

2012

## Isolation and characterization of a novel substrate for the pro-apoptotic Omi/HtrA2 protease

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Ward, Nathan, "Isolation and characterization of a novel substrate for the pro-apoptotic Omi/HtrA2 protease" (2012). *HIM 1990-2015*. 1314.

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ISOLATION AND CHARACTERIZATION OF A NOVEL SUBSTRATE FOR  
THE PRO-APOPTOTIC OMI/HTRA2 PROTEASE

by

NATHAN P. WARD

A thesis submitted in partial fulfillment of the of the requirement  
for the Honors in the Major Program in Biotechnology  
in the College of Medicine  
and in The Burnett Honors College  
at the University of Central Florida  
Orlando, Florida

Spring Term 2012

Thesis Chair: Dr. Antonis Zervos

## ABSTRACT

Omi, also known as HtrA2, is a mammalian pro-apoptotic mitochondrial protein and a member of the HtrA (high temperature requirement A) family of serine proteases. Omi promotes the caspase-dependent apoptotic pathway through cleavage of IAPs (inhibitor of apoptosis proteins); this cleavage inactivates IAPs and facilitates caspase activity. Omi's proteolytic activity is necessary and essential for its pro-apoptotic function.

This study is aimed to further understand the role of Omi in the cytoplasm by using the yeast two-hybrid system to identify novel Omi interactors/substrates. A HeLa (cervical carcinoma cell line) cDNA library was screened using Omi as a "bait" protein. One of the proteins identified in this screen as a strong Omi interactor was the S5a protein and was selected for further analysis.

S5a is a soluble cytosolic mammalian protein and a component of the proteasome's 19S regulatory subunit. The proteasome is a large cytosolic protein complex responsible for the controlled degradation of damaged or denatured cellular proteins. Further characterization of the interaction through an *in vitro* proteolytic assay demonstrated that Omi can cleave recombinant S5a protein. This data suggests that S5a is a *bona fide* substrate of Omi that is degraded upon induction of apoptosis. It also provides a new mechanism that leads to the inactivation of the proteasome during cell death.

For my parents, Jamie and Bill Ward, who instilled in me a love for learning and inquiry.

For Tiffany Smith, whose encouragement and support helped to initiate this process and who kept me on track until the very end.

## ACKNOWLEDGEMENTS

I express the sincerest gratitude to my thesis committee members, who have been an integral part of this project. I am especially grateful to my thesis chair, Dr. Antonis Zervos, for affording me the opportunity to work in his laboratory and providing the means to complete this thesis. Special thanks to Dr. Shadab Siddiqi and Dr. Swadeshmukul Santra for serving on my committee and providing specific assistance during the writing process. Additionally I would like to express my deepest appreciation for the experimental assistance provided by Dr. Lucia Cilenti and Camilla Ambivero. Their guidance was essential in my grasp and application of the molecular biology techniques performed for this project.

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## INTRODUCTION

Omi/HtrA2 is a mammalian mitochondrial serine protease that displays homology to the bacterial high temperature requirement A (HtrA) protein family (1). Omi was first identified through the use of the yeast-two hybrid system as a specific interactor of Mxi2, which is an alternatively spliced form of the stress activated kinase p38 (2). Upon induction of apoptosis, Omi translocates to the cytoplasm and activates the caspase-dependent apoptotic pathway. The pro-apoptotic activity of Omi is entirely dependent on its proteolytic activity (3).

Omi promotes the caspase-dependent apoptotic pathway through targeted binding and cleavage of IAPs (Inhibitor of Apoptosis Proteins). This irreversible cleavage of IAP's results in the inability of those proteins to inhibit caspases (4). Besides IAP proteins, Omi has been shown to cleave various other substrates including Hax-1, Ped/pea-15 (5,6). These published data suggests Omi cleaves multiple known and possibly several unknown substrate proteins.

Caspase activation has previously been shown to inhibit proteasome function during apoptosis (7). The 26S proteasome is a large cytosolic protein complex responsible for the controlled degradation of ubiquitinated-protein conjugates derived from both the nucleus and cytosol. The 26S Proteasome consists of two distinct sub-complexes, the 20S core particle (CP) and two 19S regulatory particles (RP) located on either side of the 20S core. Proteasome activity is essential in regulating cellular processes such as cell cycle progression, antigen processing and transcription. Another key role of the proteasome is the regulation of apoptosis through degradation of pro-apoptotic signals. Caspase activation results in cleavage of 3 subunits of the 19S regulatory sub-complex: S1, S2 and S5a. The cleavage of these subunits inhibits



proteasome degradation of ubiquitinated substrates including pro-apoptotic proteins that are then able to upregulate the apoptotic pathways (7,8).

S5a is a cytosolic protein that appears in a free form as well as a non-ATPase component of the 26S proteasome's 19S regulatory sub-complex. S5a is composed of two distinct functional domains: a von Willebrand A (vWA) domain and two separate Ubiquitin-interacting motifs (UIMs). These UIMs facilitate an interaction between S5a and polyubiquitinated proteasome substrates. S5a serves as a docking site for the proteasome to which the shuttling proteins, Rad23 and Dsk2, transport specific ubiquitin-protein conjugates. This docking interaction between S5a and ubiquitinated proteins facilitates the unfolding of these proteins by other 19S RP subunits, and prepares them for degradation by the 20S CP (9).

