Apoptosis in Uninfected and Baculovirus infected *Sf9* cells: Phosphorylation of alpha subunit of Eukaryotic Initiation Factor 2 (eIF2a)

Thesis submitted for the degree of
Doctor of Philosophy

By

S. Aparna

(RegdNo.98LBPH07)

Department of Biochemistry
School of Life sciences
University of Hyderabad
Hyderabad 500 046, INDIA

March, 2004

To

My Parents

And

Late Father-in-law

Department of Biochemistry
School of Life sciences
University of Hyderabad
Hyderabad 500 046, INDIA

DECLARATION

I hereby declare that the work presented in this thesis *entitled "Apoptosis in Uninfected and Baculovirus infected Sf9 cells: Phosphorylation of alpha subunit of eukaryotic initiation factor 2 (eIF2a)"* has been carried out by me under the supervision of Prof. K. V. A. Ramaiah and this has not been submitted for any degree or diploma of any university.

25/2/2004

Prof. K. V.A. Ramaiah

Supervisor

K.V.A. RAMAIAH, Ph.D.
PROFESSOR
DEPT. OF BIOCHEMISTRY
UNIVERSITY OF HYDERABAD
HYDERABAD - 500 046. INDIA

S. Aparna

SomajAparna

Candidate

Department of Biochemistry
School of Life sciences
University of Hyderabad
Hyderabad 500 046, INDIA

CERTIFICATE

This is to certify that Soma Aparna has carried out the work in the present thesis under my supervision for a full period prescribed under the Ph. D. ordinances of the University. I recommend this thesis entitled *"Apoptosis in Uninfected and Baculovirus infected Sf9 cells: Phosphorylation of alpha sub unit of eukaryotic initiation factor 2 (eIF2a)"* for submission of the award of the degree *Doctor of philosophy* of this university.

25/3/2004
Prof. K. V. A. Ramaiah
Supervisor

K.V.A. RAMAIAH, Ph.D.
PROFESSOR
DEPT. OF BIOCHEMISTRY
UNIVERSITY OF HYDERABAD
HYDERABAD - 500 046. INDIA

25/3/04
Head I/c
Department of Biochemistry

HEAD
DEPARTMENT OF BIOCHEMISTRY
SCHOOL OF LIFE SCIENCES,
UNIVERSITY OF HYDERABAD,
HYDERABAD-500 046. (A.P.)

Prof. A.S. Raghavendra
Dean

Dean
School of Life Sciences
University of Hyderabad
Hyderabad-500 134. (India)

School of Life Sciences
University of Hyderabad

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Acknowledgements

I express my deep sense of gratitude to my supervisor and mentor, Prof. K. V. A. Ramaiah for his valuable guidance and support all through my Ph. D. work.

I am thankful to Prof. A. S. Raghavendra, Dean, School of Life Sciences and Prof. C. K. Mitra, Head, Department of Biochemistry for providing the necessary infra-structure to carry out this work. I also thank Prof. Subba Rao and Prof. T. Suryanarayana, former Heads, Department of Biochemistry.

I am grateful to Prof Seyed E. Hasnain, Director. CDFD and Dr. Abani K. Bhuyan, Dept of Chemistry for providing p35 deletion mutant baculovirus and for assisting me in carrying out caspase assays respectively. I am thankful to Dr. Sudhir Sahdev for helping me in the cloning work.

I am thankful to Dr. Randal Kaufman. Howard Huges Medical Institute for providing me the PERK antibody and I am thankful to Dr. David Ron, Skirball institute of Molecular Medicine, New York for providing PERK-GST, plasmid.

I am thankful to my friends of school of life sciences, Ms. Padmavati, Zeenath, Drs. Sreelakshmi, Radha and Sangeetha. I am especially thankful to Mr. Sai Krishna. I am thankful to past and present members of my lab, Drs. Sudhakar, Lakshminarayana and Rahsekhar, Hussain, Rajesh and Pushpanjali. I also thank many other colleagues of school of life sciences.

I am thankful to all the technical staff of the School of Life Sciences.

I am greatly indebted to my parents for their support and motivation with out which this work would not have been possible. I am ever grateful to my husband for his encouragement and patience. I am thankful to my mother-in-law for her understanding and caring. I am thankful to my brother and sister for their affection.

I wish to acknowledge CSIR, New Delhi for financial support for my Ph. D. work and for awarding the travel grant to attend the international conference on Translational Control meeting, 2002 at New York.

Aparna

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
- A1F : Apoptosis inducing factor
- AP : Alkaline phosphatase
- Apaf-1 : Apoptosis protein activating factor
- ATF_4 : Activated transcription factor
- ATM : Ataxia telangiectasia mutated protein
- ATP : Adenosine 5' tri phosphate
- Bad : Bcl-2/Bcl-XL associated death promoter
- Bak : Bcl-2 homologous antagonist killer
- Bax : Bcl-2 associated X-protein
- BCIP : 5-bromo-4-chloro-3-indoyl-phoshphate
- Bcl-2 : B-cell CLL
- BH : Bcl-2 homology domain
- Bid : BH3 interacting death domain agonist
- BIM : Bcl-2 interacting mediator
- Bip : Immunoglobulin heavy chain binding protein
- BIR : Baculovirus IAP repeat
- BOD : Bcl-2 related ovarian death agonist
- BOK : Bcl-2 related ovarian killer
- BOO : Bcl-2 homologue of ovary
- CAD : Caspase activated DNase
- c-AMP : cyclic- adenosine mono phosphate
- CARD : Caspase activation recruitment domain
- CAT : Chloramphenicol acetyl transferase
- Ced : Cell death

- Met : Methionine
- mg : milligram
- MOI : Multiplicity of infection
- NACa : Nascent associated complex a
- NAIP : Nucleotide binding apoptosis inhibitor protein
- NBT : Nitro Blue Tetrazolium
- NF-kB : Nuclear factor-kappa B
- NP : Nucleo capsid protein
- Omi : Outer mitochondrial IAP binding protein
- OPIAP : Orgyia pseuditsugate IAP
- PABP : Poly A binding protein
- PAGE : Poly acry lam ide gel electrophoresis
- PAK2 : P21 activated kinase-2
- PARP : Poly (ADP-Ribose) Polymerase
- PDI : Protein disulphide isomerase
- PERK : Pancreatic endoplasrnic-resident eIF2α kinase
- PK cδ : Protein kinase c delta
- PKR : Double-stranded RNA-dependent eIF2a kinase
- PP2A : Protein phosphatase 2 A
- PUMA : p53 up regulated modulator of apoptosis
- ROCK : Rho GTPase activated serine threonine kinase
- Rpr : repear
- S51A : eIF2a mutation of ser^{51} eIF2a to Ala
- S51D : eIF2α mutation of ser^{51} eIF2α to Asp
- SDS : Sodium dodecyl sulphate
- *Sf9* : *Spodoptera frugiperda*
- Smac : Second mitochondria derived activator of caspase
- SRP : Signal recognition particle
- TEMED : N',N',N',N'-tetra ethyl methyl ethyl diamine
- Tg : Thapsigargin

- ER : Endoplasmic reticulum
- ERK : Extra cellular regulated kinase
- ERP : Endoplasmic reticulum protein
- Et : Etoposide
- FADD : Fas associated death domain
- FCS : Fetal calf serum
- FKB13 : FK506 binding protein
- FLICE : Fas associated death domain-like interleukin converting enzyme
- FLIP : FLICE inhibitory protein
- GADD : Growth arrest DNA damage
- GCN2 kinase : General nonderepressible kinase
- GRP : Glucose response protein
- GSH : Reduced glutathione
- GST : Glutathione S-transferase
- HID : Head involution defective
- HRI kinase : Heme regulated inhibitor kinase
- HSP : Heat shock promoter
- Htr A2 : High temperature requirement protein A2
- IAP : Inhibitor apoptosis of proteins
- IC AD : Inhibitor of caspase activated DNase
- ICE : Interleukin converting enzyme
- IFN : Inteferons
- IGL : Insulin growth factor
- IkB : Inhibitor of kappa B
- IL : Interleukin
- IREs : Internal ribosome entry sites
- JNK : c-JUN-NH_2 terminal kinase
- KI : Kinase insertion
- MAPK : Mitogen activated protein kinase

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- SRP : Signal recognition particle
- TEMED : N',N',N',N', N'-tetra ethyl methyl ethyl diamine
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- Tn : Tunicamycin
- TNFR-1 : Tumor necrosis factor receptor-1
- TNFa : Tumour necrosis factor -alpha
- TRAF : TNF receptor adaptor factor
- TRAIL : TNF-related apoptosis inducing ligand
- TSP : Thrombospondin
- UPR : unfolded protein response
- UV light : Ultra violet light
- wt : wild type
- XIAP : X-linked Inhibitor of apoptosis
- Z-IETD-fmk : N-carbobenzoxy-isoleucine-glutamine-[O-methyl]-threonine-apartyl-[O-methyl]-fluoromethtylketone
- z-VAD-fmk : N-carbobenzoxy-valyl-alanvl-aspartyl-[0-methyl]-fluoromethylketone
- μg : microgram

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1. An Overview of eukaryotic protein synthesis: Proteins, polymers of amino acids, occupy a large fraction of cells of an organism. They play important roles in cellular functions such as transport, catalysis, structure and regulation. Hence a large proportion of cell resources are devoted to synthesizing proteins.

Protein synthesis or translation of nucleotide sequences in messenger RNA (m RNA) to the corresponding amino acid sequence in protein is the final step in gene expression. Similar to RNA synthesis from a DNA template by a process called transcription, translation is also a key control point for regulation. Soon after articulation of central dogma of molecular biology (Watson and Crick, 1953), Jacob and Monad pointed out that 'the synthesis of individual proteins may be provoked or suppressed within a cell, under the influence of specific external agent and the relative rates at which different proteins are synthesized may be profoundly altered, depending on external conditions' (Jacob and Monad, 1961). Indeed they pointed out that such regulation 'is absolutely essential for survival of the cell'. Scant attention was focused on the efficiency of utilization of mRNA, in specifying protein synthesis in bacterial cultures. Subsequent studies in developing embryos, reticulocytes, virus and phage-infected cells and higher eukaryotic cells responding to a varied stimuli ranging from heat to hormones and starvation to mitosis-have clearly identified regulatory mechanisms in the control of protein synthesis and thus the term 'Translational control' was coined as early as in 1970.

Translational control forms a part of cellular defense and cell survival. Regulation of translation in the pathway of gene expression is economical in energetic terms and may provide a means of fine control. In some systems like reticulocytes, oocytes, RNA viruses, there is very little opportunity to control the gene expression at transcriptional level. Similarly, in the development of embryos where concentration gradients of protein determine the early development, translational control appear to play an important role.

Protein synthesis requires specific machinery similar to DNA replication and RNA synthesis, and cannot be substituted by other components coming from any other

biological processes. For convenience, this multistep and muitifactorial pathway is divided into a) initiation b) elongation and c) termination.

1.1 Initiation step in protein synthesis: One of the important prerequisites for the initiation of protein synthesis is the availability of free ribosomal subunits 40S and 60S. At the end of protein synthesis, the 80S ribosomes are dissociated into their respective subunits. The availability of 40S subunits to join the charged initiator tRNA is dependent on anti-association factors such as eIF1A, elF3 and eIF6 (Chandhuri et al., 1997, 1999; Si and Maitra, 1999). Binding of these factors to 40S subunit will prevent the joining of 60S subunit. The joining of charged initiator tRNA (Met-tRNAi) to 40S subunit is then mediated by GTP and translational initiation factor (eIF2). The latter is a heterotrimeric protein with three subunits a (~36 kDa). β (~38 kDa) and y (~59.1 kDa). eIF2 distinguishes initiator tRNA from elongator tRNA by the presence of methionyl residue and A-U base pair at the end of the acceptor stem. In physiological conditions, eIF2 has a higher affinity for GDP than for GTP. However the joining of Met-tRNAi to eIF2 and then to 40S requires the presence of GTP and this joining reaction is inhibited by GDP. The exchange of guanine nucleotides (GNE) (GDP for GTP) on eIF2 is catalyzed by a heteropentameric protein called eIF2B (Webb and Proud. 1997).

Phosphorylation of a small portion (~ 30%) of eIF2a by eIF2a kinases promotes a tight complex formation between eIF2 and elF2B in which the GNE activity between eIF2 and elF2B becomes non-functional (Sudhakar et al., 2000; Babu and Ramaiah, 1996; Pavitt et al.. 1998). In the absence of functional eIF2B, eIF2 cannot join the initiator tRNA. Functional 43S complex (eIF3-eIFl A 40S-eIF2-GTP-Met-tRNAi) will then join to 5' end of mRNA. Most of the eukaryotic mRNAs with few exceptions, contain an m^{7}G (methylated guanosine) cap at the 5' end and poly A tail at the 3' end. These features play an important role in mRNA translation, stability, transport and in mRNA processing. The joining of 43S complex to mRNA requires a heterotrimeric eIF4F complex. It consists of eIF4G a scaffolding protein (~171-176 kDa in mammals) that brings together other components of the initiation pathway and eIF4E (-25 KDa in mammals), a protein that recognizes the 5' cap structure. In addition, the eIF4F complex consists of eIF4A (~46

kDa in mammals), a RNA helicase that is capable of unwinding the secondary structure in mRNA (Conroy et al., 1990; Morino et al., 2000). The RNA helicase activity of eIF4A is stimulated by eIF4B and 4H. Poly A binding protein (PABP1) binds to poly A and also affects translation of mRNAs. The formation of eIF4F complex (4G-4A-4E) is modulated by translation repressor proteins called eIF 4E-Bps (~12 kDa) (4E binding proteins). After the formation of 48S initiation complex (mRNA-eIF4E-eIF1-eIF3-eIF2-GTP-MetRNAi). the 40S subunit scans down the mRNA to identify the 'start' AUG site (Kozak, 1989). This scanning process requires ATP. On rare instances, the ribosome instead of scanning mRNA from 5` end of mRNA, hops from one region to another by a process called 'shunting or repositioning' (La Torre et al.. 1998) or bypasses the start codon that is present in a poor context and starts initiation at another AUG which is present in a more favorable context through a mechanism called leaky scanning (Kozak, 1989).

The 48S complex thus formed will now join with the 60S subunit to form the 80S initiation complex. Prior to joining the 60S subunit, the protein factors bound to 48S complex have to be released. The release of these factors is helped by GTP hydrolysis induced by eIF5 (58~62 kDa in mammals) and eIFB (~125~168 kDa in mammals) (Chakravarti et al., 1993; Pestova et al., 2000). eIF5 induces hydrolysis of eIF2 bound GTP on 48S complex (Chakravarti and Maitra, 1993) but differs from eIF5B in that, it is not a ribosome dependent GTPase. The physiological function of eIF5B is understood when eIF5 is found insufficient to promote joining of 60S subunits to 48S complex that has been assembled as a natural mRNA using a complete set of factors (Pestovao et al., 2000). The formation of SOS initiation complex with the Met-tRNAi properly positioned on 'start' AUG marks the end of initiation.

1.2 Elongation cycle in protein synthesis: The elongation cycle has three important steps a) joining of amino acylated tRNA to the A site, b) peptide bond formation between adjacent amino acids and c) the translocation of ribosome. Depending on the sequence in mRNAs, the ribosome will add one amino acid at a time to the growing polypeptide chain. Eukaryotic elongation cycle requires three factors namely eEFIA (~50.1 kDa),

eEF1B with three subunits **a** (~24.8 kDa), β (~31.1 kDa) and y (~50 kDa) and eEF2 (~95.1 kDa) which are required to a) facilitate the joining of amino acylated tRNAs b) induce GTPase activity and facilitate the release of eEF1A. and c) in the movement of mRNA by three nucleotides from the A site to P site. respectively.

1.3 Termination of protein synthesis: Termination of protein synthesis occurs when the ribosome reaches the stop codon. In eukaryotes. release factor 1 (eRF1) recognizes all three stop codons in mRNAs. A second release factor eRF3 stimulates eRFl activity in a GTP dependent fashion (Frolova et al.. 1996; Zhouravleva et al.. 1995). eRFl protein appears equivalent to the aminacyl group attached to the CCA-3' sequence found in the amino acyl stem of a tRNA molecule (Song et al., 2000). The eRFl protein is thought to bind to the A site of the ribosome so that the Gln-185 residue of GGQ motif is in coordination with a water molecule and can modulate a nucleophile attack on the ester bond of the peptidyl t RNA molecule in the P site. resulting in the hydrolysis of nascent polypeptide chain. The occurrence of GGQ motif in all prokaryotic and eukaryotic release factors. despite the overall low sequence similarity. may reflect a similar mechanism of translation termination reaction.

1.4 Regulation of protein synthesis: Protein synthesis is a complex, multistep and multi-factorial process. in such pathways. it is always economical for the cell to control the pathway at the onset than interrupt some where in the middle. Consistent with this idea. several examples are known where protein synthesis is controlled at the initiation level than at elongation and or termination. However regulation of protein synthesis is also blocked at the elongation level as a safety measure to halt further peptide chain initiation. The best studied examples of regulation during elongation phase are found in the synthesis of secretory proteins where binding of signal recognition particle (SRP) to the signal sequence of secretory mRNAs halts peptide chain elongation (Blobel and Sabatini. 1971; Walter and Blobel, 1981).

In the initiation step of protein synthesis, regulation can occur a) at the level of met-tRNAi binding to 40S subunits. or at b) at the level of binding of ribosomes and or

proteins to the 5' and 3' ends of mRNA. In both cases, regulation is mediated chiefly through phosphorylation-dephosphorylation of translational initiation factors or regulatory proteins that bind to the 5' and 3' ends of mRNAs. The amount of information present on various aspects of translational regulation is enormous. Keeping in view of the Ph. D. work reported here it is felt to confine essentially to the regulation of protein synthesis that is mediated by changes in the phosphorylation of eIF2a by a variety of eIF2α kinases and the importance of elF2a phosphorylation in gene-specific regulation, in apoptosis and in various other cellular disorders.

1.5 Regulation of protein synthesis and **eIF2α phosphorylation**: A number of physiological conditions and environmental stresses such as heme-deficiency, virus infection. amino acid starvation, accumulation of unfolded proteins in the endoplasmic reticulum (ER), heavy metal stress, oxidizing conditions, nitric oxide. exercise. apoptosis, nutrient deprivation and many other stress conditions are known to activate eIF2a kinases and stimulate the phosphorylation of serine 51 residue in eIF2α. Indeed gradually it is becoming clearer that it is a stress signal. Phosphorylation of eIF2a promotes a complex formation between eIF2 and the guanine nucleotide exchange factor, eIF2B in which eIF2B loses its activity to exchange guanine nucleotides on eIF2. These events lead to an inhibition of protein synthesis either globally or in gene specific manner (Hinnebusch, 2000; Krishnamoorthy et aL, 2001). In addition to eIF2a phosphorylation, phosphorylation of the s-subunit of elF2B also regulates its own activity and there by elF2 activity (Webb and Proud. 1997) or its ability to join the initiator tRNA and 40S ribosomes. Recent studies further suggest that changes in eIF2 phosphorviation play important roles in glucose metabolism (Sonenberg and Newgard, 2001), malignancy and cell death (Koromilas et aL 1992; Donze et al.. 1995; Srivastava et aL, 1998). Also deregulation of elF2 function because of mutations in the guanine nucleotide exchange factor. eIF2B, can result in 'leukoencephalopathy' with vanishing white matter (VWM), an inherited brain disease (Leegwater et al., 2001). In addition, eIF2a phosphoryiation may play an important role in the regulation of several diseases that occur due to the accumulation of unfolded proteins (please see the section on eIF2a kinases).

2. eIF2a kinases and their role in apoptosis: A family of four serine/threonine kinases have been found to phosphorylate the serine 51 residue in the α-subunit of eIF2. These are i) heme-regulated inhibitor (HRI), ii) double stranded RNA-dependent protein kinase (PKR). iii) general control non-derepressible kinase 2 (GCN2) and iv) pancreatic eIF2a kinase that resembles PKR (PERK or PEK) (Dever, 2002). Although all these four kinases are known to phosphorylate serine 51 residue in eIF2a. their regulation and regulatory mechanisms are different. Heme regulated inhibitor kinase is activated when heme concentrations in maturing red blood cells become too low, switching off synthesis of globin. PKR is activated by low concentrations ($ngml^{-1}$) of double-stranded RNA produced during viral infection. Amino acid starvation that leads to the accumulation of uncharged tRNAs activates GCN2 kinase whereas. perturbed protein folding in the endoplasmic reticulum activates PERK/PEK. All these kinases contain the conserved 1-X1 domains of serine/threonine kinases and share sequence homology in their catalytic domains but differ in their regulatory domains. eIF2a kinases, in addition contain a kinase insertion (KI) sequence between the two halves of the conserved domains i.e.. between 1V and V kinase domains (Chen and London, 1995). In HRI, GCN2 and PERK the KI is about 120 amino acids while in PKR it is about 40 amino acids long. The last 20 amino acids of KI sequence of all the eIF2a kinases share significant homology suggesting a functional role. This sequence at the end of the C-terminus of Kl region is required for substrate recognition. Special sequences are found in each of this eIF2a kinase that are important in the regulation. These are for example the amino terminal 160 amino acids of PKR which contains two dsRNA binding motifs, rich in basic amino acids. Similarly GCN2 contains 530 amino acid residues at the C-terminus that has a sequence homology to histidyl tRNA synthetase. PERK has an N-terminal ER luminal domain that can sense the stress in ER. In contrast, the amino terminal region in HRI is required for stable heme-binding.

2.1 HRI kinase: Heme-regulated inhibitor is the first eIF2a kinase that was discovered in heme-deficient rabbit reticulocytes and in their lysates. Heme-deficiency causes inhibition of protein synthesis with disaggregation of polyribosomes in cell-free

transiational systems prepared from anemic rabbit reticulocytes (Hunt et al., 1972). Addition of hemin restores protein synthesis in such lysates. The inhibition of protein synthesis is accompanied by increased phosphorylation of eIF2a in lysates. Subsequently the cDNA coding HRI kinase was identified (Chen et al.. 1991). Purified HRI protein has been found active *in vitro* in phosphorylating eIF2a. Addition of hemin inhibits auto kinase and hetero kinase activities of HRI *in vitro*. From a variety of studies, it has been proposed that heme promotes inter subunit disulfide-bond formation and thus regulates the HRI kinase activity (Chen et al., 1989, Yang et al., 1992, Chefalo et aL, 1998). HRI protein and mRNA are expressed however predominantly in immature erythroid cells. In addition, HRI mRNA levels are increased during erythroid differentiation of mouse erythroleukemina (MEL) cells, and the increase in dependent on heme presence (Crosby et al., 1994). Using gene knock out studies. Chen's group has established that HRI is expressed predominantly in erythroid cells and it regulates the synthesis of both a and β globins in red blood cell precursors by inhibiting the general transiational initiation factor eIF2 (Han et 1., 2001). This inhibition occurs when intracellular concentration of heme declines thereby preventing the synthesis of globin peptides in excess of heme. The globin proteins are found aggregated within red blood cells and its precursors, resulting in a hyperchormin, normocytic anemia with decreased RBC counts, erythroid hyperplasia and accelerated apoptosis in bone marrow and spleen in mice lacking both copies of HRI genes and are suffering from iron deficiency (Han et al.. 2001). Hence HRI kinase is found to be a physiological regulator of gene expression and cell survival in the erythroid lineage. Forced expression of HRI in 3T3 cells results in loss of cell proliferation and cell death. These two effects can be counteracted by over expression of nonphosphorylatable form of eIF2a (S51A) thereby suggesting that these effects are mediated by eIF2a phosphorylation (Crosby et al.. 2000).

2.2 PKR kinase: PKR is a double stranded RNA (dsRNA) dependent eIF2α kinase. Its transcription is induced by type I interferons (Samuel et al., 1997). Unlike other eIF2α kinases, PKR phosphorylates other substrates like IκB (inhibitor of NF-κB) (Bonnet et al.. 2000). PKR is ubiquitously found in all mammalian tissues. Although some earlier reports suggested that PKR-like kinase is also present in plants (Langland et al.. 1995;

1996) and in lower eukaryotes like insects (Chong et aL, 1992; Barber et aL, 1992), the recent genomic evidence is against it. In addition to its role in translational regulation, PKR also plays a role in cell cycle, differentiation, cell growth and apoptosis (Kaufman, 1999a). Abrogation of PKR or eIF2α phosphorylation leads to malignancy or transformation in cultured cells (Donze et al., 1995). Complementing these observations expression of catalvticaily inactive mutant PKR transforms NIH-3T3 cells to form tumors in nude mice and promotes growth in the Hela cells (Donze et al., 1995; Koromilas et al., 1992; Meurs et aL. 1993). Wild type PKR acts as a tumor suppressor, and the growth inhibitory activity of PKR could be attributed to apoptosis when over expressed and/or activated in mammalian cell line (Lee and Esteban, 1994; Der et aL, 1997). Further PKR activity is found regulated during the cell cycle (Zamanian-Daryoush et al., 1999). Induction of PKR leads to accumulation of cells in Gl phase of cell cycle and increased expression of effector molecules forming death induced signaling complexes (DISC) such as Fas. the TNF a receptor (TNFR-1), Fas-associated protein with death domain (FADD) and caspase 8 (Aktas et aL, 1998; Balachandran et aL, 1998; Donze et aL, 1999). Importance of PKR in cell differentiation is highlighted from such studies where myogenesis in L8 cells inhibited with the expression of a dominant-negative PKR (Salzbcrg et al.. 2000).

PKR is activated by a variety of double stranded RNA molecules with a high degree of secondary structures. The activation curve for PKR by dsRNA is bimodal; low concentrations of dsRNA activate and high concentrations of dsRNA inhibit PKR kinase activation (Hunter et al.. 1975). PKR protein consists of two dsRNA binding domains of approximately 70 ami no acids in its amino terminus. Both these dsRNA binding motifs are required for specific and high affinity binding that is required for activation of PKR kinase activity (Patel and Sen, 1992; Patel et aL, 1996). The kinase domain is located on the C-terminal half of the protein. Binding of dsRNA is thought to disrupt inhibitory interactions between the N-terminal half of PKR and the kinase domain and promote dimerization leading to PKR activation (Nanduri et aL, 1998). In addition to dsRNA and highly structured RNAs. PKR is activated by poly anions such as heparin. This activation does not require dsRNA binding domains (Patel and Sen. 1998).

The involvement of PKR in cellular anti-viral defense mechanism has been established from the facts that it is activated by dsRNA and its expression is induced by interferons. Consistent with this idea. a large number of viruses express inhibitors of PKR. These include a) inhibition of PKR activation by dsRNA analogs such as VA1, H1V-1 TAR, EBER-1 and HDV RNA produced by the respective viruses like adenosine virus, human immunodeficiency virus. Epstein barr virus and hepatitis delta virus (Gale and Katze, 1998: Kaufman. 1999a); b) inhibitor of PKR dimerization caused by NS5A, P58 IPK and PK2 produced by hepatitis C virus, influenza and baculovirus respectively (Gale et al., 1998b. 1999; Dever et al., 1998). c) inhibition of PKR activity by K3L and Tat proteins produced by vaccinia (Davies et al.. 1993; Sharp et al., 1997, 1998) and HIV respectively (Gunnen' et al.. 1990. 1992; Brand et al.. 1997) d) sequestration of dsRNA by sigma 3. E3L. NS1 and Us11 proteins produced by reovirus, vaccinia, influenza and herpes simplex virus-1 respectively (Davies et al.. 1993; Taylor et al., 1999). e) inhibition of eIF2a phosphorylation by PKR in the presence of pseudo substrates such as K3L and E2 proteins produced by vaccinia and hepatitis C virus respectively (Davies et al.. 1993; Kawagishi-Kobayashi et al.. 1998: Taylor et al., 1999) and t) dephosphorylation of eIF2α by γ1 34.5 protein of herpes simplex virus or T antigen of SV-40 (He et al.. 1998). Thus viruses are known to produce proteins that inhibit eIF2a kinase activation and eIF2a phosphorylation which appear to be necessary for the host cells to be alive and for continued propagation of viruses.

2.3 GCN2 kinase: GCN2 is the third eIF2a kinase. It is discovered in yeast cells under conditions of amino acid starvation. GCN2 kinase activation interestingly is found to increase the synthesis of GCN4. a transcriptional activator of many genes which are involved in amino acid synthesis (Dever et al.. 1992). GCN2 stimulated GCN4 translation. This is the first example to indicate that activation of eIF2α kinase and eIF2α phosphorylation does not always lead to a diminution in protein synthesis as has been observed in reticulocytes or in their lysates and suggests that eIF2α phosphorylation regulates translation in a gene-specific manner. eIF2a phosphorylation that occurs through GCN2 kinase activation requires two additional proteins such as GCN1 and

GCN20 (Hinnebusch, 1996). Recent studies indicate that mammalian cells also contain the homologs of GCN2, GCN1 and GCN20 and mammalian GCN2 is also activated under conditions of amino acid starvation (Berlanga et al., 1999; Sood et al., 2000). Unlike PKR, GCN2 kinase is also present in lower eukaryotes such as in insects (Santoyo et al., 1997). Hence it is likely that the entire net work linking amino acid starvation through GCN2 stimulated elF2a phosphorylation has been conserved through out eukaryotic evolution.

Yeast GCN2 is a 1659 amino acid protein. One of the important features is that the C-terminus of its kinase domain (~500 residues) is related to the histidyl t-RNA synthetase (His Rs) (Wek et al., 1989). Amino acid starvation leads to the accumulation of uncharged tRNAs. It has been proposed that binding of uncharged tRNAs to the His-Rs like domain in GCN2 kinase may produce a conformational change in GCN2 which in turn allows the kinase domain to phosphorylate eIF2a. This hypothesis is consistent with the fact that the C-terminal segment of GCN2 containing His-Rs domain preferentially binds uncharged versus amino acylated tRNAs (Dong et al.. 2000) and activates elF2a kinase.

In addition to amino acid starvation, yeast GCN2 kinase can be activated by purine nucleotides (Hinnebusch et al., 2000), glucose starvation (Yang et al., 2000), high salinity (Goossens et al., 2001) or by treatment of the cells with the alkylating agent MMS (Natarajan et al., 2001).

2.4 PERK/PEK kinase: This fourth eIF2a kinase has been recently discovered in the endoplasmic reticulum (ER) of pancreatic cells (Shi et al.. 1998; Harding et al., 1999). Because of its resemblance to some extent to PKR ad its high abundance in pancreas, the kinase is referred to as PERK/PEK. It is activated in response to ER stress or unfolded proteins (Kaufman. 1999b). The N-terminal domain of PERK is located in the lumen of ER and is complexed with a chaperone GRP78/Bip. This domain monitors the stress in the ER and it resembles IRE 1, an ER-resident stress responsive kinase. The C-terminus of PERK contains eIF2a kinase domain and is located in the cytoplasm. ER lumen has

high concentrations of calcium and ATP and is an oxidizing environment. These features are essential for folding and disulfide bond formation that affect most proteins which fold in the ER. In addition, protein folding in also influenced by protein glycosylation, a post translational modification that occurs in ER. And also ER is the site for synthesis of lipids and sterols. Synthesis of lipids is important in forming new membranes whenever it is required. The folding environment in the ER is monitored continuously by a stress-response pathway that regulates the rate of transcription of chaperone encoding genes. Unfolded protein response (UPR) signaling pathway, which is conserved in all eukaryotes, results in the accumulation of malfolded proteins in ER, and, triggers an increase in chaperone transcription. One of the best examples is the immunoglobulin binding protein Bip, an ER-localized protein that is bound to heavy chain immunoglobulins and inhibits the secretion in the absence of light chains in pre-B lymphocytes (Haas and Wabl, 1983). Thus Bip prevents the secretion of incompletely assembled immunoglobulins. Independently it was observed that Bip was expressed at a higher level in virally transformed cells and in conditions of glucose deprivation (Lee, 1987). Hence the Bip and other related proteins that were induced during glucose deprivation were classified under glucose regulated protein family or GRPs. Infact Bip was shown identical to GRP78 (Munro and Pelham, 1986), the 78 KD family member of GRPs that has homology to the cytosolic Hsp70 stress protein. The GRPs include PDI (protein disulphide isomerase), FKBP13 (FK506 binding protein), GRP94, GRP170, ERP72 (endoplasmic reticulum protein) ERP59, ERP29, HSP47, calreticulin and CHOP/GADD 153 (C/EBP homology protein/growth arrest and DNA damage) genes (Kaufman, 1999b). GRP proteins are expressed constitutively in all cells and their transcription is however induced in response to various ER stresses such as depletion of calcium from the ER lumen, inhibition of N-linked glycosylation, reduction of disulphide bonds, expression of mutant proteins or protein subunits, or over expression of some wild type proteins and or viral proteins. Activation of PERK is regulated by Bip. When unfolded proteins accumulate in the ER, Bip dissociates from PERK, resulting in the activation of this kinase which then phosphorylate the serine 51 residue in eIF2a. Through halting translation initiation and protein synthesis, PERK relieves the ER stress by reducing the number of unfolded proteins The unfolded protein response was thus

demonstrated to consist of two compartments: a transcriptional regulatory component that induced chaperone expression to the needs of organelle and another component that attenuated translation in response to ER stress through phosphorylation of eIF2a. Consistent with its role in ER stress response, cells lacking PERK fail to phosphorylate eIF2α and down regulate protein synthesis under ER stress conditions (Harding et al., 2000). Interestingly mice devoid of both copies of PERK displayed a rapid decline in pancreatic function soon after birth and develop diabetes (Harding et aL, 2001). Further Walcott-Raliison syndrome, a disease marked by early on set of diabetes is traced to mutations in human PERK. This is perhaps the first human disease directly linked to defects in a general translational regulator. Genetic Diseases such as cystic fibrosis, Alzeheimer's, congenital hyper thyroid goiter, hemophila A, B and several others are caused by mutations that result in protein misfolding and accumulation within the ER (Thomas et aL. 1995). although it is not clear whether any pathology associated with defective protein folding may result from activation of ER stress response.

ER stress responses not only include activation of ER resident elF2a kinase like PERK but may induce expression and activation of caspase 12, one of the important death promoting proteases (please see section dealing with caspases).

3. Apoptosis:

3.1 History: Apoptosis is a genetically programmed active form of cell death that occurs in individual cells or group of cells. This phenomenon of cell death during development was first described during amphibian metamorphosis (Vogt, 1842). The term apoptosis literally means 'dropping off (Garfield and Melino, 1997). The term apoptosis was proposed by Kerr, Wyllie and Currie (Kerr et al., 1972). During the course of development of a multicellular organism, there is always a purpose for elimination of unwanted or superfluous cells. Nature has chosen apoptotic cell death to maintain definite cell number at various stages of development and homeostasis, as a defense mechanism, and in aging (Vaux and Strasser, 1996).

3.2 Phases during apoptosis: Cell decides itself to undergo apoptosis; rather it commits suicide, when it is exposed to negative chemical stimulus or withdrawn from growth factors or interleukin-2 (needed for mitosis) etc. An apoptotic cell has characteristic ceil morphology due to membrane blebbing. The whole process can be divided in to three phases (Vaux and Strasser, 1996). *Initiation phase:* First, the signal should be perceived by the cell. The stimulus can be external which involves surface receptor, or might originate within the cell due to toxins, drugs or radiation. The perceived signal is transduced to the cell death effector machinery. *Effector phase:* The transduced signal is amplified in order to execute cell death. *Execution phase:* This is the outcome of activation of apoptotic machinery. Specific proteins are targeted and DNA is cleaved and cleared (Fischer et al., 2003).

3.3 **Biochemical** changes in a cell during apoptosis:

Shrinkage of the whole cell: Cells undergoing apoptosis shrink remarkably as observed by an electron microscopy. Cytoplasm is extremely condensed due to isosmotic loss of water and ions with out affecting the other organelle except for the cisternae of ER which get swollen. Transition from normal to apoptotic shrunken stage is very rapid without any intermediate phase (Cohen, 1993).

Release of cytochrome c: Release of cytochrome c is the initializing event in the mitochondrial dependent apoptotic pathways. This event triggers the formation of an apoptosome complex which activates caspase 9 (Liu et al., 1996; Wang, 2001).

Caspase activation: Activation of caspases, cysteine-dependent aspartate proteases, was considered as a hall mark of apoptosis. Pro caspases, latent precursors of caspases, are converted to active proteases during apoptosis through an intricately regulated proteolytic process. The proteolytic processing occurs at critical aspartic acid residues that conform to the caspase substrate recognition consensus (see later, more about caspases). Active caspases now target a number of cytosolic proteins which are responsible for the after effects of caspase activation and cell death (Slee et al., 1999).

Development of membrane blebs on the cell surface: The characteristic cell morphology feature of an apoptotic cell is development of membrane blebs on the cell surface. As and when adherent cells enter apoptotic phase, they loose their attachment to the extra cellular

matrix as a result of loss of local adhesions (Leverrier and Ridley, 2001). This retraction stage is followed by plasma membrane blebbing, a process that depends on the actin cytoskeleton and on actinomycin-based contractility. Rho GTPases, a sub group of the Ras superfamily, regulate the organization of the actin cytoskeleton. Active caspases process many cytoskeleton components such as actin, β-catenin, gelsolin, focal adhesion kinase, and Rho GTPase activated serine threonine kinase, ROCK-1 which is implicated in membrane blebbing (Coleman et al., 2001; Leverrier and Ridley, 2001).

DNA fragmentation: DNA fragmentation is mediated by caspase-3 cleavage of DNA fragmentation factor (DFF). DFF is a cytosolic protein and is composed of two subunits-DFF45 and DFF40 or ICAD (Inhibitor of caspase activated DNase) and CAD (caspase activated DNase) (Liu et al., 1997). Formation of membrane blebs and DNA fragmentation appear to be parallel events.

