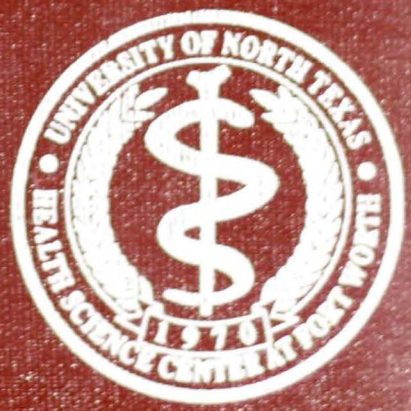
UNIVERSITY OF NORTH TEXAS
HEALTH SCIENCE CENTER AT FORT WORTH
1970

Rohini Dhar, Involvement of p70S6K in cisplatin-induced cell death. Doctor of Philosophy (Biochemistry and Molecular Biology) May 2008, 151 pp., 23 illustration, 3 tables, 153 references.

Cisplatin is used for the treatment of solid tumors; however its success is often compromised due to relapse and chemoresistance. The purpose of this dissertation is to delineate the role of p70S6K in cisplatin-induced apoptosis.

A comparison of p70S6K levels in H69 cells that acquired resistance to cisplatin (H69/CP) compared to parental H69 cells revealed that levels of phosphorylated p70S6K and S6 were elevated. Cisplatin treatment resulted in the activation of p70S6K and downregulation of total p70S6K. Inhibition of the phosphoinositide 3-kinase (PI3K) pathway by itself augmented cisplatin-induced PARP cleavage and also blocked the phosphorylation of p70S6K. Inhibition of extracellular signal-regulated kinase (ERK) pathway however attenuated cisplatin-induced PARP cleavage. These results reveal that phosphorylation of p70S6K is associated with increased cisplatin resistance, and inhibition of PI3K/p70S6K pathway could reverse cisplatin resistance.

We have found that cisplatin caused a time- and concentration-dependent downregulation of p70S6K. While the calpain and the proteasome inhibitors had no effect on the downregulation of p70S6K, the broad specificity caspase inhibitor z-VAD-fmk (z-VAD) reversed p70S6K downregulation by cisplatin. Furthermore, the caspase-3 inhibitor and knockdown of caspase-3 prevented cisplatin-induced proteolytic cleavage of p70S6K. While, cisplatin failed to induce cleavage of p70S6K in MCF-7 cells that lack functional caspase-3, overexpression of caspase-3 in these cells resulted in cisplatin-

induced cleavage of p70S6K. Thus, these results demonstrate that p70S6K is a novel substrate for caspase-3.

Examination of the role of p70S6K in cisplatin-induced death shows that rapamycin a pharmacological inhibitor of p70S6K, enhanced cisplatin-induced apoptosis in A549 cells. However, knockdown of p70S6K by siRNA resulted in a decrease in cisplatin-induced apoptosis. In addition, caspase-3 mediated cleavage of p70S6K at the aspartic acid residue at the 393 position and site-directed mutagenesis of Asp393 to Ala resulted in protection against cisplatin-mediated apoptosis. Interestingly, introduction of the N-terminal cleaved fragment [Δ(394-525)] resulted in potentiation of cisplatin-induced apoptosis. These results suggest that the proteolytic cleavage of p70S6K by caspase-3 is important for cisplatin-induced apoptosis.

INVOLVEMENT OF p70S6K IN CISPLATIN-INDUCED

CELL DEATH

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INVOLVEMENT OF p70S6K IN CISPLATIN-INDUCED CELL DEATH

DISSERTATION

Presented to the Graduate Council of the

Graduate School of Biomedical Science
University of North Texas
Health Science Center at Fort Worth
in Partial Fulfillment of the Requirements

For the Degree of

Doctor of Philosophy

By

Rohini Dhar, M. S.

Fort Worth, Texas

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CHAPTER IV

LIST OF ABBREVIATIONS

A	Alanine
ANOVA	Analysis of Variance
Apop	Apoptosis
CARD	Caspase recruitment domains
CGC	Conventional gastric carcinoma
CDK	Cyclin-dependent kinase
Cyt *c*	Cytochorome *c*
D	Aspartic acid
DED	Death effector domains
DISC	Death-inducing silencing complex
EGFR	epidermal growth factor receptor
ERK	Extracellular signal-regulated kinase
G1 Phase	Gap1 Phase
GRPR	Gastrin-releasing peptide receptor
GIST	Gastrointestinal stromal tumors
JNK	c-Jun N-terminal kinase
mTOR	Mammalian target of rapamycin
NSCLC	Non small-cell lung cancer
p70S6K	p70 ribosomal S6 kinase

PARP	PARP
PPARγ	Peroxisome proliferator-activated receptor γ
PDK-1	Phosphoinositide-dependent protein kinase 1
PI3K	phosphoinositide 3-kinase
PKC	Protein kinase C
PI	Propidium iodide
S phase	Synthesis Phase
siRNA	small interfering RNA
SCLC	Small-cell lung cancer
T389	Threonine-389
TNF	Tumor necrosis factor-α
WT	Wild Type
XA	Xanthohumol
z-VAD	z-VAD-fmk

CHAPTER I

INTRODUCTION

Lung Cancer

Lung cancer is the leading cause of cancer mortality in the United States. It is characterized by the uncontrolled growth of abnormal cells in the lungs affecting more men than women. Small-cell lung cancer (SCLC) is a distinct histological subtype of lung cancer (www.lungcancer.org) characterized by rapid growth and early dissemination [1]. On the other hand, non-small cell lung cancer (NSCLC) accounts for approximately 80% of all lung cancers (www.lungcancer.org) and grows slower than SCLC. Even when diagnosed at early stage of the disease the prognosis in NSCLC patients is poor [2]. Approximately 213,380 new cases of lung cancer were estimated to be diagnosed in 2007 (www.cancer.gov). Despite significant improvements in cancer diagnosis and treatment almost 160,390 patients were expected to have died from the disease in 2007 (www.cancer.gov). Some of the risk factors involve tobacco smoke, radon and asbestos exposure, and air pollution (www.cancer.gov, www.lungcancer.org). Thus, there is a need for the development of new targeted therapies of lung cancer.

Apoptosis

Normal cellular function is maintained by the precise regulation of signaling pathways to control the cell's decision to proliferate, differentiate or initiate programmed cell death. Apoptosis or programmed cell death is tightly regulated at the genetic and molecular level. Apoptosis gets rid of unwanted and/or damaged cells to maintain a balance between cell proliferation and cell death [3, 4]. Apoptosis is also important in chemotherapy-induced tumor cell killing [5]. DNA cleavage, nuclear polypeptide modifications and protein proteolysis are some of the molecular changes occurring during apoptosis. Morphological alterations include plasma membrane blebbing, cell shrinkage, chromatin condensation, DNA fragmentation and apoptotic body formation [6].

Caspase

Caspases are a family of cysteine proteases that are essential to the apoptotic process. Activation of the caspases in response to insult eventually ensures the elimination of either seriously damaged or no longer needed cells [7, 8]. Caspases are enzymes that have a stringent specificity for cleaving protein substrates containing a tetrapeptide sequence ending in aspartic acid residues [9-11]. Caspases are present in most healthy cells as inactive precursors often called caspase zymogens (procaspases). They share a common domain structure consisting of a prodomain, a large (p20) and a small (p10) subunit [12]. Upon receiving an apoptotic signal, the caspase zymogens

undergo proteolytic processing to generate a large and a small subunit that comprise the active enzyme [13]. Caspases are usually classified into two groups: initiators (caspase -2, -8, -9 and -10) and effectors (caspase -3, -6 and -7). The groups are divided based on their structural and functional differences. The initiator caspases have a long prodomain and can interact with signaling adaptor molecules through motifs in these prodomains [14]. The N-terminal domain of caspase-2 and -9 contains CARD or caspase recruitment domains whereas caspase-8 and -10 contain DED or death effector domains [15, 16]. The effector caspases on the other hand lack the large N-terminal prodomain [16]. Effector caspases cleave cellular proteins resulting in apoptosis [12]. Caspases can be activated through oligomerization of the initiator caspases (2, 8, 9 and 10), or through cleavage of the executioner caspases (3, 6 and7) [17] .

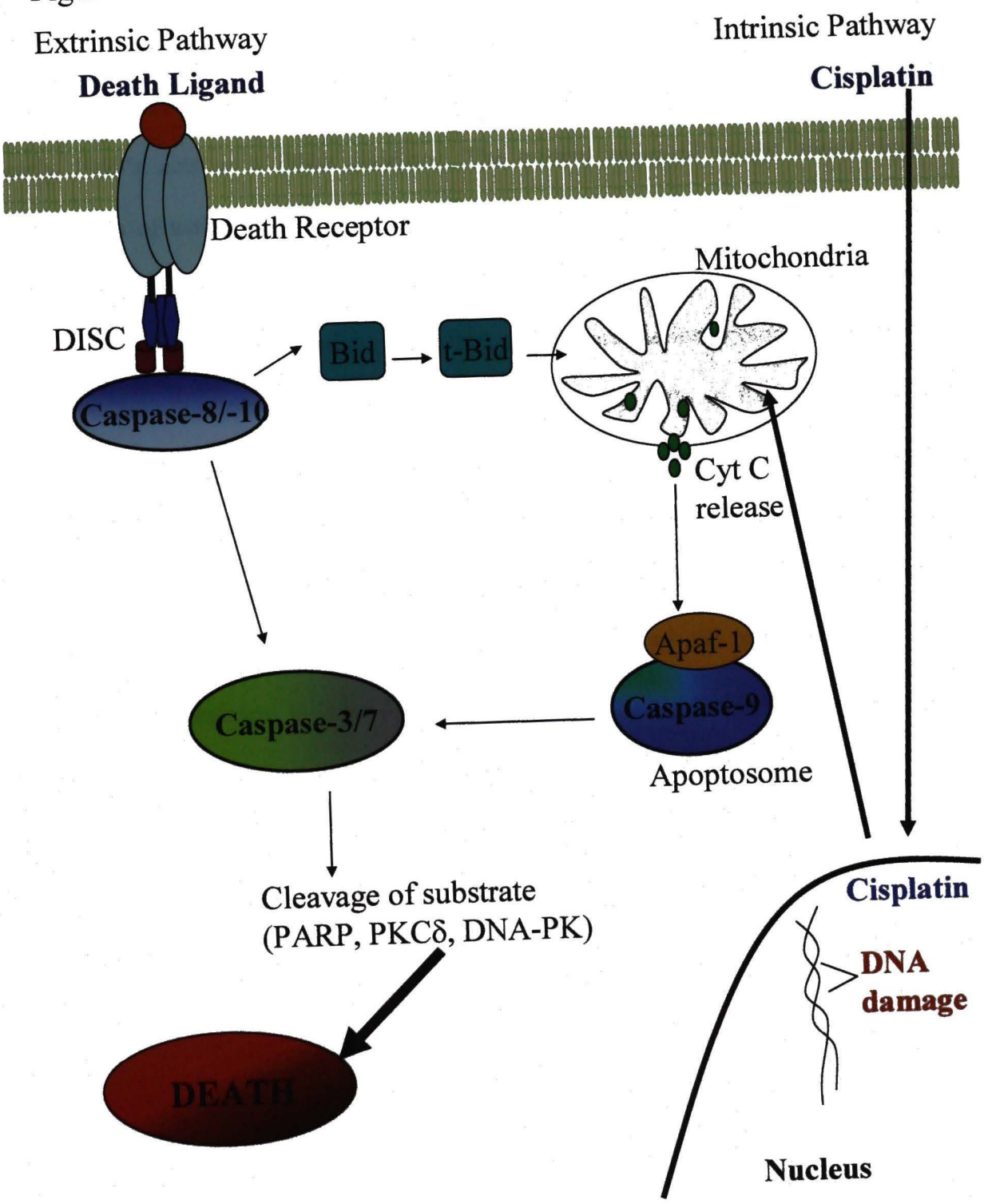
Two independent initiation pathways of the caspase cascade have been reported; the death receptor mediated extrinsic pathway and the mitochondria mediated intrinsic pathway. Both of these pathways result in activation of caspases and cleavage of critical cellular proteins to cause cell death. The extrinsic pathway is mediated by death receptors on the cell surface. It begins with the assembly of a death-inducing signaling complex (DISC) consisting of adaptor molecules and caspase-8 or -10 [4, 18]. The activated initiator caspases start the caspase cascade that results in apoptosis. Activation of the intrinsic pathway begins when DNA damaging drugs and other stress factors result in the release of apoptotic factor from the mitochondria [19]. Apoptotic factors like Cytochrome

C (Cyt c), Smac/DIABLO, HtrA2/Omi are released into the cytosol [19]. Cyt c binds to apoptotic-protease-activating factor 1 (Apaf-1) and procaspase-9 resulting in formation of the apoptosome and caspase-9 activation [4, 20]. Finally, activation of caspase -3 or -7 leads to cleavage of downstream target proteins and ultimately cell death [14, 21].

Figure 1. Extrinsic and intrinsic cell death pathways

The extrinsic pathway is activated by death ligands that bind to death receptors, resulting in adaptor proteins recruitment and activation of initiator caspases-8 or -10 leading to the activation of downstream effector caspases. The intrinsic pathway maybe activated by stress signals which causes the release of cytochrome c (Cyt c). Cyt c and the adaptor protein Apaf-1 participate in formation of the apoptosome and caspase-9 activation. Effector caspases are activated by caspase-9 leading to cell death. Further, caspase-8 can cleave Bid, which induces translocation of truncated Bid (tBid) to the mitochondria to initiate the intrinsic pathway. Crosstalk between the two pathways is mediated by the cleavage of the proapoptotic protein Bid [22].

Figure 1.


Extrinsic Pathway
Death Ligand
Intrinsic Pathway
Cisplatin
Death Receptor
Mitochondria
DISC
Bid
t-Bid
Caspase-8/-10
Cyt C
release
Apaf-1
Caspase-9
Caspase-3/7
Apoptosome
Cleavage of substrate
(PARP, PKCδ, DNA-PK)
Cisplatin
DNA
damage
DEATH
Nucleus

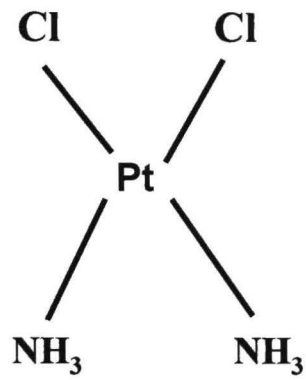
Cisplatin

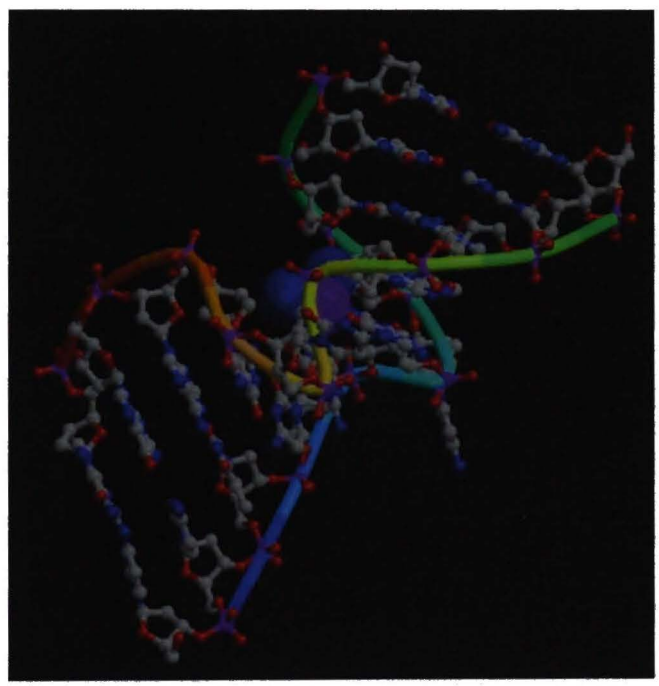
DNA damaging drugs have proved to be effective in the treatment of various carcinomas [23-25]. *cis*-diamminedichloroplatinum(II) or cisplatin discovered in the early 1960's acts as a DNA alkylator. The current accepted model about cisplatin mechanism of action is that the drug induces its cytotoxicity through binding to DNA [26] and subsequent interference with normal transcription, and/or DNA replication mechanisms. Most notable among the DNA adducts are the intrastrand cross-links with purine bases. These include guanine which form nearly 90% of the adducts and less commonly adenine. Other adducts include inter-strand crosslinks and nonfunctional adducts that may contribute to cisplatin's activity [27]. These cisplatin-DNA adducts initiate cell death. Cisplatin has been in widespread use for many years to treat several forms of cancer, including testicular, ovarian, cervical, head and neck, and lung cancer [24, 28, 29]. Treatment is limited, however, due to chemo resistance and side effects, including nephrotoxicity, emetogenesis and neurotoxicity [30-32]. Cisplatin can cause mitochondrial release of cyt *c* and caspase-3 activation resulting in cell death [33]. Cellular response to cisplatin can be mediated by several different signaling pathways some of which are Akt, extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), protein kinase C (PKC) [34-39].

Figure 2. Cisplatin and its interaction with DNA

Structure of cisplatin containing a platinum centre. Stereo-views of cisplatin bound to DNA around a vertical axis. The platinum group cross-links with guanine and protrudes in the minor groove of the DNA duplex. Taken from Coste et.al. (1999) Crystal structure of a double-stranded DNA containing a cisplatin interstrand cross-link at 1.63 A resolution: hydration at the platinated site. *Nucleic Acids Res* 27, 1837-1846.

Figure 2.





p70S6K

Tumor cell proliferation requires acceleration and upregulation in the transcription and translation rate of genes. p70 ribosomal S6 kinase (p70S6K) is a serine/threonine protein kinase responsible for the phosphorylation and activation of the 40S ribosomal subunit protein S6 [40, 41]. p70S6K can be activated by a variety of agents such as epidermal growth factor, insulin, ultraviolet rays, amino acids, thyroid hormone, hydrogen peroxide, prolactin and bone morphogenetic protein-2 [42-47].

a) Structure of p70S6K

Mammalian cells contain two similar S6 kinase proteins the p70S6K and p85S6K isoforms [48], encoded from the same transcript but differing at their initiation start sites [49, 50]. The two isoforms are coordinately regulated and differ by a 23 amino acid N-terminal extension. The amino terminus sequence of p85S6K has a nuclear localization signal and a casein kinase II site that targets the protein to the nucleus [49, 51-53]. p70S6K has a poly adenylation signal followed by a poly (A) tail and a single, centrally located catalytic domain of 267 residues. The catalytic domain has an amino-terminal segment of about 88 amino acids and a carboxy-terminal segment of 170 amino acids. The ATP-binding site of the protein is contained within the catalytic domain. The catalytic domain of p70S6K shares 40-44% identity with catalytic domains of the PKC

family. A sequence of 24 amino acids situated towards the carboxy terminus end of the protein possibly functions as a pseudosubstrate domain [50].

b) Phosphorylation and activation

p70S6K has a highly acidic N terminus, a catalytic domain and a regulatory C-terminal domain. It has also been suggested that p70S6K cannot autoactivate itself due to the presence of proline residues immediately following five of the Ser/Thr sites in the carboxy terminal region [54]. p70S6K activation is a complex process that requires multiple signaling inputs. Phosphorylation at two distinct sets of sites regulates the activation of p70S6K [41]. The autoinhibitory domain contains one set of the phosphorylation sites- S411, S418, T421 and S424. These sites are followed immediately by a proline residue [55]. The other set of phosphorylation sites T229 [56], S371 [57], T389 and S404 [56] are flanked by bulky aromatic residues on either side.

