Introduction
INTRODUCTION

Most, if not all animal cells have the ability to self-destruct by activation of an intrinsic cell suicide program when they are no longer needed or have become seriously damaged. The execution of this death program is often associated with characteristic morphological and biochemical changes, and this form of cell death has been termed apoptosis or programmed cell death (PCD). Apoptosis is the normal fate of most eukaryotic cells. The term “programmed” in PCD refers to the genetic pathways existing within each which, when stimulated in the proper context, results in cell suicide. Apoptosis is a remarkably conserved mechanism operating in multicellular organisms from nematodes to humans (Herigartner, 2000; Meier et al., 2000). The cell death mechanisms may range from necrosis, a relatively passive outcome of injury, to apoptosis, an active and ordered process. The kind of cell death depends upon the physiological status of the cell and the nature of the stimulus (Hampton and Orrenius, 1997; Fiers et al., 1999).

The process of apoptosis is controlled through the expression of an increasing number of genes conserved throughout evolution from mammals to nematodes, flies and viruses. Some gene products are activators of apoptosis and are proapoptotic in nature whereas others are inhibitors of apoptosis and are antiapoptotic. The characterization of the functions of these gene products will help to define the process of cell death at the biochemical level.

A large number of diverse agents are known to trigger apoptosis in a variety of cell types and organisms. These include: physiologic/genetic agents such as TNF (tumor necrosis factor) family members, neurotransmitters, growth factor withdrawal, calcium and glucocorticoid hormone, oncogenes, caspases, environmental agents like X-ray/gamma/UV/thermal radiation, oxidants, free-radicals, pathologic agents like viruses, bacterial toxins and therapeutic agents etc. (Thompson, 1995).
The baculovirus AcNPV was one of the first viruses known to induce and inhibit apoptosis. The study of baculovirus-induced apoptosis has led to a greater understanding of the genetic pathways of cell death and their similarities between insects and other organisms, as well as provided insight into the role of apoptosis in defence against viral pathogen.

The baculoviral p35 gene was one of the first antiapoptotic genes discovered. Till date, p35 has been expressed in organisms ranging from nematodes to human cells in order to determine whether it could block programmed cell death in diverse situations but till date no mammalian counterpart of the p35 protein has been identified. It has been shown to block developmentally programmed cell death in the nematode, Caenorhabditis elegans (Sugimoto et al., 1994; Xue and Horvitz, 1995) and in Drosophila melanogaster (Hay et al., 1994), as well as apoptotic death in mammalian neural cells (Rabizadeh et al., 1993), sympathetic neurons (Martinou et al., 1995), breast carcinoma and B-lymphocytes (Beidler et al., 1995). The death stimuli utilized in these studies also varied widely. The ability of the p35 gene to rescue cells, programmed to die, has been investigated in a number of test systems against a number of apoptotic stimuli such as growth factor withdrawal, actinomycin D, cycloheximide etc. (McCarthy et al., 1997). The ability of p35 to block many different death stimuli suggests that it acts in the effector pathway, possibly at one of the final execution steps. The ability of p35 to potently inhibit several members of the ICE family of cysteine proteases has been well established (Bertin et al., 1996; Bump et al., 1995; Xue and Horvitz., 1995).

p35 gene despite having no sequence homology with the well known bcl-2 family functions in a similar manner. Like bcl-2, baculovirus p35 gene is also an antiapoptotic gene which has been shown to arrest cell death in many systems including insects, nematodes and mammalian neurons.
Review Of Literature
REVIEW OF LITERATURE

Literally, apoptosis means "falling off" as of leaves from trees. Lockshin in 1965 coined the phrase "programmed cell death" to describe cell death in insect metamorphosis. However, a new term "apoptosis" was coined by Kerr et al., 1972 for "cell death" with a distinct morphology. The role of this evolutionarily conserved process as a cell growth regulator and "watchdog" against disturbances in the maintenance of genomic integrity and tissue homeostasis has been observed only recently and many genes controlling cell death have been identified. Apoptosis is essential for normal development of the tissue, cellular defense against viral infection and the regulation of immune system (Meier et al., 2000; Yuan and Yankner, 2000; Nicholson, 2000). In adult humans, millions of cells divide into identical daughter cells every hour. This impressive rate of cell division is accompanied by an equally impressive rate of cell destruction (Anderson, 1997). Most of the newly formed thymocytes are eliminated by this process through positive and negative selection. Hence, apoptosis is a powerful tool for the removal of cells produced in excess such as in the case of the development of certain vertebrate neurons (Jacobson et al., 1997 and Cowan et al., 1984) which have a functional importance during development but become redundant after the completion of the process (Vaux and Korsmeyer, 1999). The reduction of tadpole tail (Lockshin and Bowen, 1981) is another example of apoptosis in getting rid of unwanted cells. Some cells need to be destroyed to sculpt the body (Saunders, 1966) such as cells between developing digits in animals or cells that migrate to abnormal places e.g., lymphocytes that become useless due to failure to produce and present active antigen-specific receptors or get harmful as a result of carrying self-reactive high-affinity receptors. Therefore, the natural role of apoptosis has been ascribed to various processes such as embryogenesis (Saunders, 1966), differentiation (Fersus, 1991; Gungu, 1992), metamorphosis (Kerr et al., 1974), normal development, normal epithelial turnover like that of skin and gut lining (Bennet et al., 1984), regulation of the
immune cell population (Cohen, 1992), senescence of neutrophil polymorphs following deprivation of growth factors (Cohen, 1992) and tumor regression (Wyllie, 1985). Inappropriate, injudicious and indiscriminate apoptosis occurring under normal conditions in crucial organs results in several disease-states. Some examples of human diseases associated with the failure of or excessive apoptosis are listed in Table 1 (Thompson, 1995; Cohen and Al-Rubeai, 1995; Nicholson, 1996; William, 1991).

The cell death mechanisms may range from necrosis to apoptosis. Necrosis is characterized by cytoplasmic organelle destruction, loss of plasma membrane integrity, and spillage of the cellular contents leading to the damage of surrounding cells or tissue ultimately leading to an inflammatory response. Apoptosis is unique as it occurs without inflammation and material loss to the body, which probably underscores its importance in normal development (Wyllie et al., 1980; Vaux and Strasser, 1996; Darzynkiewicz et al., 1997; Kroemer et al., 1995). The process of apoptosis is characterized by distinct morphological characteristics such as surface blebbing, cell shrinkage, chromatin condensation, and nuclear disassembly. There are changes in the membrane of an apoptotic cell, however, that allow it to be rapidly recognized by other cells and eliminated by phagocytosis (Fadok et al., 1992; Hengartner, 2000). While the pathways for apoptosis and necrosis may be distinct, these are likely to overlap and cross-talk in-vivo. An event that produces necrosis may trigger apoptosis in surrounding tissues or conversely, induction of apoptosis could indirectly produce necrosis in some circumstances. Sometimes the same signal, e.g. treatment with calcium ionophores, could induce apoptosis under ATP rich conditions but can induce necrotic cell death if ATP level is low (Eguchi et al., 1997).

At the molecular level, the process of apoptosis can be divided into the signaling and effector phases. The signaling phase involves detection of death signal and transduction of the signal to death machinery. This signal can originate from outside the cell (through
**Diseases associated with the Inhibition of Apoptosis**

- Cancer
  - Follicular lymphomas
  - Hormone-dependent tumors
  - Breast Cancer
  - Prostate Cancer
- Autoimmune disorders
  - Systemic lupus erythematosus
  - Immune-mediated glomerulonephritis
- Viral Infections
  - Herpesvirus
  - Poxvirus
  - Adenovirus

**Diseases associated with the Increased Apoptosis**

- AIDS
- Neurodegenerative disorders
  - Alzheimer's disease
  - Parkinson's disease
  - Amyotrophic lateral sclerosis
  - Retinitis pigmentosa
  - Cerebellar degeneration
- Myelodysplastic syndromes
  - Aplastic anemia
- Ischemic injury
  - Myocardial infarction
  - Stroke
  - Reperfusion injury
- Toxin-induced disease

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**FLUOROCHROME** | **PARAMETER**
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Rhodamine 123 | ΔΨm disruption
DiOC6 (3) | Fluorogenic substrates
JC-1 | Protease activation
CMXRos | Annexin V conjugates
Merocyanin 540 | Loss of plasma -
| membrane asymmetry
Hydroethidine | Superoxide anion
Nonylacridine orange | Cardiolipin oxidation
Cisparinaric acid | Lipid peroxidation
Monobromobimane | Reduced glutathion
| Calcium-sensitive dyes | Calcium increase
| TUNEL | DNA fragmentation
| DNA-intercalating dyes | DNA loss

Table 2: Fluorochromes suitable for the determination of apoptosis-associated parameters [AIF, apoptosis inducing factor; CMXRos, chloromethyl-X-rosamine; DiOC6(3), 3,3'-dihexyloxacarbocyanine iodide; ΔΨm, mitochondrial transmembrane potential; ICE, intercell-in-18-converting enzyme; JC-1,5,6,6'-tetraethylbenzimidazol carbocyanine iodide; PS, phosphatidylinerine; ROS, reactive oxygen species] (Adapted from Kramer et al., 1997)

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**Table 1: Diseases associated with the induction or inhibition of apoptotic cell death** (Adapted from Thompson, 1995)
the binding of a ligand to a receptor) or from within the cell (for example, detection of toxins, radiation-induced damage, or virus infection). Given the magnitude of death stimuli and the fact that various stimuli can have opposite effects in different cell types, there are probably multiple signal transduction pathways that are involved in communicating the signal. The complexity of these pathways is illustrated by the identification of a variety of molecules that, when overexpressed, either enhance or reduce apoptosis (Fraser and Evan, 1996; Martin and Green, 1995; Steller, 1995; Vaux and Strasser, 1996; Williams and Smith, 1993). However, it appears that all these multiple signaling pathways converge into a common effector pathway leading to death program. The event of cell death occurs in a highly coordinated manner, with the manifestation of a series of characteristic biochemical and morphological changes including calcium flux, cytochrome c redistribution, activation of caspases, mitochondrial depolarization, intracellular acidification, plasma membrane blebbing, loss of plasma membrane asymmetry, reduction in cell volume, selective proteolysis of a subset of cellular proteins, chromatin condensation, nucleosomal DNA fragmentation and ultimately breakdown into membrane-bound apoptotic bodies that are rapidly phagocytosed (White, 1993 and 1996; Chinnaiyan and Dixit, 1996; Vaux and Strasser, 1996). These manifestations of apoptosis occur through the activation of an exquisitely-conserved intrinsic cell suicide programme that can be divided into three phases: initiation, effector and execution (Figure 1). The initiation phase comprises of extrinsic/intrinsic signaling pathways that converge into a common effector phase. Recent evidences suggest that mitochondria participate in the effector phase of the apoptotic cascade and the release of cytochrome c from the mitochondrial compartment into the cytosol represents the central death signal (Green and Reed, 1998; Kroemer, et al., 1997; Bernardi et al., 1999; Martinou et al., 2000). Caspases are crucial molecular players in the execution phase of apoptosis and are constitutively present in the cell as zymogens and are proteolytically cleaved to their active form in the
Figure 1: Process of apoptosis in animal cells showing 3 stages of the apoptotic pathway.
presence of the effector death signal. Active caspases mediate highly specific proteolytic cleavages of different target proteins (homeostasis, repair and structural proteins) and dismantle the cell for disposal (Martin and Green, 1995; Yuan, 1995; Thornberry and Lazebnik, 1998; Fraser and Evan, 1996).

**Evolutionary origins of apoptosis**

Evolution of PCD preceded the evolution of multicellularity of eukaryotes. This is documented by recent findings indicating that PCD operates in some single celled eukaryotes that emerged between 1-2 billion years ago e.g. *Trypanosoma cruzi*, *Trypanosoma brucei*, *Dictyostelium discoidium* and *Tetrahymena thermophila* (Ameisen, 1996). *Dictyostelium discoidium*, in an *in-vitro* system, exhibits non-apoptotic PCD characterized by viability loss, massive vacuolization, prominent cytoplasm condensation and focal chromatin condensation without DNA fragmentation (Comillon et al, 1994). The ciliate *Tetrahymena* loses its macronucleus during conjugation by a process involving chromatin condensation and oligonucleosomal DNA fragmentation (Davis et al., 1992). Interestingly, a protozoa, *Trypanosoma* exhibits cell death characterized by membrane blebbing, chromatin condensation and DNA cleavage into low molecular weight fragments. Most recent evidence suggests that a dying *Saccharomyces cerevisiae* cell cycle mutant (cdc48) exhibits not only chromatin condensation and DNA fragmentation but also phosphatidyl serine exposure on the outer leaflet of the plasma membrane (Madero et al., 1997; Fraser and James, 1998). Thus, a single core mechanism would underlie the phenotypic variations of PCD in these organisms. It is also possible that some of the molecules involved in the core PCD mechanism of even phylogenetically very distant organisms, e.g. *Dictyostelium* and vertebrates, are related.

**Study of apoptosis**

Apoptosis can be assayed both by morphological and biochemical techniques. Morphological features are studied by light microscopy immediately after staining cells with 0.04% trypan blue, which
selectively stains the cells being eliminated largely through non-apoptotic pathways. The apoptotic cells can be identified and scored by blebbing in cell membrane and the presence of apoptotic bodies. Biochemical changes include fragmentation of the genomic DNA into nucleosomal ladder which is usually a signature of apoptosis (Lockshin and Zakeri, 1991). The presence of DNA ladder in cells/tissues is indicative of the occurrence of apoptosis. There are two ways to show fragmentation of the genomic DNA: (i) Extraction of total DNA and subjecting them to agarose-ethidium bromide gel electrophoresis, and (ii) TUNEL (Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labelling) assay is a sensitive test of apoptosis. This test is sensitive and functions on the principle of addition of fluorescein-tagged dUTP on the 3' end of fragmented DNA by TdT. The fluorescent DNA in the cell can be identified and recorded by a fluorescent microscope at specified wave lengths. Other fluorochromes suitable for the determination of apoptosis-associated parameters are listed in Table 2 (Kroemer et al., 1997).

The biochemical assay for apoptosis is also possible through direct assay of caspase (C stands for cysteine and aspase is to denote aspartic acid protease) which was earlier known by different names such as ICE (interleukin-1β converting enzyme) protease, cysteine protease, YAMA, apopain, CPP32 (cysteine protease protein of 32 kDa) and so on. Recently, mitochondrial permeability transition (PT), which has been implicated in the activation of PCD is also used as an important tool to monitor apoptosis. Permeability transition can be quantified spectrophotometrically. The isolated mitochondria resuspended in protein-free buffer show colloidosmotic swelling due to PT which cause reduction in the OD\textsubscript{540} value. Additionally, cytochrome c release from the inner mitochondrial membrane during PT is used as a marker of apoptosis (Kantrow and Plantadosi, 1997).

**Stimuli triggering apoptosis**

A large number of diverse agents are known to trigger apoptosis in a variety of cell types and organisms. These include:
physiologic/genetic agents such as TNF (tumor necrosis factor); family members, neurotransmitters (glutamate, dopamine, N-methyl-D-aspartate), growth factor withdrawal, calcium and glucocorticoid hormone, oncogenes such as myc, rel, E1A, tumor suppressor p53, caspases (cysteine/ICE proteases) etc; environmental agents like X-ray/gamma/UV/thermal radiation, nitrogen mustard, oxidants, free-radicals; pathologic agents like viruses, bacterial toxins, β-amyloid peptide etc. and therapeutic agents e.g., cisplatin, doxorubicin, bleomycin, cytosine arabinoside, methotrexate, vincristine etc. (Thompson, 1995).

UV, ionizing and thermal radiations induce PCD in mammalian and other cells (Thompson, 1995; Verhaegen et al., 1995; Gorman et al., 1997; Warters, 1992). Glucocorticoid hormones induce apoptosis in immature thymocytes (Wyllie, 1980), whereas, IL-2 withdrawal induce apoptosis in mature T-lymphocytes (Duke and Cohen, 1986). Removal of colony stimulating factor (CSF) subjects haemopoietic precursor cells to this form of cell death. Deprivation of NGF (nerve growth factor) may induce cell death in sympathetic neurons (Martinou et al., 1995). T cells of AIDS patients undergo apoptosis due to high levels of antibodies against APO-1. Recent literature provides substantial evidence of involvement of oxidative stress in cell death in AIDS patients (Jacobson, 1996). Excess number of cells in liver parenchyma due to mitogen-induced hyperplasia is balanced by apoptosis (Columbano et al., 1985). Likewise, cell population is kept in check in the slow proliferating hepatic and adrenal cortical epithelial cells as well as in rapidly proliferating cells of internal crypt epithelium and differentiating spermatogonia. In a number of cell types, including some of those mentioned above, apoptotic signals are given out by the neighboring cells. However, they do not die unless bound by TNF through the specific cell surface receptors (Wright et al., 1992; Cohen, 1991). The function of the signaling molecule is dependent on several yet unknown environmental factors. There are several other physiological factors like androgen and estrogen which can trigger cell death in
prostate cancer (Kerr and Searle, 1973) and in the course of regression of the MCF-7 human breast carcinoma in nude mice (Kyprianou and Isaacs, 1991). Many vertebrate developing neurons require neurotrophic factors secreted by target innervated cells. In case of inadequate availability of these factors neuronal cells are eliminated by apoptosis. The same happens to epithelial cells in the ventral prostrate of adult rats and adrenal cortex cells due to inadequate supply of testosterone from testes (Kyprianou and Isaak, 1988) and ACTH hormones from the pituitary gland (Wyllie et al., 1973).

Another category of stimuli such as radiation, oxidants, free-radicals and pathogens (viruses) alter the genetic makeup of an organism. The genetic damage is then repaired by the repair machinery of host. A set of genes mostly regulated by the p53 gene are involved in this process. If the cell fails to repair DNA damage it is forced to apoptosis. This mechanism of apoptosis is thus dependent on the p53 gene, which is considered as the molecular guardian of the cell. It is generally known as a tumor suppressor gene, but now has been found to aid a number of functions including induction of apoptosis (Thompson, 1995; Elledge and Lee, 1995; White, 1993 and 1996; Steller, 1995; Shimamure and Fisher, 1996; Sionov and Haupt, 1999).

Chemotherapeutic drugs also induce substantial cell death in organisms (Thompson, 1995). Some of these drugs are known to inhibit macromolecular synthesis to induce apoptosis. Actinomycin D inhibits transcription and induces programmed cell death in Sf9 cells (Verhaegen et al., 1995). Protein synthesis inhibition by cycloheximide also activates apoptosis in Sf9 cells (Verhaegen et al., 1995). Likewise, adriamycin, bleomycin, doxorubicin, cisplatin, vincristine etc. stimulate this pathway of death via one or the other mechanisms. Sometimes the toxins produced by the body itself, under certain conditions, activate apoptosis. β-amyloid peptide produced mostly in aging individuals as a result of oxidative stress, can induce cell death in neuronal cells, resulting in Alzheimer's
type of disease symptoms (Miyata and Smith, 1996; Barinaga, 1998).