Formation of apoptotic bodies: The membrane blebs are released from the cell surface and appear as apoptotic bodies, which are sealed and maintain their osmotic gradients. There is no spillage of cellular contents and there is no provocation of inflammation. An apoptotic cell resists risk of cell lysis by strengthening its membranes through activation of cross-linking enzymes such as transglutaminases (Fesus, 1987).

Loss of mitochondria membrane potential: Loss of the **mitochondrial potential is** considered as a doubtless marker for apoptosis by which it can be differentiated from necrosis. It occurs during late phases of apoptosis (Bossy-Wetzel, et al., 1998).

Externalization of phosphatidyl serine'. Phosphatidyl serine which resides in the inner membrane of mitochondria is exposed to the outer surface of the membrane during apoptosis and acts as a 'eat me' signal for phagocytes by which apoptotic cells are cleared by phagocytes. This event is also used as a marker for apoptosis by some workers (Savill and Fadok, 2000).

Phagocytosis of apoptotic cells: These apoptotic cells employ recognition mechanisms by which they are recognized by the phagocytes. The recognition signals appear on the plasma membrane. These signals can be of three types like, loss of sialic acid on the cell surface carbohydrates, secretion of thrombospondin (TSP) by apoptotic neutrophils/ eosinophils and extemalization of phosphatidyl serine from inner plasma membrane to

outer membrane of an apoptotic cell (Savill et al., 1993; Li et al., 2003). These signals help phagocytes to recognize the apoptotic cells and phagocytose them.

3.4 Different assays for monitoring apoptosis:

Apoptosis can be monitored at various stages as described above. Morphological changes like appearance of blebs can be seen through a light microscope, and shrinkage of cell through an electron microscope. Internucleosomal DNA fragmentation can be seen on an agarose gel, and high molecular weight DNA fragmentation can be assessed by pulse-field gel electrophoresis (Rushak et al., 1996; Stewart, 1994). DNA fragmentation can also be analysed by flow cytometry (Nicoletti et al., 1991). DNA fragmentation results in leakage of DNA out of the cell giving rise to a population of cells with a sub diploid DNA content which takes up propidium iodide stain and appears as a sub G1 peak in the DNA histogram. This technique however does not allow differentiation between apoptotic and necrotic DNA degradation. Tunnel assay makes use of the exposed free -OH groups of the fragmented DNA. The DNA strand breaks can be identified by labeling free 3'-OH termini with modified nucleotides in an enzymatic reaction as observed by a light or fluorescent microscope (Gorczyca et al., 1993; 1994). Annexin V binds to exposed phosphatidyl serine of the apoptotic cells (Homburg et al., 1995). Measure of mitochondrial membrane potential is used as a marker for apoptosis (Catchpole and Stewart, 1993; Lemasters, 1998). Activation of caspases can be assayed by antibody or by substrate hydrolysis (Margolin et al., 1997). Cleavages of caspase substrates like PARP (Poly ADP ribose polymerase) (Lazebnik et al., 1994), DFF (DNA fragmentation factor) can be analysed by antibodies

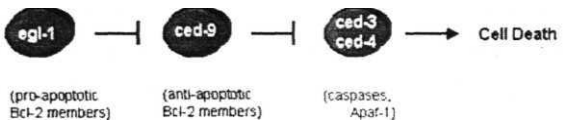
3.5 Apoptosis vs. necrosis: Apoptosis occurs in response to sub-lethal injury but necrosis occurs when cells are exposed to irreversible injury (Vaux and Strasser, 1996). Necrotic cells spill their contents which can attract potentially harmful inflammatory cells to the damaged site. Necrosis is characterized by irreversible swelling of the cell and cellular organelle like mitochondria which ultimately lead to their rupture. In apoptosis, permeability transition pore present on the mitochondria is opened to release the pro apoptotic proteins into the cytosol maintaining the mitochondrial integrity and functional

electron transport chain during the initial phases of apoptosis (Martinou and Green, 2001).

3.6 Apoptosis in invertebrates: The best studied models from invertebrates are *C. elegans,* a nematode representative and *Drosophila,* a representative of insects.

3.6.1 *Apoptosis in C. e/egans:* Most attention has been focused on the interleukin-1β (IL1-P) converting enzymes (ICE) like proteases, due partly to the enormous progress made by Horvitz and his colleagues in understanding the programmed cell death in the nematode *Caenorhabdities elegans.* These proteases are subsequently named as caspases. *C. elegans* is an evolutionarily simple prototype organism or a highly compressed version of more complex one. It is used to explore the mechanisms of apoptosis because of its invariant, lineage-restricted development. It has only 1090 somatic cells and 131 cells die by apoptosis. Three genes ced-3, ced-4 (cell death) and egl-1 (egg laying defective) have been identified in *C. elegans* that are involved in cell death. ced-3 codes for a caspase, ced-4 codes for a homologue of mammalian Apaf-1 (apoptosis protein activating factor) and egl-1 encodes pro apoptotic protein like Bcl-2 (B-cell CLL). On the contrary ced-9 gene encodes an anti apoptotic protein that antagonizes the function of pro apoptotic proteins (Figure la) (Shaham, 1998; Conradt and Horvitz, 1998; Hengartner 1998; Meier et al., 2000). Indeed using DNA sequence encoding ced-3 gene of C. *elegans,* the human caspase-3 was identified (Tewari et al., 1995a).

Figure la



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3.6.2 *Apoptosis in **Drosophila:** Drosophila* represents more complicated and evolutionarily evolved apoptotic machinery compared to *C. elegans. Drosophila* developmental cell death indicates that it is mainly determined by three pro apoptotic proteins: reaper (RPR). GRIM and head involution defective (HID). Overexpression of RPR and GRIM induces apoptosis. HID induced apoptosis is regulated by

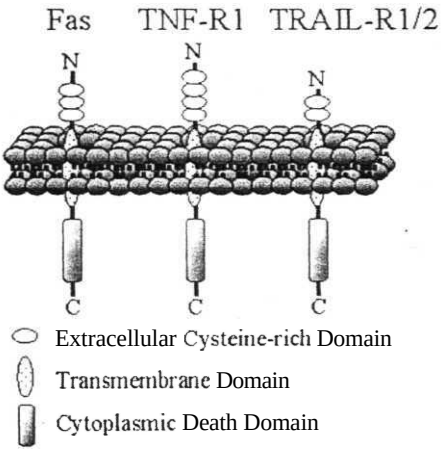
Ras/Raf/MAPK (Ras activating factor/Mitogen activated protein kinase) signaling pathways by inhibiting expression of HID protein or by promoting its phosphorylation. GRIM induces apoptosis by inhibiting the anti apoptotic dIAP1 *{Drosophila* inhibitor apoptosis protein), and RPR induces apoptosis through mitochondria by interacting with dBOK (Bcl-2 pro apoptotic member) (Chen and Abraham 2000). Five caspases have been identified in *Drosophila.* DCP-2/DREDD and DRONC (*Drosophila* Nedd-2 like caspase/Death related ced-3 like protein) act as initiator caspases. DCP-1 (Death caspase protein), drICE *(Drosophila* interleukin converting enzyme) and DECAY (death executioner caspase) act as effector caspases. DARK *(Drosophila* Apaf-1 related killer) protein functions as an Apaf-1 of mammalian counterpart (Meier et al., 2000). Existence of receptor mediated apoptosis in *Drosophila* is debatable. A homologue of FADD (Fas associated death domain) adaptor has been isolated recently (Hu et al., 2000) which interacts with the initiator caspase DREDD. Eiger is a unique homologue of TNF in Drosophila, but however eiger induces apoptosis in a DREDD (homologue of cas-8) independent manner (Kondo et al., 1997) and is thus different from TNF-a mediated apoptosis in mammalian systems.

3.7 Apoptosis in vertebrates: Vertebrate machinery is substantially homologous to that of invertebrates, although it is more elaborate and degenerate. Nonetheless mammalian apoptosis is also significantly regulated, in both development and throughout life by non-transcriptional signaling systems whose exact counterparts are either absent or have proven elusive in flies and worms. Apoptotic agents stimulate the cellular apoptotic machinery by any of the two pathways-one involving the activation of membrane bound death receptors and the other involving cytochrome c release.

3.7.1.1 ***Extrinsic pathway or Receptor mediated apoptosis:*** **Death receptors belong to a** superfamily of receptors involved in proliferation, differentiation and apoptosis called Tumor Necrosis Factor (TNF) superfamily (Krammer, 1999). Till date six death receptors (DRs) have been identified-**TNFR1** (Tumor necrosis factor receptor-1) (Gray et al., 1990; Loetscher et al., 1990; Nophar et al., 1990), Fas **(CD95/Apol)** (Itoh et al., 1991), **DR3** (TRAMP) (Marsters et al., 1996), **DR4 (TRAIL R1)** (TNF-related apoptosis

inducing ligand) (Pan et al., 1997), **DR5 (TRAIL2)** (Walczak et ah, 1997; Wu et al., 1997; Screaton et al., 1997) and **DR6** (Pan et al., 1998). Binding of ligands to the receptor protein leads to homotrimerisation of the receptors. Receptors have typically three domains: extracellular, intracellular and transmembrane. Apoptosis is initiated when the adaptor molecule binds to the cysteine rich extracellular domains (Figure 1b) (Yuan and Gores, 2002; Gruss and Dower, 1995; Curtin and Cotter, 2003). Of these six death receptors, Fas and TNFRI are well analyzed.

Figure 1b: Structure of death receptors.



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3.7.1.2 *Fas mediated apoptosis:* Fas-mediated apoptosis is the principal mechanism by which cytotoxic T lymphocytes (CTL) induce apoptosis in cells expressing foreign antigens (Medema et al., 1997). Dysregulation of Fas receptor can lead to autoimmune lympho proliferative syndrome in humans (Rieux et al., 1995), and it is involved in postischemic neuronal degeneration, traumatic brain injury (Martin et al., 1999; Herdegen et al., 1998) etc. Fas ligand is up regulated to eliminate lymphocytes in cancerous cells which are subjected to chemotherapy (Strand et al., 1996; Pinkiski et al., 2000).

3.7.1.3 ***Mechanism of*** *Fas* ***induced*** *apoptosis:* This mode of cell death begins with the formation of a protease complex-DISC (Death induced signaling complex) (Figure 1c). Binding of Fas ligand to its receptor promotes aggregation of Fas receptors on the membrane. This aggregation promotes recruitment of FADD, an adaptor protein from the cytoplasm and caspase 8 and caspase 10 are bound to FADD adaptor protein. Thus formation of DISC complex increases the concentration of these enzymes on the plasma membrane and results in auto cleavage of caspase 8 leading to its activation. Active caspase-8 directly cleaves effector caspases -3, 6, and 7. But in some cells where there is defective Fas receptor aggregation, DISC is not formed. In such cases ceramide from the phospholipids of plasma membrane is generated (von Reyher et aL, 1998) and the apoptotic signal is amplified. This amplification occurs via cleavage of a protein, BID (Bcl-2 family member-anti apoptotic) to tBid fragment that induces cytochrome c release from mitochondria and caspase 9 activation (Wang et al., 1996; Gross et al, 1999). Thus this is a model where receptor mediated apoptosis involves release of cytochrome c from mitochondria (Figure 1c). Fas mediated apoptosis is inhibited by alternative splicing of caspase-8 called splice variant caspase 8L which is catalytically inactive (Curtin and Cotter, 2003). FLIP (FLICE inhibitory protein), a virus product (Thome et al., 1997) also inhibits Fas induced apoptosis because it shares sequence homology with death domain of FADD adopter protein and acts as a competitive inhibitor for caspase -8 (Figure 1d).

3.7.1.4 ***TNFR1*** *signalling:* TNF (Tumor necrosis factor) is produced by activated macrophages and T cells in response to infection (Tartaglia and Goeddel, 1992). TNF in association with TNFR1 activates transcription factors like NF-KB and AP-1 (Activator protein), which in turn induce pro inflammatory and immunomodulatory genes. Unlike Fas receptor. TNFR1 promotes apoptosis only when coupled to a protein synthesis inhibitor like cycloheximide, suggesting that the anti-apoptotic proteins synthesized in response to TNFR1 should be shutdown for induction of apoptosis (Ashkenazi and Dixit, 1998). The expression of anti-apoptotic proteins by TNFR1 is controlled by NF-kB and JNK/AP-1 (c~JUN NH_2 terminal kinase) pathway (Beg et al., 1996; Roulston et al., 1998).

Figure 1c: Death receptor-mediated apoptosis

Oligomerization of death receptors leads to recruitment of the cytoplasmic adapter protein FADD (Fas associated death domain). FADD contains a death effector domain that mediates the recruitment of caspases through the association with a corresponding death effector domain in the prodomain of the inactive initiator caspases, caspase 8 and 10. Active caspase 8/10 cleaves the pro-apoptotic BH3 domain only protein Bid. This truncated Bid translocates to mitochondria inducing release of cytochrome *c* from mitochondria. The cytosolic cytochrome *c* binds to Apaf-1, facilitating recruitment of caspase 9 in a protein complex referred to as the apoptosome. Active caspase 9 cleaves effector caspases, caspase 3 and 7, causing apoptosis.

Figure 1d: Inhibition of death receptor-mediated apoptosis

c-FLIP (FLICE-inhibitory proteins) can bind to the DISC (death-inducing signaling complex) and either competitively inhibit procaspase 8 recruitment to the DISC or prevent proteolytic processing of caspase 8. Anti-apoptotic Bcl-2 family members, Bcl-2, Bcl-X_L, Mcl-1, etc. can associate and form complexes with pro-apoptotic Bcl-2 members, thereby inhibiting the release of cytochrome *c*. Inhibitors of apoptosis proteins (IAPs), XIAP, c-IAP-1 and c-IAP-2, bind procaspase 9 and prevent its activation, and can also directly bind and inhibit active caspases. HSP (heat shock protein) 27 binds to cytochrome *c*, and HSP70 and HSP90 bind to Apaf-1, resulting in the inhibition of apoptosome formation.

Figure 1c: Death receptor mediated apoptosis.

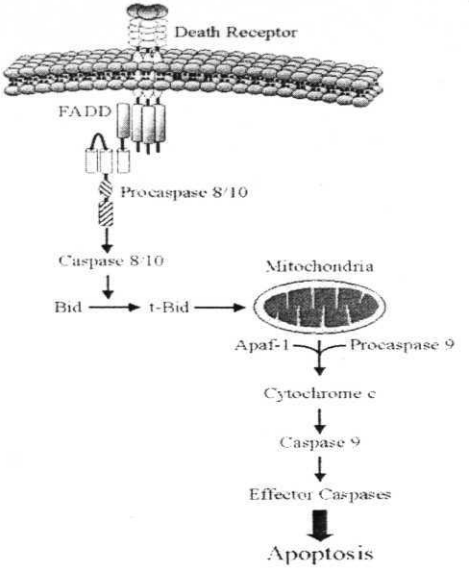

Figure Id: Inhibition of death receptor-mediated apoptosis by c-FLIP

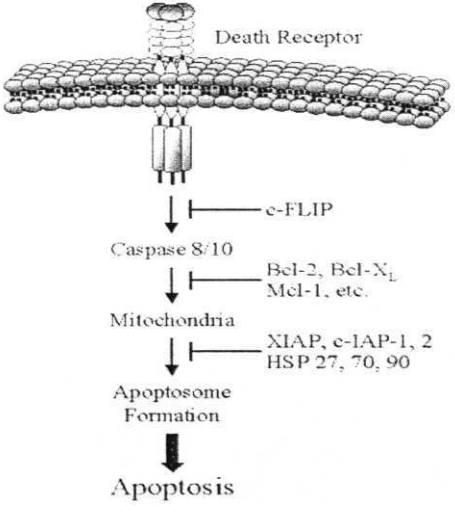

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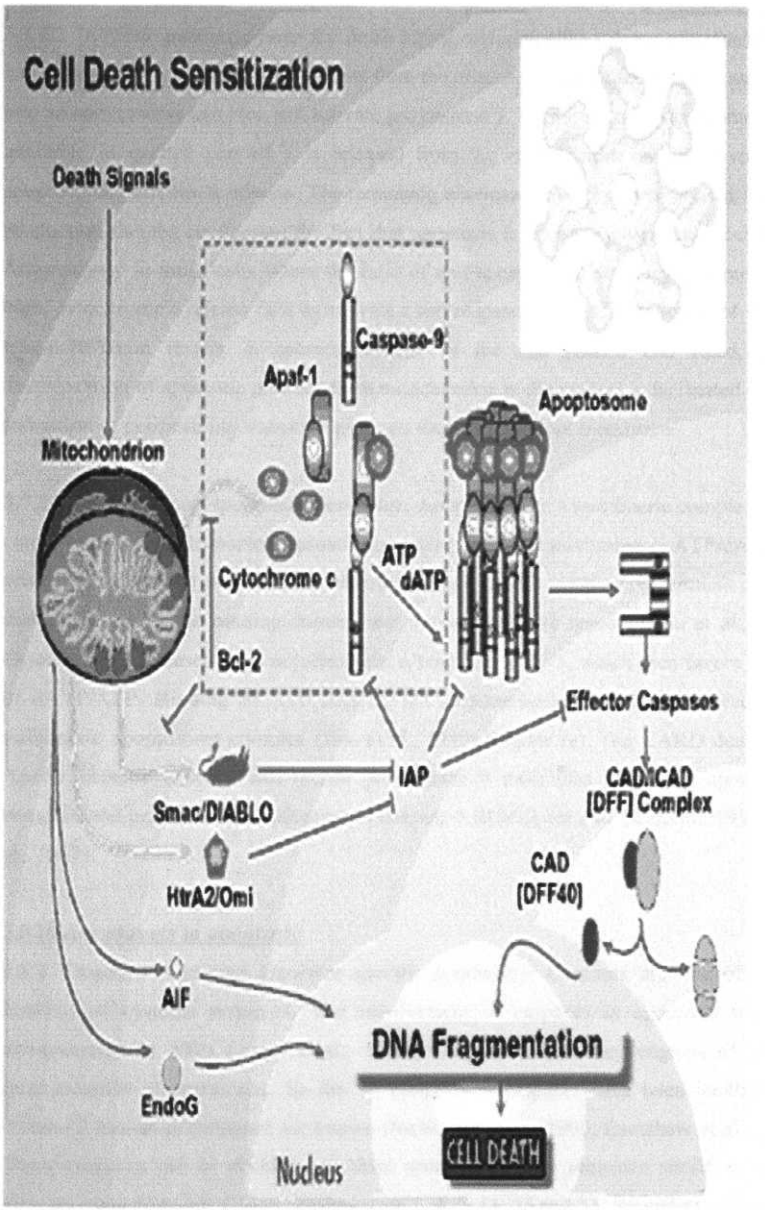
Figure 1e: Intrinsic pathway of apoptosis

Cytochrome c released from mitochondria forms apoptosome complex along with cytosolic Apaf-1 and pro caspase-1 which ultimately results in activation of pro caspase 9 to caspase 9. Caspae-9 activates effector caspases and effector active; caspase activates ICAD to CAD leading to fragmentation of DNA, IAPs inhibit caspase activation. Mitochondrial proteins like Smac and DIABLO are released from mitochondria that inhibit IAP function. Smac or DIABLO function is turn regulated by another mitochondrial protein Bcl_2 which inhibit their release from mitochondria. Endo nuclease G and AIF mitochondrial proteins can bring about DNA fragmentation with out caspase activation when released.

Figure 1e: Intrinsic pathway of apoptosis



3.7.2.1 ***Intrinsic pathway:*** Here the death signal stimulates the release of cytochrome c and various other pro apoptotic proteins from the mitochondria that recruit pro caspase 9 into an apoptosome complex and activate pro caspase 9. In cells where cytochrome c is available in excess, part of it is released from the mitochondria and is involved in apoptosome complex formation. The remaining unreleased cytochrome c is used for ATP production thereby reinforcing the fact that apoptosis is an energy dependent cell death. Alternatively in those cells where the ratio of anti apoptotic to pro apoptotic proteins is high, cytochrome c release fails to activate a pro caspase, and loss of function of electron transport chain results in necrotic demise of the cell (Green and Reed, 1998). Translocation of apoptotic proteins from mitochondria to the cytosol is facilitated through formation of permeability transition pores on the mitochondrial membrane.

3.7.2.2 ***Formation of Apoptosome complex:*** Apoptosome is a multimeric complex that is composed of Apaf-1(apoptosis protein activating factor), cytochrome c, ATP/dATP and procaspase-9. Apaf-1 is a cytosolic protein having a CARD (caspase recruitment domain) domain, a nucleotide binding domain and multiple WD-40 repeats (Zou et al., 1997). Cytochrome c released from mitochondria is bound to Apaf-1, which then favors binding of dATP/ATP. Binding of ATP triggers the oligomerisation of Apaf-1 molecules to multimeric apoptosome complex (Zou et al., 1999) (Figure 1e). The CARD domains of Apaf-1 become exposed and recruit pro-caspase 9 molecules on to the apoptosome complex and promote their activation to caspase-9 (Rodriguez and Lazebnik, 1999; Li et al., 1997).

3.8 Major players in apoptosis:

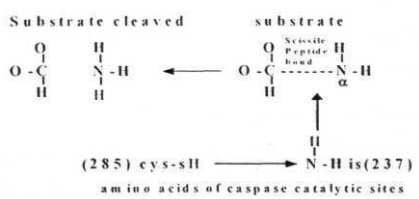
3.8.1 ***Caspases (Cysteine aspartate specific proteases)*****:** Caspases are one of the 20 families of cysteine proteases. The involvement of caspases in apoptosis was first demonstrated in 1993 (Yuan et al., 1993). Caspases act as key engines of cellular destruction in all metazoans. So far, 14 mammalian caspases have been identified, of which 12 human orthologues are known (Nicholson et al., 1999; Earnshaw et al., 1999). These caspases can be divided into three groups based on sequence similarity among their protease domains. Group I caspases are 1, 4, 5, 11, 13 and 14. Second group consists

of caspase 2, 9 and rest of the caspases comes under group-3 (Chang and Yang, 2000). Based on function, caspases 1, 4, 13 are inflammatory caspases involved in cytokine activation, caspase 9. 8. 2 and 12 are initiator caspases and caspases 3, 6, 7 are effector caspases (Nicholson. 1999). Caspase-9 acts as an initiator caspase for all intrinsic pathways, whereas caspase-8 acts as an initiator caspase for extrinsic pathways. Caspase-2 appears to have a dual role. acting as an apical caspase in stress induced apoptosis (Lassus et al.. 2002) and in CRADD (adaptor protein) involved Fas/TNF induced apoptosis (Ahmad et al.. 1997a). Caspase -12 is an apical caspase in ER stress induced apoptosis and is localized in ER (Nakagawa et al., 2000). Pro caspases 1, 8, 7 are present in cytosol. Pro caspase 3 is found in both cytosol and mitochondria whereas pro caspase 2 present in cytosol and nucleus (Colussi et al., 1998). Like most of the proteases, caspases are also synthesized as pro enzymes with little or no activity. Structurally pro caspases have two subunits large subunit-20 kDa (p20) and small subunit 10 kDa (p10) and in some pro caspases there is a short linker between these two sub units. Caspases have pro domains of 100 amino acids in inflammatory and initiator caspases and short 30 amino acid pro domains in the effector caspases at their N-terminus region. These pro domains have distinct domains like death effector domain (DED). caspase recruitment domain (CARD) and death inducing domain (DID). Caspases are strict endo peptidases. Caspases are activated either by proteolytic processing by their upstream caspases or by autocatalvsis triggered by binding of cofactors or by release of inhibitors (Thornberry and Lazebnik, 1998). Caspases with short prodomains (effector) cannot self activate and get activated by cleavage by initiator caspases (Kumar. 1999). Activation of downstream effector caspases occur in a cascade fashion. Caspase 8 cleaves pro caspases 3, 4, 7 and 9 (Srinivasula et al., 1996: Muzio et al.. 1997; Stennicke et al., 1998). Caspase 10 cleaves pro caspases 3, 7 and 8 (Fernandes et al., 1996). Caspase 9 activates caspase 3 (Srinivasula et al., 1998; Li et al.. 1997; Deveraux et al., 1998) and 7 (Srinivasula et al., 1998). The upstream caspases activate down stream caspases *in vitro* also. The effector caspases 3, 6, and 7 are capable of under going auto-activation *in vitro* when over-expressed (Fernandes et al.. 1995; 1995a; 1996; MacCorkle et al., 1998). Sometimes caspases are activated by their own activation through positive and negative feedback

amplification loops. An active caspase can activate its inactive precursor either directly or indirectly thus resulting in the exponential rate of amplification of the death signal.

Caspases recognize a tetra peptide sequence on its substrate. This tetra peptide sequence forms the basis for caspase synthetic substrate and inhibitor designing. The four amino acids in this tetra peptide sequence are named as PL P2, P3, and P4. Caspase cleaves the peptide bond on carboxyl terminal on P1. Presence of aspartic acid on P1 is the absolute requirement for all caspases. Depending on the type of amino acid present at P4 position of the cleavage site, caspases can be categorized into three groups. Group I (caspases-1, 4, 5, 13) recognize a tetra peptide sequence consisting of hydrophobic (P4)-Glu (P3)-Xxx (P2)-Asp (P1) where as group II (caspases- 2, 3, 7, CED-3 of *C. elegans)* recognize a sequence consisting of Asp (P4)-Glu (P3)-Xxx (P2)-Asp (P1). Caspases-6, 8. 9, 10 belonging to group III recognize a sequence that contains aliphatic (P4)-Glu (P3)-Xxx (P2)-Asp (P1). So the amino acid present at P4 site is the key determinant of substrate specificity (Nicholson. 1999). The active site cysteine is in the middle of a conserved QACRG motif common to all the caspases. Mutation of the cysteine in this motif inactivates these enzymes (Vaux and Strasser 1996). The active catalytic cysteine Cys285 forms a dyad with catalytic His237. The Cys285 acts as nucleophile and thus gives off its proton (H+) from its thiol ester (SH) group to amino group of His237 before binding to the substrate (please see figure 1f). Substrate binding results in the positioning of scissile peptide bond close to catalytic His237. Protonation of a amino group of scissile peptide bond (H_2N-COOH) occurs from catalytic His237, thus resulting in the cleavage of peptide bond (H_3N COOH) (Stennicke and Salvassen, 1999).

Figure 1 f: Substrate hydrolysis by caspase:



Stennicke and Salvassen. 1999

3.8.2 *Caspases **and** its substrates:* Apoptosis is executed by cleavage of many vital proteins. First substrate for caspases was identified in 1994 (Lazebnik et aL, 1994). Elucidating the consequences of this endo proteolytic cleavage will help to understand better, the pathway of cell death and other biological process. Cleavage of proteins during apoptosis is much cell type specific, which might be due to variations in the levels of expressions of various caspases. It has been reported that p-actin can be cleaved by caspases in pheochromocytoma and ovarian carcinoma cells (Kayalkar et aL 1996; Chen et aL, 1996). whereas in many other cell types no cleavage was detected (Song et al.,_ 1997) The cleavage site of a protein for caspase is not conserved among different species. Cyclin A is cleaved during apoptosis of *Xenopus* oocytes (Stack and NewPort, 1997), but the caspase cleavage site is not present in homologs of mammalian cells. Inhibition of DNA repair, for instance by the cleavage of PARP-1 (Lazebnik et al., 1994) or the kinases ATM (Ataxia telangiectasia mutated protein) (Smith et aL. 1999) and DNA-PK (DNA dependent protein kinase) (Song et al., 1996), has long been thought to promote apoptosis process. Caspases cleave several proteins that are involved in various physiological processes. These include proteins involved in RNA synthesis and splicing (Thiede et aL. 2001). cell adhesion (Bannerman et aL, 1998; Brancolin, et aL. 1997) cell cycle (Mazumder et aL, 2002; Lahti et aL. 1995: Beyaert et aL. 1997), DNA synthesis (Rheaume et aL, 1997; Song et aL. 1997). cleavage (Enari et aL, 1998) and repair (Kaufman et aL, 1993; Lazebnik et aL, 1994), DNA binding, transcription (Nyormoi et aL, 2001; Francois et aL, 2000; Barkett et aL, 2001; Zhang et aL. 1999; Bell et al., 2001), G-Protein signaling (Danley et aL, 1996; Na et aL. 1996; Cosulich et al., 1997), protein synthesis, protein degradation (Porn et aL. 1998; Wang et aL, 1998; Harvey et aL, 1998), protein modification (Kim et aL, 2001; Fabbi et aL, 1999), calcium, c-AMP. c-GMP and lipid metabolism (Mejillano et aL. 2001; Paszty et al., 2002; Atsumi et aL. 2000) and neural degeneration (Galvan et aL, 2002; Weideman et aL, 1999). Many structural proteins present in nucleus (Buendia et aL, 1999; Fernando et aL, 2001), ER, Golgi **(Mancini** et aL, 2000; Lane et aL, 2002) and also cytoskeleton (Umeda et al., 2001) are **also cleaved** by caspases. Some other substrates for caspases are membrane receptors like

TNF-R1, glutamate receptor, EGF-R (Bae et a!., 2000) etc., adaptor proteins like TRAF-1 and 3 (Irmler et al., 2000; Leo et al., 2001; Lee et al., 2001).

Induction of apoptosis alters phosphorylation status of several proteins, although there appears to be no conclusive evidence that kinases are required for the execution of the cell death programme. The kinases may be involved in amplifying or integrating incoming signals to swing the balance between cell survival and cell death. During apoptosis interestingly many kinases such as MAP Kinase/Extra regulated kinase I (MEKK-1/ERK-1), P21 activated kinase 2 (PAK2), protein kinase c-5 (PKc 5) and double stranded RNA dependent eIF2a kinase (PKR) (Widmann et al., 1998; Cardone et al., 1997; Ghayur et al., 1996; Saelens et al., 2001) are processed and the processed kinases are found to be more active than the unprocessed ones. Further, the p38 mitogen activated protein kinase (MAPK) and JNK are strongly activated in a caspase dependent manner during apoptosis and have been implicated as possible mediators of the apoptotic process (Juo et al., 1997; 1998; Xia et al., 1995). Activation of phosphoinositide-3 kinase (PI3) pathway, culminating in the Akt-mediated phosphorylation of the pro apoptotic proteins BAD (Bcl-2 associated death promoter) and caspase 9 had been demonstrated in haematopoietic cells treated with survival factor such as IGF-1 (insulin growth factor) and IL-3 (interleukin) (del Peso et al., 1997; Datta et al., 1997).

Some of the cytokines like interleukin-1β (Thornberry et al., 1992), interleukin-6 (Zhang et al., 1998), IFN-γ inducing factor are substrates for caspases. Protein phosphatases, calcineurin, PP2A (Mukerjee et al., 2000; 2001; Santoro et al., 1998) are also cleaved by caspases.

Viral proteins which are candidates for caspase proteoiytic activity are Bcl-2 homologs (Bellows et al., 2000), Crm A (Zhirnov et al., 2002), cytokine response modifier-A, serpin-like caspase inhibitor of Pox virus, virus-specific membrane channel protein of influenza virus, M2 (Zhirnov et al., 2002), nucleo-caspid protein of influenza viruses A and B, NP (Zhirnov et al., 1999; 2002) and pan caspase inhibitor of baculovirus-p35 (Bump et al., 1995; Xue et al., 1995). It is even surprising to see that both pro apoptotic,

Apaf-1 (Bratton et al., 2001; Lauber et al., 2001), Bad (Condorelli et al., 2001), Bax (Bcl-2 associated x protein) (Wood et al., 1999; Yanase et al., 2000) and anti apoptotic cellular proteins, Bid (BH3 interacting death domain agonist) (Li et al., 1998; Luo et al., 1998), Flip (Irmler et al., 1997; Clem et al., 2001), Bcl-2 (Cheng et al., 1997), XIAP (X-linked Inhibitor of apoptosis protein) (Deveraux et al., 1999; Levkau et al, 2001), c-IAP1 (CARD containing IAP) (Clem et al., 2001) are also cleaved by caspases.

3.8.3 ***Modifications of translation factors in apoptosis:*** Protein synthesis is generally down regulated following induction of apoptosis as shown by many people in many cell lines by a variety of apoptotic inducers. Mainly the initiation step in protein synthesis is targeted during apoptosis. Most of the initiation factors get modified: undergo phosphorylation, proteolytic cleavage and intra cellular translocation (Fischer et al., 2003) thereby altering the function of the initiation factors. Some of the initiation factors that are modified or cleaved are: eIF2a, elF4Gl, eIF4GII, elF3, eIF4B, 4E-BP1, eIF4E, DAP-5 (Death Associated protein of eIF4G family), and 60S ribosomal protein P0, NACa (Nascent polypeptide associated complex), PABP4 (Poly A binding protein) SRP72 (signal recognition particle) eIF2α kinases like PKR, and several other ribosomal proteins. eIF2a is both modified and cleaved. Cleavage of this initiation factor leads to a generation of C-terminally truncated cleavage product which results in protection of protein synthesis from inhibition by PKR mediated phosphorylation at ser51 residue (Satoh et al., 1999; Marissen et al., 2000b). P35 subunit of eIF3 is cleaved at 300 amino acids of C-terminus generating a truncated cleavage product (Bushell et al., 2000). eIF 4B is inactivated by caspase-mediated cleavage and the cleaved product looses Poly A binding and inhibits translation (Bushell et al., 2001). Cap-dependent translation is inhibited by dephosphorylation of elF 4E (cap-binding activity inhibited) and by inactivation of elF4E-BPl either by dephosphorylation (binding to 4E is increased) or by its cleavage after 25 amino acids (Bushell et ah, 2000b; Tee and Proud 2002). DAP-5 is death associated protein belonging to eIF4G family. Cleavage of this protein stimulates translation from IRES (Internal Ribosome Entry Sites) of some of the RNAs like c-myc, DAP-5 (Henis-Korenbelt et al., 2000, 2002) and also some of the pro-apoptotic proteins like Apaf-1 and XIAP (Holcik et al., 1999; Clemens et al., 2000). eIF4 GI is inactivated

by cleavage after 492 and 1136 amino acids. This cleavage inhibits binding of eIF4GI to 5' cap of mRNA and capped mRNAs to 40S ribosomal subunit (Marissen et al., 1998; Morley et al., 1998; Bushell et al., 2000a). Cleavage of eIF4 GII at unknown site results in shut-off of cap-dependent translation (Gradi et al., 1998; Svitkin et al., 1999; Marissen et al., 2000a). Nascent associated protein complex a (NACa) helps in correct translocation of newly synthesized polypeptides to ER. PABP4 is a poly (A) binding protein required for poly (A) shortening. Both NACa and PABP4 (Thiede et al., 2001) are cleaved at unknown sites. Cleavage of some proteins like SRP72 involved in secretory protein biosynthesis, has no effect on its function (Utz et al., 1998). Not only initiation factors but also eIF2α kinases like PKR are cleaved by caspases generating 38 kDa for the NH2 terminus and 43 kDa for the COOH terminus fragments. The cleaved fragment is found to be more active in phosphorylating its substrate eIF2a (Saleans et al., 2001).

Changes in ribosomal proteins appear to be more complex. There are two reported examples for relocation of ribosomal proteins during apoptosis. Rosen and Rosen, 1999 reported that S1, a ribosomal protein present in the 40S ribosome subunit tanslocates to membrane bleb of apoptotic cells. S19 in its dimerized form is secreted from apoptotic cells (Hofman et al., 1998; Nishiura, et al., 1999; Yamamoto, 2000) serves as a chemo attractant specific for macrophages (Hofman et al., 1998). Cleavage of 60S ribosome subunit protein P0 (Nishiura, et al., 1999) results in destruction of polysomes (Zhou et al., 1998; Brockstedt, et al., 1998; Thiede et al., 2001). S11 ribosomal protein decreases in tumor cell during staurosporine induced apoptosis. This decrease appeared to be cell type-specific as evident from four different cell lines derived from human breast carcinoma, but not in cells from other types of tumors (Nadano et al., 2001). Ribosomes dissociate from ER during apoptosis as revealed by ultra structural studies in some tissues (Pilar et al., 1976). 28S rRNA was cleaved in apoptosis induced by anti-Fas antibody in human Jurkat cells and in TNF-a induced apoptosis in human U937 cells (Nadano and Sato, 2000). Apoptosis in rat and human leukemia cells, rat thymocytes, and bovine endothelial cells was accompanied by limited and specific cleavage of polysome-associated and monosome-associated 28S rRNA, with 18S rRNA (Houge et al., 2000).

3.8.4 ***Bcl-2 family of proteins'.*** Bcl-2 and related cytoplasmic proteins are key regulators of apoptosis and tissue homeostasis. The first mammalian Bcl-2 (B-cell CLL) was discovered when this gene was activated by chromosome translocation in human follicular lymphoma (Bakhshi et al., 1985; Tsujimoto et al., 1984). The gene activation surprisingly promoted the survival of cytokine dependent quiescent haematopoietic cells in the absence of cytokines (Vaux et al., 1988). Later studies, using transgenic mice established that cell survival and proliferation were under separate genetic control and that disturbances in both were likely to contribute to neoplasia. Bcl-2 proteins contained one to four of the conserved BH (Bcl-homology) domains (BH1-BH4). At least 15 Bcl-2 family members have been identified in mammalian cells and several others in viruses (Cory, 1995; Strasser et al., 1997; Yang and Korsmeyer 1996; Chao and Korsmeyer, 1998). Some of them are pro apoptotic and some of them are found to be anti apoptotic. The pro apoptotic members have BH3 domain with BH1 or BH2 domains. These 'BH3 domain' proteins may well represent the physiological antagonists of the pro survival proteins (Oltavai and Korsmeyer, 1994). Most of the anti apoptotic members have BH1 and BH2 domains. Some of the anti apoptotic members may have additional BH domain (BH3 or BH4) (Adams and Cory, 1998). Bad (Bcl-2 associated death promoter); Bim (Bcl-2 interacting mediator) are pro apoptotic members containing BH3 domain only (EGL-1 of *C .elegans* also has BH3 domain). Other pro apoptotic members like Bak (Bcl-2 homologous antagonist killer); Bax (Bcl-2 associated x-protein) contain BH3 domain along with BH1 and BH2 domains (Meier et al., 2000) (Figure 1g). The anti apoptotic and pro apoptotic members of Bcl-2 family can physically interact (Oltavai et al., 1993; Salvotre et al., 2003).