Studies have shown that S5a is regulated at the transcriptional level during apoptosis by the Src family of kinases; this kinase activity has been implicated in the activation of transcription factors that upregulate expression of anti-apoptotic genes. The upregulation of S5a contributes to cell rescue and survival under conditions that promote cell death (10).

## MATERIALS AND METHODS

### Polymerase Chain Reaction (PCR)

For the amplification of a target DNA sequence, a PCR reaction was performed using forward and reverse primers that flank the mature Omi cDNA sequence, coding for amino acids 134-458, and contain specific restriction sites for cloning purposes. 20 ng of template DNA, 10 nM in 1  $\mu$ l of reverse and forward primers respectively, 5  $\mu$ l of 10X PCR Buffer +  $Mg^{2+}$ , 1.5 units in 1  $\mu$ l of TAQ polymerase, 10  $\mu$ M of dNTPs were brought to a final volume of 50  $\mu$ l with sterile water. This reaction mixture was made in duplicate and these were placed in a thermal cycler to facilitate PCR reaction. The program used included: denaturation for two minutes at 95°C and then 30 cycles of the following: denatured at 95°C for 30 seconds, annealed between 55-60°C (dependent on primers) for 30 seconds and elongated at 72°C for 90 seconds. The last step lasted for 7 minutes. The duplicate mixtures were combined and precipitated to isolate PCR product.

### DNA Precipitation

To precipitate DNA 3M Sodium Acetate, pH 5.5 (1/10 volume of the DNA solution) and 2.5X volume of 100 % ethanol was used. The sample was mixed well by inversion and stored at -20°C for at least 20 minutes to allow DNA to precipitate. The sample was then centrifuged (13,000 RPM) at 4°C for 20 minutes; the supernatant was discarded upon completion of centrifugation. 300µl of 70 % ethanol was added to wash any residual salt from the pellet, and the solution was centrifuged (13,000 RPM) for 5 minutes at 4°C. The supernatant was discarded and the pellet was dried for 5 minutes in a speed vacuum. The DNA was resuspended in 50µl of dH<sub>2</sub>O or TE.

### Restriction Enzyme Digestion

Restriction enzyme digestion was performed to prepare DNA for ligation into the vector. Double-stranded DNA was incubated at 37°C for a period of 1-4 hours depending on the restriction enzyme used. A reaction volume of 50µl was generated containing 5 µl of 10X Buffer (specific for the enzyme used), 0.5 µl BSA (10µg/µl), 1 µg of DNA, 1.5 µl of restriction enzyme (10 units/µl) and 42 µl of sterile water. If two sequential digestions were required, the DNA was precipitated after the first digestion and then a separate reaction mixture was generated specific for the second restriction enzyme.

## Gel Electrophoresis

Agarose gel electrophoresis was used to monitor the enzyme digestion of DNA, and to determine the yield and purity of DNA Minipreps, or PCR reaction, or to size fractionate DNA molecules for isolation. To prepare an agarose gel, 1.0 g of dried agarose (American Bioanalytical) was added to 100 ml 1X TAE (0.04 M Tris-acetate, 0.001 M EDTA). This solution was microwaved for 90 seconds until the agarose was completely dissolved. After the solution cooled, 5  $\mu$ l of ethidium bromide was added to generate a final concentration of 0.5  $\mu$ g/ml and this solution was poured into a gel tray with well combs to solidify. After the gel had solidified, the combs were gently removed and 1X TAE electrophoresis buffer was added to the tray agarose gel was covered. To prepare the samples for electrophoresis, 5  $\mu$ l of 10X gel-loading buffer (30% glycerol (Fisher), 2% Orange G - (Sigma) in 1X TAE) was added to 3-5  $\mu$ l of DNA and this was loaded into a well. DNA markers (Lambda DNA BstEII Digest) were used to determine the size and concentration of the DNA samples. Electrophoresis was performed at 50-150V until the dye migrated an appropriate distance through the agarose gel.

### DNA Ligation

A DNA insert was ligated into a specific vector following restriction enzyme digestion of both the insert and vector DNA. A ligation reaction mixture of 15  $\mu$ l was generated with 1.5  $\mu$ l of Ligation Buffer with ATP, 1  $\mu$ l DNA ligase, x  $\mu$ l of the vector and insert. The amount of DNA used was dependent on the concentration of DNA retrieved after digestion and was determined by analysis of the DNA on an electrophoresis gel. In general, a 1:3 ratio of vector to insert DNA was used and sterile water was added to bring the final volume to 15  $\mu$ l. This reaction mixture was incubated at room temperature for 5 minutes and then transferred to a 70°C water bath for 15 minutes to inactivate DNA ligase. After this step, 35  $\mu$ l of sterile water was added to bring the volume up to 50  $\mu$ l and then 500  $\mu$ l of N-butanol was added to precipitate DNA. This mixture was mixed and centrifuged at 4°C (13,000 RPM) for 10 minutes. Following centrifugation the supernatant was discarded and the sample was dried in a speedvac for 10 minutes, the dried sample was then resuspended in 4-6  $\mu$ l of sterile water.

