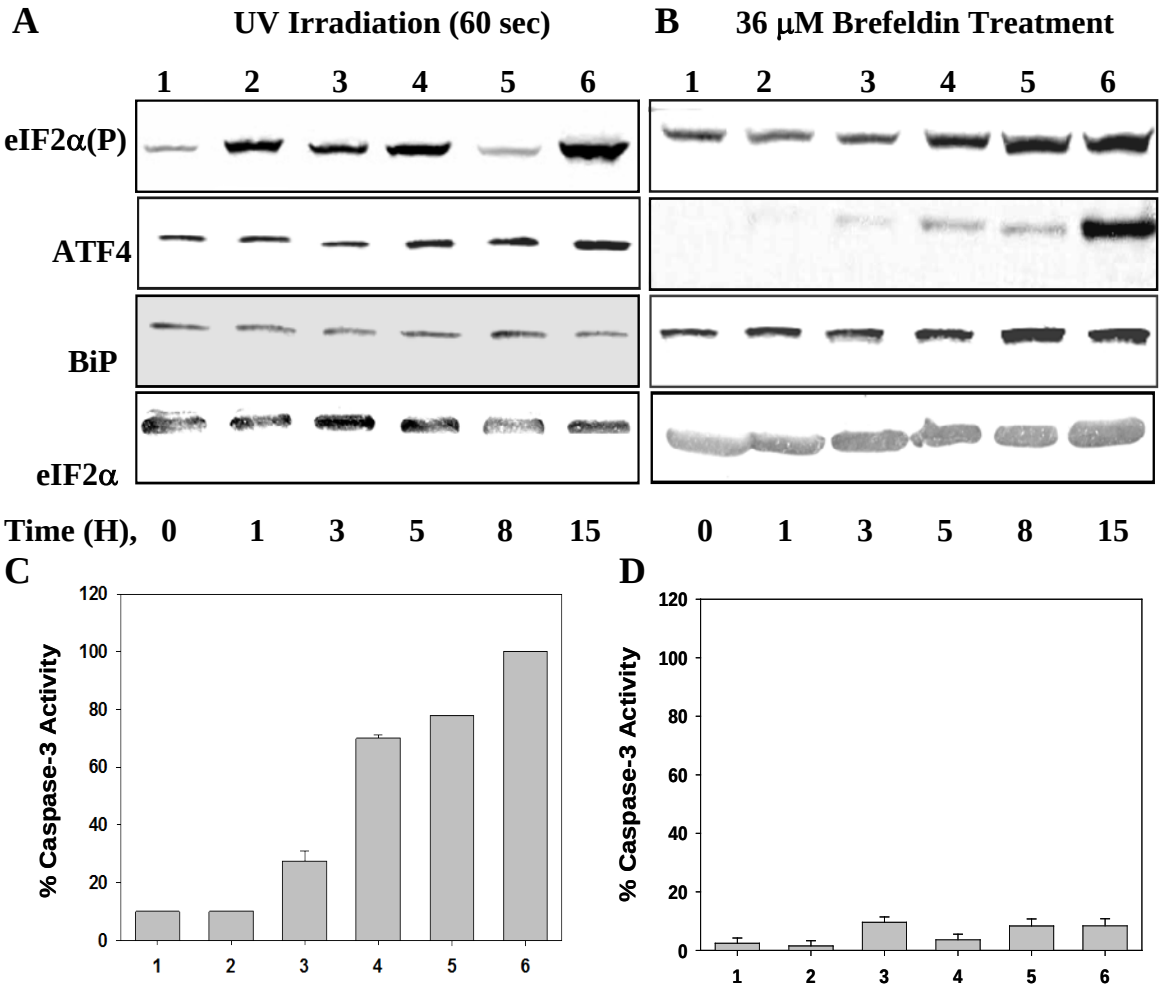
Fig. 5



Analysis of CHOP and phosphorylated JNK in UV-irradiated and brefeldin-treated cells by confocal microscopy.

Sf9 cells were treated with UV-B light or brefeldin for 5 hrs as mentioned in the legend to Fig 2, and then these were immunostained with mouse monoclonal anti-CHOP or phosphospecific anti-JNK antibodies and viewed by confocal microscopy. Panel a, depicts the comparison of CHOP protein levels and panel b, represents the phosphorylated JNK levels in control, UV-irradiated and brefeldin-treated cells respectively.

Fig. 6



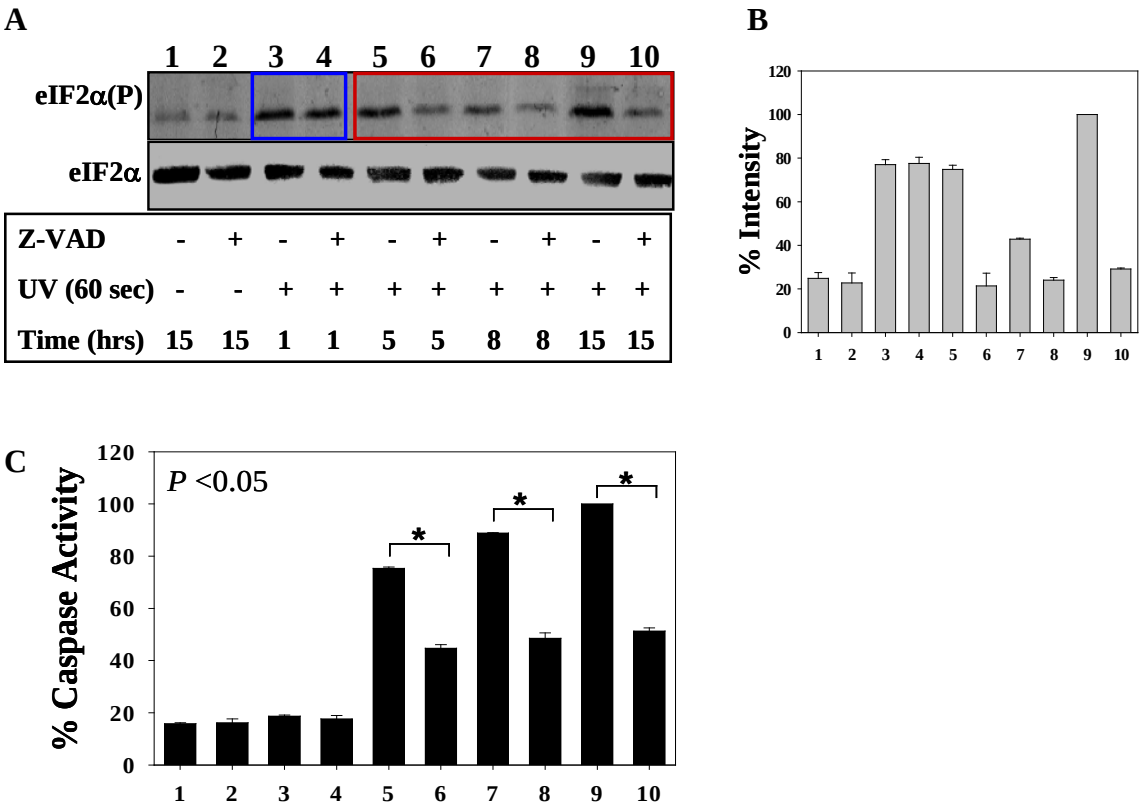
Time course analysis of eIF2α(P), ATF4 and BiP in UV-irradiated and brefeldin-treated cells.

*Sf*9 cells were irradiated with UV B light for 60 sec or treated with 36 mM brefeldin and incubated for different time periods as indicated in the figure. Cell extracts were prepared as described in 'Methods' and ~75 mg containing whole cell proteins were separated by 10% SDS PAGE and transferred onto a nitrocellulose membrane. The blot was probed with antibodies against phosphospecific anti-eIF2α, total eIF2α, ATF4 and BiP. The lysates were also used for measuring the caspase activation by spectrofluorimetrically. The various panels include: A, time course analysis of proteins in response to UV irradiation; B, time course analysis of proteins in response to brfeldin treatment; C, Caspase activation in UV irradiated cells and D, caspase activity in brefeldin treated cells. The various lanes in the blot correspond to the bars in the graph.

3.3, eIF2α phosphorylation is a cause and consequence of caspase activation

Earlier studies have shown that expression of phosphomimetic form of eIF2α or double-stranded RNA-dependent kinase, PKR, was shown to promote apoptosis in mammalian cells (Srivastava et al., 1998; Scheuner et al., 2006). However, phosphomimetic form of eIF2α was expressed in *Sf9* cells using baculovirus expression system (Sudhakar et al., 1999 and 2000). Subsequent studies have shown that caspase activation in UV-irradiated *Sf9* cells followed eIF2α phosphorylation and wt baculovirus harboring anti-apoptotic p35 gene or z-VAD-fmk, a pan caspase inhibitor mitigates UV-induced eIF2α phosphorylation and cell death in insect cells (Aparna et al., 2003). Caspases can also activate PKR kinase in mammalian cells (Saelens et al., 2001). Thus it is likely that eIF2α phosphorylation is a cause and consequence of caspase activation. To distinguish the eIF2α phosphorylation mediated by a caspase activated eIF2α kinase from an active eIF2α kinase, the UV-induced eIF2α phosphorylation was analyzed here at different time points in the presence and absence of z-VAD-fmk. Interestingly, UV-induced eIF2α phosphorylation was not affected by z-VAD-fmk within the first hr of UV irradiation **(Fig. 7A, lanes 4 vs 3).** However, the late stages i.e., at 5, 8 and 15 hrs of UV-induced eIF2α phosphorylation were significantly reduced in the presence of caspase inhibitor **(lanes 5, 7 and 9 vs 6, 8 and 10).** Since caspase activation occurs downstream to eIF2α phosphorylation within 3-5 hrs of UV-irradiation **(Fig. 6C)**, the findings here suggest that eIF2α phosphorylation is a cause and consequence of caspase activation. This suggestion is further supported by the fact that brefeldin-induced eIF2α phosphorylation which is not associated with caspase activation or cell death, is also not affected by the caspase inhibitor z-VAD-fmk *(Fig. 8).*

