DEPARTMENT OF HEALTH & HUMAN SERVICES

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October 14, 2010

To the Chairman of the thesis committee:

I have read the PhD dissertation of Jindrich Chmelar and find it to be an excellent piece of work covering a variety of disciplines. The work would be considered superior dissertation material in any university that I have been affiliated with. It clearly meets the requirements of the PhD degree.

In these studies Jindrich Chmelar describes a very complete analysis of the transcriptome of *I. ricinus* with annotation, and a comparative study among various feeding stages. Also described is the expression, purification, and structure determination of a novel salivary serpin, IRS-2. Finally, a study of the functional role of this serpin was conducted showing this protein to be a potent anti-inflammatory agent, apparently due to its ability to inhibit cathepsin G and mast cell chymase.

The transcriptome analysis published in BMC genomics represents an important contribution to the field of tick biology since it covers both the identities of the proteins found in the salivary mixture and the changes that occur in salivary protein expression during the feeding period. Because *I. ricinus* is an important vector of Borrelia in Europe, this study also represents an important comparison with the transcriptomic studies of North American *I. scapularis* and *I. pacificus* ticks that have been published previously.

The second part of the dissertation involves a mechanistic study of inhibition by the salivary serpin IRS-2. This work includes in vitro assays of enzyme inhibition, in vivo measurements of anti-inflammatory activity, cellular assays, and structure determination of a latent form of the inhibitor. A complete characterization is described here that provides a strong case for an extremely important role for these proteins in tick feeding and disease transmission. The protein appears to be targeted at mast cell chymase and cathepsin G. The discussed ramifications of this inhibition are consistent with the observations presented in the paper.

A structural characterization of IRS-2 is included in the study that ranges from crystallization of the protein to elucidation of the structure at a resolution of 1.8 Å. The paper in Acta Crystallographica describes the crystallization and initial analysis of the diffraction data. The unit cell parameters and space group are described. A molecular replacement solution is also presented, and the initial electron density map for the structure is shown. The final structure is described in the second paper along with the functional assays. A structure of the apparent latent form of the protein is presented that was produced as a result of digestion by proteases originating from the expression host. The structure of the protein verifies the identity of the reactive site loop and the fact that it is specifically cleaved by bacterial proteases.

Questions for the author:

1. You used a molecular replacement approach for determining the structure of IRS-2. What other approaches could be used? Could you provide a brief non-mathematical description of these other possibilities?
2. The serpin is cleaved in the crystal by unidentified proteases, but apparently, the cleaved serpin does not covalently modify the protease. Do you have any ideas why this might be true?
3. Is it possible that IRS-2 could use another than inhibitory mechanism for its function, e.g. binding to protease exosite or binding to some cell receptors?
4. You state that the closest homolog of the protein is antithrombin. Is there conservation of the heparin binding site? Could this have significance in the inhibitory mechanism?

Sincerely,

John F. Andersen
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ÚOCHB AV ČR

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Evaluation of the PhD. thesis of Jindřich Chmelař

"Transcriptomic and functional analysis of salivary proteins from the tick *Ixodes ricinus*"

The thesis is focused on proteins from the saliva of the castor bean tick *Ixodes ricinus*. This tick species is the most important parasite vector of infectious agents in Europe. It transmits causative agents of serious diseases including Lyme disease caused by spirochetes *Borrelia burgdorferi* and tick borne encephalitis caused by the tick-borne encephalitis virus.

The transmission of pathogens is facilitated by the modulation of host responses, mediated by the molecules present in tick saliva. The author concentrates on the identification and characterization of physiologically relevant salivary proteins of *I. ricinus*. Two complementary approaches were employed: (1) high-throughput technology that lead to the determination of *I. ricinus* sialotranscriptome and its dynamic profile during the tick blood feeding, and (2) a complex genetic and protein-level characterization of salivary proteins of serpin family. The four serpins were sequenced, and one of them, IRS-2, was biochemically and structurally characterized. Finally, anti-inflammatory and anti-platelet actions of IRS-2 were discovered.

The thesis starts with a comprehensive introduction and topic overview covering all relevant aspects of tick-host interactions. Moreover, it reviews known salivary proteins that ticks use to overcome host defense. Jindřích Chmelař has performed an elegant and original piece of work. The experimental work is technically sound and the author was able to successfully perform interdisciplinary research using variety of techniques from molecular biology and genetics, protein biochemistry, enzymology, physiology and bioinformatics.

In conclusion, results of the thesis have been published in three papers in internationally recognized, peer-reviewed journals. The work significantly contributed to the increase of our knowledge about mechanisms of tick-host interactions, and therefore, I strongly recommend Jindřích Chmelař for the awarding of the PhD. degree.

