

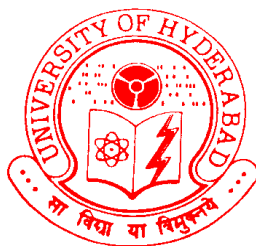
**Apoptosis in the Ovarian Cells of *Spodoptera frugiperda*:
Caspase-mediated PKC Activation and eIF2 α Phosphorylation**

A thesis

submitted to the University of Hyderabad for the award of a Ph.D. degree in
Department of Biochemistry, School of Life Sciences

by

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DECLARATION

I P. N. Pushpanjali hereby declare that this thesis entitled “Apoptosis in the Ovarian Cells of *Spodoptera fruigiperda*: Caspase-mediated PKC Activation and eIF2 α Phosphorylation” 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.

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CERTIFICATE

This is to certify that this thesis entitled “Apoptosis in the Ovarian Cells of *Spodoptera frugiperda*: Caspase-mediated PKC Activation and eIF2 α Phosphorylation” is a record of bonafide work done by P. N. Pushpanjali 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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Abbreviations:

Ac-DEVD-AFC	: N-acetyl-Asp-Glu-Val-Asp-amino-4-trifluoromethyl coumarin
Ac-DEVD-CHO	: N-acetyl-Asp-Glu-Val-Asp-aldehyde
AcNPV	: Autographa californica nuclear polyhedrosis virus
AP	: Alkaline phosphatase
ATF4	: Activating transcription factor 4
ATP	: Adenosine 5' triphosphate
BCIP	: 5-bromo-4-chloro-3-indoyl phosphate
Bcl2	: B cell leukemia/lymphoma 2
BiP	: Immunoglobulin heavy chain binding protein
bZIP	: Basic leucine zipper
C/EBP	: CCAAT-enhancer binding protein
CAT-1	: Cationic amino acid transporter
CH	: Cycloheximide
CHOP	: C/EBP-homologous protein
CREB	: CRE binding protein
CREP	: Constitutive repressor of eIF2 α phosphorylation
eIF	: Eukaryotic translation initiation factor
ERAD	: ER associated degradation
ERK	: Extracellular signal regulated kinase
GADD153	: Growth arrest and DNA damage-inducible gene153
GADD34	: Growth arrest and DNA damage-inducible gene 34
GCN	: General control non-derepressible
GDP	: Guanosine diphosphate
GRP	: Glucose regulated protein
GSH	: Reduced glutathione
GST	: Glutathione S-transferase
GTP	: Guanosine triphosphate
HRI	: Heme-regulated inhibitor
HepG2	: Human liver hepatocellular carcinoma cell line
IFN	: Interferon
I κ B	: Inhibitor of NF- κ B
IRE1	: Inositol requiring 1
IRES	: Internal ribosomal entry site
JNK	: Jun N-terminal kinase
MAPK	: Mitogen-activated protein kinase
MCL-1	: Myeloid cell leukemia sequence 1
Met	: Methionine
MOI	: Multiplicity of infection
mRNA	: messenger ribonucleic acid
NBT	: Nitro blue tetrazolium

NF- κ B	: Nuclear factor κ B
NIH3T3	: NIH 3-day transfer, inoculum 3×10^5 cells
nm	: Nanometers
NRF2	: NF-E2 related factor
PAGE	: Polyacrylamide gel electrophoresis
Pi	: Inorganic phosphate
P58 ^{ipk}	: 58 kDa inhibitor of PKR
PABP	: Poly A binding protein
PKC	: Protein kinase C
rpm	: Rotations per minute
S51A	: eIF2 α mutation of ser51 eIF2 α to Ala
S51D	: eIF2 α mutation of ser51 eIF2 α to Asp
SDS	: Sodium dodecyl sulphate
<i>Sf9</i>	: <i>Spodoptera frugiperda</i>
TEMED	: N'N'N'N'-tetra ethyl methyl ethyl diamine
Tn	: Tunicamycin
TRAF2	: Tumor necrosis factor receptor associated factor 2
tRNA	: Transfer RNA
VAIRNA	: Virus-associated (VA) RNAs of adenovirus
uORF	: Upstream open reading frame
UPR	: Unfolded protein response
UV	: Ultra-violet
wt	: Wild type
XBP-1	: X box binding protein
z-VAD-fmk (Z)	: Benzyloxycarbonyl-Val-Ala-Asp (o-methyl)-fluoromethyl ketone

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1. Introduction:

Protein synthesis is a complex biochemical process in which the nucleotide sequence of messenger RNA (mRNA) is decoded to the amino acid residues of a polypeptide chain. Protein synthesis, an integral part of gene expression defines the functionality of different cell types. As in the synthesis of other biological polymers like DNA and RNA, protein synthesis requires a special machinery comprising of an mRNA template, ribosomes, transfer RNAs (tRNAs), aminoacyl synthetases and several protein factors. The nucleotide sequence in the mRNA template is recognized as a set of three nucleotides that comprise an individual codon. Each codon codes for a specific amino acid that is brought to the protein synthetic machinery by a tRNA. While the anticodon, a three nucleotide sequence, in tRNA recognizes the codon in mRNA, the 3' end of tRNA joins a specific amino acid. Aminoacyl synthetase enzymes catalyze the addition of an amino acid to the 3' end of tRNA and the energy for the aminoacylation reaction is provided by ATP. The base pair complementarity between the nucleotides in mRNA codon and the anticodon in tRNA dictates the joining amino acid bound tRNA to the mRNA. Addition of a peptide bond between adjacent amino acids bound by peptidyl tRNA and aminoacylated tRNA is catalyzed apparently by a ribosomal RNA of the large subunit of ribosome than by any protein enzymes. The complex process of biological protein synthesis or translation is divided into four steps: initiation, elongation, termination and ribosome recycling.

Initiation:

The initiation step requires the joining of 40S ribosomal subunit carrying an mRNA to the 60S subunits to form an 80S initiation complex in which the initiator tRNA (Met-tRNA_i) carrying the amino acid methionine is positioned in the 'P' (peptidyl) site of 80S ribosome. The process requires the participation of several factors called eIFs (eukaryotic initiation factors). The initiation step is most crucial and rate-limiting.

The process of initiation can be divided into the following sub steps.

I. Joining of 40S ribosomal subunit by eIF1 and eIF1A.

Translation initiation starts with binding of eIF1 and 1A to the inter-subunit interface of 40S subunit resulting in synergistic induction of structural transition of the ribosomal subunit from a 'closed' to an 'open' state. This event concomitantly shifts the ribosomal

association-dissociation equilibrium towards free ribosomal subunits over 80S ribosomes. Presence of eIF1 sterically hinders ribosomal subunit joining and eIF1A enhances this effect by reducing the rate of eIF1 dissociation from 40S subunit (Goumans et al, 1980).

II. Formation of ternary complex, eIF2.GTP.Met tRNA.

In a parallel independent step, eIF2, heterotrimeric protein with α , β and γ subunits joins initiator tRNA (Met –tRNA_i) in the presence of GTP and forms a ternary complex (Lloyd et al, 1980). In physiological conditions, in the presence of Mg^{2+} , eIF2 has higher affinity for GDP than for GTP and GDP bound eIF2 cannot join initiator tRNA (Kapp and Lorsch, 2004; Schmitt et al, 2010). In the eIF2 complex, β and γ subunits play a role in GTP and Met- tRNA_i binding, whereas α subunit plays a regulatory role (see later). In addition, the γ subunit of eIF2 is associated with GTPase activity which is stimulated by eIF5 that interacts with eIF2 β subunit (Das et al, 2000).

III. Formation of 43S complex.

A multifactor 43S physiological complex consisting of eIF1, eIF1A, eIF3, eIF5 and the ternary complex have been identified in yeast suggesting that the eIFs form a multifactor complex (MFC) which could then bind to 40S subunits as a unit (Asano et al, 2000). Among these subunits, eIF3 plays a role in joining 43S complex to an ‘activated’ mRNA to form 48S preinitiation complex as described below.

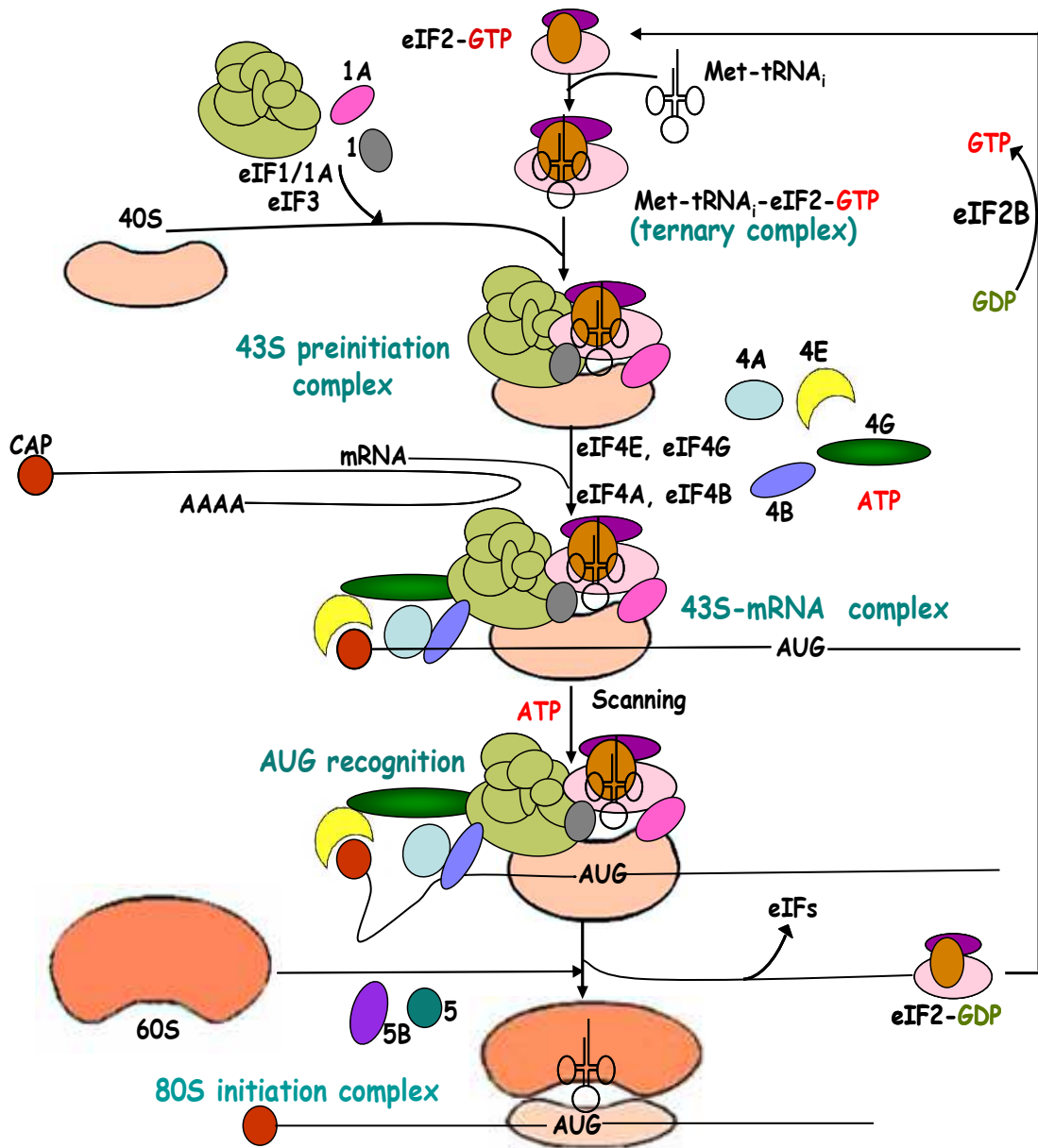
IV. Activation of mRNA.

mRNA is activated through the binding of eIF4F complex and PABP (Poly A binding protein) at its 5’ and 3’ ends respectively (Gingras et al, 1999). The eIF4F complex consists of eIF4G, a scaffolding protein that interacts with the eIF3 of 43S complex to form 48S initiation complex. The other two proteins of eIF4F complex are eIF4A and 4E (Grifo et al, 1983). eIF4A is an ATP-dependent RNA helicase that unwinds mRNA structure at the 5’ end and facilitates the scanning of 43S complex to reach the start ‘AUG’ codon (Linder et al, 1992). The 5’ methylated guanosine of typical eukaryotic mRNAs is bound by eIF4E. Poly A binding protein (PABP) recognizes the polyA tail present at the 3’ end of mRNA. Activated mRNA is pseudocircularised because of the interaction between eIF4G and PABP (Munroe et al, 1990; Gallie, 1991; Tarun and Sachs, 1995; 1996; Preiss and Hentze, 1998; Sachs and Varani, 2000). The pseudocircularisation of mRNA enhances the chances of translational continuity.

Fig. 1 Overview of translation initiation step.

The figure depicts the overview of translation initiation in eukaryotes. Eukaryotic initiation factor-2 (eIF2) forms a ternary complex (eIF2.GTP.Met-tRNA_i) by joining initiator tRNA (Met-tRNA_i). The ternary complex joins together with eIF3, eIF1 and eIF5 leading to the formation of multifactorial complex (MFC). The multifactorial complex now joins the 40S ribosomal subunit generating a 43S pre-initiation complex. The activated mRNA bound at its 5' end by eIF4F complex (consisting of eIF4E, eIF4G and eIF4A) and by PABP (Poly A binding protein) at its 3' end, joins the 43S pre-initiation complex forming 43S-mRNA complex. The 43S complex then scans the mRNA for AUG codon. Selection of legitimate AUG codon leads to the formation of 48S initiation complex which is followed by a GTP hydrolysis event that results in the release of initiation factors and subsequent joining of 60S ribosomal subunit, forming the 80S initiation complex.

Fig.1



V. Scanning of mRNA and recognition of start codon AUG.

On binding of 43S complex to 5' methylated guanosine cap, the 43S complex scans along the mRNA from 5' in an energy dependent manner to recognize AUG codon. This mechanism is referred to as "Scanning model" (Kozak, 1989; 1999). eIF1 promotes positioning of 43S complex at AUG codon. This activity is enhanced by eIF1A (pestova et al, 1998). eIF1A occupies 'A' site of small ribosomal subunit and interacts with eIF5B (Choi et al, 2000). eIF1A along with eIF5B directs the initiator tRNA to the 'P' site (Roll-Mecak et al, 2001).

VI. Joining of 60S ribosomal subunit.

Base pairing between AUG codon and Met tRNAⁱ anticodon leads to the formation of 48S initiation complex. The complex formation activates series of events that result in the formation of an active ribosome. The initiation factor, eIF5 binds to the β -subunit of eIF2 and activates GTPase function of eIF2 (Asano et al, 1999). GTP hydrolysis leads to the release of initiation factors bound to 48S complex to facilitate the joining of 60S subunit. eIF5B facilitates the subunit joining. GTP hydrolysis by eIF5B plays a regulatory role and facilitates the release of other factors such as eIF1A from either 40S or 80S complex and ensures that the final 80S complex is properly set up to elongate a polypeptide.

1.1b Elongation:

Translational elongation can be subdivided into three steps: In step 1, an incoming aminoacylated tRNA (aa-tRNA) capable of interacting with its anticodon to the next codon on mRNA in the 'A' site of the ribosome joins the 80S initiation complex. In step 2, formation of a peptide bond occurs between two adjacent amino acids positioned at 'P' and 'A' site. While the small subunit of ribosome is involved in decoding the mRNA, large subunit is involved in the catalysis of the peptide bond (Kapp and Lorsch, 2004). Apparently the peptide bond is catalyzed by ribosomal RNA than by a protein enzyme present in the 60S subunit of ribosome. The idea is supported by the findings that RNA is present in the center of the ribosomal subunit whereas proteins are present at the periphery. In step3, the ribosome translocates over the mRNA by three nucleotides equivalent to a codon. Three elongation factors eEF1A, eEF1B and eEF2 are involved in carrying these functions. eEF 1A and eEF1B, recruit the amino acylated tRNAs to the 'A' site of the

ribosome whereas eEF2 translocates the ribosome over the mRNA by one codon in a GTP dependent manner (Pape et al, 1998; Wintermeyer et al, 2001). On completion of this cycle, the free 'A' site of the ribosome is ready for another sequence of elongation until a stop codon is encountered.

1.1c Termination:

Termination of translation takes place once a stop codon or non-coding triplets UAA, UAG or UGA is encountered in the 'A' site of ribosome in the elongation step (Frolova et al, 1994). Termination in eukaryotes is mediated by the class 1 release factor (eRF1) which recognizes all stop codons and promotes hydrolysis of the ester bond linking the polypeptide chain with the 'P' site tRNA. In contrast, prokaryotic translation termination is catalyzed by two codon-specific class 1 release factors: RF1 that recognizes the UAA and UAG and RF2 that recognizes UAA and UGA. Amino acid sequence analysis reveals that GGQ sequence motif conserved in the class-I release factors is essential to recognize the stop codon and the overall shape of eRF1 resembles to a tRNA molecule. Hydrolysis of the peptide bond results in a deacylated tRNA in the 'P' site and eRF1 is evacuated by eRF3 which hydrolyzes GTP (Kisselev and Florova, 1995; Zhouravleva et al, 1995).

1.1d Recycling:

After termination of translation, the ribosome and the associated mRNA, and deacylated tRNA occurs as a complex termed post termination complex which needs to be primed before starting another round of initiation. Ribosome association is inhibited by the involvement of ribosome dissociation factors, mRNA and tRNA. Initiation factors eIF3, eIF1, eIF1A, and eIF3j, a loosely associated subunit of eIF3 promote the recycling of eukaryotic post-termination complex (Kapp and Lorsh, 2004). eIF3 promotes splitting of post termination ribosomes into 60S subunits and mRNA, tRNA -bound 40S subunits. Its activity is enhanced by eIFs 3j, 1 and 1A. eIF1 also mediates release of deacylated tRNA in P site, whereas eIF3j ensures subsequent dissociation of mRNA.

1.2 Eukaryotic Initiation Factor 2 (eIF2)

At the end of initiation of protein synthesis, the GTP bound to eIF2 is hydrolyzed and the eIF2.GDP is released. To enter into another round of initiation, the GDP bound to eIF2 is exchanged for GTP by a heteropentameric guanine nucleotide exchange protein called eIF2B (Webb and Proud, 1997; Pavitt et al, 1998; Proud, 2005). While the β and γ subunits play a role in the joining of eIF2 to GDP or GTP, Met-tRNA_i, mRNA and in the hydrolysis of GTP, the small or α -subunit that is phosphorylated on its conserved serine⁵¹ residue by several stress-induced eIF2 α kinases regulate its intersubunit and inter protein interactions, translation of global and gene-specific mRNAs and subsequent gene expression (Proud, 2005). This chapter provides the current information available pertaining to the role of eIF2 in the mechanics and regulation of protein synthesis, and the importance of phosphorylation of eIF2 α in cell physiology and metabolism.

1.2a Subunit composition and structure of eIF2: Eukaryotic initiation factor 2 (eIF2), a heterotrimer with three subunits. It plays a critical role in the formation of 80S initiation complexes in the translation of eukaryotic mRNAs. It joins initiator tRNA (Met-tRNA_i) in the presence of GTP and delivers the ternary complex, eIF2.GTP.Met-tRNA_i to 40S ribosomal subunits in the first step of translational or protein synthesis initiation. The protein has three subunits of different molecular masses and the respective genes α , β and γ were identified in humans, yeast and in plants (Lloyd et al, 1980; Barrieux and Rosenfeld, 1997). The molecular masses of the different subunits are 36.2, 39.0 and 51.8 in humans, 34.7, 31.6 and 57.9 in yeast which are encoded *sui2*, *sui3* and *gcd11* genes and 41.6, 26.6 and 50.9 in plants (Hershey and Merrick, 2000). The human subunits of eIF2 are 58%, 47% and 72% identical respectively to yeast *Sachharomyces cerevisiae* (Kimball, 1999).

eIF2 is important in various functions which include GDP-GTP binding, Met-tRNA_i binding, GTP hydrolysis and in the recognition of start codon. eIF2, in the presence of physiological concentrations of Mg²⁺ has a higher affinity for GDP than for GTP. GTP is required for eIF2 to join Met-tRNA_i and GTP promotes the joining of eIF2 to Met-tRNA_i by 20 times as studied in yeast, and 80 times in archae. However the GTP-dependence is

much higher for prokaryotic elongation factor, EF.Tu to join amino acylated tRNA (Kapp and Lorsch, 2004; Schmitt et al, 2010).

Crystal structures for the eukaryotic β and γ -subunits are not yet available. However, archeal β and γ -subunits are crystallized (Schmitt et al, 2002; Roll Meckak et al, 2004; Cho and Hoffman 2002; Gutierrez et al, 2004; Sokabe et al, 2006). The γ -subunit of eIF2, is the largest among all the three subunits. Unlike the β -subunit, the sequence of γ -subunit has all the GDP-binding elements, typical to G-binding proteins. Further γ -subunit of eIF2 is highly homologous to eEF1A or EF.Tu structurally and functionally. Since eEF1A or EF.Tu binds to aminoacylated tRNAs, it is suggested that γ -subunit plays a role in GDP/GTP binding and in the binding of initiator tRNA.

The GTPase center in γ -subunit of eIF2 hydrolyzes the GTP and eIF2 is released as eIF2.GDP at the end of initiation. The GTPase function of eIF2 is critical in the decoding process of mRNA as it involves in hydrolyzing the GTP at the appropriate time and the release of Met-tRNA_i into the P-site. While mutations in all the three subunits have shown to reduce the fidelity of initiation site selection, the mutations in γ -subunit i.e in the GTP-binding site appears to increase both the eIF5 independent GTPase activity of eIF2 and the rate of dissociation of Met-tRNA_i (Donahue et al, 1988; Cigan et al, 1989; Huang et al, 1997, Asano et al, 2001; Das et al, 2001; Hashimoto et al, 2002). Since GTP is required for the interaction between methionine in the Met-tRNA_i with eIF2, this will ensure probably unacylated tRNA_i from entering the initiation pathway and also for releasing of initiator tRNA from eIF2 upon start site recognition. A recent study further describes that a structural rearrangement in the 43S complex leading to partial GTP hydrolysis occurs prior to AUG recognition promoting the formation of eIF2.GDP.Pi. The release of Pi however occurs after the recognition of AUG codon (Sonenberg and Hinnebusch, 2009; Schmitt et al, 2010).

The β -subunit of eukaryotic eIF2 ranges from 250-333 amino acids. It is longer compared to archeal version and contains two additional domains: a longer N-terminal domain of about 125 residues and a shorter 15 residue sequence on the C-terminus. The crystal structure of eukaryotic- β is not yet available. However the archeal β -subunit structure reveals that it has an N-terminal α -helix, which is connected to the central α - β domain

through a linker. The N-terminal domain of eukaryotic β -subunit has three lysine stretches and the C-terminus has a zinc-binding domain (Pathak et al, 1988; Cho and Hoffman, 2002; Gutierrez et al, 2004; Sokabe et al, 2006). Mutational studies in β -subunit suggest that β -subunit plays a role in mRNA binding, tRNA binding, GDP/GTP binding, GTPase activity of eIF2 perhaps more indirectly, and in aiding the γ -subunit in all these functions (Hinnebusch, 2000). Although many early studies suggested that β -subunit of eIF2 is not critically required for many of the functions of eIF2 including the joining of initiator tRNA by the γ -subunit, analyses of a yeast strain harboring only β and γ -subunits but not α -subunit suggested that β -subunit of eIF2 has a major role rather than α -subunit in the joining of initiator tRNA to the γ -subunit of eIF2 (Schmitt et al, 2010). In contrast, in archaea, it is suggested that α -subunit plays a major role in the binding of initiator tRNA to the γ -subunit. However, the mechanism by which eIF2 β influences the interaction between eIF2 γ and tRNA is not understood.

Further, eIF2 β -subunit is a hub for interaction with several proteins. It interacts directly with several initiation factors such as eIF5, eIF2B ϵ , eIF1A, eIF1 and 40 S ribosomal subunits (Das et al, 1997; Kimball, 1998; Singh et al, 2004). In addition, it also interacts with cellular factors like Nck1, a cofactor of eIF2 α phosphatase. The N-terminal lysine domains in eIF2 β are involved in the interaction with the C-terminal domains of eIF5 and to the ϵ or catalytic subunit of eIF2B. Since the lysine sequences are not found in the archeal β -subunit, it is likely that archaea may not have eIF5 or eIF2B-like proteins. Moreover, eIF2 β binds directly to TIF32 (transcriptional intermediary factor 32)/eIF3a (Valasek et al, 2002) and its binding to eIF5 potentiates the association of the latter with Nip1/eIF3c, which helps to integrate the ternary complex into the multifactor complex in yeast.

Analyses of the α -subunit of archeal and eukaryotic initiation factor 2 reveals that the general structure is conserved between these species but the eukaryotic eIF2 α has an additional acidic sequence at the C-terminus which is not found in archaea. eIF2 α has two domains: an N-terminal β -barrel domain which has an S1 type oligo nucleotide/ or oligosaccharide binding fold subdomain and an α - helical subdomain. The conserved serine⁵¹ residue in eukaryotic eIF2 α is located in a loop in domain 1. This residue is a

target for phosphorylation by several eIF2 α kinases. In spite of lack of any sequence homology, the C-terminal domain with an α - β -fold is structurally similar to the C-terminal region of the eukaryotic elongation factor (eEF) 1B α , a guanine nucleotide exchange factor (equivalent to prokaryotic EF-Ts) that replaces GDP for GTP on eEF1A (equivalent to prokaryotic EF-Tu) (Ito et al, 2004; Monika et al, 2005).

The current understanding is that the α -subunit plays a role in the regulation of translation of mRNA while β and γ -subunits play a role in start site recognition, GTP hydrolysis and GTP-GTP and Met-tRNAⁱ binding. The intersubunit interactions among the eIF2 subunits of archaea and yeast suggest that α and β -subunits do not interact with each other whereas the γ -subunit interacts with α and β -subunits on either side. Based on these studies, it is suggested that γ -subunit appears to be central or core subunit in the structure of eIF2. Unlike these findings, recently, this laboratory demonstrated that recombinant human eIF2 subunits interact with each other and form α - β dimers, β - γ and α - γ dimers. An analyses of the functional significance α - β dimer formation with mammalian eIF2 suggests that it may play a role in the inhibition of GDP/GTP exchange activity of eIF2B which occurs upon phosphorylation of eIF2 α . Unlike in yeast where phosphorylated eIF2 α interacts directly with the regulatory subcomplex of eIF2B, mammalian eIF2 α does not directly interact with eIF2B and its association requires the β -subunit of eIF2. Phosphorylation of eIF2 α that promotes interaction with eIF2B is dependent on the presence of β -subunit thereby suggesting that β -subunit aids, not only in the functions of the γ -subunit as described above, but also plays a role in the regulation of eIF2B activity mediated by eIF2 α phosphorylation (Suragani et al, 2005; Rajesh et al, 2008).

Although, the physiological functions are not understood, mammalian β -subunit of eIF2 unlike its yeast counter part is phosphorylated by several kinases like PKC, PKA, DNA PK and casein kinase-II. The sites phosphorylated *in vitro* on mammalian eIF2 β have been mapped at Ser², Ser⁶⁷ (both targeted by CK2), Ser¹³ (targeted by PKC) and Ser²¹⁸ [targeted by PKA (protein kinase A)] (Welsh et al, 2002). eIF2 β is also a substrate for DNA-PK (DNA protein kinase) (Ting et al, 1998) although the phosphorylation site(s) for this kinase have not been identified yet. The studies on the phosphorylation of eIF2 β in mammalian cells have shown that it varies under different conditions such as heat shock (Duncan et al,

1984), serum deprivation (Duncan et al, 1985), diabetes (Garcia et al, 1996) and birth (Luis et al, 1993). Phosphorylation of eIF2 β by CK2 decreases the affinity of GDP binding to eIF2.

1.3 Phosphorylation of eIF2 α and translation attenuation:

The conserved ser⁵¹ residue in the α -subunit of eIF2 is a substrate for several eIF2 α kinases which are regulated by different physiological stress conditions. These include some of the well characterized eIF2 α kinases such as HRI, heme-regulated inhibitor; PKR, double stranded-RNA-dependent protein kinase (Friedman et al, 1972; Manche et al, 1992; Dey et al, 2005; Garcia et al, 2006a), general control non-derepressible kinase, GCN2 and PKR-like endoplasmic-resident kinase (PERK or PEK) (Shi et al, 1998, Schroder and kaufman, 2005; 2006). While HRI expression is limited to red blood cells, PKR and GCN2 expressions are ubiquitously observed in all mammalian cell types. In contrast, PERK expression is found high in professional secretory cells such as pancreas. The activation signals of these respective kinases are heme-deficiency, low concentration of double stranded RNA or virus infection, amino acid starvation that accumulates uncharged tRNAs and accumulation of unfolded proteins either due to excessive protein synthesis that exceeds protein folding capacity of the cell or improper degradation of proteins respectively. In addition, many other stressors such as heavy metal stress, heat shock, redox imbalance (Scheuner et al, 2001; Harding et al, 2003), DNA-damaging agents like ultraviolet radiation etc (Aparna et al, 2003), are known to promote eIF2 α phosphorylation because of the activation of one of the above eIF2 α kinases or by an undiscovered eIF2 α kinase (s). eIF2 α kinases are found from yeast to humans. In addition, some parasites are shown to contain eIF2 α kinases like PfeIK1 in *Plasmodium falciparum* (Mohrle et al, 1997) and TgIF2K in *Toxoplasma gondii* etc (Sullivan et al, 2004). While the eIF2 α kinases are regulated by different stressors, they all phosphorylate the conserved ser⁵¹ residue in the α -subunit. Since eIF2 α phosphorylation is a stress signal, it evokes adaptive responses or apoptotic responses due to the associated changes in gene expression, inter-protein interactions and coincident signalling activities. Phosphorylation of eIF2 α is considered as an integrated stress response (ISR) (Ron and Harding, 2007).