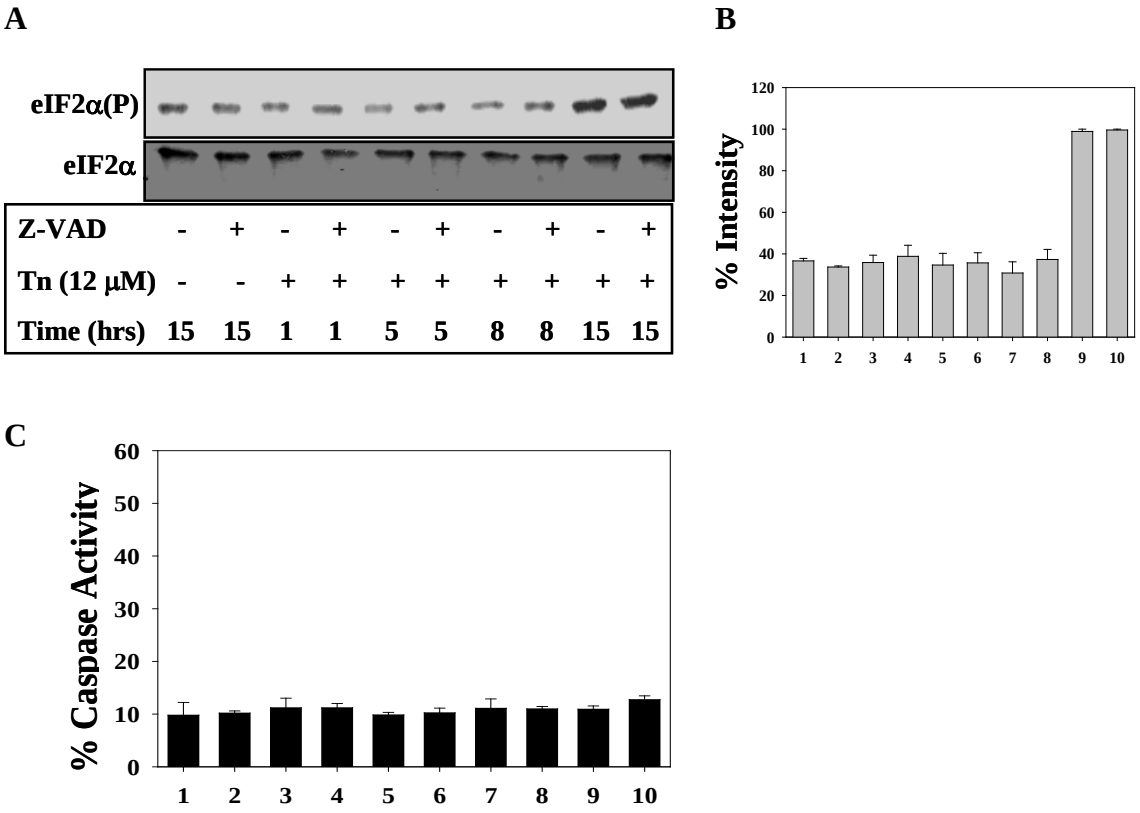
Fig. 7



Changes in eIF2α phosphorylation and caspase activity at different time points in UV-irradiated cells in the presence and absence of zVAD-fmk.

*Sf*9 cells were treated with UV-irradiation for 60 sec and then incubated at 27^0 C for different time periods in the presence and absence of z-VAD-fmk. Cell extracts were then prepared and analyzed for eIF2α phosphorylation by immunoblot analysis using a phosphospecific anti-eIF2α antibody and caspase activity of the extracts was monitored using Ac-DEVD-AFC hydrolysis. Panels a, represents the phosphorylation status of eIF2α in UV- irradiated cells in the presence and absence of z-VAD-fmk. Panel b, represents the corresponding densitometry and c, represents the corresponding caspase activity. Total eIF2α levels were used here as a loading control. (* indicates p value less than 0.05 and n=3).

Fig. 8



Changes in eIF2α phosphorylation and caspase activity at different time points in brefeldin-treated cells in the presence and absence of zVAD-fmk.

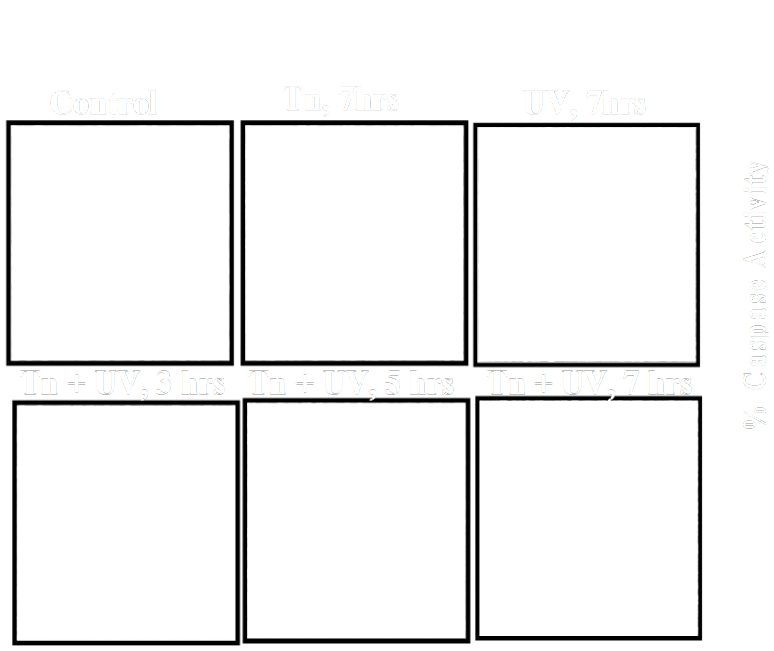
Sf9 cells were treated with 36 μM brefeldin at 27^0 C for different time periods in the presence and absence of z-VAD-fmk. Cell extracts were then prepared and analyzed for eIF2α phosphorylation by immunoblot analysis using a phosphospecific anti-eIF2α antibody and caspase activity of the extracts was monitored using Ac-DEVD-AFC hydrolysis. Panel a, represents the phosphorylation status of eIF2α in brefeldin-treated cells in the presence and absence of z-VAD-fmk. Panel b, represents the corresponding densitometry and c represents the caspase activity. Total eIF2α levels were used here as a loading control.

3.4, BiP expression resists apoptosis and interferes with caspase activation

To further understand the role of BiP in eIF2α phosphorylation mediated caspase activation and apoptosis, expression of BiP, phosphorylation of eIF2α, caspase activation and cell death were monitored in 5 hr-tunicamycin pretreated cells that were UV-irradiated for 60 seconds and incubated at 27^0 C for 3, 5 and 7 hrs **(Fig. 9)**. BiP expression and phosphorylation of eIF2α **(Fig. 10A)** were enhanced in UV-irradiated cells at 3 , 5 and 7 hrs which were pre-treated with tunicamycin compared to UV-irradiated cells alone **(lanes 2, 3, 4 vs 1)**. UV-irradiated cells which displayed higher caspase activation **(Fig. 9B)** and apoptosis **(Fig. 9A)** compared to tunicamycin pretreated UV-irradiated cells, elicited enhanced phosphorylation of eIF2α which was associated with low levels of BiP expression **(Fig. 10A)**. Small changes in BiP expression apparently can modulate eIF2α phosphorylation-mediated changes in caspase activation.

Complementing the above observations **(Figs. 9 and 10)**, addition of purified S51D, a recombinant phosphomimetic form of human eIF2α, but not the wild type eIF2α, stimulated generation of active caspase fragment in *Sf9* cell extracts prepared from healthy cells **(Fig. 11, lane 5 vs 3)** than from tunicamycin-treated cells **(lane 7)** only in the absence of a caspase inhibitor, Ac-DEVD-CHO. Consistent with these observations, addition of a recombinant phosphomimetic S51D mutant eIF2α where Ser-51 residue is replaced by an aspartic acid, enhanced the generation of an active caspase fragment and caspase activity in BiP-depleted tunicamycin-treated extracts than in undepleted extracts **(lane 9 vs 8)**. These findings therefore suggest that eIF2α phosphorylation associated with BiP expression interferes in caspase activation.

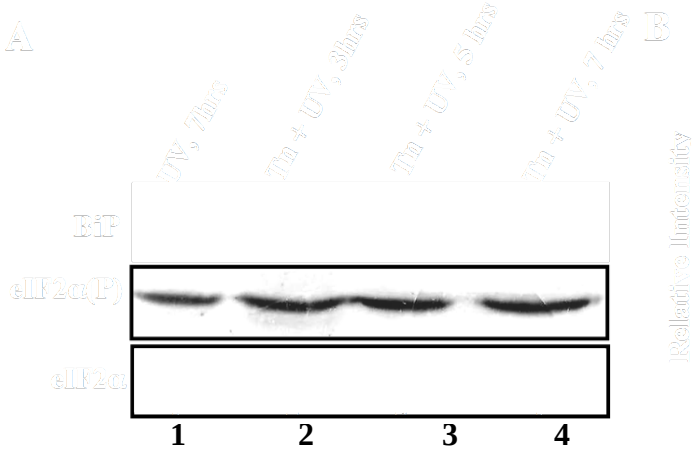
Fig. 9



Morphology of UV-irradiated cells pre-treated with tunicamycin.

