Spring 5-1999

Site-directed Mutagenesis in the PAI-1 Binding Region of Vitronectin

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UNIVERSITY HONORS PROGRAM

SENIOR PROJECT - APPROVAL

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PROJECT TITLE: Site-directed Mutagenesis in the PAI-1 Binding Region of Vitronectin

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I have reviewed this completed senior honors thesis with this student and certify that it is a project commensurate with honors level undergraduate research in this field.

Signed: Cynthia B. Peterson, Faculty Mentor

Date: 5-4-99

Comments (Optional):

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A Senior Thesis
by
Tiffany Thomas

INTRODUCTION

Vitronectin is a multifunctional adhesive glycoprotein present in mammalian plasma and the extracellular matrix of many tissues (1). This adhesive protein circulates in human plasma at concentrations of 200-400μg/ml, and serves as a regulatory protein in humoral defense mechanisms through interactions of macromolecules in cascades of coagulation and fibrinolysis (2). Some target macromolecules that interact with vitronectin include: heparin, PAI-1 (plasminogen activator inhibitor-1), proteases such as thrombin, serine protease inhibitor-protease complexes, and a sub-class of integrin receptors on the surface of cells (3). The anti-fibrinolytic protein, PAI-1, is a major inhibitor of tissue-type plasminogen activator (tPA), and urokinase-type plasminogen activator (uPA) (4-6). Like other serpins, PAI-1 has a reactive loop mimicking the substrate of its target proteases (7). The active conformation of PAI-1 is relatively unstable. Therefore, the protein undergoes a rapid conversion to a latent form, characterized by the insertion of the reactive loop into a central β-sheet within the molecule (8). Binding to vitronectin stabilizes the active conformation of PAI-1, resulting in a 2 to 3 fold increase in its half-life (9,10). This stabilization of PAI-1 is thought to arise from the restriction of β-sheet movement and thus the prevention of the insertion of the reactive loop as a result of binding to vitronectin (11). Recent work has localized the PAI-1 binding site to the N-terminal Somatomedin B region of vitronectin, consisting of the first 44 amino acid residues of the protein (12). Deng et al. (13) generated chimeras between fragments of the vitronectin Somatomedin B domain and other complementary sequences in inactive Somatomedin B homology domains. This study indicates that the essential elements for PAI-1 binding are located between residues

12 and 30 of vitronectin. In addition, alanine mutagenesis revealed that eight cysteines, Gly-12, Asp-22, Leu-24, Tyr-27, Tyr-28, and Asp-34 are vital to PAI-1 binding activity (13).

In order to gain further insight into the PAI-1 binding site of vitronectin, site-directed mutagenesis will be used to identify residues involved in particular macromolecular interactions. While most of the recombinant work has relied on fragments expressed in E. *coli*, an insect cell line has been developed in Dr. Cynthia Peterson's lab to express full-length recombinant vitronectin. Using the Bac-to-Bac Baculovirus Expression System, two full-length mutant forms of vitronectin, as well as the "wild-type" version have been expressed. Alanine mutagenesis of Tyr-27, and Tyr-28, are the two mutants manipulated during this study. Upon expression of the mutant proteins, competitive Elisa assay will be used to evaluate the specific PAI-1 binding properties of "wild-type" vitronectin, the Y27A, and the Y28A mutant forms of vitronectin. Unfortunately, the project did not progress as far as desired. Therefore, the Elisa assay analysis of the mutant vitronectin will not be discussed here.

MATERIALS AND METHODS

Two mutants of the protein vitronectin were generated using PCR by Dr. Cynthia Peterson. Both mutations are amino acid substitutions, one at position 27, substituting an alanine for a tyrosine, and the other at position 28, again substituting an alanine for a tyrosine. The procedure for expressing these mutants is described below.
Transforming bacteria. The day before the transformation, an overnight culture of MV1190 cells was prepared. An autoclaved wooden stick was dipped into the MV1190 cells and then dipped into 2mL of Luria Broth (LB) in a flame sterilized, autoclaved 13x100-glass tube. The cells grew overnight, shaking at 37°C. The following day, 20mLs of LB was inoculated in an autoclaved klett flask with 200μL of the overnight culture. This was grown with shaking at 37°C for approximately 3 hours or until a klett reading between 40 and 60 was obtained. 10mLs of cells was placed into each of two 14mL Falcon Tubes. The cells were then spun down in a Beckman centrifuge at