Fig. 2 Recycling of eIF2.

At the end of initiation of protein synthesis, eIF2 is released as eIF2.GDP binary complex which cannot join initiator tRNA (Met tRNA_i) unless the GDP is exchanged for GTP. The GDP/GTP exchange on eIF2 is catalyzed by eIF2B, a heteropentameric guanine nucleotide exchange protein. Phosphorylation of serine⁵¹ in eIF2 α sequesters the eIF2B into a 15S complex, eIF2(α P).eIF2B in which eIF2B becomes non-functional. A type 1 phosphatase dephosphorylates eIF2(α P) in physiological conditions and restores eIF2B activity. In eIF2B, the ϵ and γ subunits perform the catalytic functions whereas the α , β and δ form a regulatory complex. In yeast, phosphorylated eIF2 α interacts with the regulatory subcomplex whereas in mammalian cells, the β -subunit of eIF2 mediates the interaction between eIF2(α P) and eIF2B.

Fig. 3 eIF2 α Kinases.

The figure depicts the homology among four different eIF2 α kinases: PKR, PERK, GCN2 and HRI. The eIF2 α kinases sense diverse stress signals and converge on phosphorylation of eIF2 α at ser⁵¹ position (Proud 2005).

Fig. 2

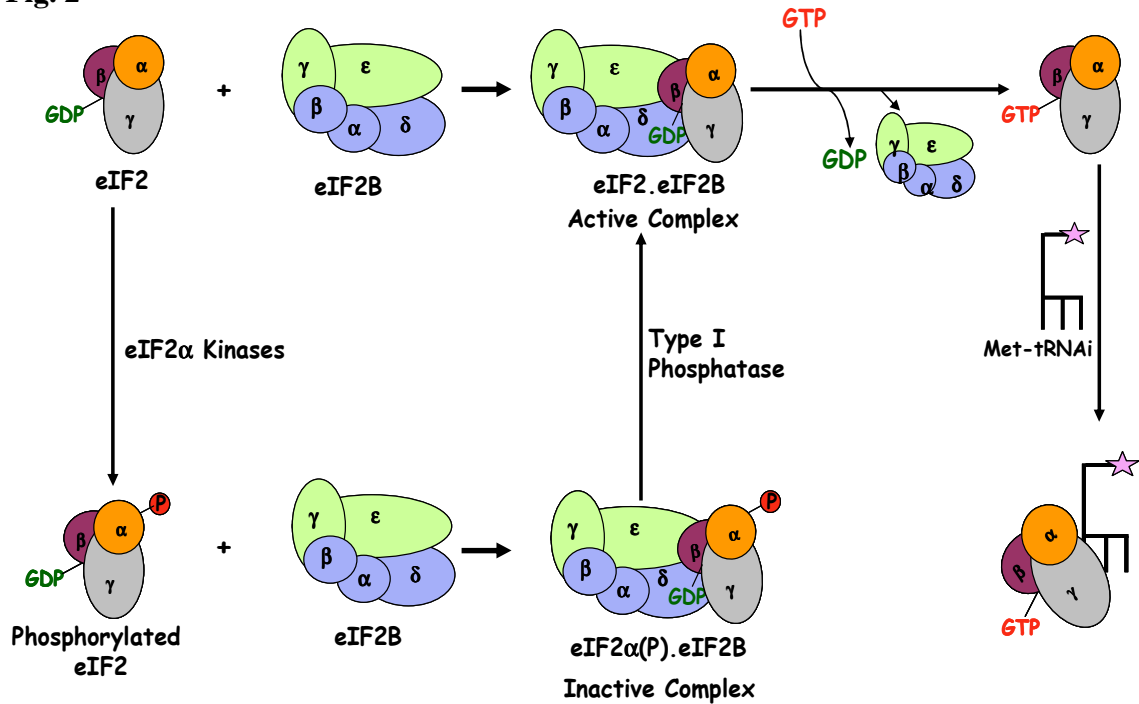
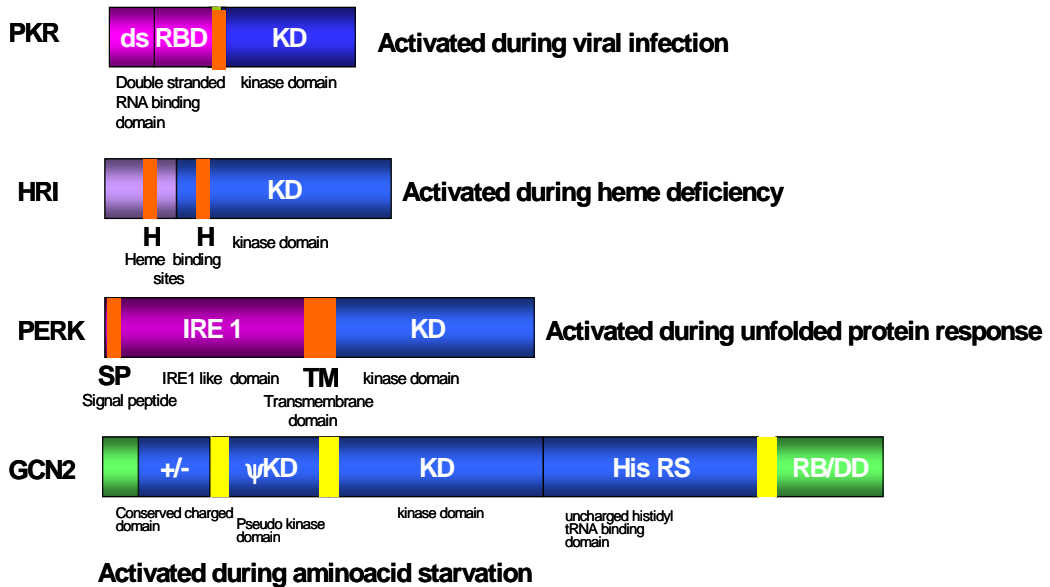


Fig. 3



PERK has about 30% sequence identity with PKR and 25% identity with HRI and yeast GCN2

Several cellular and viral regulators modulate the activation of eIF2 α kinases and subsequent eIF2 α phosphorylation (see later). Unlike human eIF2 α , the carboxy terminal of yeast eIF2 α is also a substrate for casein kinase-2 and is phosphorylated at three different sites which are not found in human. Although, the physiological significance is not yet understood, the β -subunit of human eIF2 is also phosphorylated *in vitro* and *in vivo* by several kinases as mentioned above (Suragani et al, 2006).

1.3a Cellular and Viral regulators of eIF2 α phosphorylation: Phosphorylation of eIF2 α is regulated directly through the activation and repression of the corresponding kinases/phosphatases. A type1 phosphatase activity is implicated in the dephosphorylation of eIF2 α (Babu and Ramaiah, 1996; Brush et al, 2003). Recent evidence indicates that there are many viral and cellular regulators that influence the activation and repression of the eIF2 α kinases and phosphatases and thereby influence the phosphorylation of eIF2 α . For example, the PKR activity i.e., its ability to dimerize, autophosphorylate and phosphorylate the substrate is influenced by low levels of double stranded RNA and also regulated by cellular proteins like PACT, PKR activating protein or RAX (Patel et al, 1998; Ito et al, 1999). This protein, heterodimerizes with PKR and activates it in the absence of double stranded RNAs. In mammalian cells treated with arsenite, thapsigargin, hydrogen peroxide and interleukin3 deprivation, PACT/RAX is rapidly phosphorylated and associates with PKR, which is followed by PKR activation and eIF2 α phosphorylation (Baltzis et al, 2004; Onuki et al, 2004). P58^{IPK} is another stress mediated cellular regulator of PKR (Lee et al, 1990; 1992; Polyak et al, 1996; Yan et al, 2002). In normal cells, P58^{IPK} associates with the heat shock protein 40 and forms an inhibitory complex. Cellular stress or virus infection such as influenza virus induces dissociation of p58^{IPK} from the inhibitory complex and disrupts PKR activity (Gale et al, 1996, Tan et al, 1998, Melville et al, 1999). P58^{IPK} interacts with a region of PKR that spans the ATP binding region in C-terminal catalytic domain (amino acids 244-296). Binding of p58^{IPK} with this region prevents PKR dimerization and auto-phosphorylation. P58^{IPK} is also induced during unfolded protein response (UPR) (Yan et al, 2002; Van Huizen et al, 2003) that occurs due to the accumulation of unfolded proteins in the endoplasmic reticulum. Human dihydrouridine synthetase-2, hDUS2, produced in HT1080 cells acts as a cellular PKR inhibitor. hDUS2

encodes for a human tRNA modification enzyme-dihydrouridine synthase (Mittelstadt et al, 2008).

PKR activation requires the binding of dsRNA to a RNA binding domain in PKR located in the N-terminus of the kinase. Hence many viral mRNAs and viral coded proteins can interfere into this activation of PKR by dsRNA so that the host cell eIF2 α is not phosphorylated and facilitates the virus propagation. These include adenovirus encoded VAI RNA (Katze et al, 1987, Mathews and Shenk et al, 1991, Schneider et al, 1996), reovirus induced capsid protein $\sigma 3$ (Ilyod and Shatkin; 1992, Yeu and Shatkin; 1997), influenza virus encoded non-structural NSI protein (Lu et al, 1995) and NS5A produced by hepatitis C virus (Gale et al, 1997; 98 and 99) etc. These molecules interfere with dsRNA binding to PKR, thus preventing its dimerization and autophosphorylation. Interestingly the glycoprotein E2 of hepatitis C virus has been reported to interact with PKR and PERK, inhibiting their activities (Pavio et al, 2003, Taylor et al, 1999). Cells infected with Epstein bar virus produce large amounts of small non-coding EBER RNAs that bind to PKR and prevent the activation of enzyme (Clark et al, 1990). Herpes simplex virus-1 (HSV-1) produces U(s)11 protein which acts to prevent PKR activation. The $\gamma_1 34.5$ protein, a virulence factor encoded by HSV-1, plays a critical role in mediating eIF2 α dephosphorylation (He 1997; Cheng et al, 2005). U(s)11 expression prevents eIF2 α phosphorylation and the inhibition of translation observed in cells infected with a $\gamma_1 34.5$ mutant of HSV-1. U(s)11 inhibits the activation of the cellular kinase PKR and the subsequent phosphorylation of eIF2 (Carroll et al, 1993; Cassady et al, 1998 a and b).

Vaccinia virus encodes two genes products E3L and K3L both of which counter viral resistance to interferon (Goebel et al, 1990). The E3L protein synthesized early during virus infection contains an amino terminal z-DNA binding domain and a carboxyl terminal with typical double stranded RNA binding motif which sequesters dsRNA and prevents activation of PKR and phosphorylation of eIF2 α (Chang et al, 1992). In addition, E3L also prevents the activation of 2'-5' oligoadenylate synthetase, yet another property of PKR. K3L protein has homology to eIF2 α and acts as a pseudosubstrate for PKR in competition with eIF2 α , consequently suppressing phosphorylation of eIF2 α and shut off of host protein synthesis (Davies et al, 1992, Carroll et al, 1993, Kawagishi-Kobayashi et al, 1997).

Fig. 4 Table: Regulation of PKR action by cellular and viral regulators.

S.No	Regulator	Source	References
Cellular Activators			
1	E2F-1	Carcinoma cells	Vorburger et al, 2005
2	<i>Mda7</i>	Human lung cancer cells	Pataer et al, 2005
3	RAX and PACT	Mouse and Human	Ito et al,1999
Cellular Inhibitors			
1	gp67	various organisms	Datta, 2000
2	hDUS2	HT1080	Mittelstadt et al, 2008
3	HSP 90 and HSP 70	Cancer cells	Donze et al, 2001, Pang et al, 2002
4	Nucleoplasmin	Karpas 299 cells	Garcia et al, 2006
5	P58 ^{ipk}	Influenza virus	Lee et al, 1994
6	TRBP	Expressed in response to HIV infection	Gatignol et al, 1991
Viral Regulators			
Proteins affecting the interaction between PKR and dsRNA			
1	σ 3 and σ 4	Reo virus	Yue and Shatkin, 1997; Jacob and Langland, 1998
2	E3L	Vaccinia Virus	Romano et al, 1998; Sharp et al, 1998
3	NS1	Influenza virus	Lu et al, 1995; Hatada et al, 1999
4	NSP3, NSP5	Rota virus	Langland et al, 1994
5	SM	Epstein -Barr virus	Poppers et al, 2003
6	VP35	Ebola virus	Feng et al, 2007
7	Us11	Herpes simplex virus	Peters et al, 2002
PKR: Viral protein interaction			
8	E3L	Vaccinia Virus	Romano et al, 1998, Sharp et al, 1998
9	NS5A and E2	Hepatitis C virus	Gale et al 1999, Ghosh et al, 1999
10	P58 ^{ipk}	Influenza virus	Melville et al, 1999, Goodman et al,1998
11	PK2	Baculovirus	Dever et al, 1998
12	Us11	Herpes simplex virus	Cassady and Gross, 2003
13	vIRF-2	Kaposi's Sarcoma herpes virus	Burysek and Pitha, 2001

Viral RNAs affecting dsRNA binding to PKR			
14	EBER RNA	Epstein -Barr virus	Sharp et al, 1993
15	IRES	Hepatitis C virus	Vyas et al, 2003
16	VAI RNA	Adenovirus	Mathews and Shenk, 1991
Proteins that mimick eIF2			
17	C8L	Swine pox virus	Kawagishi-Kobayashi et al, 2000
18	E2	Hepatitis C virus	Taylor et al, 1999
19	eIF2 α	Rana virus	Essbaur et al, 2001
20	HIV-1 TARBP	Human Immunodeficiency virus	Gunnery et al, 1990, Katze and Agy, 1990
21	K3L	Vaccinia Virus	Carroll et al, 1993, Davies et al, 1992
22	ReIF2H	Ambystoma tigrinum virus	Essbauer et al, 2001
Viral regulators affecting dephosphorylation of eIF2α			
23	$\gamma_{134.5}$ / PP1 α	Herpes simplex virus	He et al, 1997a, 1997b
24	E6 / GADD34/ PP1 α	Human papilloma virus	Kazemi et al, 2004
25	Large T antigen	Simian virus 40	Swaminathan et al, 1996
Viral proteins inhibiting PKR			
26	C protein	Sendai virus	Takeuchi et al, 2008
27	C protein	Measles virus	Toth et al, 2009
28	P / V protein	Simian virus 5	Gainey et al, 2008
Alteration of PKR subcellular localization by viral factors			
29	E6 and E7	Human papilloma virus	Hebner et al, 2006
30	m142 and m143	Human cytomegalovirus	Budt et al, 2009, Child and Geballe, 2009
31	TRS1, IRS1	Human cytomegalovirus	Hakki et al, 2006
Viral proteins affecting PKR degradation			
32	NSs	Rift Valley fever virus	Ikegami et al, 2009
33	protease	Polio virus	Black et al, 1989, 1993
Binding and sequestration of viral RNA by proteins			
34	B2	Betanodavirus	Fenner et al, 2006
35	E3L	Vaccinia Virus	Liu et al, 2001
Inhibition of PKR mediated Caspase activation			
36	LANA2	Kaposi's Sarcoma herpes virus	Esteban et al, 2003
37	MC159L	molluscum contagiosum virus	Gil et al, 2001
Inhibition of caspase mediated PKR activation			
38	P35	Baculovirus	Aparna et al, 2003

K3L also binds to PERK and inhibits its activation. This raises the possibility that K3L interferes with ER stress response. TRS1 protein produced by cytomegalo virus is an RNA binding protein that functions as E3L of vaccinia virus (Child et al, 2004). Another protein named HIV1- TARBP (HIV1- Transactivating RNA binding protein) produced during HIV infection, has RNA binding motifs, binds to RNA binding domains in PKR and inhibits its activity (Gunnery et al, 1990, Katze and agy; 1990).

Like virus infection regulates PKR activation and eIF2 α phosphorylation in host cells, the activation of PERK is regulated by unfolded protein response in endoplasmic reticulum and also by virus infection. PERK is one of the three ER stress sensors. The other two sensors are: IRE-1 (Inositol-requiring element-1) and ATF6. In normal inactive conditions, all the sensors are bound by BiP, an ER chaperone, stress marker and master regulator of ER stress sensors. PERK activation requires the dissociation of BiP which occurs whenever the unfolded proteins accumulate due to excessive protein synthesis that exceeds the protein folding capacity of the cell, improper modification of proteins and their degradation. The dissociated BiP binds to unfolded proteins (Bertolotti et al, 2000, Shen et al, 2002, Zhang et al, 2004). In addition, BiP is also a survival signal and its expression is enhanced in response to ER stress (Morris et al, 1997). Thus ER stress has two arms; translational attenuation by PERK-mediated eIF2 α phosphorylation and transcriptional induction of genes. XBP-1, a transcriptional factor is produced by IRE-1 activation, and, ATF4 (Activated transcription factor 4) by eIF2 α phosphorylation. In contrast, activation of ATF6 arises due to proteolytic processing during ER stress. The transcriptional induction of these b-zip transcription factors facilitates chaperone synthesis including BiP. Many viruses are also known to promote ER stress because most of their glycoproteins are synthesized in ER which can lead to PERK activation and also BiP induction. PERK is inactive when it is bound by BiP. While BiP synthesis cannot affect activated PERK-mediated eIF2 α phosphorylation, it is likely that BiP may interfere in eIF2 α phosphorylation-mediated caspase activation as has been suggested (Aarti et al, 2010). Recent studies demonstrate that a cytopathic strain of bovine viral diarrhea virus, a member of flaviviruses, activates PERK and increases eIF-2 α phosphorylation, and cell death (Jordan et al, 2002). In contrast, a similar phenotype is not found with noncytopathic strain of bovine viral diarrhea virus, which tends to cause chronic infection but not cell

death (Meyers and Thiel, 1996). Ectopic expression of E2 protein of hepatitis C virus acts inhibits PERK activation as it resembles eIF2 α and may interfere with the normal binding of cellular of eIF2 α to PERK (Pavio et al, 2003). The activation and inactivation of non-ER cytosolic eIF2 α kinases like HRI, PKR and GCN2 are also regulated by cytosolic chaperones like members of the heat shock proteins (HSPs) (Matts et al, 1992, 93, Uma et al, 1998, Thulsiraman et al, 1998, Donze et al, 1999, 2001)

Proteasome inhibition is also shown to regulate cytosolic eIF2 α phosphorylation more significantly than ER stress-induced PERK-mediated eIF2 α phosphorylation. Proteasome inhibition in mouse embryonic fibroblast (MEF) cells leads to enhanced eIF2 α phosphorylation and significant reduction in protein synthesis, concomitant with induced expression of the bZIP transcription regulator, ATF4, and its target gene CHOP/GADD153 (growth arrest DNA-damage inducible protein- 153) (Obeng et al, 2006). GCN2 appears to be the primary eIF2 α kinase activated by exposure of these fibroblast cells to proteasome inhibition which has a central role in the recognition of cytoplasmic stress signals. In contrast, ER stress is not effectively induced in MEF cells subjected to proteasome inhibition, with minimal activation of the ER stress sensory proteins, eIF2 α kinase PEK (PERK/EIF2AK3), IRE1 protein kinase and the transcription regulator ATF6 following up to 6 h of proteasome inhibitor treatment. Decline in eIF2 α phosphorylation reduces caspase activation and delays apoptosis. Since deletion of CHOP, a transcriptional regulator in MEF cells impedes apoptosis, it is likely that expression of CHOP associated with eIF2 α kinase activation plays a crucial role in eIF2 α -phosphorylation-mediated apoptosis. Hence it is suggested that eIF2 α kinases are integral to cellular stress pathways induced by proteasome inhibitors, and may be central to the efficacy of anticancer drugs that target the ubiquitin/proteasome pathway (Jiang et al, 2005).

Phosphorylation of eIF2 α , a stress signal as mentioned above inhibits general protein synthesis, an important energy saving mechanism because translation consumes upto 50% of cellular energy depending on the organism. In addition, the attenuation of translation of mRNAs reduces unwanted proteins that might interfere with the stress response pathways. However, as an adaptive or survival measure, translation of selective mRNAs can occur. Phosphorylated eIF2 α reduces the GDP/GTP exchange activity of heterpentameric eIF2B

protein and the conversion of inactive eIF2.GDP to active eIF2.GTP that can bind initiator tRNA there by affecting the formation of eIF2.GTP.Met.tRNAⁱ ternary complex of the initiation step which is common to all mRNAs (Proud, 2005; Webb and Proud, 1997). For example GCN4 mRNA in yeast has many upstream open reading frames (uORFs) preceding the main GCN4 ORF. When eIF2 α is not phosphorylated, the uORFs are translated efficiently but not GCN4 main ORF (Hinnebusch et al, 1997). However when initiation is slowed down due to eIF2 α phosphorylation, reinitiation occurs mainly at GCN4 mRNA bypassing the small uORFs. GCN4 is a transcriptional factor that is expressed efficiently when eIF2 α is phosphorylated due to the activation of GCN2 eIF2 α kinase in cells that are starved of amino acids or lack proper nutrients (Dever et al, 1992). GCN4 expression in turn ensures the expression of other genes that are involved in the amino acid biosynthetic pathways. In mammalian cells, ATF4 mRNA is upregulated in response to eIF2 α phosphorylation. ATF4 mRNA structure and its translation mechanism resembles to GCN4. ATF4, like GCN4, is a b-zip transcription factor which in turn upregulates genes involved in redox maintenance. GCN2 like kinase is also present in mammalian cells (Harding et al, 2003). Activated GCN2 in brain suppresses memory formation by phosphorylating eIF2 that in turn facilitates the expression of ATF4 (Zhang et al, 2002; Vattem and Wek, 2004). ATF4 is a memory suppressor because it inhibits transcriptional factor cyclic AMP-response element binding protein (CREB)-mediated gene expression particularly of the early-immediate gene targets that are critical for long term synaptic plasticity and memory (Costa-Mattioli et al, 2008). These findings suggest that translational control mechanisms which are involved in sensing amino acid presence in microorganisms are adapted in higher animals to control learning and behaviour. Stress conditions associated with eIF2 α phosphorylation have been reported to regulate translation initiation at internal AUGs in C/EBP family members (Calkhoven et al, 2000). However there is no evidence that eIF2 α phosphorylation affects other uORF containing genes such as CHOP or GADD-153 and GADD34, a co factor of eIF2 α phosphatase that have two or more uORFs although their basal translation is up regulated in response to eIF2 α phosphorylation.

While the 5' cap and 3' polyA tail are features of most of the eukaryotic mRNAs, there are mRNAs in eukaryotes with IRES (internal ribosome entry sequence) elements in the 5'

untranslated region (5'UTR) like in viral mRNAs (Gerlitz et al, 2002; Gebauer and Hentze, 2004). The IRES facilitates translation of mRNAs by directly recruiting the ribosomes independently of the cap structure. Interestingly, the translation of many of these IRES containing mRNA like CAT1 (cationic amino acid transporter), PDGF2 (platelet derived growth factor -2) VEGF (vascular endothelial growth factor) and c-Myc were found enhanced during differentiation or in response to cellular stress that promotes eIF2 α phosphorylation (Brewer et al, 1999; Harding et al, 2000; Scheuner et al, 2001; Gerlitz et al, 2002; Jefferson and Kimball, 2004). The mechanism by which eIF2 α phosphorylation stimulates IRES containing mRNAs is not understood. However it is likely that most of the IRES containing mRNAs may not require eIF2 or eIF4F complexes as the ribosomes recruit internally bypassing the cap structure. BiP, a chaperone of endoplasmic reticulum is yet another example whose translation is upregulated in response to accumulation of unfolded proteins that causes stress in the endoplasmic reticulum (ER) and following eIF2 α phosphorylation. However, ER stress but not eIF2 α phosphorylation appears to be the signal for increased BiP translation (Aarti et al, 2010).

Other stress signaling pathways of late have been shown to influence GCN2-mediated eIF2 α phosphorylation that occurs in response to amino acid starvation. These include for instance, the activation of MEK/ERK signaling by amino acid limitation was dependent on GCN2 and phosphorylation of eIF2 α . Of the three major pathways tested, only MEK-ERK pathway appears to be essential for activation of eIF2 α phosphorylation following amino acid starvation/limitation in HepG2 cells. Inhibition of MEK /ERK pathway in amino acid starvation reduces eIF2 α phosphorylation-ATF4 expression. Exogenous expression of ATF4 rescues AARE-driven ATF4 transcriptional inhibition. Knockdown of *GADD34* expression did not prevent the dependence of eIF2 α phosphorylation on MEK, arguing against a MEK/ERK action on the PP1-GADD34 phosphatase (Thiaville et al, 2008). The inhibition of PP1 activity possibly by JNK can also lead to the expression of ATF4 in the absence of any stressors when JNK pathway is inhibited (Monick et al, 2006). In cellular response to UVA, phosphorylation of double stranded RNA-dependent protein kinase, PKR, at threonine 451 is mediated through ERK2 (extra cellular regulated kinase 2) and RSK2 (receptor signaling kinase 2) and is involved in the regulation of ser⁵¹ phosphorylation of eIF2 α in UVA-irradiated JB6 cells (Zykova et al, 2007). PKR is a

dual specificity kinase. It can be activated and autophosphorylated on its thr-446 residue by small concentrations of double stranded RNA. It is also a substrate for tyrosine kinases such as Janus kinase 1 (Jak1) and tyrosine kinase 2 (Tyk2) which are activated by interferons (Su et al, 2007). Thus interferons not only stimulate the expression of PKR which can be activated by viral RNAs through its phosphorylation on thr 446 residue, but also induce tyrosine kinases that regulate its tyrosone phosphorylation on residues at 101 and 293. Phosphorylation of eIF2 α and inhibition of protein synthesis by PKR and PERK activation leads to the induction of PI3 kinase (phosphoinositide-3 kinase) pathway as observed by increased AKT or PKB (protein kinase B) phosphorylation in mouse embryonic fibroblasts. eIF2 α kinase activated PI3 kinase signaling pathway may offer cytoprotective action and plays a role in cell survival (Kazemi et al, 2007).

Further eIF2 α phosphorylation pathway is also connected to NF-kB activation. Expression levels of most cellular proteins will decrease with time upon eIF2 α phosphorylation. According to their respective protein half-lives, the consequential rapid decrease in the steady-state level of high-turnover proteins has recently been found to lead to the activation of NF-kB in response to eIF2 α phosphorylation, mediated by the down-regulation of I κ B proteins (Jiang et al, 2003). Proteasome inhibition in MEF cells activates efficiently cytosolic stress, GCN2 kinase activation and eIF2 α phosphorylation, but not ER stress-induced eIF2 α phosphorylation.

A recent study suggests that Src homologous domain containing adaptor Nck1 is a key component of molecular complex of protein phosphatase-1 (Latreille M and Larose L, 2006) that dephosphorylates eIF2 α and facilitates the cells to recover from stress in the endoplasmic reticulum. Subsequent study has shown that Nck-1 not only modulates eIF2 α Ser⁵¹ phosphorylation driven by stress conditions preferentially activating PERK, and also PKR and HRI, but not GCN2 (Cardin et al, 2007). Overexpression of Nck1 enhances translation through its direct interaction with the β -subunit of eIF2 (Kebache et al, 2002). Phosphorylation of eIF2 α is also accomplished by protein phosphatase 1 complex (PP1C) containing either the protein CReP (constitutively active phosphatase) or GADD-34, a cofactor of eIF2 α phosphatase which targets PP1C to eIF2. CHOP, a bzip transcriptional factor is induced in response to ER stress and downstream to eIF2 α

phosphorylation which in turn induces GADD-34, a cofactor of eIF2 α phosphatase. CHOP deletion leads to reduced levels of GADD-34 which mediates negative feedback on the levels of eIF2 α phosphorylation (Zinszner et al, 1998; Marciniak et al, 2004). This in turn would lead to recovery in ER stress induced eIF2 α phosphorylation-mediated inhibition in protein synthesis. Further glucose-induced stimulation of overall translation in beta cells depends on a protein-phosphatase 1-mediated decrease in phosphorylation of eIF2 α (Oyadomari et al, 2002). Because defects in pancreatic endoplasmic reticulum kinase (PERK)-eIF2 α signaling system lead to pancreatic β -cell failure and diabetes, deregulation of the PP1 system or other cellular regulators of eIF2 α phosphorylation like p^{58IPK} can lead to cellular dysfunction and disease in these professional secretory cells.