Sf9 cells were pretreated with 12 μM tunicamycin for 5 hrs at 27^0 C and then irradiated with UV-B light for 60 sec and incubated for 3, 5 and 7 hrs respectively at 27^0 C. Cells were monitored for morphological changes and the cell extracts prepared from these cells were used to analyze the caspase activation as mentioned in the legend to Fig. 4. The various panels are as follows: a, represents cell morphology viewed under an inverted microscope and b, represents caspase activity. The various morphological figures in panel a that correspond to the numbers in panel b represent the following: 1, control; 2, 12 μM tunicamycin treatment for 7 hrs (Tn, 7hrs); 3, UV-irradiated cells incubated for 7 hrs (UV, 7 hrs); 4, 12 μM tunicamycin pre-treatment for 5 hrs followed by UV irradiation and incubated for 3 hrs (Tn+UV, 3 hrs); 5, 12 μM tunicamycin pre-treatment for 5 hrs followed by UV irradiation and incubated for 5 hrs (Tn+UV, 5 hrs); 12 μM tunicamycin pre-treatment for 5 hrs followed by UV irradiation and incubated for 7 hrs (Tn+UV, 7 hrs); (* indicates significant decline in caspase activity in tunicamycin pre-treated group when compared to UV irradiated cells; $P < 0.05$ and n=3).

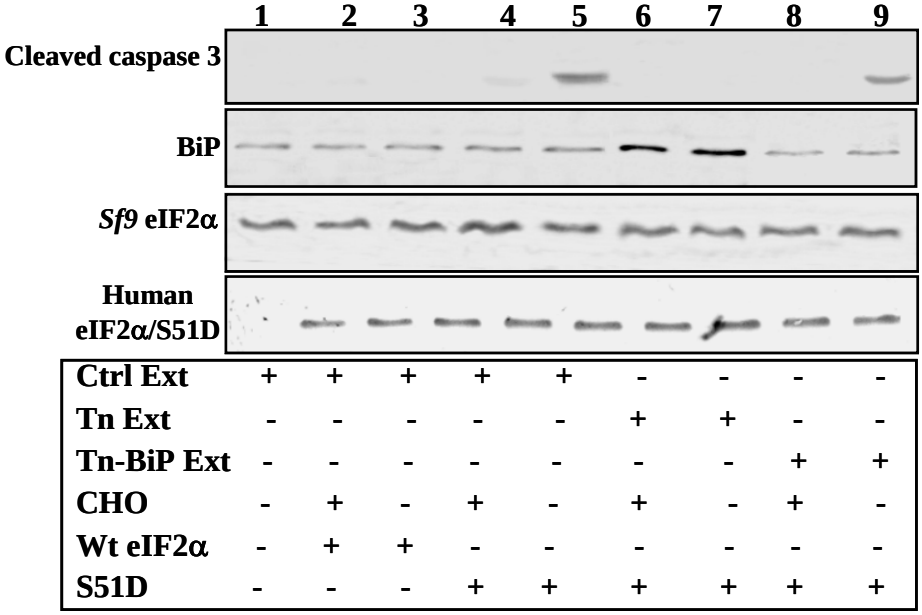
Fig. 10



Analysis of BiP and phosphorylated eIF2α in UV-irradiated cells pre-treated with tunicamycin.

*Sf*9 cells were pretreated with 12 μM tunicamycin for 5 hrs at 27^0 C and then irradiated with UV-B light for 60 sec and incubated for 3, 5 and 7 hrs respectively at 27^0 C as mentioned in the legend to figure 6. The cell extracts prepared from these cells were used to analyze the BiP, phosphorylated eIF2α and total eIF2α by western blot analysis. The various panels are as follows: a, represents western blot indicating expression of BiP, eIF2α phosphorylation and total eIF2α levels; b, bar diagram, represents corresponding densitometric analysis of eIF2α(P) and BiP detected in panel a; The various lanes in panel a that correspond to the numbers in panel b represent the following: lane 1, UV-irradiated, lane 2, tunicamycin-treated + UV irradiation- 3 hrs; lane 3, tunicamycin-treated + UV-irradiated- 5 hrs and lane 4, tunicamycin-treated + UV-irradiated- 7 hrs.

Fig. 11



Sf-caspase activation and caspase-3 like fragment release in Sf9 cell extracts: Effect of recombinant phosphomimetic form (S51D) of eIF2α.

Cell extracts were prepared from untreated and 12 μM tunicamycin-treated cells. BiP was depleted from tunicamycin-treated extracts wherever indicated (Tn-BiP) by immunodepletion as mentioned in "Materials and Methods". These extracts were then incubated with 5 μg of recombinant wt eIF2α or a phosphomimetic mutant form of eIF2α (S51D) for 1 hr at 37^{o}C in presence and absence of 5 μM Ac-DEVD-CHO, a caspase inhibitor. Caspase cleavage was monitored by immunoblot analysis using a monoclonal antibody specific for active caspase-3 and the activity was also measured by a spectrofluorimetric analysis involving hydrolysis of Ac-DEVD-AFC as described in 'Materials and Methods' . The various panels represent the following: A, represents western blots indicating caspase cleavage, levels of BiP and eIF2α; B, represents the caspase activity corresponding to the lanes in panel a; (* indicates significant increase in caspase activity in extracts devoid of BiP when compared to controls; $P < 0.0001$ and n=3).

3.5, Phosphorylation of eIF2α reduces its interaction with BiP and caspase3

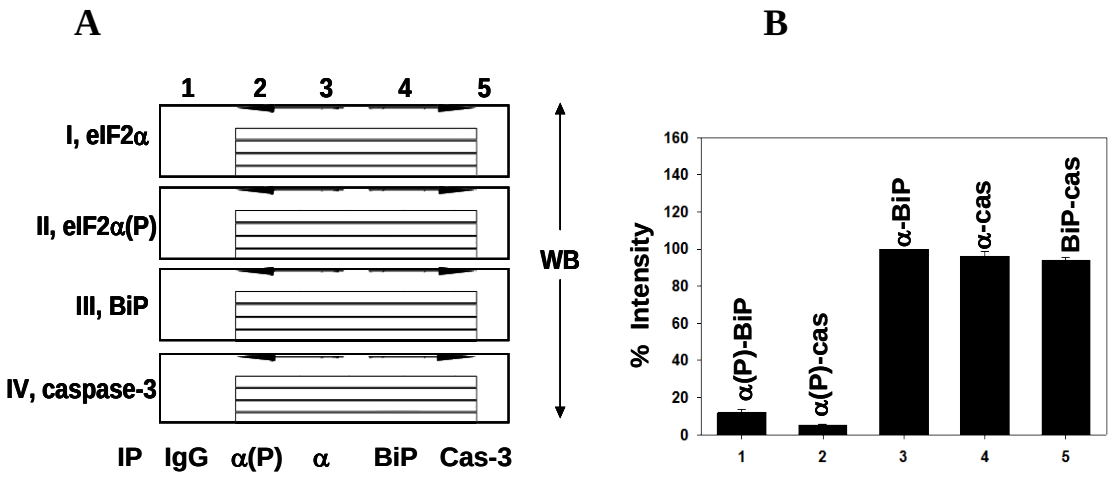
Results from the observations presented above **(Figs 1-11)** indicate that BiP probably interferes in ER stress induced eIF2α phosphorylation mediated caspase activation and apoptosis. Reports published earlier in mammalian cells indicate that, BiP can interact with an ER resident caspase such as caspase 7 (Rao et al., 2002) and knockdown of BiP using an siRNA directed against it promotes caspase activation and cell death (Luo et al., 2006). Further, eIF2α is shown to interact with mammalian caspase 3 (Marissen et al., 2000; Rajesh Kamindla, 2009) and has been shown that unlike unphosphorylated form, phosphorylated form of eIF2α is a better substrate for purified caspase 3 (Rajesh et al., 2008). *Sf9* cells are known to harbour a single caspase called *Sf*-caspase (Liu and Chejanovsky, 2006), which behaves more like a caspase 3 (Aparna et al., 2003). Suspecting there could be an interaction among eIF2α, BiP and *Sf*-caspase, which could be modulated by changes in phosphorylation of eIF2α, interactions among these proteins have been evaluated here in *Sf9* cell extracts by co-immunoprecipitation, far-western and pull down analyses **(Figs. 12-14).** ER stressed *Sf9* cells expresss BiP without caspase activation while UV irradiated cells which fail to express BiP display an active caspase. To ensure the presence of both BiP and caspase in extracts, cell extracts prepared from UV- irradiated cells and that of tunicamycin treated cells were mixed in equal proportion. Recombinant human wild type and phosphomimetic forms of alpha subunit of eIF2 were expressed and purified as described in Rajesh, 2009; to carry out the pull down and far-western analyses. In addition to *Sf9* cell extracts, pull-down analysis was also carried out in *HeLa* cell extracts prepared from tunicamycin treated cells.

The results on co-immunoprecipitation indicate that cell extracts immunoprecipitated with phosphospecific anti-eIF2α antibody **(Fig. 12, lane 2),** co-precipitates BiP and *Sf*-caspase, as evidenced by the western blot analysis with the respective antibodies **(panel A, lane 2, III and IV)**. Deducting the non-specific interaction of these proteins as evidenced in the presence of anti-IgG **(lane 1, I-IV)**, these results indicate that there exists a faint interaction among phosphorylated eIF2α, BiP and *Sf*-caspase. In contrast, antibody against eIF2α co-precipitates relatively significant amounts of BiP and *Sf*-caspase **(lane 3, III and IV).** Complimenting these observations, anti-BiP antibody co-precipitates relatively high levels of unphosphorylated form of eIF2α than

phosphorylated form **(lane 4, I vs II)**, and its interaction with *Sf*-caspase is relatively higher compared to the interaction displayed by eIF2α with caspase **(lane 4 vs 3, IV)**. Similarly, anti caspase-3 antibody co-immunoprecipitates relatively higher amount of BiP **(lane 5, I and III),** and unphosphorylated form of eIF2α than phosphorylated form **(lane 5, I vs II)**. Taken together, these results based on co-immunoprecipitation studies suggest that unphosphorylated form of eIF2α than the phosphorylated form interacts more readily with both BiP and *Sf*-caspase.

