INVOLVEMENT OF HEPATITIS C VIRUS PROTEIN NS5A IN APOPTOSIS

By

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Bachelor of Science, 2006
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Arlington, TX

Submitted to the Graduate Faculty of the
College of Science and Engineering
Texas Christian University
in partial fulfillment of the requirements
for the degree of

Master of Science

August 2009

AKNOWLEDGEMENTS

I would primarily like to thank my thesis advisor, Dr. Giri Akkaraju for his patience, enthusiasm, and wisdom over the past two years. His love for Science and his optimism for research are truly contagious, and have served as motivation and inspiration for all his students! All advisors should teach research the way he does.

Additionally, I must acknowledge the help of my fellow lab mates, especially Molli Jones Crenshaw. There was never a dull moment in lab with Molli. And through the ups and downs, her positive outlook and friendliness always made the lab a little brighter.

I could not go without thanking the Biology faculty at TCU. All of my professors have been especially kind, respectful, and very helpful. They have taken a notable interest in the education and success of each student which will forever leave my peers and me with the utmost gratitude.

Finally, I would like to thank my wonderful family, namely my parents, Ram and Uma Subramanian, for their unwavering support for everything I do, and their unconditional love for me.

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INTRODUCTION

The Hepatitis C Virus (HCV), a primary cause of the disease Hepatitis, infects approximately 170 million individuals worldwide. HCV is an enveloped, single-stranded RNA virus of the family Flaviviridae. First identified in the 1970s, HCV was not formally named until 1989 when its genome was first published. The virus is blood-borne and can therefore be contracted via blood transfusions, sexual contact, sharing needles, and can be passed to infants during childbirth. The two most common genotypes of the virus, HCV 1A and HCV 1B, cause severe liver inflammation and hinder the liver's ability to cleanse the blood of waste products (Dossett *et al.* 1999). Individuals often appear asymptomatic during the first several weeks of initial infection. However, very few are cleared of the virus at this stage, which often leads to chronic, life-long infection which can lead to liver cirrhosis or hepatocellular carcinoma.

There is no vaccine for HCV, and the current treatment is lengthy and not highly successful due to the mutations acquired by the virus during replication resulting from an error-prone viral RNA-dependent RNA polymerase, that often result in changes to antigens normally recognized by a vaccine. Infected individuals are typically injected with synthetic pegylated interferon-α, combined with orally-administered ribavirin, a nucleotide analogue that targets HCV RNA-dependent RNA polymerase (CDC 2007).

The genome of HCV has been studied extensively since its discovery. Following translation, it gives rise to a polyprotein that includes both structural and non-structural proteins. Proteins C, E1, and E2 are the structural proteins which give rise to the

nucleocapsid and envelope glycoproteins, respectively. Non-structural proteins include NS2, a transmembrane protein, NS3, which is involved in protease and RNA helicase functions, and NS4A and NS4B, which are both cofactors. NS5B is the viral RNA-dependent RNA polymerase, while NS5A, our protein of interest, provides the virus with resistance to interferon (IFN) (Gale and Foy 2005).

Interferons (IFN) are a group of cytokines responsible for inducing the anti-viral response in neighboring cells. When a virus-infected cell dies and releases the numerous virion particles within, the infectious particles will likely reach neighboring cells that have already been "warned" by interferon of a possible viral infection. Viral infection can also induce apoptosis in the host cell by interfering with mechanisms such as mRNA translation and gene transcription. Interfeon-induced apoptosis begins with the Jak-Stat signal transduction pathway. The pathway embodies rapid signaling from the plasma membrane to the nucleus of the cell. First, IFNα and IFNβ bind to their own transmembrane receptors, initiating the phosphorylation of tyrosine residues by Jak kinases. The now active Jak kinases will in turn conscript Stat proteins bound to the phosphorylated receptor by phosphorylating them as well. The newly dimerized Stat proteins are then translocated to the nucleus where they activate the transcription of several IFN-stimulated genes. One of these targets is the gene for Mx proteins, which interfere with viral polymerase activity, inhibiting the production of mRNA. Another is the gene for the enzymes 2'5'-oligo(A) synthetase and ribonuclease L, which will ultimately break down both cellular and viral mRNAs. Thirdly, Protein kinase R or double stranded RNA-dependent protein kinase (PKR), the IFN-induced gene that is of

most interest to our research, also ultimately induces the cessation of protein synthesis by the cell, leading to apoptosis.

One of the favorable characteristics of apoptosis is that it is self-contained and does not damage other cells. This is a contrast to necrosis, another form of cell death which induces the cell to lyse, releasing its contents and triggering an inflammatory response in the organism. Cells that are disintegrating from apoptosis typically come unattached from their substrate and begin to shrink as their membranes invaginate. Their chromatin then condenses and their DNA fragments. Apoptosis can be triggered when irreparable DNA or cellular damage or possible tumor formation is detected by one of the checkpoints of the cell cycle. p53 is a protein involved in arresting the cell cycle at G1 and G2 checkpoints. If cellular damage is extensive, p53 will not allow the cell cycle to continue, but will instead induce APAF, or apoptotic protease activating factors, which will in turn initiate the activation of a series of caspases (calcium-dependent cysteine proteases), known as the caspase cascade, to begin apoptosis in the cell (Alberts *et al.* 2002).