1.3b eIF2 α phosphorylation and cellular homeostasis: In cell culture experiments, abrogation of eIF2 α phosphorylation promotes transformation while PKR-mediated eIF2 α phosphorylation leads to cell death in mammalian cells (Donze et al, 1995; Srivastava et al, 1998; Scheuner et al, 2006) suggesting that eIF2 α phosphorylation determines cellular homeostasis. eIF2 α kinase signaling pathway also plays a role in starvation induced autophagy in yeast, virus and in mammalian cells (Talloczy et al, 2002). Mammalian eIF2 α kinase-dependent autophagy is antagonized by the herpes simplex virus encoded neuro virulence gene product, ICP-34.5 (He et al, 1997). PERK knock out cells display higher sensitivity towards ER stress agents like tunicamycin and readily undergo cell death (Ron, 2002) suggesting that eIF2 α phosphorylation is also required to protect cells from ER stress-induced apoptosis. PKR, an eIF2 α kinase which is activated by low concentrations of double stranded RNA is found to be a substrate for caspases and also is activated by caspases (Saleans et al, 2001; Aparna et al, 2003). Tumor-suppressor activity for PKR has also been invoked, in NIH 3T3 fibroblasts and over expression of dominant negative mutants of PKR in mouse causes malignant transformation (Koromilas et al, 1992; Meurs et al, 1993). In cell culture experiments, transformed cells lacking either PERK or its downstream effector ATF4 impair the ability of these cells to survive as tumors in vivo (Bi et al, 2005), thereby suggesting that PERK-ATF4 pathway may have a role in cancer therapy. ER stress induced eIF2 α phosphorylation by salubrinal, a thiourea

compound inhibits selectively the replication of herpes simplex virus in its host cells. Analysis of the mechanism reveals that the virus produces a protein phosphatase that dephosphorylates host cell eIF2 α and facilitates the virus replication and inclusion of salubrinal inhibits the host and virus coded CReP and GADD-34 phosphatase activities (Bryant et al, 2008). A reduction in eIF2 α phosphatase activity attenuates newly synthesized unfolded protein load and the ER stress. In fact regulation of host cell eIF2 α phosphorylation by viral and cellular proteins plays a crucial role in virus propagation. However if the stress is severe, CHOP, a transcriptional factor activates ER-oxidase 1 and elevates reactive oxygen species (ROS) and thereby promotes cell death (McCullough et al, 2001; Ikeyama et al, 2003; Marciniak et al, 2004).

Expression of wild-type PKR in yeast causes increased phosphorylation of eIF2 α and results in inhibition of cell growth (Chong et al, 1992; Dever et al, 1993). Two knock out mice developed by disruption of exons 2 and 3 of the PKR gene (N-PKR $^{-/-}$) (Yang et al, 1995) and by elimination of exon 12 (C-PKR $^{-/-}$) (Abraham et al, 1999) displayed no growth abnormalities and were virus resistant. In exception, N-PKR did not resist encephalomyocarditis virus infection and C-PKR $^{-/-}$ vesicular stomatitis virus infection. Interestingly, mouse embryonic fibroblasts from N-PKR $^{-/-}$ were defective in I κ b α phosphorylation and NF κ B activation by dsRNA (Zamanian et al, 2000) and were susceptible to apoptosis by tumor necrosis factor α (TNF α), dsRNA and lipopolysaccharide treatment (Der et al, 1997). Activation of PKR and phosphorylation of eIF2 α suppress tumors by protein PTEN, phosphatase and tensin homolog deleted chromosome 10 (Mounir et al, 2009). In parasites, eIF2 α kinases and phosphorylation play a role in stress response and in development. PfeIK1, the malrial parasite eIF2 α kinase, plays a role in response to starvation where as the eIF2 α kinase of *Toxoplasma gondii* plays a role the transition of tachyzoite to baryzoite, a quiescent stage of the organism (Sullivan et al, 2004).

Expression of nonphosphorylatable form of eIF2 α leads to malignancy in NIH 3T3 cells (Donze et al, 1995) while enhanced phosphorylation of eIF2 α that occurs due to over expression of PKR or S51D, a phosphomimetic form of eIF2 α , results in cell death (Srivastava et al, 1998; Scheuner et al, 2006). However, these observations are at variance

indicating that tumor cells often display higher level of eIF2 levels and phosphorylation of its α -subunit, as shown in ehrlich ascites cells (Clemens et al, 2004). To tolerate such higher levels of eIF2 α phosphorylation, they also have enhanced levels of other initiation factors including eIF2B, the GDP/GTP exchange factor that recycles eIF2.GDP to eIF2.GTP. It is not thus clear whether apoptosis is mediated by eIF2 α phosphorylation or due to consequent changes in mRNA translation (Perkins and Barber, 2004). A recent study describes apoptosis that occurs in response to several stressors is characterized by down regulation of a BCL-2 family protein MCL-1. Stabilization of MCL-1 blocked apoptosis initiation, while cells with reduced MCL-1 protein content were strongly sensitized to stress-induced apoptosis. Stress-induced phosphorylation of eIF2 α at Ser⁵¹ was both essential and sufficient for the down-regulation of MCL-1 protein in stressed transformed mammalian cells (Fritsch et al, 2007). A recent study by us in rats suggests that ER stress-induced eIF2 α phosphorylation declines with ageing and is associated with the expression of pro-apoptotic proteins such as GADD153 and CHOP, a cofactor of eIF2 α phosphatase (Hussain and Ramaiah, 2007a).

Further PKR is proteolyzed and eIF2 α is phosphorylated at the early stages of apoptosis induced by various stimuli. These events coincide with the caspase activity and are prevented by caspase inhibitor. These findings suggest that caspase-dependent activation mode for PKR leading to eIF2 α phosphorylation and translation inhibition in apoptosis (Saleans et al, 2001). Previously, this laboratory expressed S51D, a phosphomimetic form of eIF2 α in insect cells using baculovirus expression with out leading to cell death (Sudhakar et al, 2000; Suragani et al, 2006). An analysis of these results prompted us to investigate whether any of the baculoviral proteins are antiapoptotic and can effect eIF2 α phosphorylation. Infection of *Sf9* (*Spodoptera frugiperda*) cells, natural hosts of baculovirus by a mutant baculovirus which is devoid of its anti apoptotic gene p35 resulted in enhancement of eIF2 α phosphorylation and cell death readily. In contrast, wt baculovirus infection resists UV-induced apoptosis and eIF2 α phosphorylation. Consistent with these studies, synthetic caspase inhibitors reduced eIF2 α phosphorylation and stress-induced apoptosis. Further expression of a phosphomimetic form of eIF2 α promotes apoptosis. Complimenting these observations, non-phosphorylatable form of eIF2 α is

found to mitigate UV-induced apoptosis suggesting that eIF2 α phosphorylation plays a role in apoptosis. However, many times eIF2 α phosphorylation induced by several stressors did not lead to apoptosis suggesting that other factors as well contribute to eIF2 α phosphorylation-mediated cell death. Insect cells, however, unlike mammalian cells do not show receptor-induced apoptosis (extrinsic) and do not have several caspases. Their eIF2 α kinases are not well characterized. In addition to mediating cell death, phosphorylated mammalian eIF2 resists caspase action where as unphosphorylated eIF2 serves as a good substrate for caspases (Rajesh et al, 2008).

In animal studies, analyses of homozygous mice harboring non-phosphorylatable S51A mutant eIF2 α (where ser⁵¹ is replaced by alanine) or mice lacking PERK reveal that the former has severely reduced number of pancreatic β -cells and develop hypoglycemia compared to the latter that develop hyperglycemia shortly after birth (Harding, 2002; Scheuner et al, 2008). Heterozygous mice harboring one copy of non-phosphorylatable form of eIF2 α are born normally but become obese on high fat diet (Scheuner et al, 2005; Marciniak et al, 2006). Obesity in humans and mice results in insulin resistance which is counteracted by increased production of proinsulin in the ER of pancreatic beta cells. PERK dependent eIF2 α phosphorylation regulates the protein load and ensures the long term survival of the tissue. Moreover, the eIF2 α ^{+S51A} mice exhibit enhanced learning and memory using different training protocols (Costa-Mattioli et al, 2008). PERK knock out mice develop skeletal dysplasia and exhibit growth retardation due to defective osteoblasts (Zhang et al, 2002). Further, in humans, mutations in PERK lead to Wallcot-rallison syndrome, an infantile diabetes (Delepine et al, 2000). In iron-deficient HRI knock out mice, globin without heme was found aggregated within the red blood cells resulting in hyperchromic, normocytic anemia, decline in RBC count and accelerated apoptosis in bone marrow and spleen (Han et al, 2001). These findings suggest that phosphorylation of eIF2 α plays a role after birth, and, it is coupled with protein synthesis to protein folding, glucose metabolism and memory.

1.4 Objectives:

The objectives of the thesis are based on some of the previous studies of the laboratory. This laboratory is working on various aspects of regulation of protein synthesis mediated by eIF2 α phosphorylation (Ramaiah et al, 1992; Krishnamoorthy et al, 1998). They include the evaluation of various novel agents that promote eIF2 α phosphorylation, phosphorylated-mediated changes in gene expression (Aarti et al, 2010), mechanics and regulation of protein synthesis caused by eIF2 and phosphorylation of the α -subunit (Ramaiah et al, 1992; 1994; Sudhakar et al, 1999; 2000), expression and characterization of the recombinant subunits of human eIF2, the intersubunit and interprotein interactions, the effect of phosphorylation of the α and β subunits on these interactions (Suragani et al, 2005; 2006; Rajesh et al, 2008), the eIF2 α phosphorylation mediated cell survival and death (Aparna et al, 2003; Aarti et al, 2010), and the coincident signaling activities that influence eIF2 α phosphorylation (Pushpanjali and Ramaiah, 2010). The laboratory uses cell-free translational systems to evaluate some of these aspects and also the ovarian cells of *Spodoptera frugiperda*, a lepidopteran insect and natural hosts of baculovirus for the expression of various subunits of eIF2 and their mutants. In one of the previous studies, the insect cells, unlike mammalian cells, are found able to express phosphomimetic form eIF2 α without undergoing cell death. Analyses of these results lead to an understanding that baculovirus p35, an antiapoptotic protein may be interfering in the cell death promoted by the expression of phosphomimetic form using baculovirus expression. Further studies revealed that a mutant baculovirus devoid of its anti apoptotic gene p35 readily stimulates apoptosis. UV-induced eIF2 α phosphorylation and cell death is mitigated in *Sf9* cells infected by wt baculovirus that harbors p35 antiapoptotic gene, by a caspase inhibitor like z-VAD-fmk (Aparna et al, 2003) and also by expression of non-phosphorylatable form of eIF2 α (Aparna et al 2003). Complementing these observations, infection of *Sf9* cells by a mutant baculovirus that is devoid of its PK2 gene, an inhibitor of eIF2 α kinase, but still harbors p35 antiapoptotic gene, cannot protect cells as efficiently as cells infected by wt baculovirus (Aarti et al, 2010) suggesting that eIF2 α phosphorylation plays a critical role in apoptosis. Further studies by my colleague have revealed that ER (endoplasmic reticulum) stress-induced eIF2 α phosphorylation, as characterized by expression of BiP, an

ER chaperone does not lead to apoptosis in *Sf9* cells unlike in mammalian cells suggesting that eIF2 α phosphorylation, primarily a stress signal can lead to cell survival or suicide depending on its cellular location (Aarti et al, 2010).

However, in addition to eIF2 α phosphorylation, there are many other signaling pathways known to affect cell survival and death. These include activation of MAP kinases or PKCs etc. Like eIF2 α kinases, there are many forms of PKCs which are regulated by different conditions. Like eIF2 α kinases, PKCs can also protect cells against cell death and their activation can also lead to cell death. Like eIF2 α kinases, some of the PKCs are found substrates for caspases. Keeping in view of some such similarities, the thesis work is taken up to identify the connection or importance, if any between eIF2 α phosphorylation mediated cell survival and death to PKC activation under such conditions. Keeping in view of this objective, the following questions are addressed.

- a) Does PMA, phorbol myristate acetate, an activator of PKC stimulate stress-induced eIF2 α phosphorylation, caspase activation and cell death of *Sf9* cells? Does it have any preference between ER and non ER stressors-induced eIF2 α phosphorylation. Can stress-induced effects be reversed by calphostin, an inhibitor of PKC?
- b) Whether eIF2 α phosphorylation is a cause or consequence of caspase activation or both and what stage(s) of eIF2 α phosphorylation is affected by PKC activation?
- c) Can cell free extracts obtained from control cells be used to evaluate the importance of caspase activation-mediated eIF2 α phosphorylation and its contribution, if any, by PKC activation?

Methodology:

2.1 Materials

2.2 Cell Culture

2.3 Preparation of cell extracts

2.4 Preparation of nuclei and cell extracts devoid of nuclei

2.5 Induction of Apoptosis

2.6 Assays for Apoptosis

i) Trypan blue exclusion test

ii) Caspase activity

iii) Hoechst Staining

iv) DNA fragmentation

v) FACS Analysis

2.7 Sodium Dodecyl Sulphate-polyacrylamide gel electrophoresis

2.8 Western Blotting

2.9 Immunodepletion

2.10 PERK Cleavage assay

2.11 Protein Estimation

2.1 Materials:

Agarose, antibiotic-antimycotic solution, ATP, BCIP, bromophenol blue, BSA, cell culture grade DMSO, coomassie R 250, cycloheximide, EGTA, etoposide, glutathione, Hoechst stain, mannitol, NBT, PMSF, polystyrene tubes Protinase K, sodium bicarbonate, sucrose, tissue culture flasks and 60mm tissue culture dishes, triton-X100, trypan blue and tween-20 were obtained from Sigma,USA. Anti-Cytochrome c antibody, aprotinin, calphostin, leupeptin, pepstatin phorbol myristate acetate (PMA) and tunicamycin were obtained from Calbiochem, USA. Ac-DEVD-AFC, a caspase-3 fluorogenic substrate, Ac-DEVD-CHO a caspase inhibitor, z-VAD-fmk, a pan caspase inhibitor were obtained from BD-Biosciences, USA. Grace's insect cell medium and fetal bovine serum were obtained from Gibco BRL. 0.45µM nitrocellulose membrane, acrylamide, bis-acrylamide, glutathione sepharose 4B, BL21 cells were purchased from Amersham Pharmacia. 0.22 and 0.45 µM filter discs were purchased from Millipore. Whatmann 1 and 3mm filter papers were obtained from Whatmann, UK. Phosphospecific anti-eIF2 α antibody was obtained from Cell Signalling Technologies, USA. Anti-PERK antibody was obtained from Santa Cruz, USA. Anti-mouse IgG, anti-rabbit IgG were obtained from Promega, Inc, USA. Tris-HCl, glycine and methanol were obtained from Qualigens, India.

2.2 Cell Culture: *Spodoptera frugiperda* (*Sf9*) cells were grown in TNM-FH medium supplemented with 10 % fetal bovine serum and 1 % antibiotics. Approximately 90% confluent cells were used for the experiments. Trypan blue exclusion test was carried out to access the viability of the cells. Trypan blue (0.4 %) was added to cell suspension in 50 µl at a final concentration of 0.04 % and counted in a hemocytometer.

2.3 Preparation of cell extracts: Cells were harvested and washed with phospho saline buffer at 4° C and lysed in lysis buffer containing 20 mM Tris-HCl, 1 mM MgCl₂, 1 mM DTT and protease inhibitors : 10 µg /ml of pepstatin, 10 µg/ml of aprotinin, 250 µM PMSF. Cells were incubated for 10 mins at 4° C and centrifuged at 12000 rpm for 10 mins. Supernatant was collected and stored at -70°C.

2.4 Preparation of nuclei and cell extracts devoid of nuclei: Approximately 80% confluent cells were harvested, washed with PBS at 4°C and suspended in buffer A (50

mM Hepes-KOH pH 7.9, 10 mM KCl, 0.2 mM EDTA pH-8, 1 mM DTT, 1 µg/ml pepstatin, 1 µg/ml leupeptin, 1 mM PMSF) and incubated on ice for 15 min. After incubation 25 µl of 10% NP-40 was added, vortexed briefly and centrifuged at 1600 rpm for 3 mins. The supernatant was collected and centrifuged at 13000 rpm for 30 mins at 4°C and saved as cytoplasmic extract. The pellet was washed with lysis buffer once and centrifuged at 1000 xg for 3 mins. It is then resuspended in 500 µl of lysis buffer and layered on 30% sucrose buffer (50 mM Hepes-KOH pH 7.9, 10 mM KCl, 1 mM EDTA pH-8, 1 mM DTT, 30% sucrose, 0.5 mM PMSF) and spun at 3000 rpm for 10 mins. The pellet rich in nuclei was resuspended in buffer A containing 30% glycerol and used immediately or stored at -70°C for further use.

2.5 Induction of Apoptosis: 2×10^6 cells were seeded in each 35 mm dish and used for the experiments. Cells were treated with apoptosis inducing agents and incubated at 27° C for indicated time points as mentioned in the legend to figures. Cells pretreated with z-VAD-fmk, a caspase inhibitor, or PMA, an activator of PKC and Calphostin, an inhibitor of PKC for 1 hr at 27° C were also used to determine the inhibition or activation of PKC on stress-induced cell survival and cell death. In the case of baculovirus infected cells, *Sf9* cells were infected with wild type Ac-MNPV baculovirus at the rate of 10 MOI for 36 hrs, checked for baculovirus infection characteristic features such as enlargement of cells, contact inhibition and appearance of polyhedra. After 36 hrs of infection, cells were treated with various agents for 10 hrs at 27° C to determine the cell viability.

2.6 Assays for Apoptosis:

i) Trypan blue exclusion test: Cells were scored for apoptosis by monitoring plasma membrane blebbing by observing under phase contrast microscope and trypan blue exclusion test. A 45 µl of cell suspension was stained with 0.04% trypan blue and viewed under a VWR AE31 inverted microscope equipped with a motic digital camera and the software (20 x magnifications). Live cells exclude the dye whereas as the dead cells take up the stain and appear blue.

ii) Caspase activity: Caspase activity which is a key characteristic feature in apoptotic cells was analyzed by the hydrolysis of specific caspase 3 substrate, Ac-DEVD-AFC hydrolysis. Active caspases cleave the substrate between V-AFC, releasing fluorogenic AFC. Approximately 100 μg of protein was taken in 20 μl of lysis buffer and diluted to 475 μl of buffer containing 20 mM HEPES-KOH (pH-7.5), 10 mM DTT, 1 mM EDTA and 10% sucrose for each reaction. The final concentration of Ac-DEVD-AFC was 10.9 μM . Ac-DEVD-AFC hydrolysis was monitored by fluorescence emission of the released AFC (excitation-400 nm; emission-500 nm) using Fluoromax Spectrofluorimeter. Caspase activity is monitored in cell extracts prepared from cells treated with various agents such as UV-irradiation, cycloheximide and tunicamycin. To determine the importance of caspase activation mediated eIF2 α phosphorylation, cell extracts prepared from healthy cells have been treated with 50 μM cytochrome c and 1.0 mM ATP to stimulate the endogenous caspase activity of the extracts. Such cell extracts were also treated with agents such as PMA and calphostin to determine the importance of PKC activation or inactivation on cytochrome c- mediated caspase activation in cell extracts.

iii) Hoechst Staining: The integrity of nuclei of *Sf9* cells treated with various agents was checked by staining them with DNA binding stain-Hoechst (excitation-346 nm, emission-460 nm). 2.5 μM of dye was added to 40 μl of nuclear extract and incubated in dark for 10 mins and viewed under a Leica TCS SP2 AOBS laser scanning confocal microscope equipped with Leica confocal software.

iv) DNA fragmentation: *Sf9* cells treated with various agents were analyzed for DNA fragmentation. In brief, the cells after treatment were harvested, washed with PBS and incubated in 400 μl lysis buffer (50mM Tris-HCl, pH-8, 10 mM EDTA, 0.2% SDS and 0.5 $\mu\text{g/ml}$ proteinase K) at 37⁰ C for overnight. After incubation, 40 μl of 3 M NaOAc, pH 8.0, and 900 μl of ice cold absolute ethanol were added, mixed properly and centrifuged at 16,000xg for 20 mins at 4⁰C. The DNA pellet was air dried, and again incubated in 20 μl of TE buffer containing 0.2 mg /ml RNase A at 37⁰ C for 30 mins. The samples were run on 2% agarose gel and run at 60V for 2 hrs after the addition of 4 μl of 6X DNA loading buffer.

v) FACS Analysis: FACS analysis was performed to quantify the percentage of apoptotic cells. After treatment the cells with various agents were harvested by centrifugation at 1500rpm for 5mins at 4⁰C, washed with PBS, suspended in the residual PBS and fixed with 500 µl of 70% ethanol. The fixed cells were incubated overnight at 4⁰ C. The cells were then washed in PBS, suspended in PBS containing 50 µg/ml propidium iodide, 1% triton X-100 and 50 µg/ml RNase A and incubated in dark for 1 hr at 37⁰ C. After incubation, cells were centrifuged, washed again with PBS, resuspended in sheath fluid and analyzed by BD Biosciences flow cytometer.

2.7 Sodium Dodecyl Sulphate-polyacrylamide gel electrophoresis: Modified Laemmli method was followed to separate the proteins on 10% SDS-PAGE. The separation gel mix, 8 ml contained the following: 1.875 ml of 1.5 M Tris-HCl pH 8.8, 2.5 ml of 30:0.8 acrylamide: Bis-acrylamide, 75 µl of 10% SDS, 50 µl of ammonium per Sulphate, 8 µl of TEMED and 3.75 ml of water. The 5% stacking gel mix in a total volume of 2.5 ml contained: 1.875 ml of water, 0.375 ml of 0.5 M Tris-HCl pH 6.8, 0.375 ml of 30:0.8 acrylamide: Bis-acrylamide, 25 µl of 10% SDS, 50 µl of ammonium per Sulphate, 8 µl of TEMED. Proteins were prepared in a sample buffer containing 0.25 M Tris-HCl pH 6.8, 10% SDS, 40% glycerol, β-mercaptoethanol and bromophenol blue. Vertical slab gel electrophoresis was carried out at 120 volts with Tris-SDS-glycine buffer (0.3% Tris, 1.5% glycine, 0.1% SDS) until the dye front ran into the lower buffer.

2.8 Western Blotting: The proteins separated on SDS-PAGE were transferred on to nitrocellulose membrane electrophoretically at 70 Volts. The transfer was done for 2 hrs in a transfer buffer (25 mM Tris, 19.5 mM glycine in 20% methanol). After the transfer, membrane was stained with Ponceau S red solution and molecular proteins were marked. The membrane was destained with TBS (10 mM Tris-HCl pH 8.0, 150 mM NaCl) and soaked in blocking solution (5% milk powder in 10 ml TBS). After 1hr blocking, the membrane was probed with primary antibody diluted in TBS for 10-12 hrs at 4⁰ C. The membrane was washed thrice with TBS-T (0.05% tween 20). The membrane was then incubated with secondary anti-IgG-AP conjugate for 1hr. The membrane was again washed thrice with TBS-T. The membrane was developed with 10 ml of AP buffer (100 mM Tris-HCl pH 9.5, 100 mM NaCl, 5 mM MgCl₂) containing 66 µl of NBT (one tablet of 25 mg

dissolved in one ml of water) and 33 μ l of BCIP (one tablet dissolved in one ml of dimethyl formamide). The color development was arrested by washing the membrane with distilled water.

2.9 Immunodepletion: For cytochrome c experiments; healthy *Sf9* extracts were first immunodepleted with anti-cytochrome c antibody to deplete the endogenous cytochrome c present in the extracts. Cell extracts were incubated with anti-cytochrome c antibody for overnight at 4^o C, later 50 μ l of sepharose beads were added to the extracts and incubated for another 4 hrs at 4^o C. The beads were then collected by centrifuging at 4000 rpm. The extract depleted of cytochrome c was collected and used for further experiments.

2.10 PERK Cleavage assay: Cleavage of recombinant purified mouse PERK was monitored in *Sf9* cell extracts containing active caspase to determine a) the ability of caspases to process recombinant PERK and b) the intensity of caspase activation. PERK cleavage was carried out in a cleavage buffer containing 10 mM Tris-HCl, pH 7.5, 2.5 mM KH₂PO₄, 2 mM NaCl, 68 mM sucrose and 220 mM mannitol at 37^o C for four hours. After incubation, reactions were stopped by the addition of 4X sample buffer, boiled, electrophoresed on 10% SDS-PAGE. The processing of PERK was analyzed using stained gels or by western blots.

2.11 Protein Estimation: The concentration of proteins in the extracts was quantified by biorad Bradford reagent as described by manufacturer.

Chapter I

Chapter I

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Stress-induced Cell Death or Cell Survival: Effects of PMA and Calphostin.

3.1 Abstract:

To determine the importance of PKC activation on cell survival and cell death, the ovarian cells of *Spodoptera frugiperda* (*Sf9*), which are natural hosts of baculovirus are treated with phorbol myristate acetate (**PMA**), an activator of PKC and calphostin (**cal**), an inhibitor of PKC in the presence of diverse stressors such as UV-irradiation (**UV**), a DNA-damaging agent, cycloheximide (**CH**), an inhibitor of protein synthesis elongation and tunicamycin (**Tn**), an inhibitor of N-linked glycosylation. Further, we have also studied the effects of PMA and calphostin in baculovirus-infected cells which are subjected to UV-irradiation, cycloheximide and tunicamycin. Cell death and survival are studied here by Hoechst staining, FACS analysis, caspase activation by Ac-DEVD-AFC hydrolysis and DNA fragmentation. Our observations suggest that PMA and calphostin do not promote cell death. However, PMA enhances and calphostin mitigates partly the cell death induced by UV-irradiation and 1.0 mM cycloheximide. Low concentrations of cycloheximide (0.25 mM) do not stimulate apoptosis. However, addition of PMA promotes apoptosis in cells treated with low concentrations of cycloheximide. In contrast, PMA and tunicamycin, treated cells do not display apoptosis. Wt baculovirus infection or addition of **z-VAD-fmk** (**Z**), a synthetic pan caspase inhibitor abrogates the UV + PMA and also CH + PMA induced cell death.

3.2 Results:

PMA enhances and calphostin mitigates stress-induced cell death, DNA fragmentation and caspase activation.

Cell Morphology and DNA fragmentation: An analysis of *Sf9* cells for cell death or survival was studied by inverted microscopy, Hoechst staining (**Fig. 5**), flow cytometry (**Fig. 6**), and also by DNA fragmentation (**Fig. 7**). *Sf9* cells treated with a 60 sec UV-irradiation (200 J/m²) and incubated for 10 hrs at 27°C, or cells treated with 1.0 mM Cycloheximide undergo apoptosis. Analysis of FACS data in **Fig. 6A** reveals that UV-irradiation and cycloheximide showed 55-58% or 58-60% of apoptosis respectively (**Fig. 6B, bar # 6 and 24 vs 1**). However, treatment in the presence of 12 µM tunicamycin, an inhibitor of N- linked glycosylation and a known stressor of the endoplasmic reticulum

(ER), for 10 hrs did not promote significant amount of apoptosis (**bar # 33 vs 1**). Prior treatment for 1hr with 50 μ M z-VAD-fmk abrogates both the UV and cycloheximide-induced apoptosis (**bar # 7 and 25 vs 6 and 24**). Wt baculovirus infected cells, at 36 hrs of infection, also resisted UV or cycloheximide (CH)-induced apoptosis (**Bar # 8 and 26 vs 6 and 24**).

Low concentrations of 50 nM PMA, an activator of PKC, or calphostin, an inhibitor of PKC did not induce significant changes in cell morphology and apoptosis (**bar # 4 and 5**). PMA enhanced further both UV-irradiation and cycloheximide-induced apoptosis from 58% to 80% and 60% to 83% respectively (**bar # 9 and 27 vs 6 and 24**). In contrast, addition of 50 nM of calphostin, a known inhibitor of PKC, decreased UV and cycloheximide-induced apoptosis (**bar # 12 and 30 vs 6 and 24**). However, unlike z-VAD-fmk, calphostin inhibits only PKC-mediated apoptosis in UV and cycloheximide-treated cells and PKC activation occurs not prior to but presumably after caspase activation. Cells undergoing apoptosis display typically enhanced DNA fragmentation (**Fig.7**).