### Bacterial Transformation

Electroporation was used to transform bacteria with plasmid DNA. An electric pulse increases the permeability of the bacterial cell walls and allows for the uptake of plasmid DNA. An electroporation cuvette (0.2 cm electrode gap) was cooled to 4°C and 4-6 µl of the DNA ligation mix (10 ng) was transferred into 100 µl of competent bacterial cell suspension. This solution was mixed gently, incubated on ice for about 1 minute and then transferred into the chilled electroporation cuvette. The cuvette was placed in the Gene Pulser (BioRad) to deliver a single exponential decay pulse of 2.5 kV and then 600 µl of room temperature LB medium was added immediately. The entire mixture was transferred to a sterile 2-ml microcentrifuge tube and incubated at 37°C for 1h. Different volumes (150µl and 250µl) of the mixture were plated on LB-agar plates, containing the antibiotic that was also the selectable marker in the vector used, and were incubated overnight at 37°C. Single bacterial colonies were then picked and used to inoculate 1.5 ml of 50 µl/ml antibiotic (Amp) LB media for DNA Miniprep isolation.

### Quick Isolation of Plasmid DNA from Bacteria (Miniprep)

Individual colonies were picked and used to inoculate 2 ml of LB media + Amp. After an overnight incubation at 37°C, the suspensions were transferred to microcentrifuge tubes, and centrifuged (13,000 RPM) for 2 minutes. The supernatant was then aspirated and the pellet re-suspended in 300 µl of a STET/Lysozyme solution (8% Sucrose, 5% of 100X Triton, 50 mM of Tris-HCl, pH 8 and 50 mM of EDTA, 10mg/ml Lysozyme). Samples were placed in a boiling water bath for 1 minute and centrifuged (13,000 RPM) for 10 minutes. Following centrifugation, the pellet containing cellular debris was removed with a toothpick and 200 µl of Isopropanol was added. The samples were mixed thoroughly and centrifuged (13,000 RPM) for 10 minutes. The supernatant was then discarded and 300 µl of 70% Ethanol added to wash the pellet during a 5-minute centrifugation (13,000 RPM). The supernatant was discarded and the DNA pellet dried in a Savant speedvac for 10 minutes. The DNA was re-suspended in 50 µl of TE.

### Clean DNA QIAprep Isolation of DNA from Bacteria

This method of Plasmid isolation uses the QIAprep Spin Miniprep Kit according to the instructions provided by the manufacturer QIA. Individual colonies were chosen to inoculate 2 ml of LB media + Amp and were incubated overnight at 37°C. These overnight suspensions were mixed and transferred to microcentrifuge tubes, which were then centrifuged (11,000 RPM) for 2 minutes. The supernatant was aspirated and the pellet was resuspended in 200 µl of Resuspension Buffer P1 containing RNase A (100 µg/ml). 200 µl of Lysis Buffer P2 was then added and the tubes were then gently inverted 5-6 times to mix and ensure cell lysis. The lysate was neutralized and adjusted to high-salt binding conditions through the addition 300 µl of Neutralization Buffer N3. The sample was immediately mixed for three minutes and then centrifuged (13,000 RPM) for 10 minutes. The supernatant was transferred to a QIAprep spin column using a pipette. The column was centrifuged for 1 minute at 13,000 RPM and the flow-through was discarded. The column was washed with 0.50 ml of PB Buffer and centrifuged for 1 minute at 13,000 RPM, and the flow-through was discarded. The column was then washed with 0.75 ml of PE Buffer and centrifuged for 1 minute at 13,000 RPM. The flow-through was then discarded and the column washed for an additional 1 minute. The Column was transferred to a 1.5 ml microcentrifuge tube and 50 µl of EB buffer (10 mM TrisCl, pH 8.5) was added and the column was centrifuged for 1 minute at 13,000 RPM.



### Yeast-Two Hybrid Screening

The EGY48 yeast strain was used as the host to screen for two-hybrid interactions. This strain had been transformed with a pSH18-34 plasmid containing an upstream LexA operator, and a LacZ reporter gene that directs  $\beta$ -galactosidase synthesis. The cDNA of specific proteins that had been shown to interact previously with Omi were cloned into a pJG4-5 shuttle vector that contains a galactose inducible promoter; these served as the prey constructs. Mature Omi<sub>134-458</sub> and mature HtrA1<sub>156-480</sub> were used as bait constructs and had previously been cloned into a pGILDA vector, which also contains a galactose inducible promoter. This recombinant yeast was grown on Ura-His-Trp- Galactose/Rafinose selective media containing X-Gal; positive bait-prey interactions were identified by the presence of blue colonies. The prey protein expressed by the colony with the strongest detectable interaction was chosen for further study to characterize its interaction with Omi.