I have the following questions regarding the presented work to the author:

1, You have determined full length sequences of four *I. ricinus* serpins and functionally characterized one of them - IRS-2. Can you predict the biological role and targets for the other three serpins based on their amino acid sequences? Do you plan to express and characterize also these serpins?

2, Based on the biochemical characterization of IRS-2 inhibitory specificity, can you predict what types of immune cells can be theoretically modulated by IRS-2?

3, Serpins were originally identified as inhibitors of serine peptidases, however, this group contains homologous proteins with a wide array of biochemical functions. One of these is inhibition of peptidase of cysteine class. Did you examine IRS-2 activity against cysteine peptidases, especially against C1 (papain-like) or C14 (caspases) families?

4, Chymostatin and indomethacin displayed similar anti-inflammatory effect as IRS-2. Why were these compound selected for the experiment, and what can be deduced from this observation?

5, Interaction of several serine proteases with serpins is regulated by glycosaminoglycans. Did you test IRS-2 inhibition of targeted proteases in presence of these sulfate polysaccharides?

6, The sialotranscriptome from *I. ricinus* was determined from 600 sequenced clones from 4 different libraries. Notably, serpin family was not revealed using this approach. Can you estimate what part of the total transcriptome is covered by your analysis?

Praha, 28.10. 2010

Mgr. Martin Horn, CSc.

UNIVERSITY OF CALIFORNIA
UC RIVERSIDE | Institute for Integrative Genome Biology

October 8, 2010

To: Ph.D. Thesis Committee

Re: Jindrich Chmelar Thesis Defense
"Transcriptomic and Functional Analysis of Salivary Proteins from the Tick Ixodes ricinus"

Dear Ph.D. Thesis Committee,

I most enthusiastically support the approval of Jindrich Chmelar Thesis Defense at the University of South Boemia, Faculty of Science, Department of Parasitology. Mr. Chmelar excelled during his doctoral degree as it can be judged by: i) his publications in *Blood*, *BMC Genomics*, *Antimicrob. Agents Chemother,* and *Acta Cristalographica;* and ii) the range of topics studied during his doctoral degree. I consider his thesis as outstanding. I make this assessment based on my previous interactions with other graduate students in three major research universities: Purdue University, Yale University and the University of California.

Mr. Chmelar Ph.D. thesis has a nice blend between descriptive and mechanistic work. To shed some light into the genes that are expressed in the salivary glands of the tick *Ixodes ricinus*, he used functional genomics and genomics techniques to catalogue the sialome of the castor bean tick *I. ricinus*. Previous to this work, the expression of genes in the salivary glands of the tick *I. ricinus* was poorly understood. Mr. Chmelar and his collaborators constructed cDNA libraries from four different feeding stages of *I. ricinus* females, sequenced clones, performed bioinformatics analysis and determined that several groups of over-expressed genes were associated with feeding, tick attachment, or evasion of the host immune system.

Then, in his *Blood* article he performed mechanistic studies with one tick protein – *I. ricinus* serpin-2 (*irs2*). He showed that IRS-2 inhibits the host inflammatory response and platelet aggregation. Inhibition of these biological functions was consistent with its specificity for cathepsin G and chymase and to a much smaller degree for thrombin. Mr. Chmelar concluded that knowledge of the properties of this parasite-derived serpin and its structure could form the basis for the development of novel anti-inflammatory therapeutics. Mr. Chmelar acquired skills in bioinformatics, molecular biology, biochemistry and biophysics. This study is highly meritorious as only a few tick proteins have been extensively characterized in a mechanistic manner.

Mr. Chmelar's Ph.D. thesis is well-written and provides extensive information on the state of the field in tick immunobiology and the properties of tick saliva in the context of inflammation, host immunity and coagulation. A few questions, however, deserve to be asked by this *ad hoc* reviewer.

1. What could be the pharmaceutical applications of IRS-2?

2. Are there some notable differences between the completed *I. ricinus, I. scapularis and I. pacificus* transcriptomes?

3. What are the main differences between IRS-2 and the already described serpin IRIS in their activity and mode of function?

4. Cathepsin G and chymase are novel targets of tick salivary secretion. How cathepsin G and chymase may play a role in vector-host interaction? Can their interaction with IRS-2 occur in the intracellular or extracellular environment? Could there be a possible effect on innate immunity?

In summary, Mr. Chmelar's Ph.D. thesis accomplishments and the relevance of his research is something worth noting. I, therefore, express full confidence in approving Jindrich Chmelar Thesis Defense entitled *"Transcriptomic and Functional Analysis of Salivary Proteins from the Tick Ixodes ricinus"*.

Sincerely yours,

Dr. Joao Pedra, Ph.D. - Assistant Professor
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