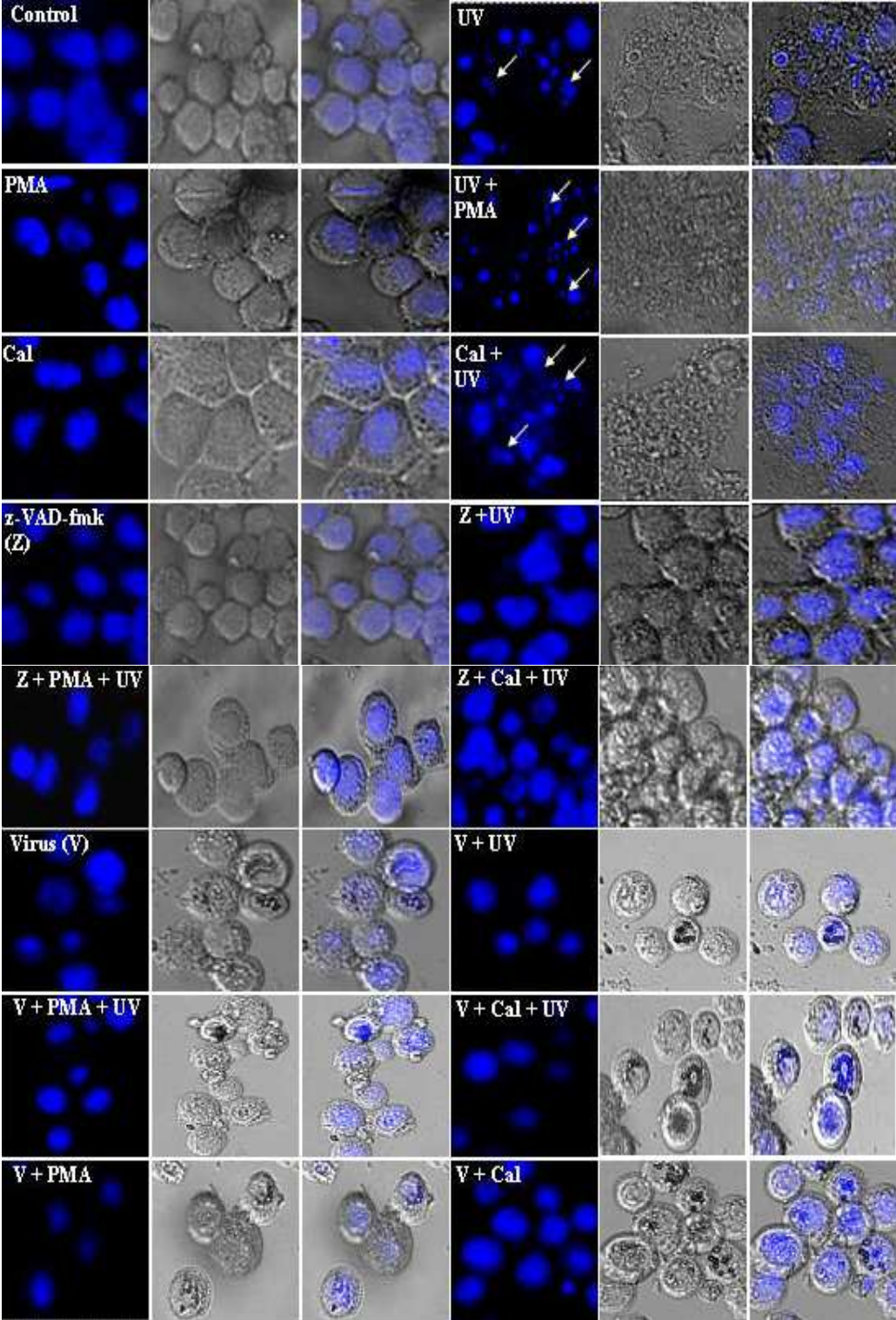
Caspase Activity:

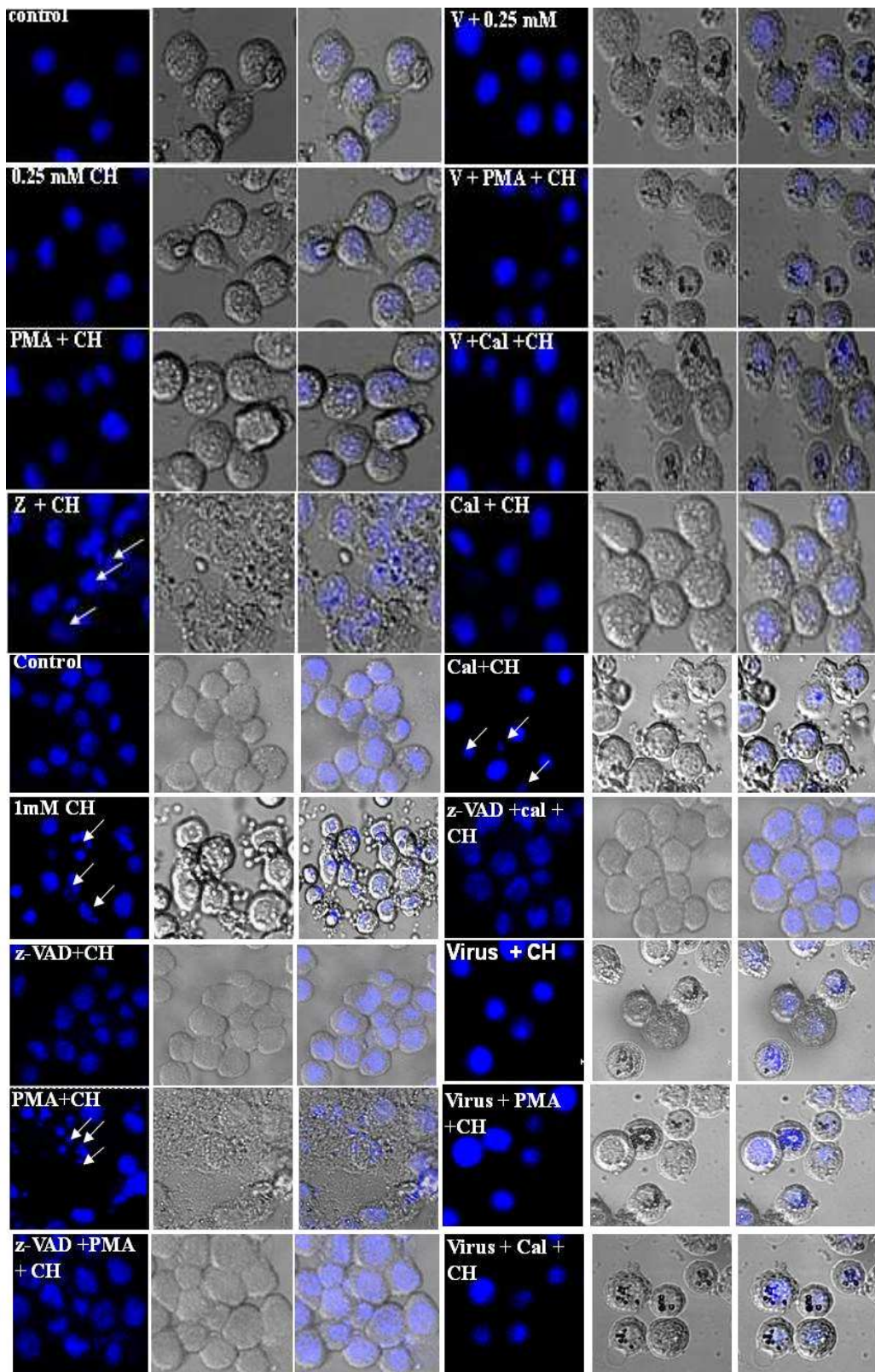
Analysis of caspase activity by AcDEVD-AFC hydrolysis reveals that UV-irradiated and 1.0 mM cycloheximide-treated cells display higher levels of caspase activity coinciding with their cell death (**Fig. 8A**). PMA, calphostin, tunicamycin, z-VAD-fmk treatments do not result in caspase activation. However PMA enhances UV-irradiation induced or cycloheximide-induced caspase activation. Addition of z-VAD-fmk, a caspase inhibitor, mitigates UV-irradiation and cycloheximide induced caspase activation. Low concentrations (0.25 mM) of cycloheximide that do not elicit apoptosis also fail to stimulate caspase activation. However, PMA stimulates caspase activation of *Sf9* cells treated with 0.25 mM cycloheximide which is proportional to the cell death observed under those conditions (**Fig. 5 and 6**). Cells treated with tunicamycin alone or with tunicamycin and PMA do not display any caspase activation (**Fig. 8A**) or cell death (**Fig. 5**). UV-irradiation or cycloheximide induced caspase activation is mitigated in wt baculovirus infected cells (**Fig. 5B**) presumably because of the expression of P35 and PK2 proteins as has been suggested previously.

Fig. 5 Apoptosis in *Sf9* cells: Hoechst Staining.

Sf9 cells uninfected or infected with wt baculovirus were treated with the following agents: UV-irradiation 200 (J/m²) for 60 secs, 0.25 mM or 1.0 mM cycloheximide, and 12 μM tunicamycin and incubated at 27⁰ C for 10 hrs. To determine the importance of caspase(s) or PKC activation in the stressed cells, the latter were treated with 50μM z-VAD-fmk, 50 nM of PMA or calphostin prior to exposing the cells to different treatments. Cells were incubated at 27⁰C for 10 hrs and then stained with 2.5 μM Hoechst dye and viewed under Leica TCS SP2 AOBS laser scanning confocal microscope equipped with Leica confocal as mentioned in 'Materials and Methods'.

Fig. 5





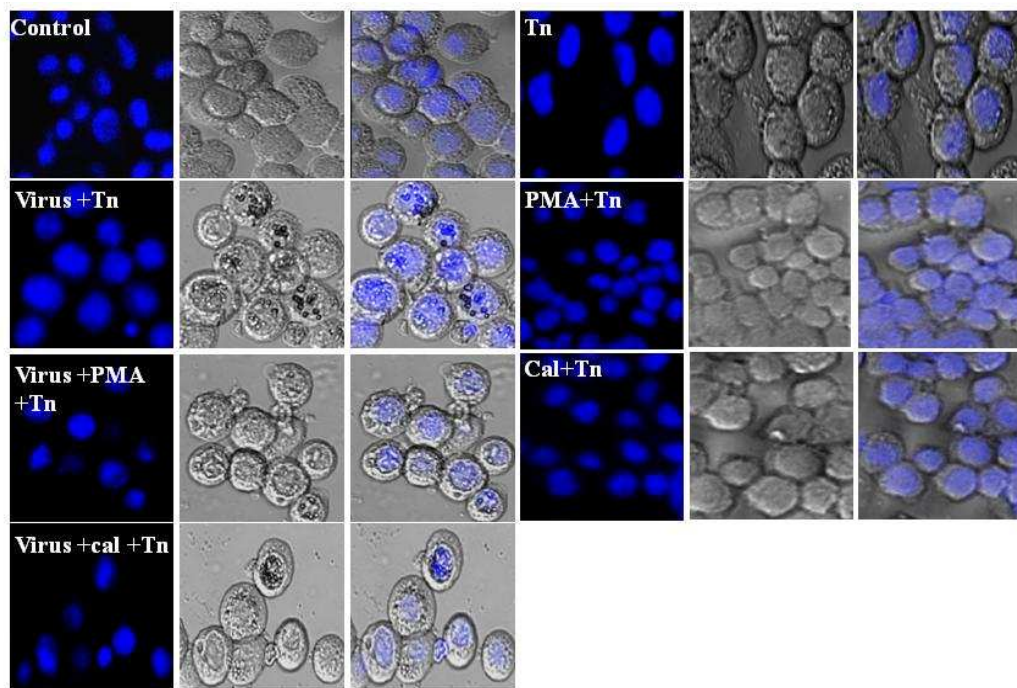
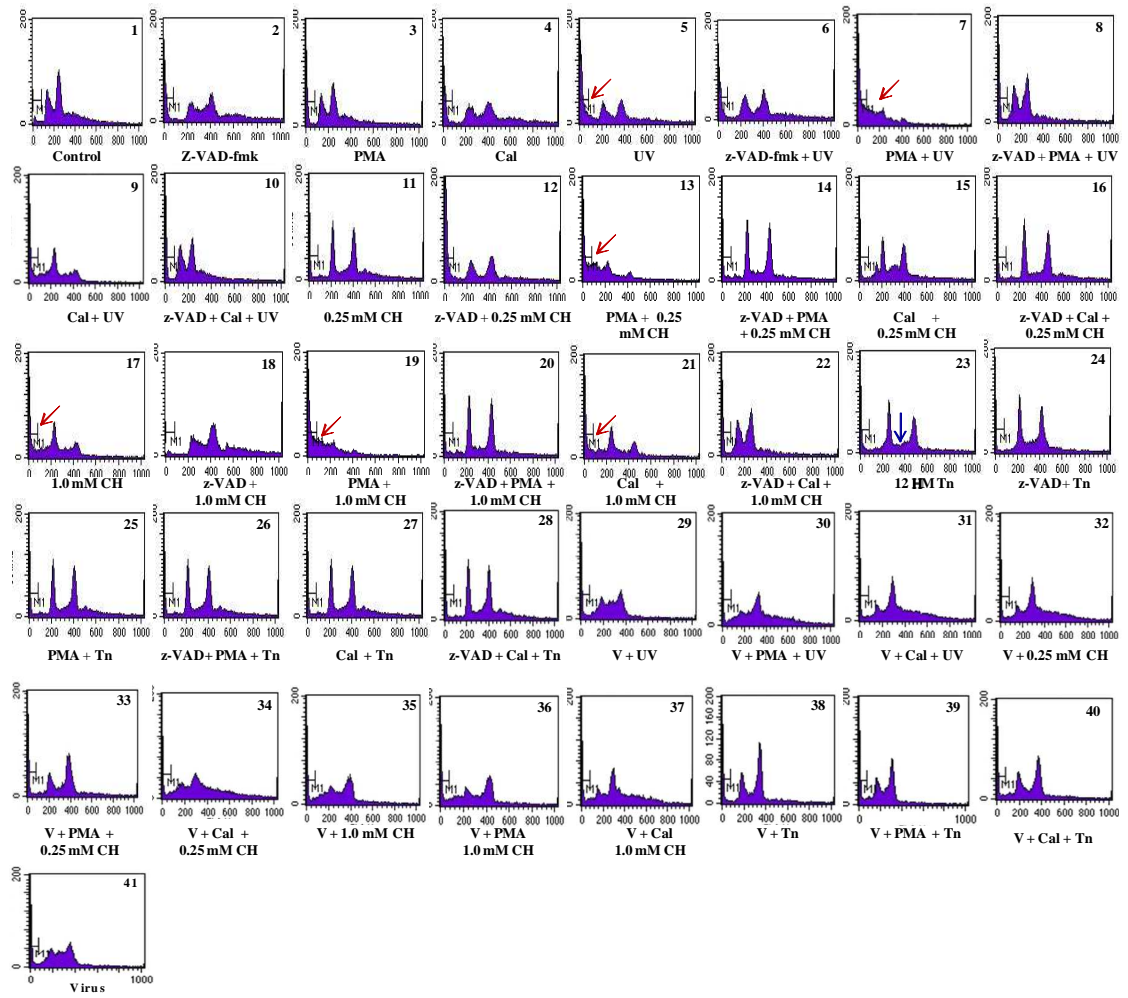


Fig. 6 Flow cytometric analysis of *Sf9* cells.

Uninfected and baculovirus infected *Sf9* cells were treated with UV-irradiation (200 J/m²) at 312 nm for 60 secs, 0.25 mM cycloheximide, 1.0 mM cycloheximide and or 12 μM tunicamycin with and without z-VAD-fmk, PMA, calphostin and incubated at 27⁰ C for 10 hrs. After the incubation, the cells were harvested, processed, stained with propidium iodide as mentioned in 'Materials and Methods' to analyze the cells for sub G0-G1 stage of cell cycle by flow cytometer. Panel A represents the graphical representation of cells in different phases. Panel B is a bar diagram and represents the percentage of cells in sub G0-G1 or apoptotic phase.

Panel. A



Panel. B

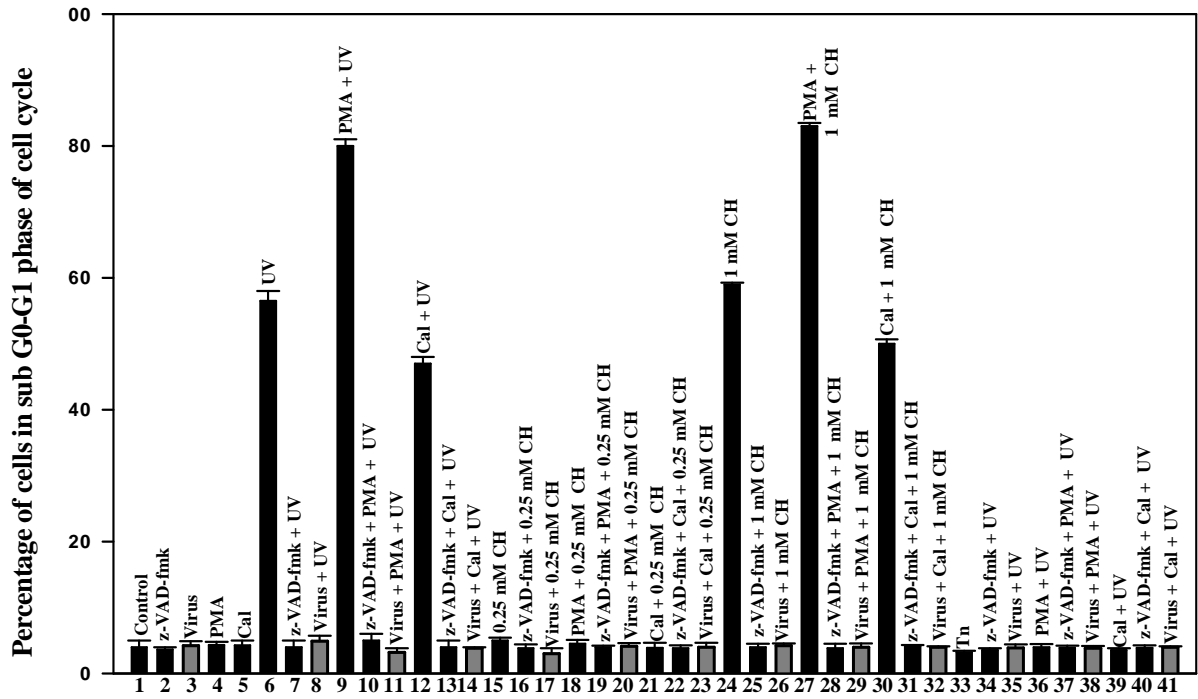


Fig. 7 DNA Fragmentation.

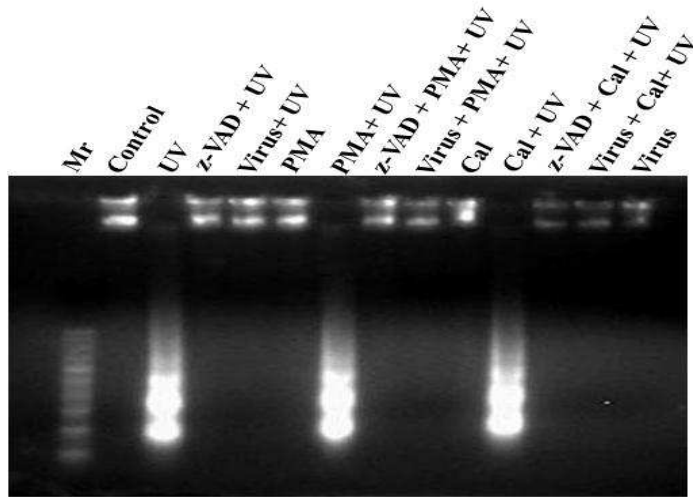
The DNA was extracted from infected and uninfected *Sf9* cells, irradiated with UV-irradiation, or treated with 0.25 mM cycloheximide, 1.0 mM cycloheximide or 12 μ M tunicamycin in the presence and absence of z-VAD-fmk, PMA and calphostin as mentioned in 'Materials and Methods'. The extracted DNA was run on 2% agarose gel to check for fragmentation of DNA.

Panel A: Represents fragmentation of DNA extracted from uninfected and baculovirus-infected *Sf9* cells treated with UV-irradiation in the presence of various agents.

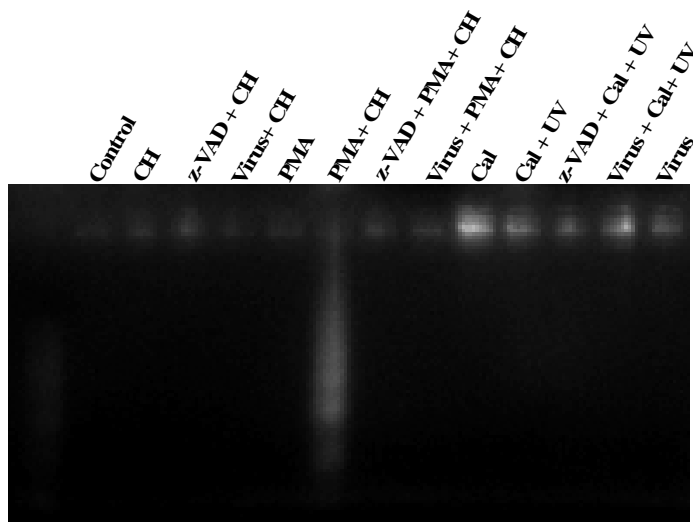
Panel B: The gel represents the fragmentation of DNA in *Sf9* cells treated with 0.25 mM cycloheximide in the presence and absence of baculovirus, 50 μ M z-VAD-fmk, 50 nM PMA or calphostin.

Panel C: Represents the fragmentation of DNA extracted in *Sf9* cells treated with 1.0 mM cycloheximide in the presence and absence of baculovirus, 50 μ M z-VAD-fmk, 50 nM PMA and 50 nM calphostin.

Panel. A



Panel. B



Panel. C

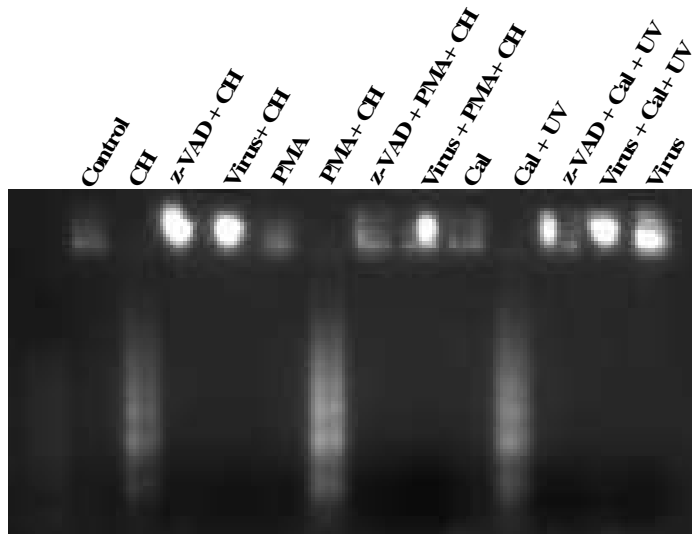


Fig. 8 Caspase activity in *Sf9* cells.

Caspase activity was studied in *Sf9* cells that were treated with UV-irradiation (200 j/m²), 0.25 mM cycloheximide, 1.0 mM cycloheximide, or 12 μM tunicamycin in the presence and absence of 50 μM z-VAD-fmk, 50 nM PMA and 50 nM calphostin. Caspase activity of the extracts was measured by using Ac-DEVD-AFC hydrolysis as described in 'Materials and Methods'.

Panel A: The bar diagram represents caspase activity in uninfected *Sf9* cells treated with various agents.

Panel B: The graph represents the caspase activity in Ac-MNP Virus infected and uninfected *Sf9* cells treated with various agents.

Chapter II

Chapter II

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Stress induced phosphorylation of eIF2 α in *Sf9* cells: Effect of PMA and calphostin.

4.1 Abstract:

Analyses of phosphorylation of conserved ser⁵¹ residue in the α -subunit of eukaryotic initiation factor 2 (eIF2 α) reveals that PMA and calphostin do not effect eIF2 α phosphorylation. However, PMA stimulates and calphostin mitigates partly UV-irradiation and also cycloheximide-induced eIF2 α phosphorylation in *Sf9* cells. A time course analyses of eIF2 α phosphorylation suggests that PMA enhances the late stages of UV-irradiation or cycloheximide induced phosphorylation but not initial stages. Late stages of eIF2 α phosphorylation are however mitigated by pancaspase inhibitor like z-VAD-fmk much more efficiently than calphostin, a PKC inhibitor. These findings therefore suggest that eIF2 α phosphorylation is a cause and consequence of caspase activation and calphostin mitigates partly caspase-mediated phosphorylation of eIF2 α that is occurring due to PKC-mediated caspase activation. These findings are consistent with the idea that PMA, calphostin and z-VAD-fmk do not effect phosphorylation of eIF2 α in tunicamycin-treated cells that do not display any caspase activation or cell death. PMA or calphostin also do not effect eIF2 α phosphorylation in baculovirus-infected *Sf9* cells. However, stress-induced eIF2 α phosphorylation is reduced in the late hrs of baculovirus infected cells both in cells treated with proapoptotic agents such as UV and cycloheximide and non apoptotic agents like tunicamycin.

4.2 Results:

PMA enhances and calphostin mitigates UV-irradiation or cycloheximide-induced eIF2 α phosphorylation:

Phosphorylation of eIF2 α is primarily a stress signal and can evoke adaptive and apoptotic responses (Hussain and Ramaiah 2007b). It can be a cause and consequence of caspase activation. Earlier, we observed that eIF2 α phosphorylation occurs prior to caspase activation in UV-irradiated cells (Aparna et al, 2003). Addition of z-VAD-fmk or infection by wt baculovirus that harbors an anti apoptotic gene like p35, mitigates the late stages of UV or cycloheximide induced eIF2 α phosphorylation effectively (Aparna et al, 2003) suggesting that caspase activation can lead to eIF2 α phosphorylation. To determine the

importance of PKC and caspase activation on eIF2 α phosphorylation, *Sf9* cells, infected or uninfected by baculovirus, were treated here with UV-irradiation (for 60 seconds), cycloheximide (0.25 mM or 1.0 mM) and 12 μ M tunicamycin in the presence and absence of pharmacological activators or inhibitors like PMA, calphostin and z-VAD-fmk.

Analyses of phosphorylation of eIF2 α in uninfected *Sf9* cells irradiated with UV-irradiation (200 j/m²) and incubated for 10 hrs (**Fig. 9**), or treated with 0.25 mM (**Fig. 10**), or 1.0 mM cycloheximide (**Fig. 11**), or with 12 μ M–tunicamycin (**Fig. 12**) for 10 hrs in the presence and absence of PMA or calphostin reveals that PMA enhances and calphostin reduces partly UV-irradiation or cycloheximide, but not tunicamycin-induced eIF2 α phosphorylation. A pan caspase inhibitor, z-VAD-fmk mitigates only UV and cycloheximide, but not tunicamycin-induced eIF2 α phosphorylation (**Figs 9-11 vs 12**). However, unlike calphostin, z-VAD-fmk inhibits UV and cycloheximide-induced eIF2 α phosphorylation much more efficiently (**Figs. 9-11; lanes 8 vs 4**). The concentrations of PMA and calphostin chosen here however do not effect eIF2 α phosphorylation (**Figs. 9-11, lanes 5 and 9**).

Stress induced eIF2 α phosphorylation is reduced in *Sf9* cells at 36 hrs of baculovirus infection.

Baculovirus-infected cells, at 36 hrs of infection display reduced eIF2 α phosphorylation compared to uninfected cells (**Fig 13-16, lane 2**) which is consistent with our earlier observations (Aparna et al, 2003) that were carried out with cells after 15 hrs of baculovirus infection. In the previous studies, UV and cycloheximide-induced eIF2 α phosphorylation but not tunicamycin-induced eIF2 α phosphorylation was effectively reduced by baculovirus infection (at 15 hrs) or by z-VAD-fmk treatment. However in the present experiments, it was observed that UV, cycloheximide and also tunicamycin induced eIF2 α phosphorylation was reduced in 36 hrs of baculovirus- infected *Sf9* cells. The reduction in eIF2 α phosphorylation in baculovirus infected cells may be occurring by caspase dependent or independent mechanisms through the expression of p35 and PK2-like proteins. While p35 reduces the caspase-mediated eIF2 α kinase activation, PK2 inhibits directly eIF2 α kinase activation and thereby eIF2 α phosphorylation (Aparna et al, 2003; Dever et al, 1999). Since PK2 is a late baculovirus protein unlike p35, the

reduction in eIF2 α phosphorylation observed in 36 hrs infected control cells or tunicamycin- treated cells may be due to the expression of PK2-like protein. In contrast, the reduction in eIF2 α phosphorylation in UV-irradiated or cycloheximide- treated cells appears to be due to the expression of p35, a pan caspase inhibitor protein. This is consistent with the idea that addition of z-VAD-fmk, a synthetic caspase inhibitor that mimics like P35, cannot affect eIF2 α phosphorylation in uninfected *Sf9* cells as shown in **lane 2 of Figs 9-12**. UV-irradiation or cycloheximide-induced phosphorylation of eIF2 α is mostly unaffected by PMA or calphostin in baculovirus infected cells as baculovirus infection mitigates UV-irradiation induced caspase activation (**Fig. 8, Panel B**), cell death (**Fig. 5**) and eIF2 α phosphorylation (**Figs. 13-15**).

PMA enhanced eIF2 α phosphorylation occurs through caspase activation.

Analysis of eIF2 α phosphorylation during early (1- 5 hrs) and late stages (at 10 hrs) of UV-irradiation indicates that the phosphorylation of eIF2 α is enhanced approximately to 45% within 1 hr (**Figs. 17, lane 3**) and to 75% at 5 hrs (**Fig. 17, lane 4**). At 5 hrs, but not during the first hr, PMA enhances by about 20% (**Fig. 17; lane, 12 vs 4**) and calphostin reduces by 20% of UV-irradiation-induced eIF2 α phosphorylation (**lane 16 vs 4**). At 10 hrs (**Fig. 9**), the increase in UV-irradiation induced eIF2 α phosphorylation by PMA (**Fig 9, lane 6 vs 3**) or its decline by calphostin (**lane 8 vs 3**) is approximately 30% and 20% respectively suggesting that PMA enhances more efficiently the late stages of UV-induced phosphorylation. A similar effect was also observed in cells treated with 1.0 mM cycloheximide (**Fig. 18**). These findings therefore suggest that the late stages of UV-irradiation or cycloheximide-induced eIF2 α phosphorylation is partly contributed by PKC activation as PMA enhances and calphostin decreases part of the phosphorylation in UV-irradiated or cycloheximide-treated cells. In contrast, addition of z-VAD-fmk, a caspase inhibitor, reduces UV-irradiation induced eIF2 α phosphorylation much more efficiently than calphostin at 10 hr time point than at 5 hrs and is unaffected during the first hour. Hence, PMA-stimulated eIF2 α phosphorylation presumably occurs after caspase activation than prior to caspase activation. This suggestion is consistent with the finding that tunicamycin-induced eIF2 α phosphorylation that does not lead to cell death is unaffected by PMA or calphostin or by z-VAD-fmk even in the late stages (**Fig. 5**).

