RESULTS AND DISCUSSION:

- Stress-Induced changes in morphology of Sf9 cells
- Pro apoptotic agents stimulate caspase 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 phosphorylation in Sf9 extracts in vitro
- Baculovirus infection and apoptosis
- eIF2a phosphorylation 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 phosphorylation
- Kinetics of apoptosis, caspase activation and Sf9 eIF2a phosphorylation in UV-B treated cells
- Cleavage of eIF2a kinases by Sf9 extracts
- Effect of purified caspases on PERK cleavage
- Caspase-cleaved PERK stimulates Sf9 eIF2α phosphorylation in vitro
- Protein synthesis in apoptotic and non-apoptotic conditions
- Discussion
The ovarian cell lines of *Spodoptera frugiperda (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; l, EGTA 50 mM; m, Calcium ionophore (Cl) 100 uM; n, Tunicamycin (Tn) 20 uM; o, Thapsigargin (Tp) 35 uM.
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 5l), etoposide (125 μM), and cycloheximide (3 mM) (60-70% apoptosis). In contrast, tunicamycin (20 μM), A23187 (100 μM) 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 μvl) (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 while 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°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 caspase 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 eIF2a 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 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 (Figures 9 and 10). Phosphospecific antibody has been found to recognize specifically the phosphorylated form of eIF2α that is formed due to the action of eIF2α kinases (Sudhakar et al., 1999; Laxminarayana et al., 2002). eIF2α 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 uM) 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 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, 3.0 mM. The bar diagram below the blot represents average values of two independent experiments.
Figure 9: Phosphorylation of 5/9 eIF2α 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 eIF2α 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 μM.
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 eIF2a 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 eIF2a phosphorylation (Figure 9C, lanes 4, 5 and Figure 10A, lane 7) but also induced apoptosis. A decrease in eIF2a 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, lane 2 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 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 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 eIF2α 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 mM; 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 eIF2α phosphorylation.

Panel 9D: Cells were treated with 100 uM calcium ionophore for 30 min and 15 hrs, extracts were prepared and status of eIF2α 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 eIF2α 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; lane 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.
Figure 10: A quick glance of eIF2α 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 μM etoposide (Et); 4, 125 μM Et; 5, 20 μM cycloheximide (CH); 6, 500 μM CH; 7, 3 mM CH.

Panel 10B: 1, control; 2, 10 mM EGTA; 3, 50 mM EGTA; 4, 20 μM calcium ionophore (CI); 5, 100 μM CI; 6, 4 μM tunicamycin (Tn); 7, 20 μM 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), eIF2α phosphorylation (dotted bars) and % 35S methionine incorporation or protein synthesis (striped bars).
A

Con  UV-B  --Etoposide--  --Cycloheximide--
20  120uM  20  500  3000uM

B

Con  --EGTA--  CI-A23187  Tunicamycin
10  50mM  20  100uM  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 eIF2α 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 eIF2α phosphorylation under such conditions *in vitro* (Figure 11) thereby suggesting that the mechanism of activation of eIF2α 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 eIF2α 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 eIF2α phosphorylation in Sf9 cells (Figure 12C) and this result is consistent with earlier observation from this lab (Sudhakar et al., 2000). Increase in eIF2α 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 eIF2α 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 eIF2α phosphorylation.

Panel A: Morphology of cells
Panel B: caspase activity
Panel C and D: eIF2α 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 eIF2α 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.
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 eIF2α phosphorylation (Figure 13C, lanes 3-5).

eIF2α 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 eIF2α such as S51A (51 serine residue is replaced by alanine) or 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 (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 eIF2α (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 eIF2α (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 eIF2α (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 eIF2α; 4, UV-B and recombinant virus with S51D eIF2α.

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 eIF2α; 2, cells expressing wt eIF2α; 3, cells expressing S51D eIF2α.
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 eIF2α showed a higher caspase activity compared to those carrying wild type eIF2α (curves 4 and 3). The cell extracts were also analyzed to determine the expression of human eIF2α protein (Figure 14C) and also the phosphorylation status of the endogenous Sf9 eIF2α, and, of the recombinant wt and mutant human eIF2α (Figure 14D). Expression of the recombinant eIF2α, both wt and mutant proteins was detected by a polyclonal anti-eIF2α 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-eIF2α antibody; the one with reduced mobility corresponds to Sf9 eIF2α 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 eIF2α showed least apoptosis with correspondingly reduced Sf9 eIF2α phosphorylation (Figure 14D, lane 1). The phosphorylation of recombinant human wt eIF2α 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 eIF2α 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 eIF2α 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 eIF2α to evaluate the importance of eIF2α phosphorylation on apoptosis in the absence of any other agents or baculoviral genes. Sf9 cells transfected with PNN1 plasmids 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 eIF2α (Figure 15A). While the expression of human eIF2α 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 eIF2α than in the presence of S51A or wt eIF2α (Figure 15C, bars 4 vs. 3, 2 and 1 respectively). These findings further endorse that eIF2α 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 eIF2α
Figure 15: Transient expression of human eIF2α wt and mutants in Sf9 cells

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

Panel B: Phosphorylation of Sf9 cellular eIF2α and recombinant human eIF2α in cells expressing recombinant human eIF2α, wt and or mutants. Sf9 cells were transfected with 5 µg of recombinant PNN1 plasmid harbouring human S51A, wt or S51D eIF2α. Cell extracts were prepared and the phosphorylation status of endogenous Sf9 eIF2α and of the expressed recombinant human eIF2α 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 eIF2α in cells expressing human wt eIF2α; lane 3, phosphorylation of eIF2α in cells expressing human S51D eIF2α.