Recently, it has become clear that integrin-mediated cell anchorage has a vital role in the control of apoptosis (Frisch and Ruoslahti, 1997; Howe et al., 1998). Indeed, a new term 'anoikis' has been coined to describe apoptosis caused by loss-of anchorage. Integrin-mediated signaling mainly occurs either via direct or collaborative signaling. It is suggested that in the direct signaling pathway, the adhesion to the extracellular matrix proteins can activate cytoplasmic tyrosine kinases (e.g. focal adhesion kinase (FAK) and serine/threonine kinases such as the mitogen-activated protein kinases (MAPK) cascade), induce ionic transients (e.g. Ca\(^{2+}\), Na\(^+/H^+\)), and stimulate lipid metabolism (e.g. phosphatidylinositol-4, 5-biphosphate (PIP\(_2\)) synthesis). In the collaborative pathway, integrin-mediated cell adhesion modulates signaling events initiated through other types of receptors, particularly receptor tyrosine kinases (RTKs) that are activated by polypeptide growth factors (Aplin et al., 1998). Recently, it has been shown that FAK has an important role in anchorage regulation of apoptosis. Thus, expression of activated forms of FAK in epithelial cells blocks anoikis (Frisch et al., 1996a). It has been shown that cell adhesion can activate phosphoinositide 3'-OH kinase (PI3-K), probably by a Ras-dependent mechanism. Further, expression of constitutively activated forms of PI3-K or Akt (a cytosolic protein kinase) blocks anoikis in epithelial cells (Khwaja, 1997). These observations suggest an antiapoptotic pathway that proceeds from integrin engagement, to FAK, to PI3-K, and thence to Akt. A recent observation shows that FAK can be cleaved by caspases thereby suggesting a positive feedback loop whereby initiation of apoptosis activates caspases and shuts down the FAK-PI3-Akt anti-apoptotic pathway (Wen et al., 1997). Another possible arm of the anoikis pathway has also been explored, one that involves caspases, bcl-2 and c-Jun amino-terminal kinase (JNK) cascade (Frisch et al., 1996b). Many of the twenty five or so known integrins recognize a tripeptide, arginine-glycine-aspartate.
(or RGD in single-letter code), in target proteins of the extracellular matrix (Ruoslahti, 1996; Ruoslahti and Reed 1999). In-vitro experiments have shown that small peptides containing RGD motif bind to integrins and when presented to a cell in a soluble form, inhibit cell attachment. The peptide-blocked integrins cannot provide the signals that the cell would receive from the matrix-bound integrin, resulting in changes in cell shape and ultimately, apoptosis (Frisch and Francis, 1994, Chen et al., 1997). The activation of caspase is the probable explanation for the pro-apoptotic activity of RGD peptides (Buckley et al., 1999).

**Death receptors: signaling and modulation**

Death receptors are cell surface receptors that transmit apoptosis signals initiated by specific "death ligands" and play a central role in instructive apoptosis. Death receptors belong to the tumor necrosis factor (TNF) receptor gene superfamily, which is characterized by cysteine-rich, similar extracellular domains. The death receptors contain a homologous cytoplasmic sequence termed the "death domains".

The best characterized death receptors are CD95 (also called Fas or Apo1) (Nagata and Golstein, 1995) and TNFR1 (also called as p55 or CD120a) (Ashkenazi and Dixit, 1998). Other death receptors include avian CAR, death receptor 3 (DR3, also termed as Apo3, WSL-1, TRAMP, or LARD), DR4, and DR5 (also known as Apo2, TRAIL-R2, or KILLER). The ligands that activate these receptors are structurally related molecules that belong to the TNF gene superfamily. It is well established that TNF mediates apoptosis by activation of multiple signals (Pellegrini and Strasser, 1999; Rath and Aggarwal, 1999) [Figure 2].

**Oxidative stress and apoptosis**

Oxidative stress develops as a results of generation of reactive oxygen species (ROS) in the course of oxygen metabolism (Imlay and Linn, 1988). ROS are known to play a role in growth regulatory pathways. In addition, a role for ROS in apoptotic pathways is also
Figure 2: The death receptors pathways of cell death. Apoptosis can be induced by death ligand-death receptor signaling through the adaptor proteins. (Modified from Roth and Apparail, 1999; Pellegrini and Strasser, 1999).
found. The mechanism of action of oxidative stress in causing pathophysiological disturbances and onset of disease-states and diseases is not very clear. Some of the recent findings nevertheless point to the involvement of apoptosis in these disorders as described earlier. It has been recently observed that stimulation with Fas ligand resulted in apoptosis through the generation of $\text{O}_2^{-}$. This process was inhibited by the expression of a dominant-negative ras gene, suggesting a role for small GTPases in the control of redox state of cell. Two recent studies suggest that ROS may mediate p53-dependent apoptosis. Overexpression of p53 resulted in a significant increase in ROS levels, while treatment of the cells with antioxidants inhibited p53-mediated apoptosis (Johnson et al., 1996; Polyak et al., 1997). Several chemical compounds are known to manifest their biological effects through the generation of oxidative stress (Cadet et al., 1999). Oxidative stress produces several unfavorable alterations in tissues and leading to the initiation and or progression of disease-state in a wide range of living systems including humans (Packer and Glazer, 1990; Greenwald, 1990; Bast et al., 1991). Precise mechanism of induction of damage by different ROS is not clearly understood. However, it has been shown that singlet oxygen has been found to attack deoxy guanine of DNA to modify it into 8-hydroxydeoxyguanosine (8-oxodG) (Floyd et al., 1989) and superoxide radicals induce single as well as double strand breaks in naked DNA molecules as well as in DNA under in-vivo conditions (Sah et al., 1995). Several anti-oxidants including those extracted from biological sources have been shown to reduce the genotoxicity of ROS. Various reports show that TNF induces mitochondrial MnSOD in different cell types (Kawaguchi et al., 1990; Wong, 1995; Xu et al., 1999) similar to the effect of ROS, which also lead to MnSOD induction. Both TNF and ROS-mediated induction can be inhibited by antioxidants (Warner et al., 1996). In the myelogenous leukemia cell line, ML-1a, TNF treatment leads to mitochondrial ROS production, which is inhibited in the presence of antioxidants such as PDTC (pyrrolidinedithiocarbamate). The mitochondrial ROS then causes
cytochrome c release and, via caspase-3 activation results in apoptosis (Higuchi et al., 1998a and 1998b). Recent evidences indicate that HIV-induced histopathological effects accrue as a result of oxidative stress (Pace and Leaf, 1995).

**Importance of apoptosis**

Apoptosis plays a vital role in many normal situations in both vertebrates and invertebrates (Wyllie et al., 1980). One of the most advantageous characteristics of apoptosis is the maintenance of cellular membrane integrity during apoptotic death, which prevents the leakage of intracellular components into the extracellular milieu and hence, prevention of inflammatory responses. Apoptosis is particularly prominent during development, in processes such as tissue involution and remodeling (Ucker, 1991). Apoptosis is also important in normal cell turnover in nearly all tissues, especially those with high turnover rates such as skin, intestinal epithelium, and blood and lymph systems. This is illustrated by the fact that defects in apoptosis in these tissues can lead to tumor development (Thompson, 1995). Apoptosis is also important for functioning of the vertebrate immune system, where it is involved in cell killing by cytotoxic T-cells and in the selection of memory B-cells (Duvall and Wyllie, 1986; Clem, 1997).

**Role of Mitochondria in cell death**

**Mitochondria and Cell Death in the Evolutionary History**

It is believed that both mitochondria and chloroplast are the descendent of symbiotic prokaryotes living within the eukaryotic cell. Prospects of huge energy benefits for both, accruing from the emerging oxygen atmosphere which was toxic to other life forms, kept them together. The result of this endosymbiosis was the emergence of protoeukaryotic cells hosting an intracellular body which became mitochondria. This alliance was frequently threatened because of the conflicts in selection between their genomes, and the fully aerobic atmosphere. The protomitochondria, however, played the lead role in oxygen
metabolism providing oxidative phosphorylation (ATP generation). However, conditions that favored the protomitochondria over the host cell would kill the cell and release the endosymbiont. The symbiosis, therefore, remained perilously fragile until essential genes for mitochondrial metabolism and biogenesis were acquired by the nuclear genome culminating in the modern 'obligate symbiosis' (Ernster and Schatz, 1981; Green and Reed, 1998).

Evidence of Mitochondrial Involvement in Cell Death

There are several biochemical and cellular evidences which directly link mitochondria with cell death. The fact that anucleate cells (cytoplasts) are capable of dying by apoptosis provide evidence that cytoplasmic factors are responsible for the control of apoptosis. Further, the mitochondrion enriched fraction from egg extracts of frog, *Xenopus laevis*, is essential for the cell-free induction of apoptosis. Bax, a mammalian cell death protein targets mitochondrial membrane to induce mitochondrial damage and cell death. Bcl-2, an anti-apoptotic protein, also exists abundantly in the mitochondrial membrane. In a cell-free system, presence of mitochondria is necessary to induce nuclear condensation and DNA fragmentation which is considered as a hallmark of apoptosis. Induction of caspases that actually executes cell death in the presence of ATP also requires cytochrome c which is released from mitochondria. These evidences unequivocally associate mitochondria with apoptosis (Kroemer et al., 1997; Green and Reed, 1998; Gross et al., 1999).

How do Mitochondria Orchestrate Apoptosis?

Three inter-related pathways are regulated in mitochondria to bring about cell death:

(i) disruption of electron transport, oxidative phosphorylation and adenosine triphosphate (ATP) production;
(ii) release of factors that trigger activation of key enzyme(s) of apoptosis i.e. Caspase family of proteases; and
(iii) change in the cellular redox potential (Green and Reed, 1998).
(i) Disruption of electron transport chain:
Several agents, such as, gamma-radiation, ceramide, and Fas (receptor) ligation cause disruption of electron transport chain at various levels (for example, reduction in ATP generation) leading ultimately to apoptosis. However, it is important to understand that a drop in ATP production occurs late in the process, hence this affects downstream events in the apoptotic pathway (Bossy-Wetzel et al., 1998; Eguchi et al., 1997). Loss of mitochondrial ATP, as such, can kill a cell by necrosis but, it may not necessarily induce apoptosis. Thus, the observed loss of ATP could be an effect of apoptosis, but not essentially a cause.

(ii) Caspase Activating Factors:
Caspase is the ultimate death executioner in most of the apoptotic programmes. Mitochondrial involvement in apoptosis cannot be explained by a mere "loss of function" due to the energy deficit in the cell. The recent impulse to mitochondrial studies in the context of cell death came with the identification of mitochondrial proteins that participate in modulating the execution phase of apoptosis. These protein are (i) pro- and anti-apoptotic members of bcl-2 family that localizing on the outer mitochondrial membrane; and (ii) proteins that may be released during apoptosis, like cytochrome c and apoptosis inducing factor (AIF) (Bernardi et al., 1999). Mitochondria releases cytochrome c upon activation, which, in turn, activate caspases in the presence of ATP in the cytoplasm (Figure 3). Involvement of cytochrome c in the death programme was demonstrated by studies on cell-free system in which spontaneous, bcl-2 mediated inhibition of nuclear condensation and DNA fragmentation were observed to depend on the presence of mitochondria (Newmeyer et al., 1994). Experiments on vertebrates demonstrate that cytosolic cytochrome c exists as an "apoptosome" which comprises cytochrome c, Apaf 1 and procaspase-9 (Li et al., 1997). Interestingly, irrespective of the apoptotic signal, redistribution of mitochondrial cytochrome c (Apaf-2) has been observed in
Figure 3: Model for caspase activation by mitochondria
(Adapted from Green and Reed, 1998)
different mammalian and *Xenopus* cells (Zou *et al.*, 1997; Kluck *et al.*, 1997a and 1997b; Yang *et al.*, 1997). From the apoptosome, active caspase-9 is released which, gears up other types of caspases to orchestrate the biochemical execution of cells. The precise pathway, however, remains unknown. Several competing models have been proposed to explain how cytochrome c is released from mitochondria during apoptosis. Two models share the common prediction that the outer mitochondrial membrane ruptures as a result of swelling of the mitochondrial matrix. The first model postulates the opening of a megachannel called the permeability-transition pore (PTP). This is poorly characterized in molecular terms, but is proposed to span both the inner and the outer mitochondrial membranes at sites at which the two membranes are apposed. The adenine-nucleotide translocator (ANT, located in the inner mitochondrial membrane) and the voltage-dependent anion channel (VDAC, found in the outer mitochondrial membrane) are considered to be major components of the PTP. According to the PTP model, PTP opening cause permeabilization of the inner membrane and mitochondrial depolarization by the binding to the ANT. This process allows the entry of water and solutes into the matrix and leads to mitochondrial swelling. The other model postulates a defect in mitochondrial ATP/ADP exchange because of closure of VDAC. This leads to hyperpolarization of inner mitochondrial membrane and subsequent matrix swelling. Both models may account for some but not all types of cell death. Three other proposed models do not predict damage of the outer mitochondrial membrane but rather the formation of a pore in this membrane that is large enough to allow the passage of cytochrome c and other mitochondrial proteins of the intermembrane space into the cytosol. Permeabilization of mitochondrial membranes appears to be a common event in many forms of cell death. However, the mechanism underlying the process may differ according to the specific 'death' stimuli, and may account for the differences in these two pictures of cell death (apoptosis verses necrosis) (Bernardl *et al.*, 1999).
Surprisingly, most of the caspase-inhibitors fail to prevent cytochrome c release induced by apoptotic agents including UV, staurosporine (inhibitor of protein kinase C) and over expression of bax (a gene product that induces apoptosis) (Green and Reed, 1998; Jurgensmeier et al., 1998). Release of cytochrome c from mitochondria may thus kill cells in two ways: (i) Release of active caspase via an express pathway involving Apaf-1, or (ii) a slow necrotic pathway due to depletion of cytochrome c in mitochondria and consequent drop in ATP-generation (Waterhouse and Green, 1999).

(iii) Change in the cellular redox potential:
O$_2^-$ are generated mainly in mitochondria (Bredesen, 1995). During electron transport in the respiratory chain, 1-5% of electrons miss their track to fall prey to O$_2$ producing O$_2^-$ ions. Agents that cause uncoupling may also enhance production of O$_2^-$. ROS produced in the course of faulty mitochondrial metabolism may induce peroxidation of membrane lipids leading to morphological changes akin to apoptosis. Anaerobic conditions may also gear up cells to undergo apoptosis by certain stimuli (Jacobson and Raff, 1995). However, ROS can be generated even under conditions of virtual anaerobiosis, and, thus, their role in apoptosis cannot be contradicted entirely on this basis.

**Redox potential and Permeability Transition pore**
In normal growing cells, a specific chemical and electrical gradient exists on both sides of the mitochondrial membrane due to an asymmetric distribution of protons and ions. Disruption in transmembrane potential ($\Delta\psi_m$) can be observed in many different cell types, irrespective of the apoptosis-inductng agent. In most of the apoptotic cells, the mitochondrial inner transmémbrane potential collapses, which indicates opening of conductance channel known as PT pore. PT pore opening results in cessation of ATP synthesis, matrix Ca$^{2+}$ outflow, depletion of reduced glutathione, etc. Although the exact structures of PT porés is not
well understood, there are reports which show the existence of complex formation between the ANT (adenine nucleotide translocator) present in the inner membrane with the outer membrane proteins in the inner-outer membrane contact sites which, in turn, interact with hexo and glycerol kinases. However, certain substances (e.g. cyclosporins) that inhibit PT pores appear to block apoptosis. In addition, a further specific inhibitor of PT, bongkrekic acid (BA), a ligand of ANT, can inhibit the pre-apoptotic $\Delta \psi_m$ disruption and subsequent apoptosis in several different experimental systems (Marchetti et al., 1996; Zamzami et al., 1996). This suggests that PT pore inhibitors could also be considered as potential therapeutic targets in future (Kroemer et al., 1997; Bernardi et al., 1999; Green and Reed, 1998).

Caspase-key players in cell death
Aspartate specific cysteine proteases i.e. caspases play a central and evolutionarily conserved role in transducing the apoptotic signal and the final execution of apoptosis in multicellular organisms (Villa et al., 1997; Porter et al., 1997; Salvesen and Dixit, 1997; Cryns and Yuan, 1998; Hu et al., 1998; Nicholson and Thornberry, 1997; Hirata et al., 1998). Caspases are restricted endopeptidases and their action on different protein substrates leads to their activation or inactivation but never degradation. The only other enzyme known to activate caspases is granzyme B, an aspartate specific serine protease (Zhivotovsky et al., 1997). In mammals, there are fourteen different caspases (Cryns and Yuan, 1998; Hu et al., 1998), whereas a single caspase has been reported from lower eukaryotes like C. elegans and Spodoptera frugiperda. C. elegans caspase (ced-3) and Sf9caspase-1 (Sf/caspase-1) have structural homology with human caspase-1 and caspase-3 respectively (Ahmad et al., 1997). It is not known whether caspases are synthesized or activated in a developmental/tissue-specific manner and/or are ordered in an apoptotic protease cascade. It is possible that caspases might differ in their subcellular localization, substrate cleavage specificity and also different caspases might respond to different stimuli. Different
alternative spliced forms or alternative proteolytic processing variants of caspases could also differ in their biological activity. All caspases are synthesized as zymogens that require cleavage at aspartates to liberate a large and a small subunit, which associate into an α2β2 tetramer to form an active enzyme (Villa et al., 1997). Recently, the mechanistic mathematical model has been reported describing key elements of receptor-mediated and stress-induced caspase activation using mass-conservation principles in conjunction with kinetic rate laws (Fussenegger et al., 2000). However, the exact molecular mechanism by which the caspases are activated in the apoptotic process is not clearly defined.

In humans, out of ten caspases, ICE subfamily i.e. caspases-1, -4 and -5 participate primarily in the activation of pro-inflammatory cytokines and inflammation (Figure 4 [1]) whereas ced-3 subfamily i.e. caspases -2, -3, -6, -7, -8, -9 and -10 participate largely in the promotion of apoptotic cell death (Figure 4 [A]). Caspases can be divided into two classes: long prodomain caspases (ced-3, caspases-1, -2, -4, -5, -8, -9 and -10) and short prodomain caspases (caspases-3, -6, -7 and -11). Long prodomains of some caspases contain death effector domains (DEDs) that mediate binding to other proteins and are thus involved in targeting and regulating their activation (Boldin et al., 1996; Fernandes-Alnemri et al., 1996; Boldin et al., 1995). Caspases with short prodomains are the foot soldiers of apoptosis, operating at the downstream end of the cascade (Figure 4) to cleave different substrates (Rao et al., 1996; Takahashi et al., 1996). Caspase-3 seems to be the workhorse caspase of apoptosis.

Four classes of caspase substrates have been identified. Class-1 substrates such as caspases, protein kinase Cδ, p21 activated kinase 2, cytosolic phospholipase A2, sterol regulatory element binding protein and the 45 kDa DNA fragmentation factor are activated as a result of caspase cleavage (Datta et al., 1997; Liu et al., 1997). Caspase cleavage also causes inactivation of class-2 substrates such as DNA protein kinase catalytic subunit, the
Figure 4: Role of caspases in inflammation and apoptosis
retinoblastoma tumor suppressor protein and GDP dissociation inhibitor type D4 (Emoto et al., 1995). Class-3 substrates are structural proteins like nuclear lamins A and B and Gas2 (Rao et al., 1996; Takahashi et al., 1996; Lazebnik et al., 1995; Orth et al., 1996; Brancolini et al., 1995) whereas, class-4 substrates (including PARP, fodrin, U1 Sn RNP 70 kDa protein, C1 and C2 HnRNP proteins, huntingtin, actin and p53 regulator-mdm2) undergo caspase cleavage with unknown consequence for apoptosis (Kumar and Lavin, 1996; Lazebnik et al., 1994; Crions et al., 1996; Kayalar et al., 1996; Song et al., 1997; Gu et al., 1995; Lippke et al., 1996; Schlegel et al., 1996; Gößberg et al., 1996; Waterhouse et al., 1996; Whyte, 1996). Thus, parallel and sequential proteolytic activation and inactivation of key substrates contribute to the execution of cell apoptosis.

Over the past decade, the significant focus of research was on calpain-mediated proteolysis, and its contribution to cell death. The two ubiquitous calpains (m- and μ-calpains) exists as pro-enzyme heterodimer (80 kDa-29 kDa) in resting cells but this is activated by Ca2+ and autolytic processing to produce a heterodimer (78 kDa-18 kDa). Physiologically, the activity of these calpains might also be regulated by the endogenous protein inhibitor calpastatin. Calpain activation in many form of apoptosis was first demonstrated in thymocytes, as measured by calpain autolysis. In addition, various calpain inhibitors were found to protect against apoptosis in immune cells (Wang, 2000).