### Protein Expression

Target DNA sequences encoded for His-Omi<sub>134-458</sub> or His-S5<sub>aFL</sub> were amplified using PCR and ligated into a pET vector respectively for use in expressing His-tagged recombinant protein. This plasmid DNA was used to transform BL21(DE3) bacterial cells. These cells were grown overnight on LB-Agar+Kanamycin plates. Individual colonies were selected and used to inoculate 20 ml of LB+Kanamycin media and incubated at 37°C overnight; Kanamycin was present at a concentration of 40 µg/ml. Half (10 ml) of the culture media was then transferred to 500ml of fresh LB+Kanamycin and grown for 4 hours at 37°C until the optical density at 600nm (OD<sub>600</sub>) reached a value between 0.8-1.2. The bacteria were induced to express the target protein through addition of 500 µl of 1 M IPTG (to a final concentration of 2 mM). The bacteria were induced for 4 hours at 25°C in a shaker to inhibit growth and promote protein expression. Bacterial cells were then collected into 250 ml centrifuge tubes and centrifuged at 7000 RPM for 10 minutes (Sorvall rotor) at 4°C. The supernatant was then discarded and the cell pellet was frozen overnight at -80°C.

### Protein Isolation

Lysis buffer was prepared: 8X Binding buffer (2M NaCl, 160 mM 8X Tris pH 8, 40 mM Imidazole, 0.4% sodium azide) and was diluted to 1X in 50 ml, bacterial protease inhibitors (SIGMA cat. #P2714) were added in a 1:100 concentration, finally 100 µl of lysozyme was also included. The frozen pellet was resuspended in 10 ml of lysis buffer; Triton was added to 1% final concentration. The lysate was incubated at 30°C for 20 minutes and vortexed every 5 minutes to release genomic DNA. The lysate was then sonicated while on ice to shear the DNA and decrease viscosity. The sonicated lysate was then transferred to a 25 ml centrifuge tube and centrifuged at 15,000 RPM (Sorvall rotor) for 40 minutes at 4°C. The supernatant was collected and added to a 50 ml falcon tube containing 1/10 the volume of Ni-NTA agarose resin. The falcon tube was rotated for 4 hours at 4°C. The protein-resin mixture was then added to a flow-through column for purification of the His-tagged protein of interest. The column was washed with 20X column volume of 1X Binding buffer, then 20X column volume of 1X Washing buffer (2 M NaCl, 160 mM Tris-HCl pH 8, 480 mM 8X Imidazole, 0.4% sodium azide). After all of the Washing buffer had passed through the column 10X column volume of 1X Elution buffer (1 M NaCl, 2 M 4X Imidazole, 80 mM Tris-HCl pH8, 0.2% sodium azide) was added and 3X 1.5 ml elution fractions were collected. Each fraction was transferred into dialysis tubing and the tubes dialyzed overnight at 4°C in a 2 L beaker containing Dialysis buffer (20 mM Tris-HCl pH8, 200 mM NaCl). An SDS-PAGE gel was used to monitor the purity of each fraction; Coomassie blue staining was used for protein visualization.

### In vitro Protein Cleavage

Aliquots of purified His-S5a and His-Omi<sub>134-458</sub> or His-Omi<sub>S/A</sub> were incubated together to monitor ant potential cleavage of S5a by Omi<sub>134-458</sub>. 3  $\mu$ l aliquots of purified His-S5a and 9  $\mu$ l of Omi assay buffer were transferred to microcentrifuge tubes and 3  $\mu$ l of purified His-Omi<sub>134-458</sub> or His-Omi<sub>S/A</sub> was added. The reactions were incubated at 37°C for either 1, 2 or 3 hours and analyzed by SDS-PAGE followed by Coomassie blue staining to visualize the proteins.