In an uninfected cell, protein synthesis is carried out by the GDP-GTP exchange of the eIF-2 cycle. When double stranded viral RNA enters the cell, two active PKR molecules will bind to the strands and phosphorylate each other. This phosphorylation will amplify PKR's kinase activity, primarily initiating the phosphorylation of the alpha subunit of the eIF-2 complex. The phosphorylation of the substrate inactivates the eIF-2 complex, and shuts down the remainder of the cycle. Normally, eIF-2 binds to GTP, and forms a

complex with mRNA and the 40S ribosomal subunit, facilitating the exchange of GTP for GDP, preparing a translation initiation complex. During a viral infection, however, when activated PKR phosphorylates the α-subunit of the eIF-2 complex, it prevents it from cycling, thereby effectively shutting down protein synthesis in the cell. Apoptosis will eventually occur due to insufficient mRNA translation and protein synthesis. This induction of apoptosis by PKR is one of the cell's preventative mechanisms from allowing the virus to proliferate (Acheson 2007).

It has been hypothesized that the ability of protein NS5A to inhibit this interferon-induced PKR block and allow viral replication is what ultimately leads to hepatocellular carcinoma of Hepatitis C patients. It is thought that PKR binds to the ISDR, or interferon sensitivity determining region, of NS5A. It has been shown that mutations to this ISDR can inhibit NS5A binding and eventually bring about apoptosis in the cell (Dossett *et al.* 1999). Additionally, the inability of HCV-infected cells to block the eIF-2 cycle has been seen by analysis of the phosphorylation of the serine51 protein residue of eIF-2α. This is the residue that PKR normally phosphorylates when activated, however, the percent of phosphorylation remained unchanged in NS5A-positive mammalian cells, demonstrating the inability of PKR to phosphorylate eIF-2 in these cells (Dossett *et al.* 1999). The oncogenic potential of HCV has been studied by groups who have injected wild-type HCV 1A strains into mice, all of which developed tumors. Furthermore, in a study where HCV-infected mammalian cells were plated on an agar medium alone, the cells appeared to lose their necessity for substrate attachment and rapidly continued to divide (Dossett *et al.* 1999).

The goal of this project is to further study the role played by NS5A in apoptosis by characterizing its behavior when it is constitutively expressed by cells, and analyzing this behavior when these cells are exposed to apoptotic stimuli. The aim is also to determine if NS5A inhibits apoptosis, and if so, what candidate apoptotic proteins it may be binding to and causing to malfunction.

Materials and Methods

Tissue Culture

Adherent HEK 293 cells were purchased from ATCC (Manassas, VA) and grown in monolayer on Corning tissue culture dishes. All cells were grown in Dulbecco's Modified Eagle Medium (DMEM) (MediaTech, Herndon, VA) and 5% CO_2 enriched air at 37°C in a tissue culture incubator (NuAire, Plymouth, MN). Added to the medium were: 10% fetal bovine serum (FBS), 1% glutamine, 1% non-essential amino acids, and 1% penicillin/streptomycin antibiotics (all from Sigma Aldrich).

Creation of 293: 10A and 293: H27 cell lines

HEK 293 cells plated into two 100-mm dishes and transfected with 1ug of either NS5A 10A or NS5A H27 plasmids using Lyovec reagent (Invivo Gen). One day post-transfection, medium was changed, and two days following transfection, cells in each 100-mm dish were trypsinised (MediaTech) and equally divided into two more 100-mm dishes. Transfected cells were treated with 0.1µg/mL of either Puromycin or Zeocin (Invitrogen). The pellet of the cells remaining from the original plate was harvested for a transient IP by centrifugation (at 8,000rpm for 5 min), and washed in 1X Phosphate

Buffered Saline (PBS) (2.7mM KCl, 1.4mM KH_2PO_4, 137mM NaCl, 4.3mM Na_2HPO_4 $7H_20$). Adherent cells were fed new medium and treated with antibiotic every 3-4 days, or until colonies of cells became visible on the plate. The colonies were selected and grown in a 6-well dish. Once the wells were 90% confluent, the cells were split into a fresh dish with some saved for a Western blot. The aforementioned pellets were lysed with SDS lysis buffer with protease inhibitors (SDS Lysis Buffer, 0.01ug/mL Leupeptin, 0.001M PMSF, 1 tablet Roache protease inhibitor) , and protein concentrations were measured by Bradford Assay (Sigma Aldrich).