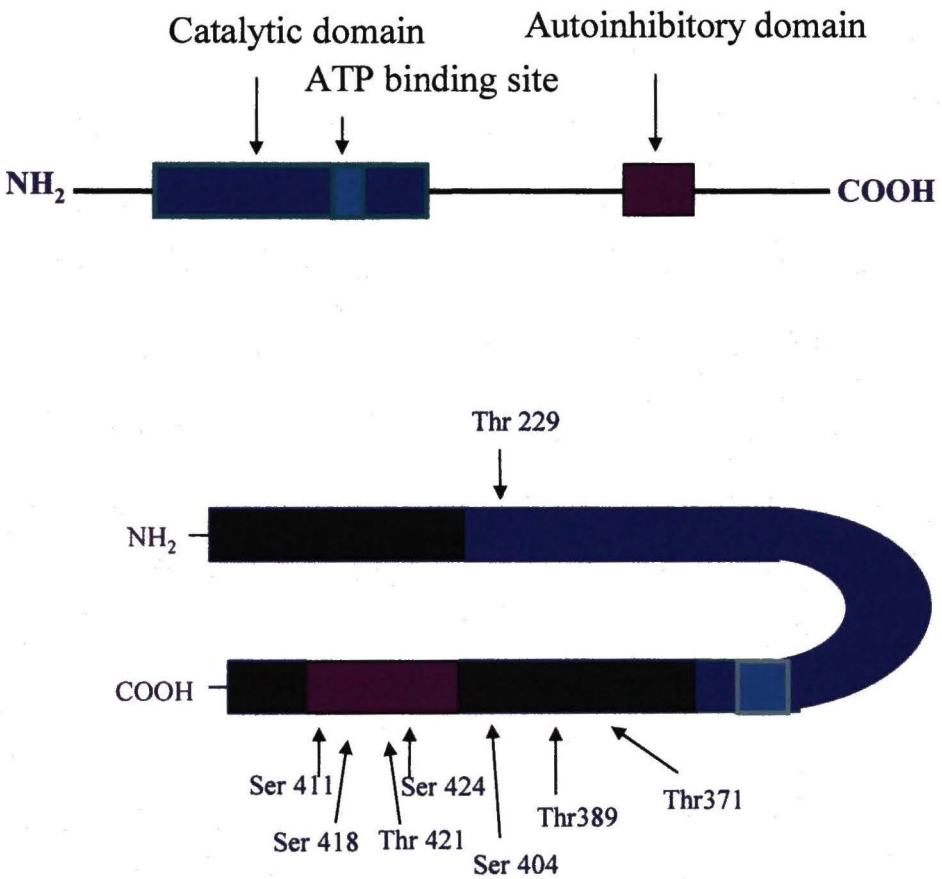
T389 is the principal target of p70S6K phosphorylation since mutation of T389 to non phosphorylatable alanine reduces kinase activity whereas mutation to phospho-mimicking glutamic acid confers constitutive kinase activation [56]. It has been shown both *in vivo* and *in vitro* that phosphoinositide-dependent protein kinase 1 (PDK-1) is also known to phosphorylate p70S6K at T229 [58]. During mitosis the Cdc2-cyclin B complex can phosphorylate p70S6K at S411 [59]. While the mammalian target of rapamycin (mTOR) phosphorylates p70S6K at T389 [60], recently it has been shown that

phosphatidic acid can phosphorylate p70S6K at T389 and T421/S424 [61]. Insulin-induced T389 phosphorylation was almost completely blocked when S411 was mutated to an alanine. This suggests that phosphorylation at S411 is required for T389 phosphorylation and activation of the protein [62]. Thus, T229 in the activation loop, as well as S371 and T389, in the linker region coupling the catalytic and autoinhibitory domains, and S411 in the autoinhibitory region are important for kinase activation [56, 58, 62].

Figure 3. Structure of p70S6K and its phosphorylation sites

Full length p70S6K is a 70-kDa protein consisting of a catalytic domain at the highly acidic amino terminal end and an autoinhibitory domain at the carboxy end. The ATP binding site is contained within the catalytic domain. The autoinhibitory domain contains one set of the phosphorylation sites S411, S418, T421 and S424. These sites are followed immediately by proline residues. The other set of phosphorylation sites T229, S371, T389 and S404 are flanked by bulky aromatic residues on either side.

Figure 3.


Catalytic domain
ATP binding site
Autoinhibitory domain
NH2
COOH
Thr 229
NH2
COOH
Ser 411
Ser 418
Thr 421
Ser 424
Ser 404
Thr389
Thr371

c) Inhibition of p70S6K

Rapamycin is a macrolide fungicide isolated from the bacteria *Streptomyces hygroscopicus* that possesses potent antimicrobial, immunosuppressant, and antitumor properties [63, 64]. Rapamycin inhibits basal as well as insulin-stimulated activation of p70S6K [65, 66]. The phosphorylation of T229, T389 and S404 is blocked by treatment of cells with the immunosuppressant rapamycin [56]. On the other hand, the activity of p85S6K is unchanged by the addition of rapamycin [65]. Though induction of apoptosis is not universal, rapamycin is in clinical trials as a chemotherapeutic drug [63, 67-70]. Numerous studies have shown it can synergize with other agents such as tamoxifen, imanitib and doxorubicin to promote apoptosis [71-73]. Similarly, rapamycin derivatives enhanced cisplatin-induced cell death in ovarian, leukemia and non small cell lung cancers (NSCLC) [74-76]. Rapamycin also prevents cyclin-dependent kinase (CDK) activation, Rb protein phosphorylation, and accelerates the turnover of cyclin D1, leading to G1 arrest [77].

d) Function of p70S6K

The activation of p70S6K and its downstream target ribosomal protein S6 mediates nutrient and mitogen-stimulated translation, which is essential for cell growth and proliferation [41]. Stem cell factor which regulates cellular proliferation can activate p70S6K via the Akt pathway in spermatogonial cells. Further, p70S6K can regulate cell

cycle by activating cyclin D3 translation in these cells to drive G1/S phase progression [78]. In addition, it has been shown that when drosophilas have a deficient p70S6K gene there is a marked retardation in development and smaller body size [79]. Though disruption of the gene in mice did not affect their viability, it resulted in smaller mice [80]. p70S6K can phosphorylate and thus inactivate BAD resulting in cell survival and growth [81]. Furthermore, association of p70S6K with the actin cytoskeleton has been demonstrated and it is known to be involved in actin cytoskeletal organization and cell migration. While actin stress fiber formation results in p70S6K inactivation, the abolition of the fiber formation increases p70S6K activation [82]. Silencing of p70S6K activity decreased cell migration, invasion, and proliferation in ovarian cancer cells [83].

Phosphorylation of S6 which is downstream of p70S6K results in the regulation of translation of a class of mRNA transcripts, which contain an oligopyrimidine tract at their transcriptional start site. This class of mRNAs encodes for many of the components of the protein synthesis apparatus including all ribosomal proteins, elongation factors and poly (A) binding proteins and can represent up to 20% of the total mRNA in the cell [83]. Failure to recruit these messages suppresses the recruitment of the translational machinery required for cell cycle progression. Thus, inhibition of p70S6K activation with either neutralizing antibodies or treatment with immunosuppressant rapamycin severely compromises the cell's ability to progress through the G1 phase of the cell cycle [65, 84]. Amongst other effects serum-induced entry into S phase of the cell cycle, inhibition of

protein synthesis activation and an inhibitory effect on DNA synthesis late in G1 phase, was associated with inhibition of p70S6K activity [84].

Figure 4. Activation of p70S6K by phosphorylation

Mitogen stimulation facilitates T389 phosphorylation resulting in a conformational change of p70S6K. This results in binding and activation of its downstream target S6. Taken from Dennis et.al. (1998) Phosphorylation Sites in the Autoinhibitory Domain Participate in $p70^{s6k}$ Activation Loop Phosphorylation. *The Journal of Biological Chemistry* 273, 14845-14852.

Figure 4.

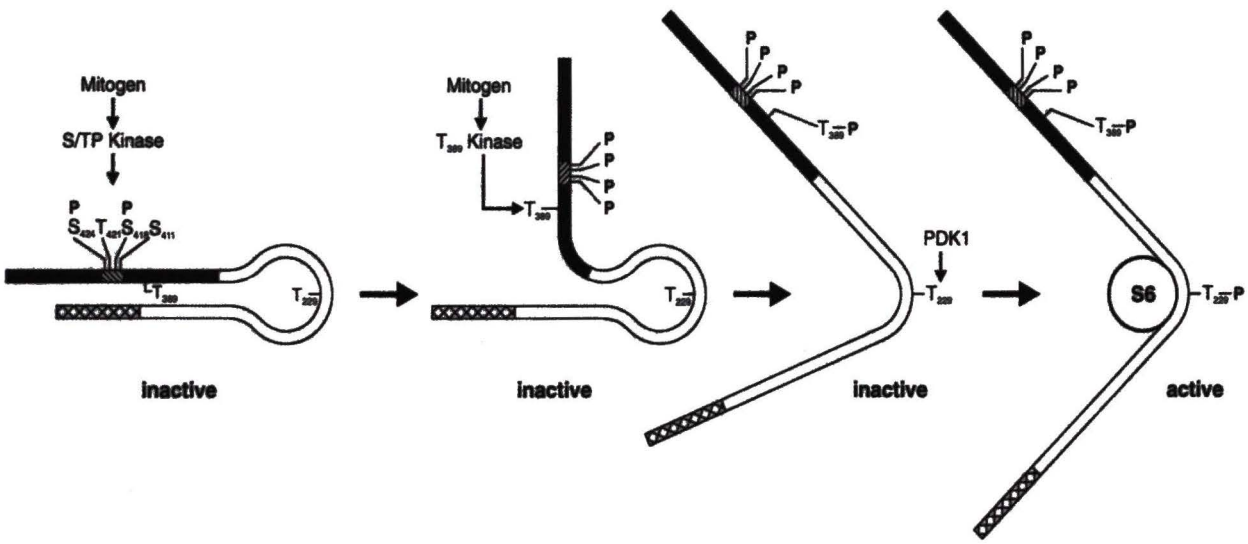
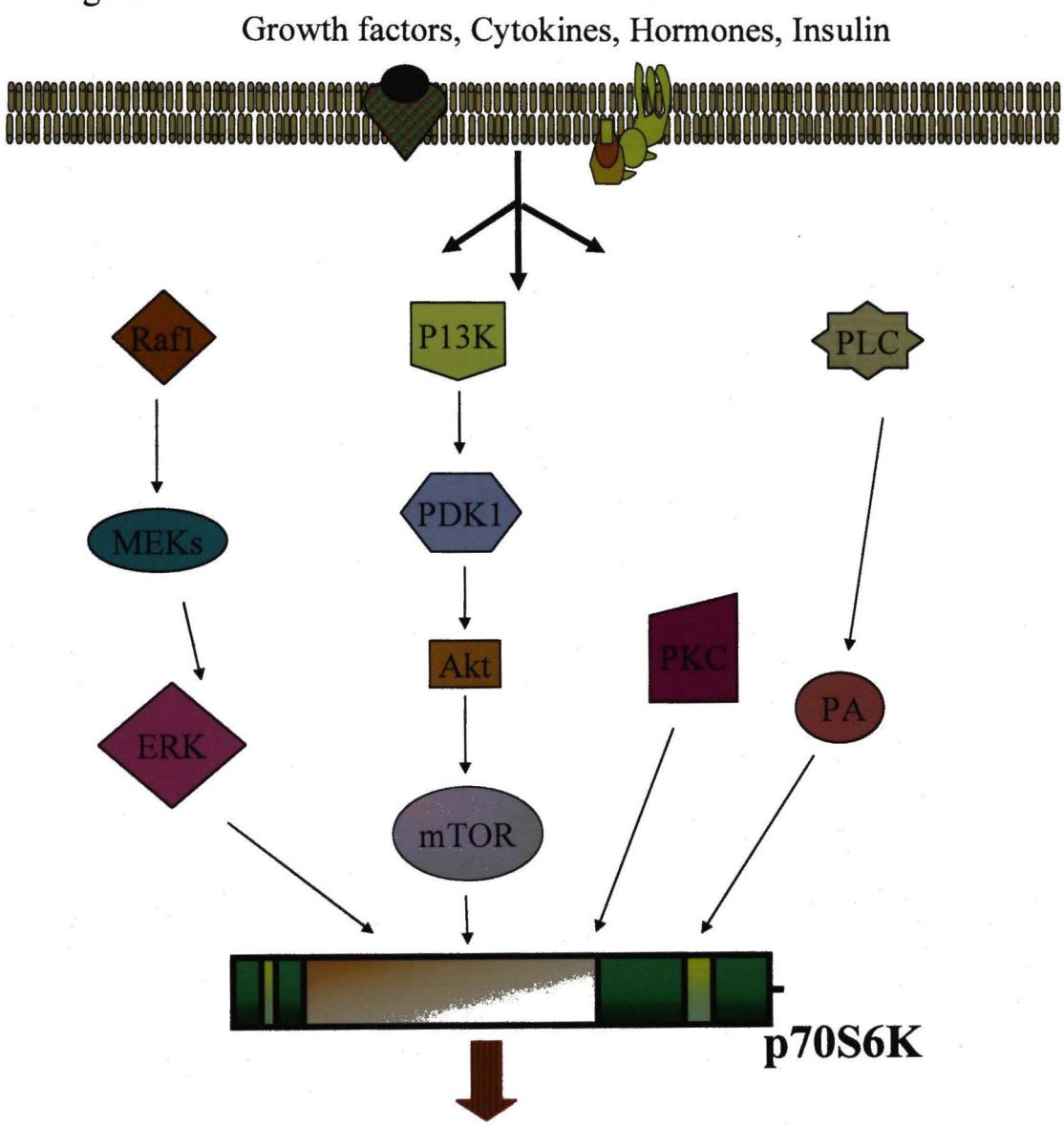

Mitogen
S/TP Kinase
P
P
Mitogen
PDK1
S6
inactive
inactive
inactive
active

Figure 5. p70S6K signaling

The p70S6K signaling pathway involves the activation of diverse upstream proteins that can phosphorylate p70S6K. Activation of p70S6K involves a sequential phosphorylation of multiple Ser/Thr sites, primarily by the rapamycin-sensitive pathway involving PDK1, Akt, and mTOR. Activation of p70S6K may also be mediated by classical and novel PKCs, phosphatidic acid and the Raf/MEK/ERKs.

Figure 5.

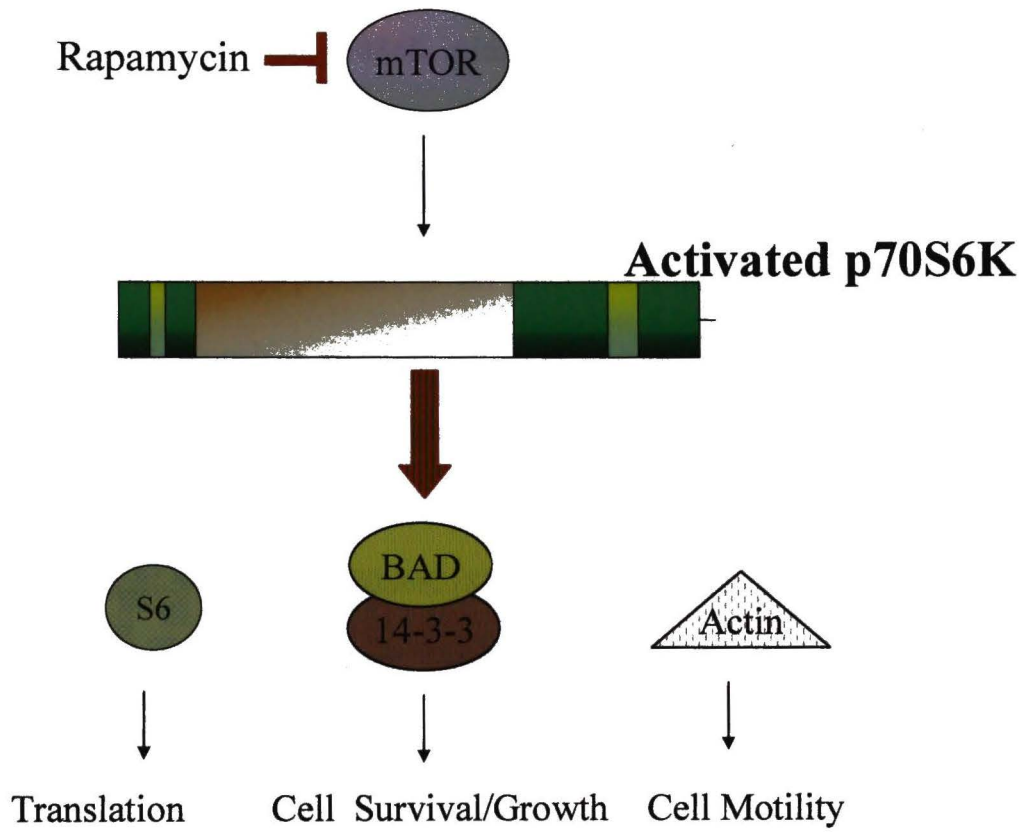


Phosphorylation And Activation Of p70S6K

Figure 6. Downstream targets of p70S6K

p70S6K promotes translation, protein synthesis, cell survival, growth and cell motility. Active p70S6K phosphorylates important downstream effectors of protein synthesis such as S6, a subunit of the 40S ribosome. Phosphorylation of S6 promotes TOP-mRNA class translation initiation. p70S6K promotes cell survival by phosphorylating BAD and cellular migration via actin fibres. Further, p70S6K phosphorylation can be inhibited by rapamycin which blocks activation of mTOR upstream of p70S6K.

Figure 6.


Rapamycin
mTOR
Activated p70S6K
S6
BAD
14-3-3
Actin
Translation
Cell Survival/Growth
Cell Motility

e) p70S6K and cancer

Due to its involvement in regulating translation, cell cycle progression, differentiation and cell motility, p70S6K is implicated as an oncogene [41, 78, 82]. Many lung cancer cells show high constitutively-active p70S6K [46, 85]. Overexpression of p70S6K has been associated with basal and growth factor-stimulated proliferation of SCLC cells [85]. Recently, p70S6K levels have also been shown to be increased in tumorigenic human bronchial epithelial cells compared to normal human bronchial epithelial cells as well as in premalignant and malignant tissue samples [86]. Further, introduction of mutations leading to oncogenic transformation resulted in elevated constitutive phosphorylation of p70S6K [87]. Thus, alterations in the p70S6K pathway can lead to cellular transformation and disrupting the p70S6K pathway maybe a strategy for tumor promoters to function.

The aim of chemotherapy is to hinder cancer cells' ability to grow and/or multiply. p70S6K may be involved in the cellular response to chemotherapy. Several reports have shown that p70S6K activation can be attenuated by DNA damaging agents in different cell lines. DNA damaging agents like etoposide and cisplatin led to the dephosphorylation and decreased activity of p70S6K in Swiss 3T3 and Rat-1 cells [70]. Since the decrease in activity could not be blocked by the broad specificity caspase inhibitor, the inactivation was not caspase mediated [70]. It has also been shown that after a 12 h treatment with cisplatin, the phosphorylation of p70S6K was inhibited while levels

of p70S6K remained unchanged in myoblasts [88]. Cisplatin downregulated phosphorylation of p70S6K in conventional gastric carcinoma (CGC) cells [89]. In bone marrow stromal cells, etoposide exposure led to downregulation of p70S6K phosphorylation [90].