**Putative Endonucleases implicated in apoptosis**

One of the biochemical hallmarks of apoptosis is the enzymatic internucleosomal degradation of chromatin, which can be viewed on agarose gels as DNA ladder of 180 base pairs (bp) and their multiples (Peitsch et al., 1993 and 1994). Two kinds of DNA fragmentation patterns have been observed in apoptosis. The first one involves production of larger fragments of DNA which are approximately 50 kbp in length, and the second involves the production of DNA fragments which are 180-200 bp in length.
and/or their multiples (Montague et al., 1997). Earlier reports suggested that the endonucleases involved in apoptosis could be the domain nuclease or/and fragmentation nuclease responsible for the generation of 50 Kb fragments and 180-200 bp fragments, respectively (Earshaw, 1995). The fragmentation nucleases have been variously identified as either DNase I, DNase II (acidic), Ca$^{2+}$/Mg$^{2+}$-dependent endonucleases, Ca$^{2+}$/Mg$^{2+}$-dependent endonucleases or cyclophilins. The ion dependence of these candidate endonucleases, their cellular localization and the capability to form DNA ladder have been documented (Table 3) (Enari et al., 1998). HeLa cytosol DNA Fragmentation Factor (DFF) is a heterodimeric protein of 40 kDa and 45 kDa subunits, which functions downstream of caspase-3 to trigger DNA fragmentation during apoptosis (Liu et al., 1998). Caspase-3 cleaves the 45 kDa subunit of DFF at two sites liberating the 40 kDa subunit, which either acts directly as a DNase or activates a DNase which further cleaves the nuclear material to give a DNA ladder. However, DFF does not show DNase activity when incubated with naked DNA suggesting that DFF activates the Ca$^{2+}$/Mg$^{2+}$-dependent nuclease(s) that resides within the nuclei (Liu et al., 1997). The most interesting report is the isolation of a caspase-activated DNase (CAD) that degrades DNA during apoptosis (Table 3). CAD is different from DNase I and DNase II and is present in the cytoplasmic fraction of mouse lymphoma cells along with its inhibitor (ICAD). CAD is a 39 kDa protein with a nuclear localization signal at its carboxyl end whereas ICAD exists in two forms i.e. the long form (331 amino acids) and the short form (265 amino acids). ICAD acts as a chaperone for CAD synthesis and it remains complexed with CAD to inhibit its DNase activity in normal healthy cells. ICAD and CAD are electrostatically linked since ICAD is an acidic protein whereas CAD is a basic protein. CAD is always produced as a complex with ICAD and caspase-3 cleavage releases CAD, which then enters the nucleus and degrades the nuclear material leading to the formation of a DNA ladder. Mouse ICAD-L has 76% homology to the human DFF45 suggesting that ICAD-L may be a counterpart of human DFF45. Hence, the DNase
<table>
<thead>
<tr>
<th>DNase (MW)</th>
<th>Ion dependence</th>
<th>Ladder formation</th>
<th>Cellular localization</th>
</tr>
</thead>
<tbody>
<tr>
<td>NUC-18 (18 kDa)</td>
<td>Ca²⁺ and Mg²⁺</td>
<td>yes</td>
<td>Nuclear</td>
</tr>
<tr>
<td>DNase I (32-37 kDa)</td>
<td>Ca²⁺ and Mg²⁺</td>
<td>yes</td>
<td>secreted</td>
</tr>
<tr>
<td>DNase II (29 kDa)</td>
<td>None</td>
<td>yes</td>
<td>ER/nuclear</td>
</tr>
<tr>
<td>CAD (39 kDa)</td>
<td>None</td>
<td>yes</td>
<td>lysosomal/nuclear</td>
</tr>
</tbody>
</table>

Table 3: DNases involved in apoptosis. (Modified from Petitsch et al., 1994)

<table>
<thead>
<tr>
<th>Caspases</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Fluorescein</td>
<td>FLIPs</td>
<td>FLIPs can form death effector filaments to initiate or amplify apoptotic stimuli independent of receptors on the plasma membrane.</td>
</tr>
<tr>
<td>Reaper</td>
<td>Drosophila death inducing gene product with homology with death domain of Fas/CD95 receptors; could act via cytochrome c release and can be antagonized by Bcl-2.</td>
<td></td>
</tr>
<tr>
<td>CrmA</td>
<td>Fas-associated via death receptor</td>
<td></td>
</tr>
<tr>
<td>IAPs</td>
<td>Inhibitor of apoptosis family of proteins, highly conserved throughout evolution; some IAPs function by inhibiting the distal cell processes without being cleaved.</td>
<td></td>
</tr>
<tr>
<td>DAP-1</td>
<td>Cysteine 15 kDa, proline-rich basic protein with two CXXC phosphorylation sites; could potentiating apoptosis.</td>
<td></td>
</tr>
<tr>
<td>DAP-2</td>
<td>A proapoptotic 160 kDa death domain containing Ca²⁺ calmodulin dependent serine/threonine kinase localized to the cytoskeleton; specifically associated with the actin microfilament assembly.</td>
<td></td>
</tr>
<tr>
<td>DAP-3</td>
<td>A 46 kDa, proapoptotic, ubiquitously expressed, nucleotide binding protein.</td>
<td></td>
</tr>
<tr>
<td>DAP-5</td>
<td>A 97 kDa translation initiation factor, eIF4G homologue; putative death promoting active form of this protein could trigger the cap-independent translation of mRNAs required for cell death.</td>
<td></td>
</tr>
<tr>
<td>Cathepsin D</td>
<td>A 49 kDa intermediate form, seen in apoptotic cells with altered cellular localization and substrate specificity.</td>
<td></td>
</tr>
</tbody>
</table>

Table 4: Non Bcl-2 protein regulators of apoptosis.
regulated by DFF may be CAD or CAD-like DNase as overexpression of ICAD inhibits DNA degradation in the apoptotic process in human cells (Enari et al., 1998; Sakahira et al., 1998).

Role of Kinases in apoptosis
A large number of related/unrelated kinases have been implicated in the apoptotic pathway. An important reason for the suggestive involvement of these kinases is the commonality of many factors required in the pathways of cell division and cell death (Anderson, 1997). Kinases are grouped into: (i) Stress-activated serine/threonine protein kinases, (ii) cyclin-dependent serine/threonine kinases, (iii) lipid-activated serine/threonine kinases, (iv) nucleic acid-dependent serine/threonine kinases, (v) cAMP-dependent serine/threonine kinases, (vi) Tyrosine-protein kinases, and (vii) Miscellaneous serine/threonine kinases as well as protein phosphatases. It is presumed that the death programme exists constitutively in all cells but, under the control of a "dead man's switch". This switch remains actively repressed to facilitate cell survival. A battery of signals are involved in regulating this programme and among these signals, the kinases are indirectly associated with the execution of cell death. The key players, caspases, are not regulated by phosphorylation/dephosphorylation switch. Evidences suggest that parallel cascades of stress-activated MAP/ERK family of kinases can regulate cell division, cell death and their arrest in G0/G1 (Verheij et al., 1996) via phosphorylation/dephosphorylation of transcription and translation factors. A common feature of stress-induced apoptosis is rapid generation of ceramide due to hydrolysis of sphingomyelin (Kolesnick and Golde, '1994; Xia et al., 1995) and ceramide is known to induce apoptosis in many cell types (Verheij et al., 1996) through activation of serine/threonine phosphatase (Dobrowsky and Hannun, 1992) and kinase (Mathias et al., 1991). On the other hand, growth factor-receptors commonly activate the RAS/RAF/MKK/ERK proliferative cascade by stimulating the release of diacylglycerol through the action of phospholipase C (Xia et al., 1995). Diacylglycerol can inhibit apoptosis induced by TNF-α.
and ceramide thereby suggesting cross talks between these antagonist lipid mediators (Jarvis et al., 1994; Obeid et al., 1993). FAST (Fas-activated serine/threonine kinase) is phosphorylated on the serine/threonine residues in Jurkat cells. In response to Fas ligation it gets activated by dephosphorylation (Tian et al., 1995). The active form of FAST phosphorylates and concomitantly activates TIA-1, an RNA binding protein involved in signaling apoptosis in response to Fas ligation. Phosphorylation of TIA-1 precedes the onset of DNA fragmentation (Tian et al., 1995; Taupin et al., 1995). Studies on kinase-dependent signaling of apoptosis presents a very complex scenario. On the whole, kinases are considered to amplify and integrate the various stimuli into the core apoptotic programme.

The influence of apoptosis on viral replication strategies

It is now widely recognized that the ability of individual cells to direct their own suicide has had an important influence on the evolution of viruses and their strategies for replication (Razvi and Welsh, 1995). This is due to the fact that many viruses provoke the suicide response very early in their replication process, and that the premature death of the host cell can have a negative influence on the ability of viruses to replicate. Thus, viruses have had to either evolve mechanisms to prevent cell suicide or find ways to cope with replicating in an apoptotic environment (Clem, 1997). The mechanisms by which viruses induce apoptosis are in most cases not well understood. However, in some cases, overexpression of a single viral gene product has been shown to induce apoptotic death, but the precise role of gene product triggering apoptosis during viral infection remains to be demonstrated (Li et al., 1995; Rao et al., 1992; Prikhod’ko and Miller, 1996). Disregulation of the cell cycle has been proposed as a potential mechanism of apoptosis induction with the transforming protein E1A of adenovirus being the best example (Debbas and White, 1993). A variety of strategies are employed by viruses to block apoptosis. For example, the adenoviruses appear to have evolved several mechanisms of counteracting host cell
apoptosis. The adenovirus early gene E1A induces p53-dependent apoptotic death, which can be counteracted by either of two polypeptides (19 K and 55 K) encoded by the E1B gene. E1B-19K is a homologue of bcl-2 gene (Chiou et al., 1994), the prototype of a cellular gene family whose members are involved in cell death regulation. E1B-55K binds to and inactivates the cellular p53 protein, another cellular protein important in signaling apoptosis in certain situations (Debbas and White, 1993). Other viral genes products that inactivate p53, such as SV40 T antigen and papillomavirus E6, are also capable of inhibiting p53-dependent apoptosis (Shen and Shenk, 1995). Finally, the E3 gene of adenovirus also encodes proteins that are able to inhibit the killing of infected cells by cytotoxic T lymphocytes or tumor necrosis factor (Wold and Gooding, 1991; Clem, 1997).

In addition to adenovirus, several other DNA viruses contain homologues of the bcl-2 family, including BHRF1 of Epstein-Barr virus (Henderson et al., 1993), ORF16 of herpesvirus saimiri (Nava et al., 1997), KSBc1-2 of human herpesvirus 8 (Cheng et al., 1997), and LMW5-HL of African swine fever virus (Neilan et al., 1993). Poxvirus also encodes genes that can inhibit or delay host cell apoptosis, including the serpins crmA (spi-2) (Ray et al., 1992) and Spi-1 (Brooks et al., 1995) and the ankyrin repeat-containing protein CHOhr (Ink et al., 1995). The crmA gene product blocks death through its ability to inhibit ICE proteases. Cytomegalovirus IE1 and IE2 are transcription factors that are able to inhibit apoptosis induced by an adenovirus mutant lacking E1B (Zhu et al., 1995). Finally, LMP-1 of Epstein-Barr virus is able to promote the survival of latently infected lymphocytes, possibly through a signal transduction mechanism (Mosialos et al., 1995).

Understanding the mechanism by which viruses manipulate normal host functions to their own advantage has been of vital importance in furthering our understanding of many cellular processes, including cell cycle regulation, tumorigenesis, RNA transcription and processing, and protein translation. In a similar
fashion, the study of baculovirus-induced apoptosis has led to a greater understanding of genetic pathways of cell death and their similarities between insects and other organisms, as well as provided insight into the role of apoptosis in defense against viral pathogens (Clem, 1997).

**Regulation of apoptosis**

Much of the current knowledge on the genes involved in the apoptotic cell death programme has emerged from genetic studies on invertebrate model organisms such as *Caenorhabditis elegans* and *Drosophila melanogaster* (Hengartner and Horvitz, 1994c and 1994d; Ellis et al., 1991; McCall and Steller, 1997; Steller and Grether, 1994). Yeast two hybrid selection screen systems led to the identification of cell surface receptor (TNF family) associated adapter proteins and enzymes (Nagata, 1997; Wallach et al., 1997). A number of mutants affecting different stages of cell death programme have been isolated and characterized from *Caenorhabditis elegans*. Altogether 14 genes have been identified by mutation studies of *C. elegans*. These genes named *ced*-1 through *ced*-14 (*ced* stands for cell death defective), regulate four different stages of the cell death programme. The stages include (i) the decision whether a cell will die or adopt another fate, (ii) death, (iii) clearance/engulfment/phagocytosis of the dead cell, and finally, (iv) degradation of the engulfed corpse. Of all these genes, the ones involved in the death execution, engulfment and degradation are responsible for all somatic cell death, while those involved in decision making influence only a few cells to undergo apoptosis. Normal healthy cells after receiving death signals will decide between life and death. Three genes *ced*-3, *ced*-4 and *ced*-9 regulate cell death programme and *ced*-3 and *ced*-4 are directly involved in the execution of cell death, whereas, *ced*-9 protects cells from undergoing apoptosis (Spector *et al.*, 1997). Ced-3, *ced*-4 and *ced*-9 are structurally and functionally homologous to mammalian caspases, Apaf-1 and *bcl*-2, respectively indicating their evolutionarily conserved nature (Thompson, 1995; Zou *et al.*, 2000).
The *bcl-2* gene family

The *bcl-2* gene was initially discovered in 1985 as a gene translocated in human follicular lymphoma and was the first recognized component of the physiological death mechanism (Bakshi *et al.* 1985; Tsujimoto *et al.*, 1985). Since then *bcl-2* has received much attention because it interferes with endogenously programmed or externally induced apoptotic cell death (White, 1996; Hockenbery *et al.*, 1990; Hockenbery, 1995; Vaux *et al.*, 1998). The oncogenic potential of the *bcl-2* lies in its ability to promote cell survival that would otherwise be programmed to die and thus *bcl-2* has become the prototype of a newly defined class of oncogenes acting in a direction opposite to tumor suppressors such as *p53* (Reed, 1997a; Kroemer, 1997). It belongs to an expanding multigene family of proteins that regulate apoptosis during development and homeostasis (White, 1996). The *bcl-2* family proteins have a putative transmembrane domain at their carboxy terminus and four conserved regions i.e., BH1, BH2, BH3, and BH4. Except BH4, the other three domains could form a hydrophobic groove on the surface of the protein in an aqueous environment that controls protein-protein interactions. Thus, homo- as well as hetero-dimerization of *bcl-2* family members influence the process of cell survival/cell death (Reed, 1997a). The *bcl-2* family comprises of at least 16 members (*Figure 5*). Six family members, *bcl-2*, *bcl-XL*, *bcl-w*, *boo*, A1 and Mcl-1 protect cells against a broad range of physiological and experimentally applied death stimuli including growth factor depletion, DNA damage, and glucocorticoids. Some of these members have COOH-terminal hydrophobic domains which are in part responsible for localization of proteins on the cytoplasmic face of the outer mitochondrial membrane, endoplasmic reticulum, and nuclear envelop (Lithgow *et al.*, 1994). The other 10 *bcl-2* family members which promote apoptosis include bax, *bcl-xS* (a splice variant of the *bcl-x* gene), bak, bok, bad, bik, bid, Hrk, blk and bim.
Figure 5: The Bcl-2 family members. There are two opposing categories in Bcl-2 family: the prosurvival cohort and the proapoptotic cohort. Family members share one or more BH1 to BH4 sequence motifs (numbered regions 1 to 4). A striped region indicates hydrophobic tail, present in some family members. (Adapted from Pellegrini and Strasser, 1999).
(O'Connor et al., 1998). These proapoptotic members can be subdivided into two groups depending on the number of BH regions they possess. Bax, bok, bak and bcl-xS have three or two BH regions, whereas bik, blk, Hrk, bim, bad, and bid have one BH domain (BH3) and are often referred to as BH3-only proteins. This BH3 domain is essential for the killer function of the proapoptotic members. Like bcl-2 some of these proapoptotic members have hydrophobic domains, but these proteins appear to remain cytosolic before an apoptotic stimulus (at least in case of bax, bad, and bim). The proapoptotic and antiapoptotic bcl-2 family members can physically interact with each other, sometimes in an antagonistic manner. The ability of these proteins to induce apoptosis is dependent on their ability to bind and antagonize prosurvival members. The converse does not always apply, the prosurvival function of bcl-xL does not require interaction with death promoters (activators) (Yin et al., 1994). It appears that the relative proportion of active suppresser to activator probably decides life or death in case of the bcl-2 family (Pellegrini and Strasser, 1999).

The mechanism by which death promoters and suppressers work is not yet well defined. One model is that the bcl-2 prosurvival homologues function by inhibiting the activity of Apaf-1 (directly or indirectly) or related adapter proteins and the subsequent activation of caspase-9 and possibly other initiator caspases (Chinnaiyan et al., 1997). The proapoptotic members may function by inhibiting this interaction allowing Apaf-1 to activate caspase-9. Another model suggests involvement of mitochondria. Perturbations in mitochondrial function may allow release of cytochrome c, which is able to activate Apaf-1 and the subsequent caspase cascade (Martinou, 1999). It has been postulated that the bcl-2 family members may act by regulating the release of cytochrome c and hence the interaction between Apaf-1 and caspase-9 (Chinnaiyan et al., 1997; Susin et al., 1996). It is not clear how the bcl-2 family members regulate cytochrome c release. One hypothesis is that bcl-2, bcl-xL, and bax form channels in the
mitochondria and thus inhibit or promote release of cytochrome c (Minn et al., 1997). However, studies monitoring the movement of cytochrome c through liposomes have shown that neither bak nor bax can form channels that allow passage of cytochrome c (Shimizu and Tsujimoto, 2000). Another hypothesis is that the bcl-2 family members regulate the opening of a pre-existing ion channel that normally would not permit passage of cytochrome c (Shimizu et al., 1999).

In addition to associating with each other, members of the bcl-2 family also associate with at least 12 other non-family proteins, including the protein kinase Raf-1, the protein phosphatase calcineurin, the GTPase R-Ras and H-Ras, the p53 binding protein (p53-BP2), the prion protein Pr-1, ced-4, cytochrome c, BAG-1, Nip-1, Nip-2 and Nip-3 (Reed, 1997b; Kharbanda et al., 1997). Interestingly, presence of BH4 domain in antiapoptotic proteins like bcl-2/bcl-XL but not in the proapoptotic proteins implies that antiapoptotic proteins could interact with the above mentioned proteins possibly through BH4 domain and retain them to intracellular membranes. Conversely, the proapoptotic protein, bax apparently does not interact with these proteins and hence may promote cell death (Reed, 1997a). Although the functional significance of the interaction of different proteins with bcl-2 is unclear, bcl-2 and its antiapoptotic homologues tethered to mitochondrial membrane could act as adapters or docking proteins which pull other proteins like Raf-1, calcineurin, p53-BP2, ced-4 and ced-3 from the cytosol and in turn inhibit apoptosis (Zou et al., 1997; Reed, 1997b; Chinmalyan et al., 1997; Golstein, 1997). bcl-XL also binds to cytochrome c and inhibits its translocation to cytosol (Figure 2) and thus prevents the apoptotic formation (Figure 4) and activation of cytosolic caspases and apoptosis (Kharbanda et al., 1997; Hengartner, 1998).

