Discussion
DISCUSSION

P35 and oxidative stress-induced apoptosis in S2 insect cells

The mechanism of action of baculoviral antiapoptotic p35 gene which acts at the execution step of the apoptotic process by stoichiometrically inhibiting various members of ICE proteases family has been reported. p35 prevents cell death, by specifically inhibiting caspase activation, induced by various stimuli. Though p35 was accepted as a general inhibitor of caspases, some recent reports show the existence of certain p35-resistant caspases. 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 also not be antagonized by baculovirus p35 (Igaki et al., 2000).

While extensive data are available on p35 and its ability to inhibit caspase activation, no report exists with respect to p35 in relation to oxidative stress-induced apoptosis. The ability of p35 to inhibit oxidative stress-induced apoptosis was the focus of the present study with a view to identify alternate pathway(s) of action of p35. The objective of establishing insect cells as a model to study oxidative stress-induced apoptosis was to provide an easily amenable system to investigate the mechanism of apoptosis inhibition by a baculovirus-encoded antiapoptotic p35 gene. With the development of insect cells as a model for oxidative stress-induced apoptosis and given the conserved nature of the basic machinery of the apoptotic pathway among the metazoans, it was possible to study important mechanistic and evolutionary issues not just relating to p35 but oxidative stress-associated signaling cascade leading to apoptosis.
To study oxidative stress-induced apoptosis in S/9 cells, H$_2$O$_2$ was selected as a stimuli to trigger apoptosis because of its biological reactivity compared to many ROS and its ability to cross membranes and diffuse away from the site of generation. Reactive oxygen species also act as signaling molecules, amongst other stimuli, for the activation of the apoptotic pathway (Suzuki et al., 1997). H$_2$O$_2$ is often generated as a by-product of oxidative metabolism in oxygen-dependent organisms. This is also central to the cytotoxic action of several drugs and pathogenic infections (Thompson, 1995; Suzuki et al., 1997). UV radiations also induce apoptosis via the generation of ROS (Halliwell et al., 1990). A time-dependent increase in the production of intracellular peroxide and superoxide anions involvement has been observed upon UV treatment (Ioannou and Chen, 1996). Besides being a major cause of oxidative stress in the cells, UV radiation induces apoptosis by a large number of unrelated pathways, such as enhanced Fas transcription and/or mRNA stability (Leverkus et al., 1998), induction of c-fos, c-jun, transcriptional factors SAP-1 and nuclear factor kB gene expression.

Our results demonstrate that S/9 insect cells undergo apoptosis by exogenous addition of H$_2$O$_2$, intracellular build up of oxidative stress caused by treatment of cells with hydroxylamine, a potent inhibitor of catalase, as well as by UV radiation. Peroxidases, catalase, superoxide dismutase etc. are the inherent defense guards which maintain the reactive oxygen species balance in normal cells. Reduction in the activity of any of these detoxifying enzymes or the antioxidants of the cells can lead to ROS buildup eventually resulting in cellular apoptosis (Wertz and Hanley, 1996; Jacobson, 1996). DMSO, which is a known scavenger of the hydroxyl radicals is able to restrict the action of these radicals in causing apoptosis of S/9 cells. Our data clearly show that p35 also operates via an antioxidant pathway in the inhibition of apoptosis. This was apparent from the ability of p35 to arrest H$_2$O$_2$-induced cell death or those induced by blocking cellular scavengers of reactive oxygen species or upon UV exposure. The action of p35 to
block apoptosis was seen both in the viral context as well as when it was present alone in the plasmid. Recombinant AcNPV carrying specific p35 deletion failed to rescue Sf9 cells from oxidative stress-induced apoptosis once again directly pointing to the involvement of p35 in this process.

The fact that the presence of p35 protein is required well before the initiation of apoptosis by oxidative stress implied that p35 could intercept oxidative stress-induced pathway at an upstream step besides the execution step of apoptosis (Bump et al., 1995; Ahmad et al., 1997). This posed an important question of whether p35 protein was acting as a sink for the ROS. This was directly addressed by ESR spectroscopy. The ESR results clearly show that p35 was able to quench the in-vitro and in-vivo generated free radicals. In-vitro experiments where the superoxide radicals (via xanthine/xanthine oxidase system) detected by the ESR signals were quenched in the presence of p35 protein not only confirmed the antioxidant action of p35 but also reinforced the notion that p35 functions at an upstream step of the apoptotic signaling cascade.