Other agents like Genistein combined polysaccharide (GCP) a nutritional supplement comprising of isoflavones can lead to growth arrest and apoptosis in prostate cancer cells. This effect of GCP was suggested to be mediated by a decrease in p70S7K phosphorylation [91, 92]. There was a modest decrease in phosphorylated levels of p70S6K when the gastrin-releasing peptide receptor (GRPR) and epidermal growth factor receptor (EGFR) pathways were blocked in head and neck squamous cell carcinoma (HNSCC) [93]. Similarly, Leucine-rich repeat C4 (LRRC4) which has been shown to inhibit glioma cell proliferation can decrease phosphorylation of p70S6K [94]. Curcumin has low toxicity in normal cells, but was shown to induce autophagy and G2/M arrest in malignant glioma cells. Autophagic stimulation was suggested to be due to inhibition of the Akt/mTOR/p70S6K pathway [95].

Rosiglitazone, a synthetic ligand for Peroxisome proliferator-activated receptors γ (PPARγ) inhibits cell growth in cancer cells which is mediated by decreased levels of phosphorylation of p70S6K [96]. Exposure to staurosporine that can cause apoptosis and inhibit protein kinases resulted in dephosphorylation and decreased activity of p70S6K [97]. Two tyrosine kinase inhibitors SU11248 and ZD6474 also downregulated

phosphorylated p70S6K levels in gastrointestinal stromal tumors (GIST) [98, 99]. Effects of the tyrosine kinase inhibitor adaphostin have been examined in human leukemia cells. Adophostin downregulated levels of phosphorylated p70S6K at T421/S424 [100]. In head and neck squamous cell carcinoma cells and multiple myeloma cells there was an inhibition of phosphorylation of p70S6K when exposed to farnesyltransferase inhibitors [101, 102]. p70S6K phosphorylation has also been shown to be inhibited by Deoxyspergualin which can induce death in mouse lymphoma cells [103].

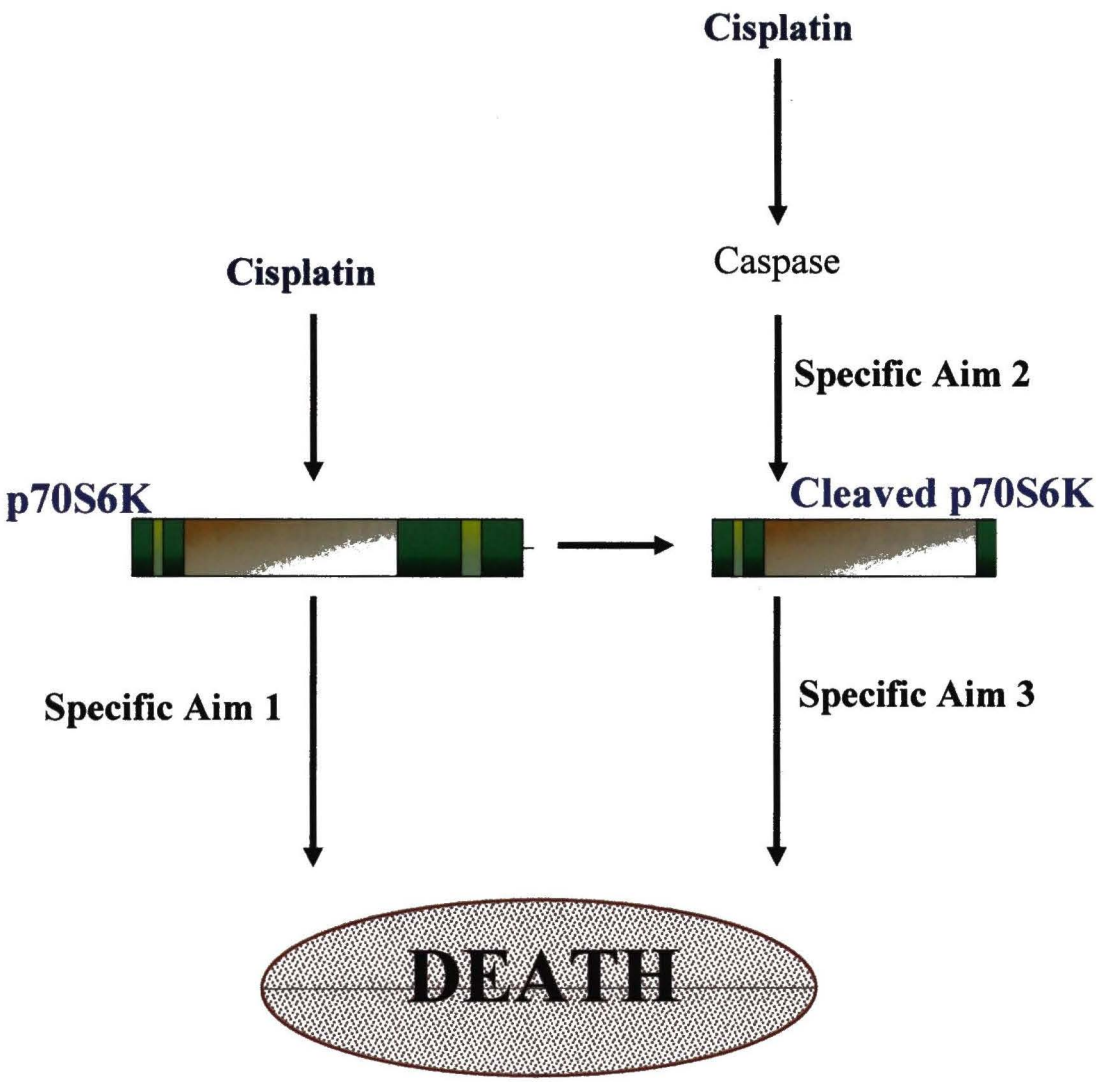
Thus there are numerous reports that show that p70S6K is downregulated when cells are treated with various chemotherapeutic drugs. However, there are also reports indicating that p70S6K activation is enhanced during chemotherapy [104, 105]. A previous report showed that although cisplatin had little effect on p70S6K phosphorylation in cisplatin-resistant SR2 cells, p70S6K phosphorylation was decreased by cisplatin in the parental sensitive cell line [104]. Ultraviolet irradiation which also causes DNA damage induced phosphorylation of p70S6K [105]. Further, activation of the extrinsic death pathway by tumor necrosis factor-α (TNF) activated the phosphorylation of p70S6K in MCF-7 cells [106]. Cisplatin and Xanthohumol (XA) did not decrease the phosphorylation of p70S6K in a highly malignant variant of gastric cancer cells [89] and in leukemia cells [107]. In fact, XA that causes apoptosis in leukemia cells as measured by PARP activation and annexinV staining activated p70S6K .

Though awareness of the role of p70S6K in cancer has been increasing it is not clear as to how p70S6K is regulated by drugs. In an attempt to address this question we are focusing our studies on cisplatin as the drug of choice. Before targeting the p70S6K pathway for therapy it is also important to establish the mechanism by which it is regulated vis a vis the caspase signaling. Further cisplatin resistance in cells is associated with amplification of the Akt1 gene, resulting in activation of its downstream target p70S6K [36]. This has led us to believe that the p70S6K pathway may have an important role in cisplatin-mediated cell death. Thus, understanding the role of p70S6K in cisplatin-induced cancer cell death will facilitate the exploitation of p70S6K as a therapeutic target.

Figure 7. Hypothetical Model of the project

Cisplatin is used in cancer therapy; however acquisition of resistance by tumors to cisplatin is a major problem. The efficacy of cisplatin not only depends on its ability to induce DNA damage but also on the cells' ability to respond to this damage. Treatment with cisplatin initiates cell death and activates the caspase cascade. The hypothesis tested in this dissertation is that cleavage of p70S6K by caspase is important for DNA damage-induced apoptosis. Our hypothesis leads us to a number of questions regarding the role of p70S6K in cisplatin-mediated cell death and resistance, the mechanism by which cisplatin induces downregulation of p70S6K and the importance of caspase-3 mediated cleavage of p70S6K.

Figure 7.


Cisplatin
Caspase
Cisplatin
Specific Aim 2
p70S6K
Cleaved p70S6K
Specific Aim 1
Specific Aim 3
DEATH

Project hypothesis and Specific Aims

Lung cancer is the leading cause of cancer mortality in the United States. It has been shown that p70S6K is constitutively expressed and activated in several cancers, including lung cancer [108]. One of the most common chemotherapies for cancers is DNA damaging agents. Cisplatin or *cis*-diamminedichloroplatinum(II) is commonly used for the treatment of many cancers, including testicular, ovarian, bladder, cervical, head and neck and small cell lung cancer [109]. Cisplatin forms complexes with DNA and exerts its cytotoxicity by directly inhibiting DNA and RNA synthesis. A major clinical setback associated with cisplatin therapy is relapse and resistance to further treatment. Perhaps, surviving cells after DNA damage can develop the ability to activate survival pathways to prepare for the next insult. Although DNA is the major target of cisplatin, other cellular factors can influence its cytotoxicity. We have made a novel observation that p70S6K is cleaved in response to apoptotic stimuli. Furthermore, inhibition of caspases central to the apoptotic machinery prevented cell death and p70S6K cleavage. Thus, we hypothesize that cleavage of p70S6K by caspase is important for DNA damage-induced apoptosis. To test this hypothesis we propose the following specific aims.

Specific Aim 1. To determine if p70S6K is important for cisplatin-induced cell death.

Specific Aim 2. To determine which caspase cleaves p70S6K.

Specific Aim 3. To determine the functional significance of p70S6K cleavage in cisplatin-induced cell death.

Note on Materials used in the Dissertation

Chapter II represents already published data (Dhar, R., and Basu, A., Int J Oncol, 2008. **32**(5): p. 1133-7.)

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CHAPTER II

CONSTITUTIVE ACTIVATION OF p70S6K IS ASSOCIATED WITH INTRINSIC RESISTANCE TO CISPLATIN

SUMMARY

Cisplatin is widely used for the treatment of solid tumors, including small cell lung cancers, but its success is often compromised due to relapse and resistance to further treatment. p70 ribosomal S6 kinase (p70S6K) has been shown to be upregulated in lung cancer cells. In the present study, we have investigated whether the p70S6K pathway contributes to cisplatin resistance in human small cell lung cancer H69 cells. The levels of phosphorylated p70S6K and its downstream target S6 but not total p70S6K or S6 were elevated in the H69 cells that acquired resistance to cisplatin (H69/CP) compared to parental H69 cells. Cisplatin treatment resulted in the activation of p70S6K and downregulation of p70S6K was associated with cisplatin-induced poly(ADP-ribose) polymerase (PARP) cleavage. While the ability of cisplatin to induce apoptosis was attenuated in H69/CP cells, inhibition of p70S6K by rapamycin enhanced cisplatin-induced apoptosis in these cells as evident by the increase in cisplatin-induced PARP cleavage. The phosphoinositide 3-kinase (PI3K) inhibitor Ly294002 alone induced PARP cleavage and further augmented cisplatin-induced PARP cleavage. In contrast, inhibition of extracellular signal-regulated kinase (ERK) by U0126 attenuated cisplatin-induced PARP cleavage. Both rapamycin and Ly294002 enhanced cisplatin-induced activation of

ERK1/2. Taken together, these results suggest that activation of p70S6K contributes to cisplatin resistance in small cell lung cancer H69 cells, and inhibition/downregulation of p70S6K as well as activation of ERK1/2 could circumvent cisplatin resistance.

INTRODUCTION

Cisplatin is widely used for the therapy of several types of cancer, including testicular, ovarian, cervical, and small cell lung cancer (SCLC) (1-3). Cisplatin treatment is, however, limited due to the acquisition of resistance by tumor cells to the drug and its toxic side effects (4-6). The mechanism(s) of cisplatin resistance is often multifactorial and an understanding of cisplatin resistance is critical to exploit this drug effectively for the treatment of cancers.

p70S6K is a serine/threonine protein kinase responsible for the phosphorylation and activation of 40S ribosomal subunit protein S6 (7, 8). It acts downstream of the mammalian target of rapamycin (mTOR). The activation of p70S6K and its downstream target ribosomal protein S6 mediates nutrient and mitogen-stimulated translation, which is essential for cell growth and proliferation (9). Activation of S6 results in the translation of a class of mRNA which contains an oligopyrimidine tract at their transcriptional start site. This class of mRNAs encodes for many of the components of the protein synthesis apparatus. Due to its involvement in regulating translation, cell cycle progression, differentiation and cell motility, p70S6K has been implicated as an oncogene (10).

A number of studies have focused on the involvement of p70S6K in influencing cellular responses to apoptotic stimuli. Treatment of Swiss 3T3 and RAT-1 cells with etoposide and staurosporine resulted in dephosphorylation and decreased activity of p70S6K (11, 12). It has also been shown that cisplatin inhibited the phosphorylation of

p70S6K in mouse myoblasts (13). Rapamycin, an immunosuppressant blocked the activation of mTOR/p70S6K, thus compromising the cell's ability to progress through the G1 phase of the cell cycle (14, 15). Rapamycin derivatives are in clinical trials for the treatment of several cancers (16) although the induction of apoptosis by rapamycin is not universal (17, 18) . It has been reported that rapamycin can synergize with other agents such as tamoxifen, imanitib and doxorubicin to enhance apoptosis (19-22). Similarly, rapamycin derivatives enhanced cisplatin-induced cell death in ovarian, leukemia and lung cancers (23-26). In contrast, it has been reported that rapamycin did not induce apoptosis in Rat-1 and PC12 cells and thymocytes (17, 18).

Cisplatin is often used for the treatment of human SCLCs yet acquisition of resistance by these tumors to cisplatin is a major problem in the therapy of SCLC (27). It has been reported that p70S6K pathway is constitutively activated in several SCLCs (28) and activation of p70S6K was associated with anchorage-independent growth of SCLC (29). In addition, rapamycin analog CCI779 restored cisplatin sensitivity in cisplatin-resistant small cell lung cancer cells (26, 30) although it is not known if p70S6K signaling pathway is affected when cells acquired resistance to cisplatin. Recently, it has been reported that cisplatin resistance of non-small cell lung cancer A549 cells was associated with amplification of AKT1 gene, resulting in activation of its downstream target mTOR pathway (31). In the present study, we have investigated if p70S6K signaling pathway is compromised when SCLC H69 cells acquire resistance to cisplatin. We have demonstrated that p70S6K is constitutively activated in SCLC H69 cells that acquired resistance to cisplatin, and inhibition of p70S6K enhanced cellular sensitivity to

cisplatin. Furthermore, inhibition of PI3K/Akt and mTOR pathway resulted in activation of ERK reversed cisplatin resistance.

EXPERIMENTAL PROCEDURES

Materials

Polyclonal antibodies to p70S6K, phospho-p70S6K (T389), Akt, phospho-Akt (S473), S6 and phospho-S6 (S235/236), were purchased from Cell Signaling Technology, Inc. (Danvers, MA). Monoclonal antibodies to GAPDH and phospho-ERK1/2 were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Monoclonal antibody to PARP was purchased from Pharmingen (San Diego, CA) and ERK1/2 antibody was purchased from BD Biosciences. Monoclonal antibody to actin was obtained from Amersham (Arlington Heights, IL). Horseradish peroxidase-conjugated goat anti-mouse and donkey anti-rabbit antibodies were obtained from Jackson ImmunoResearch (West Grove, PA). The enhanced chemiluminescence detection kit was from Amersham (Piscataway, NJ).

Cell Culture

Parental SCLC H69 cells and cells selected for resistance to cisplatin (H69/CP) were generously provided by Dr. Nagahiro Saijo (National Cancer Center Research Institute, Tokyo, Japan). Cells were maintained in RPMI 1640 (Life Technologies Inc., Grand Island, NY) supplemented with 2 mM glutamine and 10% (v/v) heat-inactivated fetal bovine serum at $37^{O}C$ in the presence of 5% CO_2.

Immunoblot Analysis

Cells were lysed in M-PER mammalian extraction buffer (Pierce, Rockford, IL) containing 1 mM DTT and protease inhibitors. Equal amounts of total protein were separated by 10% (w/v) SDS-PAGE and transferred onto a poly(vinylidene difluoride) membrane. Western blot analyses were performed as described before (10). The blot was probed with antibody to either GAPDH or actin to control for equal loading.

RESULTS

Levels of phosphorylated p70S6K and S6 are higher in cisplatin-resistance human small cell lung cancer (SCLC) H69 cells

To determine if the p70S6K pathway is affected when human H69 SCLC cells acquire resistance to cisplatin, we compared the level and activation status of p70S6K in parental H69 cells and its cisplatin-resistant variant H69/CP cells. Figure 1A shows that there was little change in the total level of p70S6K in the cisplatin-resistant H69/CP cells as compared to the parental H69 cells. Phosphorylation of p70S6K at threonine 389 (T389) is important for the activation of p70S6K (32, 33). Therefore, we compared the phosphorylation status of p70S6K at T389 in the H69 and H69/CP cells. Phosphorylation of p70S6K at T389 was increased by approximately 2-fold in H69/CP cells compared to H69 cells. In addition, the level of phosphorylated S6 (p-S6), which is a downstream target of p70S6K, was also much higher in the H69/CP cells compared to parental cells, indicating increased activation of p70S6K in the H69/CP cells. These results suggest that cisplatin resistance was associated with an increase in phosphorylation/activation of p70S6K.

Cisplatin enhances activation of p70S6K in H69 and H69/CP cells

Since the level of phosphorylated p70S6K was constitutively higher in the cisplatin-resistant cells, we compared the effect of cisplatin on p70S6K phosphorylation and cell death in H69 and H69/CP cells. Activation of caspase-3 or -7 during apoptosis cleaves full-length 115-kDa poly(ADP-ribose) polymerase (PARP) to an 85-kDa

fragment and cleavage of PARP is used as a measure of apoptosis (34). Figure 2 shows that a 48 h exposure to cisplatin caused a concentration-dependent increase in PARP cleavage in H69 cells such that the cleaved fragment of PARP appeared when cells were treated with 20 μM cisplatin and increased further when treated with 40 μM cisplatin. In contrast, 40 μM cisplatin had little effect on the cleavage of PARP in H69/CP cells. Cisplatin caused a concentration-dependent increase in the phosphorylation of p70S6K in both H69 and H69/CP cells. However, while increase in p70S6K phosphorylation was apparent when H69/CP cells were treated with 10 μM cisplatin, higher concentrations of cisplatin were required to induce p70S6K phosphorylation in H69 cells. The level of p70S6K also decreased when H69 cells but not H69/CP cells were treated with 40 μM cisplatin, suggesting that decrease in p70S6K protein level correlates with cisplatin-induced cell death.

Rapamycin enhances cisplatin-induced cell death in H69 and H69/CP cells

Rapamycin is a macrolide fungicide that possesses potent antimicrobial, immunosuppressant, and antitumor properties (16). Rapamycin leads to the dephosphorylation and inactivation of p70S6K (35). Figure 3A shows that rapamycin alone had little effect on PARP cleavage in either H69 or H69/CP cells. While rapamycin enhanced cisplatin-induced PARP cleavage when H69 cells were treated with 10 μM cisplatin, rapamycin had no additional effect when H69 cells were treated with 25 μM cisplatin. Treatment of H69/CP cells with either rapamycin or cisplatin alone had little effect on PARP cleavage. However, pretreatment with rapamycin enhanced sensitivity of

H69/CP cells to cisplatin considerably such that in the presence of rapamycin 25 μM cisplatin was equally effective in inducing PARP cleavage in both H69 and H69/CP cells. Cisplatin caused a concentration-dependent increase in phosphorylation of p70S6K at T389 site and treatment with rapamycin inhibited phosphorylation of p70S6K at T389. Cisplatin-induced phosphorylation of p70S6K correlated with phosphorylation of S6, a downstream target of p70S6K (Fig. 3B), suggesting that p70S6K is activated by cisplatin treatment. As expected, rapamycin inhibited phosphorylation of S6. Thus, dephosphorylation of p70S6K by rapamycin may be associated with increase in cisplatin-mediated cell death.