To further confirm these observations, purified recombinant subunits of human wt eIF2α and its phosphomimetic mutant form were separated on a 10% SDS-PAGE and transferred onto a nitrocellulose membrane. The membrane was then incubated with *Sf9* cell extracts containing both BiP and active caspase002C and the interaction among the proteins was determined by the use of respective antibodies in a far-western analysis **(Fig. 13)**. Consistent with the co-immunoprecipitation studies, unphosphorylated form of eIF2α interacts more efficiently with *Sf*-caspase and BiP than the phosphomimetic form **(lanes 2 and 3 in blots I and III respectively).** Similar results are also observed in the pull-down experiments, where the unphosphorylated recombinant eIF2α linked to the Ni-NTA agarose beads is able to interact efficiently with BiP and active caspase both in HeLa and *Sf9* cell extracts compared to the phosphomimetic form **(Fig. 14, lanes 3 and 9 vs 6 and 12 in blots I and III).** These studies demonstrate clearly that there exists an interaction among eIF2α, BiP and *Sf*-caspase. However, phosphorylation of eIF2α significantly reduces its affinity for BiP and caspase, suggesting that the interaction of BiP with eIF2α and *Sf*-caspase, is probably necessary for maintaining the caspase in its inactive state. Further, the reduced interaction between phosphorylated eIF2α and caspase, supports our previous observation that the phosphorylated eIF2α resists caspase action while the unphosphorylated form is a better caspase substrate (Rajesh et al., 2008). These findings therefore suggest that eIF2α phosphorylation-mediated caspase activation in indirect and is regulated by inter-protein interactions.

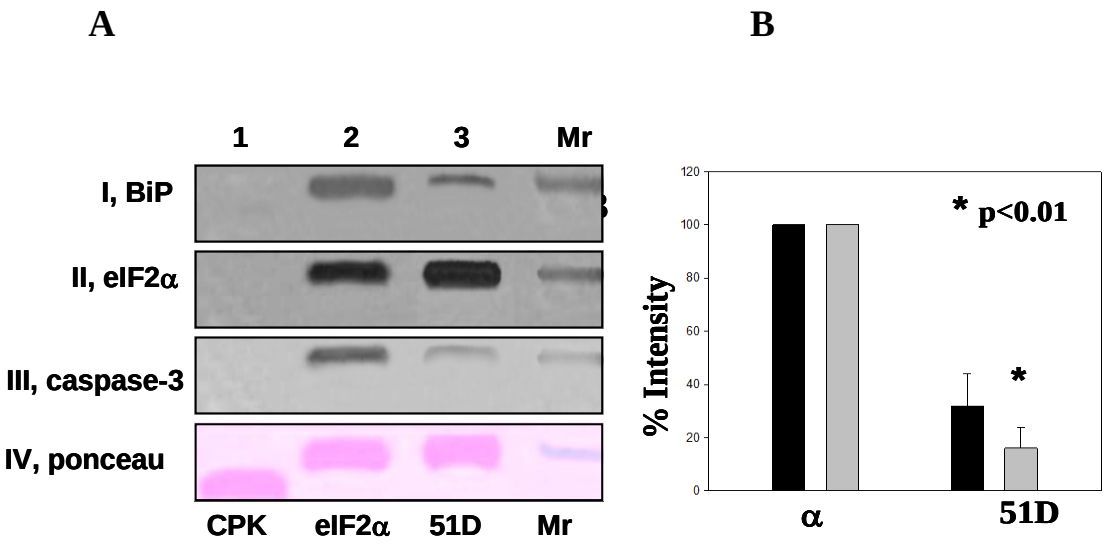
Fig. 12



Interaction among eIF2α, BiP and Sf-caspase: Co-immunoprecipitation studies.

Sf9 cell extracts were prepared by mixing equal proportion of the extracts from cells treated with 12 μM tunicamycin for 15 hrs or exposed to one minute UV-irradiation and incubated at 27^{o} C for 15 hrs. Co-immunoprecipitation studies were carried out with the following antibodies: a) anti-IgG, b)phospho specific eIF2α, c) anti- eIF2α, d) anti-BiP or e) anti-caspase 3 antibodies to determine the interaction among these proteins. Panel A represents the interaction of proteins immunoprecipitated as mentioned above with eIF2α (I); eIF2α(P) (II); BiP (III) and caspase (IV) respectively. Panel B indicates the corresponding quantification of the interaction.

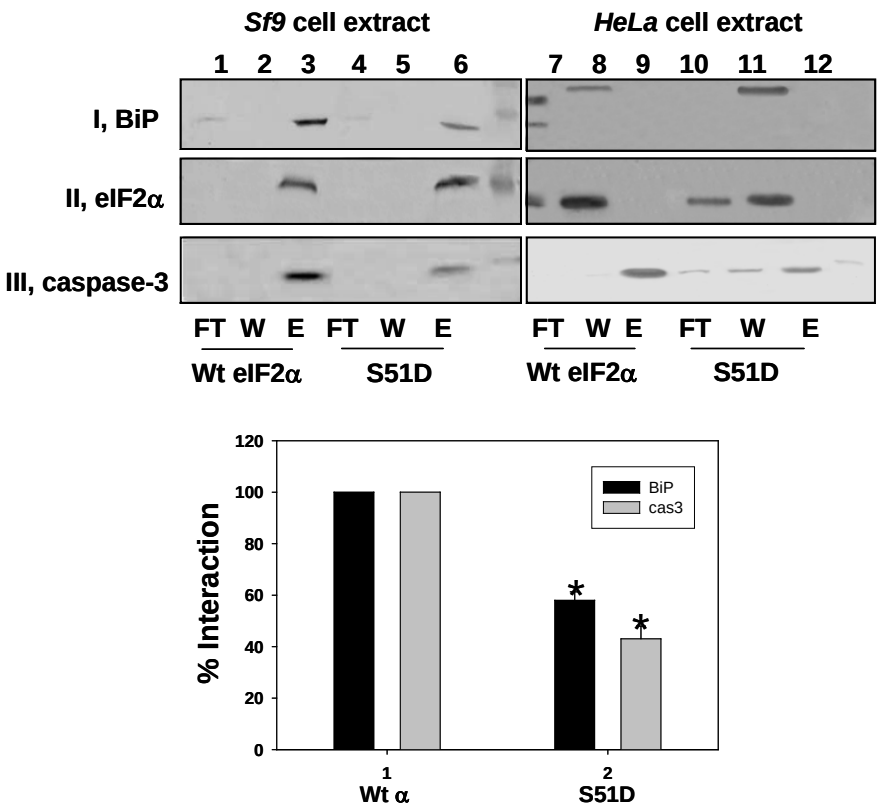
Fig. 13



Interaction among eIF2α, BiP and Sf-caspase: Far-western analysis.

Purified recombinant human wt and phosphomimetic forms were separated on a 10% SDS-PAGE and transferred onto the nitrocellulose membrane. CPK is used as a negative control. The membrane was probed with *Sf9* cell extracts which was prepared as described in the legend to Fig. 3.11 to monitor the interaction among these proteins. The blot was then probed with respective antibodies to detect the interaction. The figure in panel A, is an immunoblot and I-IV depict the interaction of eIF2α (wt) and S51D forms with: (I) BiP (II) eIF2α; (III) *Sf*–caspase and (IV) indicates the levels of the recombinant eIF2 subunits as detected by ponceau staining. Panel B, represents the quantification of interaction.

Fig. 14



Interaction among eIF2α, BiP and Sf-caspase: Pull-down analysis.

Recombinant wt and mutant S51D eIF2α-subunits were bound to Ni-NTA resin and a pull down assay was carried out as described in Materials and Methods using *Sf9* extracts prepared as mentioned previously, and 12 μM tunicamycin treated HeLa cell extracts. Various unbound and bound fractions were separated on a 10% SDS-PAGE and transferred onto a nitrocellulose membrane. The figure is a western blot. Panel A, depicts the interaction of the proteins in *HeLa* cell extract and *Sf9* cell extract respectively. The upper portion of both panels indicates the levels of BiP and the lower portion indicates the levels of cleaved caspase 3 which are co-eluted with the recombinant subunits as shown in the middle portion. Panel B represents the quantification of interaction in presence of wt and mutant forms of eIF2α. Black bar represents the interaction with BiP while the gray bar represents the interaction with active caspase 3. (* indicates significant decline in interaction of S51D with BiP and caspase 3 when compared to unphosphorylated eIF2α; $P < 0.05$ and n=3).

Stress-induced cell death in Sf9 cells: Importance of MAPKs and eIF2α phosphorylation

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The cell has the ability to choose between adaptation and apoptotic demise in response to stressful insults through multiple interacting anti-apoptotic and pro-apoptotic signaling pathways. When one pathway becomes predominant, a delicate balance is perturbed resulting in either an adaptive or an apoptotic response. In addition phosphorylation of eIF2α, cells also respond to diverse stresses by the activation of signaling cascades such as the mitogen activated protein kinase (MAPK) pathways. MAPKs comprise a family of ubiquitous serine/threonine kinases which mediate the signaling cascades involved in a variety of cellular events ranging from embryogenesis, cell differentiation, proliferation and death (Pearson et al., 2001). Three major MAPK cascades are highly conserved in many systems for balancing cell survival and death. These include: the ERK cascade, c-jun N-terminal kinase (JNK) cascade and the p38 MAPK cascade (Raman et al., 2007).