The ability of p35 protein to sequester ROS is also explained by the analysis of the amino acid composition of p35. It is likely that p35 protein has metal-binding site(s), which could enhance its anti-oxidant property and/or its three dimensional structure contains some aminoacids which confer electrodynamically stable configuration conducive to ROS-trapping. p35 protein has five scattered histidine residues, which may form a functional metal binding domain to enable it to bind to certain metal ions (e.g. Fe$^{2+}$, Mn$^{2+}$, etc.). p35 also contains six cysteine residues, which when present in other proteins or polypeptides show antioxidant/free radical scavenging action and often serve as radioprotectors, especially under oxygenated conditions (Sah and Kesavan, 1996), supporting the antioxidant role of p35.
Oxidative damage to cellular macromolecules such as nuclear and mitochondrial DNA and proteins caused by ROS is considered to be of key importance in the aging process (Yan et al., 1997). The chain of oxidative reactions initiated by ROS eventually knocks down the crucial biomolecules thereby driving the cellular machinery to undergo PCD via activation of caspases which ultimately brings about the execution of cell death. The ability of p35 to inhibit ICE proteases coupled with our demonstration of its antioxidant property additionally points to its "Swiss army knife" like action of p35, similar to bcl-2 (Hengartner, 1998)—an observation of significant therapeutic implications.

**P35 and oxidative stress-induced apoptosis in murine macrophages**

In Sf9 insect cell system, p35, which is transcribed by the host RNA polymerase-II machinery, inhibits apoptotic response mounted by the host cell to check the growth and multiplication of the invading viruses by exhibiting a multipronged strategy to effectively counter the host cell death machinery. In order to increase the relevance of our observation regarding dual mode of action of p35 in insect cells, further studies were conducted to investigate if similar pathway(s) exists in mammalian system. Rat peritoneal macrophages were selected to study the oxidative stress-induced apoptosis and to assess the role of p35 in abrogating oxidative stress-induced apoptosis in mammalian system. The objective of this study was also to assess whether ectopic expression of p35 in mammalian cells retains the functional properties of p35.

Initial experiments demonstrate that exogenous treatment of mammalian macrophages with H2O2 was able to induce apoptosis in a dose- and time-dependent manner. Macrophages undergoing H2O2-induced apoptosis follow the pathway characterized by the activation of caspase-3 preceded by the release of cytochrome c into the cytosol from the mitochondria. Cytochrome c is transcribed from a nuclear gene and is synthesized in the
cytoplasm as apocytochrome c. During apoptosis, when the holocytochrome c is released from the mitochondria and accumulated in the cytosol, it appears to be stable for at least several hours (Kluck et al., 1997a and 1997b). Once the cytochrome c concentration in the cytosol rises above a certain threshold it interacts with at least one other cytoplasmic factor to activate caspase thereby triggering apoptosis by activating downstream events (Kluck et al., 1997a and 1997b; Kuwana et al., 1998). We show that cytochrome c release and caspase activation is a function of the concentration and the duration of exposure of H$_2$O$_2$ to macrophages. Transfection of murine primary macrophages with p35 under CMV early promoter prior to oxidative shock was able to rescue macrophages from undergoing apoptosis.

In a manner similar to insect cells, p35 was unable to arrest oxidative stress-induced apoptosis in mammalian macrophages once the signal to commit cell death has already been transduced. As shown by ROS quenching data, the ability of p35 to act upstream was also evident from the experiments where p35 expression prior to oxidant exposure was able to significantly prevent mitochondrial cytochrome c release. The ROS quenching property of p35 could possibly play a role in this scenario, since in-vivo generation of ROS is related to mitochondrial membrane permeability (ΔΨm) decrease and subsequent release of cytochrome c (Jacobson, 1996). It is proposed that p35-mediated reduction in ROS level in macrophages might stabilize the mitochondrial membrane potential ΔΨm and as a result prevent cytochrome c release.

Taken in totality, the present observation on the ability of p35 to avert oxidative stress-induced apoptosis in mammalian macrophages by modulating cytochrome c release and consequent activation of caspase as well as its ability to serve as ROS quencher has contributed to our molecular understanding of mechanism of action of this antiapoptotic protein.
Caffeine and oxidative stress-induced apoptosis in Sf9 cells

In order to further authenticate these observations on the ability of p35 to abrogate oxidant-induced apoptosis, a parallel study was planned with a chemical antioxidant and a well known dietary supplement, caffeine. Caffeine, an ingredient of coffee and one of the most widely consumed drug, shows differential pharmacological activity depending on the dose and the route of exposure. Recently, caffeine has been investigated for its potential antioxidant activity against ROS-induced microsomal membrane damage, which acts by inhibiting lipid peroxidation (Devasagayam et al., 1996). The protective effect of caffeine on radiation-induced apoptosis in human TK6 lymphoblastoid cells and TP53-deficient human leukemia cell line, HL60, has also been suggested (Zhen and Vaughan, 1995; Ning and Knox, 1999). However, in murine EL4 T-lymphoma cells, caffeine tends to enhance radiation-induced apoptosis (Palayoor et al., 1995). Caffeine, with potent antioxidant abilities, is known to act differently depending on its concentration and the amount of oxygen present in the incubation medium (Kamat et al., 2000).