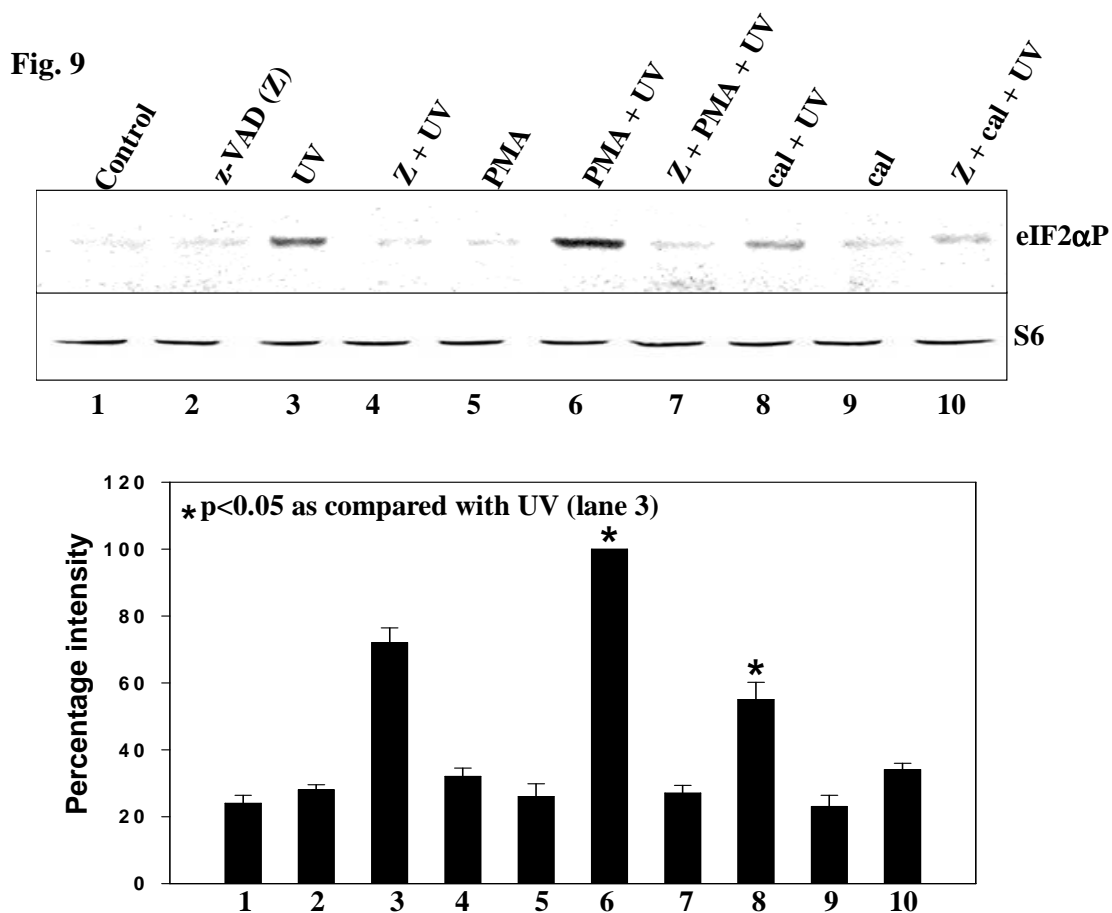


Fig. 9 UV irradiation-induced eIF2 α phosphorylation: Effect of PMA, calphostin and z-VAD-fmk.

Sf9 cells were treated with UV-irradiation for 60 sec (200 J/m²) and incubated at 27° C for 10 hrs. Cell extracts were prepared as described in ‘Materials and Methods’. Equal amount of protein (~ 100 μ g) was used from all samples and separated on 10% SDS-PAGE, transferred to a nitrocellulose membrane. Membranes were then probed by a phospho-specific anti- eIF2 α polyclonal antibody raised in rabbits. Levels of S6, a small ribosomal protein, were analyzed in samples using an anti-S6 polyclonal antibody as a loading control. The membranes were developed as described in ‘Materials and Methods’. The figure is a western blot. The blot in the upper panel represents UV-irradiation induced eIF2 α phosphorylation in *Sf9* cells and in the lower panel represents the levels of S6 protein. Blots were also quantified using Image J software from NIH and the results are shown in the bar diagram.

Fig. 10

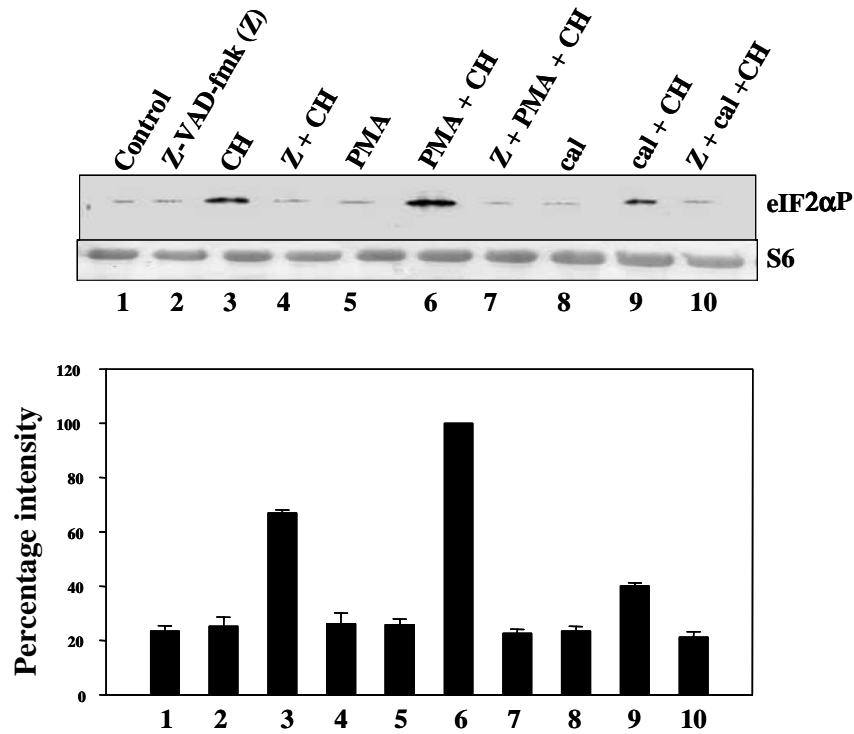


Fig. 10 0.25 mM cycloheximide-induced eIF2 α phosphorylation: Effect of PMA, calphostin and z-VAD-fmk.

Protein extracts were prepared from *Sf9* cells treated with cycloheximide for 10 hrs in the presence and absence of z-VAD-fmk, PMA and calphostin as mentioned in 'Materials and Methods'. Samples were analyzed and quantified for eIF2 α phosphorylation and S6 proteins as described in the legend to **Fig. 9**. The figure in the upper panel is a western blot representing eIF2 α phosphorylation and in the lower panel represents the loading control, S6. The bar diagram represents the quantification of eIF2 α phosphorylation.

Fig. 11

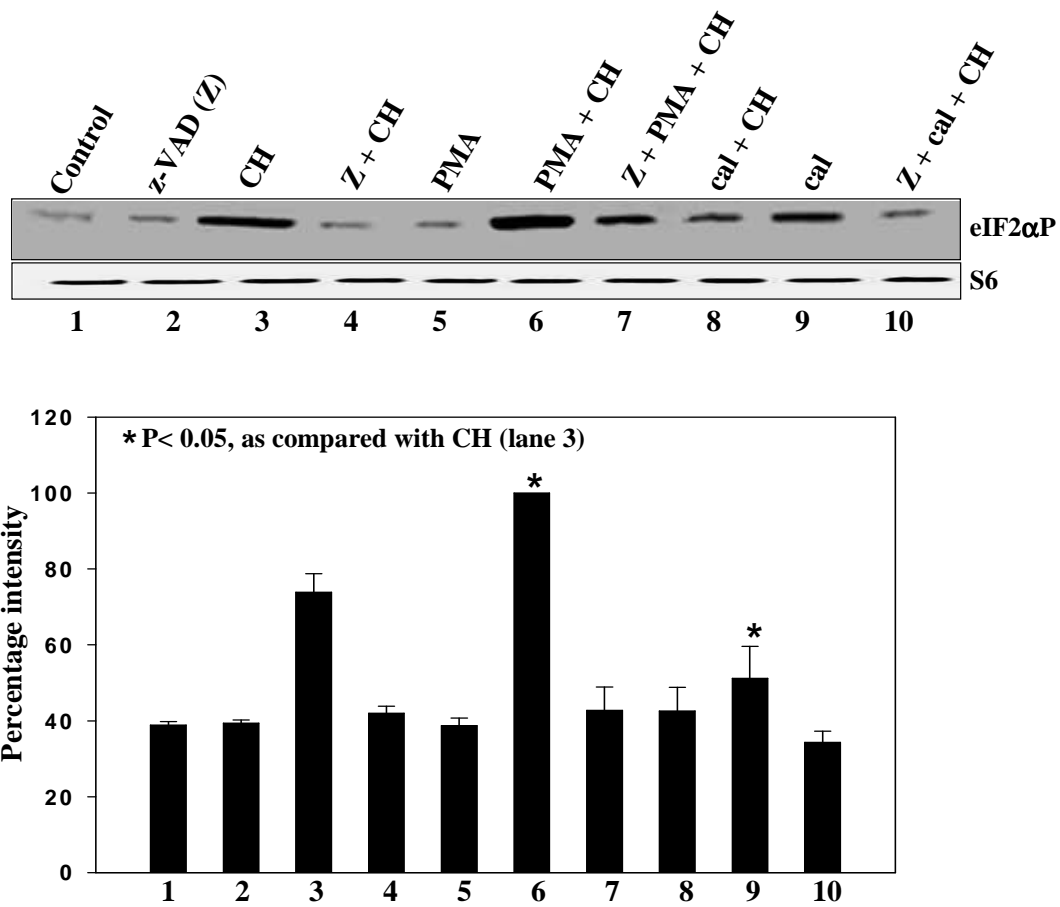


Fig. 11 1.0 mM cycloheximide-induced eIF2 α phosphorylation: Effect of PMA, calphostin and z-VAD-fmk.

Protein extracts were prepared from *Sf9* cells treated with cycloheximide for 10 hrs in the presence and absence of z-VAD-fmk, PMA and calphostin as mentioned in 'Materials and Methods'. Samples were analyzed and quantified for eIF2 α phosphorylation and S6 proteins as described in the legend to **Fig. 9**. The upper panel represents eIF2 α phosphorylation and the lower panel represents the loading control, S6. The bar diagram represents the quantification of eIF2 α phosphorylation.

Fig. 12

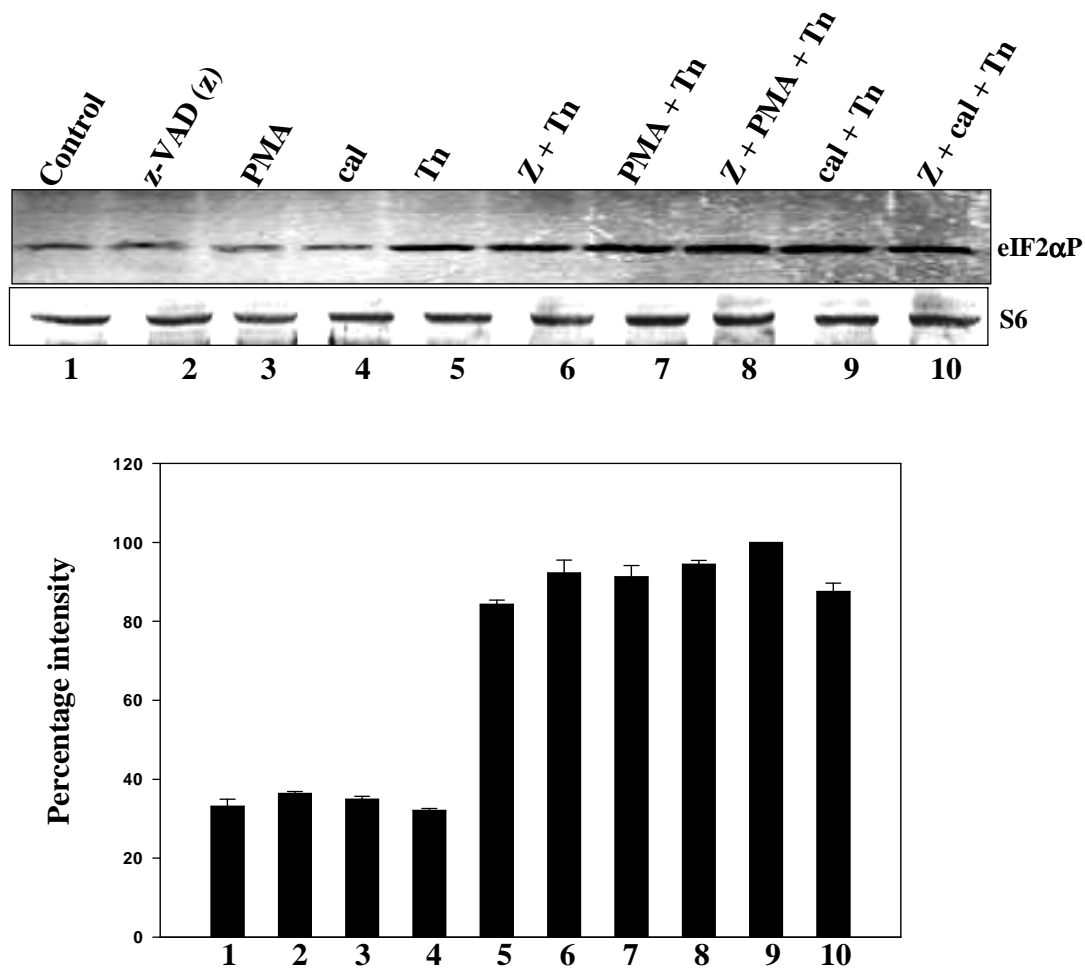


Fig. 12 Tunicamycin-induced eIF2 α phosphorylation: Effect of PMA, calphostin and z-VAD-fmk.

Protein extracts were prepared from *Sf9* cells treated with 12 μ M tunicamycin for 10 hrs in the presence and absence of z-VAD-fmk, PMA and calphostin as mentioned in ‘Materials and Methods’. Samples were analyzed and quantified for eIF2 α phosphorylation and S6 protein as described in the legend to **Fig. 9**. The upper panel in the figure is a western blot representing eIF2 α phosphorylation and the lower panel, S6. The quantification of phosphorylation of eIF2 α is shown in bar diagram.

Fig. 13

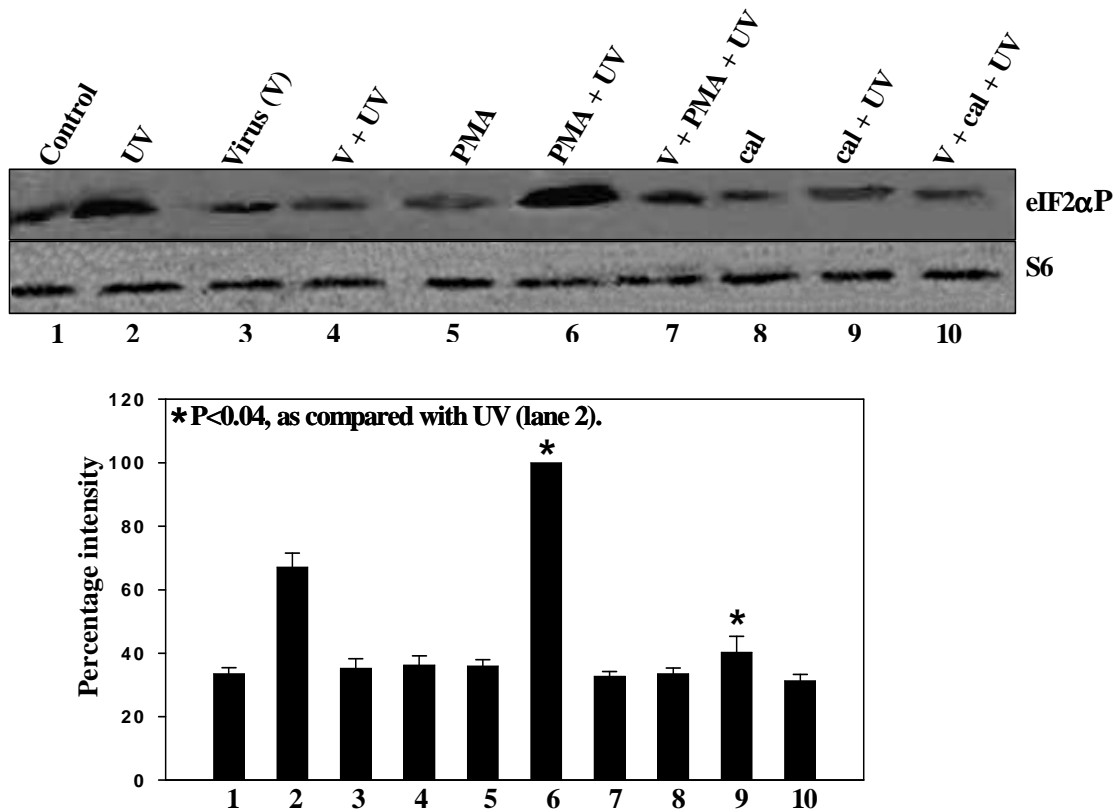


Fig. 13 UV-irradiation induced eIF2 α phosphorylation in baculovirus-infected cells: Effect of PMA and calphostin.

Sf9 cells were infected with wild type baculovirus for 36 hrs, treated with UV-irradiation for 60 sec (200 J/m^2) and were incubated at 27°C for 10 hrs. Protein samples prepared as mentioned in 'Materials and Methods' were analyzed and quantified for eIF2 α phosphorylation and S6 proteins as described in the legend to **Fig. 9**. The blot in the upper panel represents UV-irradiation induced eIF2 α phosphorylation in *Sf9* cells and in the lower panel represents the levels of S6 protein. Blots were quantified using Image J software from NIH and the results were shown in the bar diagram.

Fig. 14

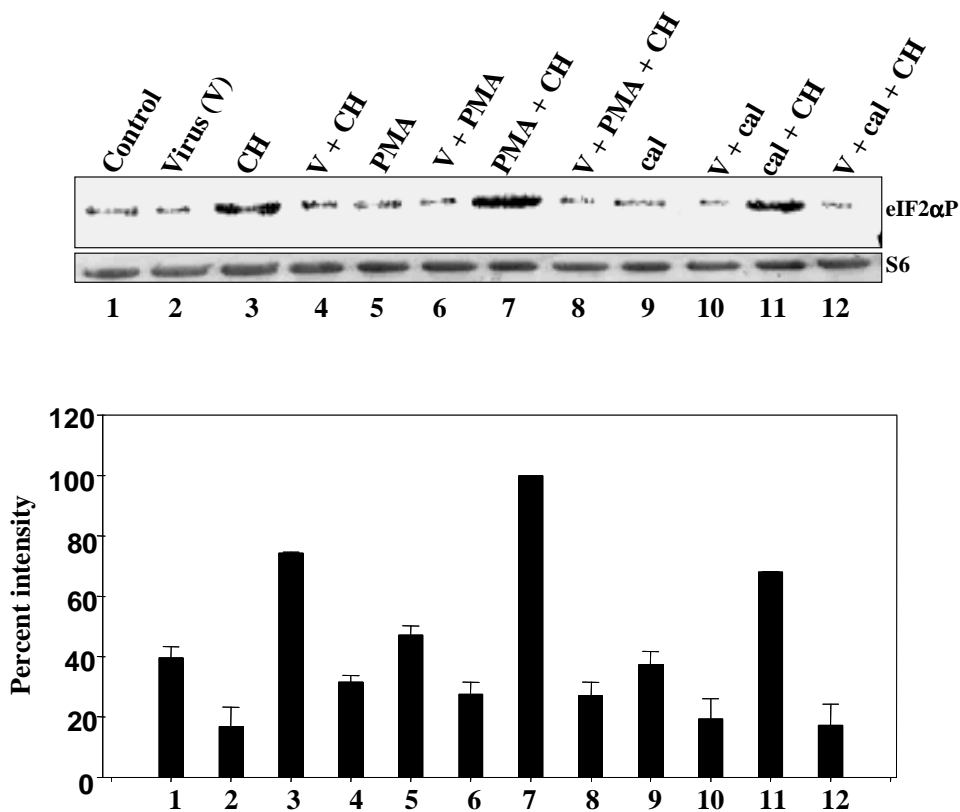


Fig. 14 0.25 mM cycloheximide-induced eIF2 α phosphorylation in baculovirus-infected cells: Effect of PMA and calphostin.

Baculovirus infected *Sf9* cells were treated with cycloheximide in the presence and absence of PMA and calphostin. Protein samples prepared as mentioned in ‘Materials and Methods’ were analyzed and quantified for eIF2 α phosphorylation and S6 proteins as described in the legend to **Fig. 9**. The figure in the upper panel is a western blot representing eIF2 α phosphorylation and in the lower panel represents the loading control, S6. The bar diagram represents the quantification of eIF2 α phosphorylation.

Fig. 15

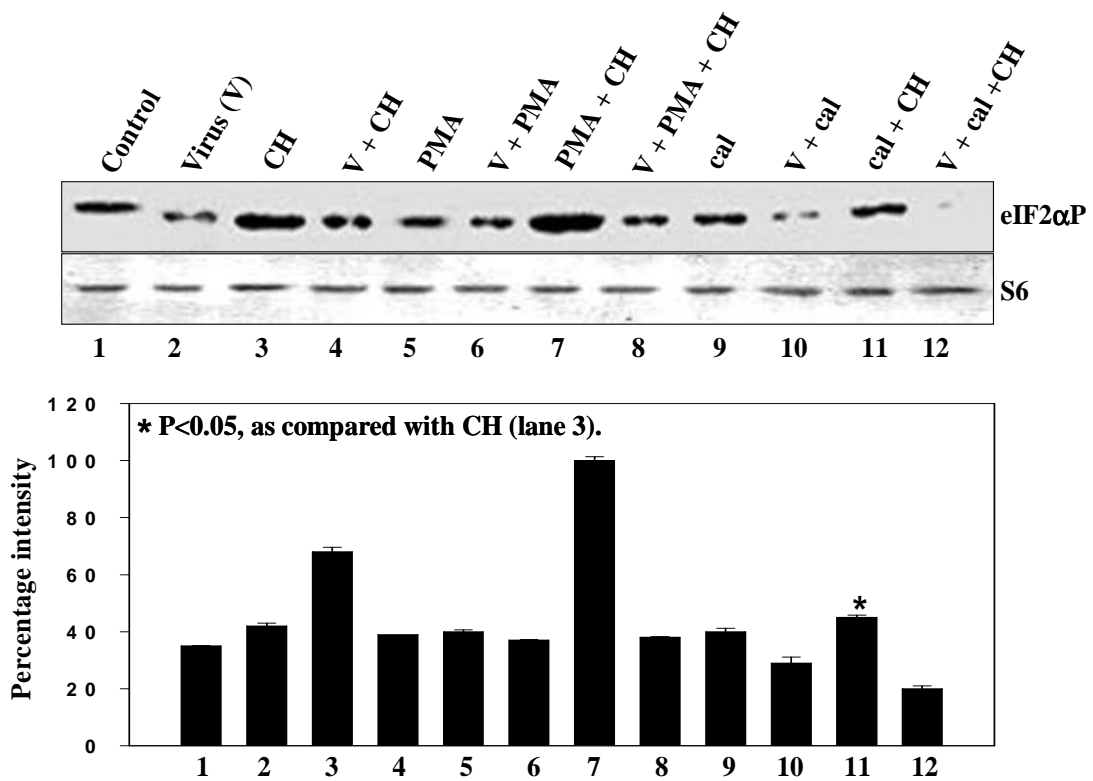


Fig. 15 1.0 mM cycloheximide-induced eIF2 α phosphorylation in baculovirus-infected cells: Effect of PMA and calphostin.

Sf9 cells infected with baculovirus for 36 hrs were treated with cycloheximide in the presence and absence of PMA and calphostin. Protein extracts were prepared as mentioned in 'Materials and Methods'. Samples were analyzed and quantified for eIF2 α phosphorylation and S6 proteins as described in the legend to **Fig. 9**. The upper panel represents eIF2 α phosphorylation and the lower one S6. The bar diagram represents the quantification of eIF2 α phosphorylation.

Fig. 16

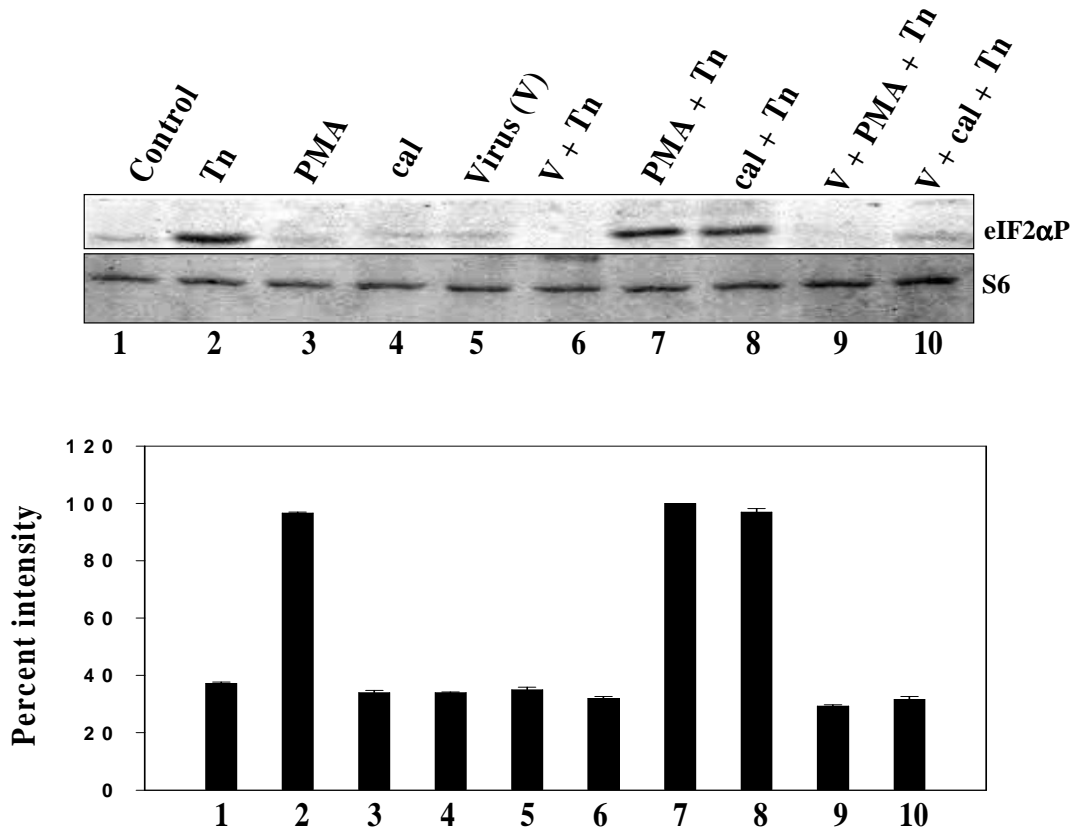


Fig. 16 Tunicamycin-induced eIF2 α phosphorylation in baculovirus-infected cells: Effect of PMA and calphostin.

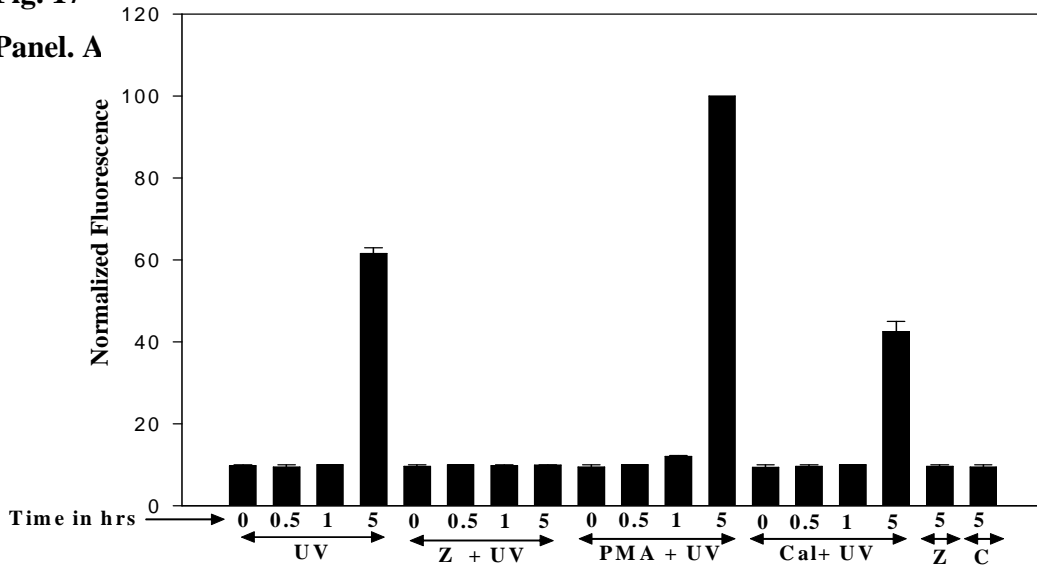
Protein extracts were prepared from *Sf9* cells infected with baculovirus and treated with 12 μ M tunicamycin in the presence and absence of PMA and calphostin as mentioned in 'Materials and Methods'. Samples were analyzed and quantified for eIF2 α phosphorylation and S6 protein as described in the legend to **Fig. 9**. The upper panel in the figure is a western blot representing eIF2 α phosphorylation and the lower panel represents the loading control, S6. The bar diagram represents the quantification of phosphorylation of eIF2 α .