Cell Growth Assay

Two 48-well dishes were plated with either HEK 293 or 293: NS5A 10A-6 cells with $1x10^3$ cells per well. Cells were counted beginning 48 hours after plating. The day of plating was treated as day 1 of the assay. Medium from the wells were aspirated and 0.5mL Trypsin was added. Once cells appeared loose, 0.5mL complete medium was added and cells were counted on a hemocytometer (Hausser Scientific). Wells were counted every other day and in triplicate.

Luciferase Assay

HEK 293 and 293: NS5A 10A-6 cells were plated in either half of a twelve-well dish at $1x10^5$ cells per well. The following day, Lyovec Reagent was used to transfect each well with 100 ng IFNβ-Luciferase and 50ng RL-CMV reporter plasmids. Twenty four hours post-transfection, selected wells were infected with Sendai Virus. All cells were lysed the following day using Passive Lysis Buffer (Promega). Luciferase Assay Reagent, followed by Stop and Glo Buffer (Promega) were added to the samples as per the

manufacturer's protocol and the samples were then read by a Luminometer (Berthold Detection Systems) to measure Luciferase activity.

Western Blot

Preparation of SDS Page Gel

A 9% SDS Page gel was prepared (4X Tris buffer pH 8.8, 30%acrylamide/0.8% bisacrylamide, 10% (w/v) ammonium persulfate, 0.01% TEMED). Gels were run in SDS Running Buffer (0.125M Tris Base, 0.96M Glycine, 0.5 w/v SDS) at 100v for 1 hour or until the bromophenol blue band reached the bottom of the gel. Proteins were transferred to Polyvinylidene difluoride (PVDF, Millipore) membranes in transfer buffer (0.04M Tris Base, 0.29M Glycine, 20% Methanol) at 100v for 1 hour.

Immunoblotting:

Equal amounts of Sample (normalized for protein concentration) and pre-stained SDS broad range ladder (Biorad) were loaded, and the gel was run. Membranes were washed in PBST (2.7mM KCl, 1.4mM KH_2PO_4, 137mM NaCl, 4.3mM Na_2HPO_4 $7H_20$, 0.1% Tween 20).

Once the transfer was completed, the membrane was incubated for 1 hour in 2% BLOTTO solution (10g Non-fat dry milk, 500mL PBST) and primary antibody was prepared (1:1000 in 2% BLOTTO)).

After one hour, the membrane was placed in a sealed tub with the primary antibody solution for 30 minutes and then incubated at 4®C overnight. The membrane was subjected to three, 10 minute washes in 2% BLOTTO.

Secondary antibody was prepared (1:10,000 in BLOTTO) and added to the membrane for one hour. The membrane was again subjected to three, 10 minute washes in 2% BLOTTO, and given a final wash in 1X PBS. Alkaline Phosphatase staining reagents, NBT and BCIP (Promega) were added, as per manufacturer's instructions, to Alkaline Phosphate Buffer (100mM Tris pH 9.5, 5mM $MgCl_2$, 100mM NaCl) for membrane staining.

Immunoprecipitation

Anti-flag M2 affinity gel (Sigma-Aldrich) was thoroughly suspended in its vial before being transferred (at 30uL per sample) to a 1.5mL microcentrifuge tube. The tube was centrifuged at 10,000rpm for 1 minute and the supernatant removed. The resin was washed twice in TBS (0.15M NaCl, 0.05M Tris HCl pH 7.4), where on the final wash, it was equally divided among the samples.

Harvested cell pellets were resuspended in hypotonic lysis buffer (10mM Tris HCl, pH 7.5, 10mM KCl, 0.5mM EGTA, 1.5mM $MgCl_2$), rocked at 4®C for 20 min, and then centrifuged at 14,000rpm for 10 minutes at 4®C. The cell lysates were transferred to fresh, chilled microcentrifuge tubes and spun again at 14,000rpm for 30 minutes at 4®C. This supernatant was drawn through at 0.45µm filter into the tubes containing the washed Anti-Flag M2 affinity gel. The suspension was incubated at 4®C for 2 hours, then centrifuged for 1 minute at 10,000 rpm. The resin was washed 3 times with TBS/0.5% NP-40 (0.15M NaCl, 0.5M Tris HCl pH 7.4, 0.5% NP-40), with 5 minutes of rocking at 4®C in between each wash.

The centrifuged resin was now twice incubated with 250μg/mL Flag peptide at 4®C for 30 minutes and the eluate saved. To the eluate 1μg anti-NS5A rabbit polyclonal antibody was added and incubated at 4®C for one hour.

Twenty microliters sample buffer was added to each sample and tapped to mix. Samples were boiled at 96®C for 3 minutes and then centrifuged at 8,000rpm for 30 seconds. The supernatant, now ready to be loaded onto a gel, was transferred to a fresh tube.