Sf9 cells were subjected to oxidative stress which was generated either by directly exposing the cells to H2O2 or by exposing them to UV radiation or by in-situ generation of free radicals by inhibiting catalase activity. Results clearly show that caffeine also operate via an antioxidant pathway in the inhibition of apoptosis in Sf9 cells. This is apparent from the ability of caffeine to arrest H2O2-induced apoptosis or those induced by blocking cellular scavengers of ROS, such as catalase or upon UV-exposure. The results obtained from the experiment designed to look at the time-dependent inhibition of oxidative stress-induced apoptosis showed increasing protection as a function of exposure time. While this could possibly be a reflection of the increased presence of caffeine within the cells on prolonged exposure, it was nonetheless clear that the observed effect of caffeine was not the result of extracellular inactivation of H2O2 but likely due to the
quenching of H$_2$O$_2$ and/or its derived free radicals and/or physiological interception in-vivo, by caffeine, of signals inducing apoptosis. Caffeine was required well before the initiation of apoptosis by oxidative stress implying that it intercepts the oxidative stress-induced pathway at an upstream step of apoptosis. Insect cells exposed to oxidative stress prior to caffeine treatment showed, in a manner similar to p35, that caffeine was unable to arrest oxidative stress-induced apoptosis once the signal to commit cell death has already been transduced. Caffeine’s action at an upstream step in apoptotic cascade was evident from the experiment where cells exposed to caffeine before subjecting them to oxidative insult were able to significantly inhibit the release of mitochondrial cytochrome c into the cytosol. Caffeine acts to induce cell death via caspase-dependent pathway. This was shown by an experiment where caffeine was able to prevent oxidative stress-induced activation of caspase specifically Sf-caspase-1 by inhibiting its processivity from an inactive form to generate active cleaved products. In fact, the data on the reduced caspase mRNA levels observed in caffeine pretreated cells with respect to control cells suggests that caffeine may be acting at the transcriptional level to inhibit the enhanced expression of caspase. Caffeine being a potent free radical scavenger can protect the cells from cell death by quenching the reactive oxygen species at an initial stage of the signalling cascade before the cells can commit suicide. Alternatively, caffeine may directly inhibit the Sf-caspase-1 thereby preventing cell death. These data while complementing the antioxidant property of caffeine with respect to its involvement in lipid peroxidation additionally demonstrate that caffeine can inhibit H$_2$O$_2$-induced apoptosis in Sf9 cells with possible therapeutic applications.

Normal cellular metabolism has to constantly face the onslaught of a continuous production of reactive oxygen species (ROS), as superoxide and hydroxyl radicals are formed as primary by-product of the mitochondrial respiratory chain. Different organisms have evolved different mechanisms to counteract this. Apart from
different antioxidative enzymes such as superoxide dismutase, glutathione S-transferase, catalase, certain other proteins too function as ROS quenchers. Among these is the p35 which apart from acting fairly downstream in the apoptotic cascade to inhibit group I, II and III caspases (Scarlett and Murphy 1997; Zhou et al., 1998; Thornberry and Lazebnik, 1998) also functions as a decisive antioxidant as shown in this work. Neuronal expression of p35 protein in transgenic mice confers functional caspase inhibitory activity and prevents apoptosis in isolated cerebellar culture (Viswanath et al., 2000). Preliminary result reported in this thesis on injecting p35 cloned under the CMV promoter, suggests that baculoviral p35 can be stably expressed for weeks when injected into mice. Microdissection, in the in-vivo context, of the molecular events associated with p35-mediated inhibition of apoptosis both at an upstream as well as downstream steps in the cell death pathway will lead to a better understanding of its antiapoptotic function. This will clearly have an implication on preventing the progress of various oxidative stress-induced degenerative diseases.
Summary & Conclusion
SUMMARY

Apoptosis or programmed cell death (PCD) is a highly conserved genetically controlled response of metazoan cells to commit suicide. PCD plays a crucial role in the regulation of cellular and tissue homeostasis and any aberrations in apoptosis leads to several diseases including cancer, neurodegenerative disorders and AIDS. The mechanisms by which apoptosis is controlled are varied. Caspases play a key role in the execution of cell death by mediating highly specific cleavages of crucial cellular proteins collectively manifesting the apoptotic phenotype. Protein inhibitors like crmA, p35 and IAPs prevent/control apoptosis induced by a broad array of cell death stimuli by several mechanisms. Among endonucleases, caspase activated DNase (CAD) plays a crucial role in DNA fragmentation, a biochemical hallmark of apoptosis. As regulation of cell death seems to be as complex as regulation of cell proliferation, multiple kinase-mediated regulatory mechanisms might control the apoptotic process.