Fig. 17 Time course (0-5 h) analysis of eIF2 α phosphorylation and caspase activity: Effect of PMA, calphostin and z-VAD-fmk on UV-irradiated cells.

Sf9 cells were treated with PMA, calphostin and z-VAD-fmk 30 mins prior to UV-irradiation. Cells were exposed to UV-irradiation (200 J/m²) for 60 sec and incubated with the above pharmacological agents at 27°C for different time periods: 0.5 h, 1 h, 5h. Cell extracts were prepared, analyzed for caspase activity and eIF2 α phosphorylation as described in the legend to Fig. 9. Panel A represents the caspase activity and panel B phosphorylation of eIF2 α and the corresponding levels of S6, loading control. The phosphorylation levels eIF2 α were quantified and the percent intensity of eIF2 α is represented in the bar diagram.

Fig. 17

Panel. A



Panel. B

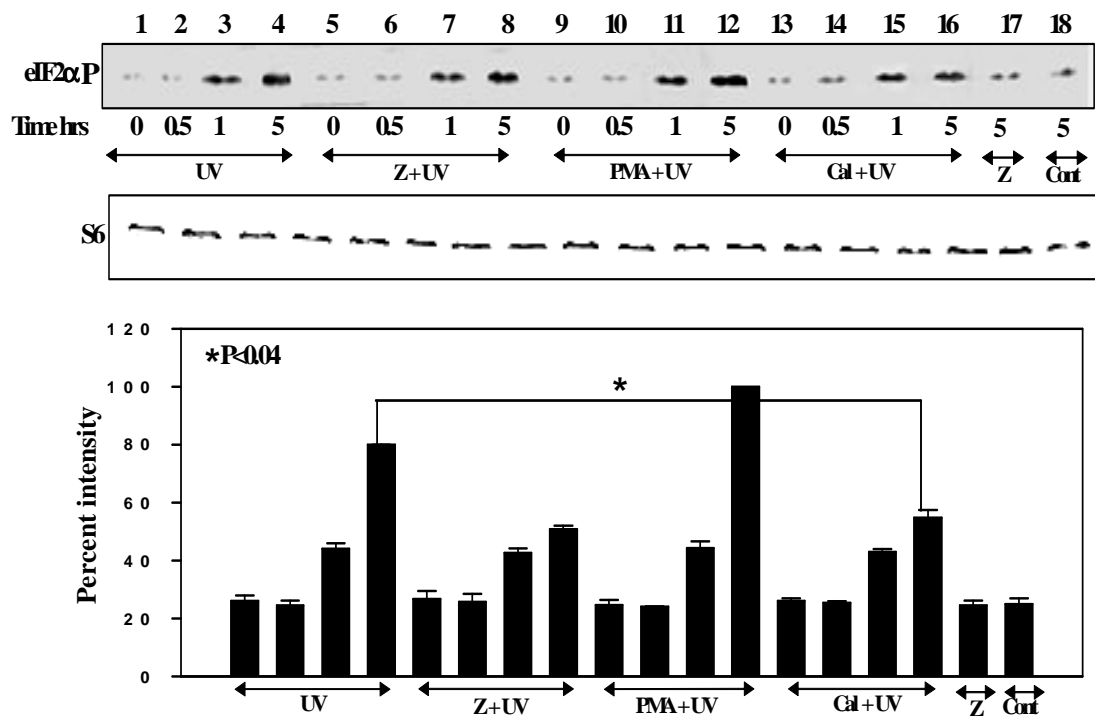
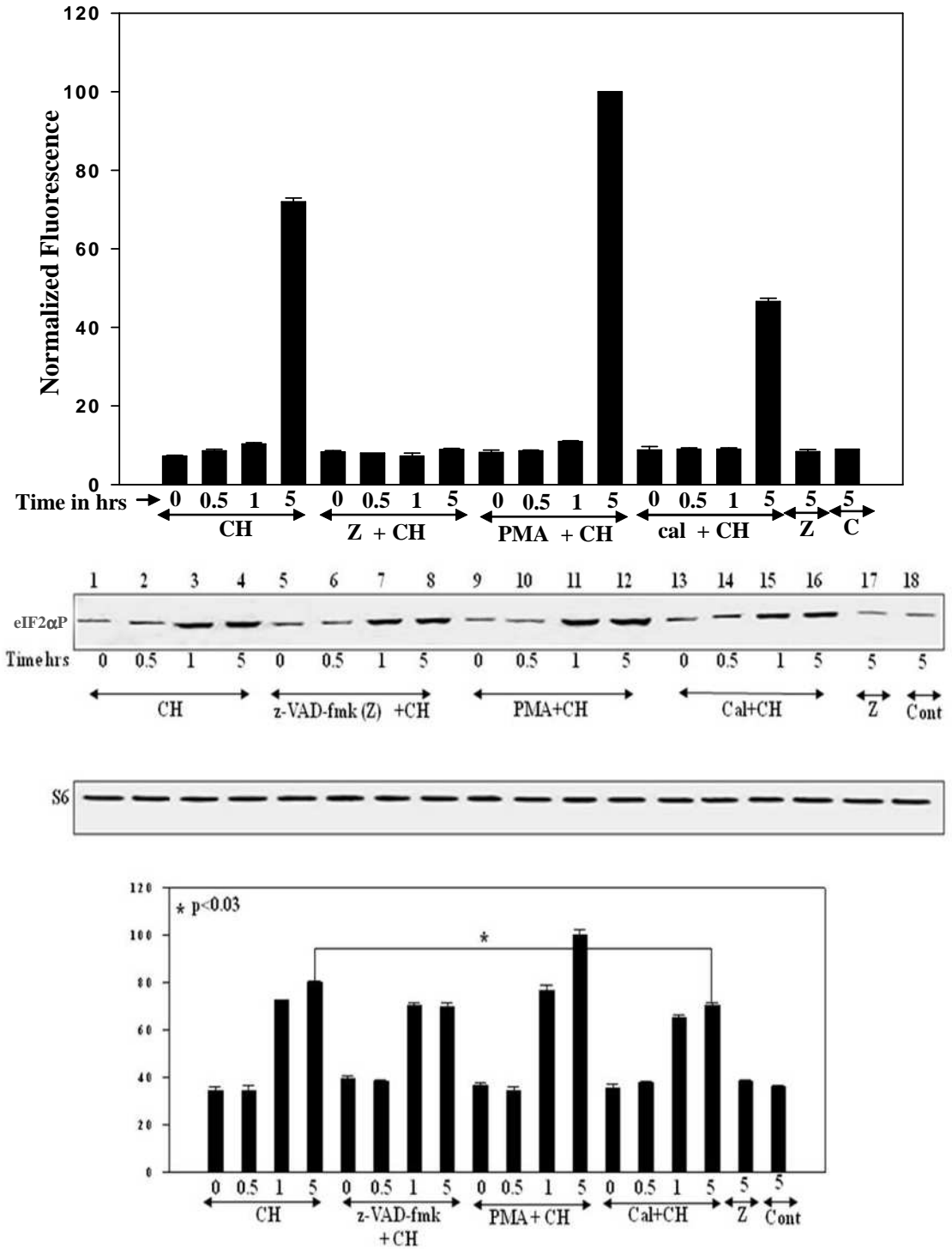


Fig. 18 Time course (0-5h) analysis of eIF2 α phosphorylation in 1.0 mM cycloheximide- treated cells: Effect of z-VAD-fmk, PMA and calphostin.

Sf9 cells were treated with PMA, calphostin and z-VAD-fmk 30 mins prior to 1.0 mM cycloheximide treatment. Cells were treated with 1.0 mM cycloheximide and incubated with the above pharmacological agents at 27°C for different time periods: 0.5 h, 1 h, 5h. Cell extracts were prepared, analyzed for caspase activity and eIF2 α phosphorylation as described in the legend to Fig. 9. Panel A represents the caspase activity and panel B phosphorylation of eIF2 α and the corresponding levels of S6, loading control. The phosphorylation levels eIF2 α were quantified and the percent intensity of eIF2 α is represented in the bar diagram.

Fig. 18

Panel. A



Chapter III

Chapter III

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Caspase activation and eIF2 α phosphorylation in cytochrome c-treated cell extracts: Effect of PMA and calphostin.

5.1 Abstract:

PKC-induced caspase activation and eIF2 α phosphorylation has also been studied here in *Sf9* cell-free extracts devoid of nuclei. Addition of 50 μ M cytochrome c along with 1.0 mM ATP stimulates caspase activation and eIF2 α phosphorylation in these extracts. Further DNA isolated from the nuclei added to these cytochrome c and ATP-treated extracts display fragmentation. Since z-VAD-fmk abrogates caspase activation and eIF2 α phosphorylation, the findings therefore suggest that cytochrome c induced caspase-activation mediates eIF2 α phosphorylation. Addition of PMA to cytochrome c-and ATP-treated extracts enhances further caspase activation and also eIF2 α phosphorylation which are reversed partly by calphostin. Unlike z-VAD-fmk, calphostin mitigates partially PMA-induced caspase activation and eIF2 α phosphorylation. Further, purified recombinant mouse PERK added to cytochrome c-treated extracts is efficiently processed compared to untreated extracts suggesting that active caspases can process PERK *in vitro*.

5.2 Results:

Caspase activation in cell-free extracts supplemented with cytochrome c and ATP.

In this study, cell free extracts devoid of nuclei have been prepared from healthy *Sf9* cells to a) study the endogenous caspase activity in the presence and absence of cytochrome c and b) to determine the importance of PKC activation or inhibition on caspase-mediated eIF2 α phosphorylation. Addition of cytochrome c and 1.0 mM ATP to both undepleted and cytochrome c depleted *Sf9* extracts resulted in caspase activation (**Fig. 19A**). ATP alone stimulated the caspase activity in the extracts which are not depleted of their endogenous cytochrome c. However addition of ATP alone did not promote caspase activation in cytochrome c depleted extracts. This finding is consistent with the earlier observations that ATP facilitates the formation of apoptosome complex and caspase activation in cytochrome c-treated extracts (Liu et al, 1997). Further, the caspase-activated cell-free extracts promoted DNA laddering of isolated healthy nuclei of *Sf9* cells (**Fig. 19B**). Earlier it was shown that PKR, an eIF2 α kinase was cleaved by purified caspases 3,

7 and 8 (Saleans et al, 2001). Here, we tested the ability of *Sf9* cell free extracts treated with cytochrome c and ATP to process the recombinant PERK, an ER-resident eIF2 α kinase. The processing of PERK was analyzed by monitoring the appearance of a processed PERK fragment and reduction in the full length protein by SDS-PAGE in cytochrome c depleted and undepleted extracts in the presence and absence of externally added cytochrome c and ATP. Exogenously added PERK was cleaved in cytochrome c and ATP-treated cell-free extracts where caspase is active (**Fig. 22**). The cleavage of PERK was found related to the caspase activity of the extracts and was mitigated in the presence of z-VAD-fmk (**Fig. 22**).

Cytochrome c-treated cell extracts display enhanced phosphorylation of eIF2 α .

Analysis of eIF2 α phosphorylation in cytochrome c supplemented extracts revealed that the basal level of eIF2 α phosphorylation both in undepleted and cytochrome c depleted extracts (**Fig. 20 left panel, lanes 1 and 4**) was enhanced significantly by cytochrome c and ATP (**lanes 3 and 6 vs. 1 and 4**). Addition of ATP alone to extracts containing endogenous cytochrome c also stimulated eIF2 α phosphorylation (**lanes 2 vs. 1**) significantly than in cytochrome c depleted extracts (**lanes 5 vs. 4**). The corresponding levels of cytochrome c, in the right panel correspond to caspase activation and eIF2 α phosphorylation. Presence of caspase inhibitor, z-VAD-fmk, abrogated cytochrome c and ATP-mediated eIF2 α phosphorylation almost completely (**Fig. 21 lanes 5 vs. 3**) suggesting that caspase activation is the primary cause for enhanced eIF2 α phosphorylation in cytochrome c- and ATP-treated extracts. The right panel in **Fig. 21** represents the levels of cytochrome c in the extracts. Cytochrome c-treated extracts displayed higher phosphorylation levels of eIF2 α as shown in **Figs. 20 and 21 (lanes 3 vs. 1)**. Addition of PMA or calphostin in the absence of added cytochrome c did not alter the phosphorylation status (**Fig. 23 B lanes 6 and 7 vs. lane 1 and the corresponding bar diagram**). However, PMA enhanced (**lanes 4 vs. 3**) and calphostin reduced cytochrome c-mediated eIF2 α phosphorylation (**lanes 5 vs. 3**) in extracts. The phosphorylation levels of eIF2 α were related to the caspase activity as observed in these extracts (**Fig 23 A**). PMA-treated cytochrome c-supplemented cell extracts displayed enhanced PERK processing (**Fig. 24 lane 10**) and calphostin reduced the PERK processing (**lane 14**) compared to cytochrome

c-treated extracts (**lane 6**). These findings therefore suggest that caspase activation by cytochrome c can lead to both eIF2 α kinase and PKC activation in cell extracts. Together, these activities can further enhance caspase activation and cell death.

Fig. 19

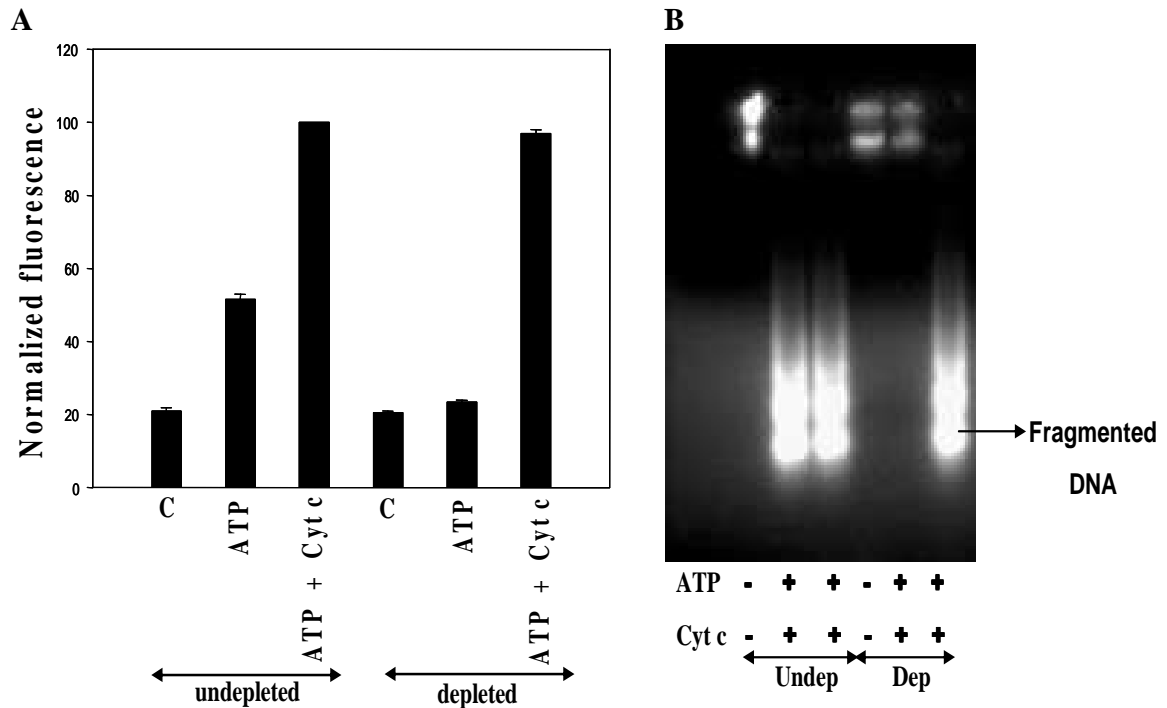


Fig. 19 Cytochrome c-mediated caspase activation in cell-free extracts devoid of nuclei.

Caspase activity was induced in *Sf9* extracts (~100 μ g) undepleted or depleted of their endogenous cytochrome c, devoid of their nuclei by the addition of externally added cytochrome c (50 μ M) and ATP (1.0 mM) as mentioned in 'Materials and Methods'. Panels A and B represent the caspase activity and DNA fragmentation in undepleted and cytochrome c depleted extracts respectively.

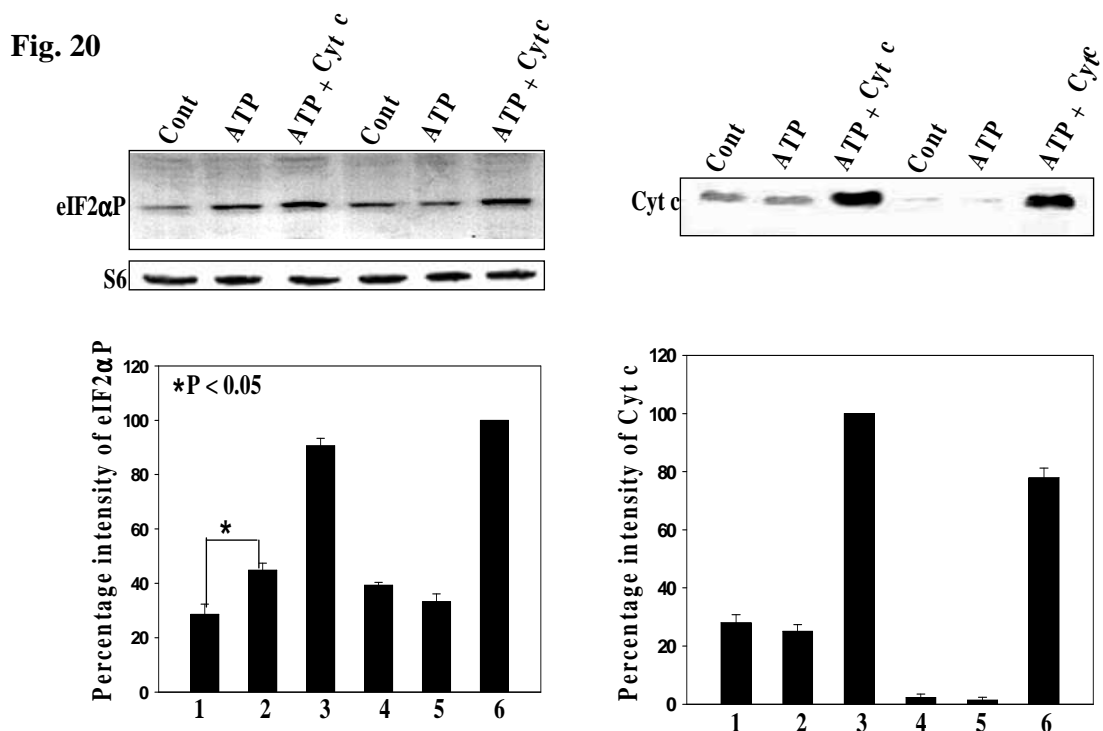


Fig. 20 Phosphorylation of eIF2α in cytochrome c-treated cell free extracts.

Phosphorylation of eIF2α was analyzed in undepleted and cytochrome c depleted cell free extracts that were treated with 50 μM cytochrome c and 1.0 mM ATP as mentioned in ‘Materials and Methods’. The levels of cytochrome c (endogenous or supplemented) using monoclonal anti-cytochrome c antibody and of S6 using polyclonal anti-S6 antibody were also monitored. The left upper panel represents the phosphorylation of eIF2α, whereas the right panel represents the levels of cytochrome c in the above mentioned extracts. The levels of phosphorylated eIF2α and cytochrome c were quantified using image J software. The results are displayed in left and right bar diagrams corresponding to the western blots above.

Fig. 21

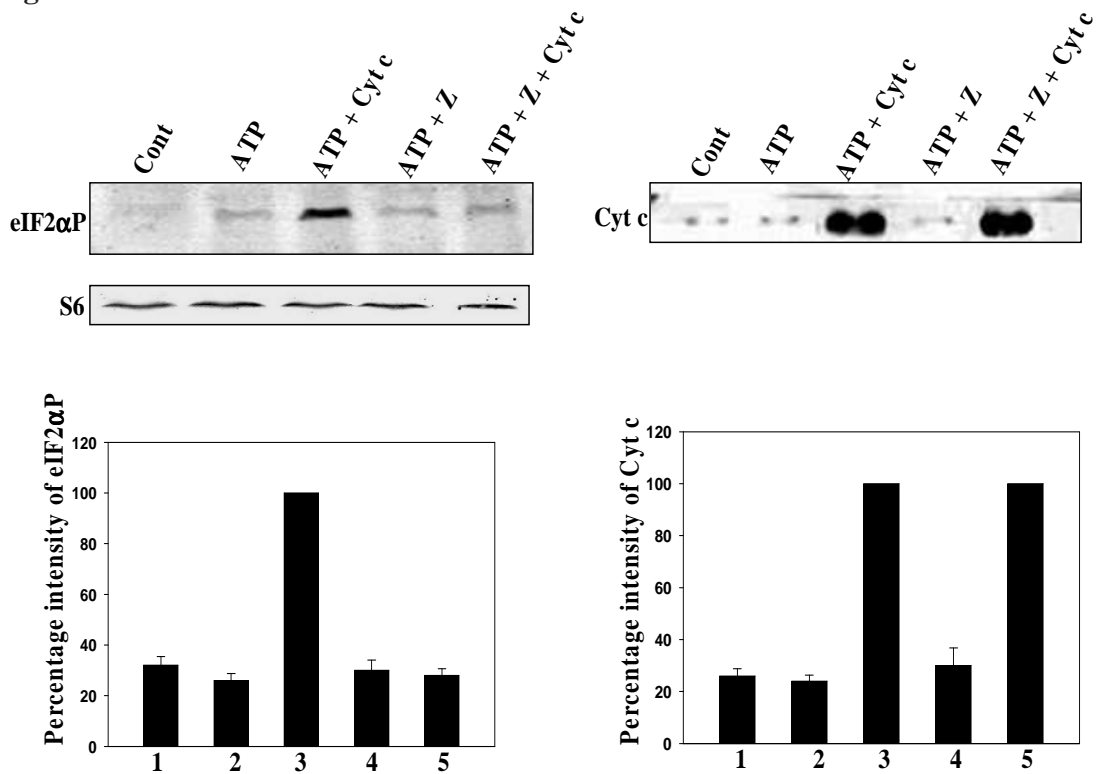


Fig. 21 Cytochrome c-mediated caspase activation in cell-free extracts devoid of nuclei: Effect of z-VAD-fmk.

Caspase activity was induced in *Sf9* extracts devoid of their nuclei and endogenous cytochrome c as mentioned in the legend to **Fig. 19**. The extracts were analyzed for phosphorylation of eIF2 α , cytochrome c and S6 as mentioned in the legend to **Fig. 20**. The levels of phosphorylation of eIF2 α and of cytochrome c were quantified as described in **Fig. 20**. The results are presented in bar diagrams below the respective western blots.

Fig. 22

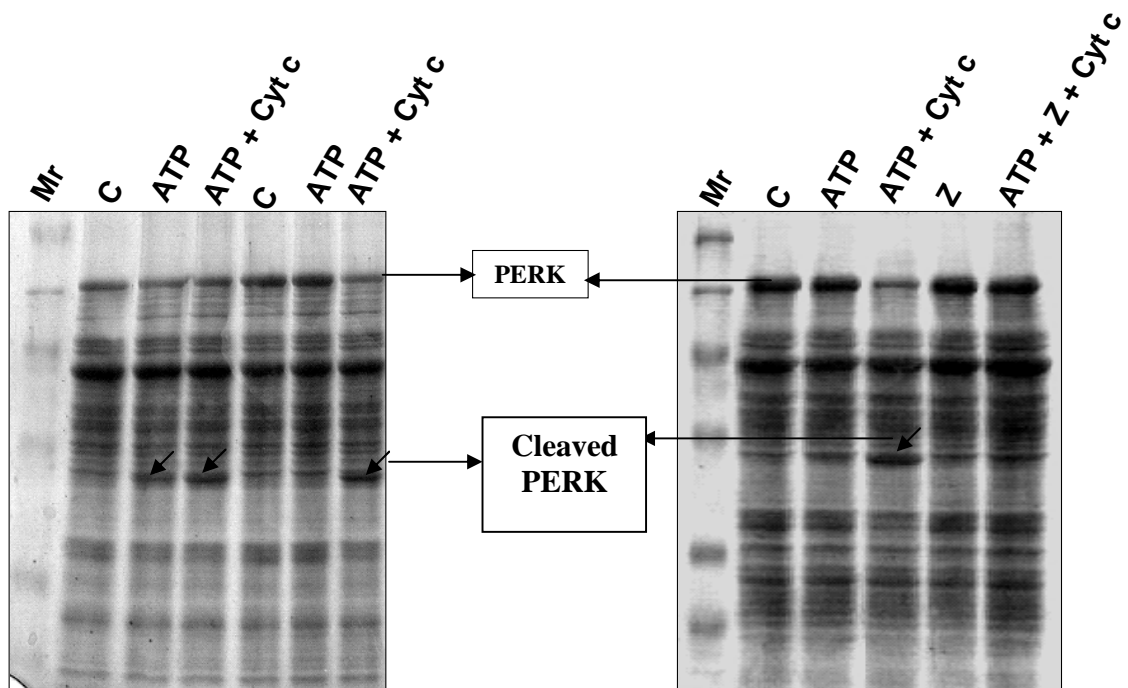


Fig. 22 PERK cleavage in cytochrome c-treated cell free extracts.

PERK cleavage assay was performed in undepleted and cytochrome c depleted cell free extracts treated with 50 μ M cytochrome c and 1.0 mM ATP as mentioned in 'Materials and Methods' (**Panel. A**). Effect of z-VAD-fmk (50 μ M) on cleavage of PERK (150 ng) was also studied in cytochrome c depleted extracts treated with cytochrome c and 1.0 mM ATP (**Panel. B**). The figures are mass stained gels. The various lanes are as follows: Mr, Molecular weight marker; c, control extracts; ATP, ATP treated extracts; cyt c, cytochrome c- treated extracts; z, z-VAD-fmk-treated extracts.

Fig. 23

Panel. A

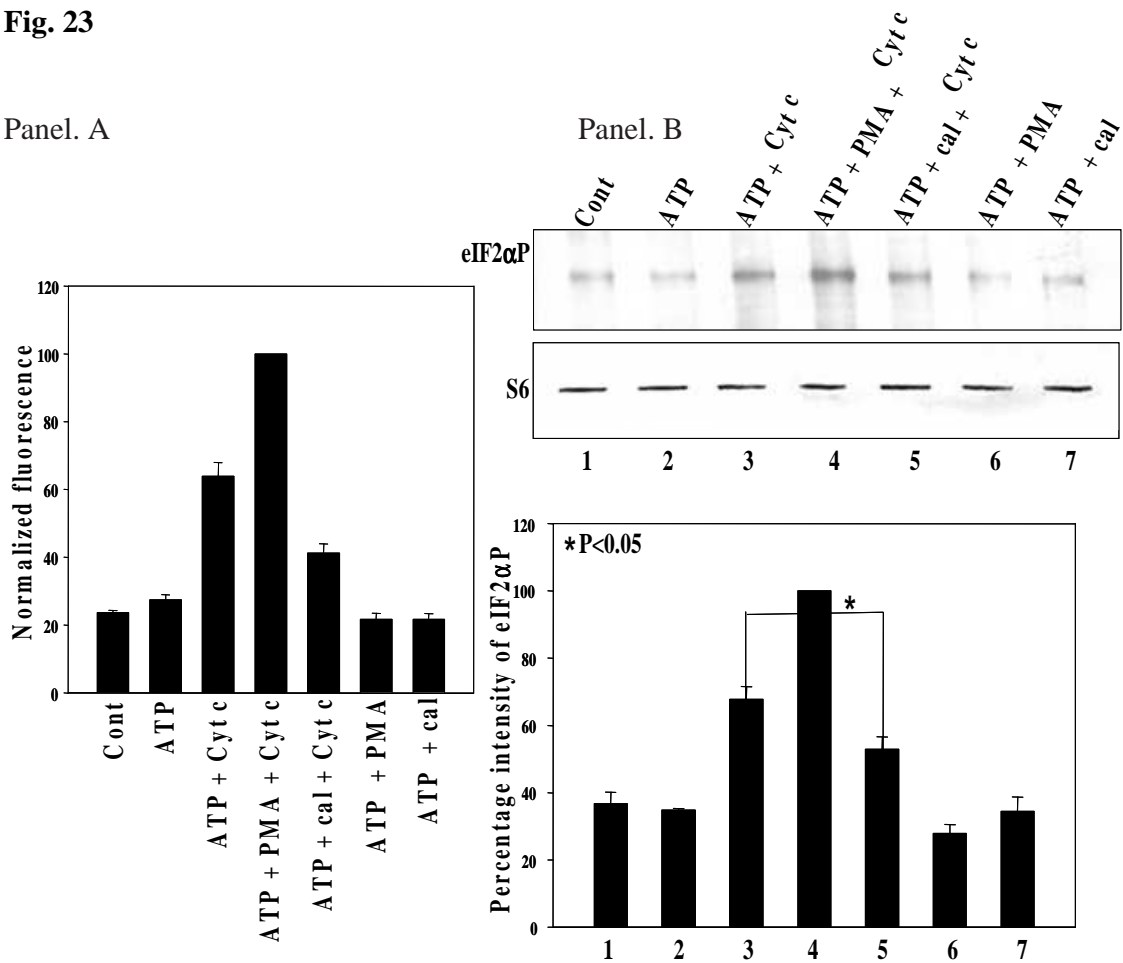


Fig. 23 Cytochrome c-mediated caspase activation in cell-free extracts: Effect of PMA and calphostin.