The bcl-2 protein is localized in the outer mitochondrial membrane, endoplasmic reticulum and nuclear membranes. The antioxidant role of bcl-2 is attributed to its localization in
mitochondria which is the center for redox reactions and ROS formation (Hockenbery et al., 1993). However, bcl-2 can also inhibit cell death in cells lacking mitochondrial DNA, and thereby respiration, suggesting that bcl-2 does not act via scavenging ROS or alternatively, utilizes other pathways (White, 1996; Jacobson et al., 1993). Differential subcellular localization of bcl-2 family members may induce apoptosis, for example, redistribution of soluble bax to mitochondria appears important for its death promoting activity (Hsu et al., 1997; Wolter et al., 1997). Bcl-2 inactivation by proteolytic cleavage is observed in HIV and alphaviral infections resulting in the loss of this key negative regulator of cell death (Strack et al., 1996; Grandgirand et al., 1998). This may lead to oxidative stress and NF-κB activation, finally leading to cell death. The three-dimensional structure of bcl-xl, an antiapoptotic member of the bcl-2 family reveals a striking similarity to the pore-forming domains of certain bacterial toxins/colicins suggesting that bcl-2 family members form channels in-vivo for either ions or proteins or both (Reed, 1997a; Muchmore et al., 1997). Thus, despite the 'Swiss army knife' like multiple independent functions of bcl-2, the precise mode of action of bcl-2 family proteins in regulating apoptosis has not yet been determined (Reed, 1997a; Hawkins and Vaux, 1997; Hengartner, 1998).

p53
The human p53 tumor suppressor is a multifunctional nuclear phosphoprotein (Bates, and Vosuden, 1996; Harris, 1996; Levine, 1997; Shimamura and Fisher, 1996) involved in regulating cell growth following exposure to various stress stimuli (Table 4 and Figure 6). p53 induces either growth arrest, which prevents the replication of damaged DNA, or apoptosis, which is important for eliminating defective cells. Whether the cell enters growth arrest or undergoes apoptosis, depends on the final integration of incoming signals with antagonistic effects on cell growth. p53 exhibits sequence specific DNA binding activity and directs interaction with different cellular and viral proteins. Mutations in
Figure 6: Induction of growth arrest by p53 and p53-mediated apoptosis through SST-dependent (solid arrows in the circle) and SST-independent (broken lines in the circle) mechanisms. (Modified from Vogt-Szem and Kupc, 1999).
p53 or inactivation through interaction with viral or cellular proteins are the most frequent alterations observed in cancer cells (Levin, 1997). This protein consists of an amino terminal activation domain, a central sequence-specific DNA binding domain, a carboxy terminal tetramerization domain and a regulatory region that controls switching from latent to the one that is active for DNA binding (Prives, 1994; Jayaraman and Privés, 1995). Although p53 is considered a "molecular guardian of the cell", it is neither essential for viability nor for apoptosis. However, p53 being a surveillance factor induces apoptosis under specific circumstances of cellular damage, whereas normal developmental pathways controlling apoptosis can operate independent of p53 and thus p53 is necessary for some, but not all forms of apoptosis (White, 1996). Elevated steady-state levels of p53 after DNA damage result in transcriptional activation of target genes such as p21 and GADD45. The p21 protein being the cell-cycle dependent kinase inhibitor, blocks the phosphorylation of retinoblastoma (Rb) and therefore prevents the cell entry into S phase (Harper et al., 1993; Sionov and Haupt, 1999). This cell cycle arrest mechanism permits repair of DNA damage (Figure 6) however, if DNA repair is inadequate, p53 promotes apoptosis (Figure 6), thereby preventing the propagation of genetic defects to successive generations (Yamaguchi et al., 1999). The role of p53-dependent transcription in apoptosis has been more controversial. p53 might activate the transcription of death genes such as bax (Miyashita and Reed, 1995) or repress the transcription of survival genes like bcl-2 (Miyashita et al., 1994), or both (Figure 6). p53 has also been reported to induce Fas expression—an alternate transcription-dependent action of p53 in regulating apoptosis. Wu et al. have demonstrated p53 mediated transactivation of cathepsin-D suggesting its role in p53-dependent apoptosis, induced by DNA damaging drug adriamycin (Wu et al., 1998). However, Caelles et al., 1994, showed that p53 can induce apoptosis in the presence of inhibitors of transcription and translation ruling out transcriptional activation of p53 as an exclusive mechanism of apoptotic induction (Caelles et al., 1994). It is possible that p53
can induce apoptosis through transcriptional repression (White, 1996). Thus, induction of bax expression promotes apoptosis and bcl-2 and E1B19K overexpression can block p53-dependent apoptosis. Simultaneous induction of apoptosis and growth arrest by p53 leads to cell death or growth arrest if bcl-2 like activity is low or high respectively. p53 also induces the transcription of its own negative regulator, mdm2 to turn off its own activity (Oliner et al., 1993). While p53 has recently been shown to be a general repressor of RNA polymerase III driven gene transcription (Cairns and White, 1998), it may also induce apoptosis through a transcription independent mechanism that has not yet been established. Also cellular p53 levels may decide between cell-cycle arrest or apoptosis, lower levels of p53 resulting in arrest whereas higher levels leading to apoptosis. Apoptosis and cell cycle arrest are two independent functions of p53 because a transcriptionally incompetent p53 can induce apoptosis but not cell cycle arrest whereas induction of p21 which is a major transcriptional target of p53 can induce arrest but not apoptosis. It seems that there is synergism between transcription dependent and independent functions of p53 in apoptosis (White, 1996). While p53-mediated growth arrest is dependent on its sequence-specific transactivation (SST) function, p53-promoted apoptosis is mediated by both SST-dependent and -independent pathways (Figure 6) (Amundson et al., 1998; Sionov and Haupt, 1998). A number of proteins are known to interact with p53 in-vitro and in-vivo which may be involved in the p53 dependent apoptotic pathway (Kubbutat and Vousden, 1998; Sionov and Haupt, 1999). However, the mechanisms by which p53 induces apoptosis is still unclear.

**Thioredoxin peroxidase**

Thioredoxin peroxidase is a member of a newly discovered family of proteins that is conserved from yeast to mammals with the gene sequence identical to the gene encoding natural killer enhancing factor-A (NKEF-A). NKEF is the major antioxidant present in the cytosol of human red blood cells protecting them from oxidative
injury. Thioredoxin peroxidase requires thioredoxin or a thiol-containing intermediate for its peroxidase function. It inhibits cell death in response to several different insults by a mechanism distinct from bcl-2 (Table 4) and probably upstream of the site of action of bcl-2 (Zhang et al., 1997). A stress protein, MSP23, known to have thioredoxin peroxidase activity have been shown to get induced in murine peritoneal macrophages exposed to oxidative stress (Ishii et al., 1999). Recently, a human and mouse peroxiredoxin V (Prx-V) have been identified which is biochemically a thioredoxin peroxidase. Overexpression of Prx-V has been shown to prevent the p53-dependent generation of reactive oxygen species thereby, inhibiting p53-induced apoptosis (Zhou et al., 2000).

Harbingers of death from Drosophila
Reaper, hid, and Grim:
The induction of apoptosis in Drosophila requires the activities of three closely linked genes, repear (rpr), grim and head involution defective (hid), whose gene products kill by activating a caspase pathway (Abrams, 1999; White et al., 1994). Reaper, hid and grim are all transcriptionally regulated by a variety of death-inducing stimuli, and the hid gene is repressed by active Ras signaling (Bergmann et al., 1998; Kurada and White, 1998). Therefore, it appears that these genes act as integrators for relaying different death-inducing signals to the core death programme (Song and Steller, 1999).

Rpr, hid and grim proteins appear to induce cell death by binding to and inhibiting the anti-apoptotic activity of DIAP1 (Wang et al., 1999). It has been proposed that IAPs act at multiple steps in the apoptotic pathway, both upstream and downstream of rpr, hid and grim (Vucic et al., 1998; Vucic et al., 1997; McCarthy and Dixit, 1998). However, these models are based on results obtained under non-physiological conditions. In particular, high-level overexpression of IAPs might protect against co-expression of rpr, hid and grim through complex formation and sequestration of pro-
apoptotic proteins, thereby suggesting that IAPs are most likely to be the downstream targets for rpr, hid and grim during the induction of apoptosis.

Recently, Drob-1, a Drosophila member of bcl-2/ced-9 family that promotes cell death has been identified. Drob-1 induced apoptosis accompanied by elevated caspase activity has been reported. However, Drob-1 induced cell death could not be antagonized by baculovirus p35, a broad caspase inhibitor suggesting that Drob-1 promotes cell death by inducing both caspase-dependent and -independent pathways at the mitochondria (Igaki et al., 2000).

**Induction of apoptosis by baculoviruses**

**Characteristics of AcNPV-induced apoptosis**

The baculovirus AcNPV was one of the first viruses known to induce and inhibit apoptosis (Clem et al., 1991; Clem, 1997). The spontaneous mutant of AcNPV known as the annihilator mutant (vAcAnh) was isolated as an occlusion negative plaque during the construction of an expression vector. Sf21 cells, derived from Spodoptera frugiperda did not produce polyhedra when infected with vAcAnh; but unexpectedly, the virus did not contain the foreign gene of interest. Moreover, vAcAnh-infected cells died much more rapidly than cells infected with wild type AcNPV, and the morphology of the dying cells was distinctly different from that of wild-type-infected cells. The first clue that cells were undergoing apoptosis was an obvious blebbing of the plasma membrane beginning at 9 hr post-infection (p.i.). The onset of blebbing in vAcAnh-infected Sf21 cells is somewhat asynchronous, i.e., most of the cells start to bleb between 12-24 h p.i. (Clem et al., 1991). After 24 h time interval, blebbing process intensifies and the cytoplasmic and nuclear content are shed into large membrane-bound vesicles called apoptotic bodies. These apoptotic bodies continue to exclude viral dyes for a number of hours after they are formed. In addition to the blebbing of cytoplasm and formation of apoptotic bodies, other characteristics typical of vertebrate apoptosis are also exhibited by vAcAnh-infected Sf21
cells. These include nuclear condensation and budding of nuclear material into the apoptotic bodies, a retention of intact mitochondria (and exclusion of vital dyes) until late in the apoptotic process, and internucleosomal cleavage of cellular DNA beginning between 6-12 h p.i. (Clem et al., 1991). The apoptotic death that these cells undergo appears to be remarkably similar to that of mammalian cells, except that the blebbing is usually much more rapid in these cells.

It seems that at least the initial stage of apoptosis is triggered by the wild type AcNPV infection of Sf21 cells, since a transient blebbing occurs that is similar to the initial stages of blebbing induced by vAcAnh (Clem et al., 1991). However, the blebbing seen in wild type infected cells is only transient, and it eventually disappears. The cells remain viable for several days before succumbing to a death that appears to be necrotic. The failure of the apoptotic response to progress in wild type infected cells suggested that a gene product either encoded or induced by AcNPV was able to intercede and prevent apoptosis from proceeding to completion. Marker rescue assays determined that the apoptotic death triggered by vAcAnh could be suppressed by a gene or genes in the EcoRI-S fragment of the AcNPV genome, and nucleotide sequencing revealed a deletion in the p35 gene of vAcAnh (Clem et al., 1991). The role of p35 gene in blocking apoptosis was confirmed by the replacement of a portion of p35 in wild type AcNPV with the lacZ gene. This p35 mutant virus had the same phenotype as vAcAnh, confirming that the p35 gene was responsible for blocking the apoptotic response during wild type infection (Clem, 1997).

Neither wild type AcNPV nor p35 mutant viruses induced apoptosis or transient blebbing in the TN-368 cell line derived from Trichoplusia ni (Clem et al., 1991). The phenotype of p35 mutant viruses appears to be completely normal in these cells. This was important as it allowed for the production of working stocks of the mutant viruses, which replicated poorly in Sf21 cells.
Even though p35 mutant infection triggered apoptosis in Sf21 cells, it was initially difficult to prove that the p35 gene product (p35) was acting directly on a cellular apoptotic pathway and not by suppressing viral initiation of apoptosis. The RNA synthesis inhibitor, actinomycin D can also trigger rapid apoptosis in these cells (Crook et al., 1993). Actinomycin D-induced apoptosis appears identical to p35 mutant-induced death, and this lead to the demonstration that p35 blocks apoptosis that is triggered by a non viral signal in the absence of other viral genes (Cartier et al., 1994; Clem and Miller, 1994).

Despite the presence of the p35 gene, wild-type AcNPV induces apoptosis in the cell lines SL2 from Spodoptera littoralis (Chejanovsky and Gersgberg, 1995) and CF-203 from Choristoneura fumiferana (Palli et al., 1996). Internucleosomal cleavage of cellular DNA is also observed in these cell lines, and AcNPV-induced apoptosis appears ultrastructurally similar in SL2 and Sf21 cells. However, the apoptosis observed in CF-203 cells appears to differ somewhat from that of Sf21 cells. While apoptosis triggered by p35 mutant virus infection in Sf21 cells is identical in appearance to that triggered by actinomycin D, there are morphological differences between the virus-induced death and actinomycin D-induced death in CF-203 cells (Palli et al., 1996). Since, CF-203 is the only one of the three cell lines that has been examined during apoptosis by electron microscopy (Palli et al., 1996), it is difficult to draw any solid conclusions at this point concerning the subtleties of the morphological differences between apoptotic death in the three cell lines.

Possible mechanisms of induction of apoptosis by AcNPV
Pretreatment of p35 mutant-infected Sf21 cells with the DNA synthesis inhibitor, aphidicolin blocks virus-induced apoptosis (Clem and Miller, 1994). This result, alongwith the timing of apoptosis induction (6-9 h p.i.), suggests that the initiation of apoptosis is somehow related to the transition from the early to
late stages of infection. During this crucial time, several important events are occurring simultaneously in the infected cells: (1) viral DNA synthesis is initiated, (2) late gene expression begins, and (3) the synthesis of cellular RNA and protein, along with the expression of early viral genes, is gradually shut down. However, it is difficult to determine which of these processes is responsible for the induction of apoptosis, since they are very closely interconnected (Clem, 1997).

1. Block in RNA synthesis:
It is clear that inhibiting RNA synthesis is sufficient to induce apoptosis in Sf21 cells (Clem and Miller, 1994). Treatment with actinomycin D, which intercalates into the DNA and prevents RNA elongation, 5, 6-dichlorobenzimidazole riboside, a nucleoside analogue, or α-amanitin, which binds to and inactivates the large subunit of RNA polymerase II, each induces rapid apoptosis in these cells (Clem and Miller, 1994). The fact that the three drugs, each of which inhibits RNA synthesis by independent mechanisms, and induce apoptosis with similar kinetics suggests that the induction is due to a specific effect. These results point to the suggestion that the cessation of post mRNA synthesis seen at late times in AcNPV infection (Ooi and Miller, 1988) could be responsible for the induction of apoptosis (Clem and Miller, 1994). Although the active process of apoptosis often requires RNA and protein synthesis, inhibition of these processes has been shown to induce apoptotic death in some situations (Raff et al., 1993). Presumably the continued synthesis of a short-lived inhibitory protein is required to prevent the initiation of death in these cells. It is puzzling that the protein synthesis inhibitor cycloheximide does not induce apoptotic death in Sf21 cells, despite the fact that it is potently active in this cell line (Clem and Miller, 1994). It could be possible that the synthesis of a specific RNA species is required to inhibit death, or perhaps the process of transcription itself along with its concomitant changes in chromatin structure is somehow involved. It also may simply be that cycloheximide is a more leaky drug that the RNA synthesis inhibitors tested, and only
low levels of an inhibitory proteins are required for inhibitory function.

2. Viral DNA replication:
It is equally possible that an unplanned round of DNA synthesis is what triggers the apoptotic death of AcNPV-infected Sf21 cells. When the viral genes necessary for DNA synthesis are transiently expressed in Sf21 cells, very little DNA can be recovered unless p35 is included in the transfections, suggesting that apoptosis is triggered by the expression of the viral DNA synthesis machinery (Lu and Miller, 1995). However, the expression of one of these genes, IE-1, has been shown to be capable of inducing apoptosis by itself (Prikhod'ko and Miller, 1996), and including p35 in transient gene expression assays that contain IE-1 also increases reporter gene expression (Gong and Guarino, 1994; Todd et al., 1995 and 1996). The ability of another antiapoptotic gene, Cp-iap, to replace p35 in these transient assays is another evidence to prove that apoptosis is involved (Lu and Miller, 1995; Prikhod'ko and Miller, 1996; Todd et al., 1995). As discussed above, it may be that either stimulus (cessation of RNA synthesis or induction of viral DNA synthesis) is sufficient to induce apoptosis in Sf21 cells, although it seems that transient expression of IE-1 or the DNA synthesis machinery is not nearly as efficient at inducing apoptosis as is inhibition of RNA synthesis (Prikhod'ko and Miller, 1996).

3. Possible apoptosis-stimulating viral genes:
Expression of the early viral transactivator IE-1 is sufficient to induce apoptosis in a sub population of transfected cells (Prikhod'ko and Miller, 1996). The observation that only about 25% of cells transfected with IE-1 undergo apoptosis suggests possible connection with the cell cycle. However, it is uncertain whether expression of IE-1 is a major trigger for apoptosis in infected cells (Prikhod'ko and Miller, 1996), since during infection IE-1 would still be expressed in the presence of aphidicolin, which blocks virus-induced apoptosis (Clem and Miller, 1994). However, these results could be consistent if IE-1
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induces apoptosis by directly stimulating an unscheduled round of cellular DNA synthesis (similar to adenovirus E1A) and aphidicolin acts by interceding at a point downstream of the IE-1 trigger. Whether or not aphidicolin blocks IE-1 induced apoptosis in uninfected cells has not been reported, but it has no effect on actinomycin D-induced death (Clem and Miller, 1994). Another piece of evidence questioning whether IE-1 is the major apoptotic trigger in infected cells is the observation that the temperature-sensitive IE-1 mutant tsB821 induces apoptosis in Sf21 cells at the nonpermissive temperature (Ribeiro et al., 1994). However, the level of apoptosis stimulated by tsB821 is not high and it is possible that a small amount of functional IE-1 is produced in these cells at the nonpermissive temperature. It is also noteworthy that the apoptosis that does occur in tsB821-infected cells coincides with the initiation of DNA synthesis, which is delayed (Ribeiro et al., 1994).

Another possible player in AcNPV-induced death is the p10 gene. Viral mutants lacking a functional p10 gene fail to lyse nuclei at late times (van Oers et al., 1993; Williams et al., 1989). This function of p10 appears to be analogous to that of the adenovirus death protein (Tollefson et al., 1996). The E3 region of adenovirus encodes a small (11.6 kDa) glycoprotein [adenovirus death protein (ADP)] that localizes in the nuclear membrane at late times in adenovirus-infected cells. The nuclei of cells infected with adenovirus mutants lacking ADP also do not lyse, similar to that of p10 mutants of AcNPV. The death of both adenovirus-infected cells and wild type AcNPV-infected Sf21 cells does not appear to be apoptotic in nature, but these deaths are in a sense programmed, since a specific viral gene product is required for final lysis of the infected cells or nuclei (Clem, 1997).

**Induction of apoptosis by other baculoviruses**

In addition to AcNPV, other baculoviruses have also been shown to induce apoptosis in lepidopteran cells. Mutants of the closely related virus, *Bombbyx mori* NPV (BmNPV) lacking functional p35
also induce an apoptotic response in BmN cells (Kamita et al., 1993), although only a portion of the infected cells undergoes apoptosis, and the apoptotic response is more delayed than vAcAnh-infected Sf21 cells. This delay may explain why virus protein synthesis and replication appeared normal despite the induction of apoptosis (Kamita et al., 1993).

The insect virus Hz-1, which was formally classified in the baculovirus family but is now temporarily unclassified, has also been shown to induce apoptosis upon superinfection of Sf9 cells (Lee et al., 1993). Although previously uninfected Sf9 cells died by necrosis, when infected with Hz-1, persistently infected Sf9 cell lines underwent extensive apoptosis when superinfected with wild type Hz-1. The few surviving cells continued to divide and eventually replaced the monolayer. The production of viral progeny in the apoptotic cells was greatly diminished, suggesting that the process of apoptosis may contribute to the establishment of persistent infection (Lee et al., 1993).

