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14. ABSTRACT

Tissue inflammation and inflammatory cytokines can positively affect breast cancer prognosis. By providing a detailed understanding of the mechanism of inflammasome formation and activation, we hope to create the potential for inflammation based cancer therapies. For each of the key protein domains in the inflammasome complex, panels of constructs have been screened for their amenity to further biochemical characterization. NALP LRR domain was found to express poorly, with poor solubility, possibly due to oxidation-related folding problems. Constructs of the adaptor CARDINAL were found to express well, but suffer proteolysis during purification. Analysis of CARDINAL partial proteolysis products may yield a proteolyticaly stable CARDINAL subdomain. NACHT constructs were successfully produced by baculovirus/SF9 expression, and will be subjected to biochemical and enzymatic characterization.

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INTRODUCTION:

Inflammation and inflammatory cytokines, including the IL-1β mediated inflammation regulated by the inflammasome complex, appear to have a positive effect on breast cancer prognosis[1,2]. This project aims to study the intra- and inter-molecular interactions that govern inflammasome assembly and activation, and to structurally characterize the constituent proteins and their interactions. By providing a thorough understanding of inflammasome-mediated inflammation at a molecular level, it may be possible to modulate beneficial inflammation events.

BODY:

Work accomplished thus far has chiefly focused on the identification of constructs amenable to recombinant expression in sufficient quantity, and exhibiting acceptable stability and solubility.

Ligand binding to the LRR domain and activation of NALP

According to the prevailing model of inflammasome activation, assembly of the complex is initiated by the binding of a stimulating ligand to the NALP LRR domain[3,4,5]. In order to biochemically investigate this interaction, initial work has focused on generation and purification of a LRR-domain construct.

The precise boundary between the NALP NACHT and LRR domains is unclear. Multiple sequence alignments of NALP family members and of related LRR-containing proteins reveal no clear boundary, based on sequence conservation. There is some suggestion that the ambiguous region of some ~100 amino acids between the canonical NACHT and LRR domains constitutes a small independent domain[3]. Alternatively, it is possible that the LRR domain requires a capping region at one or both ends, as is observed in a subset of LRR structures. Since accurate domain boundaries are likely necessary for proper protein folding, constructs of varying length were generated for recombinant expression.

All LRR constructs were found to overexpress poorly with poor solubility in *Escherichia coli.* Similar results were observed when these constructs were produced by baculovirus infection of SF9 insect cells. These observations may be attributable to a phenomenon noted during the purification of the related LRR protein, ribonuclease inhibitor (RI)[6]. The RI LRR domain contains a number of cysteine residues that make integral side chain to backbone interactions, stabilizing the fold. The purification of RI was noted to be highly sensitive to oxidation. Some steps in the purification of NALP LRR domain cannot be performed in the presence of reducing agent. It is possible that that these periods in an oxidizing environment may be sufficient to affect the structural cysteine residues and irreversibly misfold the protein.

Better results were observed in expressing full length NALP protein, as discussed below, perhaps because the LRR domain contacts the NACHT domain, thus shielding some or all cysteine residues. We continue to address the difficulties of LRR domain expression. Constructs generated thus far are derived from NALP family members NALP2 and NALP12. As the number of structurally important cysteine residues varies among family members, a construct consisting of LRR domain from a different NALP protein may be more amenable to recombinant expression.

NACHT domain oligomerization and regulation of oligomerization

Homology between NALP proteins and the well studied apoptosis protein, APAF-1, suggests that inflammasome assembly and activation involves both ligand binding and ATP hydrolysis[7]. The NACHT domain, belonging to a superfamily of oligomeric ATPases, likely plays a central role in regulation of inflammasome formation and activity.

In order to study the oligomerization and ATP-binding properties of the NACHT domain, a panel of NACHT-containing constructs was generated. As with the LRR domain, there is ambiguity in the location of the C-terminus of the folded NACHT domain. Several constructs were cloned, including full-length NALP, PYD+NACHT constructs, and NACHT-alone constructs of varying length.