DNA fragmentation assay using TES buffer

One million MEF cells were plated into two 100mm dishes, where one plate was transfected with the NS5A 10A plasmid the following day. Two days after plating, both plates were split equally into two more plates, for a total of 4 plates. The next day, one MEF NS5A plate, and one MEF plate were each treated with 1mM Staurosporine (Sigma-Aldrich). Six hours following treatment with drug, the medium was harvested to collect any floating cells, and adherent cells were harvested by scraping. The pellets were washed once in 1X PBS and centrifuged for 5 minutes at 8,000rpm. To make lysis buffer, 1.25 mg/mL Proteinase K (Sigma-Aldrich) and 10% SDS were dissolved in TES (50mM 1M Tris HCl pH 8.0, 10mM 0.5M EDTA, 0.1M 5M NaCl). Pellets were resuspended in the TES lysis buffer overnight at 37®C.

Equal volumes of Phenol: Chloroform: Isoamylalcohol (25:24:1) (Fisher BioReagents) were added to the cell lysates for 5 min before centrifugation for 2 min at 10,000rpm. The organic layer was discarded, and an equal volume of Chloroform: IAA (24:1) was added to the samples which were rocked for 5 min and then spun for 5 min

at 10,000rpm. The aqueous layers from the centrifuged samples were added to microcentrifuge tubes containing .12mM NaCl. To these tubes, 2.2X volumes of cold absolute Ethanol (Shelton Scientific) were added to the samples and mixed by inversion. For 15 minutes, the samples were stored at -80®C and spun at 14,000rpm for 15 minutes at 4®C. The pellet was washed with 70% Ethanol and spun at 13,000 rpm for 5 minutes. The supernatant was decanted, and the pellet was left to dry for 20 minutes under the fume hood. 0.1mg/mL RNase was dissolved in TE buffer (10mM Tris pH 8.0, 1mM EDTA). The dried pellets were resuspended in equal volumes of the RNase solution and incubated for 1 hour at 37®C. The DNA concentration of the samples was measured using the Nanodrop (Thermo Scientific). Equal concentrations of DNA were mixed with Loading buffer and DI water. Samples and 100bp DNA marker (Lamda Biotech) were loaded onto a 1.5% Agarose gel and run in TAE buffer (0.484% Trizma base, 0.0114% glacial acetic acid, 0.02% 0.5M EDTA) at 100V for one hour. After the samples were run, the gel was incubated in TAE buffer and 0.00015 mg/mL Ethidium Bromide for 10 minutes. The gel was then exposed to UV light (Kodak) and observed for DNA fragmentation.

RESULTS

Creation of a cell line that stably expresses NS5A

In order to conduct experiments on the behavior of NS5A, cell lines expressing NS5A mutants were created. Stable cell lines are preferred over transiently transfected cells because, unlike transiently transfected cells where expression of the transgene can be variable, they express a constant level of the transfected protein thus making it easier to compare the results between two experiments. HEK 293 cells were transfected with NS5A 10A or NS5A H27 plasmids, and over the course of several weeks, colonies were selected with either Zeocin or Puromycin and grown into cell lines. A Western blot was done on each colony to ensure the expression of NS5A (Fig. 1).

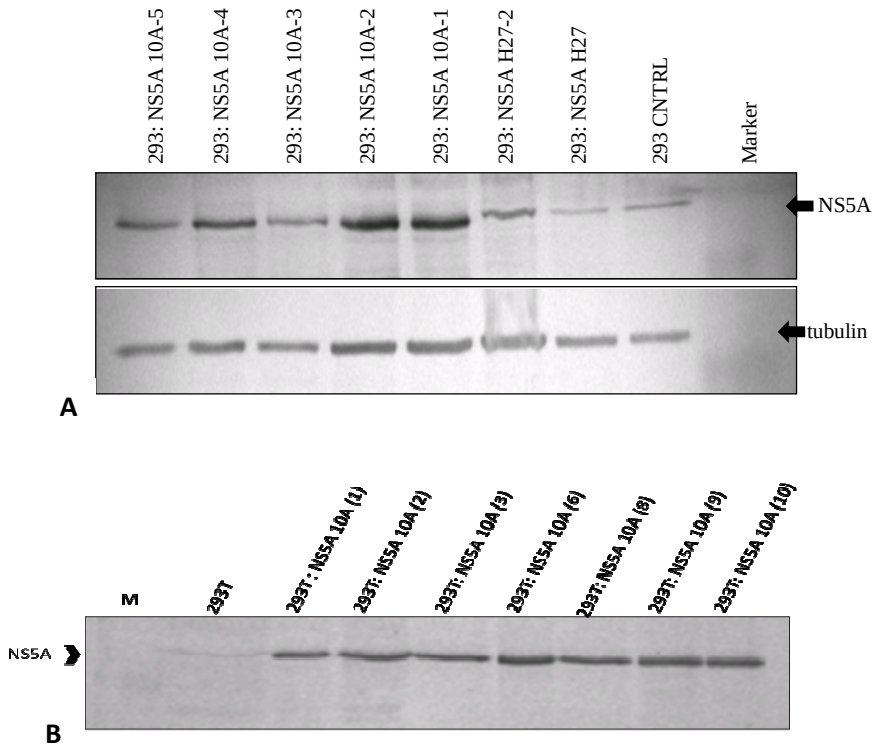

Figure 1. NS5A 10A or NS5A H27 Cell Lines

A Western blot of HEK 293 cells strongly expressing either NS5A 10A or NS5A H27

A. Western blot of lysates from various colonies of 293: NS5A 10A and 293: NS5A H27 cells stably expressing NS5A and the cellular protein Tubulin.