**Viral Protein Inhibitors of Caspases**

**Baculovirus and its antiapoptotic p35 gene**

Baculoviruses are virtually ubiquitous in the environment. They infect arthropods that inhibit terrestrial and marine ecosystems. But baculoviruses have never been found to cause disease in any organism outside the phylum Arthropoda. The Baculoviridae (Blissard and Rohrmann, 1990) is a large family of occluded viruses that are composed of two genera that are differentiated by the size of their occlusion bodies. The nuclear polyhedrosis viruses (NPVs) produce large polyhedron-shaped structure called polyhedra, which contain many virions, while the granulosis viruses (GVs) have smaller occlusion bodies called "granules", which normally contain a single virion. Baculoviruses have been used by the biotechnologists as expression vectors, as biopesticides and very recently, as mammalian cell gene transfer vectors. Their use as expression vectors originates from the fact that baculoviruses have strong promoters driving high level
expression of foreign genes at very late phase post-infection. Genes from a wide variety of sources can be expressed ranging from viruses, bacteria to higher eukaryotes (O'Reilly et al., 1992; Sridhar et al., 1994). This strong nature of baculoviral promoters as well as the versatility of baculoviruses makes them much sought after as expression vectors. Baculoviruses have large genomes with the potential to encode over 150 proteins (Ahrens et al., 1997; Ayres et al., 1994). Regulation of the 100 or more open reading frames required to accomplish productive infection is highly complex and involves sequential and coordinated expression of early, late, and very late genes. In the cascade of viral regulatory events, successive stages of virus replication are dependent on proper expression of genes within the preceding stage. Thus, critical to baculoviral replicative success is the appropriate expression and regulation of early genes. The products of early genes function both to accelerate replicative events and to prepare the host cell for the virus multiplication, which is enormously taxing for the cellular biosynthetic capacity. Specific early genes are also essential for virus-mediated regulation of the host, including the control of larval molting and evasion of host antiviral responses such as apoptosis. Thus, early baculoviral genes collectively contribute to host range determination. Transcription of strictly defined early genes usually peaks between 6 and 12 h after infection and declines thereafter when late viral transcription is vigorous. In the case of some early genes, transcription continues late into infection. Late transcription of these early/late genes, including p35, gp64 efp, 39K (pp31), and ie-1, is mediated by late promoter elements that overlap the early promoter (Miller, 1997). Since early p35 expression is involved in efficient suppression of apoptosis, p35 provides an example of an early gene that has a significant influence on viral replicative success. p35 is flanked by the early p94 gene and the hr5 transcriptional enhancer. It is transcribed within the first hour after virus inoculation to produce the polyadenylated, messenger active RNA α1 (Friesen and Miller, 1985 and 1987; Huh and Weaver, 1990). The p35 and p94 promoters are located within the 210 bp
intergenic region from which initiation of the p35 α1 and p94 α3 and α4 RNA occurs. Within the p35 promoter, the TATA element is the single-most important motif due to its contribution to promoter strength and role in determining the RNA start site (Dickson and Friesen, 1991). The p35 promoter lacks a CAGT motif. The p35 UAR (extending from position -90 to -30) confers a significant 10- to 20-fold stimulation of the basal promoter. Located immediately downstream from p35, the transcription enhancer hr5 also contributes to early p35 promoter activity. The p35 promoter also contains a consensus late promoter motif (TTAAG) at position -4 relative to the early +1 RNA start site (Pullen and Friesen, 1995). Presumably, such an arrangement prolongs expression of genes required both early and late in infection.

(i) Identification of the p35 gene:
The p35 gene was first identified in 1987 by sequence analysis of the region adjacent to the insertion of the transposable element TED (Friesen and Miller, 1987). Although the function of p35 was unknown until the mapping of the spontaneous mutation in vAcAnh in 1991 (Clem et al., 1991), the transcriptional regulation of p35 had already been studied extensively by that time.

(ii) Characteristics of the p35 polypeptide:
The protein encoded by the AcNPV p35 gene (p35) is a 299 amino acids long cytoplasmic protein and has a predicted molecular mass of 34.8 KDa. The p35 protein has no recognizable sequence motifs and lacks a signal sequence. The remarkable features of the p35 protein are several clusters of charged residues, including lysine-rich domains at the center of the molecule and at its carboxyl terminus, and additional highly charged domains in the amino terminal half of the protein (Bertin et al., 1996). Based on hydrophobicity calculations, these highly charged domains are predicted to be exposed on the outer surface of the molecule, and thus may participate in protein-protein interactions.
The p35 polypeptide is remarkably sensitive to mutation, including insertions, deletions, and amino acid substitutions. In the most comprehensive example of p35 mutagenesis to date, 11 out of 18 small (2 codon) insertions throughout p35 resulted in non-functional protein (Bertin et al., 1996). Most of the null mutations were in the amino terminal half of the proteins. However, the extreme carboxyl terminus of p35 is also important for its function, since mutations in this region also abolish anti-apoptotic activity (Bertin et al., 1996; Hershberger et al., 1992). In addition, Bertin et al., 1996, defined three charged regions in the amino terminal half of p35 that were designated CHR1, CHR2 and CHR3. Substitution of selective charged residues with alanine in these regions resulted in loss of anti-apoptotic function in 3 of 11 cases (Clem, 1997).

Several lines of evidence suggest that p35 must complex with itself or other proteins for stability and/or function. The amino terminal portion of the molecule can serve as a dominant negative inhibitor of p35 function (Cartier et al., 1994). When a construct encoding only the amino terminal 76 amino acids was stably expressed in Sf21 cells, the cells still underwent apoptosis upon wild type AcNPV infection, despite the expression of full length p35 protein. There was little or no accumulation of full length p35 protein in these infected cells despite the fact that other viral proteins accumulated to normal levels, suggesting that an amino terminal domain of p35 must interact with either itself or another protein in order to stabilize the entire p35 protein (Cartier et al., 1994). Many of the null mutants produced by Bertin et al., 1996, also exhibited decreased steady state levels of p35 protein, both in apoptotic Sf21 cells and in non-apoptotic Tn368 cells. The overall tertiary structure of p35 was not predicted to be greatly perturbed by these minor mutations, suggesting that protein-protein interactions were being disrupted (Bertin et al., 1996). Recently, Fisher et al., 1999 reported the 2.2 Å crystal structure of baculovirus p35. The p35 monomer possesses a solvent-exposed
loop that projects from the protein’s main β-sheet core and positions the requisite aspartate cleavage site at the loop’s apex.

(iii) p35 is an inhibitor of ICE-like proteases:
Given its ability to block apoptosis in a wide variety of organisms, p35 has been shown to potently inhibit several members of the ICE family of cysteine proteases (Bertin et al., 1996; Bump et al., 1995; Xue and Horvitz., 1995). The only other known protease with a requirement for aspartate in the P1 position is the serine protease granzyme B, which is involved in the killing of cells by cytotoxic T-lymphocytes (Shi et al., 1992). The ability of p35 to inhibit ICE proteases has been demonstrated in vitro using purified proteins as well as when expressed in COS cells. p35 inhibits the cleavage of prointerleukin-Iβ by ICE (Bump et al., 1995). In-vitro, purified p35 can efficiently block the activity of purified mammalian ICE homologues including ICE itself, ICH-1, ICH-2, and CPP-32 (Bertin et al., 1996; Bump et al., 1995), as well as the nematode ICE homologue, CED-3 (Bertin et al., 1996; Xue and Horvitz., 1995). As expected, p35 also inhibits an ICE-like activity induced in Sf21 cells by AcMNPV infection (Bertin et al., 1996). However, p35 has no inhibitory activity against granzyme B (Bump et al., 1995). Although both competitive (Xue and Horvitz., 1995) and non-competitive models (Bump et al., 1995) have been proposed to explain how p35 inhibits ICE, the ability of p35 to inhibit ICE at an equimolar ratio indicates that it is acting as an irreversible inhibitor rather than a competitive substrate (Bertin et al., 1996; Bump et al., 1995). p35 can be considered as a suicide inhibitor because it not only acts as a pseudo substrate but also as an irreversible inhibitor of active caspases. It has been shown by the interaction of p35 and caspase-3, as a model of the inhibitory mechanism, that it is classic slow-binding inhibition, with both active-sites of the caspase-3 dimer acting equally and independently (Zhou et al., 1998). Caspase mediated cleavage of p35 into 25 kDa and 10 kDa fragments is required for its antiapoptotic activity (Bump et al., 1995; Xue and Horvitz., 1995). The cleavage occurs between residues Asp87 and Gly88 of P35.
after an aspartate residue that is the characteristic cleavage site for the inhibition and complex formation (Bertin et al., 1996; Bump et al., 1995; Xue and Horvitz., 1995). The cleavage site is within one of the highly charged regions of p35, and thus is predicted to be exposed on the outer surface. Cleavage after residue 87 (P1 position) is necessary but not sufficient for anti-death function. Mutation of residue 84 (P4 position) results in a protein that is still cleaved but is no longer protective in Sf21 cells, providing further evidence that the cleaved protein must remain bound to the active site of ICE in order to function as an inhibitor (Bertin et al., 1996). Interestingly, the P4 mutant retains its ability to inhibit mammalian CPP-32 and ICE in vitro (Bertin et al., 1996). This tolerance at P4 position fits with the ability of p35 to inhibit ICE-like enzymes from a variety of organisms.

According to the recent observation, as mentioned earlier, the p35 crystal structure predicts that the RSL associates with the β-sheet core of P35 positioning the caspase cleavage site at the loop’s apex. Distortion or destabilization of this reactive site loop (RSL) by site-directed mutagenesis converted p35 to an efficient but non-inhibitory substrate which, unlike wild-type p35, failed to interact stably with the target caspase or block protease activity. Thus indicating that cleavage is insufficient for caspase inhibition thereby suggesting a new model wherein the p35 reactive loop participates in a unique multi-step mechanism in which the spatial orientation of the loop with respect to the p35 core determines post-cleavage association and stoichiometric inhibition of target caspases (Fisher et al., 1999). It has been shown that disruption of RSL interaction with the β-sheet by substituting hydrophobic residues caused loss of caspase inhibition, without affecting p35 cleavage. Restabilization of the helix/sheet interaction by charge compensation from within the β-sheet partially restored anti-caspase potency. Moreover, the identification of p35 oligomers in baculovirus-infected cells suggested that similar p35 interactions occur in vivo (Zoog et al., 1999).
A protease, Sfcaspase-1, has recently been identified from Sf9 (Spodoptera frugiperda) insect cells which is capable of cleaving p35 and is potently inhibited by p35 thus proving the involvement of caspase inactivation during the antiapoptotic pathway of p35 action (Ahmad et al., 1997). Though p35 was accepted as a general inhibitor of caspases, some recent reports suggest that certain p35-resistant caspases also exist. The caspase inhibitor p35 fails to rescue Drosophila caspase DRONC-induced cell death in-vivo and is not cleaved by DRONC in-vitro, making DRONC the first identified p35-resistant caspase. Furthermore, the overexpression of Drob-1, the first Drosophila member of the bcl-2/ced-9 family, induced apoptosis in Drosophila S2 cells accompanied by elevated caspase activity. This Drob-1-induced cell death, however, could not be antagonized by baculovirus p35, a broad-spectrum caspase inhibitor (Igaki et al., 2000).

p35 functions analogously to another viral inhibitor of ICE, the cowpox crmA protein however, p35 and crmA differ in several important ways. CrmA is a 38 kDa serpin like molecule from cowpox virus which upon cleavage binds stably to the active site of serine proteases leading to their inactivation (Ray et al., 1992). It functions to limit the host inflammatory response, thereby promoting viral infection. In contrast to p35, crmA is a potent inhibitor of granzyme B. CrmA expression blocks apoptosis induced by TNF-binding, CD-95-ligation, serum and nerve growth factor withdrawal, detachment from the extracellular matrix etc. By contrast, crmA is ineffective at blocking apoptosis induced by ionizing radiation, DNA damaging agents, staurosporine, ceramides etc. (Villa et al., 1997; Cryns and Yuan, 1998; Nicholson and Thornberry, 1997).

Functionally, p35 and bcl-2 are closely comparable antiapoptotic genes, but they differ in many ways: i) p35 is viral encoded, whereas, bcl-2 is of host origin; ii) Structural homology between them is negligible; iii) p35 is a soluble cytosolic protein while bcl-2 is predominantly membrane-associated, particularly where
oxygen metabolism is intense; iv) p35 is generally found in a relatively small amount and is known to inactivate ICE proteases and its homologues stoichiometrically to block apoptosis while Bcl-2 functions at the level of mitochondria i.e. upstream to caspase activation.

IAPs
A second class of antiapoptotic proteins known as inhibitors of apoptosis (IAPs) was first identified in baculoviruses and now known to be present in both mammals and Drosophila (Hay et al., 1995; Rothe et al., 1995; Duckett et al., 1996; Liston et al., 1996; Ambrosini et al., 1997). Normally the apoptotic response of the host cell to virus infection is thwarted by the AcNPV p35 protein. Mutants of AcNPV that lack functional p35 are unable to prevent the apoptotic response of the host cell which is shown to be blocked by Cp-IAP and Op-IAP present in two other baculoviruses (Cydia pomonella granulosis virus and Orgyia pseudotsugata NPV). Thus, Cp-IAP and Op-IAP presumably function by interacting with cellular components of the death pathway that are conserved between insects and mammals. Structurally IAPs are distinguished by the presence of the RING domain (zinc finger-like motif) at their C-termini and the BIRs (baculovirus IAP repeats) suggesting the zinc-binding ability requirement for the anti apoptotic activity (Crook et al., 1993; Birnbaum et al., 1994; Clem and Miller, 1994; Clem and Duckett, 1997). Several human homologues of IAPs have been identified which include survivin, NAIP, c-IAP1/hIAP-2/MIHB, c-IAP2/hIAP-1/MIHC and hILP/X-IAP/MIHA (Liston et al., 1996; Ambrosini et al., 1997), among which NAIP has BIRs but lacks RING domain. The Drosophila homologues of IAPs are DIAP1 and DIAP2/dILP. Although most IAPs appear to have antiapoptotic activity their mechanism of action seems to be different. The antiapoptotic effects of mammalian IAPs may occur by modulation of the aggregation of unknown adapter proteins associated with the initial activation of apoptosis. XIAP, c-IAP1 and c-IAP2 also seem to function by inhibiting the distal death proteases, caspase-3 and -7 without cleavage, probably by directly blocking active sites
of these caspases (Table 4) (Roy et al., 1997). Recently a Drosophila nuclear protein, DOOM produced from an alternatively spliced transcript of mod gene (regulator of chromatin structure) when overexpressed was found to induce apoptosis in S21 cells (Harvey et al., 1997). DOOM when coexpressed with IAPs (cytosolic proteins) results in translocation of IAPs to the nucleus suggesting that IAPs function by preventing the apoptotic action of DOOM.

**Chemical inhibitors of apoptosis with special reference to caffeine**

The major area of thrust in apoptosis is to identify agents that could inhibit apoptosis. These antiapoptotic agents could be used either to enhance the life span of cells or as potential therapeutic molecules in the treatment of diseases associated with enhanced apoptosis. There is strong evidence to believe that reactive oxygen species (ROS) play an important role in specific signaling pathways that can trigger PCD (Jacobson, 1996). Various antioxidants such as vitamin E, benzoate, BHA (butylated hydroxyanisol), vitamin C, glutathione can act as antiapoptotic agents. Caffeine is a well known biomolecule with a potential antioxidant and antiapoptotic activity. Caffeine is pharmacologically a very important molecule with ability to act on various cellular targets. Caffeine, with potent antioxidant abilities, is known to act differently depending on its concentration and the nature of the apoptotic stimuli etc. (McKenna et al., 1996; Kesavan et al., 1991). Several experiments suggest that caffeine can reduce the cytotoxic/cytostatic activity of aromatic agents displaying DNA intercalating property and inhibiting DNA topoisomerase II activity (Traganos et al., 1991a and 1991b; Kimura and Aoyama, 1989). Additionally, it has also been found to be effective on non-intercalating, aromatic compounds that can inhibit DNA topoisomerase I (Traganos et al., 1993). In contrast, however, caffeine also has the tendency to potentiate the cytotoxic effects of DNA damaging agents such as ionizing radiation and alkylating agents by suppression of the DNA repair mechanisms (Labanowska et al., 1988; Fingert et al., 1986). Caffeine can also induce mitotic events in early S-phase arrested
BHK cells by induction of translation or stabilization of the protein product(s) of mitosis-related RNA that accumulates in the S-phase cells when the replication of DNA is suppressed (Schlegel and Pardee, 1986; Schlegel et al., 1987). Caffeine has also been shown to induce apoptosis in S-phase-arrested HeLa cells by stimulating the cyclin A-dependent kinase activity (Meikrantz et al., 1994) which is consistent with another study which shows that cell killing by TNF-α is potentiated by caffeine in other cell types (Belizario et al., 1993). Caffeine can also enhance paclitaxel-induced apoptosis from 43% to 88% in MCF-7 breast cancer cells (Saunders et al., 1997).

**Death associated proteins**
The technical knockout (TKO) strategy based on random inactivation of functionally relevant genes involved in cell death by transfecting cells with antisense cDNA expression libraries in the presence of an apoptotic stimulus has identified seven different genes, including thioredoxin and cathepsin-D (Table 4), of which five are novel genes. These genes are termed death associated protein genes namely DAP-1 through DAP-5 (Kissil and Kimchi, 1998).
Materials & Methods
MATERIALS

Cell culture media, chemicals, enzymes, and radioisotopes were obtained from the following companies:

Amersham plc, United Kingdom: $^{32}\text{P-}\alpha\text{dCTP}$, multiprime DNA labeling kit, Rainbow protein molecular weight markers, Hyperfilm MP, phenol, Tris

Amresco Inc., USA: ampicillin, kanamycin, acrylamide, TWEEN 20

Bengal Chemicals, India: ethanol

Bangalore Genel, India: restriction enzymes

Boehringer Mannheim GmbH, Germany: deoxynucleotides, T4 DNA ligase

BRL, USA: TEMED

Difco, USA: bacto-agar, bacto-trypton, lactoalbumin hydrolysate

Gibco BRL, USA: Grace's basal insect cell culture media, 1 kb DNA molecular size marker, lipofectin, fetal calf serum

Merck, India: hydrogen peroxide, methanol

Nestle, India: non-fat dry milk

New England Biolabs, USA: DNA polymerase I-klenow fragment, restriction enzymes

Pharmacia, Sweden: Sephadex G-50, DTT
Materials and Methods

Pharmingen International, USA: purified mouse anti-cytochrome c monoclonal antibody and polyclonal rabbit anti-caspase-3 (CPP32) antibody

Promega Corporation Inc., USA: Taq polymerase, restriction enzymes

Pronadisa, Europe: agarose

Qualigens, India: chloroform, glycerol, HCl, isoamylalcohol, isopropanol, KCl, HCl, MgCl₂, NaCl, NaOH

Sigma, St. Louis, USA: ammonium persulphate, amphotericin B, bisacrylamide, bovine serum albumin, bromophenol blue, CaCl₂, caffeine, DMSO, EDTA, EGTA, HEPES, hydroxylamine, KOH, salmon sperm DNA, sucrose, Triton X-100, trypan blue dye, thioglycollate medium and partially acetylated cytochrome c from horse heart

USB, USA: mineral oil

Oligonucleotides: Most of the oligonucleotides were commercially obtained from Rama Biotechnologies, India.
METHODS

Insect cell and virus culture

*Spodoptera frugiperda* (Sf9) cells that serve as host for AcNPV were grown in complete medium (CM) (TNMFH medium (Gibco-BRL, USA) supplemented with 10% fetal bovine serum and antibiotics) as described by Summers and Smith, 1987.

**TNM-FH medium**

TNM-FH medium (Hink, 1970) is Grace's basal insect culture medium (Grace, 1962) supplemented with lactalbumin hydrolysate and yeast autolysate. It supplies basic nutrients to the cells and has a pH of 6.2 buffered with sodium phosphate. To make 1 L of TNM-FH, 46.3 g of Grace's medium was dissolved in 700 ml of water. 0.35 g of NaHCO₃ was added and the pH adjusted to 6.2 with 10 M KOH. 3.33 g each of lactalbumin hydrolysate and yeastolate were then added and the volume made up to 1 L. The medium was sterilized by passing through a 0.22 μm filter. Cells were maintained at 27 °C in complete medium prepared by adding 10% fetal calf serum, 50 μg/ml gentamycin and 5 μg/ml amphotericin B to TNM-FH medium. Sf9 cells grow as a monolayer and have a doubling time of 18-24 h at 27 °C.

**Monolayer culture**

Cells were maintained in 25 cm² tissue culture flasks (Corning, USA) and were subcultured when they were 90% confluent. The cells were dislodged by washing the surface by gentle pipetting. For each subculture, about 2 X 10⁶ cells were seeded in a 25 cm² flasks in 5 ml of CM. The viability of cells was checked by staining with 10% v/v trypan blue (non-viable cells stain blue). Only cells with viability >90% were used for experiments.

**Infection of insect cells**

During viral infection, 1.5 X 10⁶ cells growing in complete media were seeded for 30 min in 35 mm 6-well plate or 35 mm polystyrene tissue culture dishes (Nunc, Denmark). After 30 min,
TNM-FH was removed and AcNPV or δAcNPV stock, diluted in CM to achieve moi of 10 PFU/cell, was added to each dish or well. The dishes or plates were rocked gently at intervals for ~60 min to allow virus attachment and entry. The viral inoculum was removed after 1 h and 1.5 ml CM was added to each dish. The infected cells were incubated for 6-8 h to check for the expression of p35 gene.

**Preparation of rat peritoneal macrophages**

**Elicitation of macrophages**

All studies were conducted on thioglycollate-elicited macrophages from rat unless otherwise mentioned. 3% Brewer's thioglycollate medium used for this purpose was autoclaved and aged in dark for atleast one week at room temperature before use. Each animal was injected 10 ml of thioglycollate medium, i.p. On day 4, animals were sacrificed by cervical dislocation and macrophages isolated from the peritoneal cavity.