All NACHT-containing constructs were observed to overexpress poorly in *coli*, with most protein present in the insoluble fraction, suggesting difficulty folding. Better results were observed when expressed by baculovirus infection of SF9 cells. In this system, PYD+NACHT constructs express in moderate quantity, but were found to suffer extensive proteolysis during expression and purification. Intact full-length NALP was expressed, but was found to aggregate in a time dependent fashion and eventually precipitate. (This may be related to the presence of the LRR domain.) NACHT-only constructs, however, are promising. These were found to express in moderate quantities and appear to be sufficiently soluble for purification.

Initial analysis of full-length NALP and NACHT-only constructs by gel-filtration chromatography indicate that these proteins are present in discrete oligomeric states, suggesting equilibrium between monomer and oligomer. These constructs should be suitable for subsequent studies, including characterization of ATPase activity, and the effects of ligand and ATP upon oligomerization.

Protein-protein interactions among constituents of the inflammasome

During the process of inflammasome formation, the adaptor protein CARDINAL is hypothesized to be recruited to activate NALP[3]. With respect to its role in inflammasome activation, the most interesting region of the CARDINAL protein is the FIIND domain. This domain, which does not posses homology to any known protein fold, is hypothesized to interact with the activated NALP protein via the NACHT domain.

In order to study the interaction between FIIND and the NACHT domain of NALP, a series of CARDINAL constructs were generated. These constructs included full-length constructs of two naturally occurring splice isoforms of CARDINAL, as well as a panel of truncated constructs consisting only of the FIIND domain, with varying N- and C-termini. In general, CARDINAL was found to express solubly in *coli* and baculovirus, in sufficient quantity for purification. However, all constructs produced to date exhibit a strong susceptibility to proteolysis during purification. Partially purified protein has been analyzed by MALDI mass spectroscopy, in an attempt to identify proteolyticaly stable protein fragments. A stable fragment of this protein has not yet been identified, however.

Additional developments

During this research period, a competing group has published on a member of the NALP family, NALP1, which contains the standard NALP domains, as well as a C-terminal domain homologous to CARDINAL[8]. Data in this report characterize interactions, ATPase activity, and activation of NALP1. Due to the homology between these this publication and the inflammasome proteins focused on in this work, the author may wish to change the focus and objectives in this project, in the future.

KEY RESEARCH ACCOMPLISHMENTS:

- Screening of NALP LRR domain constructs in *coli* and baculovirus/SF9 expression systems.
- Screening of NALP NACHT domain constructs in *coli* and baculovirus/SF9 expression systems.
- Screening of CARDINAL and FIIND domain constructs in *coli* and baculovirus/SF9 expression systems.
- Identification of soluble and proteolyticaly stable NACHT constructs, which will be used for further enzymatic, biochemical, and structural analysis.
- Initial characterization of the size and oligomeric state of NACHT domain constructs.

REPORTABLE OUTCOMES:

none

CONCLUSION:

Tissue inflammation and inflammatory cytokines can have a positive affect on breast cancer prognosis. By understanding at a molecular level the mechanism of inflammasome formation and activation, we hope to create the potential for inflammation based therapies.

For each of the key protein domains in the inflammasome complex, panels of constructs have been screened for their amenity to further biochemical characterization. The NALP LRR domain was found to express poorly, with poor solubility, possibly due to oxidation-related folding problems. Constructs of the adaptor protein CARDINAL were found to express well, but are susceptible to extensive proteolysis during the purification process. It is hoped that analysis of the partial proteolysis products will help to identify a proteolyticaly stable CARDINAL subdomain. NACHT constructs were successfully produced by baculovirus/SF9 expression, and will be subjected to biochemical and enzymatic characterization.

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APPENDICES:

none

SUPPORTING DATA:

none