B. Western blot of lysates from colonies of 293 cells expressing NS5A 10A only.

The effect of NS5A on an anti-viral promoter

Because it has previously been shown that NS5A inhibits PKR activity (Dossett *et al.* 1999), the cell lines were tested to see if they behave as they should upon infection with a virus. Two NS5A cell lines, 293: NS5A 10A-6, and 293: NS5A 10A-10 were plated along with control HEK 293 cells, and subsequently, all cells were transfected with the reporter gene, luciferase under control of the Interferon beta promoter which is induced by viral infection. Activation of the promoter should lead to the expression of the luciferase gene. Both control cells and those expressing NS5A were infected with Sendai virus, a mouse paramyxovirus that is commonly used in laboratories to simulate viral infection and activate the antiviral pathway, to activate the IFN-β promoter, and luciferase activity was measured (Fig. 4). This result indicates that luciferase activity was much lower (~50% of control) in cells expressing NS5A, suggesting that NS5A was blocking PKR activity, and therefore reducing luciferase expression.

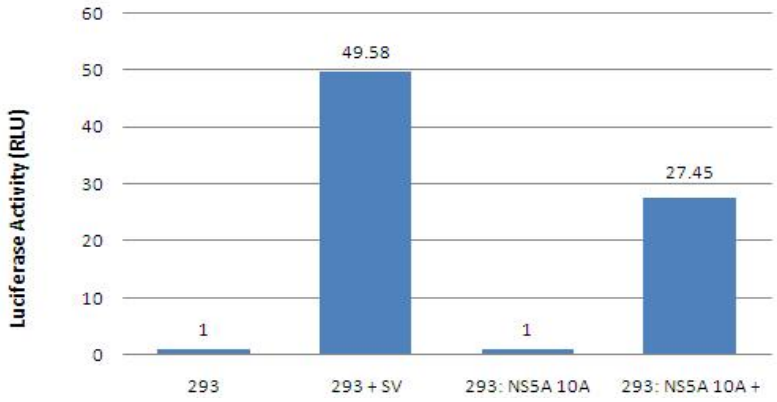

Figure 2. The effect of NS5A on an antiviral promoter: IFN-β promoter linked to a reporter gene (Luciferase)

A luciferase assay was done in order to show that NS5A inhibits PKR activity. Cells were then transfected with the NS5A gene and infected with Sendai Virus. Luciferase activity was measured.

Cell Growth Assay

To characterize the behavior of cells expressing NS5A, cell growth and cytotoxicity assays were performed (Figs. 2 and 3). Our hypothesis is that expression of NS5A in cells may deregulate the cell cycle and increase the rate of cell division. To test this we designed an experiment to study the effect of NS5A expression in our cell lines. Equal numbers of cells from the 293: NS5A 10A cell line as well as control 293 cells were plated and the number of cells from each type was counted over the course of a week. Cells expressing NS5A 10A grew rapidly compared to the control cells (Fig. 2). This evidence supports the hypothesis that NS5A may be inhibiting cell growth suppressors when expressed at high levels in cells.