The regulation of Bcl-2 family of proteins is different. Unphosphorylated pro apoptotic Bad binds to Bcl-x thus neutralizing its anti-apoptotic function, and promotes cell death. Bad is inactivated by phosphorylation by active ribosomal S6 kinase and Akt (Yang et al., 1995, Zha et al., 1996; del Peso et al., 1997; Datta et al., 1997; Harada et al., 1999). Bid is activated by caspase mediated proteolysis (Luo et al., 1998; Li et al., 1998). Bim is regulated by binding to Dynein motor complex (Puthalakath et al., 1999). Bax and Bak

Figure 1h: Regulation of apoptosis by the Bcl-2 family

In a viable cell, the pro apoptotic Bcl-2 family members Bax, Bak, and BH3-only proteins are antagonized by anti apoptotic members such as Bcl-2. In response to an apoptotic stimulus, BH3-only members are activated by transcriptional up regulation (Bax, Noxa, Puma), subcellular relocalization (Bim), dephosphorylation (Bad), or proteolysis (Bid). Activated BH3-only proteins prevent anti apoptotic Bcl-2 members from inhibiting pro apoptotic members. In addition, they might directly induce a conformational change of Bax and Bak which subsequently oligomerize and insert into the mitochondrial membrane where they form pores either by themselves or by associating with the permeability transition pore complex. In consequence, pro apoptotic factors are released from the inner mitochondrial membrane into the cytosol, such as cytochrome c which contributes to the formation of the apoptosome and the subsequent activation of the caspase cascade.

localized to ER and mitochondria. Bax and Bak are required for maintaining homeostatic concentrations of Ca^{2+} in the ER and mitochondria. Transient over expression of Bax

Figure 1g. Structure of Bcl-2 proteins:

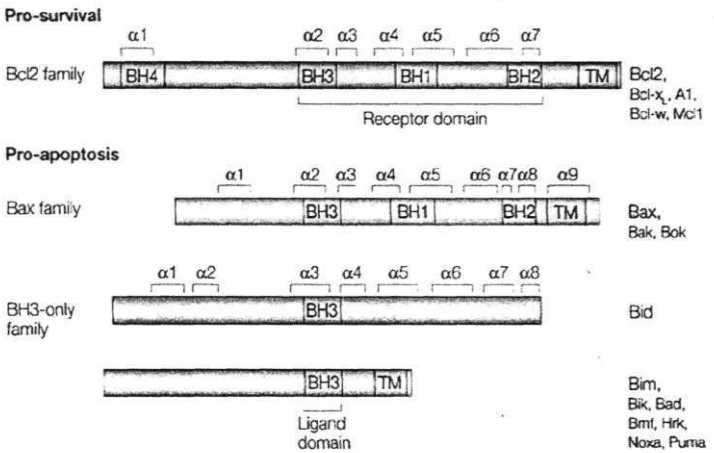
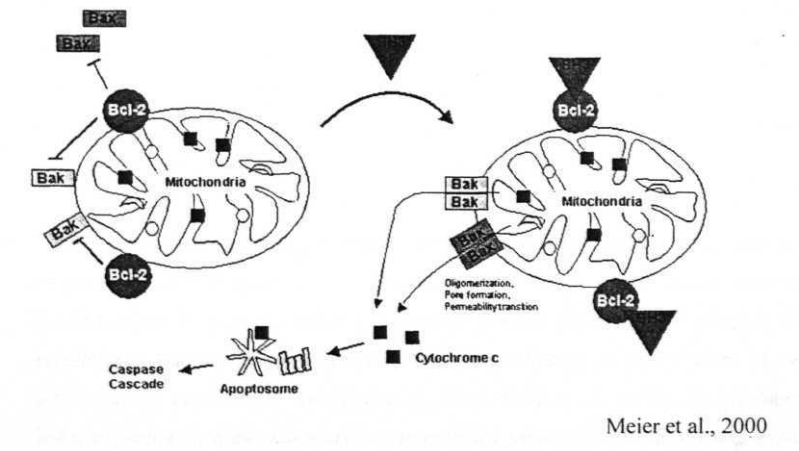


Figure 1h: Regulation of apoptosis by Bcl-2 proteins:



results in the release of ER Ca^{2+} with a subsequent increase in mitochondrial Ca^{2+} and enhanced cytochrome c release (Nutt et al., 2002; Scorrrano et al., 2003). Bax can cause mitochondrial membrane damage and cell death (distinct from apoptosis) even in the absence of caspase activation (Xiang et al., 1996). The major anti apoptotic members of Bcl-2 family, Bcl-2 and Bcl-x, are localized to the outer mitochondrial membrane and to the endoplasmic reticulum and peri nuclear membrane (Yuan and Yanker, 2000). These two proteins act by inhibiting the pro apoptotic members of Bcl-2 family through hetero dimerization (Yang and Korsmeyer, 1996; Reed, 1997). And also these anti apoptotic members are found to bind to pro apoptotic members like Bax, Bid and Bik and prevent them from stimulating the cytochrome c release from mitochondria (Antonsson et al., 1997; Schlesinger et al., 1997) (Figure 1h). Bcl-X_L is even shown to inhibit the formation of Apaf-1-procaspase-9 complex (Hu et al., 1998).

There are other mitochondrial proteins which contribute to the programmed cell death (Gurp et al., 2003). Murine Smac, second mitochondria derived activator of caspase (25 kDa) or its human homologue DIABLO (29 kDa) is a mitochondrial protein released from mitochondria after an apoptotic stimulus and it stimulates a caspase by sequestering IAPs (Van Loo et al., 2002; Verhagen et al., 2000; Du et al., 2000). Omi or HtrA2, a 49 kDa protein, is a serine protease and is also an apoptogenic factor released from mitochondria upon a stimulus (Gray et al., 2000; Faccio et al., 2000). It is proteolysed and the 37 kDa protein of proteolysis is released into cytosol and contributes to caspase independent and dependent pathways (Suzuki et al., 2001; Verhagen et al., 2002; Van Loo et al., 2002). It sequesters IAPs and promotes caspase activation, thus relieving the inhibition interaction of IAPs on caspases.

3.8.5 *Caspase independent pathways:* Two mitochondrial proteins are responsible for caspase independent apoptosis. 1) Endonuclease G is released from the mitochondria by the induction of apoptosis and translocated to nucleus where it is involved in nuclear breakdown. Endonuclease-G induced DNA fragmentation is independent of caspase activation (Li et al., 2001; van Loo et al, 2001; Gurp et al., 2003). 2) AIF, apoptosis inducing factor, is a mitochondrial intermembrane protein which translocates to nucleus

upon apoptotic stimulus. AIF is matured by removal of N-terminal mitochondrial localization signal. AIF can be activated independent of caspase activation shown in case of PARP activation. AIF is then transported to nucleus and is sufficient to induce DNA fragmentation in to high molecular weight 50 kbp fragments and membrane blebbing (Susin et al., 1999).

3.9 Physiological inhibitors of apoptosis: Induction of apoptosis is mainly inhibited by inhibiting the activation of caspases. Many caspase inhibitors are produced by the cell and also by viruses that promote their propagation. Synthetic caspase inhibitors have also been made to study the apoptotic mechanisms in various cell types and in the areas where caspases are being tried as therapeutic targets (Grobmeyer et al., 1999; Nicholson, 2000).

CrmA: This protein is a product of cowpox virus. It was discovered even before the discovery of role of caspases in apoptosis. This inhibitor structurally belongs to serine protease inhibitor (serpin) group but functions on cysteine proteases. It inhibits caspase-1 and caspase-8 (Tewari and Dixit, 1995b; Varfolomeev et al., 1998) at pico molar concentration, but most unlikely to inhibit caspases 3, 6, 7 (Zhou et al., 1997; Garcia et al., 1998) and inhibits caspase-9 *in vitro* but not *in vivo* (Kuida et al., 1998; Smith et al., 1996). Thus this viral protein has a role in defensive inflammatory responses and in TNF-a receptor mediated apoptosis as an inhibitor of caspase-1 and 8 respectively. Crm A inhibits caspases by binding to the active site and acts as a pseudo substrate. This binding region has ami no acid residues LVAD based on which synthetic inhibitors are designed (Ekert et al., 1999).

PI-9: This is the mammalian homolog of crmA but inhibits granzyme B mediated apoptosis but not receptor mediated apoptosis as crmA. It lacks the aspartate residue in its active site and thus cannot inhibit caspases (Ekert et al., 1999).

The equine herpes type II virus E8 protein and molluscum contagiosum MC159 and MC160 proteins contain DEDs (Death Effector Domain) homologous to those of

procaspase 8 and 10 and inhibit procaspase recruitment to death receptor-associated signaling complexes (Bertin et al., 1997; Hu et al., 1997).

p35: p35 is a baculovirus early gene product and targets mature caspases and serves as a suicide substrate (Bump et al., 1995). p35 is a broad-spectrum caspase-specific inhibitor and it inhibits caspases 1, 3, 6, 7, 8 and 10 (Ekert et al., 1999). No homolog for this protein has been found in mammals. Caspases cleave the P1 residue (Asp 87) in the active site of p35 and forms an inhibitory complex with the caspase. This step inhibits further activation of caspases (Manji and Friesen, 2001; La Count et al., 2000). p35 expression inhibits apoptosis in a wide variety of organisms (Hay et al., 1994; Xue and Horvitz, 1995; Biedler et al., 1995).

IAPs (Inhibitor of Apoptosis): IAPs were first described in baculovirus genomes by late Lois Miller as bacuoviral products that could suppress apoptosis of cells infected with a p35 deleted baculoviral strain in 1993 (Verhagen et al., 2001; Crook et al., 1993). Cellular orthologues are discovered in yeast, nematodes, flies and humans (Duckett et al., 1996; Liston et al., 1996; Roy et al., 1995; Uren et al., 1996; Rothe et al., 1995; Hay et al., 1995; Fraser et al., 1999; Uren et al., 1999). Two IAPS from baculovirus genome, *Cydia pomomella* IAP (Cp-IAP), *Orgyia pseudosugata* IAP (Op-IAP) were identified. Cellular IAPS are D-IAP1, D-IAP2 from *Drosophila*, Sf IAP from *Sf9* cells, eight human IAPs are identified (Huang et al., 2000). But all these IAPS have distinct targets. IAP proteins are characterized by novel domains of ~70 amino acids termed baculoviral IAP repeats (BIR). BIR domains have conserved cysteine and histidine residues in their domain Cx2Cx6Wx3Dx5Hx6. One of the BIR domains is required for suppression of apoptosis. Several of the IAPs have a RING domain located near the C-termini. In some cases both domains are required for the anti apoptotic functions of IAPs (Clem and Miller, 1994; Harvel et al., 1997b). Op-IAP and Cp-IAP of baculovirus can block apoptosis induced by actinomycin (Brinaum et a!., 1994; Crook et al., 1993) reaper expression (pro apoptotic of *Drosophila)* (Hay et al., 1995; Vucic et al., 1997) and UV treatment (Manji et al., 1997) in *Sf9* cells. Pro apoptotic genes like Reaper, Hid and Grim of *Drosophila* interact with IAPs and inhibit their anti apoptotic activity thus inducing

apoptosis (Wang et al., 1999). Of the human IAPs best characterized is CARD containing IAPs, c-IAP1, c-IAP2 and X-linked IAP (XIAP). c-IAPs are known to interact with TRAF-2 and its receptor. They also inhibit apoptosis induced by TNFR-1. XIAP is the most potent caspase inhibitor. cIAP1, cIAP2, XIAP inhibit active caspases like 9, 3 and 7 (Deveraux et al., 1997, 1998; Deveraux and Reed, 1999; Roy et al., 1997). Mammalian IAPs inhibit apoptosis in several mammalian cell lines induced by a variety of stimuli (Duckett et al., 1996; Liston et al., 1996).

IAPs expression is regulated at transcriptional, post transcriptional and at translational level. Induction of NF-KB exerts a pro survival role on the cell via increasing the transcription of IAPs (Stehlik et al., 1998; Tang et al., 2001). Apoptosis results in decline of cap-dependent translation and up regulation of cap-independent translation. XIAP is translated in a cap-independent manner through its IREs element of mRNA in response to ionizing radiation (Holcik et al., 2000). On the contrary some apoptotic stimuli are able to induce apoptosis by degrading IAPS as shown with XIAPs. The degradation is mediated by ubiquitinylation of its own RING domain (Yang et al., 2000). Another mechanism adapted by a cell to overcome the inhibitory effect of cellular IAPs is to produce regulatory proteins which can interact with these IAPs. Smac/DIABLO, mitochondrial proteins released along with cytochrome c by an apoptotic stimulus, act as competitive inhibitors for the substrate, caspase 9 of XIAP. Smac/DIABLO binds at the same pocket of XIAP where caspase 9 can bind. (Srinivasula et al., 2001; Chai et al., 2000; Liu et al., 2000; Wu et al., 2000). Similarly even *Drosophila* pro apoptotic factors like rpr, hid. grim and skl can bind to DIAPs and prevent the inhibitory action of IAPs on DRONC *{Drosophila* effector caspase) (Srinivasula et al., 2002; Christich et al., 2002; Wright et al.. 2001). Thus the action of a protein involved in apoptosis is counteracted by its antagonist and net status of the cell depends on over all interactions of all these proteins upon a stimulus imposed on a cell.

Synthetic inhibitors: Specific caspase inhibitors have been designed based on the substrate cleavage site of the caspases. These inhibitors work as pseudo substrates and are therefore competitive inhibitors for active caspases. The chemical group attached to

the peptides decides the mechanism of action of these inhibitors. Linkage of fluoro or chloro methyl ketone (-CMK, -FMK) groups to peptides yield irreversible, competitive inhibitors. The halo (fluoro or chloro) methyl ketone binds to cysteine of the active site

-FMK/-CMK + cysteine (s-s) → thiomehtyl ketone II

(fluoro or chloro methyl ketone) (Active site of caspase) (Becomes inactive)

of the caspase forming thiomethyl ketone II thus inactivating active caspase (Thornberry et al., 1994). The aldehyde group (CHO) attached to these peptide groups makes it reversible inhibitor. The -FMK adducts are more permeable than –CHO adducts. Hence -FMK adducts are used as cell permeable caspase inhibitors in *in vivo* models. The peptides/pseudo substrates can be monomers, (rimers or tetramers containing aspartate alone or aspartate with other aminoacids respectively (eg: Benzyloxycarbonyl-aspartyl (OMe)-fluoromethyl ketone, Benzyloxyl carbonyl-val-ala-asp (OMe) fluoromethyl ketone or YVAD- fluoromethyl ketone). Trimers are more permeable to cell membrane than tetramers. These cell permeable inhibitors are supposed to inhibit all other proteases such as cathepsin B both *in vitro* and *in vivo*. But CHO inhibitors inhibit caspases only (Schotte et al., 1999). DEVD-CHO strongly inhibits caspases-3, 7 and 8 (Garcia et al., 1998; Margolin et al.. 1997). z-VAD-CHO strongly inhibits caspases 1, 3, 5. 7, 8 and 9 but not 2 (Garcia et al., 1998). These cell permeable caspase inhibitors are used as a treatment in some liver injuries (Ekert et al.. 1999).

Objectives: Phosphorylation of the alpha subunit of eukaryotic translational initiation factor 2 (eIF2a) was shown to increase during apoptosis in mammalian systems (Srivastava et al., 1998). Overexpression of a phosphomimetic form of eIF2a, S51D, was found sufficient in inducing apoptosis in NIH3T3 cells (Srivastava et al., 1998). Recent work from our lab has shown that human S51D can be over expressed in *Sf9* cells using baculovirus expression system (Sudhakar et al., 2000). This expression was found to decrease cell viability with out apoptosis (Sudhakar et al., 2000). *Sf9* cells are known to be good model systems to study apoptosis (Sah et al., 1998). Baculovirus infected *Sf9* cells are resistant to apoptosis (Clem et al., 1991). Baculovirus p35 protein is a well known pan caspase inhibitor and inhibits apoptosis is *Sf9* cells and also in a wide range of other systems including mammalian systems (Hay et al., 1994; Biedler et al., 1995; Xue and Horvitz, 1995). Inspite of their suitability as good model systems to study apoptosis. the system however has not been well explored to study the involvement of signal transduction pathways. The work here is aimed to investigate the importance of phosphorylation of the alpha subunit of eukaryotic translational initiation factor (eIF2a) during apoptosis in uninfected and baculovirus infected *Sf9* cells.

Since the conditions that stimulate eIF2a phosphorylation and the relation between eIF2α phosphorylation and apoptosis is not analysed so far, here in this work, we attempted the following:

1. conditions that stimulate apoptosis
2. conditions that stimulate eIF2a phosphorylation and
3. the relation between increased eIF2a phosphorylation and apoptosis, if any, in uninfected, vvt and p35 deletion mutant baculovirus infected cells.

MATERIALS AND METHODS:

Materials and chemicals

Methods

1.1 Cell culture

1.2 Preparation of TNM-FH medium

1.3 Freezing and thawing of cells

1.4 Reconstitution of various chemicals used

1.5 Induction of Apoptosis

1.6 Assays for Apoptosis

1.7 Preparation of cell lysate

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Materials **and** Chemicals: Trypan blue, antibiotic-anitmycotic solution, ATP, cycloheximide, EGTA, etoposide, cell culture grade DMSO, NBT, BCIP, PMSF, bromophenol blue, agarose, tween 20, triton-x100, coomassie R250, sodium bicarbonate, sucrose, mannitol, glutathione, reduced glutathione, Taq Pol enzyme, BSA, tissue culture flasks, 12 well plates and polystyrene tubes were obtained from Sigma. Ac-DEVD-AFC, the substrate analog of caspase(s), recombinant human caspase-3, and caspase-8 inhibitor z-IETD-fmk were purchased from BD Pharmingen. Z-VAD-fmk, a cell permeable caspase-3 inhibitor, Ac-DEVD-CHO, bovine PARP, tunicamycin, thapsigargin, calcium ionophore, leupeptin, pepstatin and aprotenin were obtained from Calbiochem. Grace's insect cell medium, fetal calf serum and lipofectamine were obtained from Gibco BRL. Acry lam ide. bis-acrylamide were from Roche Biochemicals. 0.45 uM nitrocellulose membrane, Glutathione Sepharose 4B, BL21 cells were purchased from Amersham-Pharmacia. 0.22 and 0.45 u,M filter discs were purchased from Millipore. Whatman 1 and 3 filiter papers were obtained from Whatman, UK. Restriction enzymes were bought from New England Biolabs. A phospho specific anti-eIF2a antibody was obtained from Research Genetics, USA. and polyclonal anti-elF2a were purchased from Santacruz Biotechnology Inc., USA. Anti-PARP P85 fragment antibody, anti-mouse IgG, anti-rabbit IgG were obtained from Promega, Inc, USA. Gel extraction kit and midi plasmid isolation kit were from Qiagen. Tris-HCl, glycine, POP and POPOP were obtained from Spectrochem, India. All other chemicals were purchased from Qualigens, India.

Kind gifts: Mutant p35 virus, vAc5p35, was obtained originally from Dr. Paul D. Friesen's laboratory, Institute of Virology, University of Wisconsin, Madison, USA. PERK-GST was obtained from Prof. David Ron, Skirball Institue of Molecular Medicine, New York. USA. Anti-PERK was a gift from Randal Kaufamn, Dept. of Biological Chemistry, Howard Hughes Medical Institute, University of Michigan Medical Centre, Ann Arbor, Michigan.

Methods:

1.1 Cell culture:

Spodoptera frugiperda (*Sf9*) cells were grown in TNM-FH medium supplemented with 10% fetal calf serum and 1% antibiotics. Confluent cells with >95% viability were used in all experiments. Trypan blue exclusion test was carried out to assess the viability of the cells. Trypan blue (0.4%) is added to cell suspension in 50 µl at a final concentration of 0.04% and counted in a hemocytometer.

1.2 Preparation of TNM-FH medium:

TNM-FH medium (Hink, 1970) is Grace's basal insect cell culture medium (Grace, 1962) supplemented with lacatalbumin hydrolysate and yeastolate. The medium is enriched with all the basic nutrients for the growth of insect cells and it is buffered with sodium phosphate. To make 1 litre of TNM-FH medium, 46.3 g of Grace's medium was dissolved in 700 ml of distilled water, 0.35 g of $NaHCO_3$ was also added and the medium was adjusted to pH 6.2 with KOH. Lactalbumin hydrolysate (3.33 g) as well as yeastolate (3.33 g) were added before the volume was made to one litre. The medium was filter sterilized (0.22 µM) in the hood. 10% fetal bovine serum and antibiotics were added later to make complete medium.

1.3 Freezing and thawing of cells:

Sf9 cell stocks were made from healthy *Sf9* log-phase cultures. The cells were harvested and the cell pellet was suspended in complete medium containing 10% cell culture grade DMSO. The final cell density was kept at $4x10^6$ cells/ml. The cell suspension was aliquoted (1 ml) and frozen slowly. The cells were initially placed at 4°C for one hour, at -20° C for 2-3 hrs and then at -70° C overnight. The next day cells were transferred to liquid nitrogen. The above mentioned stocks were removed from liquid nitrogen when required and thawed by incubating at 37° C. Once the stock was thawed, the vial was wiped with 70% ethanol before taking in to the hood. The cells were transferred to a T-25 culture flask that contains 3 ml of complete medium. After seeding of cells, the medium can be changed to fresh complete medium, if necessary. Seeding capacity of the flask depends on the surface area - 96 well plate is 10,000; 24 well plate is 0.5 million, 12 well

plate is one million; 6 well plate is two million; 35 mm petri dish is two million; T-25 culture flask is four million; T-75 culture flask is twelve million cells.

1.4 Reconstitution of various chemicals used:

Etoposide, tunicamycin, thapsigargin, calcium ionophore, genestein and staurosporine were reconstituted in DMSO to a final concentration of 50 mM, 12 mM, 15.3 mM, 100 mM, 10 mM and 10 mM respectively. The final concentrations of these chemicals used in various experiments are: etoposide: 20 and 125 μM; tunicamycin: 4 and 20 μM; thapsigargin: 35 μM; calcium ionophore, A23187: 20 and 100 μM; genestein: 25 and 50 mM; staurosporine: 100 nM.

The protease inhibitor pepstatin (1.4 mg/ml) was prepared in methanol; aprotenin (5 mg/ml) and leupeptin (5 mg/ml) were prepared in water; PMSF (50 mM) was prepared in isopropanol.

1.5 Induction of Apoptosis:

*Sf*9 cells were irradiated with UV-B light (312 nm) for 30-60 seconds and incubated at 27° C for 15 hrs. Alternatively cells were treated with different agents like etoposide, cycloheximide, EGTA, A23187 and tunicamycin and incubated at 27° C for 15 hrs. Apoptosis was also studied in the presence of cell permeable caspase-3 and 8 inhibitors z-VAD-fmk, and z-IETD-fmk. For these experiments the apoptotic stimulus and caspase inhibitor were given to cells simultaneously. Effect of kinase inhibitors on UV-B induced apoptosis was studied by treating the cells with either genestein or staurosporine for 30 min, cells were exposed to UV-B light and incubated at 27^0 C for 15 hrs.

1.6 Assays for Apoptosis:

$4x10^6$ cells were taken for each experiment and scored for apoptosis by monitoring plasma membrane blebbing. A small aliquot, 45 μl of cell suspension, was stained with 0.04% trypan blue and viewed under an inverted microscope (Labovert) equipped with a digital camera and the software MV500 DEMO to score (20x magnification) the apoptosed cells.

1.7 Preparation of cell lysate:

4 million cells were pelleted at low speed, washed with ice cold PBS twice at 4° C and lysed in 100 jil of lysis buffer (20 mM Tris-HCl pH 7.8, 1 mM Mg^{2+}, 1 mM DTT, 3 μg/ml of pepstatin, 10 μg/ml of aprotenin,10 μg/ml of leupeptin, 250 μM PMSF). Cells were incubated on ice for 10 min in lysis buffer. Lysed cells are centrifuged at 12,000 rpm at 4°C for12 min and the supernatant is collected in to a fresh eppendorf and stored at-70°C.

1.8 Ac-DEVD-AFC hydrolysis:

Caspase activity of the cells undergoing apoptosis was determined using Ac-DEVD-AFC hydrolysis in the cell extracts. Approximately, 400 μg extract protein was taken in 50 μl of lysis buffer and was diluted to 750 μl with 20 mM Tris-HCl buffer (pH 7.2) containing 1 mM Mg^{2+}, 80 mM KCl and 1 mM DTT for each reaction. Ac-DEVD-AFC hydrolysis was monitored by fluorescence emission of the released AFC (excitation, 400 nm: emission, 500 nm) using Fluoromax-3, Jobin Yvon, Horiba Spectrofluori meter (Bhuyan et al., 2000). The final concentration of Ac-DEVD-AFC used in the reactions was 10.9 uM.

1.9 PARP Cleavage:

In addition to monitoring the cell morphology and caspase activity of the cells undergoing apoptosis, the relative induction of apoptosis caused by various treatments was also studied by the cleavage of pure bovine Poly ADP Ribose Polymerase Protein (PARP). Pure bovine PARP protein (150 ng) was incubated with the *Sf9* extracts (containing 60 μg of protein) at 30^0 C for 90 mins in a 40 ul reaction mixture consisting of 10 mM Tris-HCl, pH 7.5; 2.5 mM KH_2PO_4; 2 mM NaCl; 68 mM sucrose and 220 mM mannitol. The PARP reactions were processed by the addition of equal volume of 2x reducing buffer containing 62.5 mM Tris, pH 6.8, 6 M urea, 10% glycerol, 2% SDS, 5% β-mercaptoethanol and 0.005% bromophenol blue. The PARP reaction samples were boiled at 65° C for 15 minutes, and then separated on 10% SDS-PAGE, transferred to nitrocellulose membrane and probed with an antibody (anti-rabbit antibody) that recognizes the 89 kDa cleaved fragment of PARP.

1.10 Virus infection:

80-90% confluent flasks were selected for virus infection. The required MOI for infection was calculated before infection. The complete medium in which the cells were maintained was replaced by 500 µl of complete medium containing a virus for a 35 mm petri dish. The infection was given for one hour with intermittent rocking of the petri dishes or culture flasks once in 15 mins. After **one hour,** 1ml of complete **medium for** 35 mm petri dish or 3ml to T-25 **culture** flask was added and incubated in dark humid environment (preferably in a box with a moist tissue) for 48 hrs at 27° C in the incubator. In order to evaluate the importance of eIF2α phosphorylation in apoptosis, cells were treated with UV-B radiation before and after the expression of wt and mutants of human eIF2a. Expression of recombinant eIF2a wt and mutants was carried out by infecting the cells with recombinant virus expressing wt and mutants of eIF2a.

1.11 Virus amplification:

For virus amplification, cells were infected in T-75 culture flasks. Virus infected cells were left in the incubator at 27^0 C for 10-15 days for the virus to amplify. Lysis of cells occurred when ~80% **of the** cells were infected. Cell lysate was harvested and viral supernatant was collected by centrifuging the cells at 3000 rpm for 10 min at room temperature. The virus was filtered through 0.22 µ filters. The virus was stored in liquid nitrogen for long storage. It is stable **at** -70^0 C **for** 1-2 years **and** few weeks at 4°C.

1.12 Virus titre calculation by End Point Dilution assay:

The infection efficiency of the virus changes on long storage. It's necessary **that its titre** be calculated before use after long storage. Virus titre was calculated by end point dilution assay (Reilly et al., **1992).** The aim of this procedure *is* to. dilute the virus and see if such diluted inoculum is able to infect cell cultures. The titre of the virus is **obtained as pfu/ml (plaque forming** units or number of virus particles per ml). The pfu/ml value is converted to MOI *(number of virus particles per cell) during infection.*

The experiment was performed in 96 well plates. Cells were diluted to a concentration of $1x10^5$ cells/ml. Ten fold serial dilutions of the virus stock in tissue culture medium were

made. Dilutions till 10^{-5} to 10^{-8} are preferred for each virus stock. Cells were seeded at a concentration of $1x10^4$ cells per 100 ml in each well of 96 well plates. Each row was used for one dilution of the virus. In each row first well was kept as control. 10 ul of the diluted virus was added to each well except for the controls. The plate was sealed with parafilm and incubated in humid environment at 27° C in the incubator for four days. After four days of infection. virus infected cells were identified under microscope by the appearance of polyhedra particles. For each dilution number of infected wells and uninfected wells were noted and tabulated. This method is based on the assumption that (Reed and Muench, 1938) cultures infected at a particular dilution would have been infected at all lower dilutions: cultures uninfected at a particular dilution would have been uninfected at all higher dilutions.

For eg:

Dilution	infected wells	uninfected wells	Dilution	Infected	Uninfected	% infected
10^{-5}	12	0	10^{-5}	21	0	100
10^{-6}	8	4	10^{-6}	9	4	69.2
10^{-7}	1	11	10^{-7}	1	15	6.3
10^{-8}	0	12	10^{-8}	0	27	0

pfu/ml is calculated by multiplying the dose or dilution that gives 50% response ($TCID_{50}$) and concentration of virus particles at that dilution. $TCID_{50}$ is calculated by the formula: $TCID_{50}$ (dose that gives 50% response) = log of dilution giving a response greater than 50% - PD (proportionate distance) of that response.

10^{-7} respectively. The dilution that gives more that 50% of infection, 10^{-6} was taken as A in calculating the PD value and dilution that gives less than 50% of infection, 10° is taken as B. Proportionate distance (PD) of a 50% response is calculated using the formula PD=**(A-50)/(A-B). PD** = (69.2-50)/ (69.2-6.3) = **0.305.**

TCID50 (dose that gives 50% response) = log of dilution giving a response greater than 50%- the PD of that response.

Log $TCID_{50}$ = -6-0.305

-6305

$TCID_{50} = 10^{-6.305}$

The titre of the virus is the reciprocal of this = 2.02×10^6/10ul or 2.02×10^8/ml. This can be converted to pfu/ml (plaque forming units per ml) using the relationship

$pfu = TCID_{50} \times 0.69$

Thus titre of the virus is 1.4×10^8 pfu/ml. Infection to cells is given in terms of MOI (multiplicity of infection) i.e., no. of infectious virus particles or plaque forming units per cell. So if the MOI is set at 10 for 2×10^6 cells (35 mm petri dish), no. of virus particles that has to be infected is 2×10^7. This no. of virus particles is present in 143 u1 of virus which has titre value of 1.4×10^8 pfu/ml.

1.13 Purification of PKR-GST and PERK-GST proteins:

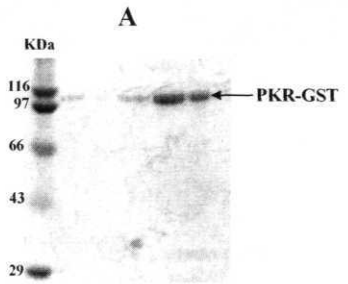
A recombinant PKR-GST construct (kind gift of Prof. Bryan Williams, Cleveland Clinic Foundation, USA) and was overexpressed in BL21 cells. PERK-GST transformed cells were obtained from Prof. David Ron, Skirball institute for molecular medicine, New York. The transformed cells (100 µl) were inoculated in a 10 ml of LB with ampicillin (100 µg/ml) and grown overnight. A secondary culture of 100 ml was set up with 2 ml (2%) of primary culture for 37° C for 10-12 hrs in LB with ampicillin. From this 50 ml was inoculated in 500 ml of LB with ampicillin and incubated in a orbital shaker at 200 rpm at 37° C for 2-3 hrs. O.D. of the cell growth was allowed to reach OD_{600} 0.6-0.7. Cells were then induced with IPTG at 0.1 mM. Induced cells were grown at 100 rpm at 27° C for 10-12 hrs. Cells were pelleted at 3000 rpm for 10 min at 4° C. Cell pellet was lysed in 10 ml of PBS lysis buffer containing 1 mM DTT, 0.1% triton x100, 10 µg/ml of leupeptin, 10 µg/ml of aprotenin, 3 µg/ml of pepstatin, 250 uM of PMSF. The lysate was then subjected to sonication at 4° C; four strokes of 10 amp of frequency were given to 3 ml of lysate each time for 15 sec with an interval of 1.5 min. The sonicated lysate was loaded on to the Glutathione-sepharose-4B affinity matrix. The column was washed with 50 mM Tris (pH 8) and flow threw was collected. The GST tagged protein was eluted with 50 mM Tris (pH 8) containing 10 mM GSH. Fractions of high O.D. were checked. The protein was eluted in the first 3-4 fractions. The eluted protein was dialyzed against 50 mM Tris buffer (pH 8) to remove reduced glutathione. The samples were stored at

Figure 2: Purification of recombinant human PKR-GST

Panel A represents coomasie gel of the purified PKR-GST protein. PKR-GST fusion construct was transformed in BL21 E.coli cells. The cells were treated with IPTG in their log phase to induce the expression. Cells were harvested, sonicated and lysed. The crude lysate was passed through glutathione sepharose 4B column. The bound protein was eluted with 10 mM GSH in 20 mM Tris-HCl, pH 8.

Panel B: Phosphorylation of human recombinant eIF2α by PKR-GST. Recombinant human wt eIF2a was expressed in *Sf9* cells and partially purified (Sudhakar et al., 2000). This purified protein (~50 ng) was incubated with *Sf9* cell extracts (~30 μg) and phosphorylated with different concentrations (~40, 80, 200, 400 ng) of PKR-GST in the presence of a reaction mixture consisting of (80 mM Tris-HCl, 2 mM Mg^{2+}, 1 mM DTT, 30 uM ATP) at 30° C for 12 min. The reaction was terminated with 4x SDS loading buffer, proteins were separated by 10% SDS-PAGE, transferred to a nitro cellulose membrane and analysed with a phosphospecific anti eIF2a antibody.

Panel C: Phosphorylation of the recombinant human wt eIF2a protein and *Sf9* (~30 μg) cell extract was carried out by of PKR-GST (~40 ng) at different time periods at 30° C as mentioned in the previous legend.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KDa
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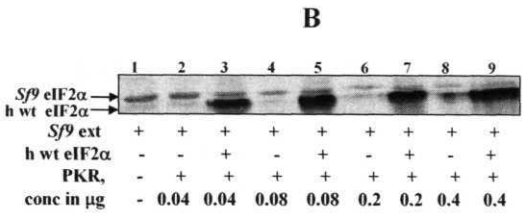
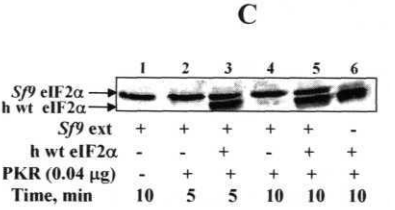
97
66
43
29
PKR-GST


B
1 2 3 4 5 6 7 8 9
Sf9 eIF2α
h wt eIF2α
Sf9 ext + + + + + + + + +
h wt eIF2α - - + - + - + - +
PKR, - + + + + + + + +
conc in μg - 0.04 0.04 0.08 0.08 0.2 0.2 0.4 0.4


C
1 2 3 4 5 6
Sf9 eIF2α
h wt eIF2α
Sf9 ext + + + + + -
h wt eIF2α - - + - + +
PKR (0.04 μg) - + + + + +
Time, min 10 5 5 10 10 10

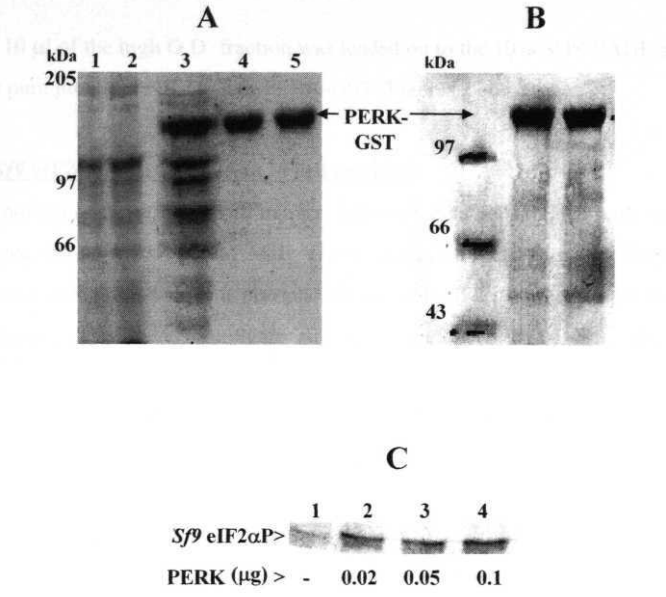


Figure 3: Purification of PERK-GST protein

PERK-GST protein was purified from transformed E. coli, BL21 strain as described in the legend to figure 3.

Panel A: Coomasie gel for PERK-GST purification. Lanes in the figure are: lanes 1 and 2, crude BL21 cell extract; lane 3, IPTG induced cell extract; lanes 4 and 5, PERK-GST protein.

Panel B: The figure is the western blot showing the immunoreactivity of the purified protein with mouse anti-PERK.

Panel C: Phosphorylation of *Sf9* eIF2α phosphorylation by purified PERK-GST. ~30 μg of *Sf9* extract prepared from control, uninfected cells was incubated with increasing concentrations of PERK (~20, 50, 100 ng) of enzyme in a phosphorylation buffer (20 mM Tris-HCl pH 7.8, 1 mM DTT, 2 mM Mg^{2+}, 80 mM KCl and 30 uM ATP) at 30° C for 10 min. The samples were terminated with 4xSDS sample buffer; proteins were separated by SDS-PAGE, transferred to a membrane probed with a phosphospecific anti-eIF2a antibody. The figure is a western **blot.**

70^0 C. 10 ul of the high O.D. fraction was loaded on to the 10% SDS-PAGE and analysed for the pure protein, PKR-GST or PERK-GST (Figures 2 and 3).