The PI3K inhibitor Ly294002 decreases p70S6K phosphorylation and augments cisplatin-induced cell death

There are controversies whether or not PI3K/Akt signaling pathway acts upstream of mTOR (36-38). In addition, it has recently been reported that amplification of AKT1 gene in cisplatin-resistant non-small cell lung cancer A549 cells could result in activation of p70S6K (31). We therefore compared the levels of total and phospho-Akt in H69 and H69/CP cells. Figure 4A indicates that levels of total and phosphorylated Akt were comparable in H69 and H69/CP cells. We also examined the effect of Ly294002, an inhibitor of PI3K/Akt, on p70S6K phosphorylation and cisplatin-induced cell death. Figure 4B shows that both H69 and H69/CP cells contained high level of phospho-Akt and Ly294002 but not rapamycin inhibited phosphorylation of Akt in these cells. However, Ly294002 was as effective as rapamycin in inhibiting phosphorylation of

p70S6K, suggesting that PI3K/Akt acts upstream of mTOR/p70S6K in these cells. Since Akt may act upstream of mitogen-activated protein kinase/extracellular signal-regulated kinase kinase (MEK/ERK) signaling pathway which may also regulate p70S6K (39), we examined the effect of MEK inhibitor U0126 on the phosphorylation of ERK1/2, Akt and p70S6K. As shown in Figure 4B, U0126 attenuated cisplatin-induced activation of ERK but not Akt. It also caused a modest decrease in p70S6K phosphorylation. Interestingly, both rapamycin and Ly294002 enhanced cisplatin-induced activation of ERK although Ly294002 was much more effective compared to rapamycin. We also compared the effects of these inhibitors on cisplatin-induced cell death. As shown in Figure 4C, Ly294002 alone induced PARP cleavage and it caused substantial increase in cisplatin-induced PARP cleavage. In contrast, U0126 inhibited cisplatin-induced apoptosis in both H69 and H69/CP cells. There was a good correlation between activation of ERK (Fig. 4B) and cleavage of PARP (Fig. 4C). These results suggest that activation of ERK was associated with cisplatin-induced cell death.

Figure 1. Levels of phosphorylated p70S6K and S6 are higher in cisplatin-resistance human small cell lung cancer (SCLC) H69 cells

A, Western blot analysis was performed with total cellular extracts using indicated antibodies. GAPDH was used as a loading control. Results are representative of three independent experiments. B, Densitometric quantification of total and phospho-p70S6K corrected for loading. Data are mean +/- SEM of three independent experiments.* $p<0.01$ versus T389 levels of control cells using paired student's t-test.

Figure 1

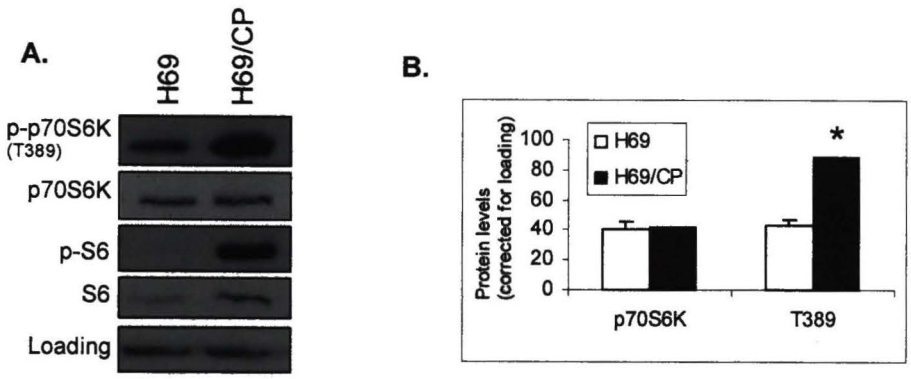

A.
H69
H69/CP
p-p70S6K
(T389)
p70S6K
p-S6
S6
Loading
B.
Protein levels
(corrected for loading)
100
80
60
40
20
0
H69
H69/CP
*
p70S6K
T389

Figure 2. Cisplatin enhances activation of p70S6K in H69 and H69/CP cells

H69 and H69/CP cells were treated with indicated concentrations of cisplatin for 48 h. Western blot analysis was performed with total cellular extracts using indicated antibodies. GAPDH was used as a loading control. Results are representative of three independent experiments.

Figure 2.

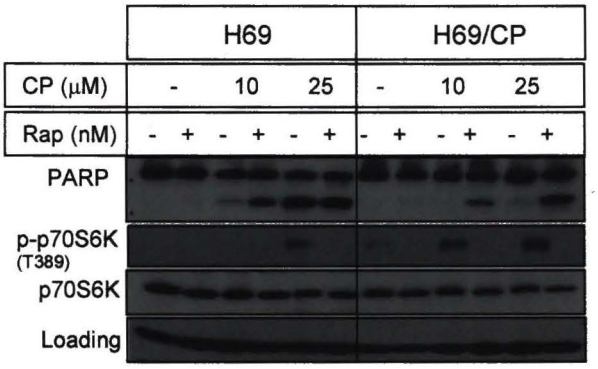

H69
H69/CP
CP (μM)
-
10
20
40
-
10
20
40
PARP
p-p70S6K (T389)
p70S6K
Loading

Figure 3. Rapamycin enhances cisplatin-induced cell death in H69 and H69/CP cells

A and B, H69 and H69/CP cells were treated with 20 nM rapamycin for 30 min prior to treatment with 10 and 25 μM CP for 48 h. Western blot analysis was performed with total cellular extracts using indicated antibodies. Actin was used as a loading control. Results are representative of two independent experiments.

Figure 3.

A.


H69
H69/CP
CP (μM)
- 10 25 - 10 25
Rap (nM)
- + - + - + - + - + - +
PARP
p-p70S6K
(T389)
p70S6K
Loading

B.

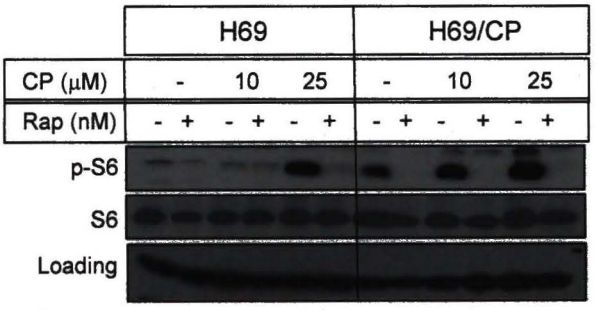
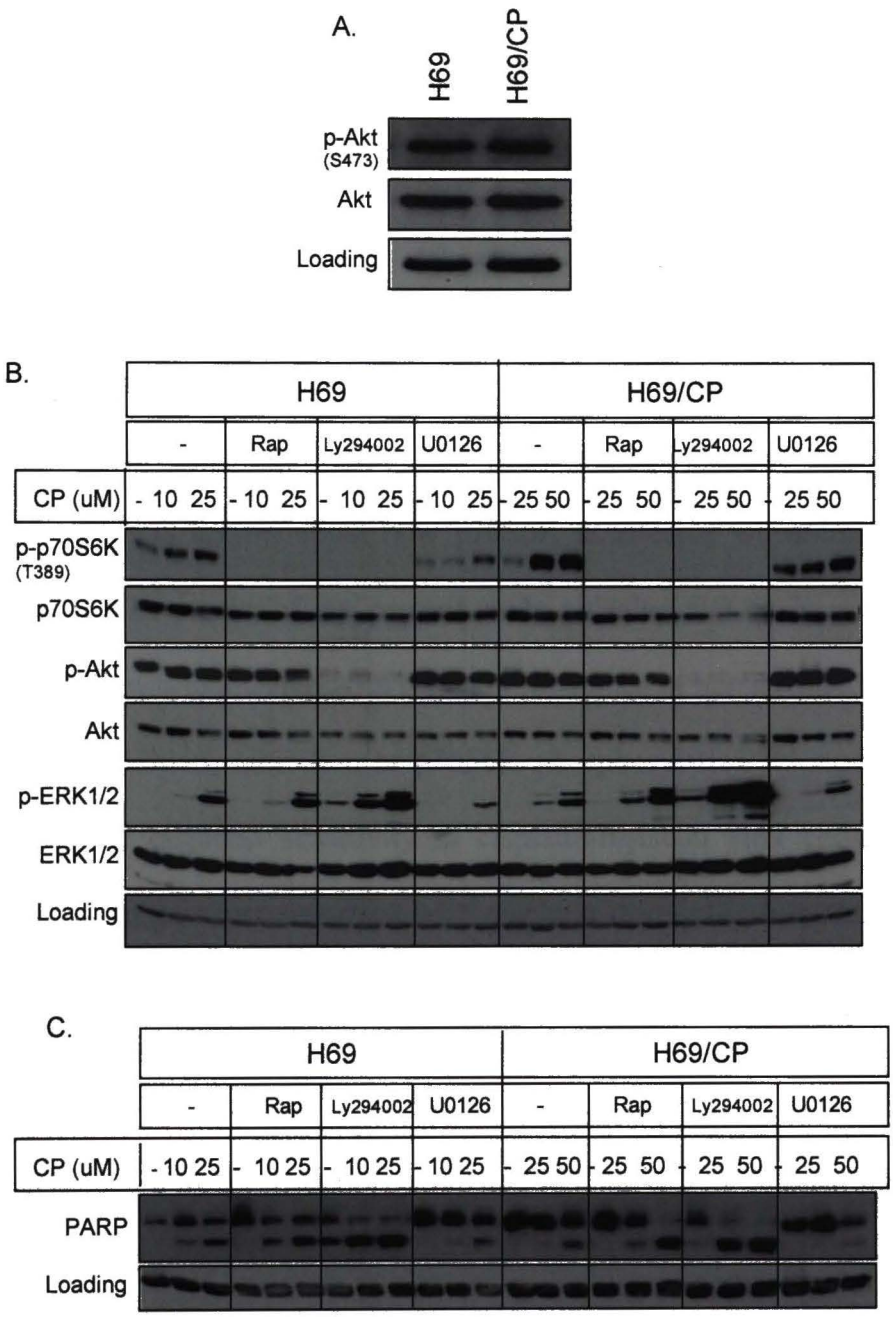

H69
H69/CP
CP (μM)
- 10 25 - 10 25
Rap (nM)
- + - + - + - + - + - +
p-S6
S6
Loading

Figure 4. The PI3K inhibitor Ly294002 decreases p70S6K phosphorylation and augments cisplatin-induced cell death

A, Western blot analysis was performed with total cellular proteins using indicated antibodies. GAPDH was used as a loading control. B and C, H69 and H69/CP cells were treated with 20 nM rapamycin, 25 μM Ly294002 and 10 μM U0126 for 30 min prior to treatment with indicated concentrations of cisplatin for 48 h. Western blot analysis was performed with total cellular extracts using indicated antibodies.

Figure 4.


A.
H69
H69/CP
p-Akt (S473)
Akt
Loading
B.
H69
H69/CP
-
Rap
Ly294002
U0126
CP (uM)
- 10 25
- 10 25
- 10 25
- 10 25
- 25 50
- 25 50
- 25 50
- 25 50
p-p70S6K (T389)
p70S6K
p-Akt
Akt
p-ERK1/2
ERK1/2
Loading
C.
H69
H69/CP
-
Rap
Ly294002
U0126
CP (uM)
- 10 25
- 10 25
- 10 25
- 10 25
- 25 50
- 25 50
- 25 50
- 25 50
PARP
Loading

DISCUSSION

The efficacy of cisplatin not only depends on its ability to induce DNA damage but also on the cells' ability to respond to this damage. The crucial point in these events may be determined by signaling events downstream of the drug-DNA interactions. Acquisition of resistance by tumor cells to anticancer drugs may involve modifications in the cells' ability to trigger downstream signaling events. It is thus the balance between the various proteins present in the cell that finally decides whether the cell should live or die. Although the involvement of p70S6K in DNA damage-induced cell death is established (11-13), little is known about how p70S6K is affected when cells acquire resistance to cisplatin. The results of our present study revealed that an increase in the levels of active p70S6K was associated with cisplatin resistance in human small cell lung cancer H69 cells. In addition, inhibition of p70S6K phosphorylation by rapamycin or Ly294002 could restore cisplatin sensitivity in cisplatin-resistant H69 cells. Taken together, these results suggest that treatment with cisplatin during the selection process caused a modification in the p70S6K signaling pathway.

We have found that cisplatin caused activation of p70S6K in both H69 and H69/CP cells as determined by its phosphorylation at T389 site and its downstream target S6 although the extent of p70S6K activation was greater in H69/CP cells compared to H69 cells. This is consistent with a recent report that tumor necrosis factor-α activated the phosphorylation of p70S6K in MCF-7 cells (40). However, this is in contrast to a previous report that showed that cisplatin had little effect on p70S6K phosphorylation in

cisplatin resistant SR2 cells whereas it in fact decreased p70S6K phosphorylation in the sensitive cell line (26). This apparent anomaly could be explained by the fact that growth factors present in the serum could affect p70S6K phosphorylation. We have found that when cells were treated with cisplatin in fresh FBS containing media, the constitutive p70S6K phosphorylation was much higher (data not shown). However, when cells were incubated with different concentrations of cisplatin for 48 h, the basal p70S6K phosphorylation was low and cisplatin caused an increase in p70S6K phosphorylation. In addition, we found that a higher concentration of cisplatin that resulted in cell death also induced downregulation of total p70S6K. Since cisplatin-resistant cells require higher concentrations of cisplatin to induce cell death, the decrease in total as well as phospho-p70S6K is less in the resistant cells compared to drug-sensitive parental cells.

p70S6K is believed to act downstream of PI3K/Akt (36, 41). A recent report attributed amplification of AKT1 gene to be the cause of p70S6K activation and cisplatin resistance in A549 non-small cell lung cancer cells (31). On the contrary, we found that there was no difference in the levels of constitutive and/or phosphorylated Akt in H69 and H69/CP cells. Furthermore, cisplatin had little effect on Akt phosphorylation in either cells. We have also found that inhibition of Akt by Ly294002 caused substantial increase in cisplatin-induced cell death. In fact, Ly294002 alone caused modest cell death, suggesting the importance of Akt signaling pathway in the survival of these SCLC cells. However, the effect of Ly294002 was much greater compared to rapamycin, suggesting that Ly294002 may act via additional pathway besides inhibiting p70S6K.

Since Akt signaling pathway can also affect mitogen-activated protein kinase (MAPK) pathway (42) we compared the effect of Ly294002 with the MEK inhibitor U0126. We have found that in contrast to Ly294002, U0126 attenuated cisplatin-induced cell death in both parental and cisplatin-resistant H69 cells. In addition, cisplatin caused activation of ERK1/2. This is consistent with our previous report that activation of ERK1/2 was associated with cellular sensitivity to cisplatin (43). In this report we found that Ly294002 caused an increase in both basal and cisplatin-induced ERK1/2 phosphorylation. In fact, the extent of ERK1/2 phosphorylation correlated with the extent of PARP cleavage. Thus, Ly294002 not only inhibits the Akt/mTOR/p70S6K pathway, it also activates the MEK/ERK pathway. In fact, rapamycin also caused a modest increase in cisplatin-induced activation of ERK1/2. Thus, activation of ERK may also contribute to cisplatin sensitization by these inhibitors. With lung cancer accounting for the largest number of cancer deaths and acquisition of resistance resulting in poor prognosis, treatment with a combination of drugs may restore cisplatin sensitivity.

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CHAPTER III

p70S6K IS A NOVEL SUBSTRATE FOR CASPASE-3

SUMMARY

We recently demonstrated that overexpression/activation of p70 ribosomal S6 kinase (p70S6K) was associated with acquisition of resistance by human small cell lung cancer (SCLC) H69 cells to cisplatin. We have inadvertently found that the levels of total p70S6K were downregulated in response to cisplatin. The objective of this study is to determine the mechanism by which cisplatin induces downregulation of p70S6K. We have shown that cisplatin caused a time-dependent decrease in full-length p70S6K in SCLC H69 as well as in non-small cell lung cancer (NSCLC) A549 cells. Concomitant to the decrease in level of p70S6K, there was an appearance of a cleaved band of about 47-kDa in cells treated with cisplatin. The broad specificity caspase inhibitor z-VAD-fmk (z-VAD) reversed p70S6K downregulation by cisplatin whereas inhibitors of calpain and the proteasome had no effect. Cell-permeable peptide inhibitors of capsase-3 and -9 but not caspase-2 or -8 inhibited cisplatin-induced proteolytic cleavage of p70S6K. *In vitro*-translated p70S6K was shown to be cleaved by human recombinant caspase-3. Furthermore, depletion of caspase-3 by siRNA blocked the cleavage of p70S6K whereas depletion of caspase -2, -8, -9 did not inhibit the downregulation of p70S6K by cisplatin. Cisplatin failed to induce cleavage of p70S6K in MCF-7 cells that lack functional caspase-3. However, ectopic expression of caspase-3 in MCF-7 cells resulted in cisplatin-induced cleavage of p70S6K. These results demonstrate that p70S6K is a novel substrate for caspase-3.

INTRODUCTION

Cisplatin is widely used for the therapy of several types of cancer, including lung cancer [1-3]. However, cancer cells become resistant to cisplatin and toxic side effects of the drug hinder its use for successful chemotherapy [4-7]. Besides the innumerable mechanisms suggested for resistance to cisplatin [8], an understanding of signaling pathways that regulate cell cycle progression and survival may be key to developing effective therapies to improve drug usage.

p70S6K is a serine/threonine protein kinase responsible for mediating nutrient and mitogen-stimulated cell growth and proliferation [9]. Activation of p70S6K and its downstream target S6 results in the translation of a class of mRNA that encodes for many of the components of the protein synthesis apparatus [9-11]. Studies have shown that dysregulation of the p70S6K pathway through alteration in its mediators can lead to cellular transformation [12, 13]. Introduction of three commonly observed mutations in the phosphoinositide 3-kinase (PI3K) that lead to oncogenic transformation also resulted in elevated constitutive phosphorylation of p70S6K [14]. Indeed various lung cancer cells have high constitutively-active p70S6K [12, 15, 16] and levels of p70S6K are higher in tumorigenic human bronchial epithelial cells compared to normal human bronchial epithelial cells [17]. In addition the activation of p70S6K and S6 plays an important role in the survival and growth of cancer cells due to its role in different

cellular processes like cell growth, cell development, cell size, cell proliferation, and cell motility [9, 18-23].

We and others have shown that p70S6K activation is linked to resistance of lung cancer cells to cisplatin [24, 25]. Several studies have demonstrated the regulation of phosphorylated p70S6K in response to apoptotic stimuli. Phosphorylation of p70S6K was inhibited by cisplatin in conventional gastric carcinoma cells, small-cell lung cancer (SCLC) and NSCLC [13, 24]. In addition, Tee *et. al.* have shown that cisplatin, etoposide, and staurosporine inhibited the phosphorylation of p70S6K [26, 27]. Moreover, although the levels of phosphorylated p70S6K were decreased, total p70S6K levels remained unchanged when mouse myoblasts were treated with cisplatin [28]. On the other hand, we have shown that p70S6K is, in fact, activated and levels of total p70S6K are downregulated when SCLC are treated with cisplatin. Similar to our p70S6K phosphorylation results, it has been shown that ultraviolet radiation that causes DNA damage induced the phosphorylation of p70S6K [29, 30].

A conserved feature of cellular response to cisplatin treatment is the activation of a family of proteases called caspases which are cysteine aspartic acid proteases [31-33]. Caspases are present in most healthy cells as inactive precursors known as procaspases which undergo proteolytic processing to generate the active enzyme when an apoptotic signal is received [34, 35]. Active caspases cleave key proteins in the cytoplasm and nucleus as well as numerous proteins involved in metabolism, cell cycle, repair, disease, and signaling pathways [36-39]. Downregulation of critical cellular proteins may reverse drug resistance. On the other hand, proteolytic activation of proteins may be necessary

for the execution of apoptosis. In the present study we have looked at the mechanism of p70S6K downregulation by cisplatin. We have shown that unequivocally p70S6K is cleaved by caspase-3.