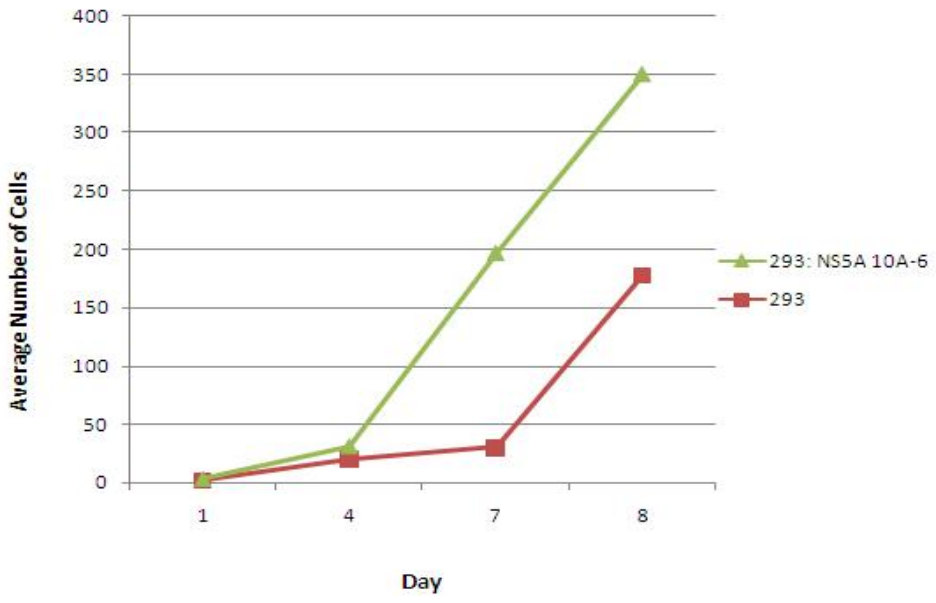

Figure 3. Cell Growth Assay
293 Cells expressing NS5A as well as HEK 293 cells were plated in equal numbers and counted in triplicate over the course of one week.

Effect of NS5A on the sensitivity of cells to the cytotoxic drug etoposide

Because we had shown that NS5A stimulates cell proliferation by interfering with PKR, we now hypothesized that it may confer some cytotoxic resistance to the host. To show this, 293: NS5A 10A-6 and 293: NS5A 10A-10 cells were again plated along with HEK 293 cells in equal numbers. By utilizing two different NS5A cell lines, we could determine any difference in cytotoxic resistance between them. The cells were then treated with increasing quantities of the cytotoxic drug Etoposide followed by the addition of the vital stain MTT. MTT is reduced by the mitochondria of living cells into an insoluble, purple stain. Therefore, only viable cells will be stained by MTT, and their quantity measured by spectrophotmometer. The proportion of living cells was measured and plotted (Fig. 3). Cells from the NS5A 10A-6 line were less sensitive to Etoposide treatment than both the control and the other NS5A 10A line. The latter, 293: NS5A 10A-10, did not behave as expected, and showed increased Etoposide sensitivity as compared to the control.

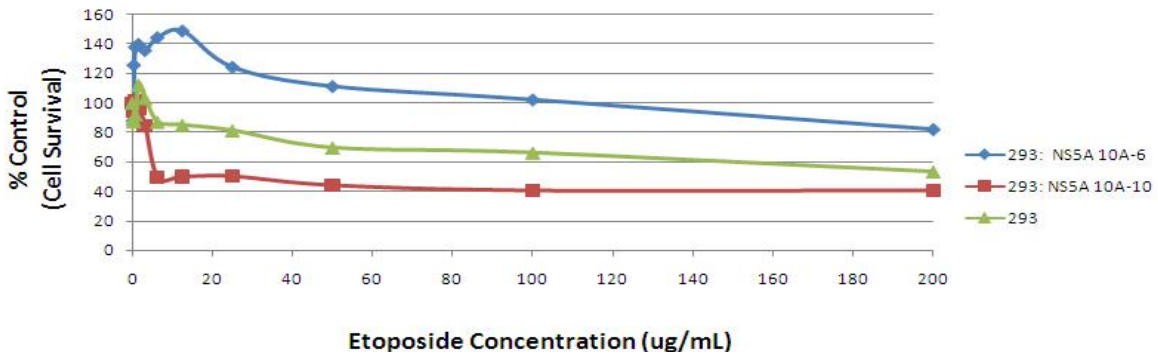

Figure 4. Cytotoxicity Assay
Cells from two 293: NS5A 10A cell lines and HEK 293 cells were plated in equal numbers and treated with increasing concentrations of Etoposide. The proportion of surviving cells was recorded.

Characterization of apoptosis by DNA Fragmentation

Given that PKR induction was blocked, and that NS5A does seem to confer some cytotoxic resistance to the host, we hypothesized that NS5A may also inhibit apoptosis. First, MEF cells transfected with the NS5A 10A gene and on MEF control cells were equally plated. The following day, cells were treated with Staurosporine to induce cell death. Both floating and adherent cells were harvested and their DNA extracted. The DNA of cells undergoing apoptosis will fragment due to the activation of nucleases. The fragments will be cleaved intra-nucleosomally into approximately 200 bp segments or multiples thereof, giving a characteristic laddering pattern when run through an agarose gel (Fig. 5). Cells expressing NS5A showed less laddering, and therefore less apoptotic activity, than those that do not.