4000rpm, 4°C for 10 minutes. After the cells were spun down, they were resuspended in 1mL TSB (solution preparation: 10mL LB, 1g PEG-8000, .5mL DMSO, .1mL MgCl2, .1 mL MgSO4, sterile filtered and stored in refrigerator). The cells were then placed on ice for 10 minutes. The cells are now competent to take up plasmid DNA. For both the Y27A and Y28A mutants, 1μL of DNA was added to 100μL of these competent cells, and mixed by pipetting up and down. The cells were then placed on ice for another 30 minutes. Afterwards, .4mL of TSBG (solution preparation: 5mL TSB, 72μL 25% glucose, sterile filtered and stored in refrigerator) was added to the cells and shaken at 37°C for 1 hour. For both the Y27A and Y28A mutants, 150μL of the transformed cells was spread on LB agar plates with ampicillin. The plates were allowed to sit upright for 5-10 minutes, and then inverted and placed in a 37°C room overnight. The next day overnight mini-preps of the colonies of Y27A and Y28A DNA was prepared. 6 mini-preps for each mutant, consisting of 2mL LB and an isolated bacteria colony (selected using a sterile stick), were placed in the 37°C room overnight. The following day the DNA for each mutant was isolated using the following procedure.

DNA isolation. 1.5mL of the cells from each of the overnight mini-preps was transferred to Eppendorf tubes. Then the cells were spun down for 5 minutes at 10,000 rpm. The supernatant was removed and the cells were spun down again for 1 minute to remove any residual supernatant. The DNA was then isolated using the QIA prep Spin Miniprep Kit(250). The protocol from pages 16 and 17 was followed from the procedural manual included with the kit in order to properly isolate the DNA for the Y27A and Y28A mutants. A restriction digest of the DNA was performed to verify that the DNA plasmid contained the gene of interest.

Restriction Digest. A 1% agarose gel was prepared using 1g of agarose and 100mL TBE (.5x). To digest the DNA, 15μL of water, 3μL of DNA, 2μL of appropriate buffer, 1μL of BamHI, and 1μL of HindIII, was added to an Eppendorf tube and placed in a 37°C room for 1 hour. The entire 20μL was loaded onto the gel, and run for approximately 1 hour. The presence of the gene of interest was verified using Ethidium Bromide staining. A picture of the gel was taken for record keeping purposes.

Transformation of DH10Bac cells with Y27A and Y28A plasmid DNA. The purpose of this procedure was to create the recombinant bacmid DNA for each mutant that was used to infect the insect cell lines. LB plates were prepared containing: 50μg/mL kanamycin, 7μg/mL gentamicin, 10μg/mL tetracycline, 300μg/mL Bluo-gal, and 40μg/mL IPTG. The procedure from page 8 of the instruction manual for the Bac-to-Bac Baculovirus Expression System from Life Technologies was followed in order to transform the DH10Bac cells with the Y27A and Y28A DNA. The cells were then plated out onto the previously mentioned LB plates. A blue/white screen was used to determine colonies containing the Y27A and Y28A bacmid DNA. The procedure manual says to incubate the plates for 24 hours, but an incubation time of 28 hours seemed to be optimal to distinguish truly white colonies from truly blue colonies.

Isolation of the recombinant bacmid DNA. Again, the procedure from pages 8 and 9 of the instruction manual for the Bac-to-Bac Baculovirus Expression System from Life Technologies was followed in order to isolate the Y27A and Y28A bacmid DNA. A restriction digest was then performed to verify the presence of the desired gene following the restriction digest procedure described above.

Transfection of the Sf9 insect cell line with the recombinant bacmid DNA. 3 60x15mm

petri dishes were seeded with 900μL of Sf9 cells at 9*10^5cells/mL, and 1100μL of ex-cell 420 without antibiotics. The cells were allowed to adhere for one hour. In the mean time, two solutions were prepared. Solution A contained 5μL of mini-prep bacmid DNA and 100μL ex-cell 420 for each transfection. Solution B contained 6μL of CellFECTIN reagent and 100μL of ex-cell 420 for each transfection. The solutions were combined and allowed to sit for 45 minutes. After the cells had adhered to the plates, they were washed with 2mL of ex-cell 420 medium. .8mL of ex-cell 420 was then added to each tube containing the mix of solution A and B. This entire mL was then overlaid onto the cells. The cells were then allowed to incubate for 5 hours at 27°C. After 5 hours, the transfection material was removed and 2ml of ex-cell 420 was added to each dish. The cells were then allowed to incubate at 27°C for 48 hours.