1. 14 ***Sf9* eIF2αphosphorylation** in cell extracts:

Equal protein (~25 μg) from cell extracts prepared from cells treated with various agents was separated on a 10% SDS-PAGE. The separated proteins were transferred to a nylon membrane and probed with a phosphospecific anti-eIF2a antibody and the membrane was developed with AP-con jugate substrate developer, NBT-BCIP. The blots were scanned at a resolution of 200 dpi by using a Hewlett Packard Scanjet 3400C. Band intensities were quantified using the Quantity 1™ Image Analysis software using Biorad Model GS-800 Calibrated Imaging Densitometer.

1.15 ***In vitro*** phosphorylation:

In vitro phosphorylation assays were carried out by incubating the *Sf9* cell extract with purified PERK-GST/PKR-GST in a 15 ul cocktail containing 20 mM Tris-HCl pH 7.8. 2 mM Mg^{2+}, 80 mM KC1 and 50 uM ATP at 30° C for a period of 12 min. The protein kinase assay was terminated by the addition of 4x SDS sample buffer (0.25 M Tris-HCl pH 6.8, 10% SDS, 10% glycerol, β-mercaptoethanol and bromophenol blue). The samples were heated for 2-3 min in boiling water and analysed on 10% polyacn lamide gel.

1.16 **Cleavage** of PKR-GST and PERK-GST *in vitro:*

Purified recombinant PKR-GST (~800 ng) and PERK-GST (~3 μg) was incubated *in vitro* at 37° C (~ 40 μg) with *Sf9* cell extracts prepared from cells undergoing apoptosis and non-apoptotic cells containing equal amount of protein for different time periods as described in figure to legends and terminated in 4x SDS-loading dye. The cleavage inhibition was demonstrated with caspase-3 inhibitor, Ac-DEVD-CHO (50 uM) which was incubated simultaneously with extracts and PERK protein. The extracts were then separated on 10% SDS-PAGE for PKR samples and 7.5% SDS-PAGE for PERK samples. The separated proteins were transferred to a nylon membrane and probed with a

polyclonal anti-PKR or monoclonal anti-PERK antibody to determine the cleavage of recombinant PKR or PERK in the extracts prepared from apoptotic cells.

1.17 Cleavage of PERK-GST by pure caspases:

~1.5 μg of PERK-GST protein in 2 μl is incubated with pure recombinant caspase-3 and caspase-6 proteins at 300 and 600 ng in the cleavage buffer (10 mM Tris-HCl, pH 7.5, 2.5 mM KH_2PO_4; 2 mM NaCl, 68 mM sucrose and 220 mM mannitol) for about 3 hrs at 30° C. The reaction was terminated with 4x SDS loading dye and proteins were separated on a 7.5% SDS-PAGE transferred to a nitro cellulose membrane and probed with monoclonal anti-PERK antibody.

1.18 *Sf9* eIF2a phosphorylation by caspase cleaved PERK:

This reaction was performed in two steps. In the first step PERK-GST was allowed to be cleaved with caspase-3 protein in the cleavage buffer (10 mM Tris-HCl, pH 7.5, 2.5 mM KH_2PO_4; 2 mM NaCl and 220 mM mannitol) for about 3 hrs at 30° C. Then the cleaved PERK was allowed to phosphorylate the *Sf9* eIF2a in the extract in the phosphorylation buffer (20 mM Tris-HCl pH 7.8, 2 mM Mg^{2+}, 30 μM ATP, 1 mM DTT and 80 mM KC1) at 30^0 C for 12 min. The reaction was terminated with 4x SDS loading dye.

1.19 *In vivo* protein synthesis:

$2x10^6$ cells were seeded in 35 mm petridish and treated with various agents as mentioned above for 15 hrs. The cells were pelleted at low speed, washed with complete media-methionine and incubated with complete medium-methionine for one hour at 27° C to deplete the endogenous methionine. Cells were then pelleted, washed, transferred to a fresh medium containing 20 uxi of labeled [^{35}S] methionine in 20 μl, and incubated again for one hour at 27° C. Cells were then washed and lysed in 60 μl lysis buffer. 15 ui (~ 20 μg protein) of the reaction mixture was spotted on a whatman filter paper, dried, and counted in a Wallac 1409 liquid scintillation counter to obtain the amount of labeled methionine taken up by the cells. A duplicate filter was processed through trichloroacetic acid (TCA) through a series of steps, first incubated on ice for 10 min in 10% TCA, boiled in 5% TCA for 3 min, and incubated at room temperature in 5% TCA for 5 min.

The filters were washed first in alcohol and then in acetone. The filters were dried, taken in scintillation fluid (50 mg POPOP, 4 g POP per litre of toluene) and measured in a liquid scintillation counter to determine the amount of radioactivity incorporated into the TCA precipitable portion of the protein. Percent incorporation of labeled methionine into the protein was calculated based on the above uptake and incorporation values. Protein synthesis with caspase inhibitors was performed in 24 well plates to minimize the usage of the inhibitor.

1.20 **Autoradiography:**

The labeled proteins were separated by 10% SDS-PAGE and the gel was dried using a Bio-rad gel drier. The dried gel was exposed to Kodak X-ray film and kept -70^0 C. The film, after exposure for the required time was developed with a set of photographic solutions as per the manufacturer's instructions. Alternatively the dried gel was exposed to the cassette of the phosphor imager and the developed image was scanned by phosphor imager.

1.21 Protein Estimation:

Cell lysate total proteins were estimated by a Bio-rad protein estimation kit as per the instructions given by the manufacturer.

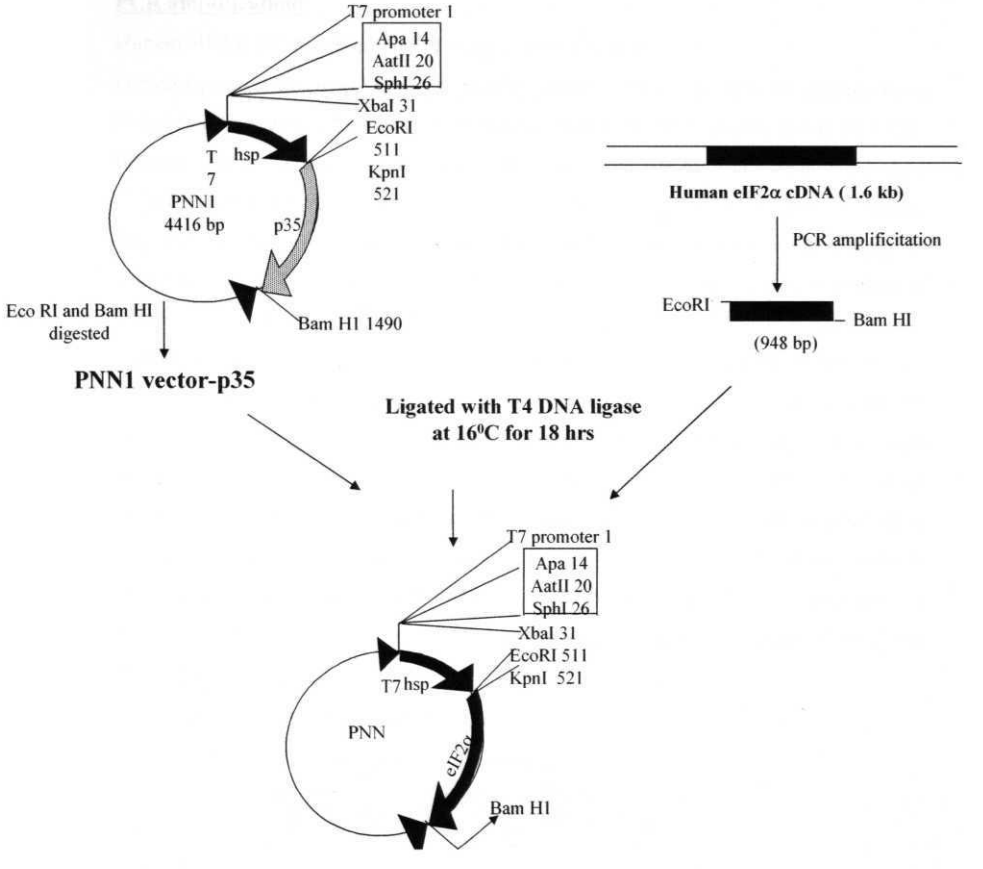
1.22 Cloning:

Wild type and mutants of human eIF2a cDNAs were cloned in to a PNN1 vector under an Hsp promoter for transient expression of the eIF2a protein in *Sf9* cells.

Steps in **cloning:**

Following steps were used: a) PCR amplification of eIF2α cDNA; b) gel elution of PCR amplified product; c) restriction digestion of gel eluted PCR product; d) ligation of PCR amplified insert and vector; e) transformation of the ligation mixture; inoculation of randomly selected transformed colonies; f) DNA isolation from the innoculum; confirmation of positive colonies; g) midi preparation of positive clone and transfection of *Sf9* cells with pure plasmid DNA (Flow chart # 1).

Flow chart for cloning:



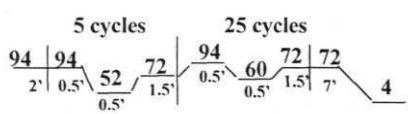
PCR amplification:

Human eIF2a cDNA was amplified using primers (32 mer).

Forward primer: cgGAATTCatgccgggtctaagttgtagattt with site for Eco RI enzyme at its 5' end (melting temp of primer with restriction enzyme: 66.73^0C; primer alone: 54.3^0C).

Reverse Primer: cgGGATCCttaatcttcagctttggcttccat with site for Bam H1 enzyme at its 5' end (melting temp with restriction enzyme: 69.02° C; primer alone: 55.4° C) (Figure 4A). Primers were reconstituted in autoclaved water at a concentration of 4 μg/μl at stored at -20° C. The method involved was step up PCR to promote proper binding of primer on the template. After denaturation of the plasmid DNA at 94° C for 120 sec, primer was annealed to its template at lower temperature, i.e., at 52° C for 30 sec. Extension of 5' and 3' overhangs was done at 72° C for 90 sec. This cycle was run for five times. Once again the plasmid was denatured at 92° C for 30 sec, primers were annealed at 60° C for 30 sec and overhangs were extended at 72° C for 90 sec. These cycles were continued for 25 times for amplification of the insert. Extension temperature for the last cycle was continued for 7 min. PCR reaction was set up with 50 ng of plasmid DNA in 1x PCR buffer, 1.5 mM of $MgCl_2$, 200 mM of dNTPs, 50 ng of forward and reverse primers, 1 enzymatic unit of Taq polymerase in a reaction volume of 50 ul and reaction is represented as:

Step up PCR for eIF2α cDNA:



Gel **elution** of **PCR** products:

PCR amplified products were checked on 0.8% agarose gel in TAE buffer by comparing with DNA marker (Figure 4B). Gel containing the DNA was carefully cut with a clean blade and DNA was eluted with gel extraction kit. Gel was solubilised with 3 volumes of gel extraction buffer with silica slurry added (1 μg of DNA=15 μl of silica slurry) and incubated at 52^0C for 15 min. Agarose was pelleted at high speed. The agarose pellet was washed with wash buffer. The supernatant containing the DNA+silica complex was

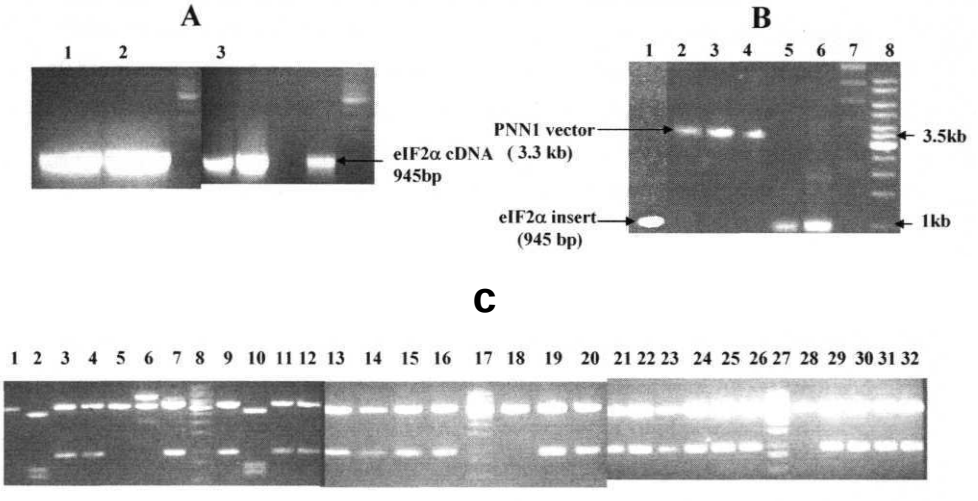
Figure 4: Cloning of eIF2a cDNA into a PNN1 plasmid

PCR amplification of human wt and mutants of eIF2α cDNA was carried out using gene specific primers as described in 'Materials and Methods'.

Panel A represents the products of PCR amplification on a 0.8% agarose gel. Lanes are as follows: Lane1, eIF2α wt; lane 2, eIF2α S51A; lanes 3, 4 and 5 eIF2a S51D.

Panel B represents vector DNA and gel eluted PCR products of eIF2a cDNA on an agarose gel. PNN1 vector DNA was isolated and digested with EcoRI and Bam H1. Vector DNA and gel eluted PCR products of eIF2a cDNA were separated on a 0.8% agarose gel. The various lanes represent different amounts of the following. Lanes 2-4 represent different amounts of vector DNA; lane 1, gel eluted eIF2α wt cDNA; lane 5 gel eluted eIF2α S51A cDNA; lane 6 gel eluted eIF2a S51D cDNA lane 7, *X* marker DNA; lane 8, 150 ng of 1 kb DNA ladder.

Panel C represents the restriction analysis of plasmid DNA isolated from transformed colonies to determine the positive colonies containing the eIF2cDNA insert. The vector DNA was ligated with eIF2a cDNA and the ligation mix was transformed as mentioned in 'Materials and Methods'. Colonies were grown in agar medium containing ampicillin (100 μg/μl) and tetracycline (15 μg/μl). Plasmid DNA was isolated from randomly selected colonies and was double digested with Eco RI and Bam H1. Digested DNA products were separated on 0.8% agarose gel to identify the 945 bp insert containing positive colonies. Lanes 8, 17 and 27 represent marker lane where as all other lanes contain plasmid DNA with or with out eIF2a cDNA insert as indicated in the figure.


A
1
2
3
eIF2α cDNA (3.3 kb)
945bp
B
1
2
3
4
5
6
7
8
PNN1 vector
3.5kb
eIF2α insert
(945 bp)
1kb
C
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32

collected. The supernatant was transferred to a fresh centrifuge tube, spun at high speed for 10 min to obtain the silica+DNA pellet. This pellet was washed thrice and the pellet was air dried. DNA was eluted in to 20 ul of autoclaved water from silica by incubating at 37^0 C for 30 min. DNA was collected in to a fresh tube and stored.

Generation of PNN1 vector and insert with compatible restriction sites:

PNN1 plasmid was double digested with Eco RI and Bam H1 in bulk and checked on 0.8% agarose gel and the vector released was gel eluted. The gel purified PCR products (insert) were also double digested with Eco RI and Bam HI and were ethanol precipitated. They were checked on an agarose gel and quantified comparing with the1 kb ladder (3 kb marker of *X* ladder is 80-90 ng per 150 ng of the total marker loaded).

Ethanol precipitation of DNA:

DNA after restriction digestion was incubated with two volumes of absolute ethanol and one tenth volume of 3 M sodium acetate pH 5.2 and incubated at -20^0 C overnight. DNA was pelleted by spinning at 13 k for 20 min. Pellet was washed with 70% ethanol and dried at 37° C for 20 min. The dried DNA was suspended in 10 ul of autoclaved water, allowed to dissolve by incubating at 37^0 C for 20 min and stored at -20^0 C.

Ligation:

Vector and insert digested with Eco RI and Bam H1 were ligated with T4 DNA ligase in a 1x ligation buffer in a final reaction volume of 10 u.1. Insert and vector were ligated at a molar ratio of 3:1. As vector is 3.5 kb and insert 948 bp, equal amount of insert and vector (100 ng) were taken for ligation. Ligation was performed at 16^0 C for 18 hrs. The entire ligation mixture was transformed in XL1 blue cells (E.coli strain with tetracycline and ampicill in resistant genes).

Transformation:

Competent XL1 blue cells were incubated with ligation mix on ice for 30 min (Sambrook et al., 1989). The cells were given heat shock at 42^0 C for 30 sec, immediately transferred to ice, 500 ul of LB with ampicillin and tetracycline was added, and incubated at 37° C

for 30 min. The transformed cells were plated on LB agar plates containing ampicillin (100 jug/ml) and tetracycline (15 fig/ml). The plates were incubated for 12 to 14 hr to allow the growth of transformed bacterial colonies. Colonies are selected randomly and inoculated in 3 ml of LB medium with ampicillin and tetracycline. Cells were allowed to grow for 10-12 hrs at 37° C and plasmid was isolated by alkaline lysis method (Sambrook et al., 1989).

Identification of positive clone:

The plasmid DNA was digested with Eco RI and Bam HI enzymes. These are the enzymes used for PCR amplification of the insert. A 948 bp insert was expected to release upon digestion of the plasmid DNA from positive clone (Figure 4C).

Midi preparation of the positive clone:

After confirmation of the positive clone; cells were transformed with 100 ml of the positive clone and were inoculated in 100 ml of LB containing 100 μg/ml ampicillin and 15 μg/ml of tetracycline. The culture was grown for 15 hrs until significant growth of culture was obtained. Plasmid was isolated using Qiagen plasmid midi preparation kit- Cells were pelted at 4000 rpm for 10 min. Cell pellet was lysed with 5 ml of lysis buffer (P1). The cell lysate was incubated with 5 ml of P2 for 10 min at room temperature. 5 ml of P3 was added, centrifuged at 15,000 rpm at 4° C for 20 min. Meanwhile the DNA columns were equilibrated with 10ml of equilibration buffer. On to this equilibrated columns the supernatant was loaded, washed with 20 ml of wash buffer. DNA was eluted with 15 ml of elution buffer in to centrifuge tubes. DNA was precipitated with 0.8 volumes of isopropanol. DNA was pelleted by centrifuging at 15,000 rpm for 10 min and washed with 70% ethanol. The DNA pellet was suspended in 500 ul of autoclaved water and stored at -20^0 C.

Transfection:

Cells were washed with incomplete medium with out serum and antibiotics twice or thrice. Around one million cells were seeded in 35 mm petri dish. Meanwhile DNA-lipofectamine complexes were made. DNA at the required concentration (5-20 μg) was

incubated in 50 µl of incomplete media for about 15 min in an eppendorf. Lipofectamine in 50 µl of incomplete media was incubated in a polystyrene tube. Volume of lipofectamine is equal to the concentration of the DNA that is transfected. DNA was added to lipofectamine vial and mixed thoroughly and incubated for about 30 min in hood. Thus 100 ul of DNA-lipofectamine mix was added on to the cells drop wise covering the whole surface of the petri dish containing 900 µl of incomplete media. Cells were incubated in the incubator at 27° C for 4-6 hrs. Incomplete medium was then changed to complete medium. After 10-12 hrs. cells were given heat shock for half an hour at 42° C. Cells were incubated in the incubator at 27° C for 48 hrs and analysed for the expression of the protein.

1.24 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE):

Proteins were separated by a modified Laemmli method. The 10% separation gel mix, **8ml.** contained the following: 1.875 ml of 2 M Tris-HCl pH 8.8, 2.5 ml of 30:0.8 acrylamide:Bis-acrylamide, 75 µl of 10% SDS. 50 ul of 10% ammonium per sulphate, 8 ul of TEMED and 3.75 ml of water (7.5% gel mix contained 2 ml of acrylamide:bisacrylamide solution. 4.25 ml of water and the rest being the same). The 5% stacking gel mix in a total volume of 2.5 ml contained: 1.875 ml of water, 0.375 ml of Tris-HCl pH 6.8, 0.325 ml ol acrylamide:bisacrylamide solution, 25 ul of 10% SDS, 50 ul of 10% ammonium per sulphate. 8 ul of TEMED. Proteins were prepared in a sample buffer containing 0.25 M Tris-HCl pH 6.8, 10% SDS, 40% glycerol, SDS, 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. The gel was stained either by coomassie or by silver nitrate.

1.25 Western blotting:

The proteins separated on SDS-PAGE were transferred on to a nitro cellulose membrane electrophoretically at 70 volts. The transfer was done for 3 hrs in a transfer buffer (25 mM Tris, 19 5mM glycine, in 20% methanol). After the transfer, membrane was stained with ponceau s red solution and molecular weight marker proteins are marked. The stain

was removed by rinsing the membrane with TBS. The membrane was thereafter soaked in blocking solution (3% blot grade BSA or 5% mild powder in TBS-10 mM Tris-HCl pH 8, 150 mM NaCl). After 1hr, the blocking solution was replaced the primary antibody diluted in TBS and left at cold room for 10-12hrs.The membrane was later washed thrice with TBST (0.05% tween 20). Membrane was incubated with secondary anti-IgG-AP conjugate for 45min. The membrane was once again washed thrice with TBST. The membrane was developed with 10 ml of AP buffer buffer (100 mM Tris-HCl pH 9.5, 100 mM Nacl, 5 mM $MgCl_2$) containing 330 u1 of NBT (one tablet of 25 mg from Sigma is dissolved in one ml of water) and 33 ul of BCIP (one tablet is dissolved in one ml of dimethyl formamide). The color development was arrested by washing the membranes in distilled water. The membrane was air dried and stored between filter papers and kept away from light. Blots were scanned at a resolution of 150 dpi by using a Hewlett Packard Scanjet 3400C. Band intensities were quantified using the Quantity 1™ Image Analysis software using Biorad Model GS-800 Calibrated Imaging Densitometer.

RESULTS AND DISCUSSION;

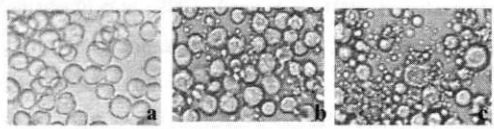
- Stress-Induced changes in morphology of *Sf9* cells
- Pro apoptotic agents stimulate 5/xaspase activity
- UV-B light also stimulated apoptosis and caspase activity in mammalian cells
- PARP Cleavage
- Pro- and non-apoptotic stresses stimulate eIF2a phosphorylation in *Sf9* cells
- Pro and non-apoptotic agents fail to stimulate eIF2a phosphoryiation in 5/9 extracts *in vitro*
- Baculovirus infection and apoptosis
- eIF2a phosphoryiation mediates apoptosis in *Sf9* cells
- Transient expression of human wt and mutant eIF2α's in *Sf9* cells
- Kinase inhibitors and apoptosis
- Inhibition of caspase activity mitigates eIF2α phosphoryiation
- Kinetics of apoptosis, caspase activation and 5/9 eIF2a phosphoryiation in UV-B treated cells
- Cleavage of eIF2a kinases by 5/9 extracts
- Effect of purified caspases on PERK cleavage
- Caspase-cleaved PERK stimulates *Sf9* eIF2α phosphoryiation *in vitro*
- Protein synthesis in apoptotic and non-apoptotic conditions
- **Discussion**

The ovarian cell lines of *Spodopterafrugiperda (Sf9)*, a lepidopteron insect, have been used here to study the relation between phosphorylation of eIF2α and apoptosis. A variety of physical, chemical and physiological agents have been used to induce apoptosis in these cells. Depending on their ability to induce apoptosis, they are classified as pro or non apoptotic stresses.

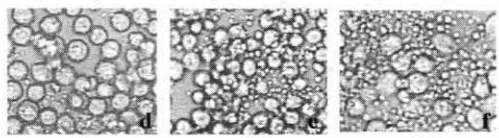
Stress-Induced changes in morphology of *Sf9* cells: In order to elicit apoptosis, uninfected and baculovirus-infected *Sf9* cells were treated with various agents such as UV-B light, etoposide (DNA damaging agents), cycloheximide (a translational elongation inhibitor). EGTA (a calcium chelator), A23187, a calcium ionophore, tunicamycin (N-linked glycosylation inhibitor) and thapsigargin (inhibitor of ER Ca^{2+} ATPase). All of them are known to promote apoptosis in many mammalian systems. Trypan blue exclusion test, formation of apoptotic bodies, and caspase activation assays were carried out to determine the process of apoptosis induced by various agents. Live and apoptotic cells excluded trypan blue. Apoptosis was assessed then by counting the live cells under microscope that are distinctly larger than apoptotic bodies under higher magnification. UV-B treatment produced a maximum of 80-85% apoptotic bodies compared to the untreated controls (Figure 5c vs. 5a). A short exposure of UV-B light for 30 seconds also resulted in 40-50% of apoptosis (Figure 5b). Etoposide and cycloheximide are known to induce apoptosis in mammalian systems (Barry et al., 1993; Alessenko et al., 1997). Etoposide induced apoptosis in *Sf9* cells in a dose dependent manner. 50 μM of etoposide induced 10-20% of apoptosis; whereas 100 μM and 200 μM induced 50-60% and 80-90% of apoptosis respectively (Figure 5, d-f). Cycloheximide inhibits translation at the elongation step resulting in the accumulation of unprocessed proteins in the endoplasmic reticulum (Gething and Sambrook, 1992). In *Sf9* cells, low concentrations of cycloheximide, 20 and 500 μM, failed to induce apoptosis (Figure 5g and 5h). On the contrary high concentrations like 1, 3 and 5 mM induced apoptosis of 30-40%, 60-70% and 80-90% respectively (Figure 5, i-k). In all our further experiments, 125 μM etoposide and 3 mM cycloheximide were used. Other agents, like EGTA, A23187, tunicamycin and thapsigargin which were known to disturb the endoplasmic reticulum and promote apoptosis in mammalian systems (McConkey and Orrenis, 1997; Ermak and

Figure 5: Apoptosis in *Sf9* cells

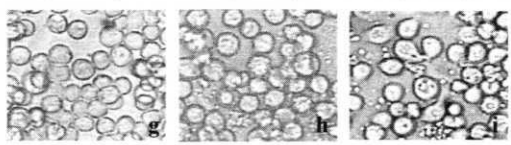
Cells were treated with the UV-B light (312 nm) for 30 and 60 seconds and incubated for 15 hrs at 27° C, or treated with different agents as mentioned below for 15 hrs at 27° C. Apoptosis was scored by looking the cells under inverted microscope as described under 'Materials and Methods'. Magnification: 20x. Various panels are as follows: a, uninfected controls cells; b, UV-B treated for 30 sec; c, UV-B for 60 sec; d, etoposide (Et) 50 uM; e, Et 100 uM; f, Et 200 uM; g, cycloheximide (CH) 20 uM; h, CH 500 uM; i, CH 1.0 mM; j, CH 3.0 mM; k, CH 5.0 mM; 1, EGTA 50 mM; m, Calcium ionophore (CI) 100 uM; n, Tunicamycin (Tn) 20 uM; o, Thapsigargin (Tp) 35 uM.



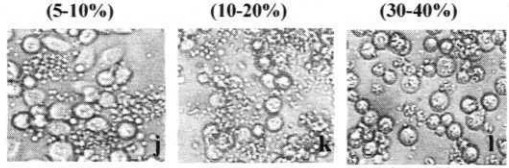
	ctl, uninfected	UVB-30 sec	UVB-60sec
% apoptosis>	(5-10%)	(40-50%)	(80-90%)



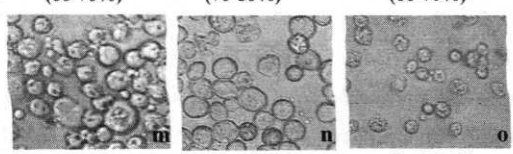
Et in μM>	50	100	200
% apoptosis>	(10-20%)	(60-70%)	(80-90%)



CH mM>	0.02	0.5	1.0
% apoptosis>	(5-10%)	(10-20%)	(30-40%)



CH mM>	3.0	5.0	EGTA-50 mM
% apoptosis>	(60-70%)	(70-80%)	(60-70%)



	CI 100 μM	Tn 20 μM	Tp 35 μM
% apoptosis>	(10-20%)	(5-10%)	(10-20%)

Davies, 2002; Zhu and Wang, 1999; Rabizadeh et al., 1993; Reimertz et al., 2003; Sala and Mollinedo, 1995; Yamaguchi et al., 2003; Jiang et al., 1994; Kaneko and Tsukamoto, 1994) have also been used. Of all the agents used here UV-B (60 sec) was the most potent inducer of apoptosis (80-85%) followed by high concentrations of EGTA (50 mM) (Figure 5I), etoposide (125 μM), and cycloheximide (3 mM) (60-70% apoptosis). In contrast, tunicamycin (20 μM), A23187 (100 u.M) and thapsigargin (35 u.M) induced very little or mild (5-10%) apoptosis here in *Sf9* cells (Figure 5, m-o).

Pro apoptotic agents stimulate *Sf* caspase activity: Ac-DEVD-AFC, a mammalian caspase-3 substrate was used to measure *Sf* caspase activity in terms of substrate hydrolysis. *Sf* caspase is 40% homologous to mammalian caspase (Ahmad et al., 1997b). Little or no caspase activity was detected in uninfected control *Sf9* cell extracts (Figure 6A and 6B, curve 1). Significant caspase activity was detected in uninfected *Sf9* cells treated with UVB light (Figure 6A, curve 2), 50 mM EGTA (Figure 6B, curve 3), 3 mM cycloheximide (Figure 6A, curve 7), and 125 μM etoposide (Figure 6A, curve 4). Low concentrations of cycloheximide, 20 and 500 μM, however, did not induce any caspase activity (Figure 6A, curves 5 and 6). High concentrations (3 mM) of cycloheximide stimulated apoptosis and caspase activity (Figure 5j and Figure 6A, curve 7). A marginal increase in caspase activity was observed in the presence of low (20 μM) and high concentrations calcium ionophore (100 μM) (Figure 6B, curves 4 and 5) and in the presence of tunicamycin (4 and 20 uJvl) (Figure 6B, curves 6 and 7). This is consistent with their inability to induce apoptosis. Overall, caspase activity was found directly related to the level of apoptosis (Figure 6A and 6B). Based on their ability to induce apoptosis in *Sf9* cells, UV-B light, etoposide (125 μM), cycloheximide (3.0 mM) and EGTA (50 mM) were classified as apoptotic agents where as A23187 and tunicamycin were classified as non apoptotic agents.

UV-B light also stimulated apoptosis and caspase activity in mammalian cells: The caspase-3 substrate, Ac-DEVD-AFC was also tested with mammalian cell extracts prepared from Raw macrophages in one experiment. It was observed that UV-B induced

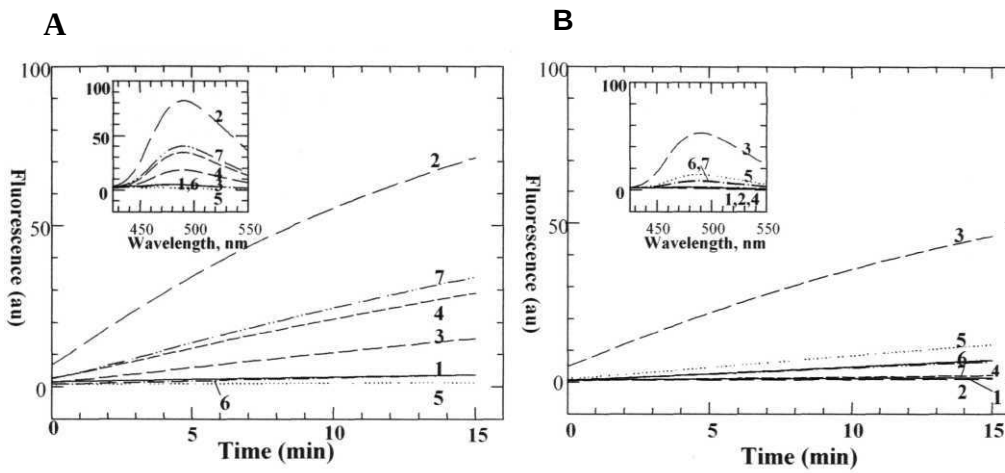


Figure 6: Caspase activity in *Sf9* cell extracts

Sf9 cells were treated with various agents and the caspase activity of the extracts (~400 ug) was measured using Ac-DEVD-AFC as described in 'Materials and Methods'. *Inset*, a fluorescence spectrum, recorded 20 min after addition of Ac-DEVD-AFC to the extracts. The two panels represent two sets of data.

Panel 6A: The curves 1-7 represent the caspase activity in the extracts prepared from cells that are treated with the following agents. 1, Control; 2, UV-B (60 sec); 3, 20 uM etoposide (Et); 4, 125 uM Et; 5, 20 uM cycloheximide (CH); 6, 500 uM CH; 7, 3.0 mM CH for 15 hrs at 27^0 C.

Panel 6B: The curves 1-7 represent the caspase activity of the cells treated with the following agents. 1, Control; 2, 10 mM EGTA; 3, 50 mM EGTA; 4, 20 uM A23187; 5, 100 uM A23187; 6, 4 uM tunicamycin (Tn); 7, 20 uM Tn.

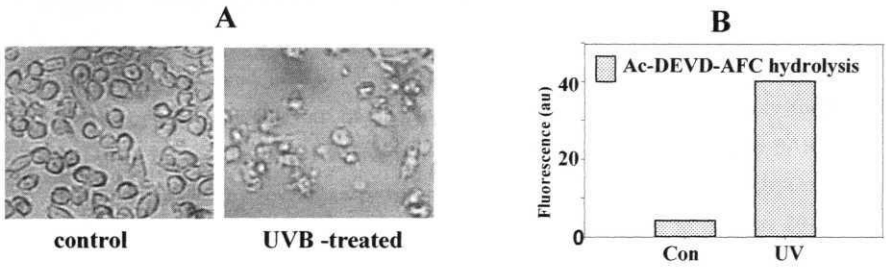


Figure 7: Morphology and easpase activity of UV-B treated mammalian Raw Macrophage cells and cell extracts

Two million mammalian raw macrophages were seeded in 35 mm petri dish and were treated with UV-B light for 60 sec and then incubated in CO_2 incubator for 15 hrs at 37° C. cell extracts were prepared and Ac-DEVD-AFC hydrolysis was measured in the extracts as described in 'Materials and Methods'.

Panel 7A: Morphology of control and UV-B treated cells

Panel 7B: caspase activity

apoptosis (Figure 7A) resulted in enhanced caspase activity (Figure 7B) which could be assayed here with the substrate, Ac-DEVD-AFC.

PARP Cleavage: In addition to measuring the caspase activity by using Ac-DEVD-AFC hydrolysis, the relative levels of apoptosis observed under microscope was further determined by studying PARP cleavage. Many earlier studies have shown that PARP is selectively cleaved by several caspases, especially by caspase-3. Caspase-3 cleaves the 113- kDa of PARP at the DEVD site between Asp214 and Gly215, to generate 89- and 24-kDa polypeptides (Gobeil et al., 2001). The cleavage of PARP here was measured by monitoring the appearance of the 89-kDa fragment of PARP that was recognized by an antibody (Figure 8). The PARP cleavage was found to correlate with the levels of apoptosis and caspase activation induced by various above agents.

Pro and non apoptotic stresses **stimulate eIF2a phosphorylation** in ***Sf9*** **cells:** Phosphorylation of elF2a is a stress signal (Kaufman, 1999b). All the agents used here are known to stimulate eIF2a phosphorylation in mammalian cell cultures. Further, eIF2a phosphorylation has been shown to mediate apoptosis in mammalian cells (Srivastava et al., 1998). However *Sf9* cells, in spite of their suitability as good model systems of apoptosis, have not so far been explored to determine the phosphorylation status of eIF2 protein in apoptotic and non apoptotic stress conditions. The phosphorylation status of elF2a as a function of various treatments was determined here qualitatively and quantitatively using phosphospecific anti-elF2a antibody, and compared with the extent of apoptosis and caspase activity for the respective treatments (Figures 9 and 10). Phosphospecific antibody has been found to recognize specifically the phosphorylated form of elF2α that is formed due to the action of eIF2a kinases (Sudhakar et al., 1999; Laxminarayana et al., 2002). elF2a phosphorylation is enhanced significantly in uninfected *Sf9* cells in response to all the agents tested (Figures 9 and 10), and is thus consistent with the notion that it is an indicator of stress. UV-B treatment and etoposide stimulated eIF2a phosphorylation in a dose-dependent manner (Figure 9A and 9B). Low concentrations of cycloheximide (20 u.M) caused a substantial increase in

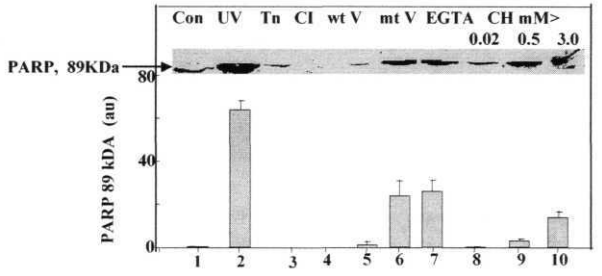


Figure 8: PARP cleavage activity of *Sf9* cell extracts

The extracts were prepared from cells treated with various agents as follows. In the case of virus infection, *Sf9* cells were infected with wt baculo virus or p35 deletion mutant virus for 48 hrs before the extracts were made. In the case of UVB treatment, cells were treated for 60 sec with UVB light and then incubated for 15 hrs at 27° C. All other treatments were carried out at 27° C for 15 hrs. Cell extracts (~ 60 μg of protein) were then incubated with ~ 150 ng of purified bovine PARP at 30° C for 90 min in a cleavage buffer as described under 'Materials and Methods'. The reactions were terminated and separated on 10% SDS-PAGE, transferred to nitrocellulose membrane and probed with an anti PARP p85 fragment antibody. The figure is a western blot. The lanes are labelled as follows: 1, Control; 2, UV-B (60 sec); 3, tunicamycin, 20 μM; 4, A23187, 100 uM; 5, wt AcNPV infection; 6, p35 deletion mutant AcNPV infection; 7, EGTA, 50 mM; 8, cycloheximide, 20 μM; 9, cycloheximide, 500 uM; 10, cycloheximide, 3.0 mM. The bar diagram below the blot represents average values of two independent experiments.

