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14. ABSTRACT The principle investigator has made substantial progress in executing the statement of work outlined in the proposal. In addition he has fulfilled the requirements mandated by his training program to date. First, the PI obtained the human C5 cDNA and substituted a PSA substrate sequence in place of the wild type activation sequence. Modifications were implemented based on results from homology modeling. Next, PSA mediated cleavage was confirmed by mass spectrometry. Recombinant production of the confirmed PSA cleavable C5 mutant was scaled up by an adenovirus for in vitro assays. Purification of the recombinant protein is ongoing. Finally, the PI has begun working on a similar project that uses a different strategy to target prostate cancer cells in an androgen and growth-independent manner.						
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Introduction:

The principle investigator (PI) has made substantial progress in fulfilling the goals outlined in the statement of work. Several major unforeseen challenges have been surmounted. The PI has completed his thesis proposal and formed a five-member thesis committee that has since met twice. The PI assisted his mentor in the application for additional funding. Finally, the PI has begun working on a similar project that uses a different strategy to target prostate cancer cells in an androgen and growth-independent manner.

Body:

Specific Aim 1 – Obtain human C5 cDNA and substitute a PSA substrate sequence in place of the wild type activation sequence; amend a purification tag to the terminus of C5 to aid in its purification. [Completed]

The C5 cDNA was mutated via site-directed mutagenesis to include a PSA recognition sequence in place of the wild type activation sequence. The resulting construct was named PAC-1. PAC-1 was transfected into COS-7 cells and the supernatant containing the recombinant protein was collected. Because of the chance the purification tag could alter the conformation and thus hinder the PSA mediated cleavage, we decided to add the tag on after cleavage was observed (in Specific Aim 2).

Specific Aim 2 – Verify PSA has the capacity to cleave PAC5. Write thesis proposal and form “Thesis Advisory Committee”. [Completed]

The recombinant protein collected above was characterized for PSA mediated cleavage by western blot analysis. Briefly, purified human PSA (Calbiochem) was incubated at 10µg/mL in the conditioned media containing the recombinant protein. The protein solution was then analyzed for cleavage by western blot using a C5 antibody (Santa Cruz Biotechnology). Cleavage would be verified by a shift in molecular weight. Unfortunately, no shift was observed.

Following the guide of Ogata and Low[1], we made a series of mutants (Figure 1) incorporating more of the semenogelin II sequence, the known physiological substrate for PSA. In all, six more mutants were generated. Meanwhile, the mutants were examined *in silico* for subtle structural changes which may render them PSA sensitive. Briefly, homology modeling was performed using the SWISS-MODEL[2] homology modeling server via the automated mode using the published crystal structure of C5 as a template (PDB 3CU7)[3]. The homology models suggested adding six more residues of semenogelin II to the left of the PSA recognition sequence and seven more residues of semenogelin II to the right would result in a α -helix containing the “HSSKLQ” PSA substrate sequence being exposed to the solvent (Figure 2). This mutant was named PAC-2.

C5a — C5b (wild type)
C5a – HSSKLQ// – C5b
C5a – **VDVREEHSSKLQ**// – C5b
C5a – VDVREEHSSKLQ//**T** – C5b
C5a – VDVREEHSSKLQ//**TS** – C5b
C5a – VDVREEHSSKLQ//**TSLH** – C5b
C5a – VDVREEHSSKLQ//**TSLHP** – C5b
C5a – VDVREEHSSKLQ//**TSLHPAH** – C5b

Figure 1: Wild type C5 and a series of mutants incorporating the semenogelin II sequence. Briefly, the sequence connecting C5a and C5b was mutated to resemble the physiological substrate for PSA.

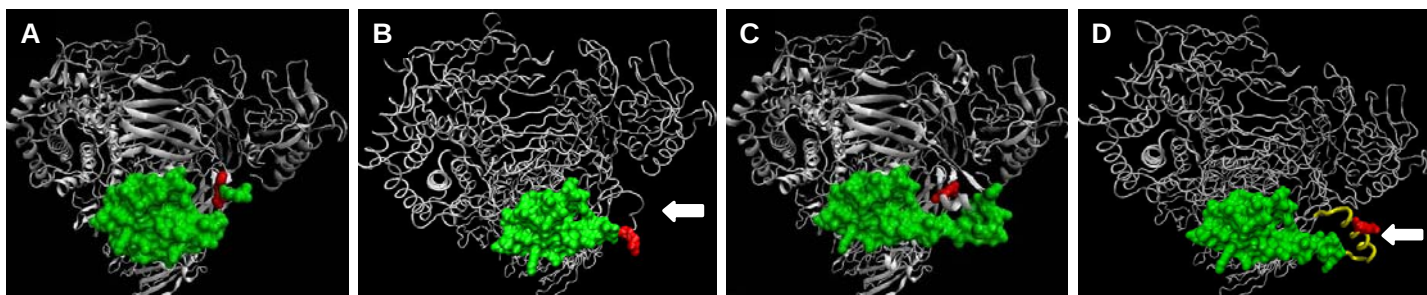


Figure 2: Models of (a) wild type C5; (b) Mut-5 protein described by Ogata et al; (c) PAC5-1 mutant with HSSKLQ at the cleavage site; (d) PAC5-2 mutant with additional flanking sequences to create a loop that may be accessible to PSA cleavage. Amino acid at cleavage site depicted in red, N-terminal fragment in green, PAC5-2 insert in yellow.