Harvesting Virus Stock. Each time the cells needed to be harvested, the volume of cells is transferred to Eppendorf tubes, since the virus stock is produced on 3mL scales, approximately 1.5mL is added to each of two Eppendorf tubes for each transfection. The cells are spun down at 5000 g for 5 minutes. The supernatant is then transferred to new Eppendorf tubes. Both cells and supernatant are stored in the freezer, protected from light.

Amplification of Virus Stock. For each mutant, more virus stock is generated by adding 25μL of previous supernatant virus stock to 3mL of Sf9 cells at 1*10^6cells/mL. The cells are incubated for three days and then amplified again. After each amplification the cells are checked for protein production.

Protein Production Verification. To verify protein production, a Western Blot procedure was used. Sf9 cells were diluted in SDS page dye, and then boiled for 5 minutes. 10μL of each sample was then loaded onto the SDS page, polyacrylamide gel. A positive control, and a molecular weight marker was also loaded onto the gel each time a Western Blot was done. A primary antibody to the vitronectin protein, followed by a secondary antibody to the primary antibody, and then developer solution produced an enzyme reaction that showed a blue band indicating the protein vitronectin. The molecular weight marker was used to verify the molecular weight of the protein to be 77kDa.

Infection of Hi5 insect cells. After a working quantity of virus stock was obtained following the repeated amplification and harvesting procedure for Sf9 cells, Hi5 cells were grown to a quantity of 1L. At 1L of 1*10^6cells/ml of Hi5 cells, 8.33mL of virus stock was added to infect the Hi5 cells. 8.33mL was used according to the concentration ratio of 25μL of stock at 3mL, and 8.33mL at 1L of cells. After three days, the Hi5 cells were harvested. This first infection of Hi5 cells was done with the Y28A bacmid DNA. However, a Western Blot revealed no presence of the protein vitronectin. The procedure for the infection of Hi5 cells was repeated for the Y27A mutant. Again, a Western Blot revealed the absence of the mutant protein vitronectin. In the shortage of time, the infection of Hi5 cells was reduced down to 20mL instead of 1L. 166.67μL of virus stock was added to the cells. This was done for both mutants. After the three day incubation period, the Western Blot procedure was used to verify the presence of mutant protein. Alas, there was no band to be seen. Unfortunately, the project did not get as far as desired, and so the actual testing of PAI-1 binding effects using a competitive ELISA assay was not done.

RESULTS

The transformation of bacteria using the Y27A and Y28A PCR generated DNA was successful. Overnight mini-preps of isolated colonies of bacteria were made in order to generate a large number of replicates of the mutant gene within the bacterial plasmid. This bacterial plasmid, containing either the Y27A gene or the Y28A gene, was isolated. This plasmid DNA was then tested by a restriction digest using restriction enzymes, BamHI and HindIII. Figure 1 represents the restriction digest results. The lower band indicates the mutant vitronectin gene. The upper band indicates the rest of the plasmid DNA. Lanes 1 through 6 are the Y27A plasmid, while lanes 7 through 13 are the Y28A plasmid. The digest verified the presence of the mutant gene for all of the samples, except for one sample of the Y28A gene in lane 10.

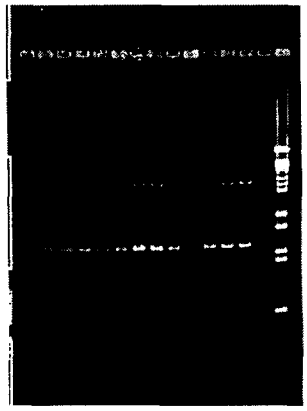

Figure 1

This plasmid DNA was then used to transform DH10Bac cells in order to generate the recombinant bacmid DNA used to infect the insect cell lines. 1μL of DNA was used to infect 100μL of competent DH10Bac cells. The cells were serially diluted in the following manner, 1:10, 1:100, and 1:1000. Undiluted cells, and all the serial dilutions were plated on the previously prepared Luria Agar plates. However, after 24 hours the undiluted plates showed little growth, while the serially diluted plates showed even less or no growth. The transformation for each mutant was redone using 3μL of Y27A plasmid DNA, and 5μL of Y28A plasmid DNA. In addition, the cells were spun down and resuspended in order to obtain a higher concentration of cells when plating. The results were an increased amount of growth for both mutants. 8 isolated colonies