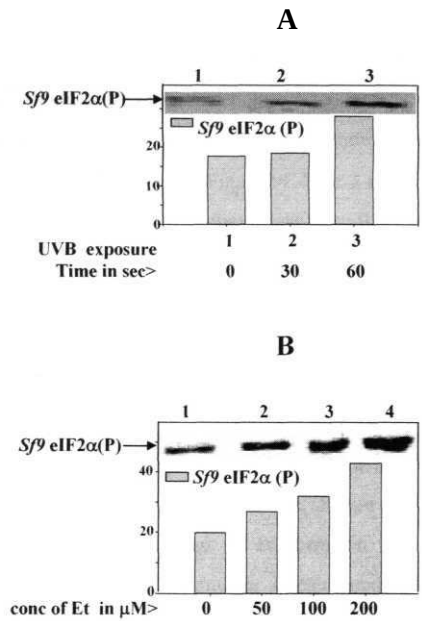


Figure 9: Phosphorylation of 5/9 eIF2a under various treatments

5/9 cells were treated with various agents as mentioned below and as described in figure 5 **and the** extracts were prepared as described in 'Materials and Methods'. ~25 μg of extract proteins was taken from each treatment and was separated by 10% SDS-PAGE and transferred to nitrocellulose membrane as described in 'Materials and Methods". The membranes were then analysed by a phosphospecific anti eIF2a antibody. Bar diagrams below the blots represent the quantification of the phosphorylated eIF2α band using Biorad Model GS-800 Densitometric Scanner. Panels are:

Panel 9A: Lane 1, control uninfected; lane 2 UV-B treated for 30 sec; lane UV-B treated **for 60** sec.

Panel 9B: Lane 1, uninfected control; lane 2 etoposide (Et) 50 μM; lane 3, Et 100 μM; lane 4, Et 200 uM.

eIF2a phosphorylation (Figure 9C, lane 2 and Figure 10A, lane 5) **without inducing** any apoptosis. An increase in the concentration of cycloheximide from 20 μM to 0.5 mM, however, decreased elF2a phosphorylation (Figure 9C lane 3 and Figure 10A lane 6). A further increase in the concentration of cycloheximide from 0.5 mM to 3 mM or 5 mM not only enhanced elF2a phosphorylation (Figure 9C, lanes 4, 5 and Figure 10A, lane 7) but also induced apoptosis. A decrease in elF2a phosphorylation between 0.02 mM and 0.5 mM cycloheximide may be due to the induction of a GAAD34-like protein that activates a protein phosphatase, PP1C (Novoa et al., 2001). Interestingly calcium ionophore, tunicamycin and thapsigargin stimulated eIF2a phosphorylation (Figure 9D and 9E) but failed to induce significant apoptosis. These agents stimulated eIF2a phosphorylation within 15 to 30 min of their treatment (Figure 9D, lane 2 and Figure 9E lanes 2, 3 and lanes 5, 6). In the case of tunicamycin and thapsigargin, eIF2a phosphorylation was reduced at 15 hr treatments as compared to the 30 min treatment (Figure 9E, lane 4 vs. 2 and lane 7 vs.6). In the presence of calcium ionophore, however, no such reduction in eIF2a phosphorylation was observed at 15 hrs (Figure 9D lane 3 vs. 2). An overall comparison of both pro apoptotic and non apoptotic treatments showed that UV-B and higher concentration of tunicamycin (20 u.M) caused a maximum increase in eIF2a phosphorylation (Figure 10A, lane2 and Figure 10B, lane 7). This was followed by calcium ionophore (100 μM), EGTA (50 mM) (Figure 10B, lanes 5 and 3 respectively), cycloheximide (3 mM), and etoposide (125 μM) (Figure 10A, lanes 7 and 4 respectively). These results suggest that both the type and magnitude of the stress play a role in eliciting apoptosis. Further, from a first glance of the data it appears as if there is no correlation between increased elF2a phosphorylation and apoptosis. However, a close analysis of the results showed clear correlation among the three parameters that we have considered i.e., caspase activation, increased eIF2a phosphorylation and apoptosis by various apoptotic agents. In contrast, the nonapoptotic agents stimulated eIF2a phosphorylation without caspase activation or apoptosis (Figure 10 bar diagram). These findings suggest that the mechanism of induction of eIF2a phosphorylation in apoptotic and non apoptotic conditions may be different.

Figure 9: Phosphorylation of 5/9 eIF2a under various treatments

Panel 9C: Lane 1, uninfected control; lane 2, cycloheximide (CH) 20 uM; lane 3, CH 500 uM; lane 4, CH 1.0 raM; lane 5, CH 3.0 mM; lane 6, CH 5.0 mM. The bar diagram below the immunoblot depicts the percent apoptosis and band quantification of the immunoblot. Open bars (□) represent the % apoptosis under those concentrations of cycloheximide and filled bars (■) are for *Sf9* eIF2a phosphorylation.

Panel 9D: Cells were treated with 100 uM calcium ionophore for 30 min and 15 hrs, extracts were prepared and status of eIF2a phosphorylation has been analysed. Lane 1, control untreated; lane 2, calcium ionophore treated for 30 min; lane 3, calcium ionophore treated for 15 hrs.

Panel 9E: Cells were treated with tunicamycin and thapsigargin and eIF2a phosphorylation was analysed in the cell extracts. Various lanes in the immunoblot indicate: lane 1, control untreated; lane 2, 20 uM tunicamycin (Tn) treated for 15 min; lane 3, 20 uM Tn treated for 30 min; 4, 20 uM Tn treated for 15 hrs; lane 5, 35 uM Thapsigargin (Tp) treated for 15 min; lane 6. 35 uM Tp treated for 30 min; lane 7, 35 uM Tp treated for 15 hrs.

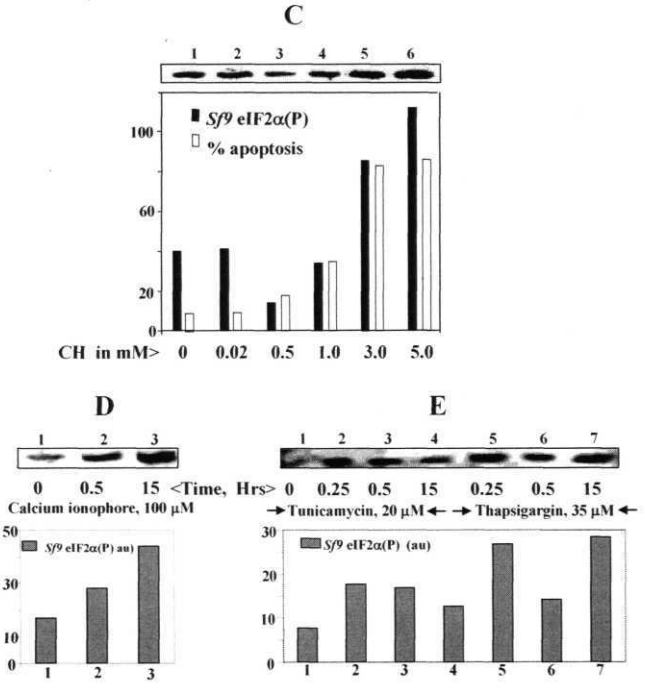
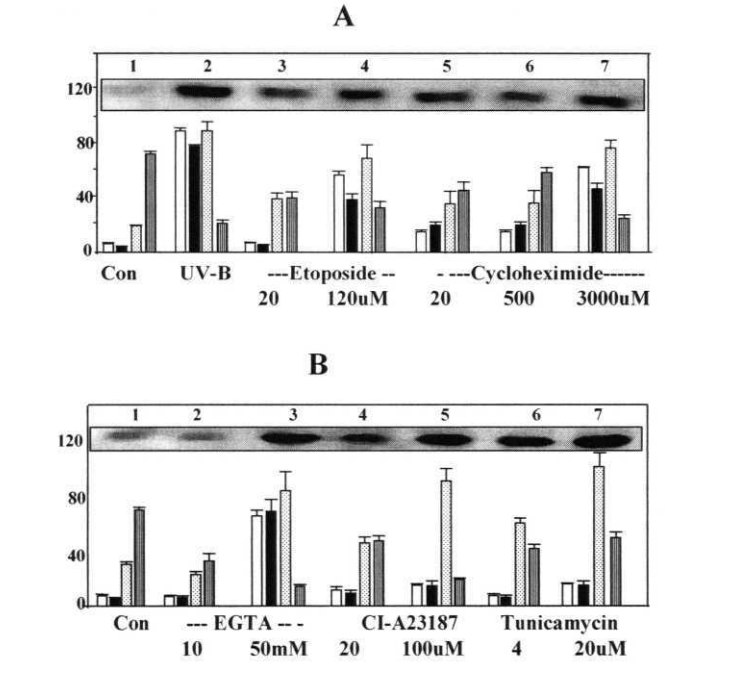

C
1 2 3 4 5 6
Sf9 eIF2α(P)
% apoptosis
100
60
20
0
CH in mM> 0 0.02 0.5 1.0 3.0 5.0
D
1 2 3
0 0.5 15 <Time, Hrs>
Calcium ionophore, 100 μM
Sf9 eIF2α(P) au)
50
30
10
0
1 2 3
E
1 2 3 4 5 6 7
0 0.25 0.5 15 0.25 0.5 15
→Tunicamycin, 20 μM← →Thapsigargin, 35 μM←
Sf9 eIF2α(P) (au)
30
20
10
0
1 2 3 4 5 6 7

Figure 10: A quick glance of eIF2a phosphorylation, caspase activity, apoptosis and protein synthesis in uninfected *Sf9* cells as a function of treatment with different agents

The various activities are measured as described in 'Materials and Methods'. A and B are two sets of data. The numbers 1 to 7 represent various treatments used and the bar diagram below for various treatments is represented.

Panel 10A: The treatments are as follows: 1, control; 2, UVB for 60 sec; 3, 20 uM etoposide (Et); 4, 125 uM Et; 5, 20 uM cycloheximide (CH); 6, 500 uM CH; 7, 3 mM. CH.

Panel 10B: 1, control; 2, 10 mM EGTA; 3. 50 mM EGTA; 4, 20 uM calcium ionophore (CI); 5, 100 uM CI; 6, 4 uM tunicamycin (Tn); 7, 20 uM Tn. The top insert represents the status of eIF2α phosphorylation as a function of different treatments. The blot was scanned densitometrically and values were plotted in the form of bar diagram depicting the various changes. The different bars show the extent of changes in % or arbitrary units for % apoptosis (□ open bars), caspase activity (■ filled bars), eIF2a phosphorylation (⁘ dotted bars) and % ^{35}S methionine incorporation or protein synthesis (▨ striped bars).


A
1
2
3
4
5
6
7
120
80
40
0
Con
UV-B
---Etoposide --
20
120uM
- ---Cycloheximide------
20
500
3000uM
B
1
2
3
4
5
6
7
120
80
40
0
Con
--- EGTA -- -
10
50mM
CI-A23187
20
100uM
Tunicamycin
4
20uM
% apoptosis
caspase activity
eIF2α (P)
35S methionine incorporation

Pro and non apoptotic agents fail to stimulate eIF2α phosphorylation in *Sf9* extracts *in vitro:* Cell extracts prepared from uninfected *Sf9* cells were treated with various agents for a period of ten minutes to determine the ability of these agents to stimulate eIF2a phosphorylation. Generally such **shorter time** periods are sufficient to activate a kinase directly in cell extracts *in vitro* (Ramaiah et al., 1997). However none of the agents could stimulate eIF2a phosphorylation under such conditions *in vitro* (Figure 11) there by suggesting that the mechanism of activation of eIF2a kinases in *Sf9* cells in the presence of various agents as mentioned above may be mediated indirectly. We have not however monitored kinase activation in the extracts for longer periods because of the possible activation of other proteases, other than caspases.

Baculovirus infection and apoptosis: Baculovirus specifically infects many of the lepidopteron insects and the virus is used to express foreign proteins in insect cells. Using baculovirus, earlier this laboratory has expressed a phosphomimetic form of human eIF2α in *Sf9* cells. However, the expression of such a toxic form of eIF2a did not result in apoptosis (Sudhakar et al., 2000). This result is different from what has been observed in mammalian cells. Hence studies have been undertaken to monitor the effect of virus infection and also the ability of various pro-apoptotic agents to induce apoptosis in virus infected cells. Baculovirus-infected cells can be recognized from uninfected *Sf9* cells by the presence of dark opaque polyhedra inclusion bodies under light microscope (Figure 12A). Wild type baculovirus-infected cells showed little or no apoptosis even after the treatment with pro-apoptotic agents such as UV-B or etoposide (125 μM) (Figure 12A). In contrast, a mutant virus that had a deletion of its p35 anti apoptotic gene promoted apoptosis (Figure 13 A). Baculovirus infection of insect cells has been shown to decrease caspase activity and apoptosis (Clem et al., 1991). In accordance with earlier reports, caspase activity was barely detected, if at all, in *Sf9* cells infected with the AcNPV, or in virus-infected cells treated with UV-B and etoposide (Figure 12B}. The wild type virus that is required for a productive infection causes a reduction in eIF2a phosphorylation in *Sf9* cells (Figure 12C) and this result is consistent with earlier observation from this lab (Sudhakar et al., 2000). Increase in eIF2a phosphorylation as a function of apoptosis was observed more significantly in UV-B and etoposide (125 μM) treated uninfected cells

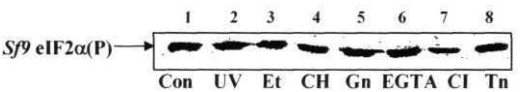


Figure 11: Effects of various agents on the phosphorylation of *Sf9* eIF2a in extracts *in vitro*

Sf9 extracts were prepared as described in 'Materials and Methods' and were treated with various agents as mentioned below. Changes in eIF2a phosphorylation was analysed by a phosphospecific anti-eIF2a antibody. The figure is a western blot. The various lanes are as follows: Lane 1, control; lane 2, UV-B (60 sec); lane 3, 125 uM etoposide (Et); lane 4, 3 mM cycloheximide (CH); lane 5. 50 uM genestein (Gn); lane 6, 50 mM EGTA; lane 7, 100 uM calcium ionophore (CI); lane 8, 20 uM tunicamycin (Tn).

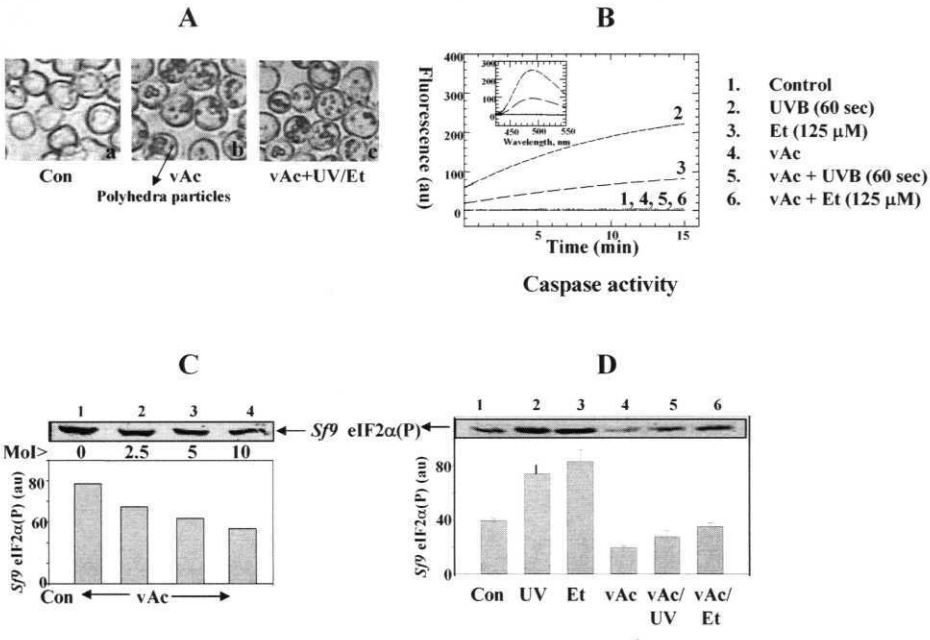


Figure 12: Morphology, caspase activity and eIF2a phosphorylation in *Sf9* cells infected with wild type baculovirus and different stress conditions such as UV-B and etoposide

Sf9 cells were infected with wt AcNP virus for 48 hrs. After the infection, the cells were treated with UV-B light for 60 sec and incubated for 15 hrs at 27° C or with etoposide (125 μM) for 15 hrs at 27° C. Cell extracts were prepared as described earlier to measure the caspase activity and for the analysis of eIF2a phosphorylation.

Panel A: Morphology of cells

Panel B: caspase activity

Panel C and D: eIF2a phosphorylation

Figure 13: Morphology, caspase activity and eIF2α phosphorylation in *Sf9* cells infected with wild type and p35 mutant AcNP virus

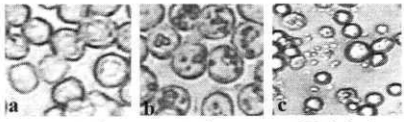
Sf9 cells were infected with different titres of wt AcNP virus or p35 deletion mutant virus. 48 hrs after infection cell morphology, caspase activity and eIF2a phosphorylation of *Sf9* cells have been analysed as described in 'Materials and Methods'.

Panel A: Morphology of cells

Panel B: caspase activity

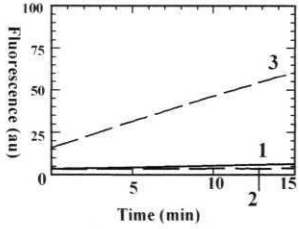
Panel C: eIF2α phosphorylation in p35 deletion mutant virus infected cells.

A



control | wt virus infection | p35 deletion mutant infection

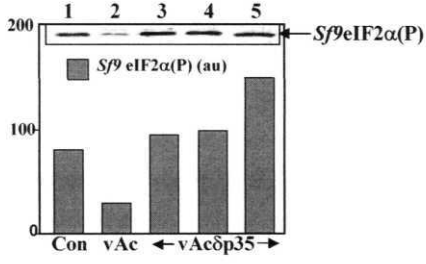
B



1. control
2. wt-virus infected
3. p35 mutant virus infected

Caspase activity

C



than in wild type virus-infected cells (Figure 12D lanes 2 and 3 vs. 5 and 6, respectively). To determine the importance of caspase involvement in stimulating eIF2α phosphorylation in cells undergoing apoptosis, we studied the effect of virus encoded p35 gene expression on eIF2α phosphorylation. This has been carried out by infecting *Sf9* cells with a deletion mutant p35 virus. Deletion mutant virus infection resulted in apoptosis (Figure 13 A), caspase activity (Figure 13B, curve, 3) and enhanced eIF2a phosphorylation (Figure 13C, lanes 3-5).

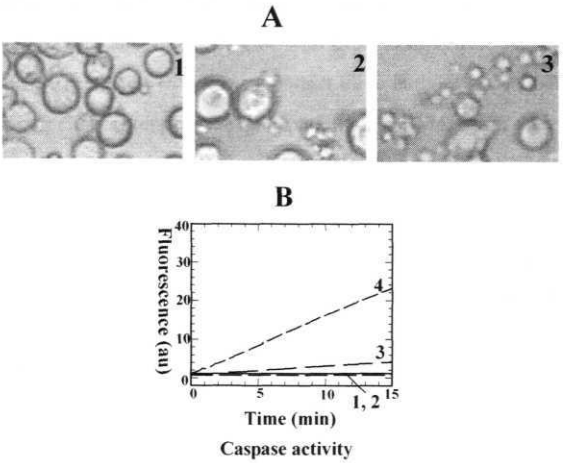
eIF2a **phosphorylation** mediates apoptosis in *Sf9* **cells:** The importance of eIF2α phosphorylation in apoptosis and in non apoptotic conditions, or during translational inhibition caused by diverse conditions/agents in mammalian systems was analyzed previously by overexpressing a nonphosphorylatable form of eIF2a such as S51A (51 serine reside is replaced by alanine) or phosphomimetic form of eIF2a, S51D (serine residue in position 51 is replaced by aspartic acid). While expression of S51A decreased both translational inhibition in heat-shocked mammalian cells (Murta Riel et al., 1993) and apoptosis (Srivastava et al., 1998), S51D was found to stimulate these processes. In order to determine the influence of eIF2α phosphorylation on apoptosis in *Sf9* cells, we expressed human wt, S51A, and S51D mutants of eIF2α using recombinant baculoviruses as described earlier (Sudhakar et al., 2000). Overexpression of recombinant human phosphomimetic form of eIF2a (S51D), did not promote apoptosis in *Sf9* cells in the absence of UV-B exposure (data not shown). Also, *Sf9* cells infected with wt virus or recombinant S51D virus were unable to undergo apoptosis in response to UV-B irradiation (data not shown). This is likely a reflection of the expression of anti apoptotic viral p35 protein that occurs during the early stages of viral infection. Consistent with this idea, apoptosis was found to be induced significantly in *Sf9* cells that were exposed first to UV-B radiation and then transfected with recombinant virus harboring human wt, S51A or S51D eIF2a (Figure 14A, 1-3). The S51D is a phosphomimetic form of eIF2α and therefore induced at least 30-35% higher apoptosis than the wild type eIF2a (Figure 14A, 2 vs. 3). However infection of *Sf9* cells with S51A virus carrying the nonphosphorylatable form of eIF2α resulted in a significant decrease in the UV-B

Figure 14: Apoptosis in UVB-treated *Sf9* cells expressing recombinant eIF2a wt and/or mutant proteins

Panel A: Morphology of cells treated first with UV-B light for 60 seconds and were transfected with recombinant virus harboring human S51A (a), wt eIF2a (b), S51D (c), forms of eIF2a.

Panel B: caspase activity in the cell extracts prepared from cells treated with the following agents. The numbers in the graph represent: 1, Wt AcNPV; 2, UV-B and recombinant virus with S51A eIF2α; 3, UV-B and recombinant virus with wt eIF2a; 4, UV-B and recombinant virus with S51D eIF2a.

Panels C and D represent eIF2α expression and phosphorylation in UVB-treated *Sf9* cells using a polyclonal anti-eIF2α antibody and a phosphospecific anti-eIF2a antibody respectively as described in 'Materials and Methods*. The various lanes are as follows: Lane 1, Cells expressing S51A mutant human eIF2a; 2, cells expressing wt eIF2a; 3, cells expressing S51D eIF2a.


A
1
2
3
B
Fluorescence (au)
40
30
20
10
0
0
5
10
15
4
3
1, 2
Time (min)
Caspase activity
C
1
2
3
S51A
wt
S51D
eIF2α
Human eIF2α Expression
40
20
0
S51A
wt
S51D
D
1
2
3
<Sf9
<H
S51A
wt
S51D
eIF2α (P)
Sf9 eIF2α(P) (au)
% apoptosis
40
20
0
S51A
wt
S51D

induced apoptosis (Figure 14, A1). Caspase activity of these cells was related to apoptosis under those conditions (Figure 14B). No caspase activity was detected in cells pretreated with UV-B and infected with wild type baculovirus, AcNPV (Figure 14B, curve 1), and or transfected with a recombinant virus harboring the S51A eIF2α (curve, 2). In contrast, cells expressing the phosphomimetic S51D form of eIF2a showed a higher caspase activity compared to those carrying wild type eIF2a (curves 4 and 3). The cell extracts were also analyzed to determine the expression of human eIF2a protein (Figure 14C) and also the phosphorylation status of the endogenous *Sf9* eIF2a, and, of the recombinant wt and mutant human eIF2α (Figure 14D). Expression of the recombinant eIF2a, both wt and mutant proteins was detected by a polyclonal anti-eIF2a antibody that recognizes the human protein but not the endogenous *Sf9* protein (Figure 14C). Cells infected with the S51D mutant virus showed relatively a lesser expression of the eIF2α protein than the S51A mutant virus (lane 3 vs. lanes 1 and 2). This was observed earlier also (Sudhakar et al., 2000), and it is a reflection of the toxic nature of the phosphomimetic form of S51D. A duplicate blot was probed with the phosphospecific antibody that recognizes the phosphorylated forms of both the human and *Sf9* proteins (Figure 14D). Two bands were detected by the phosphospecific anti-eIF2a antibody; the one with reduced mobility corresponds to *Sf9* eIF2a and the other one with increased mobility corresponds to the recombinant human eIF2α (Figure 14D). Apoptosis induced by UV-B was high in cells expressing S51D eIF2α mutant followed by the wt eIF2α. This was also reflected in the phosphorylation status of endogenous eIF2α (Figure 14D lanes 2 vs. 3, *Sf9* arrow head). The cells expressing S51A mutant of eIF2a showed least apoptosis with correspondingly reduced *Sf9* eIF2a phosphorylation (Figure 14D, lane 1). The phosphorylation of recombinant human wt eIF2a was evident in insect cells, but not with the S51A and S51D mutants expression (Figure 14D, lower band, H arrow head). These results suggest that phosphorylated eIF2α per se may not stimulate caspase activation or apoptosis but eIF2a phosphorylation is a characteristic feature of apoptotic cells and is a consequence of caspase activation.

Transient expression of human wt and mutant eIF2α's in *Sf9* cells: Since wt baculovirus infection reduces eIF2a phosphorylation and apoptosis presumably due to the production of inhibitors of eIF2α phosphorylation and apoptosis like p25 and p35 proteins respectively, we have constructed plasmids with heat shock promoters harboring human wt eIF2α, S51A (nonphosphorylatable) and S51D mutants (phosphomimetic form) of eIF2a to evaluate the importance of eIF2a phosphorylation on apoptosis in the absence of any other agents or baculoviral genes. *Sf9* cells transfected with PNN1 plasniids harboring the human eIF2α genes were heat shocked for 30 min to activate their promoters and the transcription. *Sf9* cells were then analysed 48 hrs after transfection to determine the expression of wt and mutant proteins of eIF2a (Figure 15A). While the expression of human eIF2a did take place under those conditions, apoptosis was not stimulated. Phosphorylation of eIF2α is known to decrease or inhibit protein synthesis in mammalian systems; studies have been carried out here to determine protein synthesis in *Sf9* cells expressing human wt or S51D or S51A eIF2α. Protein synthesis is reduced significantly in cells expressing S51D mutant eIF2a than in the presence of S51A or wt eIF2α (Figure 15C, bars 4 vs. 3, 2 and I respectively). These findings further endorse that eIF2a phosphorylation is not a prerequisite to stimulate apoptosis. On the contrary, cells undergoing apoptosis show increased eIF2α phosphorylation as mentioned in earlier results (Figures 9, 10, 13 and **14).**

Kinase inhibitors and apoptosis: Genestein and staurosporine are general inhibitors of tyrosine and ser/thr kinases respectively. We have used them with the rationale to determine the importance of phosphorylation cascade in general and eIF2α phosphorylation in particular on the induction of apoptosis. Although both these agents are known kinase inhibitors, previous reports document that both of them can stimulate eIF2α phosphorylation in cultured mammalian cells (Ito et al., 1999; del Vega et al., 1999). It is suggested that genestein may stimulate ER stress like thapsigargin (Shoshan et al., 1981) by inhibiting sarcoplasmic reticulum calcium ATPase that results in the release of ER calcium to cytosol and the resulted ER stress may activate an eIF2α kinase like PERK. However, the mechanism by which staurosporine stimulates eIF2a

Figure 15: Transient expression of human eIF2a wt and mutants in *Sf9* cells

Human wild type and mutants of eIF2α were cloned under Hsp promoter into a PNN 1 plasmid. *Sf9* cells were then transfected with PNN 1 plasmid harbouring human eIF2a wt, S51A, S51D eIF2a as described under 'Materials and Methods'. After transfection, *Sf9* cells were heat shocked at 42° C for 30 min to determine the expression of eIF2a wt and mutant proteins. The expression was analysed by anti eIF2a antibody. The figure is a western blot. Panel A: Immunoblot showing the expression of human eIF2a at different concentrations of plasmid transfection. Lane 1, expression of S51A mutant eIF2a in cells treated with 5 ug ofrecombinant PNN1 plasmid DNA; lane 2, expression of wt eIF2a in cells treated with 5 jug of recombinant PNN1 plasmid DNA; lane 3, expression of S51D mutant eIF2a in *Sf9* cells treated with 5, 10, and 20 μg of recombinant PNN plasmid DNA respectively.

Panel B: Phosphorylation of *S/9* cellular eIF2a and recombinant human eIF2a in cells expressing recombinant human eIF2α, wt and or mutants. *S/9* cells were transfected with 5 μg of recombinant PNN1 plasmid harbouring human S51A, wt or S51D eIF2a. Cell extracts were prepared and the phosphorylation status of endogenous *S/9* eIF2α and of the expressed recombinant human eIF2a wt and mutants were analysed using a phosphospecific anti eIF2α antibody as described in 'Materials and Methods'. Lanes are as follows: Lane 1, phosphorylation of eIF2α in cells expressing human S51A eIF2α; lane 2, phosphorylation of eIF2a in cells expressing human wt eIF2a; lane 3, phosphorylation of eIF2α in cells expressing human S51D eIF2a

Panel C: Protein synthesis was performed in *S/9* cells transfected with PNN1 plasmid expressing wt and mutants of eIFα as described in 'Materials and Methods'.

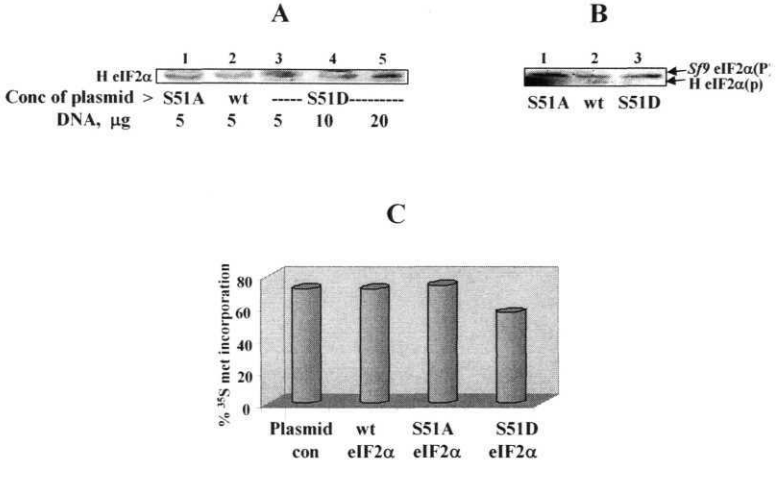

A
1 2 3 4 5
H eIF2α
Conc of plasmid > S51A wt ----- S51D---------
DNA, µg 5 5 5 10 20
B
1 2 3
Sf9 eIF2α(P
H eIF2α(p)
S51A wt S51D
C
% 35S met incorporation
80
60
40
20
0
Plasmid con
wt eIF2α
S51A eIF2α
S51D eIF2α

Figure 16: Effect of staurosporine (St) and Genestein (Gn), kinase inhibitors on morphology, eIF2a phosphorylation and caspase activity of uninfected, AcNPV-infected and of UV-B treated cells

Morphology of cells were analysed under inverted microscope, whereas, eIF2a phosphorylation and caspase activity were analysed in ceil extracts as described in the earlier figure legends. Protein synthesis was determined using [^{35}S] methionine as described in 'Materials and Methods'. Various panels represent the following.

Panel A: 1, control ceils; 2, 100 nM staurosporine treated cells (15 hrs); 3, 50 uM genestein treated cells (15 hrs); 4, 48 hrs AcNPV infected cells + 100 nM staurosporine (15 hrs); 5, 48 hrs AcNPV infected cells + 50 uM genestein (15 hrs); 6. UV-B treated for 60 sec and incubated for 15 hrs; 7. 100 nM staurosporine (30 min) + UVB (60 sec) incubated for 15 hrs; 8. 50 mM genestein (30 min) + UVB (60 sec) incubated for 15 hrs.

Panel B: A bar diagram representing percent live *Sf9* cells after 15 hrs of treatment with 100 nM staurosporine or 50 uM genestein.

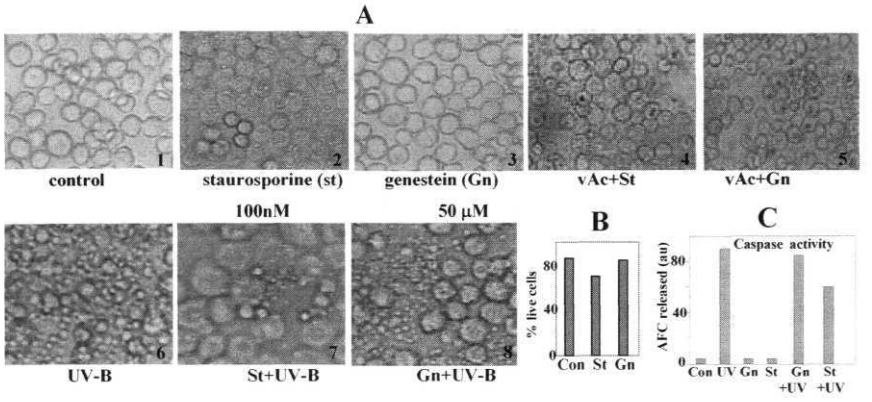
Panel C: Caspase activity in *Sf9* cells treated with 100 nM staurosporine or 50 uM genestein for 15 hrs or 30 min kinase inhibitor treated cells which are then subjected to 60 sec UV-B treatment and incubated for 15 hrs at 27° C

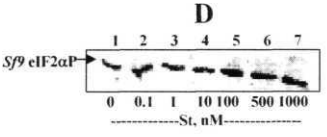
Panel D: eIF2α phosphorylation in *Sf9* cells treated with different concentrations of staurosporine for 15 hrs at 27^0 C.

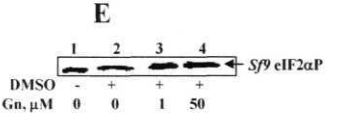
Panel E: eIF2a phosphorylation in *Sf9* cells treated with different concentrations of 50 uM genestein for 15 hrs at 27° C.

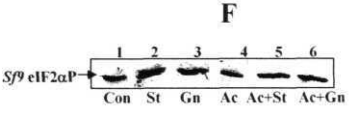
Panel F: eIF2a phosphorylation in 48 hrs of AcNPV treated *Sf9* cells treated with 100 nM staurosporine or 50 uM genestein for 15 hrs at 27° C.

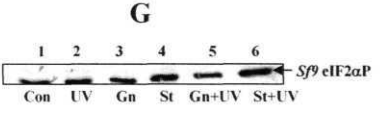
Panel G: eIF2a phosphorylation in 60 sec UV-B treated *Sf9* cells which have been pre-treated for with either 100 nM staurosporine or 50 uM genestein and incubated for 15 hrs 27^0 C.


A
1
2
3
4
5
control
staurosporine (st)
100nM
genestein (Gn)
50 μM
vAc+St
vAc+Gn
6
7
8
UV-B
St+UV-B
Gn+UV-B
B
% live cells
80
40
0
Con St Gn
C
Caspase activity
AFC released (au)
80
40
0
Con UV Gn St Gn +UV St +UV


D
1 2 3 4 5 6 7
Sf9 eIF2αP
0 0.1 1 10 100 500 1000
St, nM


E
1 2 3 4
Sf9 eIF2αP
DMSO - + + +
Gn, μM 0 0 1 50


F
1 2 3 4 5 6
Sf9 eIF2αP
Con St Gn Ac Ac+St Ac+Gn


G
1 2 3 4 5 6
Sf9 eIF2αP
Con UV Gn St Gn+UV St+UV

phosphorylation is not clear. Further staurosporine (Martin et al., 1995) and genestein are shown to induce apoptosis in many of the mammalian systems). Genestein has been shown to play a role in the regulation of plant apoptosis and cell cycle kinetics. Genestein is found to induce apoptosis in many of the animal cancer tissues (Lamartiniere et al., 1998; Park et al., 2002; Po et al., 2002).

In our experiments, both staurosporine and genestein were unable to induce membrane blebbing, an important feature of apoptotic cells (Figure 16A, 2 and 3). However cell morphology of staurosporine treated cells is distinctly different from control cells (Figure 16A, 2 vs. 1) and staurosporine treatment decreases cell viability (Figure 16B). Interestingly, both agents stimulated eIF2a phosphorylation in *Sf9* cells (Figure 16D and 16E). Further staurosporine, but not genestein, decreases UV-B mediated apoptosis (Figure 16A, 6 vs. 7 and 8). The inability of both inhibitors to stimulate apoptosis and the ability of staurosporine to reduce UV-B induced apoptosis is consistent with the decline in caspase activity under those conditions (Figure 16C). During wt baculovirus infection, only uninfected cells were influenced by staurosporine and showed the typical altered cell morphology (Figure 16A, 4). Genestein treatment did not cause any apparent change in virus infection or in UV-B mediated apoptosis (Figure 16A, 5 and 7). Wt baculovirus infection reduced staurosporine and genestein stimulated *Sf9* eIF2α phosphorylation (Figure 16E).

These findings reiterate that eIF2α phosphorylation can occur both in non apoptotic and in pro apoptotic conditions probably through different mechanisms. This suggestion is also consistent with the result here that the reduction in UVB-induced apoptosis by staurosporine is not mediated by a decline in eIF2a phosphorylation (Figure 16G). It is likely that serine/threonine kinase activation may play a role in apoptosis (Hagemann and Blank, 2001; Xia et al., 1995). This is because staurosporine, but not genestein, is able to inhibit UV-B mediated apoptosis in *Sf9* cells. It is likely that staurosporine may be inhibiting an intermediate kinase involved in UV-B mediated apoptosis.