Panel C: Protein synthesis was performed in Sf9 cells transfected with PNN1 plasmid expressing wt and mutants of eIF2α as described in ‘Materials and Methods’. 
**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 cell 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 cells; 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° 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° C.
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 eIF2α 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 μM tunicamycin or with 20 μM tunicamycin and 50 μM z-VAD-fmk, caspase-3 inhibitor.
Figure 18: Morphology, caspase activity and eIF2α phosphorylation in p35 mutant virus (vAc5p35) infected cells in the presence and absence of 50 μM z-VAD-fmk
Panel A: Morphology
Panel B: eIF2α 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°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 eIF2α phosphorylation:** Incubation of uninfected cells 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, eIF2α 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 eIF2α 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 eIF2α phosphorylation caused by the addition of apoptotic agents in Sf9 cells. These results therefore suggest that increased eIF2α 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 eIF2α phosphorylation 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 eIF2α 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 eIF2α phosphorylation and is a prerequisite for the increased eIF2α phosphorylation observed in cells that are undergoing apoptosis (Aparna et al., 2003).

**Cleavage of eIF2α kinases by Sf9 extracts:** Recent studies suggest that many kinases are activated upon cleavage by caspases (Fischer et al., 2003). eIF2α 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 μM 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 eIF2α 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, eIF2α 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 from 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$_2$PO$_4$ 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 cell 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).
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 21A) and also at 30° C (Figure 21B). We observed that like PKR, PERK was cleaved by UV-B (60 sec) 125 uM etoposide and also by 3.0 mM cycloheximide treated extracts at 37° C and 30° 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 uM 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$_2$PO$_4$, 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 immunoblot. 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: 5/9 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.
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 eIF2α 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 eIF2α 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 eIF2α. Addition of caspase alone did not stimulate the 5/9 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 eIF2α (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 eIF2α (lane 3 vs. 1).
**Figure 23:** Phosphorylation of 5/9 eIF2α 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°C 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 Mg²⁺, 80 mM KCl 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 eIF2α in Sf9 extracts by cleaved and uncleaved PERK. The phosphorylation of eIF2α was monitored by a phosphospecific anti-eIF2α 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 extract + 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.
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 eIF2α phosphorylation that occurs in apoptosis may be due to the activation of an endogenous eIF2α kinase by active caspase (s).

Protein synthesis in apoptotic and non apoptotic conditions: Phosphorylation of serine 51 residue in eIF2α inhibits protein synthesis globally and in gene-specific manner (Hinnebusch, 1996). Indeed eIF2α 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 Sf9 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 eIF2α 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 eIF2α 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 $[^{35}\text{S}]$ 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 μM 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 μM 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 μM etoposide, 6. 125 μM etoposide+i, 7. 3.0 mM cycloheximide, 8. 3.0 mM cycloheximide+i, 9. 50 mM EGTA, 10. 50 mM 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 $[^{35}\text{S}]$ 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 $[^{35}\text{S}]$ methionine was carried out for 60 min at the end of 3, 6, 18 and 25 hrs and the cells were washed and 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.
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 onset 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: SJ9 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). In fact, 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
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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 eIF2α phosphorylation in apoptosis. These cells have not been studied so far to analyze the signals that stimulate the phosphorylation of eIF2α or that activate eIF2α kinases. Hence we have analysed the ability of a variety of agents to stimulate eIF2α 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 eIF2α 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 eIF2α phosphorylation in Sf9 cells (Figure 6 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 eIF2α phosphorylation in Sf9 cells thereby suggesting that Sf9 cells contain an eIF2α 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 eIF2α kinase(s) occurs through some intermediates. So far the eIF2α 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 al., 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 p19 (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 eIF2a (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 eIF2a than with wt or the nonphosphorylatable form of eIF2a (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 Sf9 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 eIF2a phosphorylation. Expression of wt PKR or phosphomimetic form of eIF2a in a transient transfection system was found sufficient to promote apoptosis similar
to TNF-a. Our results though agree with the idea that eIF2α 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 eIF2α 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 eIF2α phosphorylation (Sudhakar et al., 1999). Despite suggestions by Blair et al., 1995 that Sf9 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.

Interestingly, many viruses are known to produce proteins that interfere with PKR-mediated activation and inhibit host cell eIF2α 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 eIF2α kinases in vitro. Consistent with this observation, we have always observed that wt baculovirus infected cells displayed low levels of eIF2α 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 eIF2α 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 eIF2α 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 eIF2α phosphorylation (Figures 13, 17 and 18). In contrast, tunicamycin-induced eIF2α 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 eIF2α 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 eIF2α phosphorylation, the findings here suggest that the mechanism of activation of eIF2α kinase(s) or stimulation of eIF2α 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 eIF2α 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 eIF2α 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 eIF2α kinases such as PKR and HRI, it is suggested that flavonoids like genestein promote ER stress (Ito et al., 1999). Thus the increased eIF2α 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 eIF2a phosphorylation (Figure 10 bar diagram). However the reduction in protein synthesis in the presence of various agents does not correlate always to increased eIF2a 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-IETD-fmk reduced apoptosis and eIF2a 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.