were streaked onto new plates to verify that the colonies were really white colonies, and so contained the desired bacmid DNA. 8 colonies were selected, 4 from the Y27A plates and 4 from the Y28A plates. After incubation at 37°C for 24 hours, an overnight mini-prep of a large white isolated colony for each of the two mutants was prepared. The mini-preps seemed to contain a good concentration of cells, and so the bacmid DNA was isolated for each of the mutants from these preps. Again, a restriction digest, using enzymes BamHI and HindIII, was used to verify the presence of the mutant gene in the bacmid DNA. The presence of the gene was indeed detected by the restriction digest. However, the bands were very faint. A representation of this restriction digest is not shown because the faint bands could not be seen clearly when the picture of the restriction digest was scanned in.

The isolated bacmid DNA for each mutant was used to infect the insect cell lines. Initially, three infections of Sf9 cells was done, one of the Y27A DNA and two of the Y28A DNA. After three amplifications of these infections, a Western Blot was used to verify the presence of the mutant protein vitronectin. The bands were faint, although they seemed to become darker with the increasing amplifications, indicating the increasing concentration of the mutant protein. After the fourth amplification, a Western Blot showed a distinct band at 77kDa for the infections, the mutant vitronectin protein. Figure 2 represents the Western Blot after the fourth amplifications. Lane 1 is the Y28A mutant protein. Lane 2 is the Y27A mutant protein. Lanes 3 and 4 are deglycosylation mutants of vitronectin. Lanes 5 and 7 show leakage

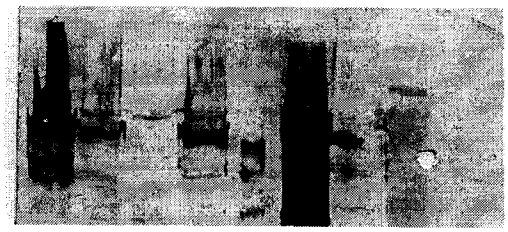

Figure 2

from lane 6 which is the vitronectin positive control. Finally, lane 8 is the molecular weight marker.

A test infection of Hi5 cells on a 3mL scale, using the Y28A DNA, was done to determine the concentration of virus stock needed to produce the desired amount of protein. Since Hi5 cells secrete protein much better than Sf9 cells. A Western Blot of the Hi5 supernatant was done, as opposed to the Western Blot of the actual cells. The amounts of virus stock used to infect the Hi5 cells were 10μL, 25μL, 50μL, and 100μL. Figure 3 represents the Western Blot of the Hi5 test infection. Lane 1 is the molecular weight marker. Lane 3 is the vitronectin positive control. Lane 5 is a deglycosylation mutant of vitronectin. Lane 7 is the 10μL infection of the Hi5 cells. Lane 8 is the 25μL infection. Lane 9 is the 50μL infection. Lane 10 is the 100μL infection. From the Western, 25μL seemed to be the optimal volume of virus stock to infect 3mL of cells. Scaling this ratio up, 8.33mL of virus stock was needed to infect 1L of Hi5 cells. After the infection of 1L of Hi5 cells with Y28A DNA, a Western revealed the absence of mutant vitronectin protein. Figure 4 represents the Western Blot of the absence of the mutant vitronectin protein. Lanes 1 and 2 were supposed to show the mutant protein. Lane 4 is the vitronectin positive control. Lane 5 is the molecular weight marker. The procedure was repeated for the Y27A mutant, and again after the infection