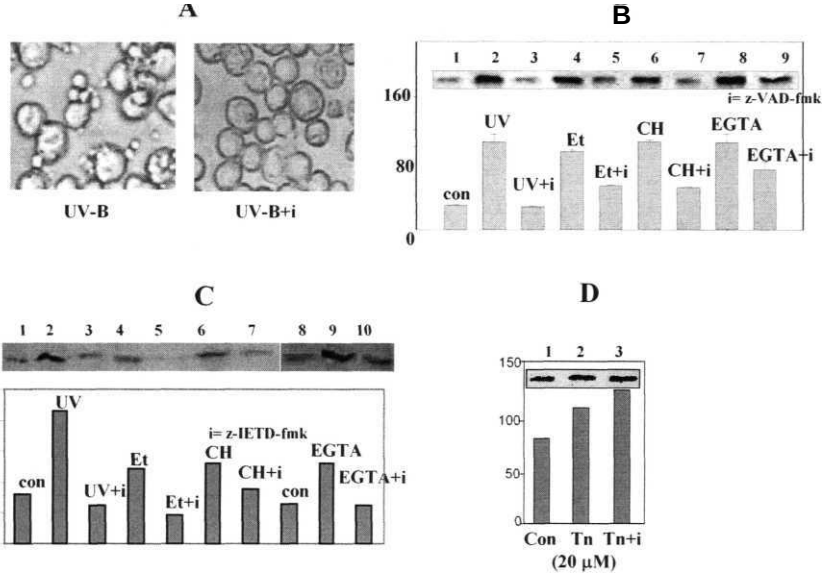
Figure 17: Effect of caspase inhibitors on morphology of *Sf9* cells and eIF2a phosphorylation of *Sf9* cells treated with various stress agents

Panel A: Morphology of UV-B treated *Sf9* cells in the presence and absence of 50 μM z-VAD-fmk (i)

Panel B: eIF2a phosphorylation in *Sf9* cell extracts prepared from cells treated with various agents in the presence and absence of 50 μM z-VAD-fmk, cell permeable caspase-3 inhibitor.

Panel C: eIF2a phosphorylation in *Sf9* cell extracts prepared from cells treated with various agents in the presence and absence of 50 μM z-IETD-fmk, cell permeable caspase-8 inhibitor.

Panel D: eIF2a phosphorylation in *Sf9* cell extracts prepared from cells treated with 20 uM tunicamycin or with 20 μM tunicamycin and 50 μM z-VAD-fmk, caspase-3 inhibitor.


A
UV-B
UV-B+i
B
1 2 3 4 5 6 7 8 9
160
80
0
i= z-VAD-fmk
con
UV
UV+i
Et
Et+i
CH
CH+i
EGTA
EGTA+i
C
1 2 3 4 5 6 7 8 9 10
50
10
con
UV
UV+i
Et
Et+i
i= z-IETD-fmk
CH
CH+i
con
EGTA
EGTA+i
D
1 2 3
150
100
50
0
Con
Tn
Tn+i
(20 μM)

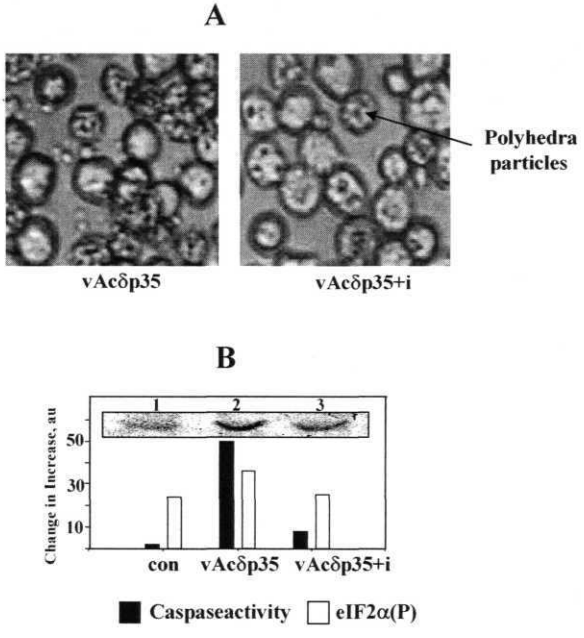


Figure 18: Morphology, caspase activity and eIF2a phosphorylation in p35 mutant virus (vAc5p35) infected cells in the presence and absence of 50 μM z-VAD-fmk

Panel A: Morphology

Panel B: eIF2a phosphorylation (□) and caspase activity (■).

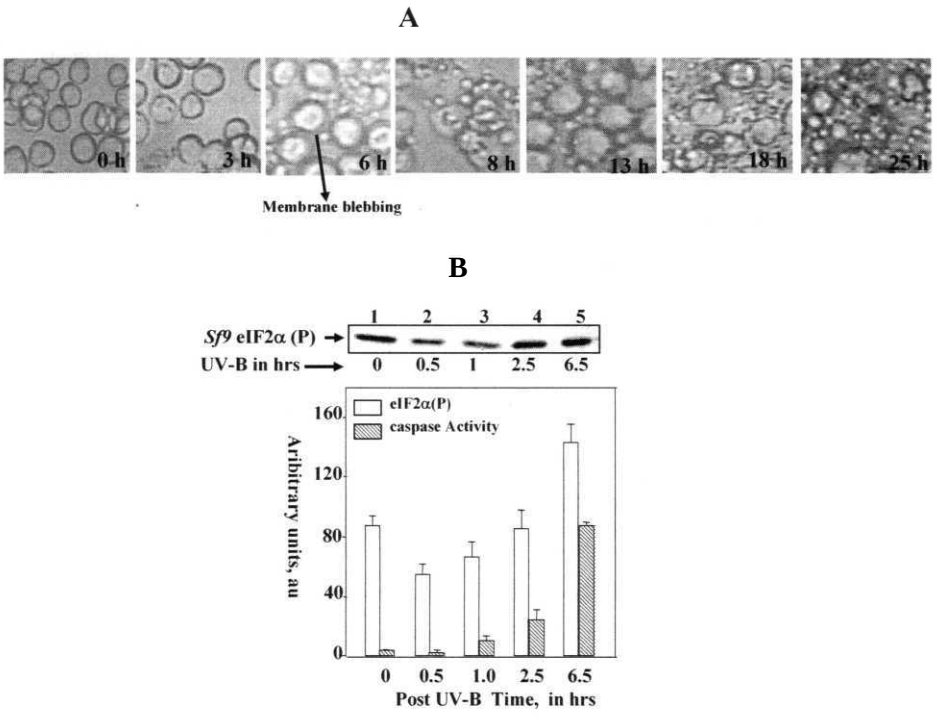


Figure 19: Kinetics of apoptosis, caspase activation and eIF2a phosphorylation in UV-B treated *Sf9* cells

Panel A: Morphology of *Sf9* cells treated with 60 sec of UV-B light and incubated at 27^0 C for 0, 3, 6, 8, 13 18 and 25 hrs.

Panel B: Caspase activity (▨) and eIF2a phosphorylation (☐) in *Sf9* cells treated with 60 sec UV-B light and incubated at 27° C for different time periods, 0-6.5 hrs.

Inhibition of caspase activity mitigates eIF2a phosphorviation: Incubation of uninfected ceils with the pro apoptotic agents along with cell permeable z-VAD-fmk, a caspase-3 inhibitor or cell permeable z-IETD-fmk, caspase-8 inhibitor, resulted in almost total loss of apoptosis in these cells. and the morphology of these cells resembled that of the control *Sf9* cells (Figure 17A). Complementing the above results. eIF2a phosphorylation and apoptosis were reduced in *Sf9* cells treated with z-VAD-fmk (Figure 17B) or z-IETD-fmk (Figure 17C) and then exposed to UV-B (60 sec), etoposide (125 μM), cycloheximide (3 mM) and EGTA (50 mM) (Figure 17B, lanes 2, 4, 6 and 8 without inhibitor vs. lanes 3. 5. 7 and 9 with inhibitor; Figure 17C, lanes 2, 4. 6 and 9 without inhibitor vs. lanes 3, *5. 1* and 10 with inhibitor). Interestingly, tunicamycin-induced eIF2a phosphorylation was not affected in the presence of z-VAD-fmk (Figure 17D lanes 2 and 3 vs. 1). This finding complements the observation on the inability of tunicamycin to stimulate caspase activity and apoptosis (Figure 10 bar diagram), and suggests that tunicamycin-induced eIF2α phosphorylation is different from the increased eIF2a phosphorylation caused by the addition of apoptotic agents in *Sf9* cells. These results therefore suggest that increased elF2a phosphorylation in apoptosis is a consequence of increased caspase activity. This conclusion is further reinforced by demonstrating that the caspase inhibitor. z-VAD-fmk, reduced eIF2α phosphorylation, caspase activity and apoptosis in p35 mutant virus infected cells (Figure 18A and B).

Kinetics of apoptosis, caspase activation and *Sf9* eIF2a phosphorviation in UV-B treated cells: *Sf9* cells were treated with UV-B light for 60 seconds and apoptosis was scored at different time periods of the treatment. Microscopic observation reveals that membrane blebbing occurs after 5 to 6 hrs of UV-B treatment (Figure 19A). However caspase activation takes place around 1.0 hr after UV-B treatment (Figure 19B bar diagram bar 3 vs. 1). Analysis of eIF2α phosphorylation indicates that it decreases initially before the onset of caspase activation and increases 2.5 hrs after UV-B treatment (Figure 19B, lanes 4 and 5 vs. 1 and the corresponding bar diagram). Maximum increase in eIF2a phosphorylation has been noticed 15 hrs after treatment (as shown in Figures 9,

10 and 17). These results together with the earlier results in Figures 9, 10 and 17 indicate that caspase activation occurs prior to increased eIF2a phosphorylation and is a prerequisite for the increased eIF2α phosphorylation observed in cells that are undergoing apoptosis (Aparna et al., 2003).

Cleavage of eIF2a kinases by *Sf9* extracts: Recent studies suggest that many kinases are activated upon cleavage by caspases (Fischer et al., 2003). eIF2a kinases also form one of the examples wherein the cleavage of the kinase renders additional catalytic activity to the cleaved kinase. PKR was shown to be cleaved by mammalian caspase and the cleaved PKR was active in phosphorylating its substrate (Saelens et al., 2001). To determine if a similar mechanism exists in *Sf9* cells, we tested the ability of *Sf9* cell extracts, prepared from cells undergoing apoptosis, to cleave the recombinant human PKR or mouse PERK. Cleavage of a recombinant human PKR (97 kDa) protein by *Sf9* extracts prepared from cells that were treated with one minute UV-B light or with 125 uM etoposide was apparent from a reduction in the 97 kDa band and the appearance of two protein bands of approximately 38 kDa and 27 kDa (as shown by arrows in Figure 20). This cleavage of PKR was noticed around 8 hrs after incubation and increased as a function of time only in extracts prepared from UV-B and etoposide-treated cells, but not in control extracts (Figure 20, lanes 7 and 8, 11 and 12 vs. 3 and 4 respectively). The cleavage of PKR by extracts prepared from apoptotic cells and increase in eIF2α phosphorylation in such cells supports the notion that increased eIF2α phosphorylation seen during apoptosis is the result of caspase-dependent cleavage and subsequent activation of such an endogenous eIF2a kinase (Saelens et al., 2001).

The eIF2α kinases present in *Sf9* insect cells have not been characterized so far, although GCN2 and PERK have been characterized in *Drosophila* (Berlanga et al., 1999; Sood et al., 2000). Moreover, eIF2a in *Sf9* extracts is found to be a poor substrate of human recombinant PKR in our studies (Figure 2B and 2C). Since PKR encoding sequences have not been recognized in any invertebrate for which complete genome sequence is available, we have also tested here the ability of *Sf9* cell extracts prepared from cells

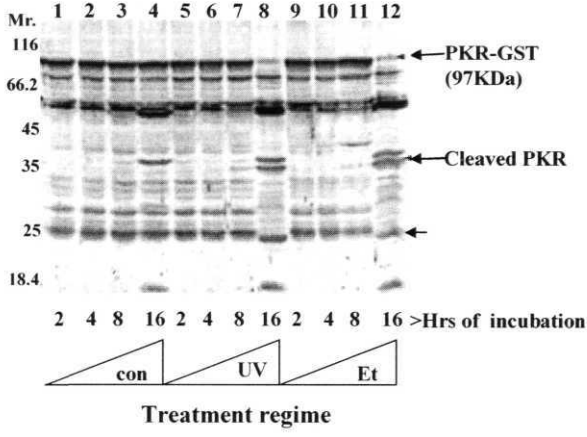
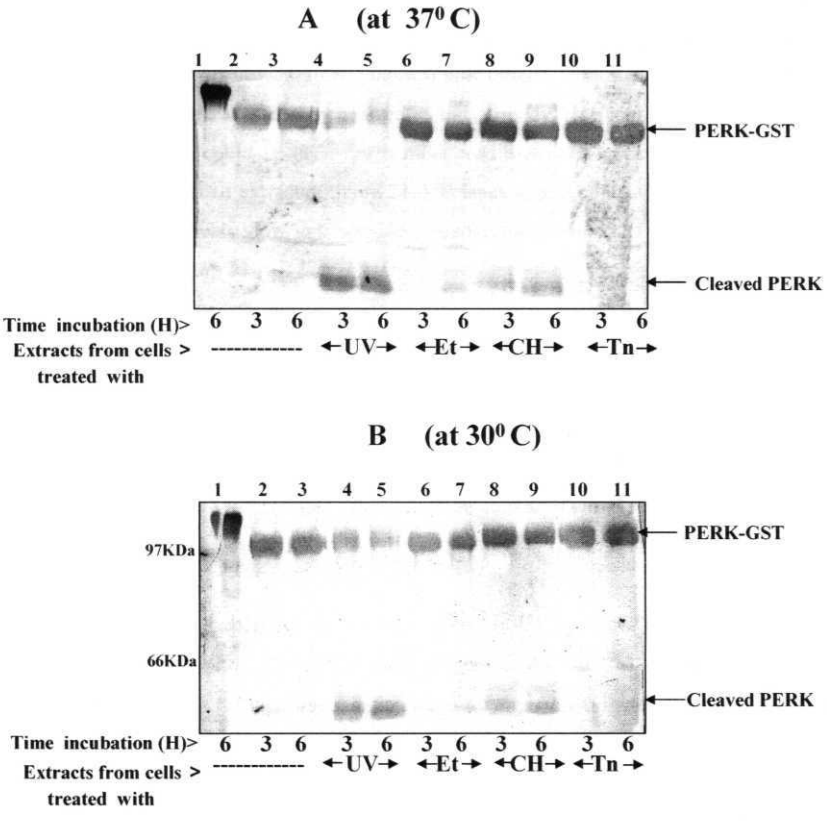


Figure 20: Cleavage of recombinant human PKR cleavage in extracts prepared from *Sf9* cells undergoing apoptosis

Recombinant PKR, prepared as described under 'Materials and Methods', was incubated with control extracts and apoptotic extracts prepared from cells treated with UV-B or 125 μM etoposide at 37° C for different time periods. The extracts were then separated by 10% SDS-PAGE. PKR (cleaved and uncleaved) were detected using a polyclonal anti-PKR antibody. The figure is a western blot. The various lanes represent the following. Lanes, 1–4, represent PKR incubation in control extracts for 2, 4, 8 and 16 hrs. Lanes, 5-8, represent PKR incubation in UVB-treated extracts for 2, 4, 8 and 16 hrs. Lanes, 9-12, represent PKR incubation in etoposide-treated extracts for 2, 4, 8 and 16 hrs.

Figure 21: Recombinant mouse PERK cleavage by 5/9 extracts

Panels A and B; Recombinant mouse PERK cleavage was studied in cell extracts prepared form *SJ9* cells treated with various agents. Reactions were carried at 37° C (Panel A) or at 30° C (Panel B) for 3 and 6 hrs in the presence of ~60 μg of extract protein and ~200 ng of PERK in a cleavage reaction mixture containing 220 mM Mannitol, 68 mM sucrose. 2 mM NaCl, 2.5 mM KH_2PO_4 and 10 mM Tris-HCl, pH 8. The lanes are as follows: Lane 1, PERK (without extract, 6 hrs); lane 2, PERK + control cell extract(3 hrs); lane 3, PERK+ control ceil extract (6 hrs); lane 4, PERK + UV-B treated (60 sec) extract (3 hrs); lane 5, PERK + UV-B treated (60 sec) extract (6 hrs); lane 6, PERK+ etoposide treated (125 uM) extract (3 hrs); lane 7, PERK + etoposide treated (125 uM) extract (6 hrs), lane 8, PERK + cycloheximide treated (3 mM) extract (3 hrs); lane 9, PERK + cycloheximide treated (3 mM) extract (6 hrs); lane 10, PERK + tunicamycin treated (4 uM) extract (3 hrs); lane 11, PERK + tunicamycin treated (4 uM) extract (6 hrs).


A (at 37⁰ C)
1 2 3 4 5 6 7 8 9 10 11
PERK-GST
Cleaved PERK
Time incubation (H)> 6 3 6 3 6 3 6 3 6 3 6
Extracts from cells > ---------- ←UV→ ←Et→ ←CH→ ←Tn→
treated with
B (at 30⁰ C)
1 2 3 4 5 6 7 8 9 10 11
97KDa
66KDa
PERK-GST
Cleaved PERK
Time incubation (H)> 6 3 6 3 6 3 6 3 6 3 6
Extracts from cells > ---------- ←UV→ ←Et→ ←CH→ ←Tn→
treated with

undergoing apoptosis or purified human active recombinant caspase-3 and caspase-6 proteins to cleave purified mouse recombinant PERK (Figures 21 and 22). The cleavage of PERK protein by *Sf9* cell extracts was carried out at 37° C (Figure 21 A) and also at 30° C (Figure 21B). We observed that like PKR, PERK was cleaved by UV-B (60 sec) 125 μM etoposide and also by 3.0 mM cycloheximide treated extracts at 37° C and 30^0 C and the cleavage product was also recognized by the antibody. However the cleavage reaction was efficient and could be seen much more readily within 3 hrs in UVB-treated and cycloheximide-treated extracts (Figure 21 A/B lanes 4, 5, 8 and 9 vs. 2 and 3). Cleavage reaction was relatively slow in etoposide-treated extracts and could be seen only at 6 hrs of incubation (Figure 21 A/B lane 7 vs. 6).

The efficiency of cleavage of recombinant PERK or PKR by extracts is related to the degree of apoptosis induced by various agents. Hence it is likely that the cleavage of eIF2a kinase in extracts prepared from cells undergoing apoptosis must be due to the presence of an active caspase. This is substantiated by the result that tunicamycin-treated extracts are not able to carry out the cleavage of PERK *in vitro* (Figure 21 A/B lanes 10 and 11).

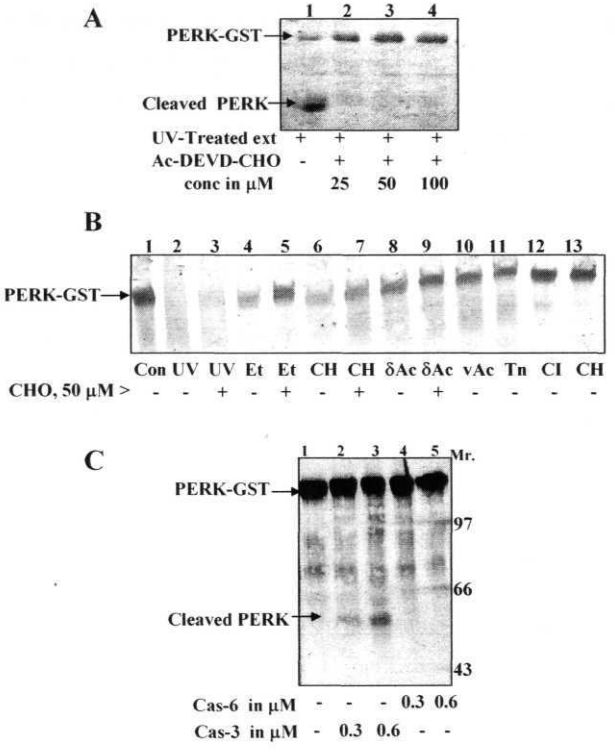
Additionally, it was observed that PERK cleavage in UV-B treated extracts was inhibited by caspase-3 inhibitor, Ac-DEVD-CHO (Figure 22A). 50 μM of the inhibitor was sufficient to inhibit the cleavage reaction almost completely (Figure 22A lane 2 vs. 1). In all these cases, the cleavage of PERK was monitored by an antibody that recognizes both the uncleaved (higher mass, ~116 kDa or ~97 kDa in the presence of extracts) and cleaved PERK (lower mass, ~58 kDa) forms. Wherever PERK was cleaved, the signal in the higher mol. wt. form of PERK was found decreased and it resulted in the appearance of a lower mol. wt. form. However in one of the experiments (Figure 22B), we used an antibody that did not recognize the lower molecular wt form or the cleaved product that occurred in the presence of extracts prepared from cells treated with different pro apoptotic agents. The result of this experiment was analysed by the decrease in the signal intensity of the higher molecular weight form of PERK. The decline in PERK signal was observed essentially in extracts prepared from cells treated with pro apoptotic agents such

Figure 22: Inhibition of PERK cleavage in 5/9 extracts *in* vitro and cleavage of PERK by pure caspases

Panel A: Purified recombinant PERK (~200 ng) was incubated in the presence and absence of caspase-3 inhibitor, Ac-DEVD-CHO (CHO) in cell extracts (~ 60 μg) prepared from 60 sec UV-B treated cells at 30° C for 3 hrs. The reaction mixture consists of cleavage buffer (220 mM Mannitol, 68 mM sucrose, 2 mM NaCl, 2.5 mM KH_2PO4, and 10 mM Tris HC1 pH 7.8). Cleavage of PERK is monitored by an antibody that recognises strongly both the cleaved and uncleaved PERK. The figure is an immnoblot. Various lanes are as follows. Lane 1, PERK + UV-B treated extract; lane 2, PERK+UV-B treated extract + CHO, 25 uM; lane 3, PERK + UV-B treated extract + CHO, 50 uM; lane 3, PERK + UV-B treated extract + CHO, 100 uM.

Panel B: *Sf9* extracts were prepared from cells treated with UV-B, etoposide (Et), cycloheximide (CH), p35 mutant AcNPV (δvAc), wild type AcNPV (vAc), tunicamycin (Tn) and calcium ionophore (CI). Extracts were then incubated with PERK protein in a reaction mixture as described in the previous legend to figure 21 at 30° C for 3 hrs in the presence and absence of 50 uM caspase inhibitor. The cleavage of PERK was monitored by a different antibody which does not recognise the cleaved product. The cleavage of PERK in the presence of inhibitor is analysed by the decline in the PERK signal. The figure is an immunoblot. The various lanes are as follows: Lane 1, PERK + control extract; lane 2, PERK + UV-B treated (60 sec) extract; lane 3, PERK + UV-B treated extract + CHO; lane 4,PERK + Et (125 uM) extract; lane 5, PERK + Et (125 uM) extract + CHO; lane 6, PERK + CH (3mM) extract; lane 7, PERK + CH (3mM) extract + CHO; lane 8, PERK + p35 mutant AcNP virus extract; lane 9, PERK + p35 mutant AcNP virus extract + CHO; lane 10, PERK + wt AcPN virus extract; lane 11, PERK + tunicamycin (20 uM) extract; lane 12, PERK + calcium ionophore (100 uM) extract; lane 14, PERK + cycloheximide (500 μM) extract.

Panel C: PERK cleavage by pure caspases. Approximately ~200 ng of PERK was incubated with pure caspase-3 and caspase-6 at a concentration of 300 and 600 ng in the cleavage buffer and cleavage is analysed by western blot analysis. Lanes in the western blot are: lane 1, PERK; lane 2, PERK + caspase-3, 300 ng; lane 3, PERK + caspase-3, 600 ng; lane 4, PERK + caspase-6, 300 ng; lane 5, PERK + caspase-6, 600 ng.


A
1 2 3 4
PERK-GST
Cleaved PERK
UV-Treated ext + + + +
Ac-DEVD-CHO - + + +
conc in μM 25 50 100
B
1 2 3 4 5 6 7 8 9 10 11 12 13
PERK-GST
Con UV UV Et Et CH CH δAc δAc vAc Tn CI CH
CHO, 50 μM > - - + - + - + - + - - - -
C
1 2 3 4 5 Mr.
PERK-GST
97
66
Cleaved PERK
43
Cas-6 in μM - - - 0.3 0.6
Cas-3 in μM - 0.3 0.6 - -

as UV-B, etoposide, higher concentrations of cycloheximide and p35 deletion mutant virus (Figure 22B lanes 2, 4, 6 and 8) but not in extracts prepared from cells treated with wt AcNPV. tunicamycin, calcium ionophore and low concentrations of cycloheximide (lanes 10, 11. 12 and 13). The reduction in PERK signal observed in lanes 2, 4, 6 and 8 by extracts prepared from cells undergoing apoptosis was reduced in the presence of Ac-DEVD-CHO. a caspase inhibitor suggesting that the cleavage reaction was facilitated by an active caspase.

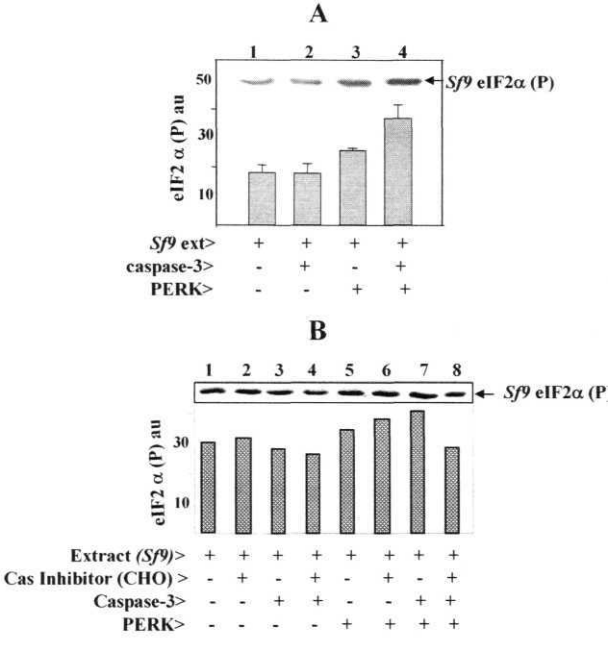
Effect of purified caspases on PERK cleavage: To demonstrate that indeed 5/9 caspases are responsible for PERK cleavage in extracts, the kinase is treated with purified human caspases 3 and 6 *in vitro* (Figure 22C). Interestingly caspase 3 was found to cleave PERK but not caspase 6 (Figure 21 lane 2 and 3 vs. 4 and 5) and the cleavage by caspase 3 was dependent on the concentration of caspase protein present in the reaction mixture (Figure 22C. compare lanes 2 and 3). These findings suggest that an active caspase -3 or caspase -3 like protein is responsible for PERK cleavage in 5/9 cell extracts as mentioned above (Figure 22B).

Caspase-cleaved PERK stimulates *Sf9* eIF2a **phosphorylation** *in vitro:* After establishing the fact that PERK can be cleaved by purified caspase-3. healthy 5/9 cell extracts were incubated with purified caspase-3 alone (Figure 23A lane 2) or PERK (lane 3) or with caspase-3 and PERK (lane 6) and analyzed the phosphorylation of 5/9 eIF2a in a two step-reaction that will allow the cleavage of PERK to occur in the first step as described in 'Materials and Methods'. Addition of PERK alone to 5/9 extracts stimulated the phosphorylation of *Sf9* eIF2α (Figure 23A lane 3 vs. 1) suggesting that the recombinant mouse PERK is active in phosphorylating 5/9 eIF2a. Addition of caspase alone did not stimulate the *Sf9* eIF2α phosphorylation *in vitro* (Figure 23A lane 2 vs. 1). However caspase-3-cleaved PERK was more efficient than caspase alone or intact PERK in phosphorylating the insect cell eIF2a (Figure 23A compare lanes 4 vs. 1. 2 and 3). Further, the increased 5/9 eIF2α phosphorylation caused by caspase-3 cleaved PERK was blocked by Ac-DEVD-CHO, a caspase inhibitor (Figure 23B). As shown in figure 23A, caspase alone did not alter the phosphorylation status of *Sf9* eIF2a (lane 3 vs. 1).

Figure 23: Phosphorylation of 5/9 eIF2a *in vitro* by PERK. Effect of purified caspase 3 and caspase inhibitor, Ac-DEVD-CHO

In step 1 purified recombinant PERK (~ 75 ng) was incubated with pure recombinant caspase 3 (50 ng) in a cleavage reaction mixture as described in the previous legend at 30^0C for 3 hrs or in the presence of caspase buffer containing 100 mM NaCl, 50 mM imidazole and 50 mM Tris-HCl pH 7.0. In the second step, *Sf9* extracts were prepared from control cells and supplemented with a phosphorylation buffer containing 20 mM Tris-HCl, pH 7.8, 2 mM Mg^{2+}, 80 mM KC1 and 30 uM ATP. Afterwards the reactions were incubated with the reaction products in step 1 at 30° C for 12 min to determine the phosphorylation of eIF2a in *Sf*9 extracts by cleaved and uncleaved PERK. The phosphon lation of eIF2α was monitored by a phosphospecific anti-eIF2a antibody. The figure is an immunoblot. Various lanes are as follows: lane 1, control untreated extract, lane 2, control extract + caspase-3; lane 3, control extract + PERK; lane 4, control extract + caspase-3 + PERK.

Panel B: The reactions were carried out in two steps as described in the legend to Figure 23A. In step 1, PERK and caspase or caspase buffer were incubated at 30° C for 3 hrs in the presence and absence of 50 uM caspase inhibitor, Ac-DEVD-CHO. In the second step, *Sf9* extracts were incubated with step 1 reaction mixtures, to determine the effect of caspase inhibitor on the caspase stimulated *Sf9* eIF2α phosphorylation by PERK. The various lanes are as follows: lane 1, control extract; lane 2, control ext + Ac-DEVD-CHO; lane 3, control extract + caspase-3; lane 4, control extract + caspase-3 + Ac-DEVD-CHO; lane 5, control extract + PERK; lane 6, control extract + PERK + Ac-DEVD-CHO; lane 8, control extract + caspase-3 + Ac-DEVD-CHO + PERK.


A
1 2 3 4
50
30
10
eIF2 α (P) au
Sf9 eIF2α (P)
Sf9 ext> + + + +
caspase-3> - + - +
PERK> - - + +
B
1 2 3 4 5 6 7 8
Sf9 eIF2α (P
30
10
eIF2 α (P) au
Extract (Sf9)> + + + + + + + +
Cas Inhibitor (CHO) > - + - + - + - +
Caspase-3> - - + + - - + +
PERK> - - - - + + + +

Addition of PERK alone stimulated eIF2α phosphorylation (lane 5) once again as shown in the figure 23B. Addition of caspase inhibitor did not affect *Sf9* eIF2α phosphorylation significantly in caspase-3 or PERK treated reactions (Figure 23B, lanes 4 and 6). However the increase in *Sf9* eIF2α phosphorylation that was observed in the presence of caspase-3 and PERK (Figure 23B, lane 7 vs. lanes 3 and 5) was reduced in the presence of caspase inhibitor (lane 8 vs. lane 7). These findings therefore suggest that caspase processed PERK is more active than unprocessed PERK and the increased eIF2a phosphorylation that occurs in apoptosis may be due to the activation of an endogenous eIF2a kinase by active caspase (s).

Protein synthesis in apoptotic and non apoptotic conditions: Phosphorylation of serine 51 residue in eIF2a inhibits protein synthesis globally and in gene-specific manner (Hinnebusch, 1996). Indeed eIF2a phosphorylation is a crucial regulatory mechanism at the translational level of gene expression. Further recent studies suggest that apoptosis mediated by nutrient deprivation and sterols require new protein synthesis (Burela et al.. 1996; Chow et al., 1995). Hence we studied protein synthesis in *Sf9* cells subjected to pro and non apoptotic stresses. Our findings indicate a) a drastic reduction in protein synthesis of *S/9* cells occurs in the presence of all stress conditions, including wt virus infection and is irrespective of whether the stress is able to promote apoptosis or not (Figure 10A and B; 24 A, B and C). The reduction in protein synthesis is not found proportional to the percent of apoptosis or to eIF2a phosphorylation. For example, UV-B radiation was observed to promote a higher amount of apoptosis compared to EGTA treatment but the reduction in protein synthesis is more severe in EGTA-treated cells compared to UV-B treatment (Figure 24A, bars 5 vs. 2). Further, caspase inhibitors which are found to reduce eIF2a phosphorylation significantly (Figure 17B and 17C) are not able to restore the stress-induced or apoptosis-mediated inhibition in protein synthesis (Figure 24B and 24C).

Figure 24: Protein synthesis in *Sf9* cells treated with various agents

Cells were treated with various agents for 15 hrs at 27° C. Then cells were washed and changed to medium containing [^{35}S] methionine (30μci/2x10^{6} cells) and incubated for 60 min at 27° C. Afterwards, cells were washed and incubated in methionine free medium for another 60 minutes at 27° C before the extracts were prepared in 60 ul of lysis buffer. **Protein** synthesis was estimated by taking labelled (S-35) methionine incorporation in to 5 ml of the TCA precipitable extracts as described in 'Materials and Methods'. The various agents used in the panel A are 1. none, 2. 60 sec UV-B light, 3. 125 uM etoposide, 4. 3.0 mM cycloheximide, 5. 50 mM EGTA, 6. 48 hrs vAC infected, 7. 48 hrs vAc infected and 60 sec UV-B treated, 8. 48 hrs vAc infected and 125 uM etoposide, 9. 48 hrs vAc infected and 3mM cycloheximide, 10. 48 hrs vAc infected and 50 mM EGTA. Panel B and C represent protein synthesis in *Sf9* cells treated with various agents for 15 hrs in the presence and absence of caspase- 3 and caspase-8 inhibitors, z-VAD-fmk and z-IETD-fmk respectively. The various agents used in these panels are: 1. none, 2. none + i, 3. 60 sec UV-B light, 4. 60 sec UV-B+ i, 5, 125 uM etoposide, 6. 125 uM etoposide+i, 7. 3.0 mM cycloheximide, 8. 3.0 mM cycloheximide+i, 9. 50 mM EGTA, 10. 50 mM EGTA+i.

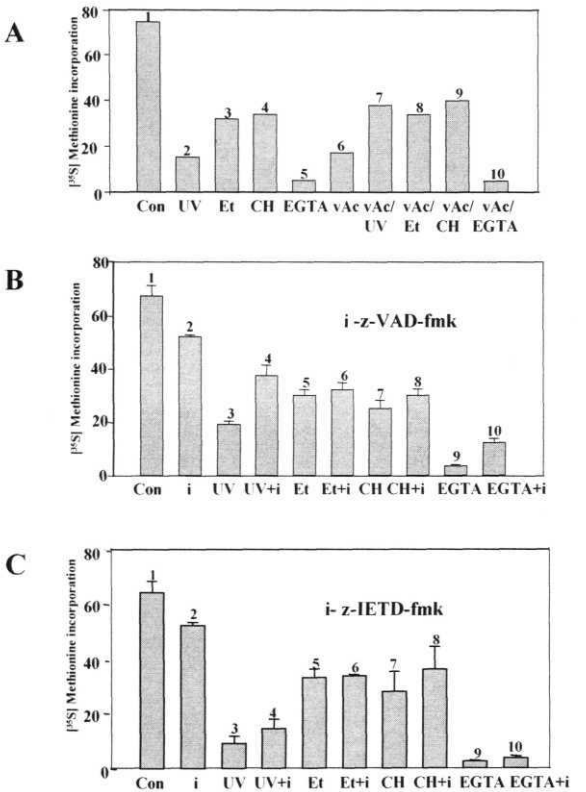
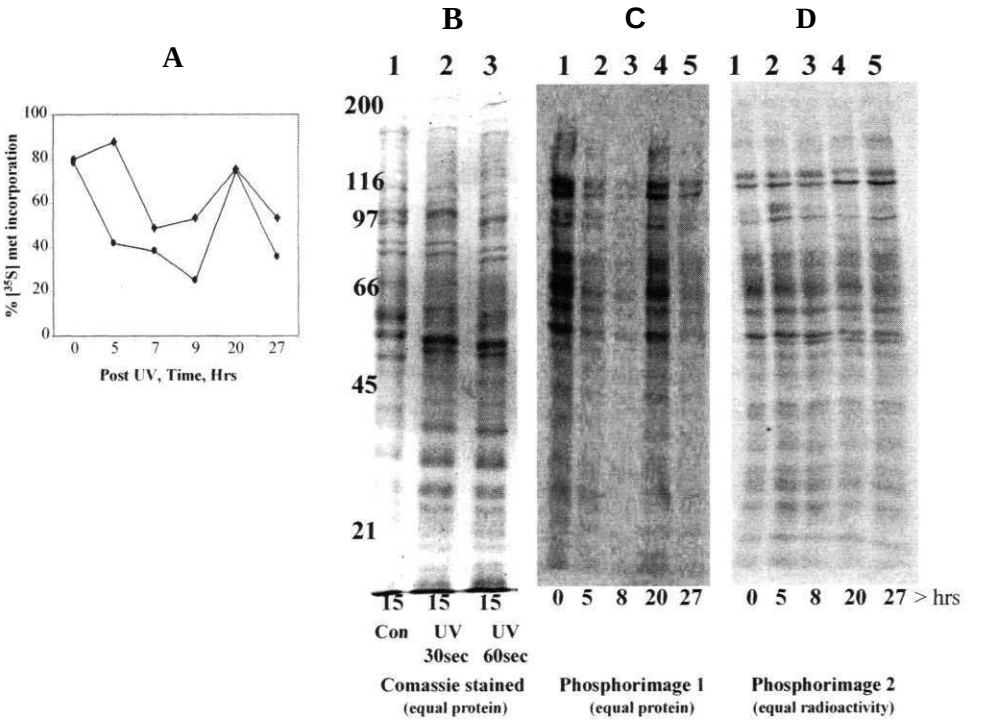

A
B
C
[35S] Methionine incorporation
0
20
40
60
80
Con
UV
Et
CH
EGTA
vAc
vAc/ UV
vAc/ Et
vAc/ CH
vAc/ EGTA
i -z-VAD-fmk
Con
i
UV
UV+i
Et
Et+i
CH
CH+i
EGTA
EGTA+i
i- z-IETD-fmk
Con
i
UV
UV+i
Et
Et+i
CH
CH+i
EGTA
EGTA+i

Figure 25: Kinetics of protein synthesis in UV-B treated *Sf9* cells

Panel A: *Sf9* cells were treated with 60 sec UV-B light and incubated at 27° C for different time periods. Protein synthesis was measured using labelled [^{35}S] methionine as described in the legend to Figure 24.