EXPERIMENTAL PROCEDURES

Materials

Polyclonal antibodies to p70S6K, phospho-p70S6K (T389), were purchased from Cell Signaling Technology, Inc. (Danvers, MA). Monoclonal antibody to GAPDH was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Monoclonal antibody to PARP, caspase-2 and polyclonal antibody to caspase-9 was purchased from Pharmingen (San Diego, CA). Polyclonal antibody to caspase-3 and monoclonal antibody to caspase-8 were obtained from BioSource/Invitrogen (Carlsbad, CA). MG132 and calpeptin were obtained from Calbiochem (San Diego, CA). Monoclonal antibody to actin was obtained from Amersham (Piscataway, NJ). siRNA SMARTpool against caspase-2, and non-targeting SMARTpool siRNA were obtained from Dharmacon (Lafayette, CO). Horseradish peroxidase-conjugated goat anti-mouse and donkey anti-rabbit antibodies were obtained from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). Polyvinylidene difluoride membrane was from Millipore (Bedford, MA), and enhanced chemiluminescence detection kit was from Amersham (Piscataway, NJ).). Lipofectamine 2000 transfection reagent was obtained from Invitrogen (Carlsbad, CA).

Cell Culture

A549 cells were a kind gift from Dr J. K. Vishwanatha. (University of North Texas Health Science Center, Fort Worth, TX). A549 and MCF-7 cells were maintained in RPMI 1640 (Life Technologies Inc., Grand Island, NY) supplemented with 2 mM

glutamine and 10% (v/v) heat-inactivated fetal bovine serum at 37^{O}C. Cells were kept in a humidified incubator at 37^{0}C with 95% air and 5% CO_2.

Transfection

Control siRNA or targeted siRNA were introduced into A549 cells using Lipofectamine 2000 (Invitrogen) and manufacturer's protocol. Briefly, cells were seeded 1 day before transfection. 48 h following siRNA transfection, cells were treated as indicated in the text and processed for Western blot analysis.

Immunoblot Analysis

Cells were lysed in M-PER mammalian extraction buffer (Pierce, Rockford, IL) containing 1 mM DTT and protease inhibitors. Equal amounts of total protein were electrophoresed by SDS-PAGE and transferred electrophoretically to a poly(vinylidene difluoride) membrane. Western blot analyses were performed as described before. The blot was probed with antibody to either GAPDH or actin as loading control.

In Vitro-Translation and Caspase Cleavage of p70S6K

Full-length p70S6K tagged with EE was cloned into pcDNA3. [^{35}S]Met-labeled wild-type p70S6K was synthesized by *in vitro* coupled transcription and translation with the T7 Quick TNT kit (Promega). Labeled proteins were incubated with human recombinant caspases in 50 mM Hepes, pH 7.5, 0.1% CHAPS, 5 mM dithiothreitol, 10% glycerol, and

0.1 mM EDTA at 37 °C for 1 h. Proteins were separated by SDS-PAGE, and autoradiography was performed with the dried gel.

Statistical analysis

Data are represented as mean ± Standard error of mean (SEM) and $n \geq 3$ unless otherwise specified. Statistical significance was determined using GraphPad Prism Software (GraphPad Software, La Jolla, CA).

RESULTS

Cisplatin induces downregulation of p70S6K in lung cancer cells

p70S6K is known to regulate cell survival and growth and is upregulated in lung cancer cells [15, 16]. Surprisingly, we observed that cisplatin treatment not only altered the phosphorylation of p70S6K [25], it also caused a time-dependent decrease in the level of total p70S6K in H69 cells (Fig. 1A). Cisplatin also induced downregulation of p70S6K in non-small cell lung cancer A549 cells (Fig. 1B).

As shown in Figure 2, cisplatin also induced a concentration-dependent downregulation of p70S6K in both H69 (Fig. 2A) and A549 cells (Fig. 2C). Along with the loss of the full-length p70S6K there was a concomitant increase in the smaller molecular weight cleaved fragment of about 47 kDa. On the basis of the densitometric analysis of three independent experiments there was a fourfold decrease in levels of total p70S6K following treatment with 50 μM cisplatin in the H69 and 60 μM cisplatin in the A549 cells (Fig 2B and 2C). These results suggest that cisplatin treatment may induce proteolytic cleavage of p70S6K.

Cisplatin induced p70S6K downregulation is mediated by the caspase pathway

To understand the mechanism of p70S6K downregulation, we examined the effect of cell-permeable inhibitors of proteasomes (MG132), calpains (calpepetin) and caspases (zVAD) on cisplatin-induced p70S6K downregulation. As shown in Figure 3A MG132 and calpeptin had no effect on the processing of p70S6K. However, the broad specificity

caspase inhibitor z-VAD completely blocked the cleavage of p70S6K. It also resulted in the loss of the cleaved band. Further, to elucidate which particular caspase(s) is involved in the downregulation of p70S6K we used cell-permeable peptide caspase inhibitors. As seen in Figure 3B, in addition to the pan-caspase inhibitor z-VAD, the caspase-3 inhibitor z-DEVD and the caspase-9 inhibitor z-LEHD blocked the cleavage of p70S6K by cisplatin, though to a lesser degree. These results suggest that cisplatin induced the activation of caspase which resulted in processing of p70S6K.

***In vitro* translated p70S6K is cleaved by caspase-3**

To determine which caspase directly cleaves p70S6K, we examined the effect of human recombinant caspases on the cleavage of ^{35}S-labeled *in vitro*-translated p70S6K. As seen in Figure 4A, p70S6K is cleaved with the appearance of a 47-kDa cleaved fragment when treated with human recombinant caspase-3. Further, human recombinant caspase-2, -7, -8 and -9 were unable to cleave p70S6K (Fig 4B) suggesting that it is caspase-3 that is responsible for cleaving p70S6K.

Depletion of caspase-3 blocks cisplatin-mediated p70S6K downregulation

Since the peptide caspase inhibitors lack absolute specificity and active recombinant caspases may have overlapping specificities in an *in vitro* assay, we used small interfering RNA (siRNA) to deplete a specific caspase to determine which caspase cleaves p70S6K in intact cells. Figures 5A and 5C show that siRNA against caspase-8, -9, -2 and -3 effectively depleted the levels of the respective caspase as compared to levels in

cells transfected with control non-targeting siRNA. Also, levels of the other caspases in the cell were not decreased (data not shown). While depletion of caspase-2, -8, and -9 did not prevent downregulation of p70S6K, depletion of caspase-3 was associated with inhibition of cisplatin-induced proteolytic cleavage of p70S6K. Thus, cisplatin-induced cleavage of p70S6K is mediated by caspase-3.

Caspase-3 cleaves p70S6K

To further confirm the involvement of caspase-3 on the cisplatin-induced proteolytic cleavage of p70S6K we used MCF-7 breast cancer cells which lack functional caspase-3. As shown in Figure 6A, when MCF-7 cells were treated with 10, 25 or 50 μM cisplatin for 24 h there was no decrease in levels of p70S6K. However, ectopic expression of caspase-3 in these cells resulted in downregulation of p70S6K with the emergence of a cleaved band (Fig 6B). These results unambiguously demonstrate that p70S6K is indeed a substrate for caspase-3.

Figure 1. Cisplatin induces downregulation of p70S6K in lung cancer cells

A, H69 cells were treated with concentration of cisplatin for indicated time periods. B, A549 cells were treated with the indicated concentrations of cisplatin for the indicated time periods. Western blot analysis was performed with total cellular extracts using indicated antibodies. GAPDH was used as a loading control. Results are representative of three independent experiments.

Figure 1.

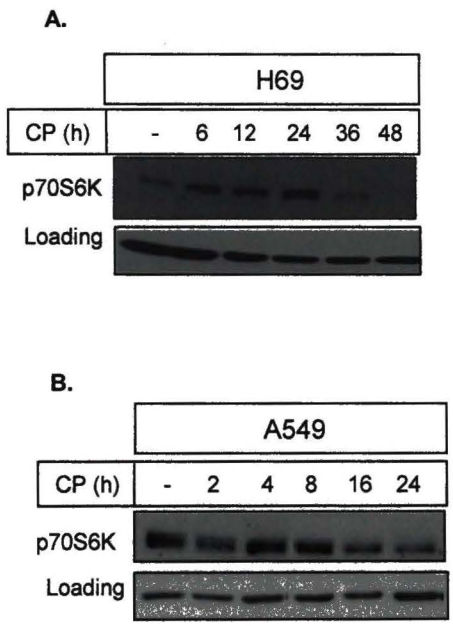
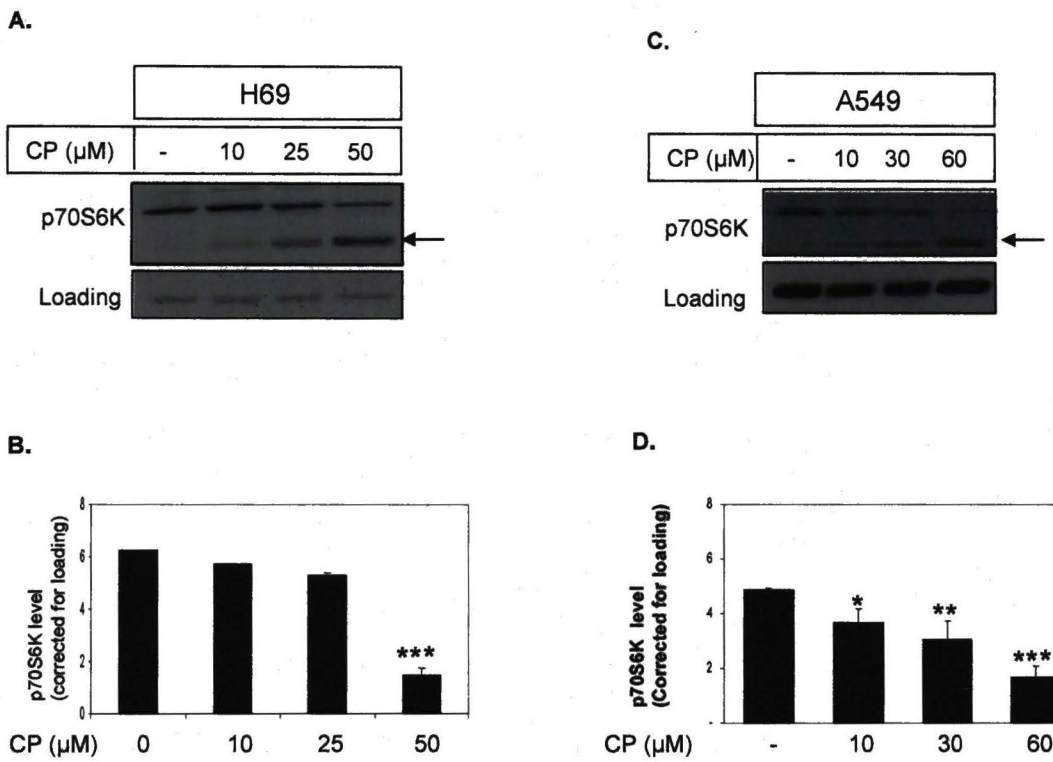

A.
H69
CP (h)
-
6
12
24
36
48
p70S6K
Loading
B.
A549
CP (h)
-
2
4
8
16
24
p70S6K
Loading

Figure 2. Cisplatin induces downregulation of p70S6K in lung cancer cells

A, H69 cells were treated with the indicated concentrations of cisplatin for 48 h. C, A549 cells were treated with the indicated concentrations of cisplatin for 24 h. Western blot analysis was performed with total cellular extracts using indicated antibodies. GAPDH was used as a loading control. Results are representative of three independent experiments. Arrow indicates cleaved p70S6K band. B and D, Densitometric quantification of total-p70S6K corrected for loading. Data are mean +/- SEM of three independent experiments. *** $p<0.0001$, ** $p<0.001$, * $p<0.01$ versus control cells using paired student's t-test.

Figure 2.


A.
H69
CP (μM)
-
10
25
50
p70S6K
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C.
A549
CP (μM)
-
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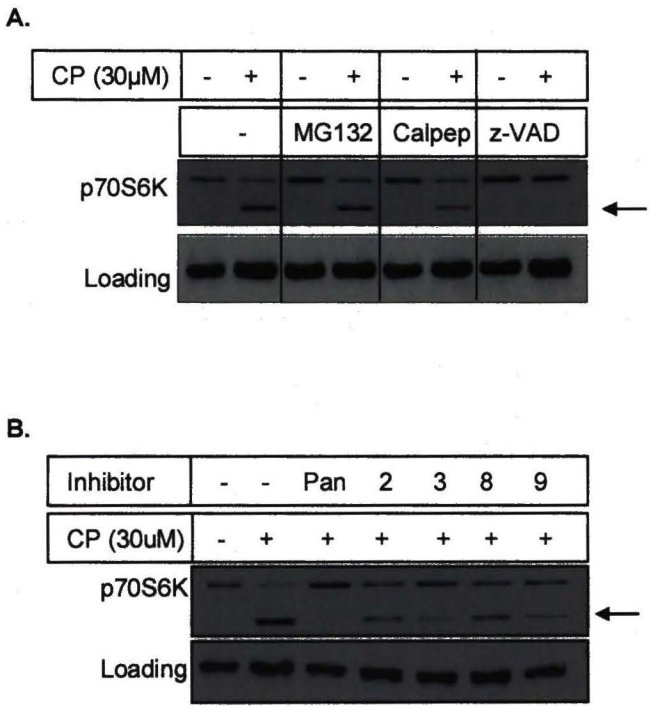
30
60
p70S6K
Loading
B.
p70S6K level
(corrected for loading)
***
CP (μM)
0
10
25
50
D.
p70S6K level
(Corrected for loading)
*
**
***
CP (μM)
-
10
30
60

Figure 3. Cisplatin induced p70S6K downregulation is mediated by the caspase pathway

A, A549 cells were treated with 10 μM MG132, 20 μM Calpeptin and 20 μM z-VAD for 30 min prior to incubation with 30 μM cisplatin. B. A549 cells were treated with 20 nM inhibitors 30 min prior to incubation with 30 μM cisplatin. z-VAD the broad specificity caspase inhibitor, z-VDVAD the caspase-2 inhibitor, z-DEVD the caspase-3 inhibitor, z-IETD the caspase-8 inhibitor and z-LEHD the caspase-9 inhibitor were used. Cells were processed after 24 h. Western blot analysis was performed with total cellular extract using indicated antibodies. GAPDH was used as a loading control. Arrow indicates cleaved p70S6K band. Results are representative of two independent experiments.

Figure 3.


A.
CP (30μM)
- + - + - + - +
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MG132
Calpep
z-VAD
p70S6K
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B.
Inhibitor
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CP (30uM)
- + + + + + +
p70S6K
Loading

Figure 4. *In vitro*-translated p70S6K is cleaved by caspase-3

A and B, (35S) Met-labeled *in vitro*-translated p70S6K was treated at 37^0C for 1 h with or without indicated human recombinant caspase. The reaction products were separated by SDS-PAGE and analyzed by autoradiography. Arrow indicates cleaved p70S6K band.

Figure 4.

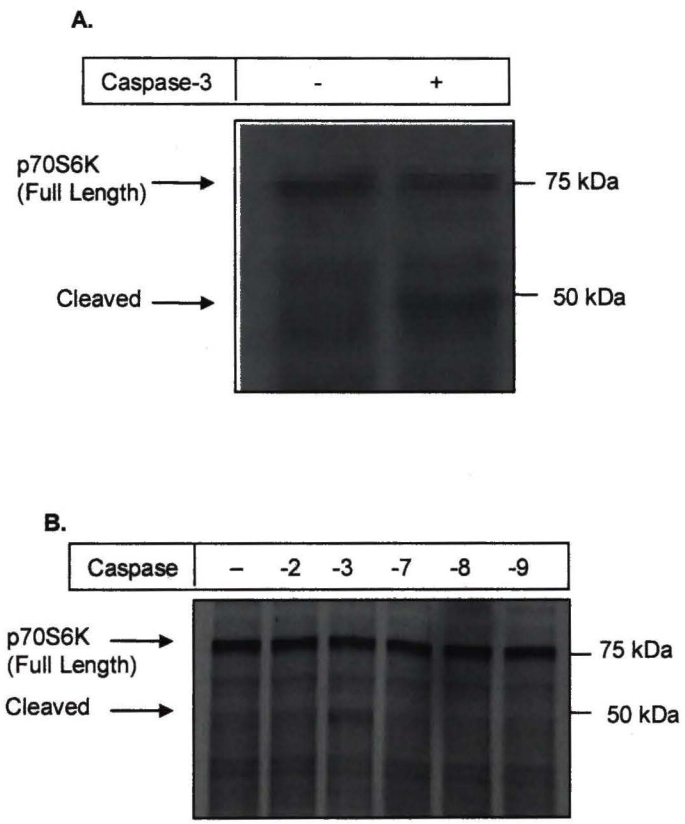
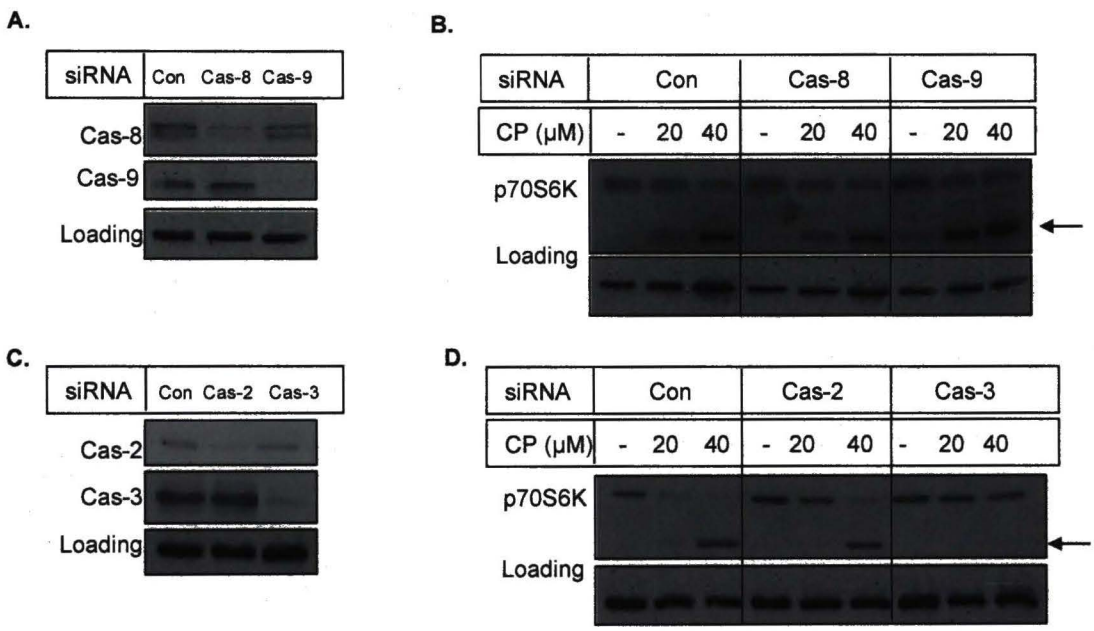

A.
Caspase-3
-
+
p70S6K
(Full Length)
Cleaved
75 kDa
50 kDa
B.
Caspase
–
-2
-3
-7
-8
-9
p70S6K
(Full Length)
Cleaved
75 kDa
50 kDa

Figure 5. Depletion of caspase-3 blocks cisplatin-mediated p70S6K downregulation

A and C, A549 cells were transfected with control or caspase siRNA as indicated. B and D, A549 cells were treated with 20 or 40 μM cisplatin after transfection with siRNA as indicated. 24 h after treatment cells were processed and western blot analysis was performed with total cellular extracts using indicated antibodies. GAPDH was used as a loading control. Arrow indicates cleaved p70S6K band. Results are indicative of two independent experiments.