Caspase activity was induced in *Sf9* extracts devoid of their nuclei and endogenous cytochrome c as mentioned in legend to **Fig. 19** in the presence and absence of PMA and calphostin. The extracts were analyzed for caspase activity, phosphorylation of eIF2 α and S6 as mentioned in 'Materials and Methods'. The levels of phosphorylation of eIF2 α were quantified. Panel A represents the caspase activity observed in these extracts. The upper blot in panel B represents eIF2 α (P) and the lower one represents the levels of S6. The bar diagram in Panel B represents the intensity of eIF2 α phosphorylation.

Fig. 24

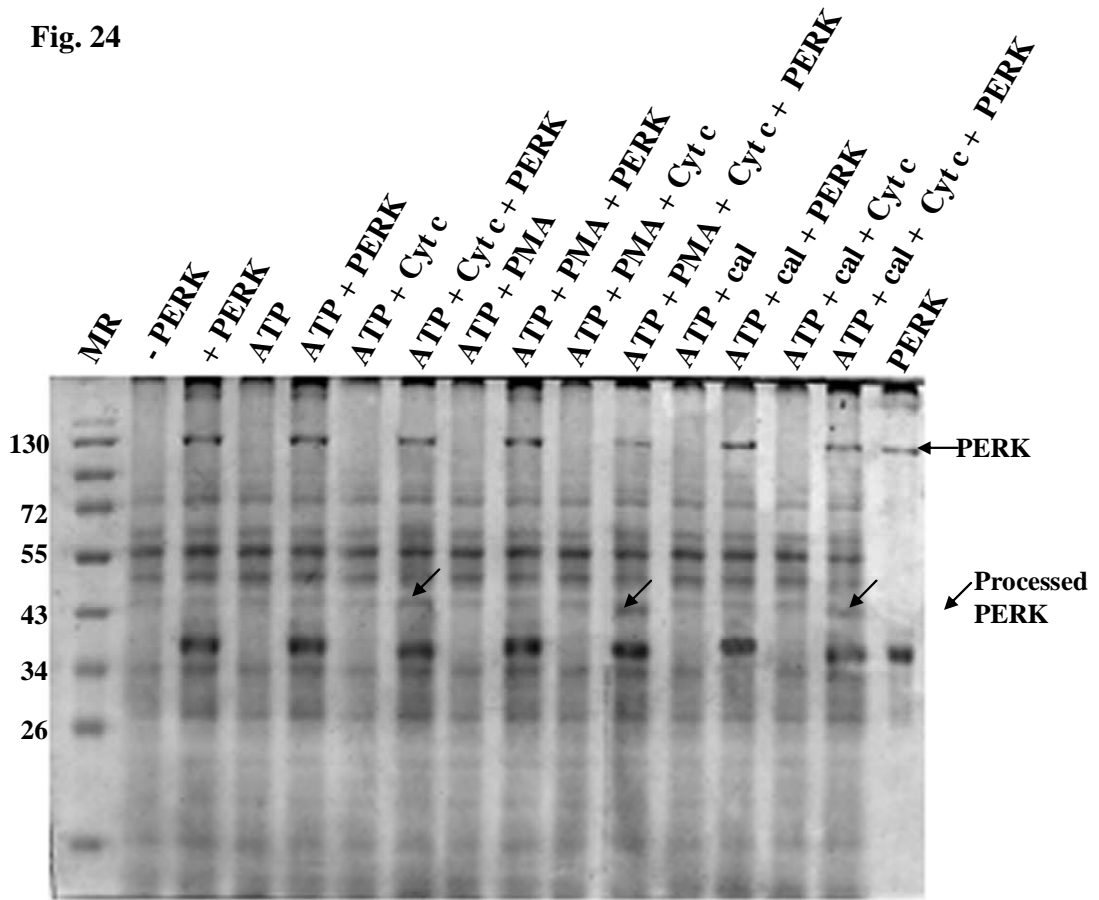


Fig. 24 PERK cleavage in Cytochrome c-treated cell free extracts: Effect of PMA and calphostin.

PERK cleavage assay was performed in *Sf9* cell free extracts treated with 50 μ M cytochrome c and 1.0 mM ATP in the presence and absence of PMA and calphostin as mentioned in 'Materials and Methods'. The figure is commissie stained gel.

Summary

Cell death or apoptosis is mediated by complex signaling pathways. Cell death may be intrinsic or extrinsic i.e., receptor mediated (Yan et al, 2005). Intrinsic cell death occurs through cytochrome c release by mitochondria, activation of Apaf-1 and formation of apoptosome that triggers caspase activation. Different forms of stress can promote cell death when the adaptive or survival mechanisms fail. Stress can affect any of the cellular organelles. Recent studies in mammalian systems indicate that the intrinsic cell death can occur due to a stress in the endoplasmic reticulum or in the cytosol (Hussain and Ramaiah, 2007b). Both of them can communicate to mitochondria. We have shown previously that the phosphorylation of eIF2 α is a stress signal (Aparna et al, 2003). It inhibits general protein synthesis and is a typical mechanism in cells to save cellular energy. In addition, this mechanism plays an important role in unfolded protein response (UPR) that is elicited by endoplasmic reticulum due to the accumulation of unfolded proteins. Accumulation of unfolded proteins occurs typically when the synthesis exceeds protein folding capacity of the cells or due to improper covalent modifications and degradation of proteins. In addition a release in ER bound calcium to cytosol can evoke UPR because many of the ER chaperones require calcium for their functioning (Hussain and Ramaiah, 2007b). Interestingly both ER and cytosol are involved in the synthesis of proteins and contain their own chaperones, caspase(s) and eIF2 α kinases. Earlier studies from this laboratory have also suggested that phosphorylation of eIF2 α plays a role in cell survival and death (Aparna et al, 2003). Baculovirus p35, an anti-apoptotic protein, or caspase inhibitor like z-VAD-fmk mitigate eIF2 α phosphorylation and cell death suggesting that caspases can also affect eIF2 α kinase activation (Aparna et al, 2003). An analysis of eIF2 α phosphorylation-mediated cell death and survival in *Sf9* cells in our laboratory reveals that ER-stress induced eIF2 α phosphorylation accompanied by expression of BiP, an ER chaperone protects the cells from stress-induced death where as non-ER stress that is not accompanied by BiP expression leads to cell death (Aarti et al, 2010). Reduced ER stress as evidenced by lack of BiP expression and eIF2 α phosphorylation coincides with increased proapoptotic proteins in most of the rat tissues during chronological ageing (Hussain and Ramaiah, 2007a).

Many recent studies have shown that several coincident signalling mechanisms like phosphoinositidyl kinase 3 (PI3K) (Kazemi et al, 2007) pathway, mitogen activated protein kinases (MAP Kinases that include ERK, p38 and JNK) pathways are known to effect cell survival and also eIF2 α (Dent et al, 2003) phosphorylation. Protein kinase C activation is also implicated in UV-irradiation induced caspase activation and cell death. However there are no reports indicating that PKC activation effects eIF2 α phosphorylation or the cell death mediated by eIF2 α phosphorylation. UV-irradiation or DNA damage is known to elicit both PKC activation, phosphorylation of eIF2 α and cell death in different cell types (Sakaki et al, 2002; Aparna et al, 2003). Hence here we have studied the connection, if any, between these two pathways in *Sf9* cells which are natural hosts of baculovirus.

Consistent with our previous studies (Aparna et al, 2003) non-ER stressors that are characterized by lack of BiP expression (Aarti et al, 2010) as in the case of UV-irradiation and cycloheximide treatments promote phosphorylation of eIF2 α and cell death. Time course analysis of eIF2 α phosphorylation reveals that z-VAD-fmk, a pan caspase inhibitor mitigates the late stages (at 10 h) (**Fig. 9, 17, 11 and 18**), but not the early stages (**Fig. 17 and 18**) of UV-irradiation or cycloheximide-induced eIF2 α phosphorylation thereby suggesting that caspase activation which follows eIF2 α phosphorylation can also lead to enhanced eIF2 α phosphorylation in a feed back loop manner. Low concentrations of cycloheximide that leads to eIF2 α phosphorylation without inducing cell death results in enhanced caspase activation and eIF2 α phosphorylation in the presence of PMA (**Fig. 5**). Tunicamycin, a typical ER stressor that induces BiP expression, promotes eIF2 α phosphorylation but not cell death (**Fig. 5**). Non-ER stress induced cell death is related to the caspase activity which is estimated by Ac-DEVD-AFC hydrolysis (**Fig. 6**). The fact that eIF2 α phosphorylation in non-ER stressed conditions stimulates caspase activation is further substantiated by the observation that tunicamycin-induced eIF2 α phosphorylation does not lead to caspase activation and is not affected by z-VAD-fmk (**Fig. 5**).

PMA, phorbol 12-myristate 13-acetate also referred to as 12-O-tetradecanoylphorbol 13-acetate (TPA) is commonly used phorbol ester ligand to activate PKC in biological systems. Addition of PMA, stimulates UV-irradiation or cycloheximide-induced eIF2 α

Fig. 25

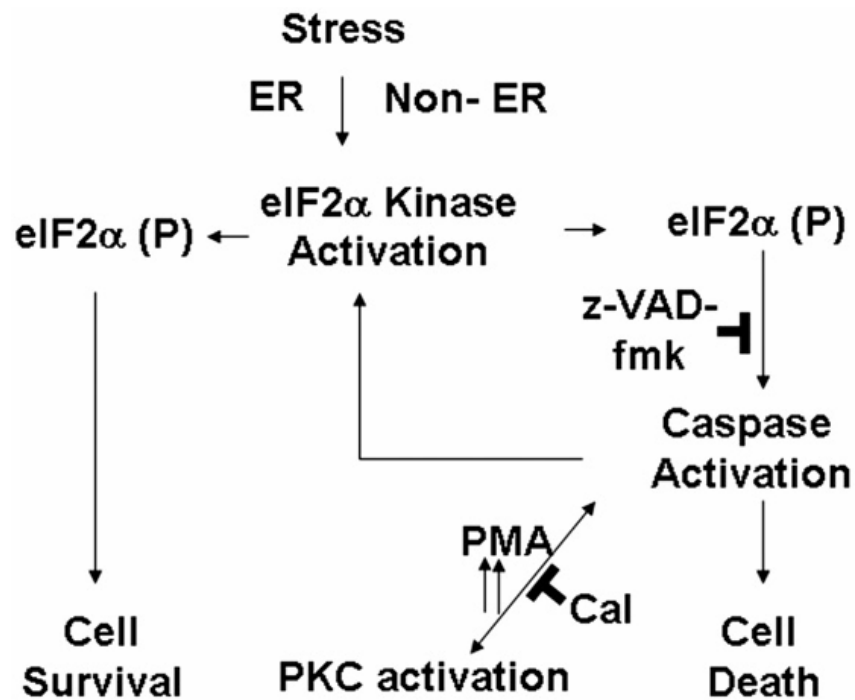


Fig. 25 Model: Effect of PKC activation on eIF2 α phosphorylation.

Analyses of non-ER stress induced eIF2 α phosphorylation in *Sf9* cells or cytochrome c induced eIF2 α phosphorylation in cell-free extracts suggests that caspase activation leads to enhanced eIF2 α phosphorylation. PMA, an activator of PKC enhances caspase-mediated eIF2 α phosphorylation. Caspase-mediated eIF2 α phosphorylation is mitigated partially by calphostin and completely by z-VAD-fmk thereby suggesting that caspases can activate multiple signalling pathways.

phosphorylation and cell death but does not affect tunicamycin-induced eIF2 α phosphorylation thereby suggesting that caspase activation stimulates PKC activation and further enhancement in eIF2 α phosphorylation. This notion is further supported by the finding here that PMA enhances very efficiently and calphostin, reduces albeit less efficiently, the late stages but not the early stages of the UV-irradiation or high concentrations of cycloheximide induced eIF2 α phosphorylation than the caspase inhibitor z-VAD-fmk. These findings therefore suggest that calphostin inhibits PMA mediated PKC activation that occurs in response to caspase activation mediated by eIF2 α phosphorylation. The above results are further substantiated by the observation that *Sf9* cells infected with baculovirus resisted caspase activation and eIF2 α phosphorylation induced by UV-irradiation and cycloheximide. Addition of PMA and calphostin did not affect the virus infected *Sf9* cells. eIF2 α phosphorylation was also found reduced in UV-irradiated (**Fig. 13**), cycloheximide (**Fig. 14 and 15**) and tunicamycin- (**Fig. 16**) treated cells that were infected with baculovirus. This may be due to the fact that baculovirus-infected cells produce PK2 and P35 proteins to regulate host cell eIF2 α phosphorylation, caspase activation and cell death to facilitate the propagation of virus. This interpretation is consistent from the previous findings of this laboratory (Aparna et al, 2003; Aarti et al, 2010). The above results are further substantiated by the observations that cytochrome c-mediated-caspase activation in cell-free extracts as analyzed by Ac-DEVD-AFC hydrolysis (**Fig. 19A**), DNA fragmentation of healthy nuclei (**Fig. 19B**), and the cleavage of PERK (**Fig. 22A and B**), leads to eIF2 α phosphorylation (**Fig. 20 and 21**) which is further enhanced by PMA and reduced by calphostin (**Fig. 23**).

A recent study suggests that eIF2 α phosphorylation, which is known to inhibit general protein synthesis, however upregulates translation of PKC η (Raveh et al, 2009) as other gene-specific mRNAs like ATF4. PKCs are implicated in amplifying the apoptotic response in variety of cell types undergoing apoptosis (Mizuno et al, 1991; 1997; Emoto et al, 1995; Basu et al, 2003; Denning et al, 2002). Among PKCs, PKC δ is implicated in cell death in diverse apoptotic stimuli (Emoto et al, 1995; Mizuno et al, 1997; Denning et al, 1998; Reyland et al, 1999). It is a known caspase substrate specifically cleaved by caspase-3 and the processed catalytic domain is shown to be constitutively active (Basu, 2003; Kato et al, 2009). eIF2 α kinases are also shown to be processed and activated in cells

undergoing apoptosis (Saleans et al, 2001; Deng et al, 2002). However, neither the PKCs nor eIF2 α kinases have been characterized in *Sf9* cells. Interestingly PKCs are implicated in ER stress mediated autophagy (Sakaki et al, 2007). Our studies point out that non-ER stress induced eIF2 α phosphorylation-mediated caspase activation can enhance PKC activation, eIF2 α phosphorylation (in a feedbackmanner) and subscribes to the notion that caspases can activate multiple signaling pathways to enhance cell death as described in our model (**Fig. 25**).

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PKC activation contributes to caspase-mediated eIF2 α phosphorylation and cell death

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ABSTRACT

Back ground: Stress-induced phosphorylation of the alpha-subunit of eukaryotic initiation factor 2 (eIF2 α), involved in translation, promotes cell suicide or survival. Since multiple signaling pathways are implicated in cell death, the present study has analyzed the importance of PKC activation in the stress-induced eIF2 α phosphorylation, caspase activation and cell death in the ovarian cells of *Spodoptera frugiperda* (Sf9) and in their extracts.

Methods: Cell death is analyzed by flow cytometry. Caspase activation is measured by Ac-DEVD-AFC hydrolysis and also by the cleavage of purified recombinant PERK, an endoplasmic reticulum-resident eIF2 α kinase. Status of eIF2 α phosphorylation and cytochrome *c* levels are analyzed by western blots.

Results: PMA, an activator of PKC, does not promote cell death or affect eIF2 α phosphorylation. However, PMA enhances late stages of UV-irradiation or cycloheximide-induced caspase activation, eIF2 α phosphorylation and apoptosis in Sf9 cells. PMA also enhances cytochrome *c*-induced caspase activation and eIF2 α phosphorylation in cell extracts. These changes are mitigated more efficiently by caspase inhibitor, z-VAD-fmk, than by calphostin, an inhibitor of PKC. In contrast, tunicamycin-induced eIF2 α phosphorylation that does not lead to caspase activation or cell death is unaffected by PMA, z-VAD-fmk or by calphostin.

Conclusions: While caspase activation is a cause and consequence of eIF2 α phosphorylation, PKC activation that follows caspase activation further enhances caspase activation, eIF2 α phosphorylation, and cell death in Sf9 cells.

General significance: Caspases can activate multiple signaling pathways to enhance cell death.

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1. Introduction

The ovarian cells of *Spodoptera frugiperda* (Sf9) are natural hosts of baculovirus and are good model systems to study the process of apoptosis [1,2]. Previously we used these cells to express all the three subunits of human eukaryotic initiation factor 2 (eIF2) and some of the variants of the alpha-subunit using baculovirus expression system to determine the importance of inter-subunit and protein interactions on the function and regulation of eIF2 [3–5]. The trimeric eIF2 protein plays an important role in delivering initiator tRNA (Met-tRNA_i) to 40S ribosomal subunits in the presence of GTP and in the identification of authentic translational start site in the initiation step of eukaryotic protein synthesis [6–8]. After ensuring correct base pairing between Met-tRNA_i and the start AUG codon in mRNA, the GTPase activity of eIF2 is stimulated by eIF5, the GTPase activating protein and the eIF2.GDP is released from ribosome. Phosphorylation

of the conserved ser⁵¹ residue in the alpha-subunit of mammalian eIF2 by a family of stress-activated eIF2 α kinases [9] occurs at different cellular locations: cytoplasm and on the surface of endoplasmic reticulum (ER). Phosphorylation of ser⁵¹ in eIF2 α inhibits eIF2B, a heteropentameric GDP/GTP exchange factor that recycles eIF2 GDP to eIF2.GTP [5]. As a consequence, general protein synthesis is inhibited. Phosphorylation of eIF2 α also upregulates translation of certain gene-specific mRNAs and evokes adaptive or apoptotic signaling pathways [10–14]. Abrogation of eIF2 α phosphorylation leads to transformation and severe phosphorylation leads to cell death in cultured cells [15–17]. Homozygous mice harboring nonphosphorylatable form of eIF2 α hypoglycemic and hypoinsulinemic and die soon after birth [18]. In contrast, heterozygous mice harboring one copy of nonphosphorylatable form of eIF2 α are prone to obesity on high fat diet [19].

Expression of S51D, a phosphomimetic mutant of human eIF2 α or double-stranded RNA-dependent kinase (PKR), an eIF2 α kinase, promotes apoptosis in mammalian cells [16]. However in our earlier studies, we could express a phosphomimetic form of eIF2 α in Sf9 cells using baculovirus expression system [3,5]. Subsequent studies by us have shown that wt baculovirus infection or addition of z-VAD-fmk, a caspase inhibitor mitigates UV-induced eIF2 α phosphorylation, caspase activation and apoptosis. In contrast, p35 deletion baculovirus that lacks its antiapoptotic p35 gene readily stimulates eIF2 α phosphorylation and apoptosis [1]. UV-irradiation-induced apoptosis

Abbreviations: PMA, Phorbol myristate acetate; PKC, Protein kinase C; UV, Ultraviolet; ER, Endoplasmic reticulum; eIF2 α , alpha-subunit of eukaryotic initiation factor-2; z-VAD-fmk, Benzyloxycarbonyl-Val-Ala-Asp (OMe) fluoromethylketone; PERK, ER-resident eIF2 α kinase; Sf9, *Spodoptera frugiperda*

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is partly mitigated by the expression of nonphosphorylatable S51A mutant of eIF2 α and enhanced by S51D, a phosphomimetic mutant eIF2 α . These studies together with the time course studies on caspase activation and eIF2 α phosphorylation raised the possibility that eIF2 α phosphorylation is a cause and consequence of caspase activation [30]. Consistent with these studies in insect cells, an earlier study also suggested that the double-stranded RNA-dependent eIF2 α kinase (PKR) is proteolyzed *in vitro* by recombinant caspases-3, -7 and -8, and the kinase domain efficiently phosphorylates the conserved ser⁵¹ residue in eIF2 α , thereby suggesting that PKR can also be activated by caspases in addition to low concentrations of dsRNA [20]. Interestingly, certain stressors like tunicamycin or A23187 that promote stress in the endoplasmic reticulum (ER), as evidenced by BiP expression induce eIF2 α phosphorylation but not apoptosis in *Sf9* cells suggesting that phosphorylation of eIF2 α is a stress, survival and suicidal signal [30].

In addition to PKR, several other kinases including conventional and novel PKCs are activated by caspases [21,22]. Like the family of eIF2 α kinases, the family of PKCs that comprises at least 11 isoforms is implicated in cell survival and death [23]. Here we show for the first time that caspase-mediated eIF2 α phosphorylation in UV-irradiated or cycloheximide-treated *Sf9* cells and in cytochrome *c*-treated extracts is further enhanced by PMA, a pharmacological activator of PKC and inhibited by calphostin, an inhibitor of PKC.

2. Materials and methods

2.1. Materials

All reagents otherwise mentioned below were purchased from Sigma, USA. Anti-cytochrome *c* antibody, calphostin, phorbol myristate acetate (PMA) and tunicamycin were obtained from Calbiochem. Ac-DEVD-AFC, Ac-DEVD-CHO and z-VAD-fmk were purchased from BD-Biosciences. TNM-FH medium and fetal bovine serum were purchased from Gibco BRL. Phosphospecific anti-eIF2 α and S6 antibodies were obtained from Cell Signaling Technologies.

2.2. Cell culture, morphology and flow cytometry

Sf9 cells were cultured in TNM-FH medium supplemented with 10% fetal bovine serum and 1% antibiotics as described earlier [1]. Trypan blue exclusion test using hemocytometer was performed to score the viable cells. Cells were scored for apoptosis by monitoring plasma membrane blebbing by observing under a VWR AE31 inverted microscope equipped with a motic digital camera and the software (20 \times magnification). Percentage of cells undergoing apoptosis was quantified by flow cytometry. After each treatment, cells were harvested by centrifugation at 1500 rpm for 5 min at 4 °C, washed with PBS (phosphobuffer saline), resuspended in the residual PBS, fixed with 500 μ l of 70% ethanol and were incubated overnight at 4 °C. The cells were then washed in PBS, suspended in PBS containing 50 μ g/ml propidium iodide, 1% Triton X-100 and 50 μ g/ml RNase and incubated in dark for 1 h at 37 °C. Cells were then centrifuged, washed again with PBS, resuspended in sheath fluid and analyzed by flow cytometer FACS CALIBUR 3CS from Becton Dickinson Biosciences.

2.3. Induction of apoptosis

2×10^6 cells were used for the experiments. Cells were treated with UV-irradiation (200 J/m²) for 60 s, 1.0 mM cycloheximide and 12 μ M tunicamycin in the presence and absence of 25 μ M z-VAD-fmk, or 50 nM PMA or calphostin 1 h prior to the treatment and incubated at 27 °C for 10 h. To induce caspase activity *in vitro*, the endogenous cytochrome *c* of the healthy *Sf9* extracts was first immunodepleted using anti-cytochrome *c* antibody as mentioned [24]. Then the extracts (~100 μ g protein) were incubated with 50 μ M horse heart

cytochrome *c* and 1.0 mM ATP. To determine the importance of PKC activation on caspase activity, Ac-DEVD-AFC hydrolysis was monitored in cytochrome *c*-treated extracts in the presence and absence of PMA or calphostin.

2.4. Preparation of cell extracts

Cells treated with various agents were harvested and lysed as mentioned earlier [1]. Protein was estimated using Bradford reagent from Biorad. For the preparation of cell-free extracts devoid of nuclei, healthy *Sf9* cells were harvested, washed with PBS and suspended in lysis buffer containing 50 mM Hepes-KOH pH 7.9, 10 mM KCl, 0.2 mM EDTA (pH 8.0), 1 mM DTT, 1 μ g/ml pepstatin and leupeptin, and 1 mM PMSF. After incubation on ice for 15 min, 0.25% NP-40 was added, vortexed briefly and centrifuged at 1600 rpm for 3 min. The supernatant was collected and centrifuged at 13,000 rpm for 30 min at 4 °C. The supernatant was saved as cytoplasmic extract. The pellet was washed once with lysis buffer and centrifuged at 1000 \times g for 3 min. It is then resuspended in 500 μ l of lysis buffer and layered on 30% sucrose buffer containing 50 mM Hepes-KOH pH 7.9, 10 mM KCl, 1 mM EDTA pH-8.0, 1 mM DTT, 0.5 mM PMSF, and spun at 3000 rpm for 10 min. The pellet rich in nuclei was stored in buffer A containing 30% glycerol at -70 °C or used immediately.

2.5. Caspase activity and eIF2 α phosphorylation

Caspase activity was analyzed by the hydrolysis of specific caspase 3 substrate, Ac-DEVD-AFC as mentioned [1]. Fluorescence emission of the released AFC (excitation—400 nm; emission—500 nm) was measured using a Fluoromax-3, Jobin Yvon Horiba Spectrofluorimeter. Phosphorylated eIF2 α was analyzed in various conditions by western blots as described previously using equal amount of protein (~100 μ g) [1]. The processing of recombinant mouse PERK, an eIF2 α kinase, was studied in *Sf9* cell extracts prepared from cells undergoing apoptosis as a measure to determine caspase activity. Recombinant PERK was incubated in PERK cleavage buffer (10 mM Tris-HCl pH 7.5, 2.5 mM KH₂PO₄, 2 mM NaCl, 68 mM sucrose and 220 mM mannitol) along with the extracts or purified caspase-3 at 37 °C for 4 h. After incubation, the reaction was stopped by the addition of 4 \times sample buffer; the samples were boiled, electrophoresed on 10% SDS-PAGE and stained with coomassie blue.

2.6. Integrity of nuclei and DNA fragmentation

The integrity of nuclei was checked by hoechst dye (excitation—346 nm, emission—460 nm). 2.5 μ M of dye was added to 40 μ l of nuclei and incubated in the dark for 10 min and viewed under a Leica TCS SP2 AOBs laser scanning confocal microscope equipped with Leica confocal software. Healthy *Sf9* nuclei were incubated with cell-free extracts treated with cytochrome *c* for 4 h at 37 °C and were analyzed for DNA fragmentation as mentioned [24].

3. Results

3.1. PMA enhances and calphostin mitigates stress-induced cell death and caspase activation

An analysis of *Sf9* cells for cell death by flow cytometry and caspase activation by Ac-DEVD-AFC hydrolysis in the presence of various agents reveals that caspase activation is directly proportional to cell death (Fig. 1). Cells incubated for 10 h at 27 °C after 60 s of exposure to UVB irradiation (200 J/m²) or treated with 1 mM cycloheximide, an inhibitor of polypeptide chain elongation, showed 55–58% or 58–60% of apoptosis respectively (Fig 1, white bars, bar # 5 and 11 vs. 1) and displayed higher caspase activity (black bars). Treatment in the presence of 12 μ M tunicamycin, an inhibitor of N-linked glycosylation and a known stressor of the endoplasmic reticulum (ER), for 10 h however

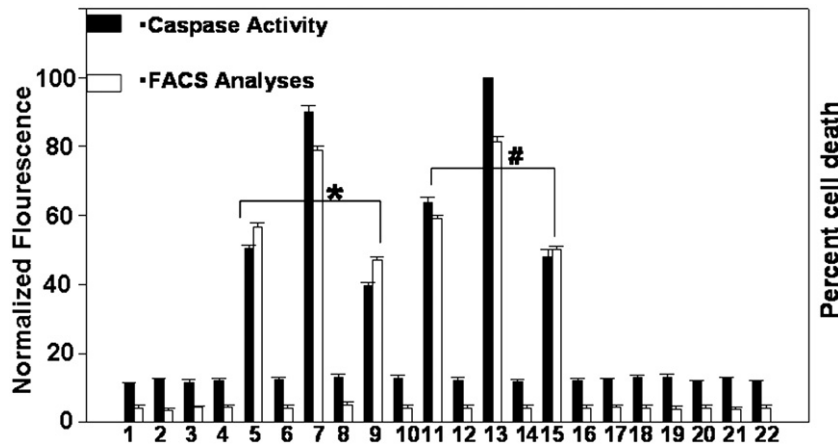


Fig. 1. Caspase-3 activity and cell death. *Sf9* cells are treated with various agents for 10 h at 27 °C, except in the case of UV-irradiation. UV-irradiation (200J/m²) is given for 60 s and then the cells are incubated at 27 °C for 10 h. Cells are analyzed for cell death and caspase activity as described in Section 2. The black bars indicate the relative levels of caspase activity and the white bars indicate the percentage of cell death as measured by flow cytometry. The various bars represent the following: 1) control, 2) 50 μM z-VAD-fmk, 3) 50 nM PMA, 4) 50 nM Cal, 5) UV, 6) z-VAD-fmk + UV, 7) PMA + UV, 8) z-VAD-fmk + PMA + UV, 9) Cal + UV, 10) z-VAD-fmk + Cal + UV, 11) 1.0 mM cycloheximide (CH), 12) z-VAD-fmk + CH, 13) PMA + CH, 14) z-VAD-fmk + PMA + CH, 15) Cal + CH, 16) z-VAD-fmk + Cal + CH, 17) 12 μM, tunicamycin (Tn), 18) z-VAD-fmk + Tn, 19) PMA + Tn, 20) z-VAD-fmk + PMA + Tn, 21) Cal + Tn, and 22) z-VAD-fmk + Cal + Tn. The bar represents mean ± standard error. * *P*<0.01 and # *P*<0.02 *n*=4.

did not promote significant amount of apoptosis or any caspase activation (bar # 17 vs. 1). Prior treatment for 1 h with 50 μM z-VAD-fmk, a caspase inhibitor, abrogates both the UV and cycloheximide-induced apoptosis and caspase activation (bar # 6 and 12 vs. 5 and 11). Wt baculovirus infected cells also resisted UV or cycloheximide-induced apoptosis (data not shown).

