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TITLE: Obstructing Androgen Receptor Activation in Prostate Cancer Cells through Posttranslational Modification by NEDD8

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The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.
In this study, we aim to investigate post-translational modification of the androgen receptor (AR) by a small ubiquitin-like protein, Nedd8. We planned to analyze the consequence of Nedd8 modification on AR activity, and to identify the neddylation sites on AR. We also planned to determine enzymes involved in AR neddylation and de-neddylation. We have completed all proposed tasks in the first year. Key accomplishments so far include the development of a protocol to enrich neddylated form of AR, the demonstration that a portion of endogenous AR is modified by Nedd8, and the finding that androgen reduces AR neddylation. Reportable outcomes include the preparation of a manuscript and the award of a PhD degree to a graduate student. We expect to continue the research into the second year and no major changes on future work are necessary.
# Table of Contents

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Introduction</td>
<td>4</td>
</tr>
<tr>
<td>Body</td>
<td>4</td>
</tr>
<tr>
<td>Key Research Accomplishments</td>
<td>5</td>
</tr>
<tr>
<td>Reportable Outcomes</td>
<td>6</td>
</tr>
<tr>
<td>Conclusion</td>
<td>None</td>
</tr>
<tr>
<td>References</td>
<td>None</td>
</tr>
<tr>
<td>Appendices</td>
<td>None</td>
</tr>
</tbody>
</table>
Introduction

In this study, we aim to investigate whether the male sex hormone androgen receptor (AR) is post-translationally modified by a small ubiquitin-like protein, Nedd8. We planned to investigate the consequence of Nedd8 modification on AR activity. We will also identify the neddylation sites on AR, and determine enzymes involved in AR neddylation and de-neddylation. In Aim 1, we plan to establish Nedd8 modification of AR, and determine the effects of androgen on AR neddylation. In Aim 2, we will identify neddylation sites on AR and determine the E3 neddylation and de-neddylation enzymes. Aim 3 will investigate the roles of AR neddylation on its transcriptional activity and on prostate cancer cell growth. Aim 4 will determine the effects of silencing a potential de-neddylation enzyme Jab1 on the growth of prostatic cells. The knowledge obtained from this study will have impact on understanding the regulation of prostate cancer growth and may provide a potential new target for treating prostate cancer.

Body

Task 1-1. Month 1-3: We will develop protocols to enrich neddylated form of endogenous AR. Ultimately, we will demonstrate evidence that a portion of endogenous AR is modified by NEDD8.

We have successfully developed a protocol to enrich neddylated form of endogenous AR. We have demonstrated evidence that a portion of the endogenous AR is indeed modified by Nedd8. We tested various protocols to enrich neddylated form of endogenous AR. First, large quantity of endogenous AR from LNCaP cells was purified by double immunoprecipitation with anti-AR and anti-NEDD8 antibodies. The neddylated form of AR was detected on Western blot using anti-AR and anti-NEDD8 antibodies (Figure 1). We have also tested various growth conditions to determine their effects on neddylation of endogenous AR in LNCaP cells. Preliminary data suggest that AR can be neddylated under several conditions.

Figure 1. Endogenous AR is modified by NEDD8.

LNCaP cells were maintained in stripped media for at least four days and endogenous neddylated AR was detected by immunoprecipitating neddylated proteins with mouse anti–NEDD8 antibody and immunoblotted by mouse anti–
AR antibody (Figure 1A). We found that neddylation of endogenous AR is enhanced in Jab1 knockdown cells (Figure 1B). In LNCaP cells with Jab1 silencing, immunoprecipitation was conducted by mouse anti-AR antibody and detected by rabbit anti-NEDD8 antibody. Knockdown of endogenous Jab1 was verified by Western blotting. β-actin serves as a loading control. Furthermore, we found evidence of association of Jab1 and CSN1 with AR upon DHT treatment (Figure 1C). CWR22Rv1 prostate cancer cells stably transfected with S-tag hAR were treated with solvent (ethanol) or DHT (10 nM) for indicated time period. AR complexes were purified using S protein column, resolved on SDS-PAGE and detected by goat anti-Jab1 or rabbit anti-CSN1 antibodies, respectively.

**Task 1-2.** Month 3–6: We will compare AR neddylation in various prostate cancer cell lines including LNCaP and PC3. Ultimately, we will provide data showing how AR neddylation may vary among different type of cell lines.

Preliminary data suggest that AR neddylation may occur in different cell types. We have not observed significant differences among different prostate cancer cell types.

**Task 1-3.** Month 6–9: We will determine the effect of androgen treatment on neddylation of endogenous AR. Ultimately, we will answer whether androgen treatment indeed blocks neddylation of AR, and we will provide discussion of how this might occur.

We have completed this task. As shown in Figure 1A and B, AR neddylation shows clear reduction upon treatment with DHT.

**Task 2-1.** Month 9–12: We will identify potential neddylation consensus sequences within AR. Ultimately, we will identify potential amino acid sequences in AR that may serve as conjugation sites for NEDD8.

We have identified K630 as a potential neddylation site on AR. The results are shown in Figure 2.

Figure 2. PIASy–mediated neddylation of wild type AR (lane 5) was greatly diminished in both K630R and 3KR mutants (lanes 6 and 7). His-ISG15 was used as a negative control (lane 8). The Western blots of ectopic expression levels of AR and PIASy were determined, as well as β-actin as a loading control (lower panel).

**Key Research Accomplishments:**

- We have developed a protocol to enrich neddylated form of endogenous AR.
• We have demonstrated evidence that a portion of endogenous AR is modified by NEDD8.
• We have compared AR neddylation in various prostate cancer cell lines including LNCaP and PC3.
• We have found no evidence that AR neddylation varies among different type of cell lines.
• We have determined the effect of androgen treatment on neddylation of endogenous AR.
• We found that androgen treatment may block neddylation of AR.
• We have identified potential neddylation site within AR.

Reportable Outcome:

• A manuscript has been submitted and reviewed by Molecular Cell Biology. The manuscript is pending revision.
• A PhD degree has been awarded to a student named Kai–Hsiung Chang supported by this award.

Conclusion:

In summary, we have completed all proposed tasks in the first year. Several key accomplishments and reportable outcomes are listed above. We expect to continue the research into the second year. No changes on future work are necessary. A change of personnel support from students to postdoctoral fellow or research teaching specialist may be necessary due to graduation of the involved student.

References:
None

Appendices:
None

Supporting Data:
None