Panel B, C and D: *Sf9* cells were treated with 60 sec of UV-B light and incubated at 27° C for 5, 8, 20 and 27 hrs. Labelling with [^{35}S] methionine was carried out for 60 min at the end of 3, 6, 18 and 25 hrs and the cells were washed ad incubated in methionine free medium for another 60 min prior to making the extracts. Extracts were made in lysis buffer as described in 'Materials and Methods'. Proteins were precipitated in 10% cold TCA and were separated by 10% SDS-PAGE. Proteins were analysed B) by comassie stain; C and D were analysed by Phosphor imager.


A
B
C
D
% [35S] met incorporation
100
80
60
40
20
0
0 5 7 9 20 27
Post UV, Time, Hrs
1 2 3
200
116
97
66
45
21
15 15 15
Con UV 30sec UV 60sec
Comassie stained
(equal protein)
1 2 3 4 5
0 5 8 20 27
Phosphorimage 1
(equal protein)
1 2 3 4 5
0 5 8 20 27 > hrs
Phosphorimage 2
(equal radioactivity)

These findings therefore suggest that inhibition of protein synthesis is not exclusively due to eIF2a phosphorylation. It appears that both type and magnitude of the stress also play an important role in the reduction of protein synthesis.

We also analysed the kinetics of protein synthesis and synthesis of S-35 labeled protein in UV-B treated *SJ9* cells. Interestingly, we observed a transient increase in protein synthesis after the on set of apoptosis and at 20 hrs of UV-B treatment (Figure 25A). An analysis of the labeled products indicates that there is indeed a transient increase in the synthesis of several proteins 20 hrs after UV-B treatment (Figure 25C and 25D, lanes 2 and 3 vs. 4 or 5). Preceding and following this increase, the protein synthesis is greatly reduced during UV-B treatment. Although these results are preliminary, they suggest strongly that perhaps new proteins are indeed made during the course of apoptosis. It is not known however whether transcription or translation or a combination of both regulate the synthesis of these new proteins.

Discussion: *Sf9* cells are the natural hosts of baculovirus. Several earlier studies have used this system for the expression of heterologous recombinant proteins (Luckow and Summers, 1988) and also as model systems to study the process of apoptosis or programmed cell death (Ahmad et al., 1997, Hasnain et al., 1999, Manji et al., 1997, Vucic et al., 1997). Infact, earlier studies have identified that baculovirus produces a potent p35 anti apoptotic protein (Clem et al., 1991). The p35 protein, also called pan caspase inhibitor, prevents programmed cell death in phylogenetically diverse organisms such as *C. elegans*, *D. melanogaster* and humans (Hay et al., 1994; Biedler et al., 1995; Xue and Horvitz, 1995; Rabizadeh et al., 1993). In addition, the earlier results from this laboratory indicate that a) baculovirus infection reduces *Sf9* eIF2a phosphorylation and b) *Sf9* cells, unlike mammalian cells, are found suitable to express a phosphomimetic form of human S51D eIF2a where the serine 51 residue is replaced by an aspartic acid (Sudhakar et al., 2000). It was observed by others that apoptosis induced by tumor necrosis factor alpha (TNF-α) in mammalian systems, is characterized by increased eIF2a phosphorylation. Further transient expression of a phosphomimetic form of eIF2a, S51D, using a CAT (chloramphenicol acetyl transferase vector) expression

These findings therefore suggest that inhibition of protein synthesis is not exclusively due to eIF2a phosphorylation. It appears that both type and magnitude of the stress also play an important role in the reduction of protein synthesis.

We also analysed the kinetics of protein synthesis and synthesis of S-35 labeled protein in UV-B treated *Sf9* cells. Interestingly, we observed a transient increase in protein synthesis after the on set of apoptosis and at 20 hrs of UV-B treatment (Figure 25A). An analysis of the labeled products indicates that there is indeed a transient increase in the synthesis of several proteins 20 hrs after UV-B treatment (Figure 25C and 25D, lanes 2 and 3 vs. 4 or 5). Preceding and following this increase, the protein synthesis is greatly reduced during UV-B treatment. Although these results are preliminary, they suggest strongly that perhaps new proteins are indeed made during the course of apoptosis. It is not known however whether transcription or translation or a combination of both regulate the synthesis of these new proteins.

Discussion: *Sf9* cells are the natural hosts of baculovirus. Several earlier studies have used this system for the expression of heterologous recombinant proteins (Luckow and Summers, 1988) and also as model systems to study the process of apoptosis or programmed cell death (Ahmad et al., 1997, Hasnain et al., 1999, Manji et al., 1997, Vucic et al., 1997). Infact, earlier studies have identified that baculovirus produces a potent p35 anti apoptotic protein (Clem et al., 1991). The p35 protein, also called pan caspase inhibitor, prevents programmed cell death in phylogenetically diverse organisms such as *C. elegans*, *D. melanogaster* and humans (Hay et al., 1994; Biedler et al., 1995; Xue and Horvitz, 1995; Rabizadeh et al., 1993). In addition, the earlier results from this laboratory indicate that a) baculovirus infection reduces *Sf9* eIF2a phosphorylation and b) *Sf9* cells, unlike mammalian cells, are found suitable to express a phosphomimetic form of human S51D eIF2a where the serine 51 residue is replaced by an aspartic acid (Sudhakar et al., 2000). It was observed by others that apoptosis induced by tumor necrosis factor alpha (TNF-a) in mammalian systems, is characterized by increased eIF2a phosphorylation. Further transient expression of a phosphomimetic form of eIF2a, S51D, using a CAT (chloramphenicol acetyl transferase vector) expression

plasmid was sufficient to induce apoptosis in N1H3T3 cells (Srivastava et al., 1998). Keeping in view of these observations, in mammalian cells, we have used *Sf9* cells to determine the importance of kinase activation or eIF2a phosphorylation in apoptosis. These cells have not been studied so far to analyze the signals that stimulate the phosphorylation of eIF2a or that activate eIF2α kinases. Hence we have analysed the ability of a variety of agents to stimulate eIF2a phosphorylation and apoptosis in uninfected and virus-infected *Sf9* cells.

A major observation of this study is that uninfected, but not the wt AcNPV-infected *Sf9* cells are very sensitive and respond to different signals by altering the phosphorylation status of cellular eIF2α. We demonstrate that in addition to UV-B light and etoposide, higher concentrations of cycloheximide and EGTA promote apoptosis (Figures 5 and 10), stimulate the caspase activity (Figure 6) and also eIF2a phosphorylation in uninfected *Sf9* cells (Figures 9 and 10). In contrast, tunicamycin, A23187, and low concentrations of cycloheximide failed to activate caspase and consequent apoptosis (Figures 6 and 10). Nevertheless, these agents do stimulate eIF2a phosphorylation in *Sf9* cells (Figure 9 and 10) thereby suggesting that the stress signaling pathway is important in the induction of apoptosis. The results presented here suggest that both apoptotic and non apoptotic agents stimulate eIF2a phosphorylation in *Sf9* cells there by suggesting that *Sf9* cells contain an eIF2a kinase. However this kinase activity is not triggered directly by the addition of any of these agents to the cell extracts (Figure 11) thereby suggesting that the activation of the eIF2a kinase(s) occurs through some intermediates. So far the eIF2a kinases present in *Sf9* insect cells have not been characterized, although GCN2 and PERK have been characterized in Drosophila (Sood et al., 2000; Berlanga et ai., 1999; Williams et al., 2001). Since agents such as UV-B light, cycloheximide, EGTA, A23187 and tunicamycin are known to stimulate unfolded protein response (UPR) or stress in the endoplasmic reticulum (ER) (Wu et al., 2002; Nozaki et al., 2001; Kaufman 1999b, Williams et al., 2001) both in mammalian systems and in *Drosophila*, it is likely that *Sf9* cells contain an ER-resident kinase like PERK which is stimulated in response to all of these agents as evidenced by increased eIF2α phosphorylation.

Wt AcNPV-infected *Sf9* cells resist apoptosis caused by various agents as mentioned above. In contrast a mutant virus that is devoid of its p35 gene promotes apoptosis readily and stimulates eIF2a phosphorylation in *Sf9* cells (Figure 13). The mechanism of activation of caspases in *Sf9* cells vis-a`-vis the role of p35 in inhibiting the maturation of caspase (s) is not well understood. It has been proposed that an unidentified apical caspase cleaves the pro-*Sf*-caspase-1 to p25 and p12, and subsequently the p25 caspase product gives rise to p 19 (Manji and Friesen, 2001). While cellular IAPs block the activation of an apical unidentified caspase and inhibit the cleavage of Pro-*Sf*-caspase-1 to p25 and p12 products, baculovirus p35 blocks the cleavage of p25 caspase to p19, thereby inhibiting the maturation of caspase (s). This is further substantiated by the fact that apoptosis in insect cells, induced by UV-B radiation and p35 deletion virus infection, is prevented by pro-*Sf*-caspase-1 inhibitors (Manji and Friesen, 2001). We show here that p35 viral protein not only inhibits the formation of an active caspase and consequent apoptosis but also mitigates eIF2a phosphorylation in *Sf9* cells (Figure 12).

The importance of baculovirus p35-mediated caspase inhibition leading to diminished eIF2a phosphorylation is substantiated further by the observation that wt virus-infected *Sf9* cells are unable to undergo apoptosis in response to UV-B irradiation (Figure 12) or through the overexpression of the phosphomimetic form of eIF2α (data not shown). On the contrary, apoptosis is stimulated readily in *Sf9* cells that are exposed first to UV-B light and then transfected with the recombinant baculovirus carrying the phosphomimetic form of eIF2α than with wt or the nonphosphorylatable form of eIF2α (Figure 14). These observations in *Sf9* cells indicate that eIF2a phosphorylation alone does not stimulate apoptosis or caspase activity, but it can enhance the apoptotic effect of caspase, thereby suggesting that eIF2a phosphorylation is necessary but not a sufficient condition for the induction of apoptosis in 5/9 cells.

The above results in *Sf9* cells are thus somewhat different from what has been observed in mammalian cells. In mammalian cells, TNF-a induced apoptosis is characterized by increased eIF2α phosphorylation. Expression of wt PKR or phosphomimetic form of eIF2α in a transient transfection system was found sufficient to promote apoptosis similar

to TNF-a. Our results though agree with the idea that eIF2a phosphorylation is a characteristic feature of cells undergoing apoptosis, it has been observed that it is not sufficient to induce apoptosis as has been demonstrated here by overexpressing the phosphomimetic form of eIF2a alone (data not shown) using baculovirus or non baculovirus PNN1 plasmid construct carrying a heat shock promoter (Figure 15). This may be due to the a) lack of receptor mediated apoptosis in insect cells and b) the absence of double stranded RNA dependent enzyme like PKR in insect cells. These suggestions are also consistent with many other personal observations such as that TNF-a does not induce apoptosis in insect cells (personal observations of Dr. Ramaiah's laboratory) and addition of dsRNA to insect cell extracts does not lead to the activation of any PKR like kinase or eIF2a phosphorylation (Sudhakar et al., 1999). Despite suggestions by Blair et al., 1995 that *Sf9* cells contain a protein that is antigentically related to PKR, PKR-encoding sequences have not been recognized in any invertebrate for which the complete genome sequence is available.

Interestingly, many viruses are known to produce proteins that interfere with PKR-mediated activation and inhibit host cell eIF2a phosphorylation (Kaufman, 1999a). A recent study has shown that baculovirus also produces pk2 protein that resembles the C-terminal half of a protein kinase domain and is found to inhibit activation of GCN2, PKR and HRI eIF2a kinases *in vitro.* Consistent with this observation, we have always observed that wt baculovirus infected cells displayed low levels of eIF2a phosphorylation (Figure 12). It is not known however if pk2 interferes with the host apoptosis. Our findings here suggest that baculovirus encoded p35, an anti-apoptotic or a caspase inhibitor protein also interferes with the activation of host cell eIF2a kinase albeit, in an indirect manner. This is substantiated by the observation that p35 deletion virus stimulates eIF2α phosphorylation and apoptosis in *Sf9* cells (Figure 13). These observations prompted us to investigate further the relation between caspase activation to increased eIF2a phosphorylation in apoptosis. In this pursuit, we have used cell permeable caspase inhibitors such as z-VAD-fmk and z-IETD-fmk. Both of them inhibited not only apoptosis induced by agents such as UV-B, etoposide, cycloheximide

and EGTA but also the eIF2a phosphorylation (Figures 13, 17 and 18). In contrast, tunicamycin-induced eIF2a phosphorylation was not affected by the presence of caspase inhibitor, which is found to be consistent with its inability to stimulate caspase activity or apoptosis (Figure 17D). These findings reinforce the conclusion that caspase activation is a prerequisite for increased eIF2a phosphorylation in cells undergoing apoptosis. In other words eIF2α phosphorylation is a consequence of caspase activation in apoptosis. The fact that both stressed cells and cells undergoing apoptosis display higher level of eIF2a phosphorylation, the findings here suggest that the mechanism of activation of eIF2a kinase(s) or stimulation of eIF2a phosphorylation is different in general stress conditions and in conditions that specifically promote apoptosis.

In order to understand the signaling mechanisms involved in the activation of eIF2α kinases, *Sf9* cells have been treated with broad-spectrum tyrosine kinase or ser-thr kinase inhibitors such as genestein (50 μM) and staurosporine (100 nM) respectively. Interestingly both agents stimulated eIF2α phosphorylation but not caspase activation or apoptosis. On the contrary, staurosporine, but not genestein, decreased partially UV-B induced caspase activation and apoptosis (Figure 16). Although it is surprising to observe increased cellular eIF2α phosphorylation in the presence of kinase inhibitors, the data is consistent with earlier findings that a) staurosporine cannot inhibit eIF2a kinases in cultured neuronal cells (del Vega et al., 1999) and b) genestein, a flavonoid suppresses protein synthesis and tumor cell growth *in vitro* and *in vivo* by activating eIF2a kinases (Ito et al., 1999) probably by a stress-induced signaling mechanism. Since genestein is found to activate ER-resident eIF2α kinase like PERK more strongly than other cytosolic eIF2a kinases such as PKR and HRI, it is suggested that flavonoids like genestein promote ER stress (Ito et al., 1999). Thus the increased eIF2a phosphorylation of *Sf9* cells in the presence of staurosporine and genestein suggest that both inhibitors promote stress in general, but not apoptosis in *Sf9* cells. The finding that staurosporine prevents UV-B mediated apoptosis in *Sf9* cells, suggests the probable involvement of some intermediate phosphoproteins belonging to mitogen-activated protein kinase (MAPK) signaling pathways.

Virus infection and caspase inhibitor studies have shown that inhibition of caspase activation leads to reduction in *Sf9* eIF2α phosphorylation in cells undergoing apoptosis. Further insight into the chronological events of caspase activation and eIF2a phosphorylation in *Sf9* cells has been provided by a time course experiment wherein these activities have been analyzed in cell extracts prepared from UV-B treated cells at different time periods (Figure 19). This experiment demonstrates that caspase activation precedes eIF2α phosphorylation and reiterated the above finding that enhanced eIF2a phosphorylation during apoptosis is caspase dependent.

The phenomenon of enhanced eIF2α phosphorylation during apoptosis can be directly related to its kinase activity. Caspases have been shown to activate or inactivate several kinases by site specific cleavages which are involved in signal transduction pathways like MEKK-1, protein kinase C-5, (PKC-8) and p21 activated kinase 2 (PAK2) (Widmann et al., 1998; Fischer et al., 2003). Here it has been shown that both eIF2a kinases, recombinant human PKR, and mouse PERK are cleaved *in vitro* by cell extracts prepared from cells undergoing apoptosis (Figure 20 and 21). Indeed a recent study has shown that mammalian PKR can be cleaved by caspases (Saelens et al., 2001). However, PKR like enzyme is not found in insect cells as has been mentioned above. Since the agents used here are to known to disturb ER and can cause ER stress, and PERK like kinase has been reported to be present in insects like Drosophila (Williams et al., 2001) it is likely that the increased eIF2a phosphorylation is due to the activation of PERK like kinase in *Sf9* cells. As of date, there are no reports indicating that PERK (from any source) can be cleaved by any caspases. Our studies have shown here that the cleavage of PERK occurs in extracts prepared from cells treated with pro apoptotic agents but not with non-apoptotic agents like tunicamycin (Figure 21). The cleavage of PERK is further confirmed by pure human recombinant caspase-3 but not by caspase-6 (Figure 22). And the cleavage of PERK in extracts is inhibited by caspase-3 inhibitor, Ac-DEVD-CHO (Figure 22). Interestingly, cleavage of PERK by caspase has conferred additional catalytic activity in terms of its substrate; eIF2a phosphorylation as has been demonstrated in figure 23. These results thus suggest that the enhanced eIF2a

phosphorylation observed during caspase dependent cell death in *Sf9* cells is due to the cleavage of an eIF2a kinase.

The significance of enhanced eIF2a phosphorylation in a cell is attributed to a decline in general protein synthesis, although it is not the sole contributor for the inhibition of protein synthesis during any stress condition. Paradoxically, the translation of transcriptional factor such as GCN4 in yeast and ATF4 (activated transcription factor) in mammalian system is increased in response to eIF2a phosphorylation (Hinnebusch, 1996; Ron 2002). In this study, it is noticed that all stresses whether apoptotic or non apoptotic are able to decrease protein synthesis in *Sf9* cells and promote increased eIF2α phosphorylation (Figure 10 bar diagram). However the reduction in protein synthesis in the presence of various agents does not correlate always to increased eIF2α phosphorylation. For example baculovirus infection reduced host cell eIF2a phosphorylation and also the protein synthesis (Figure 24). The caspase inhibitors, z-VAD-fmk and z-lETD-fmk reduced apoptosis and elF2a phosphorylation almost to the control level but are unable to reverse protein synthesis inhibition completely (Figure 17). These findings therefore suggest that the inhibition of protein synthesis and apoptosis involve different signaling pathways. The reduction in protein synthesis during apoptosis may be mediated partly through eIF2a phosphorylation and also by other mechanisms as well. These suggestions are consistent with the finding that z-VAD-fmk does not relieve the inhibition in translation caused by etoposide in MCF-7 breast cancer cell (Jeffery et al., 2002). Protein synthesis, during a time course experiment, showed a significant decrease in protein synthesis (4-5 hr) after caspase activation (1-2 hr) (Figures 19 and 25). Interestingly the translational inhibition was recovered during mid phase of apoptosis i.e., at around 15-18 hrs of UV-B treatment (Figure 25). Further, it has been observed that UV-B induced apoptosis leads to an increased synthesis of labeled proteins during this period (Figure 25). This is consistent with the earlier findings that stress induced apoptosis requires new protein synthesis in mammalian systems (Burela et al., 1996; Chow et al., 1995). Cellular stress in mammalian systems activates intra cellular signaling pathways that affect a sizeable number of transcriptional factors leading to alterations in the gene expression. ER is a repository for both pro and anti apoptotic

molecules. The activation of transcriptional factors such as ATF4, GCN4, and NF-kB that occurs in response to ER stress, in turn, would stimulate the expression of several pro apoptotic (CHOP/GADD-153) and pro survival proteins including GADD 34, Bip/GRP78, calreticulin, protein disulfide isomerase, NF-kB, etc. (Kaufman, 1999b; Ron, 2002). Posttranslational modifications of CHOP can lead to the transcriptional activation of novel stress-induced genes called DOC 6 and DOC 4 (downstream of CHOP) that share homology with the mammalian actin binding proteins such as villin and gelsolin or a mammalian orthologue of a *Drosophila* gene, *Tenm/Odz*, respectively. While Doc 6 is implicated in cell death. Doc 4 is important in cellular regeneration (Wang et al., 1998).

Prolonged ER stress leads to cell death. Activation of ER-resident caspase-12 occurs by different mechanisms such as the caspase-7/caspase-12 pathway and caspase 12/calpain pathway (Rao et al., 2002). A number of stimuli that disrupt protein folding such as tunicamycin and the calcium ionophore can activate both unfolded protein response (UPR) and ER overload response (EOR). These two signaling pathways respond to different and overlapping types of ER stresses, and their common feature for their induction is not known (Kaufman, 1999b). While UPR induces transcription of pro apoptotic genes such as CHOP. EOR induces anti apoptotic genes such as NF-kB (Kaufman. 1999b). A recent study further describes that NF-kB, an anti apoptotic protein, inhibits activation of GADD 153/CHOP in breast cancer cells exposed to tunicamycin or the calcium ionophore (Nozaki et al.. 2001). It is likely that such paradoxical interactions of anti- and pro apoptotic transcription factors in *Sf9* cells may be responsible for the specific differences in stress-induced apoptosis. The micro dissection of the signaling events leading to stress-induced apoptosis in insect cells and the players involved in this process remain to be elucidated. Nonetheless, the degree of conservation in these processes between the vertebrates and the invertebrate systems renders *Sf9* insect cells an indispensable model system to study apoptosis.

Summary:

- These results obtained with a wide range of stresses demonstrate that eIF2a phosphorylation is a stress signal and an increase in the eIF2a phosphorylation does not always lead to apoptosis.
- Wild type baculovirus infection abrogates apoptosis and elF2a phosphorylation.
- P35 deletion mutant baculovirus induces apoptosis with enhanced *Sf9* eIF2a phosphorylation indicating that p35, an anti apoptotic protein down regulates elF2a phosphorylation albeit indirectly.
- Synthetic caspase-3 and 8 inhibitors inhibit apoptosis and *Sf9* eIF2a phosphorylation in cells treated with pro apoptotic agents but not with non apoptotic agent like tunicamycin.
- Active caspase produced in *Sf9* cells during apoptosis, was able to cleave recombinant mammalian eIF2a kinases, PKR and PERK *in vitro.* The processed eIF2a kinase than the unprocessed kinase is found to be more efficient in phosphorylating its substrate, eIF2a.
- Overall, these findings suggest that the mechanism of activation of eIF2a kinases or eIF2a phosphorylation in *Sf9* cells in apoptotic conditions is different compared to stressed cells that are not undergoing apoptosis. Further eIF2α phosphorylation enhances the process of apoptosis.

Future Scope: The above results clearly suggest uninfected and virus infected *Sf9* cells continue to be the good model systems not only to identify and evaluate the mechanism(s) of anti apoptotic factors like IAPs and p35 like proteins but also to investigate the various players involved in the stress induced signaling pathways, interactions between kinases(s) and caspase(s) and to determine the proteins that are specifically synthesized in response to apoptosis and to investigate the importance of these proteins to promote or inhibit the process of apoptosis.

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Stress-Induced Apoptosis in *Spodoptera frugiperda* (*Sf*9) Cells: Baculovirus p35 Mitigates eIF2a Phosphorylation†

Gunda Aparna,‡ Abani K. Bhuyan,§ Sudhir Sahdev,|| Seyed E. Hasnain,||,⊥,# Randal J. Kaufman,△ and Kolluru V. A. Ramaiah*,+

Department of Biochemistry and School of Chemistry, University of Hyderabad, Hyderabad 500 046, India, Laboratory of Molecular and Cellular Biology, CDFD, Nacharam, Hyderabad 500 076, India, National Institute of Immunology, New Delhi 110 067, India, JNCASR, Bangalore, India, and Department of Biological Chemistry, Howard Hughes Medical Institute, University of Michigan Medical Center, Ann Arbor, Michigan 48109

Received June 2, 2003; Revised Manuscript Received November 3, 2003

ABSTRACT: *Spodoptera frugiperda (Sf9)* ovarian cells, natural hosts for baculovirus, are good model systems to study apoptosis and also heterologous gene expression. We report that uninfected *Sf*9 cells readily undergo apoptosis and show increased phosphorylation of the a subunit of eukaryotic initiation factor 2 (eIF2a) in the presence of agents such as UVB light, etoposide, high concentrations of cycloheximide, and EGTA. In contrast, tunicamycin, A23187, and low concentrations of cycloheximide promoted eIF2a phosphorylation in *Sf*9 cells but without apoptosis. These findings therefore suggest that increased eIF2a phosphorylation does not always necessarily lead to apoptosis, but it is a characteristic hallmark of stressed cells and also of cells undergoing apoptosis. Cell death induced by the above agents was abrogated by infection of *Sf*9 cells with wild-type (wt) AcNPV. In contrast, 5/9 cells when infected with vAcδ35, a virus carrying deletion of the antiapoptotic p35 gene, showed increased apoptosis and enhanced eIF2a phosphorylation. Further, a recombinant wt virus vAcS51D expressing human S51D, a phosphomimetic form of eIF2a, induced apoptosis in UVB pretreated *Sf*9 cells. However, infection with vAcS51A expressing a nonphosphorylatable form (S51A) of human eIF2a partially reduced apoptosis. Consistent with these findings, it has been observed here that caspase activation has led to increased eIF2α phosphorylation, while caspase inhibition by z-VAD-fmk reduced eIF2a phosphorylation selectively in cells exposed to proapoptotic agents. These findings therefore suggest that the stress signaling pathway determines apoptosis, and caspase activation is a prerequisite for increased eIF2α phosphorylation in 5/9 cells undergoing apoptosis. The findings also reinforce the conclusion for the first time that the "pancaspase inhibitor" baculovirus p35 mitigates eIF2a phosphorylation.

Changes in gene expression at the translational level occur in eukaryotic cells in response to a wide variety of conditions (*7*). that are known to promote or inhibit cell growth, cell transformation, and programmed cell death. Eukaryotic initiation factor 2 (eIF2), a key regulatory protein involved in polypeptide chain initiation, is a heterotrimer with three subunits: a, *(3,* and *y.* It facilitates the joining of initiator tRNA (Met-tRNAi) to the 40S ribosomal subunit to form the 43S preinitiation complex (reviewed in refs *1* and 2). Phosphorylation of the serine 51 residue in the a subunit of eIF2 promotes a complex formation between eIF2 and eIF2B, a rate-limiting heteropentameric guanine nucleotide exchange (GNE) factor *(3—6)* and inhibits the GNE activity of eIF2B These events lead to impairment in the recycling of eIF2 followed by inhibition of protein synthesis either globally or in a gene-specific manner (2, 5, *9).* Abrogation of eIF2α[1] phosphorylation promotes transformation in NIH 3T3 cells (*10*), and eIF2a phosphorylation levels increase in mammalian cells that are undergoing apoptosis (*11*), suggesting that a critical balance in eIF2a phosphorylation is important for the survival or maintenance of cells in culture.

The four eIF2a kinases identified and characterized so far phosphorylate the serine 51 residue in eIF2a and share sequence and structural features that are distinguishable from other serine/threonine kinases. These are heme-regulated eIF2α kinase (HRI), general control nonderepressible 2

† This work was supported by grants from CSIR (37/1120/02/EMR-II), New Delhi, India, to K.V.A.R., DST (4-1/2003-SF) to A.K.B., and DBT to S.E.H. G.A. acknowledges the support of a SRF fellowship from CSIR, New Delhi. A.K.B. is a recipient of the Swarnajayanthi Award from DST, New Delhi.

* To whom correspondence should be addressed. Fax: 011-91-40-23010120, -23010451, or -23010145. E-mail: kvarsl@uohyd.ernet.in.

+ Department of Biochemistry, University of Hyderabad.

§ School of Chemistry, University of Hyderabad.

|| Laboratory of Molecular and Cellular Biology, CDFD.

⊥ National Institute of Immunology.

JNCASR.

△ University of Michigan Medical Center.

[1] Abbreviations: eIF2α, a subunit (38 kDa) of eukaryotic translational initiation factor 2; eIF2α(P), phosphorylated eIF2α; *Sf*9, *Spodoptera frugiperda*; AcNPV, *Autographa californica* nuclear polyhedrosis virus; PERK, pancreatic endoplasmic-resident eIF2α kinase; PKR, double-stranded RNA-dependent eIF2a kinase; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; wt, wild type; Ac-DEVD-AFC, *N*-acetyl-Asp-Glu-Val-Asp-amino-4-(trifluoromethyl)coumarin; S51A eIF2α, mutation of Ser51 eIF2a to Ala; S51D eIF2α, mutation of Ser51 eIF2α to Asp; Mol, multiplicity of infection; z-VAD-fmk, carbobenzoxyvalylalanylaspartyl(*O*-methyl) fluoromethyl ketone; PARP, poly(ADP-ribose) polymerase.

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Published on Web 12/03/2003

(GCN2) kinase that is activated upon amino acid starvation or accumulation of uncharged tRNAs (reviewed in ref *12*), dsRNA-dependent protein kinase called PKR, and an endoplasmic-resident (ER) kinase called PEK or PERK that is activated during the accumulation of malfolded proteins (reviewed in refs *2* and *13*−*16*).

Induction of apoptosis in mammalian systems leads to changes in translational machinery that include cleavage of the 28S rRNA of the 60S subunit (*17*) and initiation factors such as eIF2a *(18, 19)*, eIF4B, eIF4GI, eIF4GII, and the j subunit of eIF3 *(20—23)*. Additionally, apoptosis also alters the phosphorylation of translational initiation factors such as eIF2a (*11*) and eIF4E BP1 (*24*) and various other cellular proteins (reviewed in ref 25), A recent study demonstrated caspase-dependent activation of PKR leading to eIF2a phosphorylation during Fas-mediated apoptosis in Jurkat cells *(26)*. PKR induces the activation of caspase-8 and/or other components of the signaling pathway involving TNF family receptors (*27*−*29*).

The ovarian cells of the lepidopteran insect *Spodoptera frugiperda (Sf*9 and *Sf*21), natural hosts of baculovirus, have been extensively used to express recombinant proteins *(30)* and also as a model system for studying apoptosis (*31*−*35*). The significance of the present work is further augmented by the fact that both the death-ligand receptor pathway of apoptosis and PKR-mediated eIF2a phosphorylation that are well-known in mammalian cells are unknown in insect cells. A specific baculovirus gene product, p35, was identified as being responsible for blocking the apoptotic response in host cells *(33)*. The p35 protein, also called pancaspase inhibitor, prevents programmed cell death in phylogenetically diverse organisms such as *Caenorhabditis elegans*, *Drosophila melanogaster*, and humans (*36*−*38*). We previously showed that eIF2α phosphorylation is reduced typically in wild-type virus-infected S/9 cells (3, *6)*. Further, expression of S51D (a phosphomimetic form of human eIF2a in which the serine 51 residue is replaced by aspartic acid) in S/9 cells using recombinant baculovirus, although with reduced cell viability, did not induce apoptosis (3). However, the relationship between apoptosis and eIF2α phosphorylation or phosphorylation of any other protein has not been demonstrated in S/9 cells.

The present study is undertaken to determine the importance of eIF2a phosphorylation in mediating apoptosis in *Sf*9 cells and to delineate the role of the antiapoptotic p35 baculovirus gene on elF2a phosphorylation. Our findings with a variety of pro- and nonapoptotic agents suggest that eIF2a phosphorylation in S/9 cells need not always lead to apoptosis, but cells undergoing apoptosis are characterized by increased eIF2a phosphorylation. Further, S/9 cells treated with a synthetic caspase inhibitor such as z-VAD-fmk or wt baculovirus infection resist apoptosis and show decreased eIF2a phosphorylation, whereas infection of p35 mutant baculovirus leads to increased eIF2a phosphorylation and apoptosis. The findings therefore suggest that the "pancaspase inhibitor* p35 protein produced by baculovirus mitigates eIF2a phosphorylation and apoptosis. The mechanism of induction of eIF2α phosphorylation in the presence of pro- and nonapoptotic agents appears to be different.

MATERIALS AND METHODS

Materials. A recombinant baculovirus vector harboring wt or S51A or S51D mutants of human eIF2a was prepared from the parent vector pETFVA⁻ as described earlier *(3, 6)*. A phosphospecific anti-eIF2a antibody was obtained from Research Genetics, and polyclonal anti-eIF2a was purchased from Santacruz Biotechnology Inc. Ac-DEVD-AFC, the substrate analogue of caspase(s), and recombinant human caspases-3 and -6 were purchased from BD Pharmingen. z-VAD-fmk, a cell-permeable caspase inhibitor, was obtained from Calbiochem. Glutathione−Sepharose 4B and BL21 cells were purchased from Amersham-Pharmacia. Mutant p35 virus, ∂p35AcNPV, was obtained originally by one of us (S.E.H., CDFD, Hyderabad) from Dr. Paul D. Friesen's laboratory in the Institute of Virology, University of Wisconsin, Madison, WI. Bovine PARP was obtained from Calbiochem, and the anti-PARP p85 fragment antibody was obtained from Promega, Inc.

Cell Culture. S. frugiperda (*Sf*9) cells were grown in TNM-FH medium supplemented with 10% fetal calf serum and antibiotics as described earlier *(3, 32)*. Confluent cells with >95% viability were used in all experiments. The trypan blue exclusion test was carried out to assess the viability of the cells.

*Induction of Apoptosis. Sf*9 cells were irradiated with UVB light (312 nm) for 30—60 s as described earlier *(32)* and incubated at 27 °C for 15 h or treated for 15 h with different agents such as etoposide, cycloheximide, EGTA, A23187, and tunicamycin. Apoptosis was also studied in the presence of a cell-permeable z-VAD-fmk, a caspase inhibitor, with wt AcNPV and with p35 deletion mutant virus *(32)*. To evaluate the importance of eIF2a phosphorylation in apoptosis, cells were treated with UVB radiation before and after the expression of wt and mutants of human eIF2α. Expression of recombinant eIF2a wt and mutants was carried out using a recombinant baculovirus as described earlier (3, *6)*.

Assays for Apoptosis. A total of 4 x 10^6 cells were taken for each experiment and scored for apoptosis by monitoring plasma membrane blebbing. A small aliquot, 45 *u*L of cell suspension, was stained with 0.04% trypan blue and viewed under an inverted microscope (Labovert) equipped with a digital camera and the software MV500 DEMO to score (20× magnification) the apoptosed cells. Caspase activity of the cells undergoing apoptosis was determined using Ac-DEVD-AFC hydrolysis in the cell extracts as described (39). Approximately, 400 *μ*g of extract protein was taken in 50 *uL* of extract buffer and was diluted to 750 *uL* with 20 mM Tris-HCl buffer (pH 7.2) containing 1 mM Mg^{2+}, 80 mM KC1, and 1 mM DTT for each reaction. Ac-DEVD-AFC hydrolysis was monitored by fluorescence emission of the released AFC (excitation, 400 nm; emission. 500 nm) using a Fluoromax-3, Jobin Yvon, Horiba spectrofluorometer as described earlier *(39)*. The final concentration of Ac-DEVD-AFC used in the reactions was 10.9 *μ*M. Phosphorylation of eIF2α in cell extracts containing 25 *μ*g of protein was monitored using a phosphospecific anti-eIF2a antibody. Blots were scanned at a resolution of 200 dpi by using a Hewlett-Packard Scanjet 3400C. Band intensities were quantified using the Quantity Image Analysis software using a Bio-Rad Model GS-800 calibrated imaging densitometer.

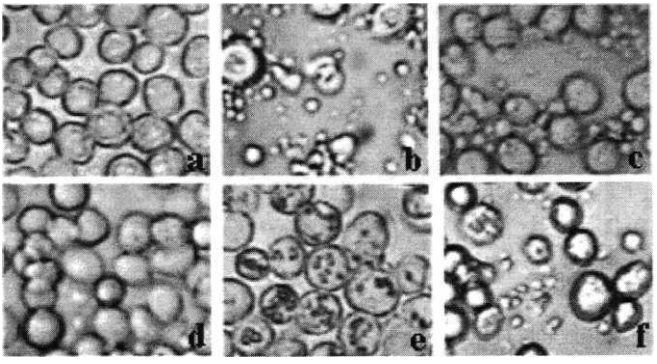

FIGURE 1: Apoptosis in *Sf*9 cells. Cells were treated with the UVB light for 1 min as described earlier *(32)* and incubated for 15 h or with different agents for 15 h at 27 °C. Apoptosis was scored by microscopic observation as described under Materials and Methods. Magnification: 20 x. Panels: a, uninfected cells; b, UVB-treated cells; c, etoposide-treated (125 μM) cells; d, tunicamycin-treated (20 μM) cells; e, wt AcNPV-infected cells treated with UVB radiation; f, p35 mutant virus-infected cells.

PARP Cleavage. In addition to monitoring the cell morphology and caspase activity of the cells undergoing apoptosis, the relative induction of apoptosis caused by various treatments was also studied by the cleavage of pure bovine poly(ADP-ribose) polymerase protein (PARP). Pure bovine PARP protein (150 ng) was incubated with the 5/9 extracts (containing 60 μg of protein) at 30 °C for 90 min in a 40 μL reaction mixture consisting of 10 mM Tris-HCl, pH 7.5, 2.5 mM KH_2PO_4, 2 mM NaCl, 68 mM sucrose, and 220 mM mannitol. The PARP reactions were processed by the addition of equal volume of 2 x reducing buffer containing 62.5 mM Tris-HCl, pH 6.8, 6 M urea, 10% glycerol, 2% SDS, 5% β-mercaptoethanol, and 0.005% bromophenol blue. The PARP reaction samples were boiled at 65 °C for 15 min, then separated on 10% SDS-PAGE. transferred to a nitrocellulose membrane, and probed with an antibody (anti-rabbit antibody) that recognizes the 89 kDa cleaved fragment of PARP.