Low concentrations of PMA (50 nM), an activator of PKC, or calphostin (50 nM), an inhibitor of PKC did not induce significant changes in cell morphology, apoptosis or caspase activation (bar # 3 and 4). PMA enhanced further both UV-irradiation and cycloheximide-induced apoptosis from 58% to 80% and 60% to 83% respectively (bar # 7 and 13 vs. 5 and 11) and accordingly the caspase activity. In contrast, addition of 50 nM of calphostin, a known inhibitor of PKC decreased UV and cycloheximide-induced caspase activity and apoptosis (bar # 9 and 15 vs. 5 and 11). However, unlike z-VAD-fmk, the reduction in caspase activity and cell death in UV or cycloheximide-treated cells in the presence of calphostin is partial (bar # 6 and 12 vs. 9 and 15). These findings therefore suggest that calphostin, unlike z-VAD-fmk, inhibits only PKC-mediated apoptosis in UV and cycloheximide-treated cells and PKC activation occurs not prior to but after caspase activation.

3.2. PMA enhances and calphostin reduces the late stages of stress-induced phosphorylation of eIF2α in *Sf9* cells

Phosphorylation of eIF2α is primarily a stress signal and can evoke adaptive and apoptotic responses. It can be a cause and consequence of caspase activation [14,15,17,30]. Earlier, we observed that eIF2α phosphorylation occurs prior to caspase activation in UV-irradiated cells [1]. Addition of z-VAD-fmk or infection by wt baculovirus that harbors an anti apoptotic gene like p35, mitigates the late stages of UV or cycloheximide-induced eIF2α phosphorylation effectively [1] suggesting that caspase activation can lead to eIF2α phosphorylation. Here we analyzed the effects of PMA and calphostin on the late and early stages of eIF2α phosphorylation in UV-irradiated, cycloheximide- or tunicamycin-treated *Sf9* cells in the presence and absence of z-VAD-fmk (Figs. 2 and 3).

An analysis of eIF2α phosphorylation reveals that none of the pharmacological agents PMA, calphostin or z-VAD-fmk can induce eIF2α phosphorylation (Fig. 3A, lanes 9, and 2) at the concentrations that we have used or can promote caspase activation and cell death (Fig. 1). UV-irradiation-induced eIF2α phosphorylation is

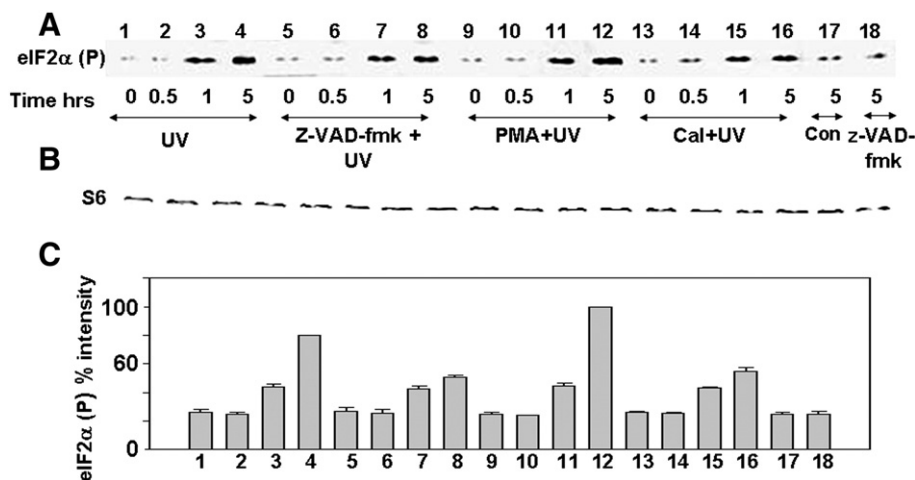


Fig. 2. Time course analysis of eIF2α phosphorylation. The effects of PMA, calphostin and z-VAD-fmk are studied in *Sf9* cells irradiated with UV light (200 J/m²) for 60 s and incubated for different time periods. eIF2α phosphorylation and levels of S6 are analyzed using phosphospecific anti-eIF2α and anti-S6 antibodies as mentioned in Section 2. Panel A represents the phosphorylation of eIF2α and panel B represents the levels of S6, 40S ribosomal protein, as loading control. The blot is a representative of three independent experiments. Panel C represents the percent intensity of eIF2α. The bar diagram represents mean ± standard error. * *P*<0.01. The numbers in various panels represent the following: 1–4, UV-treated; 5–8, UV + z-VAD-fmk; 9–12, PMA + UV; 13–16, cal + UV; 17, control (5 h) and 18, z-VAD-fmk (5 h).

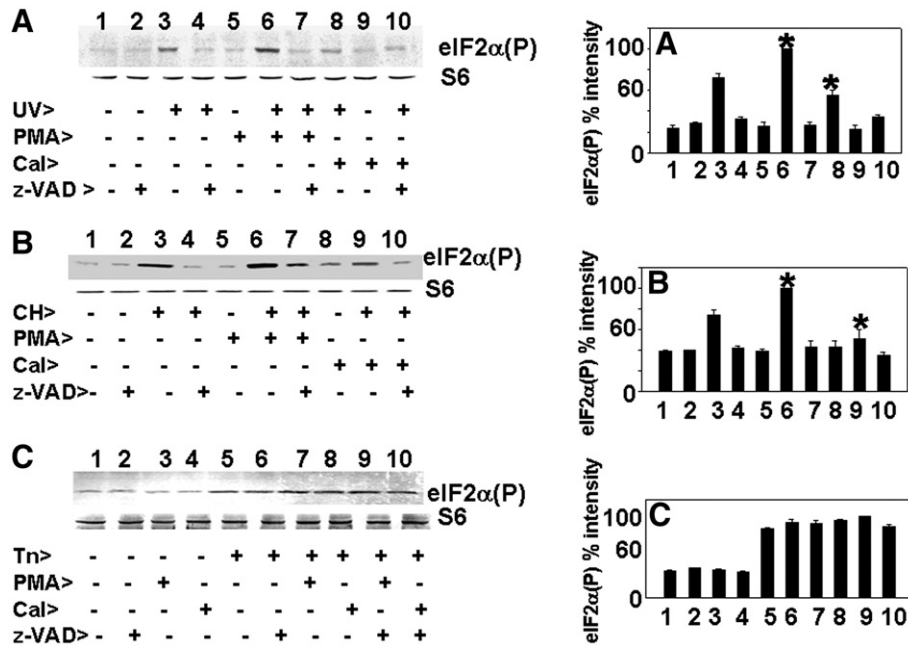


Fig. 3. Phosphorylation eIF2 α in Sf9 cells. eIF2 α phosphorylation and levels of S6 are analyzed using a phosphospecific anti-eIF2 α and anti-S6 antibodies in extracts prepared from cells exposed to various agents as mentioned in Section 2. The figures are western blots and densitometric graphs. The various panels represent the effects of PMA, calphostin or z-VAD-fmk on eIF2 α phosphorylation in cells treated with UV-irradiation (200J/m²), (panel A), 1 mM cycloheximide (panel B) and 12 μ M tunicamycin (panel C) and the corresponding bar diagrams are presented next to the blots. The blots are representative of three independent experiments. The bar diagram represents the percentage intensity of eIF2 α phosphorylation. The bar represents mean \pm standard error. Significance * $P < 0.01$ compared to UV-irradiation treatment in panel A, lane # 3, and cycloheximide in panel B, lane # 3 respectively. The lane numbers in bar diagrams correspond to the lane numbers in respective blots.

enhanced approximately to 45% within 1 h (Fig. 2, lane 3) and to 75% at 5 h (Fig. 2, lane 4). At 5 h, but not during the first hour, PMA is enhanced by about 20% (Fig. 2; lanes 12 vs. 4) and calphostin is reduced by 20% of UV-irradiation-induced eIF2 α phosphorylation (lanes 16 vs. 4). At 10 h (Fig. 3), the increase in UV-irradiation-induced eIF2 α phosphorylation by PMA (Fig. 3, panel A, lanes 6 vs. 3) or its decline by calphostin (lanes 8 vs. 3) is approximately 30% and 20% respectively suggesting that PMA enhances more efficiently the late stages of UV-irradiation-induced phosphorylation. A similar effect was also observed in cells treated with 1.0 mM cycloheximide (Fig. 3B). These findings therefore suggest that the late stages of UV-irradiation or cycloheximide-induced eIF2 α phosphorylation is partly contributed by PKC activation. In contrast, addition of z-VAD-fmk, a caspase inhibitor, reduces UV-irradiation-induced eIF2 α phosphorylation much more efficiently than calphostin at the 10 h time point than at 5 h and is unaffected during the first hour. Hence, PMA-stimulated eIF2 α phosphorylation presumably occurs after caspase activation than prior to caspase activation. This suggestion is consistent with the finding that tunicamycin-induced eIF2 α phosphorylation that does not lead to cell death is unaffected by PMA or calphostin or by z-VAD-fmk even in the late stages (Fig. 3C).

3.3. Cytochrome c stimulates caspase activation in cell-free extracts

To further study the caspase activation-mediated eIF2 α phosphorylation and its connection to PKC pathway, cell extracts devoid of their nuclei or depleted of their endogenous cytochrome c were prepared and treated with cytochrome c to determine whether caspase activity can be stimulated in such cell-free extracts.

Caspase activity is stimulated by the addition of cytochrome c and 1.0 mM ATP to both undepleted and cytochrome c depleted Sf9 extracts (Fig. 4A). ATP alone stimulates the caspase activity of the extracts which are not depleted of their endogenous cytochrome c. Addition of ATP alone however was unable to promote caspase activation in cytochrome c depleted extracts. This finding is consistent with the earlier observations that ATP facilitates the formation of

apoptosome complex and caspase activation in cytochrome c-treated extracts [25]. The caspase activated cell-free extracts are able to promote DNA laddering of isolated healthy nuclei of Sf9 cells (Fig. 4B).

A previous report demonstrated that PKR can be cleaved by purified caspases 3, 7 and 8 and the released kinase domain phosphorylates efficiently eIF2 α on its ser⁵¹ residue. We have also tested here the ability of Sf9 cells to process recombinant PERK, an ER-resident eIF2 α kinase in cell extracts to determine if it could serve as a substrate for active caspase(s) produced in UV-irradiated and cycloheximide-treated cells (Fig. 5A). Processing of purified recombinant PERK in these cell extracts was analyzed by anti-PERK-antibody which identifies both the full length and cleaved fragment of PERK in western blots. Recombinant PERK is cleaved efficiently in the extracts that are prepared from UV-irradiated (Fig. 5A, lanes 4 and 5) or cycloheximide-treated cells (lanes 8 and 9) than in untreated control extracts (lanes 2 and 3) or in etoposide (lanes 6 and 7) and tunicamycin-treated (lanes 10 and 11) extracts. The cleavage of PERK is found related to the caspase activity of the extracts. The cleavage of PERK is mitigated by Ac-DEVD-CHO, a caspase inhibitor, in UV-irradiated extracts that contained an active caspase (Fig. 5B, lanes 2–4 vs. 1).

Complementing these observations, the processing of PERK is also analyzed in cytochrome c depleted and undepleted extracts that are supplemented with cytochrome c and ATP (Fig. 5C) by monitoring the appearance of a processed PERK fragment and reduction in the full length protein by SDS-PAGE. Exogenously added PERK is cleaved in cytochrome c and ATP-treated cell-free extracts where caspase is active, but not in control extracts (Fig. 5C).

3.4. Cytochrome c-treated cell extracts display enhanced phosphorylation of eIF2 α

To determine the importance of caspase activation on phosphorylation of eIF2 α , extracts treated with or without cytochrome c are analyzed for eIF2 α phosphorylation (Fig. 6A upper panel) and levels of cytochrome c (middle panel). Levels of S6 have been used as loading controls (lower panel). Basal level of eIF2 α phosphorylation both in

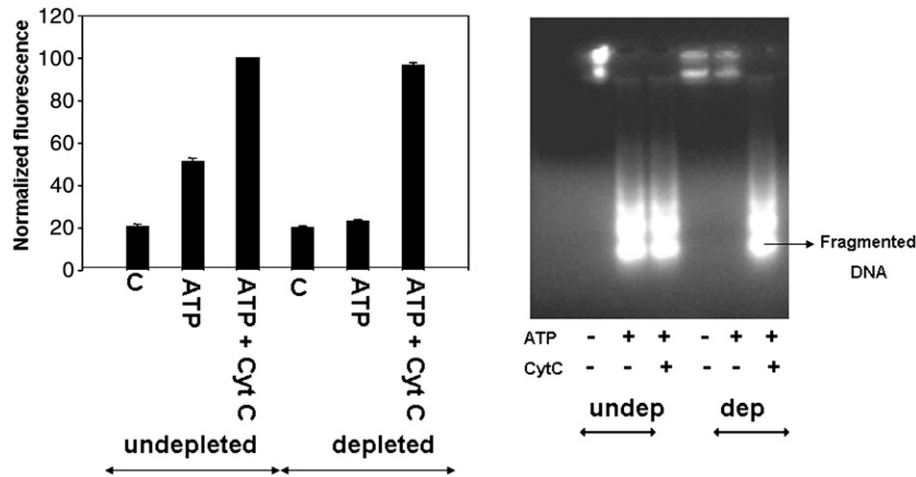


Fig. 4. Caspase activity and DNA fragmentation in cytochrome *c*-treated *Sf9* extracts. Panels A and B represent the caspase activity and DNA fragmentation in undepleted and cytochrome *c* depleted extracts respectively. The figures are representatives of three independent experiments. The bar represents mean \pm standard error.

undepleted and cytochrome *c* depleted extracts (Fig. 6A, lanes 1 and 4) is enhanced significantly in the presence of added cytochrome *c* and ATP (lanes 3 and 6 vs. 1 and 4). Addition of ATP alone to extracts containing endogenous cytochrome *c* also stimulated eIF2 α phosphorylation (lanes 2 vs. 1) more significantly than in cytochrome *c* depleted extracts (lanes 5 vs. 4). The corresponding levels of cytochrome *c*, in the middle panel correspond to caspase activation and eIF2 α phosphorylation. Presence of caspase inhibitor, z-VAD-fmk, abrogated cytochrome *c* and ATP-mediated eIF2 α phosphorylation almost completely (Fig. 6C lanes 5 vs. 3) suggesting that caspase activation is the primary cause for enhanced eIF2 α phosphorylation in cytochrome *c*- and ATP-treated extracts. The middle panel in Fig. 6C represents the levels of cytochrome *c* in cytochrome *c* depleted extracts.

3.5. PMA enhances and calphostin reduces cytochrome *c*-mediated caspase activity and eIF2 α phosphorylation

Cytochrome *c*-treated extracts display higher phosphorylation of eIF2 α as shown in Figs. 6 and 7 (lanes 3 vs. 1). Addition of PMA or

calphostin in the absence of added cytochrome *c* does not alter the phosphorylation status (Fig. 7A lanes 6 and 7 vs. lane 1 and the corresponding bar diagram). However, PMA enhances (lanes 4 vs. 3) and calphostin reduces cytochrome *c*-mediated eIF2 α phosphorylation (lanes 5 vs. 3) in extracts. PMA-treated cytochrome *c*-supplemented cell extracts display enhanced PERK processing (Fig. 7C lane 10) and calphostin reduces the PERK processing (lane 14) compared to cytochrome *c*-treated extracts (lane 6). These findings therefore suggest that caspase activation by cytochrome *c* can lead to both eIF2 α kinase and PKC activation in cell extracts. Together, these activities can further enhance caspase activation and cell death.

4. Discussion

Cell suicide and survival are mediated by complex signaling pathways. Although not very many studies are carried out, few recent studies suggest that eIF2 α phosphorylation, primarily a stress signal, is a cause and consequence of caspase-3 activation [1,16,18,20,30]. Protein kinase C isozymes, a family of serine-threonine kinases, also

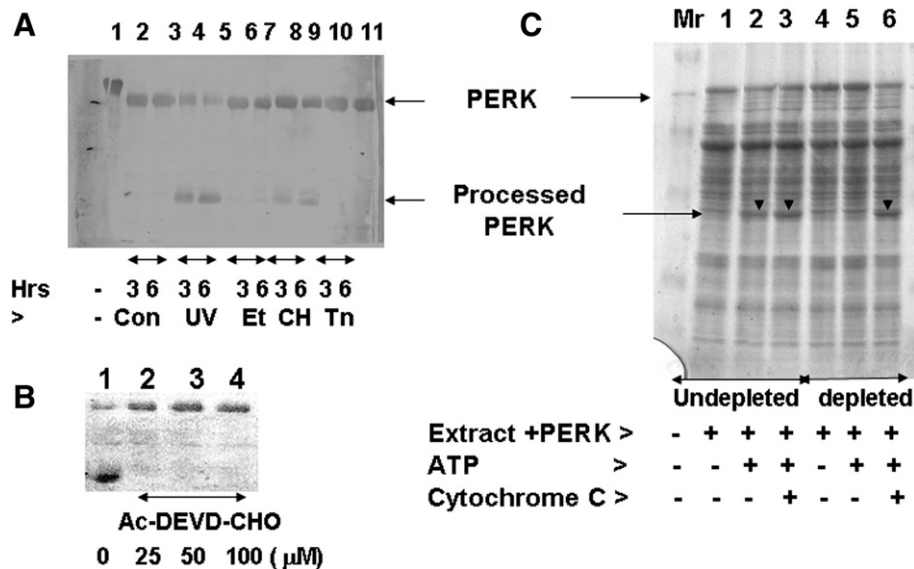


Fig. 5. PERK cleavage. PERK cleavage assay was performed in cell extracts (~ 150 μ g of protein) prepared from cells treated with UV, etoposide, cycloheximide and tunicamycin (panel A) as mentioned in Section 2. PERK cleavage was studied in the presence of different concentrations of Ac-DEVD-CHO, a caspase inhibitor in cell extracts prepared from UV-irradiated cells (panel B), and also in cell-free extracts (~ 150 μ g of protein) that were treated with cytochrome *c* and ATP (panel C). Panels A and B are western blots. Panel C is a stained gel. The figures are representative of three independent experiments.

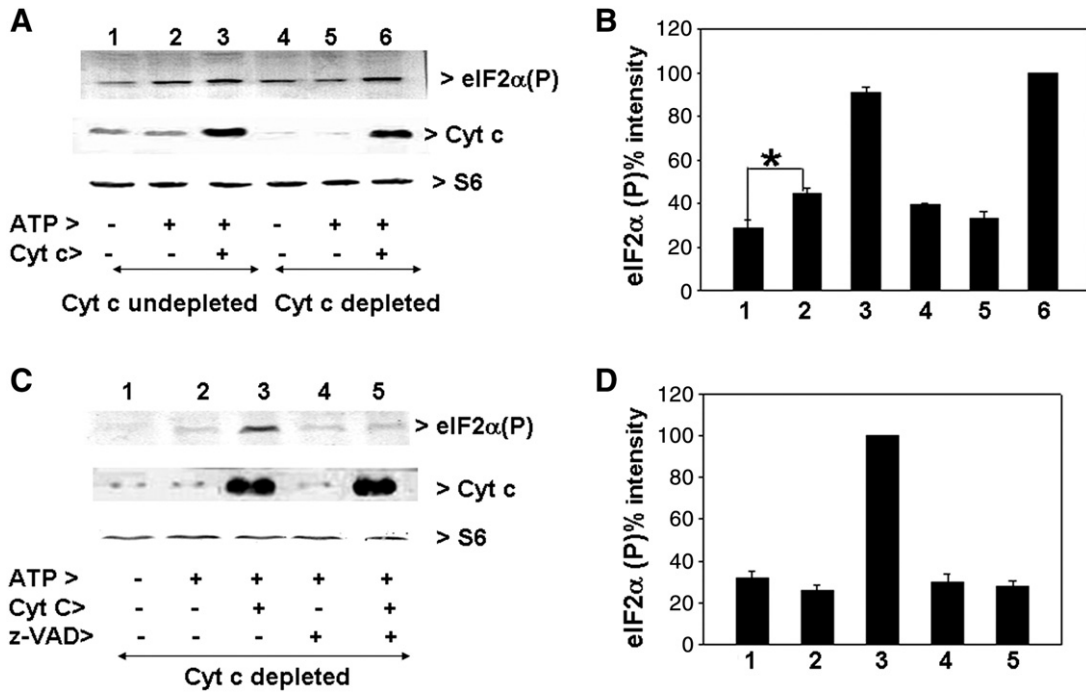


Fig. 6. Phosphorylation of eIF2α in cytochrome c-treated cell-free extracts. Phosphorylation of eIF2α is analyzed in undepleted and cytochrome c depleted cell-free extracts that are treated with 50 μM cytochrome c and 1.0 mM ATP (panel A). Phosphorylation is also monitored in cytochrome c depleted extracts treated with cytochrome c and ATP in the presence and absence of z-VAD-fmk (panel C). The levels of Cyt c and S6 are also studied. Panels B and D represent the corresponding cytochrome c levels respectively. The figures are western blots and are representatives of three independent experiments. The bar diagrams represent the percent intensity of phospho eIF2α. The lane numbers in bar diagrams represent the lane numbers in corresponding blots. The bar represents mean ± standard error. * P<0.01.

play a role in cell survival and death [26] like eIF2α kinases. PKCδ [27], like PKR, one of the eIF2α kinases, is found to be a substrate for caspases. Although phosphorylation of eIF2α occurs in response to

different stressors, the mechanisms leading to cell death and survival are not well understood and very little information is available on the coincident signaling activities. UV-irradiation or DNA damage is

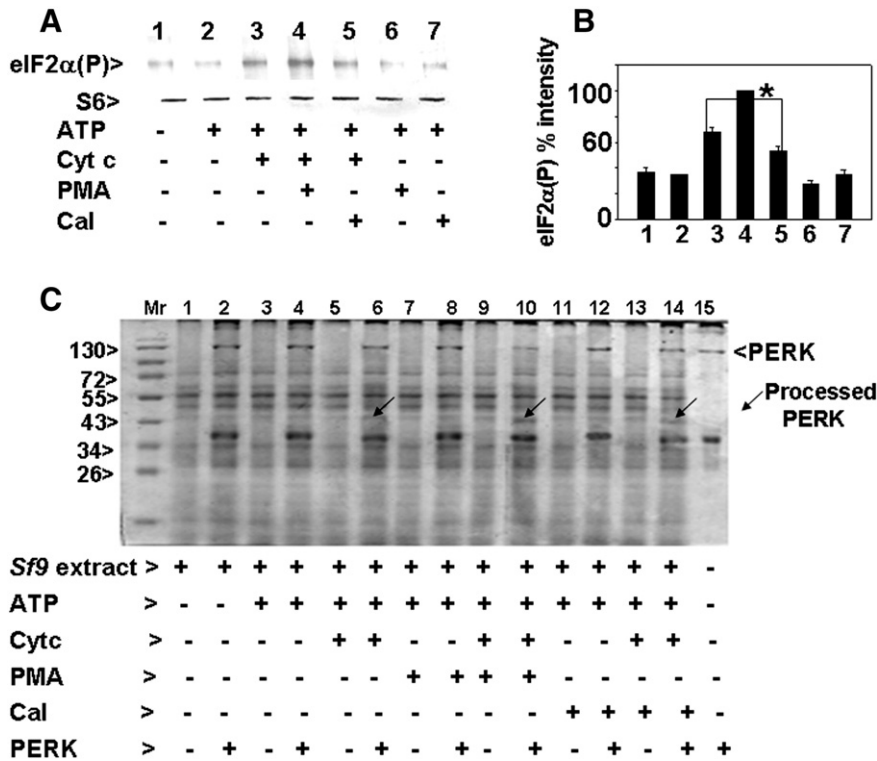


Fig. 7. Effect of PMA and calphostin on cytochrome c-treated Sf9 extracts. Effect of PMA and calphostin on PERK cleavage and phosphorylation of eIF2α is studied in cytochrome c depleted cell-free extracts supplemented with cytochrome c. Panel A represents the phosphorylation of eIF2α and levels of S6, and panel B represents corresponding densitometric graph. The blot is representative of three independent experiments and the bar represents the percent intensity of phospho eIF2α * P<0.01. Panel C represents PERK cleavage in these extracts. Panel A is a western blot and panel C is a coomassie stained gel.

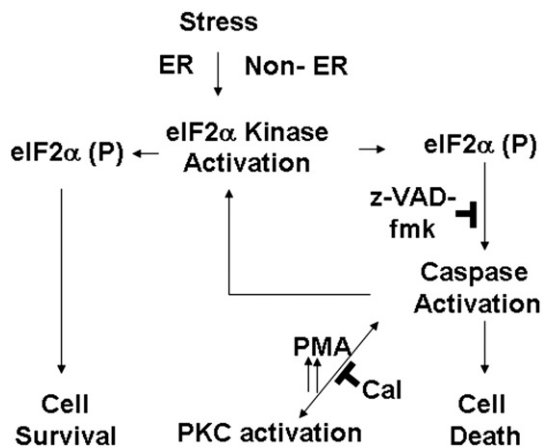


Fig. 8. Model depicting contribution of caspase-mediated PKC activation to eIF2 α phosphorylation. Analyses of non-ER-stress-induced eIF2 α phosphorylation in *Sf9* cells or cytochrome *c*-induced eIF2 α phosphorylation in cell-free extracts suggests that caspase activation leads to enhanced eIF2 α phosphorylation. PMA, an activator of PKC enhances caspase-mediated eIF2 α phosphorylation. Caspase-mediated eIF2 α phosphorylation is mitigated partially by calphostin and completely by z-VAD-fmk thereby suggesting that caspases can activate multiple signaling pathways.

known to elicit both PKC activation, phosphorylation of eIF2 α and cell death in different cell types. Hence here we have studied the connection, if any, between these two pathways in *Sf9* cells which are natural hosts of baculovirus.

Consistent with our previous studies [1] non-ER stressors that are characterized by lack of BiP expression [30] as in the case of UV-irradiation and cycloheximide treatments promote phosphorylation of eIF2 α and cell death. Tunicamycin, a typical ER stressor, promotes eIF2 α phosphorylation but not cell death (Fig. 1). Non-ER stress-induced cell death is related to the caspase activity which is estimated by Ac-DEVD-AFC hydrolysis (Fig. 1) and also to the processing of PERK, an ER-resident eIF2 α kinase, in such cell extracts (Fig. 5A and B). Time course analysis of eIF2 α phosphorylation reveals that z-VAD-fmk, a pan caspase inhibitor mitigates the late stages (at 10 h) (Fig. 3A and B), but not the early stages (Fig. 2) of UV-irradiation-induced eIF2 α phosphorylation thereby suggesting that caspase activation which follows eIF2 α phosphorylation can also lead to enhanced eIF2 α phosphorylation in a feed back loop. The fact that eIF2 α phosphorylation in non-ER stressed conditions stimulates caspase activation is further substantiated by the observation that tunicamycin-induced eIF2 α phosphorylation does not lead to caspase activation and is not affected by z-VAD-fmk (Fig. 3C).

Addition of PMA, a pharmacological activator of PKC, stimulates UV-irradiation or cycloheximide-induced eIF2 α phosphorylation and cell death but does not affect tunicamycin-induced eIF2 α phosphorylation thereby suggesting that caspase activation stimulates PKC activation and further enhancement in eIF2 α phosphorylation. This notion is further supported by the finding here that PMA enhances very efficiently and calphostin, an inhibitor of PKC, reduces albeit less efficiently the late stages but not the early stages of UV-irradiation-induced eIF2 α phosphorylation than the caspase inhibitor z-VAD-fmk. These findings therefore suggest that calphostin inhibits PKC activation that occurs in response to caspase activation mediated by eIF2 α phosphorylation. Unlike z-VAD-fmk, which inhibits caspase activation directly and efficiently, calphostin reduces caspase activation that is stimulated by PKC and thereby eIF2 α phosphorylation. The above results are further substantiated by the observations that cytochrome *c*-mediated-caspase activation in cell-free extracts as analyzed by Ac-DEVD-AFC hydrolysis (Fig. 4A), DNA fragmentation of healthy nuclei (Fig. 4B), and the cleavage of PERK (Figs. 5C and 7C), leads to eIF2 α phosphorylation (Fig. 6A and C) which is further enhanced by PMA and reduced by calphostin (Fig. 7A).

A recent study suggests that eIF2 α phosphorylation, which is known to inhibit general protein synthesis, however upregulates translation of PKC η [28] as other gene-specific mRNAs like ATF4. However, its ability to stimulate cell death is not clear because, we could study the PKC-regulated caspase activation in cell-free extracts. Among PKCs, PKC δ is implicated in cell death in mammalian cells in response to UV-irradiation. It is also processed and activated by caspase activation [22,27]. UV-irradiation is shown to activate GCN2 (general control non-repressible), one of the members of eIF2 α kinases in mammalian cells [29]. However, neither the PKCs nor eIF2 α kinases have been characterized in *Sf9* cells. Our studies point out that non-ER stress-induced eIF2 α phosphorylation-mediated caspase activation can enhance PKC activation, eIF2 α phosphorylation (in a feedback manner) and subscribes to the notion that caspases can activate multiple signaling pathways to enhance cell death as described in our model (Fig. 8).

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