A plasmid containing PAC-2 was transfected into COS-7 and HEK293T cells and conditioned media containing the recombinant protein was collected. For unknown reasons expression levels of PAC-2 were substantially lower in both cell lines. The recombinant PAC-2 was analyzed for PSA mediated cleavage by western blot as described above. Unfortunately, no shift was observed, although signal was weak and irreproducible because of low protein levels. We suspected some cleavage might actually be occurring, but at subprime levels. This combined with the small amount of PAC-2 protein made analysis by western blot problematic.

As a more sensitive way to probe for PSA mediated PAC-2 cleavage, we analyzed PSA treated conditioned media by MALDI-TOF mass spectroscopy. In this experiment, wild type C5 was completely resistant to cleavage by PSA and no release of 11 kDa fragment was observed (Figure 3). In contrast, incubation of the PAC5-2 protein resulted in production of an ~11kDa fragment corresponding to the modified N-terminal portion of the PAC5-2 protein. Based on the MALDI results, high resolution LTQ nanoHPLC/Orbitrap mass spectroscopic analysis was performed to obtain high resolution mass. Sequencing of this mass fragment using the Sequest sequencing algorithm confirmed the correct sequence corresponding to the 80 amino acid N-terminal C5 cleavage product

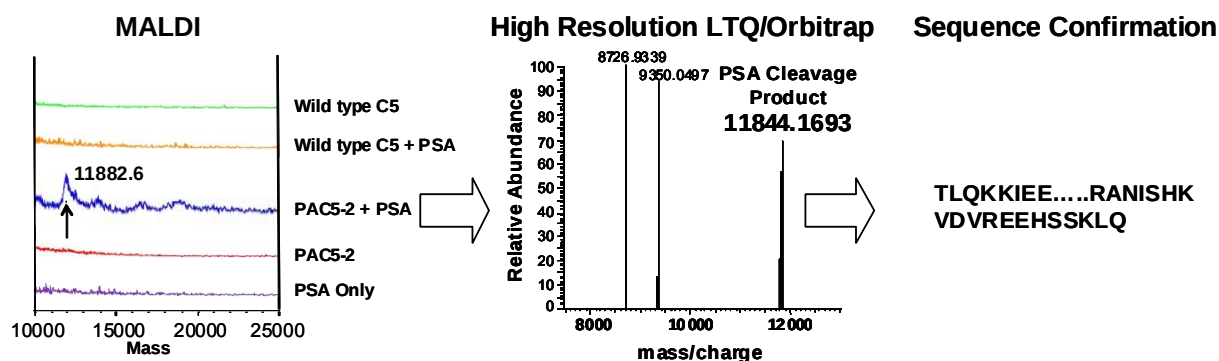


Figure 3: Comparison of PSA cleavage of wild type C5 vs. PAC5-2 mutant. Lower resolution MALDI-ToF demonstrates generation of 11kDa peak corresponding to N-terminal fragment released from C5. High resolution mass spectroscopy using LTQ/Orbitrap gives correct mass confirms the correct sequence.

Over this time period the PI wrote his thesis proposal and formed a thesis committee consisting of five faculty members at the Johns Hopkins School of Medicine:

Dr. R. Brodsky
Dr. S. Denmeade
Dr. S. Kachhap

Dr. S. Lupold

Dr. H. Wade

The committee has since met twice.

Specific Aim 3 – Demonstrate PAC5's selective toxicity to PSA producing prostate cancer cells but not PSA-null prostate cancer cells in presence of purified complement components C6-C9. [In progress]

To demonstrate selective toxicity in a tissue culture model we first needed to scale up production of PAC-2. Transient expression resulted in very low yields of protein, so it was decided another expression system would have to be used. A recombinant adenovirus expressing PAC-2 was made as described by He et al[4]. Briefly, PAC-2 was cloned into pAdTrack-CMV. pAdTrack-CMV-PAC-2 was transformed into electrocompetent AdEasier cells already harboring pAdEasy-1. Homologous recombination occurs in the AdEasier cells resulting in a ~40kB adenovirus expressing the PAC-2 DNA. This DNA was purified and transfected into HEK293T cells. Virus production was monitored by GFP expression. After a series of viral amplifications a high titer virus stock was made.

PAC-2 can be made by adding viral stock to feeder HEK293 cells. We are currently purifying this protein for tissue culture experiments.

Specific Aim 4 – Inject tumor bearing mice with PAC5 and measure tumor regression. [Not yet begun]

Key Research and Training Accomplishments:

- With the help of computer modeling a series of C5 mutants were made that led to the discovery of PAC-2, a PSA cleavable C5 as verified by mass spectroscopy.
- An adenovirus expressing PAC-2 has been made to scale up recombinant production of the PSA cleavable C5.
- The PI has written his thesis proposal, organized his thesis committee, and held two committee meetings.

Reportable Outcomes:

The discovery of PAC-2 was central to funding we applied for in June 2009.

Conclusion:

We have made substantial progress in fulfilling the goals outlined in our original statement of work. Over the next few months we hope to purify large amounts of PAC-2 and set up *in vitro* activity assays. I am up to date on all institutionally mandated training requirements. Finally, by beginning an additional project I am both expanding my knowledge base and learning additional techniques.

References:

1. Ogata, R.T. and P.J. Low, *Complement component C5: engineering of a mutant that is specifically cleaved by the C4-specific C1s protease*. J Immunol, 1995. **155**(5): p. 2642-2651.
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3. Fredslund, F., et al., *Structure of and influence of a tick complement inhibitor on human complement component 5*. Nat Immunol, 2008. **9**(7): p. 753-760.
4. He, T.-C., et al., *A simplified system for generating recombinant adenoviruses*. Proceedings of the National Academy of Sciences of the United States of America, 1998. **95**(5): p. 2509-2514.

Appendices: (none)