Figure 5.


A.
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Cas-2
Cas-3
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D.
siRNA
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Cas-2
Cas-3
CP (μM)
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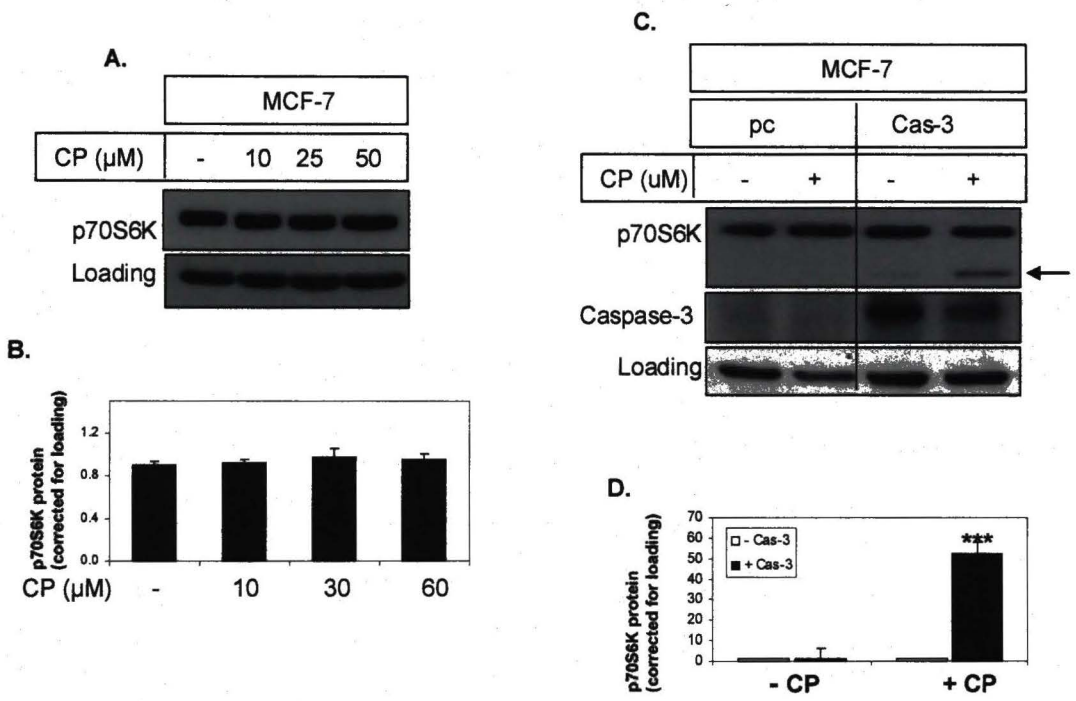
p70S6K
Loading

Figure 6. Caspase-3 cleaves p70S6K.

A, MCF-7 cells were treated with 10, 25 and 50 μM cisplatin. B, MCF-7 cells were transfected with vector alone pcDNA3 or vector containing caspase-3 construct. Cells were treated with 25 μM cisplatin. 24 h after treatment cells were processed and western blot analysis was performed with total cellular extracts using indicated antibodies. GAPDH was used as a loading control. Arrow indicates cleaved p70S6K band. B and D, Densitometric quantification of total-p70S6K corrected for loading. Data are mean +/- SEM of three independent experiments. *** $p<0.0001$ versus control cells using paired student's t-test.

Figure 6.


A.
MCF-7
CP (μM)
-
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25
50
p70S6K
Loading
B.
p70S6K protein (corrected for loading)
1.2
0.8
0.4
0.0
CP (μM)
-
10
30
60
C.
MCF-7
pc
Cas-3
CP (uM)
-
+
-
+
p70S6K
Caspase-3
Loading
D.
p70S6K protein (corrected for loading)
70
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30
20
10
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- Cas-3
+ Cas-3
***
- CP
+ CP

DISCUSSION

Cellular proteins can modulate how cells respond to DNA damage and their interactions with chemotherapeutic agents and p70S6K is one such protein. Several studies have demonstrated that in various cell lines cisplatin can downregulate levels of phosphorylated p70S6K [13, 24, 26-28], while levels of the total protein remained unaltered. The results of our present study made a novel observation that p70S6K is a substrate for caspase-3. First, caspase inhibitors but not proteasome or calpain inhibitors prevented cisplatin-induced downregulation of p70S6K. Second, inhibition of caspase-3 by peptide caspase inhibitor or depletion of caspase-3 by siRNA prevented proteolytic cleavage of p70S6K. Third, cisplatin failed to downregulate p70S6K in MCF-7 cells that lack functional caspase-3. Finally, introduction of caspase-3 in MCF-7 cells resulted in the cleavage of p70S6K.

There are conflicting reports as to whether the downregulation of p70S6K is mediated by caspases or not [26, 40]. While it has been shown that caspases did not mediate the inhibition of p70S6K phosphorylation in response to etoposide and staurosporine treatment [26, 27], the activation of caspase-8 through the Fas receptor resulted in downregulation of p70S6K [40]. In wild type Jurkat cells activation of the Fas receptor has been shown to result in downregulation of p70S6K and in caspase-8 mutant Jurkat cells this downregulation was attenuated. The authors suggested that the downregulation of p70S6K is mediated by caspase-8 [40]. We have, however, found that cisplatin induced cleavage of p70S6K in H69 cells which lack caspase-8, suggesting that

caspase-8 is not directly involved in the cleavage of p70S6K. Since caspase-8 is an apical caspase and acts upstream of caspase-3 [41] it is possible that activation of caspase-8 results in the activation of caspase-3 resulting in cleavage of p70S6K. In mutant caspase-8 cells there will also be no activation of caspase-3. Our *in vitro* cleavage assay with human recombinant caspase-3 demonstrated that caspase-3 is capable of cleaving p70S6K directly. Furthermore, in MCF-7 cells that lack functional caspase-3, treatment with as high as 50 μM cisplatin did not cleave p70S6K but overexpression of caspase-3 in MCF-7 cells resulted in proteolytic cleavage of p70S6K in response to cisplatin.

p70S6K is responsible for regulating cellular growth and proliferation and in its native confirmation has an inhibitory pseudosubstrate site that binds to the substrate binding region thereby preventing its activation [42]. Cleavage of p70S6K by caspase is important because it may possibly either activate or inactivate the molecule depending on where p70S6K is cleaved. Many kinases act as antiapoptotic proteins (e.g., Akt) and the cleavage of these proteins by caspases is one way to relieve their negative regulation on cell death [43]. On the other hand, proteolytic activation of proteins (e.g., PKCδ, etc.) has been associated with apoptotic induction [44]. Thus, proteolytic cleavage of p70S6K may have a significant impact on cell death by apoptosis.

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CHAPTER IV

FUNCTIONAL SIGNIFICANCE OF CISPLATIN-MEDIATED p70S6K CLEAVAGE

SUMMARY

p70 ribosomal S6 kinase (p70S6K) is responsible for cell growth and proliferation and its phosphorylation has been shown to be regulated by DNA damaging drugs like cisplatin. We made a novel observation that p70S6K is cleaved by caspase-3 during cisplatin-induced apoptosis. In the present study, we have examined the role of p70S6K in cisplatin-mediated cell death and further, investigated whether cleavage of p70S6K by caspase-3 is important for cisplatin-mediated cell death. Rapamycin, a pharmacological inhibitor of p70S6K, enhanced cisplatin-induced apoptosis in A549 cells. In contrast, knockdown of p70S6K by siRNA resulted in a decrease in cisplatin-induced apoptosis. p70S6K was primarily cleaved after the aspartic acid residue at the 393 position. Site-directed mutagenesis of Asp393 to Ala resulted in protection against cisplatin-mediated apoptosis. In addition, introduction of the N-terminal cleaved fragment [Δ(394-525)] resulted in potentiation of cisplatin-induced apoptosis. These results suggest that proteolytic cleavage of p70S6K is important for cisplatin-induced apoptosis.

INTRODUCTION

Cisplatin is widely used for the therapy of several types of cancer, including lung cancer [1-3]. However, the problem with cisplatin therapy is that cancer cells become resistant to cisplatin and toxic side effects of the drug hinder its use for successful chemotherapy [4-7]. Understanding of signaling pathways that regulate cell cycle progression and survival may be instrumental in developing effective therapies to improve drug usage. Cellular responses to cisplatin can be mediated by several different signaling pathways, such as Akt, extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK) and protein kinase C (PKC) [8-13]. In addition, we have shown that p70S6K phosphorylation is upregulated in response to cisplatin treatment [14].

p70S6K is responsible for mediating survival and growth of cells due to its role in different cellular processes like cell growth, cell development, cell size, cell proliferation, and cell motility [15-21]. A number of different studies have focused on the involvement of p70S6K in influencing cellular responses to apoptotic stimuli. Treatment of Swiss 3T3 and RAT-1 cells with etoposide and staurosporine induced apoptosis and resulted in dephosphorylation and decreased activity of p70S6K [22, 23]. It has also been shown that cisplatin treatment led to cell death and was associated with inhibition of p70S6K phosphorylation in mouse myoblasts [24]. Further, cell death induced by TGF-beta and curcumin is also regulated by p70S6K [25, 26].

Rapamycin, an immunosuppressant that blocks the activation of mTOR/p70S6K, compromises the cell's ability to progress through the G1 phase of the cell cycle [27, 28].

Though the induction of apoptosis by rapamycin is not global [29, 30], it has been reported that rapamycin can synergize with other agents such as tamoxifen, imanitib and doxorubicin to enhance apoptosis [31-34]. Similarly, rapamycin derivatives enhanced cisplatin-induced cell death in ovarian, leukemia and lung cancers [35-38].

Besides, initiation of apoptosis by caspases, substrate recognition by activated caspases is also important for apoptosis. The substrate specificity of the caspases is determined by binding pockets formed by four active-site loops on each of the caspases. Caspases recognize a set of four neighboring amino acids in their substrate termed P4-P3-P2-P1 and have a stringent requirement for aspartic acid at the P1 position [39-42]. Preference for the P2 and P4 positions varies greatly among initiator and effector caspases [40] but all caspases prefer, to a varying degree, the presence of glutamate in the P3 position [39].

Active caspases cleave key proteins in the cytoplasm and nucleus and the best recognized caspase substrates are caspases themselves [41]. Some of the other caspase substrates are proteins like retinoblastoma-associated protein, DNA-dependent protein kinase, protein kinase C, amyloid precursor protein, Bid, poly(ADP-ribose) polymerase (PARP) [43, 44]. Cleavage of these and other downstream substrates by caspase regulates key cellular events such as metabolism, cell cycle, cell death, repair, diseases, and signaling pathways [39, 45-47]. However, it has also been suggested that many caspase substrates are just cleaved as 'innocent bystanders' [43, 46]. A few of the proteins that are cleaved by caspases without a definite purpose are alpha-tubulin 1, sterol regulatory element binding protein-2, septin-6 and proteasome subunit p58 [48]. Since, we have

recently identified p70S6K as a novel substrate for caspase-3, the objective of the present study is to determine whether this cleavage of p70S6K has a role in cisplatin-induced cell death or if it is cleaved as an 'innocent bystander'. We have demonstrated that although cisplatin-induced cell death was enhanced by rapamycin, knockdown of p70S6K rescued cells from cisplatin-induced cell death. Further, introduction of caspase cleavage-mutant p70S6K in A549 cells inhibited whereas overexpression of N-terminal cleaved fragment potentiated cisplatin-mediated cell death.

EXPERIMENTAL PROCEDURES

Materials

Polyclonal antibodies to p70S6K, phospho-p70S6K (T389), were purchased from Cell Signaling Technology, Inc. (Danvers, MA). Monoclonal antibody to GAPDH was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Monoclonal antibody to PARP, caspase-2 and polyclonal antibody to caspase-9 were purchased from Pharmingen (San Diego, CA). Horseradish peroxidase-conjugated goat anti-mouse and donkey anti-rabbit antibodies were obtained from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). Polyvinylidene difluoride membrane was from Millipore (Bedford, MA), and enhanced chemiluminescence detection kit was from Amersham (Piscataway, NJ). Lipofectamine 2000 transfection reagent was obtained from Invitrogen (Carlsbad, CA).

Cell Culture

A549 cells were a kind gift from Dr J. K. Vishwanatha. (University of North Texas Health Science Center, Fort Worth, TX). A549 cells were maintained in RPMI 1640 (Life Technologies Inc., Grand Island, NY) supplemented with 2 mM glutamine and 10% (v/v) heat-inactivated fetal bovine serum at $37^{O}C$. HeLa cells were maintained in DMEM supplemented with 2 mM glutamine and 7% (v/v) heat-inactivated fetal bovine serum at $37^{O}C$. Cells were kept in a humidified incubator at $37^{0}C$ with 95% air and 5% CO_2.

Transfection

For DNA transfection, cells were transfected with 1 μg of pcDNA3 or vector containing WT p70S6K or mutant p70S6K using Lipofectamine 2000 (Invitrogen). Briefly, cells were seeded 1 day before transfection. 48 h following transfection, cells were treated as indicated in the text and processed for Western blot analysis. For analyzing apoptotic cells after transfecting the cells with empty vector, wild-type p70S6K (WT-p70S6K), the D393A mutant and the Δ(394-525) mutant we added a selection drug for 4 days to select for a pool of stable cells. These cells were then used for flow cytometry analysis.

Immunoblot Analysis

Cells were lysed in M-PER mammalian extraction buffer (Pierce, Rockford, IL) containing 1 mM DTT and protease inhibitors. Equal amounts of total protein were electrophoresed by SDS-PAGE and transferred electrophoretically to a poly(vinylidene difluoride) membrane. Western blot analyses were performed as described before. The blot was probed with antibody to either GAPDH or actin to control for equal loading.

Assessment of Apoptosis by Flow Cytometry

Cells were treated with varying concentrations of cisplatin for 72 h. After treatment, cells were harvested and washed with phosphate-buffered saline and fixed in 70% ethanol

overnight. Nuclei were then stained with propidium iodide and DNA content was analyzed using the Beckman Coulter Cytomics FC 500 flow cytometer.

Site-directed Mutagenesis

Site-directed mutagenesis was performed using the QuikChange kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions. Primers were purchased from Integrated DNA technology (IDT Coralville, IA). All constructs utilized for our studies were sequenced (SeqWright, Houston TX). Mutagenesis was performed for each reaction using complimentary PCR primers D322A: 5' - CCC TAC CTC ACA CAA GAA GCC AGA GCT CTG CTT AAA AAG C - 3', D342A: 5' - GGA GCT GGT CCT GGG GCC GCT GGA GAA GTT C - 3', D378A: 5' - CCT CTG TTG CAA TCT GAA GAG GCT GTA AGT CAG TTT G - 3', D383A: 5' - GAG GAT GTA AGT CAG TTT GCT TCC AAG TTT ACA CGT CAG - 3', D393A: 5' - GTC AGA CAC CTG TCG CCA GCC CAG ATG AC - 3', D396A: 5' - CCT GTC GAC AGC CCA GCT GAC TCA ACT CTC - 3', D397A: 5' - GTC GAC AGC CCA GAT GCC TCA ACT CTC AGT G - 3', D455A: 5' - CAA ATT TTC TCC TGG GGC TTT CTG GGG AAG AGG TGC - 3'. For the mutant with amino acids 393 to 397 deleted 5' - AAG TTT ACA CGT CAG ACA CCT GTC TCA ACT CTC AGT GAA AGT GCC AAT - 3' primer was used and for the double mutation of 378 and 383 we used the D383A template and the D378A primer. The concentration of the primers was 125 ng/μl. The reaction volumes were set up according to the Stratagene QuikChange kit. The PCR cycle was setup as follows: 1 cycle at 95 ^{O}C for 30 seconds (sec), then 16 cycles each at 95 ^{O}C for 30 sec, 55 ^{O}C for 1 min, 68 ^{O}C for 8

min and finally 1 cycle at 68 ^{O}C for 10 min. Following the temperature cycling the reaction was placed on ice for 2 minutes. The reaction was then digested with 1 μl Dpn1 for 1 h at 37^{O}C and 10 μl of the reaction was transformed into XL1-Blue Supercompetent cells. Following transformation single colonies were picked and DNA was extracted using the QIAGEN plasmid extraction kit.

RESULTS

Rapamycin enhances cisplatin-mediated cell death in A549 NSCLC cells

To determine the importance of p70S6K on cisplatin-induced cell death we first examined the effect of rapamycin, a pharmacological inhibitor of mTOR/p70S6K, on cisplatin-mediated apoptosis in A549 cells. Cells were treated with 20 nM rapamycin which was sufficient to cause dephosphorylation of p70S6K [14]. We monitored the number of apoptotic cells by the appearance of the sub-G1 peak during flow cytometric analysis. Treatment of A549 cells with 2 μM cisplatin had little effect on apoptosis. Although rapamycin alone had little effect on apoptosis, pretreatment of cells with rapamycin prior to cisplatin treatment increased apoptotic cells from 12% to 26%. 5 μM cisplatin caused appearance of 28% cells in the Sub-G1 peak and this was increased to 40% by rapamycin. As seen in Table 1, treatment with rapamycin caused a G1 phase arrest compared to control cells, with a concomitant decrease in number of cells in the S phase. While, the combination of rapamycin and cisplatin enhanced apoptosis, we did not see a significant increase in G1 phase cells.

Depletion of p70S6K in A549 cells results in a decrease in cisplatin-mediated apoptosis

Since pharmacological inhibitors are known to be somewhat non-specific, we determined the effect of p70S6K depletion on cisplatin-mediated cell death. Figure 2A shows that while control non-targeting siRNA had no effect on p70S6K compared to untransfected

cells, siRNA against p70S6K effectively depleted p70S6K. Cisplatin caused approximately 3-fold increase in apoptotic cells from 9% to 35% in non-transfected cells and from 14 to 31% in cells transfected with control siRNA. Knockdown of p70S6K siRNA caused arrest of cells in the G1 phase and decrease in cells in the S phase (Table 2). In p70S6K-depleted cells, cisplatin treatment increased the percentage of apoptotic cells only two-fold, from 10 to 20%. Further, apoptosis is increased in cells that are transfected with control siRNA compared to non transfected cells due to the effect of Lipofectamine 2000 which is used as the transfection agent (Fig. 2B). Thus, depletion of p70S6K resulted in protection from cisplatin-induced apoptosis at both 2 and 5 μM cisplatin concentration.

Caspase-3 cleaves p70S6K after the aspartic acid residue at 393

To determine the significance of caspase-3 cleavage of p70S6K, we wanted to identify the site at which p70S6K is cleaved. We first overexpressed p70S6K with an EE tag in cells so that we could differentiate between the endogenous and overexpressed p70S6K. Cisplatin induced downregulation of p70S6K in cells transfected with empty vector pcDNA3, accompanied by the appearance of a 47-kDa band (Fig. 3A). p70S6K overexpression can be observed by the presence of an additional band above the endogenous p70S6K band in lanes 4-6 in Figure 3A. In addition, we also see two cleaved bands (as indicated by the arrows); the upper band being the cleaved fragment of overexpressed p70S6K. The lower band is probably the cleaved product of endogenous p70S6K. As seen in Figure 3B, there is a time-dependent increase in downregulation of

endogenous and exogenous p70S6K in response to cisplatin and a corresponding increase in the cleaved band. The appearance of cleaved products of both endogenous and overexpressed p70S6K are diminished in the presence of the caspase inhibitor.