**Isolation of peritoneal macrophages**

30 ml of ice-chilled, sterile Hanks Balanced Salt Solution (HBSS) was injected into the peritoneal cavity by a 21 gauge needle without striking the viscera. Abdominal wall of the animal was shaken and the exudates from the peritoneal cavity withdrawn using 18 gauge needle. The peritoneal exudate cells were collected in 50 ml ice-chilled sterile tubes. Cells were washed twice by centrifugation at 3,500 rpm for 5 min and resuspended in HBBS. Cells were resuspended in ice-cold RPMI-1640 medium after the final wash.

**Culture of macrophages**

Cells were cultured in RPMI-1640 medium (Gibco BRL, USA) supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin at cell density 1X10^6/ml in 6 well plates. Cells were initially incubated at 37 °C in humidified atmosphere of 5% CO₂ for 2 h to allow macrophages to adhere to the surface culture plates, after this the non-adherent cells were
removed by vigorously washing with HBBS prewarmed at 37 °C. Adherent monolayer of macrophages was further cultured by adding fresh RPMI medium and was used for various experiments.

**Assay for apoptosis in S99 cells**

Cell number was assessed using a haemocytometer. About $10^4$ cells assayed for apoptosis microscopically by cellular blebbing or by oligonucleosomal DNA ladder assay.

**Morphological assay**

For microscopic examination for apoptosis, the cell viability and cellular blebbing were determined by the ability of live cells to exclude trypan blue. An aliquot of cells were stained with 10% trypan blue and apoptosed cells, characterized by cellular blebbing, were visualized (20X magnification) under a light microscope (Nikon TMS Model, Japan).

**Biochemical assay**

Oligonucleosomal DNA ladder assays were carried out following a procedure adapted from Ishida et al., 1992. Briefly, cells were harvested after 24 h post-treatment by low speed centrifugation at 4,000 rpm (Heraus Biofuge, Germany) for 10 min. The apoptotic bodies present in the supernatant were collected by centrifugation at 14,000 rpm for 20 min at 4 °C in F-20/MICRO rotor in a Sorvall High Speed Centrifuge. The cells and the apoptotic body pellets were pooled and suspended in ice cold lysis buffer (10 mM Tris-Cl, pH 7.5, 10 mM EDTA, 0.2% Triton X-100) and processed for isolation of low molecular weight DNA as described previously (Ishida et al., 1992). The DNA samples were treated with RNaseA (10 μg/μl for 10 min) prior to electrophoresis. Oligonucleosomal DNA was electrophoresed in 2% agarose TBE (0.5 X; 0.045 M Tris-borate, 10 mM EDTA) gel and the ladder visualized by ethidium bromide staining (Hershberger et al., 1994, Sambrook et al., 1989).

**Induction of apoptosis**
**Oxidative stress-induced apoptosis in insect cells**

Apoptosis was induced in cells by subjecting them to oxidative stress generated by $H_2O_2$ treatment or by exposing them to UV radiations. Different concentrations of $H_2O_2$ were used to induce apoptosis in Sf9 cells and the dose showing more than 50% apoptotic response was selected. $1.5 \times 10^6$ cells growing in complete media were seeded for 30 min in 35 mm 6-well plate or 35 mm polystyrene tissue culture dishes. To the media was added different concentrations of $H_2O_2$ (0-1.5 mM) and cells were incubated at 27 °C for 24 h. After the treatment, the cells were assayed for apoptosis by nucleosomal DNA ladder formation as well as cellular blebbing. For the dose-dependent induction of apoptosis by hydroxylamine, cells were pretreated with different doses of hydroxylamine and monitored for apoptosis after 24 h. DMSO-mediated inhibition of $H_2O_2$ induced apoptosis was conducted by pretreating the cells with 75 mM DMSO and apoptosis was scored after 24 h. For studying UV radiation-induced apoptosis, Sf9 cells seeded in complete media were exposed to UV for 35 sec at room temperature using a UV transilluminator (Ultra Violet Products, Upland, CA, USA) equipped with 6 W (312 nm) bulbs. The exposure was given from the bottom of the dishes by placing them directly over the UV lamps fixed in the UVP transilluminator. After UV radiation, cells were incubated at 27 °C for 24 h under dark conditions and assayed for apoptosis.

**Hydrogen peroxide-induced apoptosis in macrophages**

Apoptosis was induced in macrophages by exposure to different concentrations of hydrogen peroxide as described earlier in insect cell system. $1.5 \times 10^6$ cells were seeded for 2 h in 35 mm tissue culture plates and exposed to $H_2O_2$ ranging from 100-750 μM. Cells were microscopically observed at 6, 12, and 24 h post-treatment for cell membrane blebbing and the appearance of apoptotic bodies followed by nucleosomal DNA ladder assay. The LC-50 value was calculated after carrying out a concentration- and time-dependent exposure of macrophages to $H_2O_2$. The treated plates were covered with aluminium foil to prevent light induced
DNA repair. Macrophages were examined for time- and dose-dependent induction of apoptosis at specified duration.

**Plasmid construction**

The baculovirus *p35* gene was cloned in pGem7zf(+) vector under the *Drosophila* heat shock 70 gene promoter (*hsp 70*). A 480 bp *hsp 70* gene fragment carrying the promoter was excised from the pAcDZ1 (Zuidema et al., 1990) by *XbaI* and *EcoR1* and cloned in the multiple cloning region of pGem7zf(+) to generate the construct pNU1. The baculovirus *p35* gene was PCR-amplified using low error *Pfu* polymerase (Stratagene, Germany) and forward (5' *Kpn1*GGGGTACCTTGTA'TTAAGTGAGCATGAG) and reverse (AAATTATTGCCTAATATTATTTTGAGATCCCGBamHI 3') primers obtained from Rama Biotechnology, Secunderabad, India. PCR was carried out for 30 cycles comprising of: denaturation 94 °C, 1 min; annealing 50 °C, 1 min; extension 72 °C, 2 min. The amplified *p35* DNA was restricted with *Kpn1* and *BamHI* and cloned in pNU1. This construct possessing the *p35* gene downstream to the *hsp 70* promoter was sequence verified and designated as pNN1 (Figure 2.1). A third plasmid, pNN2 was constructed where *p35* was placed without *hsp* to serve as a control. For this, pNN1 was restricted with *XbaI* and *EcoR1* to remove the 480 bp *hsp* fragment. The rest of pNN1 (without *hsp*) was end-filled and ligated to generate pNN2.

pCMVp35, a pGEM7Zf(+) based recombinant plasmid, carrying the baculovirus *p35* gene under the transcriptional control of *CMV* early promoter was also constructed for expression of *p35* gene in mammalian system (Figure 2.2). pNN2 was linearized by digesting it with *Kpn1*. The fragment containing *CMV* promoter was retrieved as a *Kpn1* fragment from a recombinant plasmid, pBVLuc, containing luciferase gene under the control of CMV promoter (Raman et al., 1998) and cloned in pNN2. The new construct was verified by restriction digestion. The plasmids were propagated in competent *E. coli* DH5α cells and isolated and purified on a Qiagen column.
Figure 2.1. Schematic representation of p35 cloning (pNN1)
Linearize the plasmid by Digestion with KpnI

Ligation

Figure 2.2. Schematic representation of pCMVp35 cloning
DNA extraction and PCR analyses from mice tissue

Swiss albino mice were injected (both i.d. and i.m.) with a single dose of 100 μg of pCMVp35. Three weeks later, mice were sacrificed and total DNA was isolated from the tissues by overnight digestion at 65 °C in homogenization buffer (50 mM Tris, pH 8.0; 100 mM EDTA, pH 8.0; 100 mM NaCl; 1% SDS; 0.5 mg/ml proteinase K). Samples were extracted once with phenol and chloroform, washed with 70% ethanol, dried, and resuspended in 50 μl of TE buffer. PCR reactions were done using forward and reverse primers (5’ KpnI and 3’ BamHI), designed for the cloning of p35 gene. PCR reactions were run under similar conditions as mentioned earlier.

Preparation of competent cells

*E. coli* strain, DH5α was used for all transformation experiments. *E. coli* DH5α was inoculated into 5 ml of LB medium (10 g of tryptone, NaCl each and 5 g of yeast extract dissolved in 800 ml of distilled water and the mixture made upto 1 L) and grown at 37 °C overnight with constant shaking at 250 rpm. The fully saturated culture (1.25 ml) of the above culture was inoculated into 150 ml of LB medium and incubated at 37 °C for 1-2 h till an OD₆₅₀ of 0.25-0.3 was attained. The culture was centrifuged at 6,000 g for 10 min at 4 °C and the pellet resuspended gently in 50 ml of ice-cold 0.1 M CaCl₂. After incubating for 20 min on ice, the suspension was centrifuged at 6,000 g for 10 min at 4 °C and resuspended again in 8 ml of ice-cold 0.1 M CaCl₂. Glycerol was added to a final concentration of 15%. The suspension was aliquoted and stored at -70 °C.

Gene injection

With a 1 ml syringe and a 28 gauge needle, Swiss albino mice were injected either intradermally (i.d.) or intramuscularly (i.m.). Intradermal inoculations were given 1-2 cm distal from the tail base, with 100 μg of free pDNA (pCMVp35) in normal saline making a total volume of 100 μl (Raz et al., 1994)
**Transformation of competent cells**

100 μl of competent E. coli DH5α cells were mixed with 100 ng of plasmid DNA and incubated on ice for 30 min. After heat-shock at 37 °C for 5 min, 500 μl LB medium without antibiotics, was added and the cells were incubated at 37 °C for 30 min. Thereafter, the cells were pelleted by centrifuging in a microfuge at 12,000 rpm for 1 min. The cells were resuspended in 100 μl of LB without antibiotics and then plated onto LB agar plate containing ampicillin (100 μg/ml). Colonies of transformed cells were checked for the presence of plasmid DNA.

**Preparation and isolation of plasmid DNA**

A single colony of transformed cells was picked up and inoculated into 250 ml of LB medium containing ampicillin (100 μg/ml) and grown in an incubator-shaker at 37 °C overnight. Plasmid DNA was isolated from these cells using Qiagen columns (Qiagen, GmbH, Germany).

**Northern hybridization of total RNA extraction from Sf9 cells and tissues**

RNA was isolated from cultured cells and tissues for Northern blot analysis. Insect cells from a 35 mm culture dish were washed twice with 1 ml of ice cold PBS. The cell (1.5 x 10⁶) pellet was resuspended in 1 ml of denaturing solution (4 M GITC; 25 mM sodium acetate, pH 7.0; 0.5% sarcosyl and 0.1 M β-ME) followed by the addition of 50 μl of 2 M sodium acetate (pH 4.0), 500 μl water saturated phenol and 100 μl chloroform-isoamylalcohol (49:1). However, in case of tissue samples from control and injected mice (both i.d. and i.m.) samples were extracted 14 days post injection. Extracted tissue samples were minced on ice and then homogenized in 1 ml of denaturing solution in a glass-Teflon homogenizer. Similar protocol of isolation was followed both in case of cultured insect cells and tissue samples from mice (Chomczynski and Sacchi, 1987). Briefly, the homogenized suspension was mixed thoroughly after the addition of each
reagent, vortexted for 10 sec and cooled on ice for 15 min. The aqueous and phenol phase were separated by centrifugation at 12,000 rpm for 20 min in a refrigerated microfuge. The aqueous phase was transferred to a fresh tube followed by the addition of 500 μl isopropanol. RNA was allowed to precipitate at -20 °C for 1 h and then centrifuged at 12,000 rpm for 20 min in a refrigerated microfuge. The RNA pellet was dissolved in 300 μl denaturing solution followed by the addition of 300 μl isopropanol. RNA was reprecipitated at -20 °C for 1 h, centrifuged at 12,000 rpm for 10 min in a refrigerated microfuge, washed with 75% ethanol and the pellet collected by centrifugation at 12,000 rpm for 15 min. RNA was dissolved in 25 μl of 0.5% SDS by heating at 65 °C for 10 min and stored at -70 °C. Total RNA was subjected to Northern hybridization using 32P labeled gene specific probe (Sambrook et al., 1989).

**Colony hybridization**
The bacterial colonies obtained after CaCl₂-mediated transformation of the ligation mix were transferred onto a nylon membrane (Hybond-N+, Amersham plc, UK) and grown overnight at 37 °C. The filter was treated as follows by keeping it on a piece of polythene sheet with colonies facing upwards:

The DNA was denatured twice for 2 min each with 0.5 N NaOH. The alkali was neutralized with 1 M Tris-Cl (pH 7.5) for 5 min followed by treatment with 0.5 M Tris-Cl (pH 7.5) and 1.5 M NaCl for 5 min. The filter was air dried and the DNA immobilized by baking at 80 °C for 2 h before processing for prehybridization and hybridization.

The blot was kept in prehybridization solution at 55 °C containing 6X SSC (pH 7.0), 5X Denhardt's solution, 200 μg/ml sonicated salmon sperm DNA, and 0.05% SDS (Sambrook et al., 1989). (175.3 g of NaCl and 88.2 g of sodium citrate was dissolved in 1 L water and the pH adjusted to 7.0 with 100 mM citric acid to prepare 20X SSC. 10 g each of Ficoll, PVP, and BSA was dissolved.
in 500 ml water to prepare 50X Denhardt's solution. DNA probe (50 ng, $10^6$ cpm) was added after 4 h of prehybridization and the blot was hybridized at 55 °C for 16 h. The membrane was washed with following concentrations of SSC and SDS:

- 2X SSC - 2 X 10 min at RT
- 0.2X SSC with 0.1% SDS - 2 X 10 min at 55 °C
- 0.1X SSC with 0.1% SDS - 1 X 10 min at 65 °C

The membrane was then dried, and subjected to autoradiography. Positive clones were then identified using labeled p35 gene fragment.

**Preparation of radiolabeled p35 gene fragment (969 bp)**

50 ng of DNA fragment was labeled by random priming using a multiprime DNA labeling kit (Amersham, UK). DNA was denatured by boiling for 5 min and chilled on ice for 10 min. Random hexanucleotide primers, dNTPs (-dCTP) in a concentrated buffer solution [containing Tris-Cl (pH 7.8), MgCl$_2$, and β-mercaptoethanol], $^{32}$P-αdCTP (30 μCi) and Klenow fragment of E. coli DNA polymerase I (2 units per reaction) were added and the reaction volume made up with water to 50 μl. The reaction was incubated at 37 °C for 30 min. The enzyme was inactivated by heating at 65 °C for 5 min and the probe was separated from unincorporated nucleotides on spun-columns.

For spun-column chromatography, a 1 ml syringe was packed with a slurry of sephadex G-50, equilibrated with TE buffer. The column was placed in 1.5 ml microcentrifuge tube and centrifuged for exactly 1 min at 1,600 rpm in a microfuge to pack the column tightly. The volume of the labeling reaction was made upto 100 μl with TE buffer and loaded onto the column which was again spun for 1 min at 1,600 rpm. The unincorporated nucleotides were retained in the column while the labeled probe was eluted out. The specific activity of the probe was determined by liquid scintillating counting.
Rescue of apoptosis in Sf9 cells by p35
For p35-mediated inhibition of oxidative stress-induced apoptosis, 1.5 x 10^6 Sf9 cells were infected with wild type or p35 deletion mutant AcNPV (~10 moi) and incubated for 0-6 h before treating them with 1 mM H_2O_2 or exposing them with UV (35 sec). Cells were harvested at 24 h post-treatment. A small aliquot (45 μl) of the cell suspension was used to score for the percentage apoptotic cells and the rest was subjected to oligonucleosomal DNA ladder analysis. For transfection of Sf9 cells with the recombinant plasmid, 1.5 x 10^6 cells were transfected in a 35 mm dish with 8-12 μg of pNN1 DNA for 6 h using lipofectin (Gibco BRL, USA) as described (Habib and Hasnain, 1996). After transfection, cells were thoroughly washed with complete medium and incubated in the same for 24 h at 27 °C. Heat shock treatment was given at 42 °C for 30 min followed by incubation for 4 h at 27 °C for the expression of p35 gene. Cells were then subjected to H_2O_2 treatment and were monitored for apoptosis 24 h post-treatment.

Rescue of hydrogen peroxide-induced apoptosis in macrophages by p35 transfection
1.5 x 10^6 cells were seeded for 45 min in RPMI medium and transfected with various concentrations (1-10 μg) of pCMVp35 plasmid construct for different time periods (10 min-24 h) using lipofectin (Gibco BRL, USA) as described earlier (Habib and Hasnain, 1996). After transfection of the macrophages for different time periods, the culture medium was removed and fresh media was added. This was followed by addition of H_2O_2 (100-750 μM) in dark conditions. The treated plates were covered with aluminium foil to avoid light-dependent DNA repair. The cells were processed for further apoptotic parameters after 24 h following H_2O_2 treatment.

Rescue of apoptosis in Sf9 cells by caffeine
For caffeine-mediated inhibition of apoptosis, Sf9 cells (1.5x10^6) were seeded for 45 min and then incubated for 4 h in Grace's
insect culture media containing different concentrations of caffeine before subjecting the same to \( \text{H}_2\text{O}_2 \) (750 \( \mu \text{M} \)) treatment or UV exposure (30 sec). Cells were then harvested for morphological observation and processed for nucleosomal DNA fragmentation assay.

**ESR monitoring of ROS generation in Sf9 cells**

*In vitro* generation of \( \text{O}_2^\cdot \) was mediated by incubating cells with the xanthine/xanthine oxidase system (X+XO) (Sigma, St. Louis, USA) at 37 \( ^\circ \text{C} \) for 30 min in 50 mM phosphate buffer (pH 7.5) with or without purified recombinant p35 protein. *In vivo* generation of ROS (OH\(^\cdot \)) was maintained through the Fenton reaction. For this, cells were loaded with iron by incubating them for 10 min with 10 \( \mu \text{g}/\text{ml} \) aqueous solution of iron-sorbitol obtained from Rallis, India. The iron-loaded cells were treated with 1 mM \( \text{H}_2\text{O}_2 \) and immediately exposed to UV (~312 nm, \( 1.8 \times 10^{-6} \) watts/cm\(^2 \) at lamp to target distance) for 30 min and then subjected to ESR spectrometry at room temperature with Field 3280±100 G, sweep time 4 min, frequency modulation 100 KHz 0.63x10 G, amplitude 5x10\(^3 \), power 20 mW, RF 9.44 and response 0.3 sec. DMPO (5,5 dimethyl-1-pyroline N-oxide) obtained from Sigma, USA was used as a spin-trap in all these experiments. ESR integrated absorption intensity (I) was calculated by the formula \( I=K\cdot W^2\cdot h \), where K=line shape constant \((6.5 \times 10^{-10})\), W=width of the lines, h=height of the peak lines.

**Preparation of cell lysate and western blotting for detection of released cytochrome c**

Released cytochrome c was detected in the cytosolic fraction as described (Liu *et al.*, 1996 and Ramasarma, 1992). Sf9 cells were pelleted by low speed centrifugation and washed with 1X PBS. The pellet was then suspended in 100 \( \mu \text{l} \) lysis buffer (20 mM HEPES-KOH; pH 7.5, 10 mM KCl, 1.5 mM MgCl\(_2\), 1 mM Na-EDTA, 1 mM Na-EGTA, 0.1 mM PMSF, 1 mM DTT and 250 mM sucrose) and homogenized (20 strokes) in a Dounce homogenizer using B pestle. The cytosolic fraction was collected after differential
centrifugation by pelleting down mitochondria at 1,00,000 X g for 1 h in Sorval Ultra pro8. The supernatant (cytosolic fraction) was dialyzed for 24 h against 20 mM NaH₂PO₄. Protein was estimated from the dialyzed fractions using the BCA protein assay kit (Pierce, Rockford, USA) and equal amounts were fractionated by electrophoresis on 15% SDS-PAGE, and processed for Western Blot analysis by transferring the gel to a nylon membrane (Hybond-C Extra protein transfer membrane, Amersham, UK) at 300 mA for 2 h at 4 °C [Transfer buffer-25 mM Tris, 190 mM glycine, 20% methanol]. The membrane was blocked with 3% non fat dry milk for 2 h and then incubated with commercial mammalian cytochrome c antibody (Pharmingen, USA) (1 μg/ml, dilution) for 1 h followed by HRP-conjugated anti-mouse IgG as the secondary antibody. The blot was developed using the ECL protein detection kit (Amersham Pharmacia Biotech UK Limited, England).