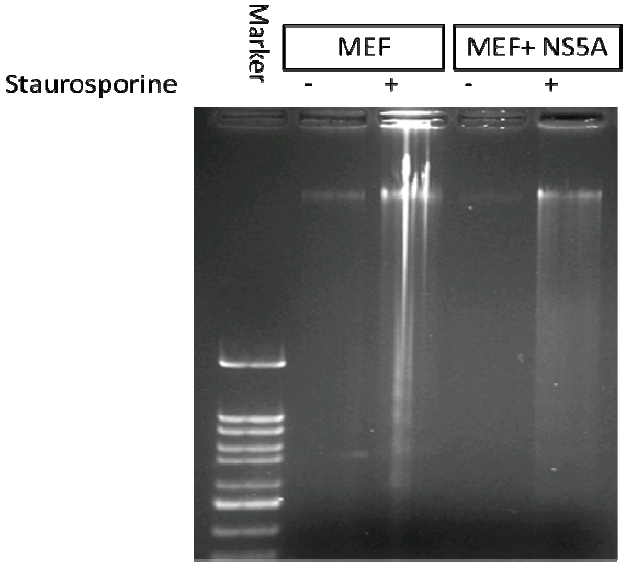

Figure 5. Determining the Effect of NS5A on Apoptosis
MEF cells were transfected with NS5A and then treated with a drug, Staurosporine, to induce apoptosis. The DNA of all cells was extracted and measured before being run through a 1.5% agarose gel. Apoptotic activity was determined.

Immunoprecipitation of NS5A

Our ultimate goal is to identify what proteins NS5A is binding to during the activation of cell growth and the inhibition of apoptosis . To do this, we plan to utilize a technique known as Co-Immunoprecipitation (Co-IP). Cells expressing NS5A will be exposed to apoptotic stimuli and then harvested. Their lysates will be incubated along with heavy agarose beads that are bound to NS5A-specific antibodies. During centrifugation, the antibody-bound beads will pull down NS5A, and any protein NS5A is bound to. A Western blot, when probed for candidate proteins, will reveal whether or not they are present. However, before identifying the proteins bound to NS5A by Co-IP, an initial immunoprecipitation was done to ensure that NS5A could be pulled down. Lysates from 293: NS5A 10A and HEK 293 cells were incubated with Flag-tagged M2 antibody beads and centrifuged. A Western blot was done on the samples at different stages during the immunoprecipitation to determine the efficiency of pull-down and to verify the presence of NS5A in the final bead eluate. The beads appear to have successfully pulled down NS5A as evidenced by the presence of a band of the appropriate size in the gel on the right hand side of figure 6, in the lane labeled eluate.

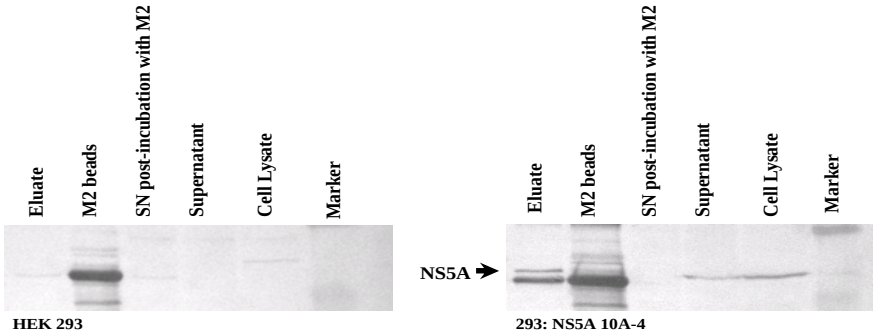

Figure 6. Immunoprecipitation of Flag-tagged NS5A
Immunoprecipitation was done as a precursor to Co-Immunoprecipitation. Cell lysates from HEK 293 and 293: NS5A 10A cell lines were incubated with beads containing Flag-tagged NS5A. The beads, now bound to NS5A, were pulled down by centrifugation before being analyzed for NS5A by immunoblot.

DISCUSSION

HCV protein NS5A is involved in genomic RNA replication and has been shown to interfere with the cellular antiviral protein PKR. NS5A is also thought to manipulate the physiology of the host cell, therefore allowing the virus to evade detection by the immune system. The ability of NS5A to inhibit PKR and to deregulate growth control genes could also lead to inhibition of apoptosis and transformation of the host cell. My research objectives were to first create and characterize the behavior of cell lines that stably express NS5A. I then determined the effect of NS5A on cell growth and cell death before characterizing the interactions between NS5A and other proteins.

To characterize the NS5A cell line, a Luciferase Assay was done to see if NS5A behaves as it should. It has been previously shown that expression of NS5A in cells prevents the activation of the Interferon-b promoter after viral infection. This is believed to be mediated by the ability of NS5A to bind to and inhibit PKR. Following the creation of