Although, the human p70S6K protein sequence has no caspase-3 consensus cleavage site (DEVD), we see a smaller cleaved fragment of about ~47-kDa when we use the antibody that targets the amino terminus of the protein. Thus, based on the molecular size of the cleaved fragment we targeted a stretch of putative amino acids and mutated a number of different aspartic acid residues to alanine in this region to determine the potential caspase cleavage site. As shown in Figure 4A, mutation of TPVDS to TPVAS (D393A) inhibited the cleavage of p70S6K. When we used p70S6K-specific antibody, we did not see complete inhibition of the cleaved p70S6K band though it was comparable to the cleaved band seen in the D393A cells without cisplatin treatment. However, when we used the EE antibody, we were unable to detect the cleaved fragment of overexpressed p70S6K. Densitometric analysis indicates levels of the ~47-kDa cleaved p70S6K normalized with total p70S6K levels (Fig. 4B). These results suggest that p70S6K is primarily cleaved at the TPVD site in intact cells.

Cleavage of p70S6K by caspase-3 results in augmentation of cell death

Since, we see contrasting results with rapamycin and p70S6K depletion on cisplatin-mediated cell death; we wanted to examine the effect of the caspase-3 mediated cleavage of p70S6K on cell death. To create the fragment that is generated when caspase-3 cleaves p70S6K, we deleted amino acids 394 to 525 of p70S6K [Δ(394-525)] to create

the larger N-terminal fragment of the protein. Besides the empty vector and wild-type p70S6K (WT-p70S6K), we overexpressed the D393A mutant and the Δ(394-525) mutant. We quantified cell death by flow cytometric analysis as shown in Figure 5B. There was an 18% increase in the apoptotic cells when control cells were treated with cisplatin and an increase of 20% when wild type p70S6K was overexpressed in the cells. On the other hand, cells overexpressing the D393A mutant showed only a 12% increase in cell death. Overexpression of the Δ(394-525) mutant alone increased cell death 3-fold over untreated vector-control cells, and cisplatin treatment further increased the percentage of apoptotic cells to 33%. There was approximately 2-fold sensitization of these cells to cisplatin when compared to cells expressing vector-control or WT-p70S6K. There were no significant changes in the percentage of cells in the G1 and S phase with the different transfections either in the presence of cisplatin or in the absence of cisplatin treatment (Table. 3). In the presence of cisplatin there was an increase in G2/M cells in the D393A cells and a decrease in the Δ(394-525) transfected cells. Thus, cleavage of p70S6K by caspase-3 results in enhanced cisplatin-mediated apoptosis.

Figure 1. Rapamycin enhances cisplatin-mediated cell death in A549 NSCLC cells

A549 cells were treated with 20 nM rapamycin 30 min prior to treatment with indicated concentrations of cisplatin. Cells were treated with cisplatin for 72 h with 2 and 5 μM cisplatin. Nuclei were stained with propidium iodide and analyzed using a flow cytometer.

Figure 1.

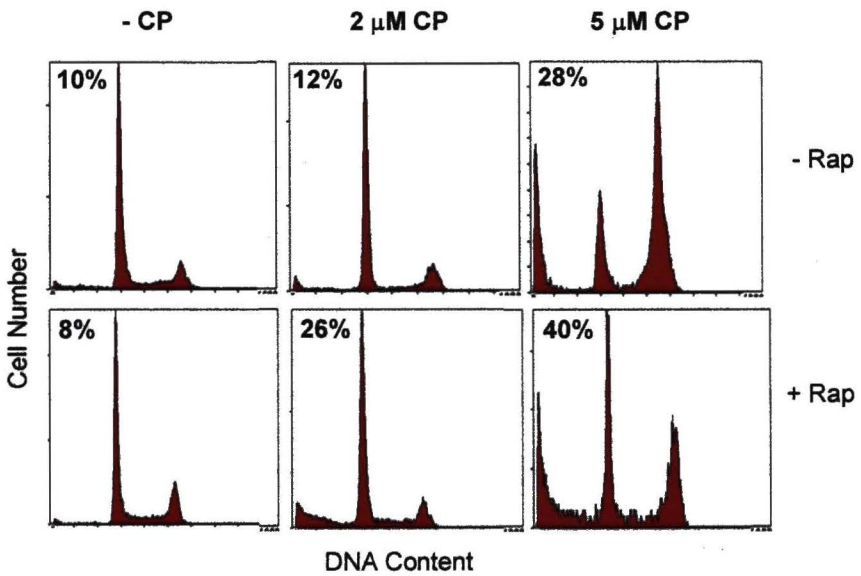

Table 1. Rapamycin enhances cisplatin-mediated cell death in A549 NSCLC cells

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Table 1.

	Apop SubG0 (%)	G1 (%)	S (%)	G2/M (%)
Con	10 ± 0.7	41.2 ± 2.3	27.3 ± 4.6	20.2 ± 6.4
CP 2 μM	11.8 ± 3.1	58.7 ± 9.8	10.6 ± 2.5	19.9 ± 5.4
CP 5 μM	27.9 ± 1.7	15 ± 4.3	11.7 ± 4.9	46.2 ± 4.7
Rap	8.4 ± 2.4	50.5 ± 5.4	17.4 ± 2	23.7 ± 1.3
Rap + CP 2 μM	25.5 ± 1.7 *	45.2 ± 7.1	13.7 ± 2.4	15.1 ± 3.2
Rap + CP 5 μM	39.6 ± 0.5 **	22.8 ± 0.9	13.5 ± 6.6	24.2 ± 6.4

Figure 2. Depletion of p70S6K in A549 cells results in a decrease in cisplatin-mediated apoptosis

A549 cells were transfected with control or p70S6K siRNA. Non transfected cells were also used as a control. Cells were treated with indicated concentration of cisplatin for 72 h. A, Western blot analysis was performed with total cellular extracts using indicated antibodies. GAPDH was used as a loading control. B, Nuclei were stained with propidium iodide and analyzed using a flow cytometer.

Figure 2.

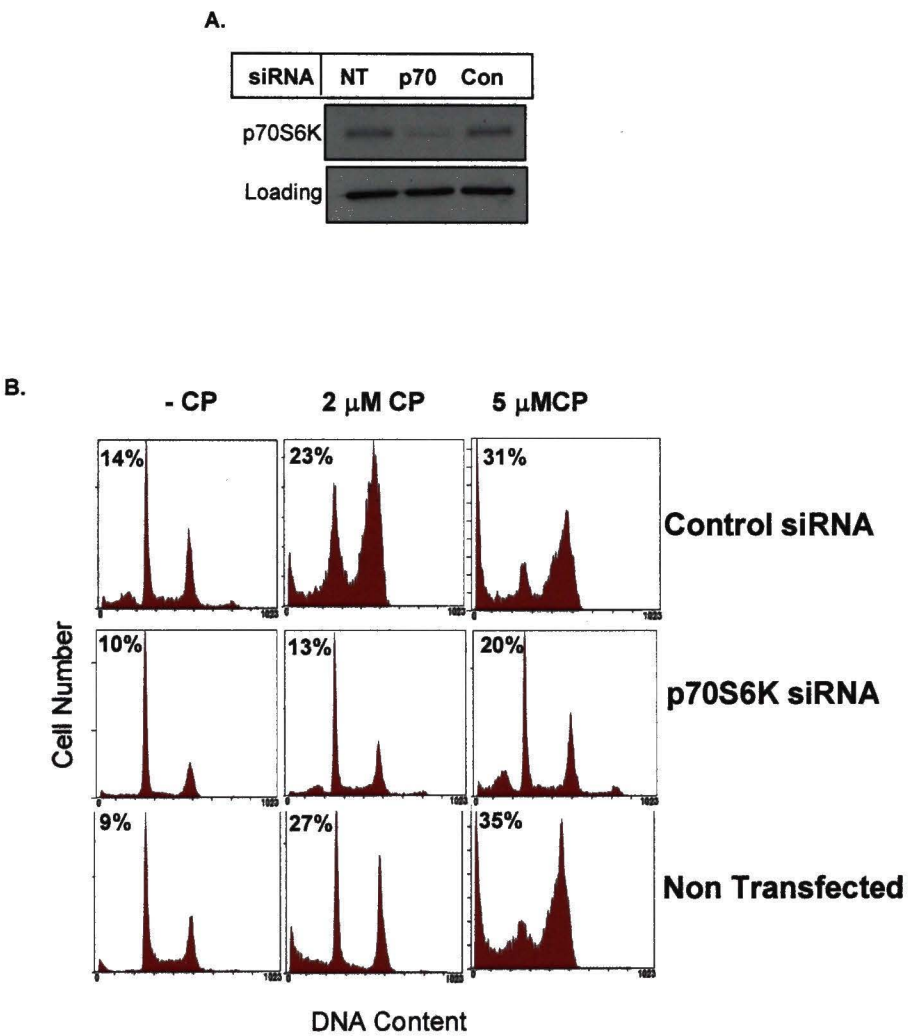

A.
siRNA
NT
p70
Con
p70S6K
Loading
B.
- CP
2 μM CP
5 μMCP
14%
23%
31%
10%
13%
20%
9%
27%
35%
Control siRNA
p70S6K siRNA
Non Transfected
Cell Number
DNA Content

Table 2. Depletion of p70S6K in A549 cells results in a decrease in cisplatin-mediated apoptosis.

A549 cells were transfected with control or p70S6K siRNA. Non transfected cells were also used as a control. Cells were treated with indicated concentration of cisplatin for 72 h. Nuclei were stained with propidium iodide and analyzed using a flow cytometer. The table shows a comparison of the different cell cycle stages. Data are mean +/- SEM of three independent experiments. ** $p<0.01$ versus cells treated with 2 μM cisplatin, * $p<0.001$ versus cells treated with 5 μM cisplatin using ANOVA.

Table 2.

		Apop Sub G0 (%)	G1 (%)	S (%)	G2/M (%)
Con siRNA	**Con**	13.9 ± 1	30.6 ± 7	15.6 ± 2	35.7 ± 4
	CP 2 μM	22.9 ± 3	26.9 ± 6	12.8 ± 1	37.1 ± 3
	CP 5 μm	31 ± 3	12.7 ± 1	26.3 ± 5	32.8 ± 4
p70S6K siRNA	**Con**	8.9 ± 3	51.8 ± 5	11.2 ± 2	26.8 ± 1
	CP 2 μM	13.2 ± 3 **	36.4 ± 11	17.1 ± 3	33.1 ± 5
	CP 5 μM	20.6 ± 2 *	10.7 ± 5	22.9 ± 8	46.7 ± 7

Figure 3. Cisplatin causes an increase in levels of endogenous and exogenous cleaved p70S6K

HeLa cells were transfected with empty vector pcDNA3 and wild type-p70S6K using lipofectamine 2000. A, 24 h after transfection cells were treated with 10 or 30 μM cisplatin for 24 h. B, Cells were treated with 5 μM cisplatin for indicated time periods. Cells were also treated with 20 μM z-VAD for 30 min prior to incubation with cisplatin for 18 h as indicated. Western blot analysis was performed with total cellular extracts using indicated antibodies. GAPDH was used as a loading control. Arrow indicates cleaved p70S6K band.

Figure 3.

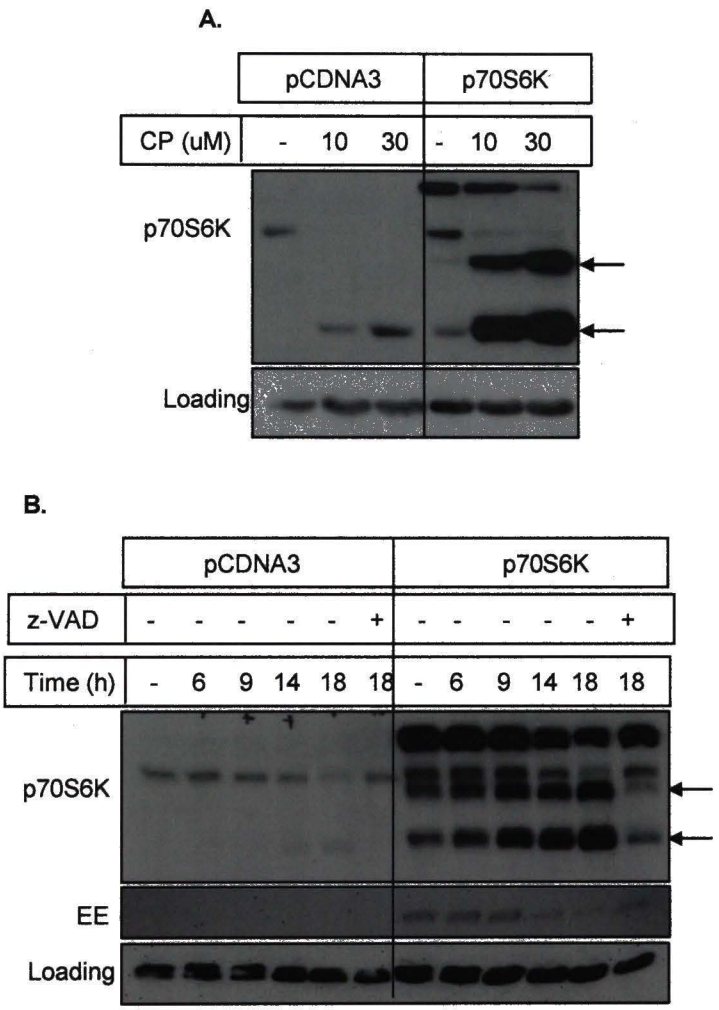
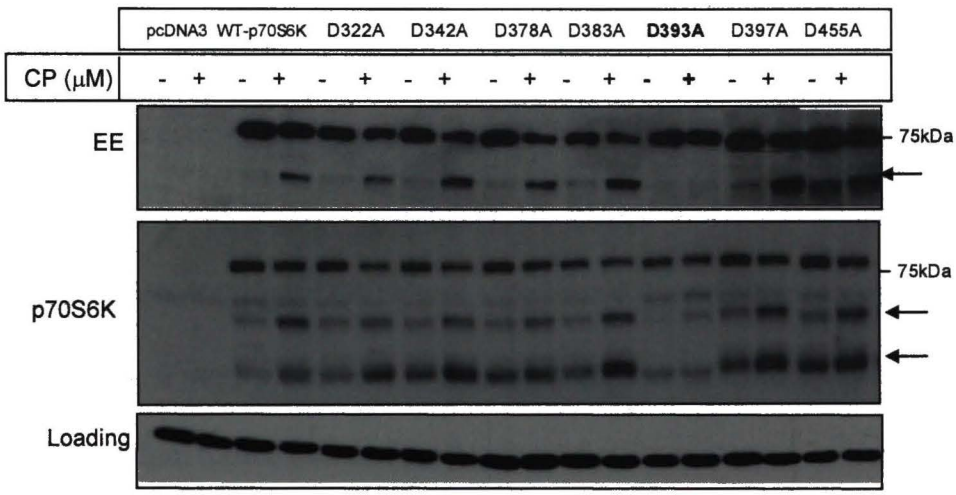

A.
pCDNA3
p70S6K
CP (uM)
- 10 30 - 10 30
p70S6K
Loading
B.
pCDNA3
p70S6K
z-VAD
- - - - - + - - - - - +
Time (h)
- 6 9 14 18 18 - 6 9 14 18 18
p70S6K
EE
Loading

Figure 4. Caspase-3 cleaves p70S6K after the aspartic acid residue at 393

Hela cells were transfected with empty vector pcDNA3, wild type-p70S6K and the mutants: D322A, D342A, D378A, D383A, D393A, D397A, D455A, double mutation 378&383. 24 h after transfection cells were treated with cisplatin for 18 h. A, Western blot analysis was performed with total cellular extracts using indicated antibodies. GAPDH was used as a loading control. Arrow indicates cleaved p70S6K band. B, Densitometric quantification of the cleaved p70S6K normalized with full length p70S6K.

Figure 4.

A.


pcDNA3 WT-p70S6K D322A D342A D378A D383A D393A D397A D455A
CP (μM) - + - + - + - + - + - + - + - + - +
EE
75kDa
p70S6K
75kDa
Loading

B.

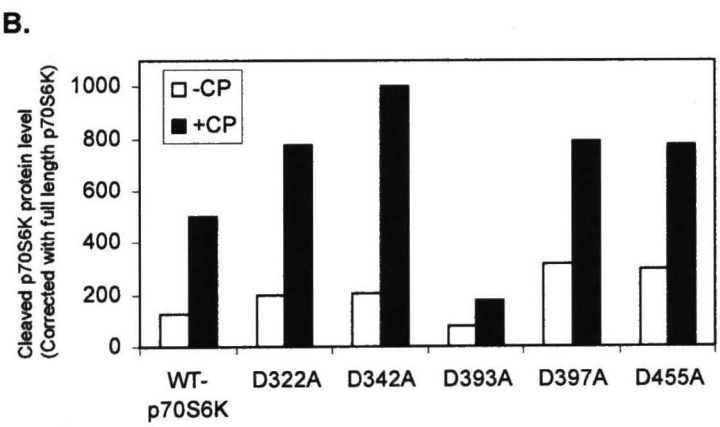

Cleaved p70S6K protein level (Corrected with full length p70S6K)
1000
800
600
400
200
0
-CP
+CP
WT-p70S6K
D322A
D342A
D393A
D397A
D455A

Figure 5. Cleavage of p70S6K by caspase-3 results in augmentation of cell death

A, A representative cartoon showing the p70S6K mutants that were generated. B, HeLa cells were transfected with empty vector pcDNA3, WT-p70S6K, D393A and Δ(394-525). 24 h post transfection 400 μg/ml G418 was added for 4 days. Cells were then allowed to grow and plated in 6 well plates and 2 μM cisplatin was added for 72 h. Nuclei were stained with propidium iodide and analyzed using a flow cytometer.

Figure 5.