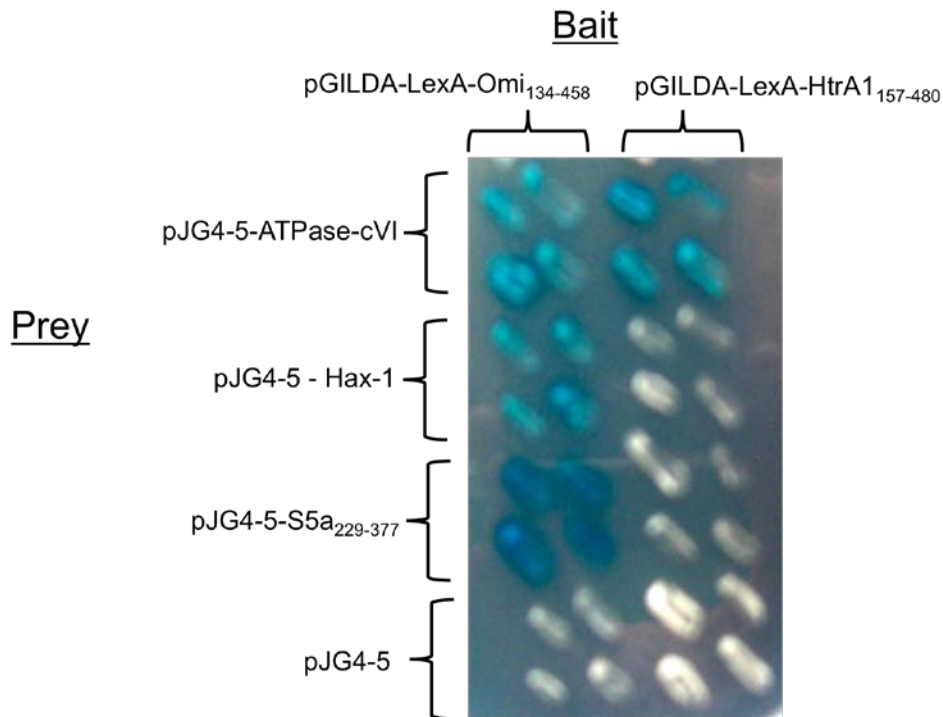
## RESULTS

### Yeast-Two Hybrid Assay to Test Selectivity of Interactions

The yeast-two hybrid system was used to identify novel interactors for the “bait” protein Omi that could also serve as its substrate. This system is based on the principle of structural exclusivity of transcription factor domains; transcription factors have distinct DNA binding and functional domains that can function in the absence of the other. The vector constructs used for the bait and prey proteins are designed to generate fusion molecules, each containing one of the separate transcription factor domains. The bait proteins fused to the DNA binding domain and the prey proteins fused to the functional domain of the transcription factor. Upon co-transformation of these constructs in yeast, an interaction between the bait and prey proteins will generate a complex containing the separated transcription factor domains thus eliciting its activity and the transcription of a LacZ reporter gene.

The related proteins, HtrA1 and Omi, were used as the “bait” and the C-terminus of S5a (S5a<sub>229-377</sub>), HAX-1, and ATPase cVI served as separate “prey” proteins for this screen. Co-transformed yeast cells were cultured and plated on U<sup>+</sup>H<sup>+</sup>W<sup>-</sup> + Glucose media. Individual colonies for each bait-prey pair were selected for plating on U<sup>+</sup>H<sup>+</sup>W<sup>-</sup> + Galactose/Raffinose/X-Gal media. Positive interactions were identified through blue/white screening. Presence of blue colonies indicates activation of the LacZ gene that codes for  $\beta$ -galactosidase, which cleaves the X-Gal present in the media into a product that upon dimerization and oxidation forms a blue pigment. All three bait proteins interacted with pGILDA-LexA-Omi, however pjG4-5-S5a<sub>229-377</sub> displayed the strongest interaction, which was determined by the intensity of the colony’s blue

color. Additionally, pJG4-5-ATPase cVI displayed an interaction with pGILDA-LexA-HtrA1, which was not seen with the other prey proteins screened. This suggested a lack of specificity for the interaction with Omi and thus the protein was not chosen for further characterization.



**Figure 1: Screen for Omi Interactors in Yeast**

Yeast-two hybrid screen of pGILDA-LexA-Omi<sub>134-458</sub> and pGILDA-LexA-HtrA1<sub>157-480</sub> bait proteins with pJG4-5-S5a<sub>229-377</sub>, pJG4-5-Hax-1, and pJG4-5-ATPase-cVI prey proteins. pJG4-5-S5a<sub>229-377</sub>, pJG4-5-Hax-1 and pJG4-5-S5a<sub>229-377</sub> are shown to interact with LexA-Omi<sub>134-458</sub>, pJG4-5-S5a<sub>229-377</sub> displayed the strongest interaction; pJG4-5-ATPase-cVI is only prey protein to show an interaction with pGILDA-LexA-HtrA1<sub>157-480</sub> in this screen.

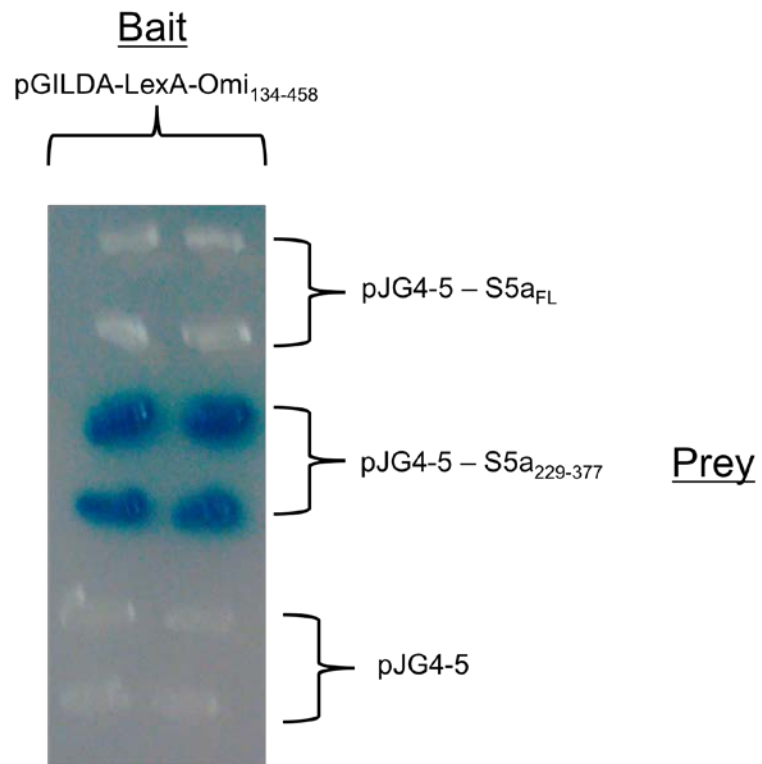
### Generation of Full Length Recombinant S5a Protein

Sequence analysis of the S5a clone isolated from the HeLa cDNA library revealed that it only encoded the c-terminal portion of the S5a protein. This part included amino acids 229-377 of S5a, and will be further referred to as S5a<sub>229-377</sub>.