cells lines expressing NS5A, we first tested the effect of the expressed NS5A on the activation of PKR and subsequently the activation of the IFN-β promoter. Cells expressing NS5A showed approximately half the luciferase activity as control cells, due to the inhibition of the antiviral promoter, suggesting that the expressed NS5A behaves as expected (Fig. 4). Since PKR induces apoptosis, then rationally the inhibition of PKR by NS5A should hinder the onset of apoptosis. Next, we employed the use of the cytotoxic drug etoposide to determine if NS5A could inhibit apoptosis that is induced via a PKR-independent pathway. To see the effect of NS5A on cell growth and cell death, both cell growth and cytotoxicity assays were done (Figures 2 and 3) using cells that constitutively express the NS5A gene (Fig. 1). The results demonstrate relatively rapid growth in cells expressing NS5A as compared to control cells. This provides further evidence of the ability of NS5A to disrupt growth-control genes in the cell, and increase cellular proliferation. However, the cytotoxicity assay revealed that NS5A did not behave as expected, as one of the NS5A-expressing cell lines appeared more sensitive to Etoposide than the control. This departure from our expectations could have resulted due to my own error. If I miscounted, and plated fewer cells than planned from the NS5A-expressing cell line that showed more resistance to etoposide than my control, but still proceeded to treat all cells with equal concentrations of etoposide, this may have induced more cell death than I had anticipated, producing the abnormal result. This assay should be repeated to determine the validity of these results.

The above series of experiments suggest that NS5A may confer some resistance against cytotoxic drugs on the host cell and may inhibit cell death. To see if NS5A specifically

inhibits apoptotic cell death, a DNA fragmentation assay was done. During apoptosis, the DNA and cell nucleus will fragment and experience intra-nucleosomal cleavage into lengths of roughly 200 base pairs or multiples thereof. Both NS5A and control cells that were treated with the apoptotic-inducing drug Staurosporine and observed for a distinct laddering pattern, a hallmark of apoptosis. This laddering pattern was more apparent in control cells than in cells expressing NS5A, suggesting that NS5A does deter apoptosis (Fig 5). The assay should be repeated, perhaps with different harvesting times, to confirm the result and to see when the inhibition of apoptosis by NS5A is greatest.

Since the previous experiment indicated that NS5A may be inhibiting apoptosis, Immunoprecipitation (IP) was performed to see what apoptotic proteins NS5A binds to. Before this Co-IP could be done, we needed to optimize the technique to demonstrate that we can reliably immunoprecipitate NS5A. This experiment proved to be successful. The immunoblot reveals the presence of NS5A after elution from the Protein A-agarose beads used in the final step (Fig 6). Now that it appears that NS5A was successfully pulled down, we can proceed to the next leg of the experiment and perform a Co-IP, using antibodies against candidate apoptotic proteins. PKR would be used as a positive control protein for the co-immunoprecipitation, since it is known to bind to NS5A which interferes with its regulatory functions. Following this, other candidate genes known to be involved in apoptosis can be examined for binding to NS5A.

What I have found supports the evidence that NS5A confers compound advantageous effects to HCV by deregulating cell growth proteins and inhibiting apoptotic cell death in

the host organism. The inhibition of transcription of antiviral genes by NS5A suppresses normal responses by the host immune system, allowing HCV to establish a chronic liver infection. Moreover, because NS5A stimulates cell proliferation, and because rapidly dividing cells have an increased likelihood of acquiring a mutation in growth-control genes, the virus is capable of potentially transforming cells. Additionally, the inhibition of apoptosis by NS5A could potentially prevent the elimination of these mutated cells and allowing them to divide uncontrollably, increasing the probability of the formation of liver cancer.

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VITA

Shilpi Subramanian was born in Bangalore, India but moved to Arlington, Texas at the age of two. She is the daughter of Ram and Uma Subramanian, and the sister of fellow Horned Frog Gokul Subramanian.

Shilpi received her B.S. in Biology from The University of Texas at Arlington in May of 2006. Following graduation, she became a Summer Intern for the U.S. Environmental Protection Agency in Atlanta, Georgia. There, her intern project was to create an informative brochure on the cultural uses of mercury. She was also selected to present her work to the director of EPA at the end of the summer.

Shilpi is a professional Classical Indian Dancer and has taught for the Arathi School of Indian Dance in Dallas for the past several years. Recently, Shilpi became a founding member of the Indique Dance Company whose primary mission is to expose Indian Dance to Dallas-Fort Worth community.

Besides Science and dancing, Shilpi loves to learn about marine animals, especially squids! In her free time, she enjoys reading, exercising, cooking and baking, listening to all types of music, spending time with her beautiful nephews, and travelling.

ABSTRACT

INVOLVEMENT OF HEPATITIS C VIRUS PROTEIN NS5A IN APOPTOSIS

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Hepatitis C, the disease caused by the Hepatitis C Virus (HCV), infects over three million people in the United States alone. In the majority of infected individuals, the virus sets up chronic infection and can ultimately result in liver cancer. The NS5A protein of HCV is thought to provide the virus with means to evade detection by the host immune system and provide resistance to interferon and inhibit apoptosis, or programmed cell death. These mechanisms of apoptotic interference by HCV may be what ultimately lead to hepatocellular carcinoma, the 3rd leading cause of cancer death. This project characterizes the behavior of cell lines that stably expresses NS5A, and their response to apoptotic stimuli. The research aims are to understand how NS5A interferes with apoptosis by identifying what apoptotic proteins it binds to.