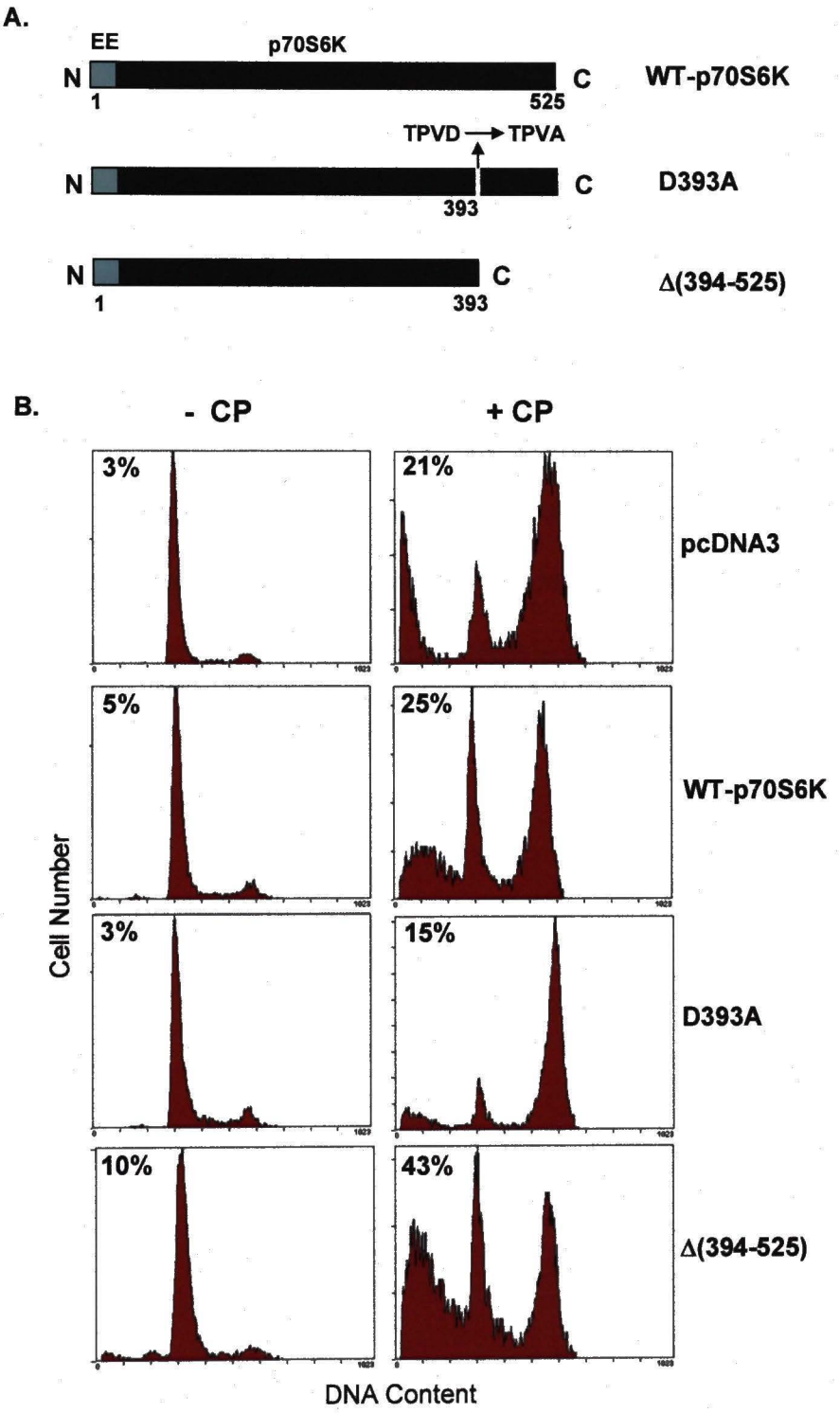

A.
EE
p70S6K
N
C
1
525
WT-p70S6K
TPVD → TPVA
N
C
393
D393A
N
C
1
393
Δ(394-525)
B.
- CP
+ CP
3%
21%
pcDNA3
5%
25%
WT-p70S6K
3%
15%
D393A
10%
43%
Δ(394-525)
Cell Number
DNA Content

Table 3. Cleavage of p70S6K by caspase-3 results in augmentation of cell death

HeLa cells were transfected with empty vector, wild type-p70S6K, D393A and Δ(394-525). 24 h post transfection 400 μg/ml G418 was added for 4 days. Cells were then allowed to grow and plated in 6 well plates and 2 μM cisplatin was added for 72 h. Nuclei were stained with propidium iodide and analyzed using a flow cytometer. The table shows a comparison of the different cell cycle stages. Data are mean +/- SEM of three independent experiments. * $p<0.001$ versus cells transfected with pcDNA3 treated with 2 μM cisplatin , ** $p<0.001$ versus cells transfected with pcDNA3, WT-p70S6K and D393A treated with 2 μM cisplatin using ANOVA.

Table 3.

		Apop Sub G0 (%)	G1 (%)	S (%)	G2/M (%)
	Con	3±2	78.8±12	7.6±4	10.7±6
pCDNA3	CP	20.6±5	13.4±2	17.7±4	47.8±5
	Con	4.6±2	71.2±11	11.5±2	13.3±7
WT-p70S6K	CP	24.9±4	24.3±3	17.8±4	32.6±3
	Con	3.4±1	69.7±8	13.6±5	13.9±3
D393A	CP	15.4±1	11.4±5	8.6±4	64.5±7
	Con	11.3±1**	65.4±7	13±1	11.1±5
Δ(394-525)	CP	42.7±4 *	21.9±1	11.5±3	23.9±3

DISCUSSION

p70S6K regulates cellular growth and proliferation and can influence cell death [22, 23, 26]. Several studies have shown that treatment with cisplatin regulates not only the phosphorylation of the p70S6K but also levels of the total protein [8, 14, 22, 24, 49]. To develop improved drug therapies it is important to elucidate the role of p70S6K in mediating cisplatin-induced cell death. The data presented here show that knockdown of p70S6K results in inhibition of cisplatin-mediated cell death. In addition, we have shown recently that p70S6K is a novel substrate for caspase-3 and results from our present study demonstrate that cleavage of p70S6K enhances cisplatin-mediated cell death.

Flow cytometry analysis shows that in A549 cells, a combination of cisplatin and rapamycin enhanced the number of apoptotic cells. Rapamycin by itself did not augment the number of apoptotic cells, which is in agreement with a previous report [50]. Since rapamycin is an inhibitor of p70S6K, we expected that depletion of p70S6K will also enhance cellular sensitivity to cisplatin. However, we saw striking differences in the cellular response to cisplatin when treated with rapamycin or with the knockdown of p70S6K. This could be explained by the fact that rapamycin may act on other targets besides mTOR/p70S6K. While rapamycin was shown to downregulate p21 resulting in tumor growth [51] it also increased Bcl-2 which is known to prevent apoptosis in various cell lines [52]. Further, when we deplete p70S6K from the cells we are not only depleting the full-length protein we also deplete the cleaved fragment that is generated in response

to cisplatin treatment. Thus, decrease in proapoptotic cleavage product of p70S6K may result in attenuation of cell death.

To find the caspase cleavage site in p70S6K, we mutated several different aspartic acid residues to alanine. We found that in the D393A mutant, levels of endogenous as well as exogenous p70S6K cleaved fragment were considerably reduced compared to wild-type in the EE blot (Fig. 4A). However, we do not see complete loss of the cleaved p70S6K band when we use the p70S6K antibody, suggesting either the presence of minor cleavage sites or nonspecific proteolysis of the endogenous protein. Further, in response to cisplatin treatment when we overexpress WT-p70S6K, the lower p70S6K cleaved band is increased substantially compared to the pcDNA3 bands. This suggests that the lower cleaved band may be from the endogenous as well as exogenous p70S6K. However we do not detect the lower cleaved band when we use the EE antibody, possibly because p70S6K is further cleaved resulting in the removal of the EE tag.

Thus, the cleavage of p70S6K by caspase-3 generates a larger fragment and probably a smaller C-terminal fragment. Whether the smaller fragment of about 132 amino acids remains intact or is further cleaved is a matter of speculation since we do not have an antibody that recognizes the C-terminal domain of p70S6K. In response to cisplatin treatment we demonstrate that in the presence of the D393A mutant cisplatin-induced apoptosis is decreased compared to vector control and WT-p70S6K. In the cells overexpressing the Δ(394-525) mutant however, there is an increase in apoptotic cells both in the presence and absence of cisplatin compared to the pcDNA3 cells. Although we do not know if the larger cleaved fragment is catalytically active, our data clearly

suggests that once p70S6K is cleaved the fragment that is generated has apoptosis inducing capabilities. Similar to our results it has been shown that caspase-mediated cleavage of PKC causes enhanced apoptosis [53]. In conclusion, these results help us to understand the mechanism of p70S6K downregulation by caspase-3 and will hopefully facilitate the exploitation of p70S6K as a therapeutic target in cancer therapy.

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CHAPTER V

DISCUSSION

Cisplatin is widely used for cancer therapy. However, cancer cells become resistant to cisplatin and toxic side effects of the drug hinder its use for successful chemotherapy [1-4]. The efficacy of cisplatin therapy depends not only on its ability to induce DNA damage but also on the cells' ability to respond to this damage. The purpose of this dissertation was to investigate whether the p70S6K pathway is important for cisplatin-mediated cell death and how the p70S6K pathway is regulated during cisplatin-mediated death. Results described in this dissertation demonstrated that (i) activation of p70S6K was associated with resistance to cisplatin, (ii) p70S6K is a novel substrate for caspase-3, and (iii) proteolytic cleavage of p70S6K is important for cisplatin-induced apoptosis.

p70S6K is responsible for mediating nutrient- and mitogen-stimulated cell growth and proliferation, and plays an important role in other cellular processes like cell development, cell size, and cell motility [5-11]. Previous studies have shown that various cancer cells have high levels of constitutively-active p70S6K [12-16]. In addition, it has been shown that there was a decrease in phosphorylation and activity of p70S6K when cells were treated with cell death-inducing agents. [17-19]. The first part of this dissertation focuses on the hypothesis that activation of p70S6K is associated with resistance to cisplatin. The results demonstrate that acquisition of resistance by human

small-cell lung cancer (SCLC) H69 cells was associated with an increase in p70S6K phosphorylation/activity. Further, inhibition of mTOR and PI3K which act upstream of p70S6K reversed cisplatin resistance.

Cisplatin treatment not only caused phosphorylation of p70S6K it also led to the downregulation of total p70S6K. Thus, the second part of the dissertation focuses on elucidating the mechanism of p70S6K downregulation. Downregulation of p70S6K by cisplatin was associated with the appearance of a cleaved fragment. Based on our studies with pharmacological inhibitors, siRNA and *in vitro* cleavage assay, we demonstrated that caspase-3 was responsible for cisplatin-induced cleavage of p70S6K. There is a report suggesting that p70S6K is downregulated in the presence of Fas ligand in Jurkat cells [20]. Though the authors suggested that p70S6K downregulation is mediated by caspase-8, they did not provide any direct evidence to support their conclusion. [20]. In addition, we demonstrated cleavage of p70S6K in H69 cells that do not express caspase-8.

Since, p70S6K is cleaved by caspase-3 in response to cisplatin, the third part of the dissertation focuses on determining the functional significance of proteolytic cleavage of p70S6K is cisplatin-mediated cell death. We have shown that in the A549 non small-cell lung cancer (NSCLC) cells, inhibition of p70S6K by rapamycin enhanced cisplatin-mediated apoptosis. On the other hand, depletion of p70S6K by siRNA resulted in protection from cisplatin-mediated cell death. It is known that rapamycin can inhibit the mTORC1 complex of mTOR which act upstream of p70S6K, 4EBP, CLIP-170 [21]. However, since p70S6K inhibition by rapamycin and knockdown of p70S6K by siRNA had opposite effects on cisplatin-induced cell death, it suggests that rapamycin has additional targets that add to the already complex picture. p21 and Bcl-2 are two proteins

that can be regulated by rapamycin affecting cell death [22, 23]. This suggests that when we use pharmacological drugs in laboratory studies we should take into consideration the caveat that multiple signaling pathways may be altered. In addition, the siRNA technique may have drawbacks since it has been shown to induce the non-specific interferon response in mammalian cells [24]. Further, the decrease in cisplatin-mediated cell death after siRNA maybe due to the depletion of both the full length and cleaved p70S6K fragments.

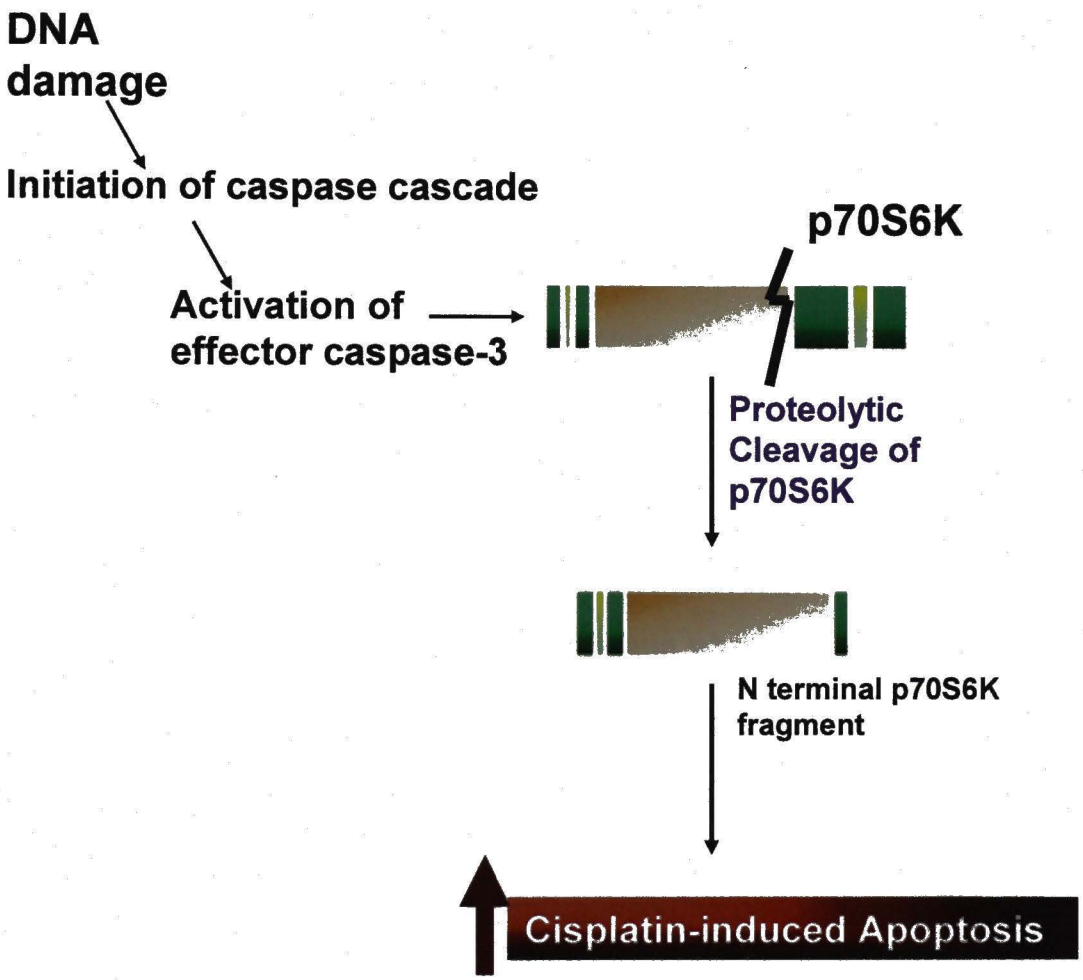
Cleavage of cellular substrates by caspases may result in the regulation of cellular events such as metabolism, cell cycle, death, repair and disease [25-27]. However, cleavage of substrates by caspases may result in their activation or inactivation and further there are proteins that are cleaved with the cleavage having no effect on their functions [25, 26, 28, 29]. Thus, to examine the significance of caspase-3-mediated p70S6K cleavage, we first wanted to determine the site at which p70S6K is cleaved. Though the preferred site at which caspase-3 cleaves proteins is DEVD we found that in p70S6K the caspase-3 cleavage site is TPVD. In fact, discovery of substrate cleavage by caspase at non-canonical sites is now becoming increasingly common [30, 31]. Moreover, we have seen that p70S6K is cleaved in a number of different lung cancer cell lines (H69, A549, H358) suggesting that it is not cell type specific due to variations in the expression of individual caspases.

Interestingly our data shows that while the caspase cleavage mutant inhibits cisplatin-mediated cell death, the cleaved fragment Δ(394-525) augments cisplatin-mediated cell death. Since, in its native conformation full-length p70S6K remains in an inactive state, its cleavage by caspase-3 probably relieves this inhibition. Thus, it is

possible that the cleaved fragment of p70S6K gets phosphorylated and translocates to a different cellular region or that the cleaved fragment maybe competing with the full length p70S6K in the cell to mediate its effect on cell death.

In summary, this dissertation demonstrated that activation of p70S6K is associated with resistance of lung cancer cells to cisplatin and treatment with cisplatin increased the phosphorylation of p70S6K. Furthermore, caspase-3 which is activated during cisplatin treatment cleaves p70S6K resulting in the generation of a fragment that has pro-apoptotic function (Fig 1.). This dissertation provides novel information regarding the mechanism by which p70S6K is regulated by caspases and provides valuable insights that may be exploited for therapeutic benefits.

Figure 1.


DNA
damage
Initiation of caspase cascade
Activation of
effector caspase-3
p70S6K
Proteolytic
Cleavage of
p70S6K
N terminal p70S6K
fragment
Cisplatin-induced Apoptosis

FUTURE STUDIES

Our data with the H69 cells shows that rapamycin enhances cisplatin-mediated cell death and combining agents that have different targets in the cell may be a good approach for chemotherapy. In fact, clinical trials that use this strategy are already being evaluated. Using combinations drugs may help in problems such as resistance and/or toxicity. Clinical studies in tumors with enhanced p70S6K activity may also be appropriate to explore these combination drugs. Moreover, the status of ATM, p53, PTEN are known to be associated with resistance to rapamycin [32]. Screening of tumor biopsy specimens by techniques like immunohistochemistry or fluorescence *in situ* hybridization (FISH) to look at the status of these proteins may provide the basis for selecting cancer patients most likely to benefit from inhibitor therapy. To determine a pharmacologically active dose that results in optimum inhibition of the target dose-response experiements in animal models need to be performed.

Since, we do not see enhanced apoptosis by cisplatin when cells are depleted of p70S6K it maybe worthwhile to look at other forms of cell death. Autophagy has been shown to be regulated by p70S6K [33] and the role of p70S6K in cisplatin-mediated autophagy needs to be investigated. In addition, deficiency of p70S6K is known to play a role in decreased cell size in drosophila [10] and motility in ovarian cancer cells [8]. Thus, it is reasonable to hypothesize that p70S6K regulates endpoints like tumor burden and metastasis in response to cisplatin treatment. Caspase-3 is primarily involved in apoptosis, however it may also play a role in differentiation of various cell types [34-37]. Since, p70S6K is also known to regulate smooth muscle differentiation [38], strategies looking at the involvement of caspase-3 regulated p70S6K on smooth muscle differentiation

would be meaningful and relevant. In addition, caspase-3 is also known to regulate proliferation [37] and since the role of p70S6K in cell proliferation is well established; its cleavage by caspase-3 may also play a role in cellular proliferation.

Caspase-3 can cleave a large number of downstream targets resulting in a large noise to signal ratio when we want to look at the effect of an individual substrate. In those instances, animal studies with non-cleavable knock-in mice could provide a connection between cleavage of an individual substrate like p70S6K and a cell death endpoint. Further, cleavage of some proteins by caspases results in their translocation to different cellular compartment to regulate apoptosis [39]. It has been shown that p70S6K after stimulation with cytokine moves to the nucleus and, insulin has been shown to stimulate nuclear p70S6K [40, 41] suggesting that both the cytoplasmic and nuclear forms of the protein may be regulated. In addition, since my studies have shown that cleavage of p70S6K results in enhanced cisplatin-mediated death, it is possible that cleavage of p70S6K alters its localization, resulting in activation/inactivation of yet unkown downstream targets. Moreover, cleavage by caspase-3 at the Asp393 site results in the autoinhibitory domain close to the carboxy terminus of p70S6K to be released. The phosphorylation sites in the autoinhibitory domain are the first to get phosphorylated, facilitating the phosphorylation of other p70S6K sites [42]. We anticipate that during cleavage the autoinhibitory domain is released, allowing phosphorylation of the other p70S6K (T389, T229) sites. Future experiments should aim at understanding whether or not p70S6K gets activated in response to cisplatin. Though, our data with the H69 cells show that phosphorylation of p70S6K is associated with resistance to cisplatin, it is not very clear whether or not the phosphorylation of p70S6K modulates its cleavage by

caspase-3 during cisplatin treatment. Mutating the phosphorylation sites of p70S6K to non-phosphorylatable mutants would help address this issue by examining the effect of these mutants on the cleavage of p70S6K.

Additionally, to utilize the cleaved fragment for therapy, introducing it by nanoparticles or high density lipoproteins is a viable future option, since this fragment enhanced cisplatin-induced apoptosis. However, due to the size of this fragment (about 47-kDa) it may not be possible to package it for delivery. Generation of deletion mutations of the fragment will help to identify the particular segment of this fragment that has the apoptosis inducing ability. The work discussed here is primarily limited to lung cancer cell lines and should be expanded to primary cultures from cancer patients and further onto animal studies.

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