**Western blot analysis for assaying caspase-3 activation**
pCMVp35 transfected cells were pelleted, washed twice with ice-cold 1X PBS and isolated the nuclei. The supernatant containing apoptotic bodies was pelleted at 13,000 rpm for 20 min in Sorval SS34 rotor at 4 °C. Both nuclear and apoptotic body pellets were pooled together and incubated in lysis buffer (1% TritonX-100, 320 mM sucrose, 5 mM EDTA, 1 mM PMSF, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 2 mM DTT, 10 mM Tris/HCl, pH 8) for 15 min at 4 °C, The lysate was centrifuged at 20,000 X g for 2 min (Dimmeler *et al.*, 1997) and the supernatant was assayed for caspase activation by western blotting as described above. For caspase-3 immunoblotting, the membrane was incubated with anticaspase-3 antibody (1:2000 dilution) followed by incubation with HRP-conjugated anti-rabbit IgG. Detection of chemiluminescence was carried out using an ECL Western Blot detection kit (Amersham), according to the manufacturer's instructions.
Results
With an extensive data available on the caspase inhibitory action of p35, we tried to decipher the mechanism of action of p35 in preventing oxidative stress-induced apoptosis.

3. p35 and oxidative stress-induced apoptosis in Sf9 insect cells:

3.1. Insect cells undergo apoptosis as a function of oxidative stress
Experiments were first designed to establish the ability of Sf9 insect cells to enter apoptosis as a direct function of oxidative stress. This was achieved by directly treating cells with hydrogen peroxide (H$_2$O$_2$) (Figure 3.1. A and B) or by increasing the intracellular build up of H$_2$O$_2$.

Concentration-dependent induction of apoptosis by H$_2$O$_2$-treatment in insect cells
Exogenous addition of H$_2$O$_2$ to in-vitro cultured Sf9 cells induced apoptosis in a dose-dependent manner, as measured by cellular blebbing (Figure 3.1. C) and nucleosomal DNA ladder formation (Figure 3.1. D). The percentage of Sf9 cells undergoing apoptosis in response to oxidative stress increased with the concentration of hydrogen peroxide. The AD$_{50}$ (concentration of H$_2$O$_2$ inducing about 50% apoptotic response) varied with the duration of treatment, batch to batch variation of H$_2$O$_2$ and the physiological state of cells. At 24 h post-treatment with H$_2$O$_2$, AD$_{50}$ for cells was estimated to be about 475 µM. For all subsequent studies, 1 mM of H$_2$O$_2$, which elicited about 70-80% apoptotic response in 24 h, was used.

Intra-cellular buildup of oxidative stress by hydroxylamine also leads to apoptosis
To complement the observations on the induction of apoptosis upon direct exposure of cells to H$_2$O$_2$, apoptosis was induced by an intracellular build up of ROS. Cells treated with hydroxylamine, a known inhibitor of catalase, should show increased accumulation of intracellular ROS ultimately culminating in apoptosis. Sf9 cells were treated with hydroxylamine, washed and assayed for
Figure 3.1. Sf9 cells undergo apoptosis as a function of H$_2$O$_2$-stress.
A. Light microscopic view of untreated Spodoptera frugiperda (Sf9) cells.
B. Sf9 cells treated with 1 mM H$_2$O$_2$ for 24 h showing cellular blebbing and formation of distinct apoptotic bodies. Magnification: 20 X.
Figure 3.1. Dose-dependent induction of apoptosis in Sf9 cells by H$_2$O$_2$ treatment.

C. In-vitro cultured Sf9 cells were treated with increasing concentrations (0-1.5 mM) of H$_2$O$_2$ for 24 h and assayed for apoptosis. The percentage of apoptotic cells was calculated by counting the cells after trypan blue staining.

D. Nucleosomal DNA ladder formation as a function of H$_2$O$_2$ concentration. Sf9 cells were processed for the isolation of apoptotic bodies and subsequently assayed for DNA ladder formation. Lane 1, untreated cells; lanes 2, 3, 4, 5 and 6 are cells treated with the increasing concentrations of H$_2$O$_2$ (0.2, 0.5, 0.75, 1.0 and 1.5 mM respectively).
apoptosis. Cells exhibited dose-dependent apoptosis upon treatment with hydroxylamine. Percentage of cells undergoing apoptosis increased from 10% to 78% as the concentration of hydroxylamine increased from 1.5 mM to 5.0 mM (Figure 3.1. E and F) with an AD\textsubscript{50} of 2.2 mM for a 24 h treatment regime. To evaluate a synergy, if any, between hydroxylamine and H\textsubscript{2}O\textsubscript{2}, cells were treated with 2.0 mM hydroxylamine prior to an exposure to H\textsubscript{2}O\textsubscript{2} (0.75 mM). Apoptosis caused by H\textsubscript{2}O\textsubscript{2} was significantly increased upon pretreatment with hydroxylamine (Figure 3.1. G). These results demonstrate that an increase in the intracellular build up of ROS can also induce apoptosis in a manner similar to that effected by the exogenous treatment with H\textsubscript{2}O\textsubscript{2}.

Inhibition of H\textsubscript{2}O\textsubscript{2}-induced apoptosis by DMSO treatment

To confirm the involvement of these reactive oxygen species in inducing apoptosis, dimethylsulfoxide (DMSO), a known scavenger of hydroxyl radicals (OH\textsuperscript{-}), was used. It was evident (Figure 3.1. G) that pretreatment of Sf9 cells with dimethylsulphoxide prior to H\textsubscript{2}O\textsubscript{2} exposure significantly reduced the apoptotic response from 55% to 25%. These results therefore point to the direct involvement of hydroxyl radicals in the H\textsubscript{2}O\textsubcript{2}-mediated apoptosis of in-vitro cultured Sf9 cells.

3.2. Expression of the baculovirus p35 gene inhibits ROS-induced apoptosis in insect cells

Having demonstrated the utility of insect cells as a model to study oxidative stress-induced apoptosis, the role of the baculovirus encoded p35 gene in inhibiting H\textsubscript{2}O\textsubscript{2}-mediated cell death was investigated. In-vitro cultured Sf9 cells were infected with wild type AcNPV at a multiplicity of infection (moi) of 10 and then treated with apoptotic dose of H\textsubscript{2}O\textsubscript{2} (1 mM) at different time periods post-infection. Apoptosis was scored by monitoring cellular blebbing (Figure 3.2. A) as well as the formation of nucleosomal ladder (Figure 3.2. B). Protection against H\textsubscript{2}O\textsubscript{2}-induced apoptosis was observed as a direct function of viral infection time. Treatment of Sf9 cells immediately after viral
Figure 3.1. Sf9 cells also enter apoptosis as a function of intracellular buildup of reactive oxygen species.
E. Dose-dependent induction of apoptosis in Sf9 cells by *in situ* generation of H₂O₂ after catalase inhibition. Sf9 cells were treated with increasing concentrations of hydroxylamine (HA) for 24 h and assayed for apoptosis by counting % of apoptotic cells.
F. Nucleosomal DNA ladder formation as a function of hydroxylamine concentration. Lane 1, untreated cells; lanes 2, 3, 4, 5 and 6 are cells treated with different concentrations of hydroxylamine (1.5, 2.0, 2.5, 3.0 and 5.0 mM respectively).
Figure 3.1. G. Hydroxylamine acts synergistically with $\text{H}_2\text{O}_2$ to induce apoptosis while DMSO inhibits the same. Cells were treated with 2.0 mM hydroxylamine (HA) or 75 mM DMSO (DM) prior to 0.75 mM $\text{H}_2\text{O}_2$ (HP)-exposure. Apoptosis was scored microscopically after 24 h treatment.
Figure 3.2. The AcNPV p35 gene inhibits H₂O₂-induced apoptosis.

A. Sf9 cells infected with wild type baculovirus at 10 moi and then exposed to 1 mM concentration of H₂O₂ (HP) at different time periods post-infection (0-6 h). Percentage cells undergoing apoptosis was scored.

B. Nucleosomal ladder assay for the cells infected with wild type virus prior to H₂O₂-exposure at different time periods post-infection. Lane 1, uninfected cells; lanes 2 and 3 are cells treated with hydrogen peroxide (HP) and AcNPV (Ac) alone; lanes 4, 5, 6, 7 and 8 are cells treated with H₂O₂ at different hpi (0, 1, 2, 4 and 6 hpi respectively).
infection (i.e. 0 hpi) yielded about 10% reduction in apoptosis as compared to uninfected cells. This decrease in the apoptotic response is possibly due to the presence of a minute quantity of p35 protein in the virions at the time of infection (Hershberger, 1994). Exposure of cells to H2O2 at increasing times post-infection caused reduced apoptosis which could be due to the early expression of the p35 gene in the cells. The presence of nucleosomal ladder complemented the microscopic cellular blebbing observations.

To convincingly document the role of p35 in arresting oxidative stress-induced cell death, a p35-deletion mutant of AcNPV (Δp35 AcNPV) was used. Infection of Sf9 cells with mutant AcNPV (at moi of 10) followed by apoptotic induction with H2O2 failed to protect cells against apoptotic onslaught pointing to the direct involvement of p35 in this process (Figure 3.2, C and D). Expectedly, the mutant virus alone induced about 79% apoptosis at 24 h in Sf9 cells which was a reflection of the apoptotic response mounted by the insect cells in response to viral infection. Cells treated with H2O2 at 0, 1, 2, 4 and 6 h after infection with Δp35 AcNPV registered about 68, 80, 88, 91, 94% apoptosis, respectively. The microscopy observations were complemented by the appearance of DNA ladder (Figure 3.2, D). It was apparent that both H2O2 and the p35-deletion mutant of AcNPV could synergystically act as apoptotic stimuli. In complementary experiments, Sf9 cells were exposed to varying doses of UV radiation and monitored for apoptosis after 24 h. The dose of UV (35 sec) showing 70-80% apoptosis was selected for further experiments. Results show that wild type AcNPV infected cells could completely abrogate apoptosis induced by UV radiation whereas mutant virus (Δp35 AcNPV) failed to do so (Figure 3.2, E).

The ability of p35 to similarly provide protection against cell death induced by intracellular accumulation of ROS was also confirmed. Sf9 cells were infected with wild-type AcNPV carrying the p35 gene and 6 h later treated with hydroxylamine (2.5 mM). The
Figure 3.2. Infection with p35 deletion mutant AcNPV (ΔAcNPV) fails to inhibit H₂O₂-induced apoptosis.
C. Sf9 cells were infected with 10 moi of mutant virus (ΔAc) and then exposed to 1 mM concentration of H₂O₂ (HP) for different time periods. Percentage cells undergoing apoptosis was scored morphologically by cellular blebbing.
D. Nucleosomal DNA ladder formation assay for cells infected with p35 deletion mutant Δp35AcNPV prior to the H₂O₂-exposure at different time periods post-infection. Lane 1, uninfected cells; lanes 2 and 3 are cells treated with HP and ΔAc alone; lanes 4, 5, 6, 7 and 8 are cells infected with ΔAcNPV and treated with H₂O₂ at different hpi (0, 1, 2, 4 and 6 hpi respectively).
Figure 3.2. E. Infection of cells with wild type AcNPV but not the p35 deletion mutant AcNPV (ΔAcNPV) inhibits UV-induced apoptosis. Sf9 cells were infected with 10 moi of wild type or p35 mutant virus and then exposed to UV light for 35 sec. Percentage cells undergoing apoptosis was scored morphologically by cellular blebbing. The different lanes are: lane 1, control cells (C); lane 2, cells infected with wild type AcNPV (Ac); lane 3, cells treated with UV and lane 4, cells infected with mutant virus (ΔAc); lanes 5 and 6 are cells infected with AcNPV and Δp35AcNPV (ΔAc) prior to UVB irradiation.

F. p35 can also inhibit cell death induced by intracellularly generated ROS. Sf9 cells were infected with wild type AcNPV (Ac) before treating with 2.5 mM hydroxylamine (HA) and scored for apoptosis after 24 h. C represents control cells.
apoptotic response was scored as usual. As expected, 2.5 mM hydroxylamine caused about 60% apoptosis. Infection of cells with wild type AcNPV prior to the treatment with hydroxylamine resulted in significant reduction of apoptosis (Figure 3.2, F). These results demonstrate the ability of the p35 gene to inhibit apoptosis induced by oxidative stress irrespective of the source of the same.

3.3. A cloned copy of the p35 gene can also intercept H2O2-induced apoptosis
To investigate the possible involvement of other viral genes besides p35 in arresting H2O2-induced apoptosis, a recombinant plasmid construct (pNN1) carrying the p35 gene placed under the transcription control of Drosophila hsp70 gene promoter was used. hsp70 promoter was earlier shown to be activated as a function of oxidative stress. SJ/9 cells were transfected with 8-12 μg of pNN1, incubated for 24 h and then treated with H2O2. Cells transfected with the p35 gene construct registered nearly 40% protection against H2O2-induced apoptosis (Figure 3.3). The reduced levels of protection observed against H2O2-induced apoptosis in p35 transfected cells compared to that obtained with wild type AcNPV may be attributed to the involvement of other viral genes such as the IAP which also have antiapoptotic activity (Manji et al., 1997; Clem and Miller, 1994; Harvey et al., 1997). It is nonetheless evident that a cloned copy of the p35 gene, outside the viral context, is able to significantly intercept oxidative stress-induced apoptosis.

3.4. p35 acts very upstream in the pathway of oxidative stress-induced apoptosis
In order to determine the stage at which p35 actually arrests H2O2-induced apoptosis, cells were first exposed to oxidative stress followed by interception with p35. At different time intervals after H2O2-exposure (0, 1, 2, 4 and 6 h), cells were infected with AcNPV (Figure 3.4, A and B). It was apparent that p35 was unable to intercept oxidative stress-induced apoptosis in SJ/9 cells once the same has already begun. These results therefore
Figure 3.3. Transfection of Sf9 cells with p35 gene construct significantly inhibits H$_2$O$_2$-induced cell death. Sf9 cells were transfected with 8-12 µg of pNN1, carrying the p35 gene under the hsp70 control, and incubated for 24 h before subjecting them to H$_2$O$_2$ (HP) treatment. Cells were scored microscopically for apoptosis 24 h after H$_2$O$_2$-treatment.
Figure 3.4. p35 acts very upstream in preventing oxidative stress-induced apoptosis in insect cells. 
A. p35 is unable to rescue cells from apoptosis after pre-treatment with H$_2$O$_2$. Cells were infected with wild type AcNPV after different time periods of H$_2$O$_2$ (HP) treatment and then assayed for apoptosis 24 h after viral infection. The number of apoptosed cells were scored microscopically. 
B. Apoptosis was assayed by the generation of nucleosomal DNA ladder formation in cells infected with wild type AcNPV (Ac) at different time periods after H$_2$O$_2$ treatment. Lane 1, C (control cells); lane 2, cells infected with AcNPV (Ac) alone; lane 3, cells treated with HP alone; lane 4, 5, 6, 7 and 8 are cells were infected with virus at different time periods (0, 1, 2, 4 and 6 h respectively) after H$_2$O$_2$ treatment.
point to a very upstream intervention of apoptosis by the baculovirus p35 gene.

3.5. Mechanism of action of p35: p35 protein acts as a sink for reactive oxygen species

To investigate the mechanism of action of p35 in inhibiting ROS-mediated apoptosis, in-vitro and in-vivo experiments involving ESR spin-trapping were carried out. ESR spectrophotometry, which records specific signals for a given paramagnetic molecule (free radical), was exploited to determine whether p35 acts as a 'sink' to sequester reactive oxygen species. It was argued that the ESR spectrum generated by free radical should disappear in the presence of p35. In-vitro studies were performed using purified recombinant p35 protein while in-vivo experiments were carried out on Sj9 cells transfected with the plasmid pNN1. The spectra generated by the free radicals in the presence and the absence of p35 were analyzed. The generation of ESR signals by the superoxides was standardized using the in-vitro xanthine/xanthine oxidase system. Results clearly showed that the specific peaks obtained for the superoxide generated by the xanthine/xanthine oxidase system (Figure 3.5. A, middle panel) was greatly reduced in intensity in the presence of purified p35 protein. ESR integrated absorption intensity (I) (integrated line intensity of the first derivation signal) was determined (Table 3.1). Attenuation of I was found out to be almost 100% by the purified p35 protein (Figure 3.5. A, lower panel).

Similarly, the ESR spectrum of ROS generated in-vivo was analyzed in the absence and presence of transiently expressed p35. Sj9 cells transfected with pNN1 were subjected to intracellular generation of hydroxyl radicals through the Fenton reaction (Lloyd et al., 1997) under UV radiation and the specific peaks were monitored by ESR spectrum. Although the ESR spin-trapped spectra was not as well resolved due to the low level of free radical generation, it was nonetheless evident that the specific peaks generated for the free radicals in the absence of p35 (Figure 3.5. B.
Figure 3.5. Mechanism of action of p35: Quenching of free radicals.
A. p35 acts to sequester reactive oxygen species. ESR spectrum of the superoxides generated using in-vitro xanthine/xanthine oxidase system. The basal level of the spectra generated by the xanthine oxidase (panel I) or those by the superoxides generated by the xanthine/xanthine oxidase system in the absence (ii) and the presence (iii) of the p35 protein are displayed.
B. ESR spectrum of ROS generated in-vivo through the Fenton reaction in S9 cells. pNN1 transfected cells were subjected to intracellular ROS generation through Fenton reaction and monitored for the peaks generated by ESR spectrum. These spectra generated by hydroxyl radical in the absence of p35 (i) or after heat shock induction of pNN1 transfected cells (ii) are presented.
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**TABLE 3.1.** ESR quenching of free radicals *in-vitro* and *in-vivo* by p35. Attenuation of the ESR integrated absorption intensity (I) by p35 protein *in-vitro* and *in-vivo*. HP-hydrogen peroxide; X-xanthine; XO-xanthine oxidase; Fe-Iron; α-cells were heat-shocked for activating p35 gene expression.
upper panel) were attenuated to almost basal level upon the expression of the p35 gene in transfected cells (Figure 3.5. B. lower panel). Attenuation (I) value was determined to be about 95% in the transfected cells. Thus, these data unequivocally demonstrate that specific ESR spectra corresponding to the free radicals generated in-vitro or in-vivo were abolished in the presence of p35 pointing to the action of p35 as a 'sink' to sequester reactive oxygen species.
4. **p35 and oxidative stress-induced apoptosis in mammalian system**

Having conclusively demonstrated the ability of p35 to intercept oxidative stress-induced apoptosis in insect cells by acting as a "sink", investigations were carried out to evaluate a similar action of p35 in non-insect system. Studies were conducted to investigate the pathway of action of p35 in murine macrophages.

4.1. **Concentration-dependent induction of apoptosis by \( \text{H}_2\text{O}_2 \)-treatment in rat peritoneal macrophages**

In order to establish macrophages as a model to study oxidative stress-induced apoptosis in mammalian system, rat peritoneal macrophages were exposed to varying concentrations of \( \text{H}_2\text{O}_2 \) and assayed for apoptosis. It was apparent that apoptosis, as monitored by the appearance of nucleosomal DNA ladder, starts after 12-14 h of \( \text{H}_2\text{O}_2 \) treatment in a concentration-dependent fashion. Complete cellular breakdown and apoptosis sets in by 18-20 h time span in >60% of the macrophage population (AD60) at \( \text{H}_2\text{O}_2 \) concentration of 625 μM. There was increase in the percentage of peritoneal macrophages undergoing apoptosis in response to increasing doses of oxidative stress as measured by cellular blebbing and nucleosomal DNA ladder formation (Figure 4.1. A and B).