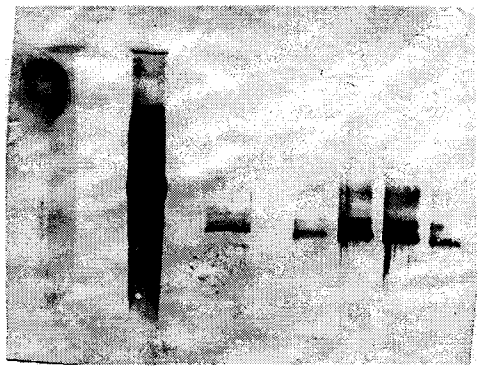

Figure 3

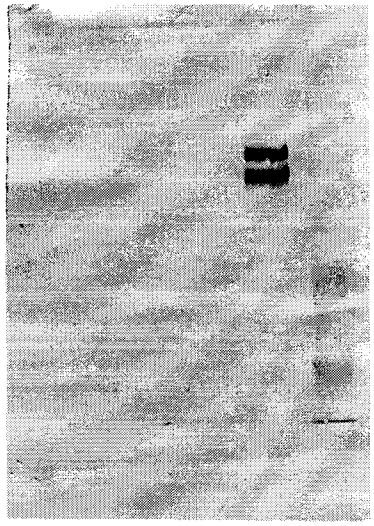

Figure 4

procedure of 1L of Hi5 cells, a Western revealed the absence of mutant vitronectin protein. In the interest of time, the infection of Hi5 cells was repeated on a 20mL scale. However, after infection, Western Blots showed no expression of the mutant vitronectin protein. Figures for these Western Blots are not represented because they all look the same as Figure 4.

DISCUSSION

The goal of this project was to further understanding of PAI-1 and vitronectin interactions using site-directed mutagenesis. Previous bacterial expression of the Somatomedin B domain of vitronectin revealed a number of essential amino acids required for binding including, Tyr-27, and Tyr-28 (13). Dr. Cynthia Peterson's lab has developed a way to express the full-length recombinant protein using a baculovirus expression system, as opposed to bacterially expressed fragments. Alanine mutants of Tyr-27, and Tyr-28, are the two mutants manipulated during this study. Unfortunately, large-scale expression of the two mutants was never achieved. Therefore, the Elisa competitive analysis of PAI-1 binding to vitronectin cannot be discussed in this paper.

There are a number of hypotheses as to why the mutant vitronectin expressed well on a 3mL scale, but would not express on a larger scale, 20mL or 1L. One reason why the mutant vitronectin would not express on a large scale is the failure of the protease inhibitors to protect the protein. After the 1L infected Hi5 cells were infected with the mutant bacmid DNA, the cells were spun down and the supernatant was stored in the refrigerator. A number of protease inhibitors was added to this supernatant including, PMSF, pepstatin, leupeptin, aprotinin, TPCK, and EDTA, in order to preserve the mutant

vitronectin protein. One of these protease inhibitors may have been defective. In other words, one of the protease inhibitors may not have inhibited its target protease, resulting in the degradation of the mutant vitronectin protein. In addition, the concentration of protease inhibitors may not have been large enough to prevent all the proteases from degrading the mutant protein. Another possibility is that there was a protease degrading the mutant vitronectin that is unknown. Therefore, no protease inhibitor was added to prevent the unknown protease from denaturing the mutant vitronectin.

In addition to the hypothesis that the protease inhibitors were defective in some way, other reasons could result in the lack of expression of the protein. The concentration of virus stock needed to express the protein on a 3mL scale, may not have been a large enough concentration of virus stock to express the protein on a 1L scale. A simple ratio was performed indicating 8.33mL of virus stock needed to express the protein on a 1L scale. In addition, this same ratio was used to determine that 166.67μL of virus stock was needed to infect 20mL of cells. These calculations could have been made in error, or the volume of virus stock was not large enough in order to infect the volume of Hi5 cells.

One other hypothesis as to why the mutant vitronectin would not express on a large scale is contamination. There is a possibility that each time a large-scale infection was performed the Hi5 cells were contaminated somehow. This contamination may have resulted in the lack of protein.

In conclusion, there are a number of reasons that could result in the lack of large-scale expression of the mutant vitronectin protein. The protease inhibitors may have been defective. The Hi5 cells could have been contaminated each time. The volume of virus

stock may not have been large enough to infect a large quantity of Hi5 cells. All of these factors may have inhibited the project, and prevented the expression of protein. As a result of the lack of expression of mutant vitronectin on a large scale, ELISA assays were not performed. Although the project did not progress as far as desired, a great deal of knowledge was gained regarding restriction digest procedures, Western Blot procedures, manipulating bacteria as well as insect cells, and protein expression. Great thanks goes to the Peterson lab for harboring me for a year and their patience in teaching me is greatly appreciated. I also want to thank the Threshold program for providing me with such a wonderful learning opportunity.

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