Full length S5a cDNA was generated by PCR using primers specific to the S5a nucleotide sequence that also contained restrictions sites that permitted cloning into the pJG4-5 vector. Upon cloning, this full-length construct was used in a secondary yeast-two hybrid screen to determine if the full-length pJG4-5-S5a would interact with pGILDA-Omi<sub>134-458</sub>.

### Secondary Yeast-Two Hybrid Interaction

EGY48 yeast was co-transformed with a pGILDA-LexA-Omi<sub>134-458</sub> “bait” construct and a full-length pJG4-5-S5a “prey” construct. The recombinant yeast were cultured and plated on U<sup>-</sup> H<sup>+</sup> W<sup>-</sup> + Glucose media. Individual colonies were selected to inoculate U<sup>-</sup> H<sup>+</sup> W<sup>-</sup> + Galactose/XGal media. Four individual clones of full-length S5a cDNA were used in the generation of “prey” constructs for yeast transformation. The c-terminal pJG4-5-S5a<sub>229-377</sub> was included as a positive control and was the only clone species that displayed an interaction with pGILDA-LexA-Omi<sub>134-458</sub> in this screen.



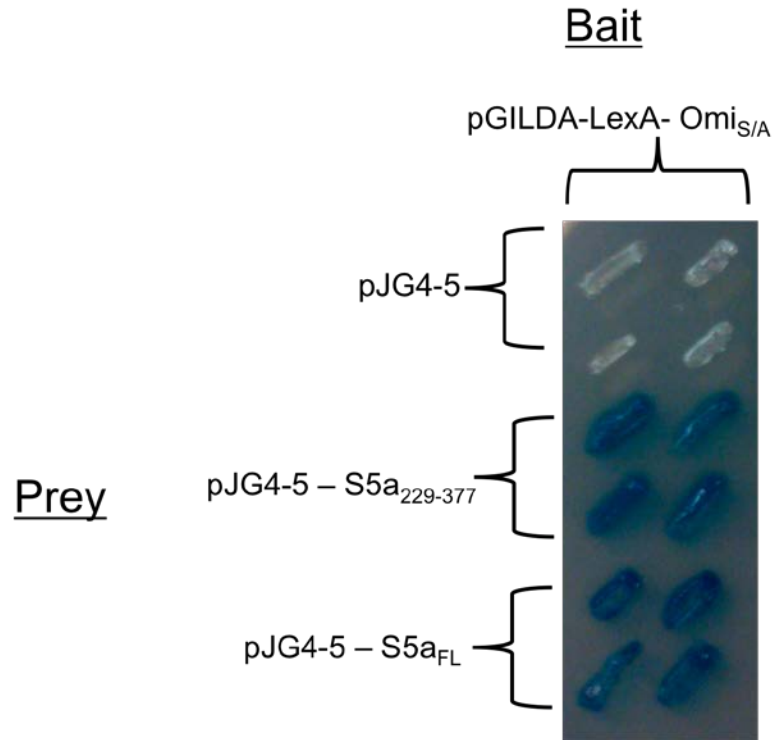
**Figure 2: Interaction of Omi<sub>134-458</sub> and S5a in Yeast**

Yeast two-hybrid test for the interaction between LexA-Omi<sub>134-458</sub> bait protein and full-length S5a prey protein. Interaction between LexA-Omi<sub>134-458</sub> and full-length S5a was not detected in the screen, C-terminal S5a<sub>229-377</sub> continues to display interaction with LexA-Omi<sub>134-458</sub>.



### Yeast Two-Hybrid Interaction with Inactive Omi Protease

Omi<sub>S/A</sub> cDNA, representing an inactive form of Omi that lacks serine protease activity, was cloned in to a pGILDA vector to generate a “bait” construct for use in yeast-two hybrid screening. Full-length S5a cDNA was used in the generation of a “prey” construct for yeast transformation. The recombinant yeast were cultured and plated on U<sup>-</sup>H<sup>-</sup>W<sup>-</sup> + Glucose media. Individual colonies were selected to inoculate U<sup>-</sup>H<sup>-</sup>W<sup>-</sup> + Galactose/XGal media. The full-length S5a showed an interaction with Omi<sub>S/A</sub>. Additionally, the C-terminal S5a<sub>229-377</sub> protein also displayed an interaction with the inactivated Omi<sub>S/A</sub>.