ERK1 and 2 are proteins of molecular weight 43 and 41 kDa and are nearly (~85%) identical to each other. Both proteins are ubiquitously expressed; however, their relative abundance varies. They are activated in response to various stimuli such as serum, growth factors, cytokines, stresses, ligands for G-protein coupled receptors (GPCRs) etc., Although ERK activation has been linked to apoptosis in few cases (Zugasti et al., 2001), it is predominantly linked to cell survival (Monick et al., 2004; Luciano et al., 2003). ERK contributes to cell survival by multiple mechanisms such as the inhibition of a number of pro-apoptotic proteins such as caspase 9 and BimEL which serve as substrates for ERK (Allan et al., 2003; Luciano et al., 2003) or through the induction of various anti-apoptotic proteins such as Bcl-2 family of proteins and BiP (Wang et al., 2007; Zhang et al., 2009). Mek 1 and Mek 2 represent the only known upstream activators of ERK1/2. Mek1/2 double knock out mice die within 24 hrs after birth (Scholl et al., 2007). Erk2 knockout mice are embryonically lethal due to placental defects (Hatano et al., 2003; Saba-El-Leil et al., 2003), whereas Erk1 knockout mice are viable (Nekrasova et al., 2005) but display hyperplastic cutaneous lesions, suggesting that the ERK signaling is necessary for cell survival.

P38 MAPKs along with JNK are described as stress activated protein kinases (SAPKs), as they are activated in response to diverse stresses and cytokines. Studies using knockout

mice have demonstrated diverse roles of SAPKs in cellular processes including regulation of cell cycle, induction of cell death, differentiation and senescence. SAPKs are known to regulate the activity of many transcription factors, kinases, cytosolic and nuclear proteins. Numerous evidences demonstrate that the SAPKs function as pro-apoptotic kinases. For instance, mice deficient in both jnk1 and 2 had reduced apoptosis in the brain during development (Sabapathy et al., 1999; Kuan et al., 1999). Similarly, cardiomyocytes and fibroblasts lacking p38α, are more resistant to apoptosis induced by different stimuli (Porras et al., 2004).

Since the MAPK are shown to be activated in response to stress and can modulate apoptosis, the importance of phosphorylation of MAP kinases such as ERK, p38 and JNK in regulating eIF2α phosphorylation mediated cell survival and death are analyzed here in the ovarian cells of *Spodoptera frugiperda (Sf9)* cells. The observations from this part of the work indicates that phosphorylation of ERK and eIF2α are enhanced both in response to ER and non-ER stressors. However, the ER stressors which are typically associated with enhanced BiP expression stimulate ERK phosphorylation more readily than non-ER stressors, which activate the pro-apoptotic kinases like p38MAPK and JNK and cause cell death. Interfering into ERK phosphorylation by U0126, a pharmacological inhibitor, reduces BiP expression, enhances phosphorylation of eIF2α, p38 and JNK, along with caspase activation, and aggravates cell death. In contrast, inhibitors of JNK and p38MAPK, reduce eIF2α phosphorylation and caspase activation to a small extent. A pan caspase inhibitor like z-VAD-fmk inhibits stress-induced cell death and eIF2α phosphorylation in the presence and absence of ERK inhibitor. However, z-VAD-fmk along with 5 mM EGTA mitigates completely the stress-induced cell death in the presence of ERK inhibitor, whereas, absence of EGTA leads to ~70% protection only, thereby suggesting that the cell death which results due to the inhibition of ERK phosphorylation is partially caspase independent. Together, these findings suggest that enhanced ERK phosphorylation and reduced eIF2α phosphorylation associated with BiP expression, serve as pro-survival signals.

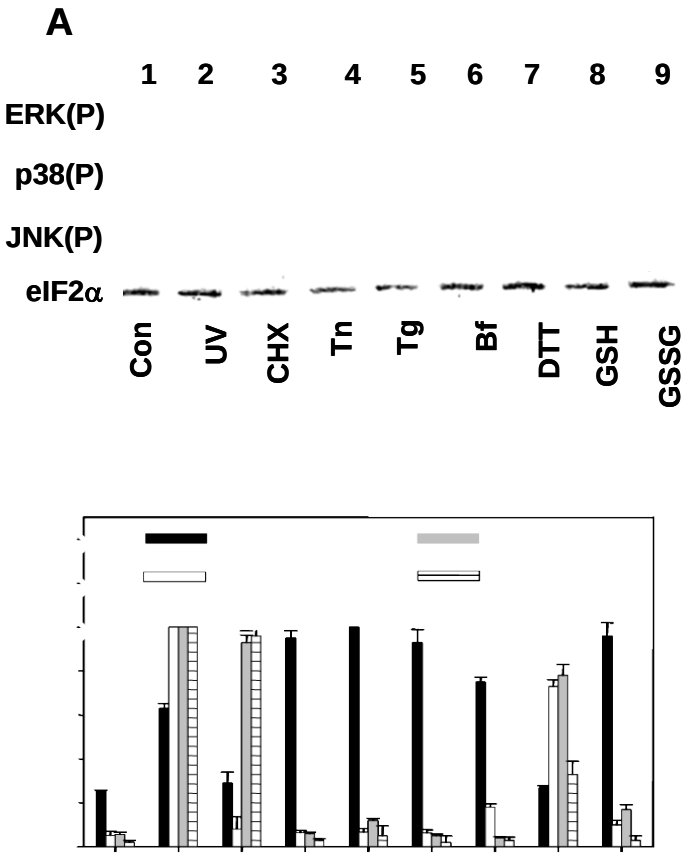
3.6, *Differential activation of MAPKS in response to pro- and non-apoptotic stressors*

Molecular mechanisms involving the paradoxical effects of eIF2α phosphorylation in cell survival and death are not resolved. It appears to be dependent on cellular location of eIF2α phosphorylation, changes in inter-protein interactions mediated by eIF2α phosphorylation as mentioned above and also on the activation of co-incident signaling pathways as suggested previously (Aarti et al., 2010). Since mitogen activated protein kinases (MAPKs) modulate variety of cellular processes including cell growth, differentiation and apoptosis (Pearson et al., 2001) and are activated in response to stress, role of MAPKs in modulating the effects of eIF2α phosphorylation mediated cell survival and death are analyzed here. MAPK subfamilies include (1) p44 and p42 MAPKs, also known as extracellular signal regulated kinase 1 and 2 (ERK1 and 2); (2) p54 and p46 c-Jun N-terminal kinase 1 and 2 (JNK1/2); (3) p38MAPK (Raman et al., 2007). Previous reports have shown that JNK/SAPK leads to cell death, while MEK/ERK activation contributes to cell differentiation, proliferation and survival. Similarly, activation of the ERK pathway is a cytoprotective mechanism and offers resistance to both extrinsic and intrinsic pathways of apoptosis (Hersey et al., 2006; Roberts and Der, 2007).

Results from this study indicate that treatment with both ER and non ER stress inducers caused an increase in phosphorylation of ERK **(Fig. 15, lane 1 vs 2-9)** and ER stressors induce comparitively higher ERK phosphorylation **(lanes 4-7 and 9)** than non-ER stressors **(lanes 2, 3 and 8)**. However, unlike ER stressors, non ER stressors which stimulate cell death, induce p38 and JNK phosphorylation in *Sf9* cells **(lanes 2, 3 and 8)**. Although both non-ER and ER stressors induce eIF2α phosphorylation immediately within one hour of treatment **(Fig. 16)**, brefeldin is however observed to take longer time (about 4-5 hrs) to induce significant eIF2α phosphorylation **(panel B, lanes 4-6 vs 2 and 3)**. This caveat has been exploited here to determine whether phosphorylation of ERK occurs prior to or after eIF2α phosphorylation. Time course analysis of ERK phosphorylation **(Fig. 16)** further reveals that ERK phosphorylation, in the case of brefeldin, an ER stressor **(panel B)**, is maintained for at least 15 hrs. However, in case of UV-irradiation, a non-ER stressor **(panel A)**, ERK phosphorylation seems to be apparent from 1 hr after treatment which then declines at later time points. These results suggest

that both ERK and eIF2α phosphorylation occur in response to stress, however, ERK phosphorylation appears to be more sensitive and occurs prior to eIF2α phosphorylation in case of brefeldin treatment. The differential phosphorylation and activation of the MAPKs in apoptotic and non-apoptotic stressors suggest a possible involvement of these kinases in eIF2α phosphorylation mediated cell survival and death mechanisms.

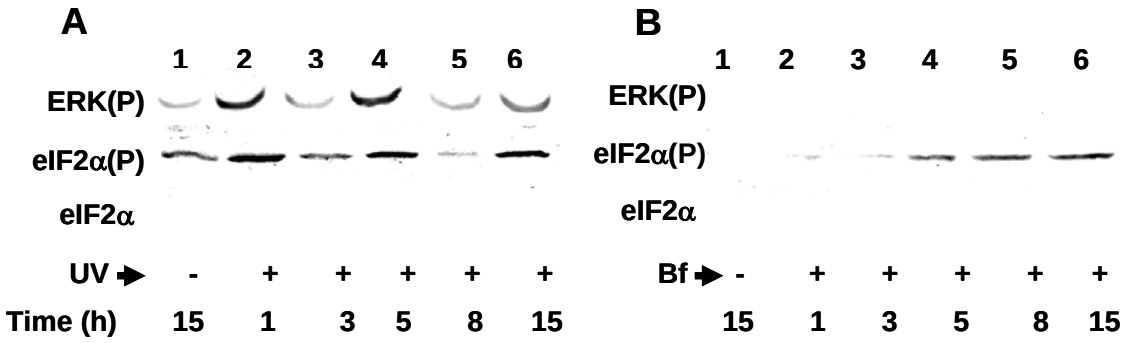
Fig. 15



Changes in phosphorylation of MAPK and Sf-caspase activation in response to different stressors.

Sf9 cells were treated with the following agents and incubated at 27^{o} C for 15 hrs. Untreated *Sf9* cells incubated for 15 hrs at 27^{o} C were used as controls. Cell extracts were prepared as described in the 'Materials and Methods'. Lysates containing 75 μg protein / lane were separated on a 10% SDS-PAGE and the levels of phosphorylated ERK, JNK and p38 MAP kinases and total eIF2α were determined by immunoblotting using respective antibodies. Panel A, represents western blots indicating the levels of different proteins as mentioned in the figure. The various lanes are as follows: 1) control, 2) 60 sec UV-B irradiation (312 nm), 3) 1 mM cycloheximide (CHX), 4) 12 μM tunicamycin (Tn), 5) 2 μM thapsigargin (Tg), 6) 36 μM brefeldin A(BF), 7) 5 mM DTT, 8) 10 mM GSH, 9) 5 mM GSSG. The bar diagrams in panel B, represent the quantification of the blots corresponding to panel A.