RESULTS

Pro- and Nonapoptotic Stresses. To elicit apoptosis. uninfected and baculovirus-infected *Sf*9 cells were treated with DNA damaging agents such as UVB light, etoposide (DNA damaging agent), cycloheximide (a translational elongation inhibitor), EGTA (a calcium chelator), calcium ionophore, and tunicamycin (a N-linked glycosylation inhibitor) which are known to promote apoptosis in many mammalian systems. Cells were assayed for apoptosis by the trypan blue exclusion test, formation of apoptotic bodies, and caspase activation. Live and apoptotic cells excluded trypan blue. Apoptosis was assessed then by counting the live cells under the microscope that are distinctly larger than apoptotic bodies under higher magnification. The morphology of the cells undergoing apoptosis in the presence of UVB and etoposide is shown in Figure 1b,c. UVB treatment produced a maximum of 80−85% apoptotic bodies compared to the untreated controls (Figure 1b vs Figure 1a). A short exposure of UVB light for 30 s also resulted in 50-60% apoptosis (data not shown). We then compared the percent of apoptosis that occurred in the presence of different concentrations of various other agents such as etoposide (20 and 125 μM), cycloheximide (0.02, 0.5, and 3.0 mM), EGTA (10 and 50 mM), calcium ionophore A23187 (20 and 100 μM), and tunicamycin (4 and 20 μm). Of these agents, UVB was the most potent inducer of apoptosis (80—85%) followed by high concentrations of EGTA, etoposide, and cycloheximide (60—70% apoptosis). In contrast, high concentrations of tunicamycin (Figure 1d) and the calcium ionophore A23187 (data not shown), which were known to induce apoptosis in mammalian systems, produced very little or mild (5−10%) apoptosis. To monitor the effect of viral infection on the ability of these agents to induce apoptosis, *Sf*9 cells were infected with baculovirus and then treated with some of these apoptotic agents. Baculovirus-infected cells can be recognized from uninfected *Sf*9 cells by the presence of dark opaque polyhedral inclusion bodies under a light microscope. Wild-type virus-infected cells showed little or no apoptosis even after the treatment with proapototic agents such as UVB (Figure 1e). In contrast, a mutant virus that had a deletion of its p35 antiapoptotic gene promoted apoptosis (Figure 1f). Incubation of uninfected cells with the proapototic agents along with cell-permeable z-VAD-frnk, a caspase-3 inhibitor, resulted in almost total loss of apoptosis in these cells, and the morphology of these cells resembled that of the control 5/9 cells (data not shown). These results thus indicate that not all stresses would lead to apoptosis. While UVB light, etoposide, cycloheximide (high concentrations), and EGTA were found to be proapoptotic agents, the calcium ionophore A23187 and tunicamycin were nonapoptotic. 5/9 cells treated with baculovirus resist apoptosis presumably because of the expression of p35 protein, which is well-known as a caspase inhibitor.

Proapoptotic Agents Stimulate Caspase Activity. Since caspase activation is a characteristic event of cells undergoing apoptosis, the action of the above proapoptotic agents was monitored in terms of caspase activity in *Sf*9 cells. The activity was measured in extracts of apoptotic cells by monitoring the hydrolysis of Ac-DEVD-AFC, a substrate specific for mammalian caspase-3 (Figure 2A). Little or no caspase activity was detected in uninfected control cell

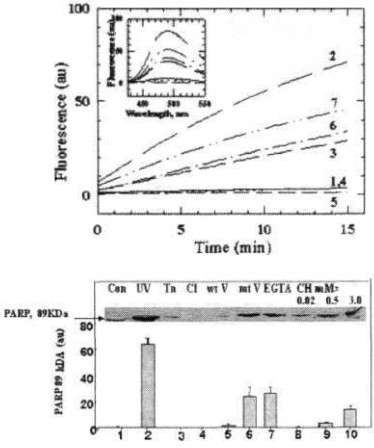

FIGURE 2: (A) Caspase activity in *Sf*9 cell extracts. Cells were treated with various agents, and the caspase activity of the extracts was measured by using Ac-DEVD-AFC as described under Materials and Methods. Inset: Fluorescence spectrum recorded 20 min after addition of Ac-DEVD-AFC to the extracts. Curves 1−7 represent caspase activity in cells treated with the following agents: 1, control; 2, UVB; 3, 125 μM etoposide; 4, 20 μM cycloheximide; 5, 500 μM cycloheximide; 6, 3.0 mM cyclohexamide; 7, 50 mM EGTA. (B) PARP cleavage activity of *Sf*9 cell extracts. The extracts were prepared from cells treated with various agents as follows. In the case of virus infection, *Sf*9 cells were infected with wt baculovirus or p35 deletion mutant virus for 48 h before the extracts were made. In the case of UV treatment, cells were treated for 60 min with UVB light and then incubated for 15 h at 26 °C. All other treatments were carried out at 26 °C for 15 h. Cell extracts (~60 μg of protein) were then incubated with ~150 ng of purified bovine PARP at 30 °C for 90 min in the cleavage buffer described under Materials and Methods. The reactions were terminated and separated on 10% SDS-PAGE, transferred to a nitrocellulose membrane, and probed with an anti-PARP p85 fragment antibody. The figure is a western blot. The lanes are labeled as follows: 1, control; 2, UVB; 3, tunicamycin, 20 μM; 4, A23187, 100 μM; 5, wt AcNPV infection; 6, p35 deletion mutant AcNPV infection; 7, EGTA, 50 mM; 8, cycloheximide, 20 μM; 9, cycloheximide, 500 μM; 10, cycloheximide, 3 mM. The bar diagram below the blot represents average values of two independent experiments.

extracts (curve 1). Significant caspase activity was detected in uninfected cells treated with UVB light (curve 2), 50 mM EGTA (curve 7), 3 mM cycloheximide (curve 6), and 125 μM etoposide (curve 3). Low concentrations of cycloheximide (20 and 500 μM), however, did not activate caspase (curves 4 and 5). High concentrations (1.0—3.0 mM) of cycloheximide stimulated apoptosis and caspase activity. A marginal increase in caspase activity was observed in the presence of low and high concentrations of tunicamycin (4 and 20 μM) and calcium ionophore (20 and 100 μM) and is consistent with their inability to induce apoptosis (data not shown). Overall, caspase activity was found to be directly related to the level of apoptosis (Figure 2A).

PARP Cleavage. In addition to measuring the caspase activity by using Ac-DEVD-AFC hydrolysis, the relative levels of apoptosis observed under the microscope were further determined by studying PARP cleavage. Many earlier studies have shown that PARP is selectively cleaved by several caspases, especially by caspase-3. Caspase-3 cleaves the 113 kDa PARP at the DEVD site between Asp214 and Gly215 to generate 89 and 24 kDa polypeptides. The cleavage of PARP here was measured by monitoring the appearance of the 89 kDa fragment of PARP that was recognized by an antibody (Figure 2B). The PARP cleavage was found to correlate the levels of apoptosis and caspase activation induced by various above agents (Figure 2A).

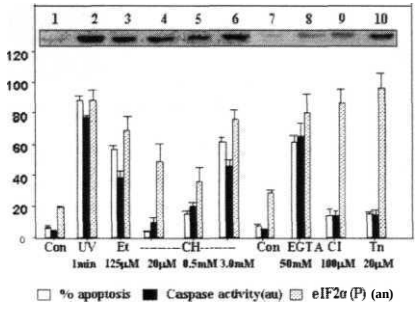

FIGURE 3: Status of eIF2a phosphorylation, caspase activity, and apoptosis in uninfected *Sf*9 cells as a function of treatment with different agents. Labels 1 and 7 represent two controls and are used to obtain relative changes in various activities as depicted in the bar diagram below for various treatments represented in labels 2—6 and 8−10, respectively. The bar diagram represents average values of three independent experiments. The treatments are as follows: 2, UVB for 60 s; 3, etoposide (Et), 125 μM; 4, cycloheximide (CH), 20 μM; 5, CH, 500 μM; 6, CH, 3 mM; 8, EGTA, 50 mM; 9, calcium ionophore (CI), 100 μM; 10, tunicamycin (Tn), 20 μM. The top insert represents the status of eIF2a phosphorylation as a function of different treatments. The western blot was scanned with a densitometer, and values were plotted in the form of a bar diagram depicting the various changes. The different bars show the extent of changes in percent or arbitrary units for percent apoptosis (open bars), caspase activity (filled bars), and eIF2α phosphorylation (dotted bars).

Pro- and Nonapoptotic Stresses Stimulate eIF2α Phosphorylation in Sf9 Cells. Phosphorylation of eIF2a is a stress signal. All of the agents used here are known to stimulate eIF2a phosphorylation in cultured mammalian cells. Further, eIF2a phosphorylation has been shown to mediate apoptosis in mammalian cells (*11*). However, 5/9 cells, despite their suitability as good model systems of apoptosis. have not so far been explored to determine the phosphorylation status of eIF2 or for that matter phosphorylation of any other protein in apoptotic and nonapoptotic stress conditions. The phosphorylation status of eIF2a as a function of various treatments was determined here qualitatively and quantitatively using phosphospecific anti-eIF2a antibody and compared with the extent of apoptosis and caspase activity for the respective treatments (Figure 3 bar diagram). The phosphospecific antibody was earlier shown to specifically recognize the phosphorylated form of eIF2a not only in 5/9 cell extracts but also from plant and mammalian cell types (5, *40*). eIF2a phosphorylation was enhanced significantly in uninfected *Sf*9 cells in response to all of the agents tested (Figure 3) and is thus consistent with the notion that it is an indicator of stress. UVB treatment and higher concentration of tunicamycin (20 μM) caused a maximum increase in eIF2a phosphorylation (lanes 2 and 10). This was followed by calcium ionophore (100 μM), EGTA (50 mM), cylco-

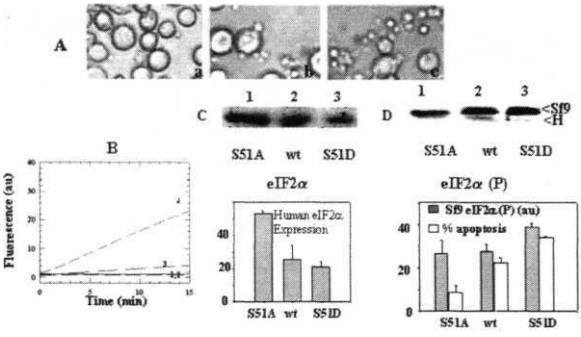

FIGURE 4: Apoptosis in UVB-treated *Sf*9 cells expressing recombinant eIF2α wt and/or mutant proteins. Morphology of cells (panel A) treated first with UVB light for 60 s and then transfected with recombinant virus harboring human S51A (a), wt eIF2α (b), S51D (c) forms of eIF2α. Cells were analyzed after 48 h to estimate caspase activity (panel B), eIF2a expression (panel C), and phosphorylation (panel D). For caspase activity of *Sf*9 cells (panel B) the curves are labeled as follows: 1, wt AcNPV; 2, UVB and recombinant virus harboring S51A eIF2a; 3, UVB and recombinant virus with wt eIF2a; 4, UVB and recombinant virus with S51D eIF2a. Panels C and D represent eIF2α expression and phosphorylation in UVB-treated 5/9 cells using a polyclonal anti-eIF2α antibody and a phosphospecific anti-eIF2a antibody, respectively, as described under Materials and Methods. Lane labels are as follows: 1, cells expressing S51A mutant human eIF2a; 2, cells expressing wt eIF2α; 3, ceils expressing S51D eIF2α. The bar diagrams below panels C and D represent average values of two independent experiments.

heximide (3 mM), and etoposide (125 μM) (lanes 9, 8, 6, and 3, respectively). Both calcium ionophore and tunicamycin stimulated eIF2a phosphorylation (lanes 9 and 10) but failed to induce significant apoptosis. Similarly, low concentrations of cycloheximide (20 μM) caused a substantial increase in eIF2a phosphorylation (lane 4) without inducing any apoptosis. An increase in the concentration of cycloheximide from 20 μM to 0.5 mM, however, decreased eIF2a phosphorylation (lane 5). A further increase in the concentration of cycloheximide from 0.5 to 3.0 mM not only enhanced eIF2a phosphorylation (lane 6) but also induced apoptosis. A decrease in eIF2a phosphorylation between 0.02 and 0.5 mM cycloheximide may be due to the induction of a GAAD34-like protein that activates a protein phosphatase, PP1C (*41*). These results suggest that the type and magnitude of the stress play a role in eliciting apoptosis. Further, from a first glance of the data it appears as if there is no correlation between increased eIF2a phosphorylation and apoptosis. However, a close analysis of the results showed clear correlation among the three parameters that we have considered i.e., caspase activation, increased eIF2a phosphorylation, and apoptosis by various apoptotic agents. In contrast, the nonapoptotic agents stimulated eIF2a phosphorylation without apoptosis (Figure 3 bar diagram). These findings suggest that the mechanism of induction of eIF2a phosphorylation in apoptotic and nonapoptotic conditions is different.

eIF2α Phosphorylation Mediates Apoptosis in Sf9 Cells. The importance of eIF2a phosphorylation in apoptosis and in nonapoptotic conditions or during translational inhibition caused by diverse conditions/agents in mammalian systems was analyzed previously by overexpressing a nonphosphorylatable form of eIF2a such as S51A (the serine 51 residue is replaced by alanine) or a phosphomimetic form of eIF2α, S51D (serine residue in position 51 is replaced by aspartic acid). While expression of S51A decreased both translational inhibition in heat-shocked mammalian cells (*42*) and apoptosis (*11*), S51D was found to stimulate these processes. To determine the influence of eIF2a phosphorylation on apoptosis in *Sf*9 cells, we expressed human wt and the S51A and S51D mutants of eIF2a using recombinant baculoviruses as described earlier (*3*). Overexpression of the recombinant human phosphomimetic form of eIF2a (S51D) did not promote apoptosis in 5/9 cells in the absence of UVB exposure (data not shown). Also, *Sf*9 cells infected with wt virus or recombinant S51D virus were unable to undergo apoptosis in response to UVB irradiation (data not shown). This is likely a reflection of the expression of antiapoptotic viral p35 protein that occurs during the early stages of viral infection. Consistent with this idea, apoptosis was found to be induced significantly in 5/9 cells that were exposed first to UVB radiation and then transfected with recombinant virus harboring wt human eIF2α or S51D (Figure 4A, panels b and c). The S51D is a phosphomimetic form of eIF2a and therefore induced at least 30—35% higher apoptosis than the wild-type eIF2a (Figure 4A, panel b vs panel c). However, infection of *Sf*9 cells with S51A vims carrying the nonphosphorylatable form of eIF2a resulted in a significant decrease in the UVB-induced apoptosis (Figure 4A, panel a). Caspase activity of these cells was related to apoptosis under those conditions (Figure 4B). No caspase activity was detected in cells pretreated with UVB and infected with wild-type baculovirus, AcNPV (Figure 4B, curve 1), as was the case with the recombinant virus harboring the S51A eIF2a (curve 2). In contrast, cells expressing the phosphomimetic S51D form of eIF2a showed a higher caspase activity compared to those carrying wild-type eIF2a (curves 4 and 3). The cell extracts were also analyzed to determine the expression of human eIF2a protein (Figure 4C) and also the phosphorylation status of the endogenous 5/9 eIF2α and of the recombinant wt and mutant human eIF2α (Figure 4D). The expression of recombinant eIF2α wt and mutant proteins was detected by a polyclonal anti-eIF2α antibody that recognizes the human protein but not the endogenous *Sf*9 protein (Figure *AC).* Cells infected with the S51D mutant virus showed relatively lesser expression of the eIF2a protein than the

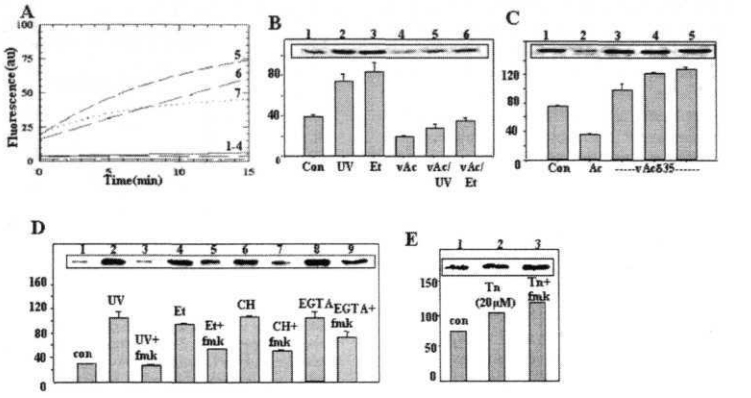

FIGURE 5: (A) Caspase activity in wt and p35 mutant baculovirus-infected extracts. Ac-DEVD-AFC hydrolysis in the extracts was measured as described in the legend to Figure 2 under the following conditions: 1, uninfected cells; 2, wt AcNPV-infected cells; 3, wt AcNPV-infected UVB-treated (1 min) cells; 4, wt AcNPV-infected etoposide-treated (125 μM) cells; 5, UVB-treated uninfected cells; 6, etoposide-treated uninfected cells; 7, p35 mutant virus-infected cells. Panels B-E represent eIF2a phosphorylation in extracts prepared from cells treated with various agents described below. Corresponding bar diagrams represent changes in phosphorylation in arbitrary units (au). The bar diagrams represent average values of two independent experiments. Panel B: lane 1, uninfected; lane 2, UVB-treated (60 s); lane 3, etoposide-treated (Et, 125 μM); lane 4, wt AcNPV-infected (vAc); lane 5, vAc-infected and UV; lane 6. vAc-infected and etoposide-treated. Panel C: lane 1, uninfected; lane 2, wt AcNPV-infected; lanes 3-5, p35 deletion mutant virus (vAcδ35) infected cell extracts with increasing virus titer. Panel D: lane 1, uninfected control; lane 2, UV-treated; lane 3, UVB + 50 μM z-VAD-fmk; lane 4, 125 μM etoposide; lane 5, 125 μM etoposide + 50 μM z-VAD-fmk; lane 6, 3 mM cycloheximide (CH); lane 7, 3 mM cycloheximide + 50 μM z-VAD-fmk; lane 8, 50 mM EGTA; lane 9, 50 mM EGTA-treated + 50 μM z-VAD-fmk. Panel E: lane 1, uninfected control; lane 2, 20 μM tunicamycin (Tn); lane 3, 20 μM tunicamycin + 50 μM z-VAD-fmk.

S51A mutant virus (lane 3 vs lanes 1 and 2). We observed this earlier also (J), and it is a reflection of the toxic nature of the phosphomimetic form of S51D. A duplicate blot was probed with the phosphospecific antibody that recognizes both the human and 5/9 proteins but only their phophorylated forms (Figure 4D). Two bands were detected by the phosphospecific anti-eIF2a antibody; the one with reduced mobility corresponds to *Sf*9 eIF2a, and the other one with increased mobility corresponds to the recombinant human eIF2a (Figure 4D). Apoptosis induced by UVB was high in cells expressing the S51D eIF2a mutant followed by the wt eIF2a. This was also reflected in the phosphorylation status of endogenous eIF2a (Figure 4D, lanes 2 vs 3, 5/9 arrowhead). The cells expressing the S51A mutant of eIF2a showed the least apoptosis with correspondingly reduced 5/9 eIF2a phophorylation (Figure 4D, lane 1). The phosphorylation of recombinant human wt eIF2a was evident in insect cells but not in the S51A and S51D mutants (Figure 4D, lower band, H arrowhead). These results suggest that phosphorylation of eIF2a that occurs during apoptosis as a function of UVB exposure is likely due to caspase activation.

p35 Deletion Mutant Baculovirus Stimulates eIF2α Phosphorylation and Promotes Apoptosis in Sf9 Cells. Baculovirus infection of insect cells has been shown to decrease caspase activity and apoptosis *(33)*. In accordance with earlier reports, caspase activity was barely detected, if at all, in 5/9 cells infected with the AcNPV or in vims-infected cells treated with UVB and etoposide (Figure 5A). The wild-type virus that is required for a productive infection causes a reduction in eIF2a phosphorylation in 5/9 cells as described by us earlier (*3*). We further observed that wt virus-infected cells resist apoptosis in the presence of proapoptotic agents. Increase in eIF2α phosphorylation as a function of apoptosis was observed more significantly in UVB- and etoposide - (125 *uM)* treated uninfected cells than in wild-type virus-infected cells (Figure 5B, lanes 2 and 3 vs lanes 5 and 6, respectively). To determine the importance of caspase involvement in stimulating eIF2a phosphorylation in cells undergoing apoptosis, we studied the effect of virus-encoded p35 gene expression on eIF2α phosphorylation. This was carried out by infecting *Sf*9 cells with a deletion mutant p35 virus. Deletion mutant virus infection readily resulted in apoptosis (Figure 1f), caspase activity (Figure 5A, curve 7). and enhanced eIF2a phosphorylation (Figure 5C, lanes 3—5).

Inhibition of Caspase Activity Mitigates eIF2α Phosphorylation. Complementing the above results, eIF2a phosphorylation and apoptosis (data not shown) were reduced in *Sf*9 cells treated with z-VAD-fmk and then exposed to UVB (1 min), etoposide (125 μM), cycloheximide (3.0 mM), and EGTA (50 mM) (Figure 5D. lanes 2, 4, 6, and 8 without inhibitor vs lanes 3,5,7, and 9 with inhibitor). Interestingly, tunicamycin-induced eIF2a phosphorylation was not affected in the presence of z-VAD-fmk (Figure 5E, lanes 2 and 3 vs lane 1). This finding complements the observation on the inability of tunicamycin to stimulate caspase activity and apoptosis (Figure 3 bar diagram) and suggests that tunicamycin-induced eIF2α phosphorylation is different from the increased eIF2a phosphorylation caused by the addition of apoptolic agents in 5/9 cells. These results therefore suggest that increased eIF2a phosphorylation in apoptosis is a consequence of increased caspase activity.

Caspase Activation Occurs Prior to Enhanced eIF2α Phosphorylation in UV-Induced Apoptosis. The kinetics of caspase activation and eIF2a phosphorylation in UV-induced

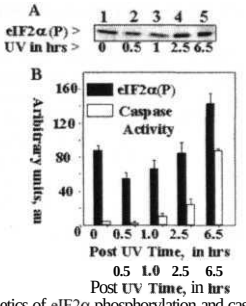

FIGURE 6: Kinetics of eIF2α phosphorylation and caspase activation in UV-induced apoptosis in 5/9 cells. Panel A represents eIF2a phosphorylation as judged by a phosphospecific antibody at different time periods of UV-treated 5/9 cells. The figure is a western blot. Lane labels are as follows: 1, control; 2-5, cell extracts prepared after 0.5, 1. 2.5, and 6.5 h of UVB irradiation. Panel B represents a bar diagram of an average of three independent experiments showing the relative densities of phosphorylated eIF2a levels and caspase activity of UV-induced apoptotic cells at different time periods. Filled and open bars represent phosphorylation and caspase activity, respectively.

apoptotic cells indicate that caspase activation occurs around 1.0 h after UV treatment (Figure 6B bar diagram, bar 3 vs bar 1). The phoshorylation of eIF2a was found to be decreased initially before the onset of caspase activation. However, the phosphorylation of eIF2a was found to be increased 2.5 h after UV treatment (Figure 6A. lanes 4 and 5 vs lane 1 and the corresponding bar diagram). The maximum increase in eIF2a phosphorylation was observed 15 h after treatment (as shown in Figures 3 and 5B,D) while the maximum caspase activation occurs around 6-8 h (Figure 6B bar diagram). These results together with the earlier results in Figure 5 indicate that caspase activation occurs prior to increased eIF2α phosphorylation and is a prerequisite for the increased eIF2a phosphorylation observed in cells that are undergoing apoptosis.

DISCUSSION

A major observation of this study is that uninfected, but not the wt AcNPV-infected, 5/9 cells are very sensitive and respond to different signals by altering the phosphorylation status of cellular eIF2a. We demonstrate that, in addition to UVB light and etoposide, higher concentrations of cycloheximide and EGTA promote apoptosis (Figures 1 and 3) and stimulate the caspase activity (Figure 2) and also eIF2a phosphorylation in uninfected *Sf*9 cells (Figure 3). In contrast, tunicamycin, A23187, and low concentrations of cycloheximide fail to activate caspase and consequent apoptosis (Figures 1-3). Nevertheless, these agents do stimulate eIF2a phosphorylation in 5/9 cells (Figure 3), suggesting that the stress signaling pathway is important in the induction of apoptosis. In addition, we have demonstrated that p35 mutant virus promotes apoptosis readily and stimulates eIF2a phosphorylation in Sf9 cells.

The mechanism of activation of caspases in 5/9 cells vis-a-vis the role of p35 in inhibiting the maturation of caspase-(s) is not well understood. It has been proposed that an unidentified apical caspase cleaves the pro-*Sf*-caspase-1 to p25 and p12, and subsequently the p25 caspase product gives rise to p19 *(34)*. While cellular IAPs block the activation of an apical unidentified caspase and inhibit the cleavage of Pro-*Sf*-caspase-1 to p25 and p12 products, baculovirus p35 blocks the cleavage of p25 caspase to p19, thereby inhibiting the maturation of caspase(s). This is further substantiated by the fact that apoptosis in insect cells, induced by UVB radiation and p35 deletion virus infection, is prevented by pro-*Sf*-caspase-1 inhibitors *(34)*. We show here that p35 viral protein not only inhibits the formation of an active caspase and consequent apoptosis but also mitigates eIF2a phosphorylation in *Sf*9 cells (Figure 5A,C).

The importance of baculovirus p35-mediated caspase inhibition leading to diminished eIF2a phosphorylation is substantiated further by the observation that wt virus-infected 5/9 cells are unable to undergo apoptosis in response to UVB irradiation (Figure 1) or through the overexpression of the phosphomimetic form of eIF2a (data not shown). On the contrary, apoptosis is stimulated readily in 5/9 cells that are exposed first to UVB light and then transfected with the recombinant baculovirus carrying the phosphomimetic form of eIF2a than with wt or the nonphosphorylatable form of eIF2a (Figure 4). These observations in 5/9 cells indicate that eIF2a phosphorylation alone does not stimulate apoptosis or caspase activity, but it can enhance the apoptotic effect of caspase, suggesting thereby that eIF2a phosphorylation is necessary but not a sufficient condition for the induction of apoptosis. The importance of increased eIF2α phosphorylation as a function of increased caspase activity was further reinforced by using a cell-permeable inhibitor, z-VAD-fmk. The inhibitor decreases eIF2α phosphorylation in cells exposed to proapoptotic agents such as UVB and etoposide and high concentrations of cycloheximide and EGTA (Figure 5D). In contrast, tunicamycin-induced eIF2a phosphorylation was not affected by the presence of the caspase inhibitor (Figure 5E), which is found to be consistent with its inability to stimulate caspase activity or apoptosis. These findings also suggest that caspase activation is a prerequisite for increased eIF2α phosphorylation in apoptotic cells. Consistent with these observations, an analysis of the kinetics of eIF2a phosphorylation and caspase activation in UV-induced apoptotic cells indicates that caspase activation occurs prior to increased eIF2α phosphorylation (Figure 6). In addition, we have also observed that recombinant eIF2a kinases, such as mammalian PKR and PERK, are cleaved in vitro by cell-free extracts prepared from apoptosed 5/9 cells and by purified caspase-3 (data not shown). Furthermore, caspase-3-treated PERK, when compared to caspase or PERK alone, can stimulate eIF2α phosphorylation in extracts prepared from healthy insect cells (data not shown). The mechanism of eIF2a phosphorylation observed in cells undergoing apoptosis thus appears to be different from the increased eIF2a phosphorylation that occurs in response to various nonapoptotic stress conditions. These interpretations also find support from a recent study that demonstrated caspase-dependent PKR activation and eIF2a phosphorylation *(26)*. This notion that eIF2a phosphorylation can be stimulated by an active eIF2a kinase or active caspase draws further support from the viral proteins which can inhibit eIF2α phosphorylation either by inactivation of the eIF2α kinase *(46)* or by inhibition of maturation of caspases as has been shown here. Caspases are also known to process and enzymatically activate other kinases that include MEK

kinase-1 (MEKK-1), protein kinase C-d, (PKC-δ), and p21 activated kinase-2 (PAK2) *(45)*.

Interestingly, many viruses are known to produce proteins that interfere with PKR-mediated activation and inhibit host cell eIF2a phosphorylation *(46)*. A recent study has shown that baculovirus produces PK2 protein that resembles the C-terminal half of a protein kinase domain and is found to inhibit activation of GCN2, PKR, and HRI eIF2α kinases in vitro *(47)*. It is not known, however, if PK2 interferes with the host apoptosis. Our findings here suggest that baculovirus-coded p35, an antiapoptotic or a caspase inhibitor protein, interferes with the activation of host cell eIF2a kinase, albeit indirectly through the inactivation of a host cell caspase. Despite suggestions by Blair et al. *(48)* that *Sf*9 cells contain a protein that is antigenically related to PKR, PKR-encoding sequences have not been recognized in any invertebrate for which the complete genome sequence is available. Also, the eIF2α kinases present in 5/9 insect cells have not been characterized, although GCN2 and PERK have been characterized in *Drosophila (43, 44)*. Since agents such as UVB light, cycloheximide, EGTA, A23187, tunicamycin, and DNA damaging agents are known to stimulate unfolded protein response (UPR) or stress in the endoplasmic reticulum (ER) (*49*−*52*),it is likely that 5/9 cells have an ER-resident eIF2a kinase-like PERK which is stimulated in response to all of these agents as evidenced by increased eIF2a phosphorylation. The activation of PERK or other eIF2a kinases by an active caspase is obviously essential to promote apoptosis.

Phosphorylation of eIF2a is known to inhibit translation of several mRNAs leading to a decline in protein synthesis globally (53). Paradoxically, the translation of transcriptional factors such as GCN4 (general control nonderepressible) in yeast and ATF4 (activated transcription factor) in mammalian systems is increased in response to eIF2a phosphorylation (reviewed in refs *54* and *57*). Although tunicamycin and the calcium ionophore are known to cause ER stress and promote apoptosis in mammalian systems, these agents did not induce apoptosis here in *Sf*9 cells. This may be related to the type and magnitude of the stress, the physiological response of the cells toward each of these stress conditions, and also the cell type *(50)*. Further, most of the work on ER stress signaling is carried out in mammalian systems, and very little is known in insect cells. In mammalian systems, cellular stress activates intracellular signaling pathways that affect a sizable number of transcriptional factors leading to alterations in the gene expression. ER is a repository for both pro- and antiapoptotic molecules. The activation of transcriptional factors such as ATF4, GCN4, and NF-kB that occurs in response to ER stress, in turn, would stimulate the expression of several proapoptotic (CHOP/GADD-153) and prosurvival proteins including GADD 34, Bip/GRP78, calreticulin, protein disulfide isomerase, NF-kB, etc. *(51, 57)*. Posttranslational modifications of CHOP can lead to the transcriptional activation of novel stress-induced genes called DOC 6 and DOC 4 (downstream of CHOP) that share homology with the mammalian actin binding proteins such as villin and gelsolin or a mammalian orthologue of a *Drosophila* gene, *Tenm/Odz*, respectively. While Doc 6 is implicated in cell death, Doc 4 is important in cellular regeneration *(56)*. Prolonged ER stress leads to cell death. Activation of ER-resident caspase-12 occurs by different mechanisms such as the caspase-7/caspase-12 pathway and caspase 12/calpain pathway *(58)*. A number of stimuli that disrupt protein folding such as tunicamycin and the calcium ionophore can activate both unfolded protein response (UPR) and ER overload response (EOR). These two signaling pathways respond to different and overlapping types of ER stresses, and their common feature for their induction is not known *(51)*. While UPR induces transcription of proapoptotic genes such as CHOP, EOR induces antiapoptotic genes such as NF-kB (57). A recent study further describes that NF-κB, an antiapoptotic protein, inhibits activation of GADD 153/CHOP in breast cancer cells exposed to tunicamycin or the calcium ionophore (50). It is likely that such paradoxical interactions of anti- and proapoptotic transcription factors in *Sf*9 cells may be responsible for the specific differences in stress-induced apoptosis. The microdissection of the signaling events leading to stress-induced apoptosis in insect cells and the players involved in this process remain to be elucidated. Nonetheless, the degree of conservation in these processes between the vertebrates and the invertebrate systems renders 5/9 insect cells an indispensable model system to study apoptosis.

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ERRATA SUBMITTED FOR THE ERRORS IN THE THESIS

Page No.	Paragraph	Line	Error	Correction
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1	third	last	appear	appears
3	second	eighth	Pestovao	Pestova
9	first	fifth	adenosine	adeno
11	first	seventeenth	infact	in fact
12	first	eighth	on set	onset
12	first	eleventh	hyper thyroid	hyperthyroid
13	3.3	third	with out	without
13	3.3	last	extra cellular	extracellular
13	3.3	second	an electron microscope	electron microscope
14	first	third	Actinomycin	Actin-myosin
16	3.6.1	fourth	These proteases are subsequently named	These proteases have subsequently been named
18	first	sixth	Yuan and Gores	Yoon and Gores
22	first	tenth	procaspase 2	procaspase 2 is
26	3.8.3	fifteenth	looses	loses
28	3.8.4	second	CLL	Chronic lymphoblastic leukemia (CLL)
29	first	first	localized	are localized
35	First	fifth	our lab	our laboratory
40	1.12	first	it's necessary	It is necessary
42	1.13	second	and was overexpressed	was overexpressed
42	1.13	third	Molecular medicine	Molecular Medicine
42	1.13	eleventh	triton	Triton
42	1.13	tweleth	A ptoteinin	Aprotinin
42	1.13	fifteenth	Glutathione sepharose	Glutathione Sepharose
42	1.13	sixteenth	flow threw	flow through
43	1.13	first	70	-70
43	1.13	first	on to the 10%	on to a 10%
43	1.15	third	(missing)	1mM DTT
44	1.19	fifth	Mci	u Ci
44	1.19	seventh	Whatman	Whatman
45	1.20	second	kept -70	kept at -70

46	first	sixth	4 μg/μl at	4 ug/ul and
47	third	third	13k	13.000rpm
49	1.24	ninth	glycerol. SDS	glycerol
63	second	fourth	Figure 21 lane 2 and 3	Figure 22 lane 2 and 3
63	third	fourth	PERK(lane 6)	PERK (lane 4)
65	third	fifth	infact	in fact
77	seventh ref		Bulera	Burela
78	seventh ref		J. Biol Chem	J. Biol. Chem.
81	last ref		De la Vega	Del Vega
97	fourth ref		Mortinou	Martinou

Clarifications regarding some sentences:

Page No 7 line 17: The globin proteins and its precursors are found aggregated within red blood cells, resulting in hyperchromic, normocytic anemia with decreased RBC counts, erythroid hyperplasia and accelerated apoptosis. This condition is observed in spleen and bone marrow of mice lacking both copies of HRI genes and in patients suffering from iron deficiency (Han et al., 2001).

Objectives: The present study has been undertaken a) to determine the activity of various known apoptotic agents to stimulate apoptosis, and/or eIF2a phosphorylation, in uninfected and baculovirus-infected *Sf9* cells and b) to understand the importance of caspase activation on eIF2α phosphorylation.

Figure legends:

Figure	Line/panel	Error	Correction
Figure 1e	Line 3	caspae	caspase
		DIABLO function turn	DIABLO function inturn
		Bcl2 which inhibit	Bcl2 which inhibits
Figure 2	Panel A Panel B	20 mM Tris-HCl 80 mM Tris	50 mM Tris-HCl 20 mM Tris and 80 mM KCl
Figure 3	Panel C	eIF2a phosphorylation	eIF2a
Figure 14	PanelA	a, b, and c	1, 2 and 3
Figure 23	Panel B, lane 7		Control extract + caspase + PERK

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Correction of authors cited in **the text:**

Page No.	Error	Correction
14	Fesus, 1987	Fesus et al., 1987
15	Rushak	Rusnak
15	Catchpole	Catchpoole
17	Hu et al., 2000	Hu, S. and Yang, X. 2000
18	Pinkiski et al., 2000	Pinkiski, M. J. and Green, D. R. 2000
19	Beg et al., 1996	Beg, S. S. and Baltimore, D. 1996
21	Nicholson et al., 1999	Nicholson, 1999
22	Kumar, 1999	included in the above section
24	Weidman	Weidemann
25	Bae et al., 2000	Bae, S. S. et al.,2001
25	Xue and et al.. 1995	Xue. D. and Horvitz, R. H. 1995
26	Wood et al., 1999	Wood. D. F. and Newcomb, E. W. 1999
26	Heniz-Korenbelt	Henis-Korenbilt
27	Saleans	Saelens
27	Pilar et al., 1976	Pilar. G. and Landmesser, L. 1976
27	Houge et aL, 2000	Houge et al.. 1995
33	Wright et al., 2001	Wright, C. W. and Clem, R. J. 2001
58	Shoshan et al., 1981	Shoshan. V. and MacLennan, D. H. 1981
77	Bulera	Burela
81	De la Vega	Del Vega
97	Mortinou	Martinou

Apoptosis in Uninfected and Baculovirus infected *Sf9* cells: Phosphorylation of alpha subunit of Eukaryotic Initiation Factor 2 (eIF2a)

Thesis submitted for the degree of
Doctor of Philosophy

By

S. Aparna

(RegdNo.98LBPH07)

Department of Biochemistry
School of Life sciences
University of Hyderabad
Hyderabad 500 046, INDIA

March, 2004