**Figure 3: Interaction of Omi<sub>S2A</sub> and S5a in Yeast**

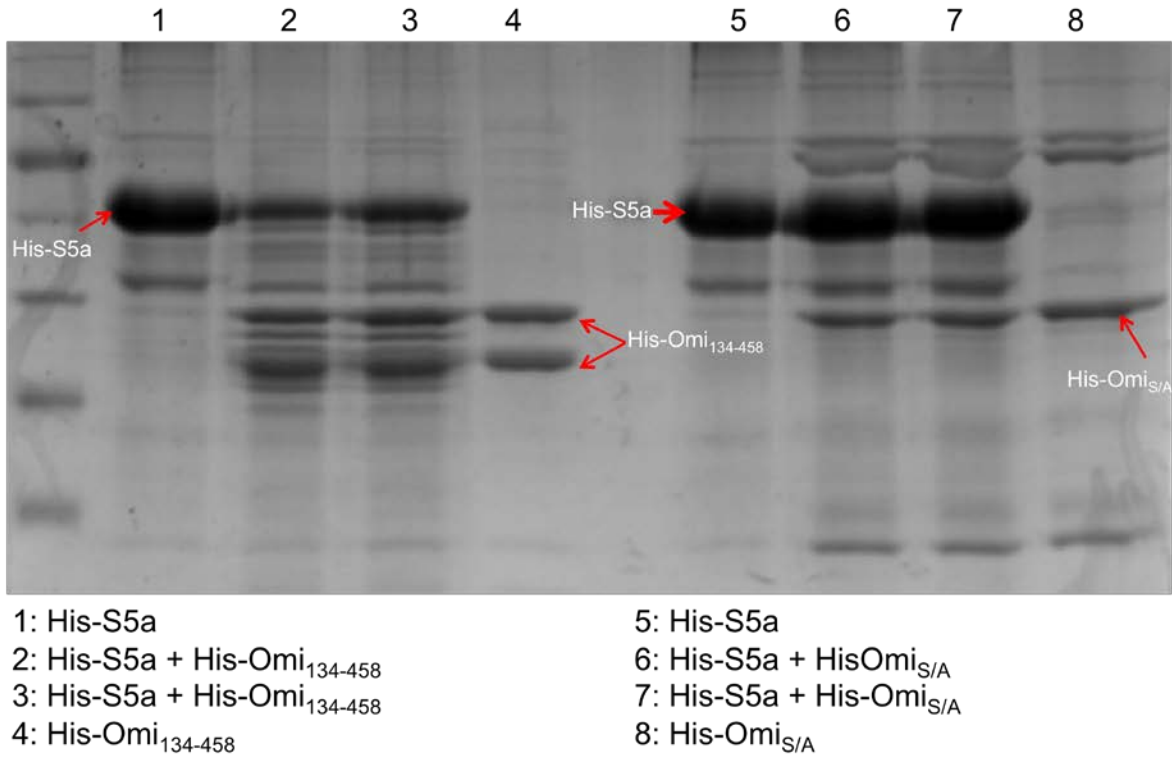
Yeast two-hybrid system test of inactive LexA-Omi<sub>S/A</sub> bait protein and full-length S5a prey protein. Interaction between LexA-Omi<sub>S/A</sub> and full-length S5a<sub>FL</sub> was detected; C-terminal S5a<sub>229-377</sub> also displays strong interaction with LexA-Omi<sub>S2A</sub>.

### In vitro Proteolytic Assay of S5a and Omi

Purified His-S5a and His-Omi<sub>134-458</sub> were incubated together at 37°C to determine if mature His-Omi<sub>134-458</sub> cleaves His-S5a in vitro. Samples of denatured and native His-S5a were incubated with active His-Omi<sub>134-458</sub> respectively and then were subject to SDS-PAGE. Samples containing solely His-S5a or His-Omi<sub>134-458</sub> were used as controls. The His-S5a control sample displayed no cleavage, where as both the denatured and native His-S5a incubations with active His-Omi<sub>134-458</sub> showed reduction of full-length His-S5a and evidence of degradative products. The denatured His-S5a and His-Omi<sub>134-458</sub> incubation displayed greater degradation than did the native His-S5a and His-Omi<sub>134-458</sub> incubation, however substantial degradation of native His-S5a was still present.

### In vitro Proteolytic Assay of S5a and Omi<sub>S/A</sub>

Another assay was also performed using His-S5a and inactive His-Omi<sub>S/A</sub> to show that active Omi<sub>134-458</sub> is responsible for the observed in vitro cleavage. Purified denatured or native His-S5a were incubated with His-Omi<sub>S/A</sub> respectively at 37°C to identify His-Omi<sub>S/A</sub> cleavage of S5a and then analyzed by SDS-PAGE and Coomassie Blue staining. The control samples containing His-S5a and His-Omi<sub>S/A</sub> alone displayed no auto-cleavage. Incubations of both denatured and native His-S5a displayed no His-Omi<sub>S/A</sub> cleavage of S5a.