Baculoviruses encode two mechanistically distinct apoptotic suppressers, inhibitor of apoptosis (IAP) and p35. Both viral proteins prevent premature insect cell death and thereby promote virus multiplication. p35 is a suicide inhibitor of group I, II and III caspases when assayed in-vitro. Despite, its effectiveness as a universal apoptotic suppresser, the intracellular targets of p35 and its effect on caspase activation are still not very clear. The objective of this study was to find out the role of p35 in inhibiting oxidative stress-induced apoptosis.

The results of this study are summarized below:

Mechanism of action of p35 in abrogating oxidative stress-induced apoptosis in insect cells

It was seen that insect cells undergo apoptosis as a function of oxidative stress generated by H₂O₂ or by inhibiting catalase activity by hydroxylamine treatment, which leads to in-situ accumulation of
H2O2. In this model system, oxidative stress-induced apoptosis can be inhibited by DMSO treatment. Baculovirus p35 gene is able to inhibit H2O2- and UV-induced apoptosis in insect cells both in viral context (AcNPV) as well as when transfected in cells in plasmid form containing p35 alone under heat shock promoter. Recombinant AcNPV carrying specific p35 deletion failed to rescue Sf9 cells from oxidative stress-induced apoptosis directly pointing to the involvement of p35 in this process. p35 is unable to intercept oxidative stress-induced apoptosis once the signal to induce apoptosis has been triggered. p35 acts very upstream in the pathway of oxidative stress-induced apoptosis by acting as a sink for reactive oxygen species.

**Mechanism of action of p35 in abrogating oxidative stress-induced apoptosis in murine macrophages**

It was observed that H2O2 can induce apoptosis in rat peritoneal macrophages in a concentration-dependent manner. Also, it is evident that p35 can intercept hydrogen peroxide-induced apoptosis in mammalian system by acting at an upstream step in the pathway of oxidative stress-induced apoptosis. Similar to insect cells, p35 cannot abrogate oxidative stress-induced apoptosis once the stimuli for the apoptotic induction is already given. Upstream action of p35 is also confirmed by its ability to inhibit H2O2-induced cytochrome c release from mitochondria in macrophages. p35 also inhibits H2O2-induced caspase-3 activation in macrophages.

A preliminary data, in mice, on the stable expression of p35, cloned under mammalian CMV promoter, provide a basis for its utility in higher organism under the predisposed conditions of oxidative stress.

**Mechanism of action of a chemical antioxidant, caffeine, in abrogating oxidative stress-induced apoptosis in insect cells**

It was demonstrated that caffeine is also able to inhibit oxidative stress-induced apoptosis in dose- and time-dependent manner in
insect cells. The study of mechanism of action of caffeine reveals that caffeine, similar to p35, acts very upstream in the pathway of oxidative stress-induced apoptosis. Caffeine is shown to inhibit mitochondrial cytochrome c release and caspase activation in Sf9 cells. Caffeine inhibits UV-induced upregulation of caspase mRNA levels thereby inhibiting oxidative stress-induced apoptosis.

CONCLUSION

The existence of an alternate pathway of action of p35 in inhibiting oxidative stress-induced apoptosis makes this protein of possible therapeutic importance. The following are the conclusion of experiments described in this thesis.

- Insect cells as well as murine macrophages undergo oxidative stress-induced apoptosis which can be abrogated by baculoviral p35 gene.
- Baculoviral p35 deletion mutant fails to rescue both insect cells and macrophages from undergoing oxidative stress-induced apoptosis.
- p35 is unable to abrogate oxidative stress-induced apoptosis in insect cells and murine macrophages once the apoptotic stimuli is given before the transfection with p35 suggesting that p35 acts upstream in the apoptotic cascade.
- p35 serves as a sink for free radicals.
- p35 inhibits H$_2$O$_2$-induced mitochondrial cytochrome c release and caspase activation in murine macrophages.
- CMV-p35 show stable expression of p35 in mice till 14 days after injection (i.d. and i.m.)
- Caffeine inhibits oxidative stress-induced apoptosis in insect cells by inhibiting mitochondrial cytochrome c release and caspase activation. Caffeine also inhibits the UV-induced enhancement in caspase mRNA levels.
Diagramatic representation of the interception of p35 at two different stages of apoptotic pathway.
REFERENCES