Fig. 16



Time course analysis of phosphorylation of eIF2α and ERK: Effect of UV-irradiation and brefeldin treatment.

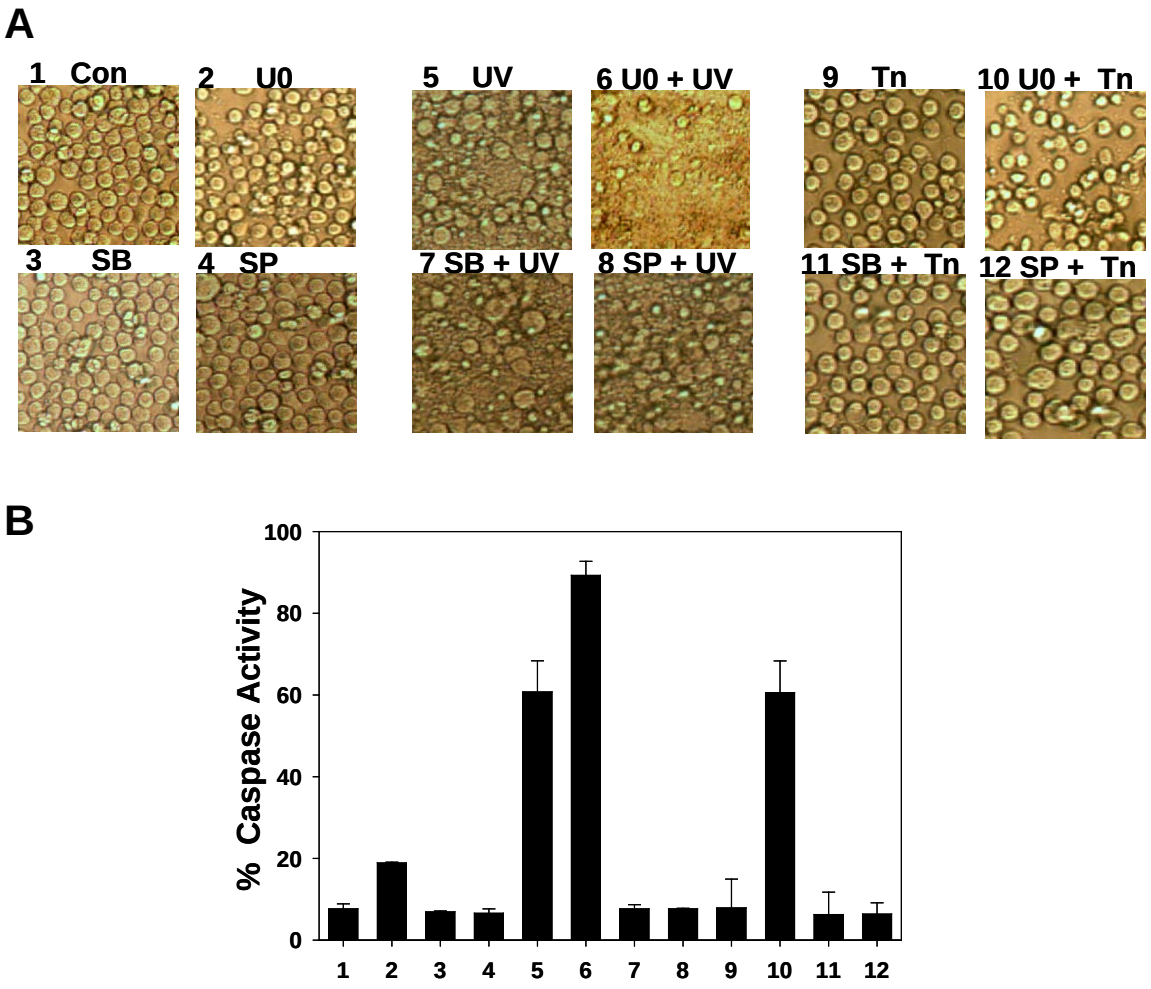
*Sf*9 cells were irradiated with UV-B light for 60 sec or treated with 36 μM brefeldin and incubated at 27^{o} C for different time periods as indicated in the figure. Cell extracts were prepared as described in "Materials and Methods" and 75 mg total cell proteins were separated by 10% SDS-PAGE and transferred onto a nitrocellulose membrane. The blot was then probed with antibodies against phosphospecific ERK and eIF2α. Panel A, depicts the phosphorylation of ERK and eIF2α in UV-irradiated cells and Panel B, depicts the phosphorylation of ERK and eIF2α in brefeldin treated cells.

3.7, Inhibition of ERK but not p38 and JNK, reduces cell survival in ER stress and hastens cell death in non-ER stress.

ER stress induced eIF2α phosphorylation is fundamentally a cytoprotective response. However, unlike *Sf9* cells, mammalian cells display ER stress induced apoptosis (McCullough et al., 2001; Yamaguchi and Wang, 2004; Boyce and Yuan, 2006). In contrast, *Sf9* cells do not display cell death in response to ER stressors or persistent ER stress-induced eIF2α phosphorylation (Aarti et al., 2010). BiP is believed to play a crucial role in counteracting the apoptosis inducing potential of ER stressors in mammalian cells by multiple mechanisms. Our above observations indicate that like BiP expression there exists a differential activation of MAPKs in pro- and non-apoptotic stresses. Therefore, a logical approach was to inhibit MAP kinases to determine the downstream effects especially with respect to eIF2α phosphorylation and BiP expression. Hence, pharmacological inhibitors such as, U0126, an inhibitor of MEK/ERK; SB203580, an inhibitor of p38MAPK and SP600125, an inhibitor of JNK, were evaluated for their effects on cell survival, death and caspase activation.

20 μM ERK inhibitor U0126, stimulates around 15-20% cell death **(Fig. 17, panel A, #1 vs 2)** and enhances a proportional caspase activity **(panel B, bar 1 vs 2)** in healthy *Sf9* cells. Low concentrations do not promote any cell death **(data not shown)**. However, the inhibitor further enhances significantly UV-irradiation induced cell death **(panel B, #5 vs 6)** and caspase activity **(panel B, bar 5 vs 6)**. Interestingly, the inhibitor also reduces the survival of tunicamycin treated cells and promotes cell death **(panel A, #9 vs 10)** and caspase activity **(panel B, bar 9 vs 10)**. ER stress coupled with inhibition of ERK signaling stimulates ER stress-induced cell death in *Sf9* cells. In contrast, no significant or obvious changes in cell survival or death have been observed in response to p38 or JNK inhibitors in healthy or stressed cells **(panel A, #3, 4 vs 1; 7, 8 vs 5; 11, 12 vs 9)**. However, caspase activation is partially reduced under these conditions **(panel B)**. These results therefore suggest that inhibition of ERK sensitizes cells to death while inhibition of p38 and JNK may reduce caspase activation and serve to protect the cells from non-ER stress induced apoptosis.

Fig. 17



Stress-induced apoptosis: Effect of MAPK inhibitors.

Sf9 cells were treated with inhibitors of MAPKs such as; 20 μM U0126, an ERK inhibitor; 10 μM SB203580, an inhibitor of p38MAPK, and 10 μM SP600125, an inhibitor of JNK, for a period of 2 hrs wherever it is indicated. After 2 hrs at 27^{o} C, cells were either UV-irradiated for 60 sec or treated with 12 μM tunicamycin or left alone for 5 hrs at 27^{o} C. The cells were then monitored under an inverted microscope for the signs of apoptosis. The cell extracts were used to measure the caspase activity as described in "Materials and Methods". Panel A represents the morphology of cells; Panel B, represents the caspase activity corresponding to panel A. Con = Control; U0 = U0126; SB = SB203580; SP = SP600125; Tn = tunicamycin.

3.8, Inhibition of ERK but not p38 and JNK, reduces BiP expression and promotes eIF2α phosphorylation.

Low concentrations of ERK inhibitor do not affect ERK and eIF2α phosphorylation or expression of BiP in healthy *Sf9* cells **(Fig. 18, panel A, lanes 2 and 3 vs 1)**. However, 20 μM U0126 which promotes cell death and caspase activation as mentioned above, reduces endogenous ERK phosphorylation completely and BiP expression partially, but promotes eIF2α phosphorylation **(panel A, lane 5 vs 1 and panel B lane 1 vs 2)**. Consistent with the above observations, inhibition of ERK in UV irradiated **(panel C)** or tunicamycin treated **(panel D)** cells, coincides with reduced BiP expression and enhanced eIF2α phosphorylation **(lane 1 vs 2)**. The decline in BiP levels in response to ERK inhibition is not complete and therefore suggests that other mechanisms can also contribute to BiP expression in response to ER stress. In contrast, inhibition of p38 and JNK does not affect BiP expression or eIF2α phosphorylation in healthy cells **(panel D, lanes 3, 4 vs 1)** or in tunicamycin treated cells. However, UV-irradiation induced-phosphorylation of eIF2α, is reduced in response to p38 and JNK inhibitors, thereby suggesting that p38 and JNK may be activated downstream to caspases, and affects the caspase mediated eIF2α kinase activation and thereby serves to promote cell death. Although it remains elusive how the MAP kinases modulate eIF2α phosphorylation, these observations indicate that probably the MAP kinases modulate caspase mediated eIF2α kinase activation and the ERK pathway plays a critical role in antagonizing the apoptosis inducing potential of eIF2α phosphorylation by inducing BiP expression, and is consistent with our previous suggestion that eIF2α phosphorylation is both pro-survival and pro-apoptotic depending on the levels of BiP, an ER chaperone (Aarti et al., 2010) which is regulated by multiple mechanisms.