**Figure 4: Differential Proteolytic Activity of Active and Inactive Omi on Recombinant S5a Protein**

Proteolytic assays conducted to demonstrate that an active serine protease, His-Omi<sub>134-458</sub>, is responsible for the observed in vitro cleavage of a His-S5a substrate. Upon incubation with His-S5a, His-Omi<sub>134-458</sub> displayed an ability to degrade the substrate in both its native and denatured form. Evidence of degradation was absent from the incubations containing the inactive form of the serine protease, His-Omi<sub>S/A</sub>, thus suggesting that active His-Omi<sub>134-458</sub> is solely responsible for the observed degradation of the His-S5a substrate in this assay.

## DISCUSSION

Omi is a mitochondrial mammalian serine protease that is implicated in both caspase-dependent and caspase-independent apoptosis. Omi selectively binds and cleaves IAP, eliciting caspase action and the activation of the caspase-dependent apoptotic pathway. Omi's complete mechanism of action in the caspase-independent pathway is still unknown, however studies have shown that it cleaves the cytosolic anti-apoptotic protein Ped/pea-15 upon induction of the pathway (5,6).

To further elucidate the function of Omi in the caspase-independent apoptotic pathway a yeast-two hybrid screen was performed to identify interactors of Omi that also serves as potential substrates. Figure 1 reveals the results of this screen, in which the prey protein *S5a*<sub>229-377</sub> displayed the strongest interaction with Omi. The intensity of blue color associated with the *S5a*<sub>229-377</sub> colonies prompted its selection for further study in characterizing an interaction with Omi. The lack of an observed interaction with a second protein bait, LexA-HtrA1, suggests that the interaction observed with LexA-Omi<sub>134-458</sub> is specific given the high homology observed between the catalytic domains of HtrA1 and Omi.

A sequence analysis of the *S5a*<sub>229-377</sub> cDNA used to generate the "prey" construct revealed that only the C-terminal amino acids (229-377) were coded for by this partial cDNA. The interaction between this C-terminal *S5a* fragment and Omi does not provide sufficient support for an interaction between full-length *S5a* and Omi. However it does suggest that a domain localized on the C-terminus of *S5a* would facilitate such an interaction with Omi.

Full length S5a cDNA was generated by PCR and then cloned into pJG4-5 to create a “prey” construct for a second yeast-two hybrid test with Omi. The results of this test are presented in Figure 2 and display a lack of interaction between the full-length S5a and LexA-Omi<sub>134-458</sub>. It was hypothesized that if mature full-length S5a was a substrate of Omi then their interaction would result in the cleavage of S5a, thus preventing the formation of the bait-prey complex and the transcription of the LacZ reporter gene. The interaction between C-terminal S5a<sub>229-377</sub> with LexA-Omi<sub>134-458</sub> further suggests that if S5a is a substrate of Omi, its cleavage site is located upstream of amino acid 229 or that the C-terminal fragment does not assume a conformation that permits cleavage.

A “bait” construct was generated using the cDNA of Omi<sub>S/A</sub>, an inactive form of the protein with a missense mutation that changes the 306 serine residue to an alanine, which abolishes its protease activity. A yeast-two hybrid test was performed with this “bait” and full-length S5a as the “prey”. The results of this screen are presented in Figure 3 and display an interaction between S5a and LexA-Omi<sub>S/A</sub>. These results support the hypothesis that active Omi cleaves S5a preventing detection of an interaction during the yeast-two hybrid screen presented in Figure 2. S5a<sub>229-377</sub> also displayed an interaction with the inactive LexA-Omi<sub>S/A</sub>, suggesting that the mutation of the Omi sequence did not disrupt the binding interaction of the two proteins, solely Omi’s ability to cleave the S5a substrate.

To further characterize the biological significance of an Omi and S5a interaction, recombinant proteins were used in an in vitro proteolytic assay. Both native and denatured forms of His-S5a were used to better understand the physiological implications of this protein-protein interaction. There was significant degradation of both forms of His-S5a upon incubation with

active His-Omi<sub>134-458</sub> at physiological temperatures, suggesting that cleavage of S5a by Omi is possible under in vivo conditions.

To discount for the possibility of the observed degradation might be the result of sample contamination, the inactive form of the mitochondrial serine protease, His-Omi<sub>S/A</sub>, was also used for a second proteolytic assay. No degradation of either form of His-S5a was observed in the presence of His-Omi<sub>S/A</sub>. Thus the observed degradation of His-S5a is solely attributed to the serine protease activity of His-Omi<sub>134-458</sub>, given that the purification steps and reaction conditions for both proteolytic assays were identical. This further supports the initial yeast-two hybrid test that suggests a protein-protein interaction between LexA-Omi<sub>134-458</sub> and S5a<sub>FL</sub>.

The data generated from this study support the notion that S5a is a protein substrate for the mitochondrial serine protease, Omi. The cytosolic localization of S5a in both its free form and as a proteasome component suggests that this protein-protein interaction requires the release of Omi from the mitochondria. This translocation occurs during induction of apoptosis, which suggests that Omi plays a role in the inactivation or degradation of the proteasome during apoptosis. Further studies are required to characterize the complete mechanism of Omi's action on S5a and to further elucidate the role of Omi in the caspase independent apoptotic pathway.

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