4.2. **p35-mediated interception of \( \text{H}_2\text{O}_2 \)-induced apoptosis in mammalian system**

In order to investigate whether p35, a viral encoded protein, also functions in a similar way in mammalian cells, the ability of p35 to intercept \( \text{H}_2\text{O}_2 \)-induced apoptosis in murine primary macrophages was evaluated. Having established oxidant-dependent induction of cell death in macrophages, the ability of p35 to intercept the same was investigated. For this, macrophages were transfected with various concentrations of pCMVp35 plasmid carrying the p35 gene under the transcriptional control of CMV early promoter for different time periods to enable the expression of the p35 gene in...
Figure 4.1. Rat peritoneal macrophages undergo apoptosis as a function of H$_2$O$_2$-concentration.
A. Peritoneal macrophages were exposed to different concentrations of H$_2$O$_2$ and the percentage of the cells undergoing apoptosis was scored morphologically 24 h post-treatment.
B. H$_2$O$_2$-induced nucleosomal ladder formation in peritoneal macrophages. Lane 1, untreated macrophages; lanes 2, 3, 4, 5 and 6, macrophages treated with increasing concentrations of H$_2$O$_2$ (125, 250, 325, 500 and 625 mM, respectively) and assayed 24 h post H$_2$O$_2$-treatment.
these cells. The transfected cells optimally expressing the p35 gene were subsequently treated with H$_2$O$_2$ (AD$_{60}$). p35 expression could rescue the macrophages from apoptosis in a dose-dependent manner [Figure 4.2. A and B], where 5 µg of pCMVp35 plasmid DNA/1.5 X 10$^6$ cells was found to be the most effective concentration compared to other concentrations tried. Transfection of macrophages with this concentration of pCMVp35, 4-5 h prior to oxidant treatment rescued 40-45% of the cells from undergoing apoptosis. This concentration of the pCMVp35 plasmid was therefore selected for further studies.

4.3. p35 also acts very upstream in the pathway of oxidative stress-induced apoptosis

Experiments were designed to dissect the mechanism of action of p35 with respect to the kinetics of the apoptotic pathway. It was investigated if p35 could arrest apoptosis after the signal for the same has already been triggered following H$_2$O$_2$-exposure in macrophages. Rat macrophages were pretreated with H$_2$O$_2$ for various time periods, washed with fresh culture media and then transfected with pCMVp35. In pCMVp35, the p35 was cloned downstream to the CMV promoter. DNA ladder pattern as well as the percent cell death plot shows that p35 is unable to rescue macrophages from apoptosis once triggered to undergo the same (Figure 4.3. A and B). These results suggest that p35 acts very upstream in the pathway of oxidative stress-induced apoptosis of macrophages.

4.4. p35 inhibits cytochrome c release from mitochondria in H$_2$O$_2$-induced macrophages

Mitochondrial cytochrome c release is a marker for membrane permeability transition and is associated with very early events of apoptosis induced by various stimuli (Scarlett and Murphy, 1997). We monitored cytochrome c release from the mitochondria of macrophages as a consequence of H$_2$O$_2$-treatment. Cytosolic and mitochondrial fractions were analyzed by western blot analysis using anti-cytochrome c antibody. Time dependent release of
Figure 4.2. Transfection of macrophages with pCMVp35 gene construct rescues H$_2$O$_2$-induced apoptosis.

A. Macrophages were transfected with pCMVp35 construct for 6 h and treated with H$_2$O$_2$ (AD$_{50}$) for 24 h. The percentage of cells undergoing apoptosis was scored morphologically.

B. Nucleosomal ladder pattern of pCMVp35 transfected macrophages treated with H$_2$O$_2$. Lane 1, untreated macrophages; lane 2, macrophages transfected with p35 construct (5 µg); lane 3, macrophages treated with H$_2$O$_2$ alone; lanes 4, 5 and 6 are macrophages transfected with pCMVp35 (1, 2.5 and 5 µg for 6 h respectively), washed with fresh medium and treated with H$_2$O$_2$ (AD$_{50}$) for 24 h.
**Figure 4.3.** p35 is unable to rescue macrophages from apoptosis after the cells have been exposed to H$_2$O$_2$.

A. Cells were transfected with pCMVp35 after different time periods of H$_2$O$_2$ (HP) treatment and then assayed for apoptosis 24 h after transfection. Apoptosis were scored by light microscopic assay.

B. Nucleosomal DNA assay showing different lanes. Lane 1, untreated control peritoneal macrophages; lanes 2 and 4, macrophages treated with H$_2$O$_2$ alone (625 μM, 2 and 4 h respectively), washed with fresh medium and incubated in dark for 22 and 20, h respectively; lanes 3 and 5 shows macrophages treated with H$_2$O$_2$ (625 μM, 2 and 4 h respectively), washed with fresh medium and transfected with p35 construct (5 μg) and incubated for 22 and 20 h, respectively.
mitochondrial cytochrome c into the cytosolic fraction was observed which was about two fold higher after one hour of $\text{H}_2\text{O}_2$-treatment as compared to 2 h, as evident by densitometric quantitation of the western blot (Figure 4.4. A and B: lane 2 with lane 3). Transfection of macrophages with pCMVp35, 4 h prior to $\text{H}_2\text{O}_2$-treatment showed significant reduction (about 3 fold) in the levels of cytosolic cytochrome c (Figure 4.4. C and D; compare lane 5 with lane 6) after 1 h as well as after 2 h (Lanes 7 and 8). Interestingly, the cytochrome c level within the mitochondrion was higher in the p35 transfected macrophages as compared to those treated with $\text{H}_2\text{O}_2$ alone (Figure 4.4. C and D; compare lane 1 with 2 and lane 3 with 4).

4.5. p35 inhibits $\text{H}_2\text{O}_2$-induced caspase-3 activation in macrophages

Having shown a significant inhibition of cytochrome c release from the mitochondria into the cytosol, the consequence of such inhibition on caspase activation was investigated. Macrophages (1.5 x $10^6$) were treated with $\text{H}_2\text{O}_2$ for various time periods and processed for western blot analysis using anticaspase-3 antibody to assay for caspase activation. The inactive form of caspase-3 upon activation is proteolytically cleaved into 18 kDa and 12 kDa subunit which together constitutes the active enzyme. The anticaspase-3 antibody used in our assay is also able to recognize the larger 18 kDa cleaved fragment. The appearance of the 18 kDa proteolytic fragment is a measure for caspase activation. Experiments were first designed to score for caspase activation in peritoneal macrophages treated with $\text{H}_2\text{O}_2$. It was apparent that $\text{H}_2\text{O}_2$ treatment (AD$_{60}$) resulted in a time-dependent activation of caspase-3 (Figure 4.5. A and B; lanes 2-5). The activation showed maximal increase after 24 h of $\text{H}_2\text{O}_2$ exposure at the AD$_{60}$ dose. These results categorically demonstrate the activation of caspase-3 as an essential component of the cascade of events associated with $\text{H}_2\text{O}_2$-induced apoptosis in primary rat peritoneal macrophages.
Figure 4.4. Mitochondrial cytochrome c release is an upstream event in H₂O₂-induced apoptosis in macrophages. A. Western blot analysis showing lane 1, untreated peritoneal macrophages; lanes 2 and 3, cytochrome c release from macrophage mitochondria after treatment with H₂O₂ (625 μM) for 1 and 2 h, respectively; lane 4, commercial horse cytochrome c (2 μg) B. Densitometric analysis of the same western blot.
Figure 4.4. p35 expression inhibits H₂O₂-induced mitochondrial cytochrome c release in rat peritoneal macrophages.

C. The different lanes are: lanes 1 and 3, mitochondrial fraction of macrophages treated with H₂O₂ alone (625 µM, 1 and 2 h, respectively); lanes 2 and 4, mitochondrial fraction of macrophages transfected with p35 construct (5 µg) and then treated with H₂O₂ (625 µM, 1 and 2 h respectively); lanes 5 and 7, macrophage cytosolic fraction treated with H₂O₂ alone (625 µM, 1 and 2 h, respectively); lanes 6 and 8, cytosolic fraction of macrophages treated with pCMVp35 construct (5 µg) and then exposed to H₂O₂ (625 µM, 1 and 2 h, respectively). Lane 9 represents commercial horse cytochrome c (1 µg) used as a protein control, position of the cytochrome c is shown by an arrowhead on the left.

D. Densitometric quantitation of the western blot (C) showing the effect of p35 transfection for 4 h prior to H₂O₂ treatment resulting in reduction of the cytochrome c release into the cytosol.
Figure 4.5. Exposure of rat peritoneal macrophages to H$_2$O$_2$ results in caspase activation.
A. The appearance of the 18 kDa fragment of caspase-3 constituting the active enzyme in a western blot analysis using anticaspace-3 antibody was scored to monitor caspase activation. The different lanes are: lane 1, untreated macrophages; lanes 2-6, activated caspase-3 detected after induction with H$_2$O$_2$ (625 μM) for 2, 4, 8, 16 and 24 h, respectively. Lane 7 represents low molecular weight marker.
B. Densitometric analysis of the western blot (A) showing increasing levels of activated caspase-3.
The effect of $p35$ expression on caspase activation was investigated by following a similar experimental regime where macrophages, transfected with $p$CMVP35 construct, were exposed to AD60 dose of $H_2O_2$ and subsequently assayed for caspase activation. $p35$ transfection prior to $H_2O_2$ treatment resulted in three-fold inhibition of caspase activation (Figure 4.5. C and D: compare lane 1 with 2) in 16 h treatment group. However, the inhibition was strikingly more (about ten fold) in case of 24 h treatment group (Figure 4.5. C and D: compare lane 3 with 4). These results unequivocally establish the effect of $p35$ expression in down regulating caspase-3 activation in $H_2O_2$-treated rat peritoneal macrophages.

4.6. Stable expression of pCMVP35 in mice

An attempt was made to check if $p35$ shows stable expression, if injected in saline, in Swiss albino mice. Injected pCMVP35 (100 $\mu$g) was checked for its stability by PCR analyses, using internal primers of $p35$. Detectable levels of $p35$ fragment were found till 21 days post injection (both i.d. and i.m) (Figure 4.6 A). Besides looking at the stability of pCMVP35, tissue samples from control (i.d.) and injected mice (both i.d. and i.m.) were also analyzed for the expression of $p35$. Mice were injected with similar amounts of pCMVP35 and tissue samples were processed two weeks later for RNA isolation and checked for expression using $p35$ probe by northern blot analyses. $p35$ expression was observed in mice injected intradermally and intramuscularly (Figure 4.6. B; lane 2 and lane 3 respectively). The long term expression of $p35$ in mice both in dermis tissue and muscles confer a therapeutic value to this protein.
Figure 4.5. *p35* gene expression results in the inhibition of caspase activation in macrophages.

C. Macrophages were treated with H$_2$O$_2$ (625 μM) alone for 16 h (lane 1) and 24 h (lane 3) and then assayed for caspase activation by western blot using anticaspase-3 antibody. Macrophages were transfected with pCMVp35 construct (5 μg) and treated with H$_2$O$_2$ (625 μM) for 16 h (lane 2) or 24 h (lane 4) before processing for caspase activation assay. The position of the 18 kDa fragment represents the large subunit of the active caspase-3 as indicated on the left.

D. Densitometric analysis of the western blot (C) showing the levels of activated caspase-3.
Figure 4.6. Stable expression of recombinant plasmid containing p35 gene under the transcriptional control of CMV promoter in mice till 14 days.
A. PCR amplification of p35 gene, using internal primers, from the DNA extracted from untreated control mice (lane 1) and mice injected intradermal (lane 2) and intramuscular (lane 3), with CMVp35 construct in PBS saline whereas lane 4 shows positive control i.e. Qiagen purified CMV-p35 plasmid.
B. Northern blot analysis of the RNA extracted from the untreated control mice (lane 1) and mice injected intradermal (lane 2) and intramuscular (lane 3), with CMV-p35 construct in PBS saline, using p35 probe.
5. Caffeine and oxidative stress-induced apoptosis in Sf9 insect cells

A parallel study was planned with a chemical antioxidant, caffeine, in dissecting oxidative stress-induced apoptosis.

5.1. Caffeine-mediated inhibition of oxidative stress-induced apoptosis

Experiments were conducted to determine if caffeine is able to intercept H$_2$O$_2$-induced apoptosis. Sf9 cells were treated with H$_2$O$_2$ in order to elicit >50% apoptotic response in 24 h. Pretreatment of Sf9 cells with increasing concentrations of caffeine (1, 3 and 5 mM) for 4 h followed by treatment with 750 µM H$_2$O$_2$ for 24 h showed significant reduction in cells undergoing H$_2$O$_2$-induced apoptosis (Figure 5.1. A). The percentage of cells showing apoptosis decreased to about 19% in cells pre-treated with 5 mM caffeine. Caffeine could similarly protect cells from UV-induced apoptosis (Figure 5.1. B). Experiments were designed to rule out the possibility of caffeine-mediated inhibition of H$_2$O$_2$-induced apoptosis simply being a reflection of neutralization of H$_2$O$_2$ by caffeine in the culture medium. For this, Sf9 cells were first treated with caffeine and then extensively washed and incubated in fresh culture media. These pre-treated and washed cells showed significantly reduced apoptosis upon exposure to 750 µM H$_2$O$_2$. Pre-exposure of cells to caffeine (5 mM) for different time periods (10, 30, 60 and 120 min) showed increasing protection as a function of exposure time with maximum inhibition of apoptosis observed at 120 min (Figure 5.1. C).

Sf9 cells were triggered to apoptotic cell death by hydroxylamine (HA), which is a specific inhibitor of catalase activity. Exposure of cells to HA leads to the endogenous accumulation of free radicals within the cells causing cell death. Cells were pre-treated with 5 mM caffeine for 4 h, exposed to 2.5 mM HA and assayed for apoptosis 24 h later. A protective effect is clearly visible (Figure 5.1. D and E), reiterating the role of caffeine as an antioxidant through its ability to quench free radicals.
Figure 5.1. Concentration-dependent inhibition of oxidative stress-induced apoptosis by caffeine.
A. Sf9 cells were incubated for 4 h with different concentrations of caffeine (1, 3 and 5 mM) before subjecting them the same to 24 h H$_2$O$_2$ treatment. Cells were harvested after 24 h for scoring % apoptotic cells by morphological observation.
B. Sf9 cells were similarly treated with different doses of caffeine (5, 10 and 20 mM) before subjecting the same to UV (30 sec) exposure and incubated for 24 h. Cells were harvested after 24 h for morphological observation.
Figure 5.1. C. Time-dependent inhibition of \( \text{H}_2\text{O}_2 \)-induced apoptosis by caffeine pre-treatment. 
Sf9 cells were treated with caffeine (5 mM) for different time periods (10, 30, 60 and 120 min) and then washed with the culture medium before treating them with \( \text{H}_2\text{O}_2 \). Apoptosis was assayed by counting the number of apoptosed cells.
Figure 5.1. *In-situ* generated oxidative stress-induced apoptosis by hydroxylamine treatment is inhibited by caffeine pretreatment.

D. S/3 cells were pretreated with caffeine (5 mM) for 2 h before exposing them to hydroxylamine (2.5 mM). Cells were checked for apoptosis 24 h after the hydroxylamine treatment by trypan blue exclusion assay under the light microscope.

E. Apoptosis was also assayed by the generation of nucleosomal DNA ladder. Lane 1, untreated cells; lanes 2 and 3, cells treated with hydroxylamine (2.5 mM) and caffeine (5 mM) alone; lane 4, cells pretreated with caffeine (5 mM) and then subjected to hydroxyamine treatment (2.5 mM).
5.2. Caffeine acts very upstream in the pathway of oxidative stress-induced apoptosis

In order to dissect the mechanism of action of caffeine with respect to its ability to intercept the apoptotic pathway at the initial or later stages, cells were first triggered to undergo apoptosis by exposure to 750 μM H$_2$O$_2$ for 8 h, and subsequently incubated with 5 mM caffeine for 24 h before assaying for apoptosis. The percentage of cells undergoing apoptosis in H$_2$O$_2$-treated cells was almost similar to cells treated with H$_2$O$_2$ followed by caffeine (Figure 5.2. A and B), clearly suggesting that caffeine cannot intercept oxidative stress-induced apoptosis once already triggered but only acts upstream in the pathway of H$_2$O$_2$-induced apoptosis.

5.3. Caffeine inhibits cytochrome c release from mitochondria during oxidative stress-induced apoptosis in Sf9 cells

Experiments were designed to elucidate the molecular mechanism of action of caffeine in preventing oxidative stress-induced apoptosis. Sf9 cells were analyzed for cytochrome c release after UV-exposure. Sf9 cells showed the release of mitochondrial cytochrome c (14.5 kDa) into the cytosolic fraction after 2 h of UV-treatment. Pre-treatment of insect cells with caffeine (5, 10 and 20 mM) for 4 h before subjecting them to a 30 sec UV-exposure resulted in the inhibition of cytochrome c release into the cytosol, as evident from the western blot analysis using commercial mammalian antibody against cytochrome c (Figure 5.3. A and B).

5.4. Caffeine-mediated inhibition of oxidative stress-induced caspase activation

To establish the role of downstream executors of apoptosis and to look at the ability of caffeine in regulating these steps, Sf9 cells were treated with UV to study caspase activation. Sf9 cells were pre-treated with similar doses of caffeine (5, 10 and 20 mM) for 4 h and then exposed to UV for 30 sec. Cells were processed after 12 h of UV-exposure and assayed for caspase activation. It was
Figure 5.2. Caffeine acts upstream in the cascade of events in H₂O₂-mediated apoptosis.

A. Sf9 cells were pretreated with H₂O₂ for 8 h before treating with caffeine treatment (5 mM). Apoptosis was assayed 24 h after caffeine treatment by counting the number of apoptosed cells as observed under light microscope. The % apoptotic cells in C (control), CAF, HP treated alone or treatment with CAF or HP for the same is shown.

B. The generation of DNA nucleosomal ladder for the same is shown. Lanes 1 and 2 represents the intact genomic DNA of control cells and caffeine treated (CAF, 5 mM) cells, respectively; lane 3 shows cells treated with H₂O₂ (HP, 750 μM) and lane 4 represents H₂O₂ treatment (HP, 750 μM) prior to caffeine treatment (5 mM).
Figure 5.3. Caffeine-mediated inhibition of UV-induced mitochondrial cytochrome c release in Sf9 cells.

A. Western blot analysis showing lane 1, untreated control insect cells; lane 2, cytochrome c release from insect cell mitochondria after treatment with UV (30 sec); lane 3, 4, and 5 show inhibition of cytochrome c release in UV-induced apoptosis by the pre-treatment with caffeine (5, 10 and 20 mM respectively).

B. Quantitation of cytochrome c release by densitometric scanning.
evident that caffeine was able to inhibit UV-induced activation of Sf\textsuperscript{caspase-1} in a dose-dependent manner as evident from Figure 5.4, A and B.

5.5. Caffeine inhibits UV-induced upregulation of caspase mRNA levels

The ability of caffeine to inhibit apoptosis lies much upstream in the apoptotic cascade as shown by its ability to inhibit cytochrome c release from the mitochondria. Besides, caffeine is able to inhibit oxidative stress-induced transcriptional activation of Sf\textsuperscript{caspase-1} as assessed by the Northern blot analysis. Sf9 cells pre-treated with 10 mM caffeine for 4 h before exposing them UV for 30 sec showed basal level of caspase as opposed to the cells exposed to UV (30 sec) alone. Densitometric analysis of the RNA blot demonstrates that Sf9 cells exposed to UV showed 10-fold increase in mRNA levels as compared to the untreated control cells and cells pretreated with caffeine (Figure 5.5, A and B).
Figure 5.4. Inhibition of UV-induced Sf caspase-1 activation in insect cells by the pre-treatment of caffeine.

A. Western blot analysis showing lane 1, high molecular weight marker; lane 2, untreated insect cells; lane 3 shows activation of Sf caspase-1 to active form (18 kDa) upon UV-treatment (30 sec); lanes 4, 5, and 6 show the inhibition of caspase activation by caffeine pre-treatment (5, 10 and 20 mM respectively).

B. Quantitation of activated caspase-1 by densitometric scanning.
Figure 5.5. Caffeine-mediated inhibition of UV-induced transcriptional upregulation of *Sf caspase-1*.

A. Northern blot analysis, using *Sf caspase-1* probe, showing lane 1, untreated control insect cells; lane 2 shows enhanced transcription of *Sf caspase-1* in insect cells exposed to UV (30 sec). Lane 3 shows the inhibition in UV-induced transcriptional activation of caspase by caffeine pre-treatment (10 mM).

B. Densitometric analysis of the above northern blot.