Fig. 18

Changes in BiP, eIF2α(P) and ERK(P): Effect of MAPK inhibitors.

Sf9 cells were treated with inhibitors of MAPKs at indicated concentrations for a period of 2 hrs as mentioned in the legend to figure 17. After 2 hrs at 27^{o} C, cells were either UV-irradiated for 60 sec or treated with 12 μM tunicamycin or left alone for 5 hrs at 27^{o} C. The cell extracts were subjected to western blot analysis using antibodies against ERK(P), eIF2α(P), BiP and eIF2α. Panel A indicates the effect of various concentrations of ERK inhibitor on *Sf9* cells. Panels B-D represent the effect of MAPK inhibitors on untreated cells (B), UV-irradiated cells (C) and 12 μM tunicamycin treated cells (D)

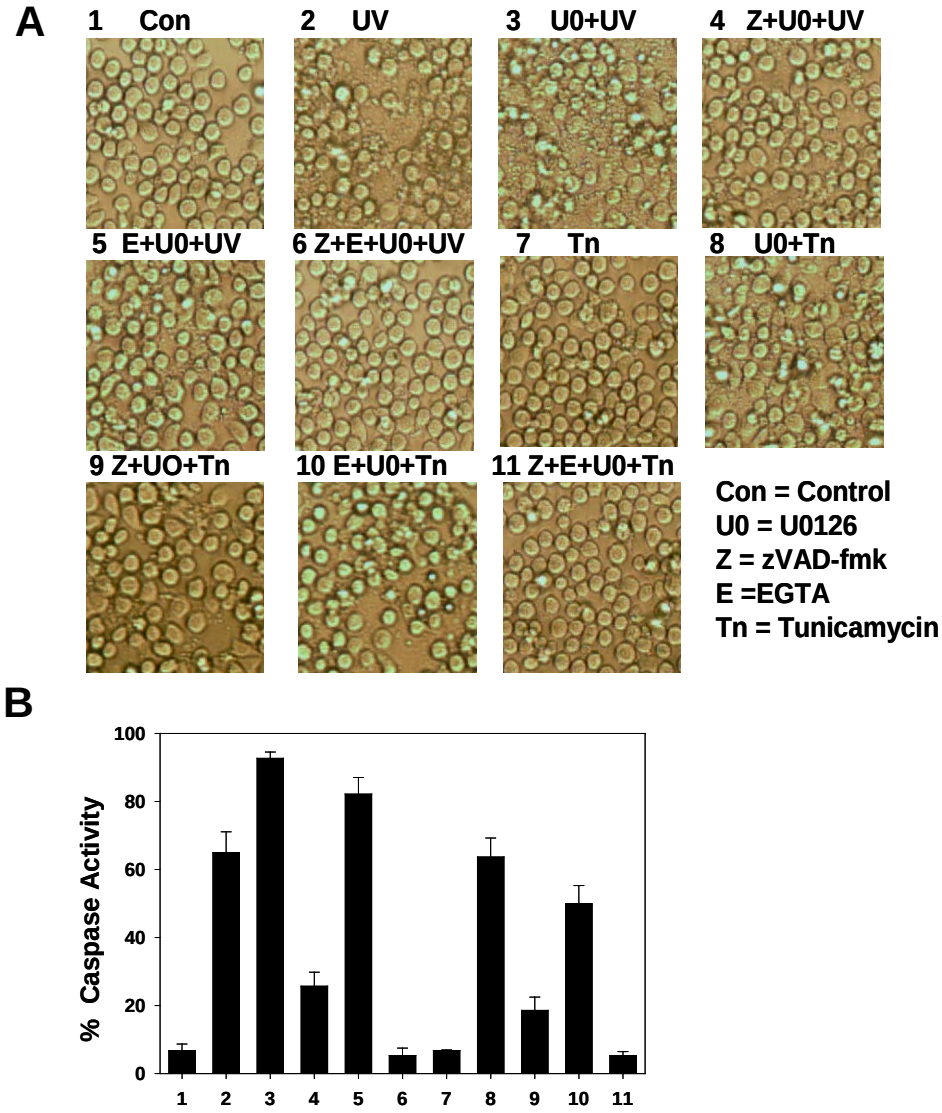
3.9, Caspase inhibitor affects eIF2α phosphorylation, BiP expression and MAPK signaling

Consistent with the above observations, the caspase inhibitor z-VAD-fmk enhances BiP expression, ERK phosphorylation and reduces eIF2α phosphorylation in UV irradiated and tunicamycin treated cells both in the presence **(Fig. 20, lanes 5 vs 3 and 9 vs 7)** and absence **(lane 4 vs 2 and 8 vs 6)** of ERK inhibitor. While caspase inhibitor enhances stress-induced ERK phosphorylation, it reduces phosphorylation of p38 and **JNK (lane 5 vs 3 and 9 vs 7)**, confirming our above mentioned suggestion that p38 and JNK may be activated downstream to caspase activation. The caspase inhibitor not only interferes in caspase activation but its presence apparently shifts the balance towards pro-survival signals like ERK phosphorylation and BiP expression. Consistent with this suggestion, inhibition of ERK promotes cell death both in the absence of stress **(Fig. 17, panel A, #1 vs 2)** and also enhances cell death in the presence of UV irradiation **(#5 vs 6)** or tunicamycin treatment **(# 9 vs 10)**.

Inhibition of ERK by U0126 stimulated activation of caspase 3 like activity in *Sf9* cells **(Fig. 19, panel B, bars 3, 8)**. z-VAD-fmk protects completely the UV-induced caspase activation and cell death. However, despite the activation of caspase 3 like protein in response to ERK inhibition, z-VAD-fmk was able to rescue only ~70% of the cells from apoptosis induced by pre-treatment with U0126 **(Fig. 19)**. These results indicate that although the caspase cascade plays a critical role in apoptosis induced by ERK inhibition, there exists an alternative mode of cell death. Studies implicate a role for calcium in activating a calpain like protease activity and most of the stresses are known to affect the calcium levels, therefore to determine if a calcium chelator like EGTA could affect the apoptosis induced in response to ERK inhibition. 5 mM EGTA, a calcium chelator, which did not affect the cell survival or death in healthy or stressed cells, was used here in these experiments. Although, EGTA alone could partially protect cells (~20%) from ERK inhibition induced apoptosis **(Fig. 20)**, it could completely protect the cells from ERK inhibition induced apoptosis when used along with z-VAD-fmk. Consistent with our above observations, the decline in apoptosis corresponds to a decline in eIF2α phosphorylation **(Fig. 21, lanes 4-6 and 9-11)**. However, EGTA unlike z-VAD-fmk, does not affect ERK phosphorylation or BiP expression **(Fig. 21, lanes 5 and 10),**

suggesting that the protease like activity essentially serves to enhance eIF2α phosphorylation in response to ERK inhibition which is the cause for reduced cell survival. These results therefore support the fact that ERK inhibition promotes predominantly a caspase mediated apoptosis which is reversed by z-VAD-fmk. However, a small but significant proportion of apoptosis is also mediated by a caspase-independent mechanism which is insensitive to z-VAD-fmk.

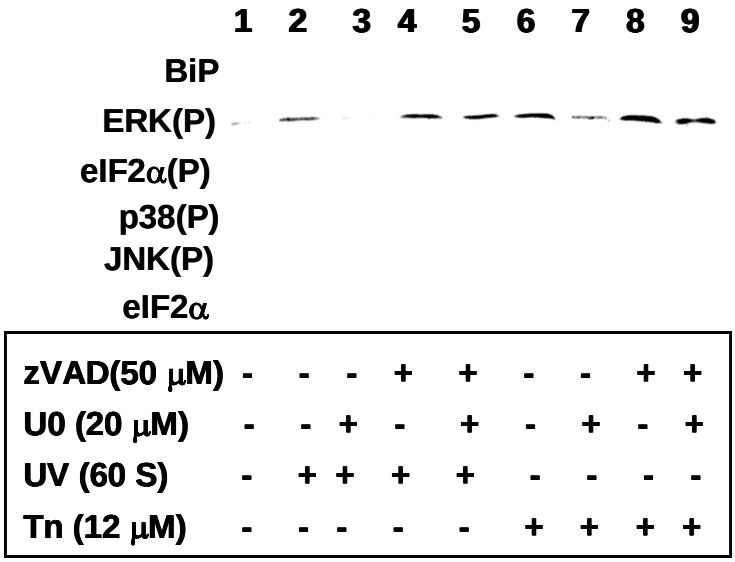
Fig. 19



Stress induced apoptosis: Effect of z-VAD-fmk and EGTA.

*Sf*9 cells were pre-treated for 1 hr with 50 μM z-VAD-fmk or 5 mM EGTA or both wherever indicated, prior to the addition of 20 μM U0126. After 2 h at 27^{o} C, cells were UV-irradiated for 60 sec UV or treated with 12 μM tunicamycin, and incubated further at 27^{o} C for 5 h. Cells were observed under the inverted microscope for the signs of apoptosis. Caspase activity for the extracts was also determined spectrofluorimetrically. Panel A, represents the morphology of cells; Panel B, represents the caspase activity corresponding to panel A. Con = Control; U0 = U0126; Z = zVAD-fmk; E = EGTA; Tn = tunicamycin.

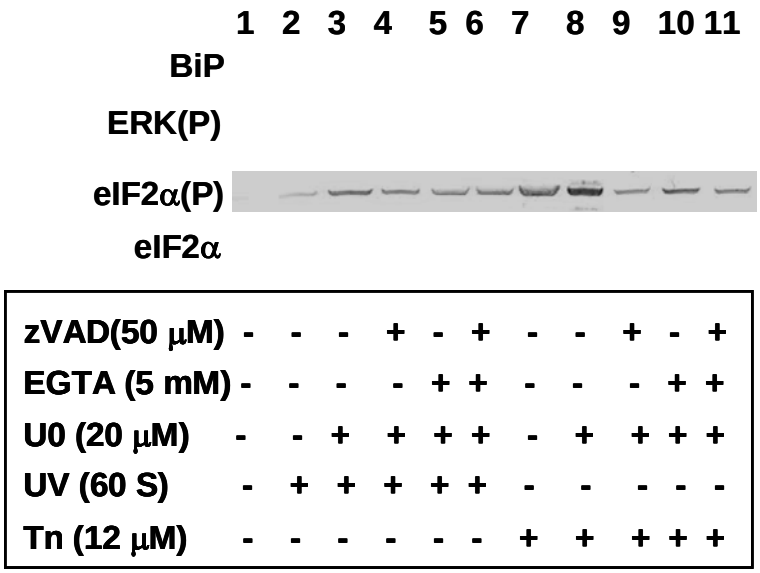
Fig. 20



Stress-induced changes in BiP, phosphorylation of eIF2α and MAPKs in response to ERK inhibitor: Effect of z-VAD-fmk.

The whole cell extracts prepared from the treated cells as mentioned in the legend Fig. 3.18, were subjected to western blot analysis with antibodies against ERK(P), p38(P), JNK(P), eIF2α(P), eIF2α and BiP.

Fig. 21



Stress-induced changes in BiP, phosphorylation of eIF2α and ERK in response to ERK inhibitor: Effect of EGTA.

The whole cell extracts prepared from the treated cells as mentioned in the legend Fig. 3.18, were subjected to western blot analysis with antibodies against phosphorylated ERK, and eIF2α, total eIF2α and BiP.

Summary

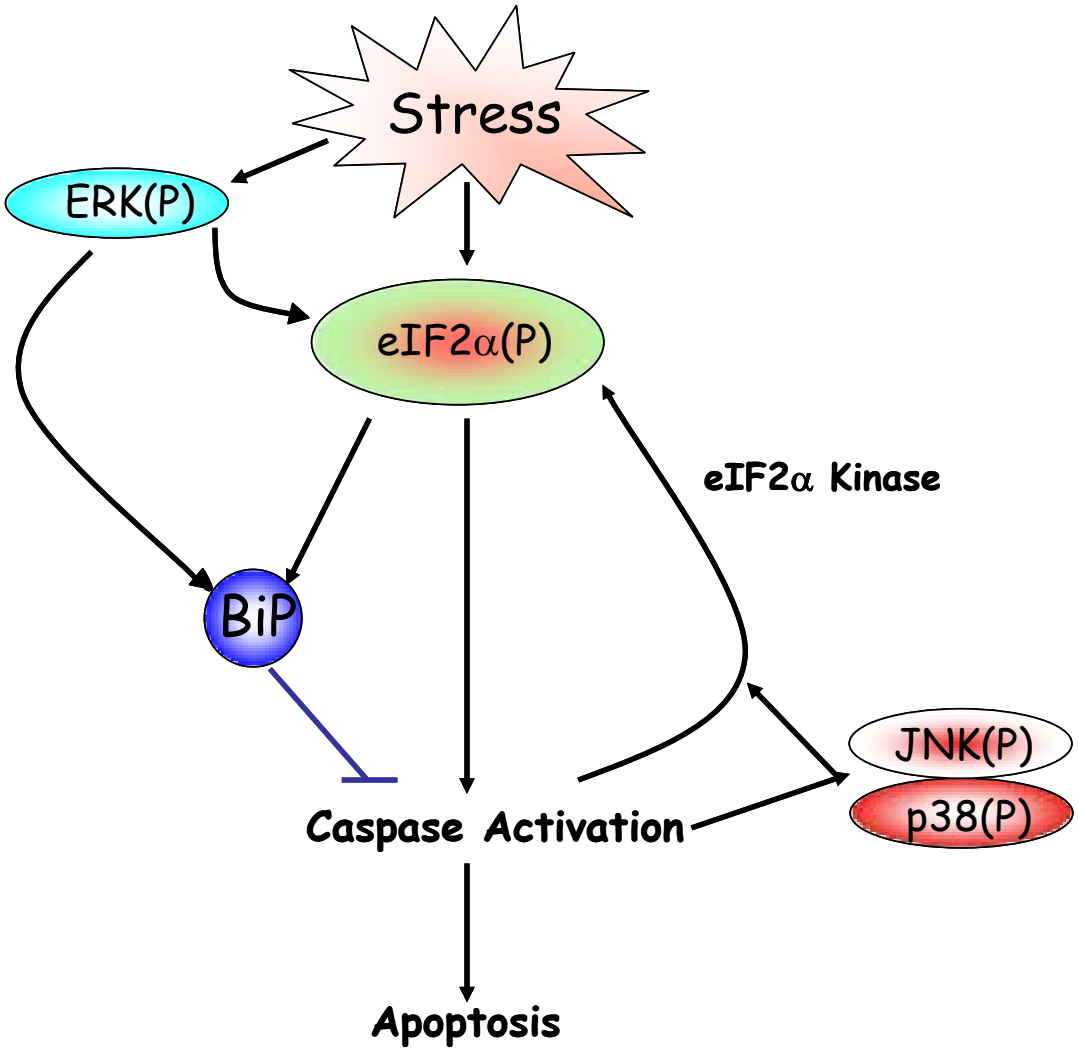
Summary

Results from this work suggest that the expression of BiP, an ER (endoplasmic reticulum) chaperone and ER stress marker associated with enhanced phosphorylation of eIF2α phosphorylation serve as a pro-survival signal, while enhanced eIF2α phosphorylation in the presence of reduced BiP expression is associated with enhanced caspase activation and cell death as observed in non-ER stressors. Further, z-VAD-fmk, a pan caspase inhibitor that cannot inhibit brefeldin, an ER stressor-induced eIF2α phosphorylation, reduces the late stages, but not the initial stages of UV-irradiation, a non-ER stressor-induced eIF2α phosphorylation. These findings therefore suggest that caspase activation is a cause and consequence of non-ER stress induced eIF2α phosphorylation. Consistent with this notion, addition of recombinant S51D mutant eIF2α, a phosphomimetic form, stimulates efficiently caspase activity in the cell extracts prepared from control cells than tunicamycin-treated cells and is further supported by the observation that 5 hr-tunicamycin treated cells resist UV-induced apoptosis. These findings therefore suggest that eIF2α phosphorylation is a cause for caspase activation and expression of BiP may play an important role in interfering with eIF2α phosphorylation mediated caspase activation. Further, the results from inter-protein interaction studies reveal that phosphorylation of eIF2α modulates its interaction with *Sf*-caspase and BiP. This is consistent with our previous report (Rajesh et al., 2008) that the unphosphorylated form of eIF2α serves as a better substrate for caspases than the phosphorylated form. These findings together with our earlier observations that phosphorylated form of eIF2α interacts with cytochrome c (Rajesh Kamindla, 2009), suggests that the changes in eIF2α phosphorylation mediated inter protein interactions some how regulates the ability of phosphorylated eIF2α to promote caspase activation and cell death. In addition, the role of MAP kinase pathways in modulating eIF2α phosphorylation mediated cell survival and cell death are also evaluated here. Like eIF2α kinases, MAPKs are highly conserved in many systems for balancing cell survival and death. Irradiation of *Sf9* cells with UV-B light promotes phosphorylation of eIF2α, ERK, JNK and P38 MAPK. In contrast, treatment with ER stressors, like tunicamycin, enhanced phosphorylation of eIF2α and

ERK kinase alone. Targeting the ERK pathway using a specific pharmacological inhibitor like UO126 sensitizes cells to apoptosis. The enhanced apoptosis in response to ERK inhibition is associated with enhanced phosphorylation of eIF2α and a reduction in the levels of BiP. These observations strongly suggest that ERK activation facilitates BiP expression under ER stress conditions and serves predominantly as a pro-survival signal both in ER and non-ER stress conditions and probably delays eIF2α phosphorylation-mediated caspase activation. This is consistent with our observations in UV-irradiated cells, where phosphorylation of eIF2α precedes caspase activation and promotes cell death. In contrast, SP600125, a specific JNK inhibitor and SB203580, a specific p38 MAPK inhibitor, are able to reduce eIF2α phosphorylation but have no effect on BiP expression and apoptosis, indicating that these pathways may be activated downstream to caspase activation and can affect the caspase mediated eIF2α kinase activation. These results together with the time course analyses of ERK and eIF2α phosphorylation, strongly suggest that ERK activation may occur upstream or parallel to eIF2α phosphorylation and promotes cell survival by delaying the eIF2α phosphorylation mediated caspase activation and the overall cellular fate depends on the balance between the phosphorylation of pro-survival ERK and the phosphorylation of pro-apoptotic proteins like eIF2α, JNK and P38. Further, z-VAD-fmk which mitigates the non-ER stress induced caspase activation, cell death and eIF2α phosphorylation, is able to partially protect the cells in response to ERK inhibition-induced apoptosis suggesting that a caspase-independent mechanism may be operative in cells undergoing apoptosis in response to ERK inhibition. .

In conclusion, our results here, indicate that the stress induced survival or suicidal response mediated by eIF2α phosphorylation will depend on the delicate balance between various pro-survival and pro-apoptotic signaling pathways, its cellular location, the changes in gene expression, and the inter protein interactions.

Fig. E



Model representing the possible involvement of MAPKs in eIF2α phosphorylation mediated cell survival and death

Phosphorylation of eIF2α is a stress signal and occurs both in response to ER and non-ER stressors. ER stressors which fail to promote apoptosis are shown to promote phosphorylation of ERK and BIP, with a concomitant decline in pro-apoptotic JNK(P), p38(P), as opposed to non-ER stressors. The ability of eIF2α phosphorylation to promote cell survival or death, probably depends on the delicate balance between the pro-survival ERK(P) and BiP, and pro-apoptotic p38(P) and JNK(P).

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