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Review of the PhD Thesis "Mechanisms Involved in Sodium Uptake Activation by the Tumor Necrosis Factor-Derived TIP Peptide" by Alexander DULEBO.

In his doctoral thesis, Alexander Dulebo reports studies aimed to elucidate the mechanism of action of the cyclic "TIP"-peptide that consists of a part of a cytokine, the Tumor Necrosis Factor (TNF). TNF is a multifunctional so called "moonlight protein". It's best known roles are the receptor mediated (TNF R1 and TNF-R2) inflammatory and anti-apoptotic effects (via NF-κB).However, under certain conditions TNF (viaTNF-R1) is also able to induce apoptosis.
TNF also possesses a Lectin like activity that could be mapped to amino acid residues 100-116. This activity is (in principle) retained by a circularized peptide consisting of these residues, the TIP peptide.
The peptide is of potential therapeutic interest because in pulmonary edema it seems to be able to support the edema fluid clearance (lung liquid clearance).

In his thesis Alexander Dulebo tried to answer four specific questions related to the TIP-peptide:

1. Does the TIP peptide physically interact with molecules on the surface of the apical side of lung epithelial cells?

The author used Single Molecular Force Microscopy (SMFS) to analyze whether the TIP-peptide interacts with surface molecules of a lung epithelial cell line (H441). He was able to detect interactions that were blocked by a oligosaccharide (*N',N'*-diacetylchitobiose) that was known to interact with the TIP peptide. Thus he could answer this question with "yes".

2. Is the peptide internalized (and is this internalization necessary to trigger the effects)?

To answer this question H441 cells were incubated with TIP-peptide that was labeled with a fluorescent dye and the fluorescence distribution was analyzed. The author could show that there was virtually no fluorescence inside the cells. Together with the observation that the labeled TIP-peptide retained its biological activity, he concluded (as far as I can judge, correctly) that internalization is not necessary for the TIP-peptide mediated physiological response and is unlikely to occur at all.

3. What is the nature of the TIP-peptide target interaction in terms of amino acid residues and structures involved?

Concerning this question Alexander Dulebo used two different approaches: (i) Molecular modeling of TIP-peptide's interaction with it's artificial binding partner *N',N'*-diacetylchitobiose (and the probably non interacting cellobiose) and (ii) determination of the structure of the TIP-peptide via crystallization. Unfortunately the "gold standard", crystallization, did not yet give rise to crystals suitable for X-ray diffraction analysis. However, the experiments the author carried out can serve as basis for more refined strategies to obtain "better" crystals. Approach (i), combined with the analysis of molecular dynamics, however lead to a plausible structural model that pointed out the importance of several critical residues for the TIP-peptide / receptor interaction and also confirmed the importance of several hydrophobic residues for the TIP-peptide - target (oligosaccharide) interaction.

and finally: *4. What is/are the TIP peptide receptors(s)?*

To address this question the author performed affinity chromatography to isolate proteins that bound to the immobilized TIP peptide. He found several putative binding proteins but was unfortunately not able to identify them due to the low amount of bound protein. This approach is obviously still somewhat preliminary and requires additional experiments to obtain conclusive results.

The results obtained during the thesis work were published in two peer reviewed scientific articles. Furthermore, one additional paper is submitted and one in preparation. Apart from the work described in his thesis Alexander Dulebo is co-author in another publication in press and four papers that are either submitted or in preparation. In three of these articles Alexander Dulebo is (or will be) first author. This clearly demonstrates his scientific capabilities.

The thesis is subdivided into three major parts. The introduction gives a good overview/review on what is known about TIP peptide effects and mechanisms of action. Additionally in this section the presented work is motivated and the aims are clearly defined. The second part is not only a detailed description of the methods and techniques used. It also introduces these techniques and reviews their applications, advantages and disadvantages. This might be somewhat unusual for a *sensu strictu* method part but nicely shows that the author was not only able to use these methods but also has a profound knowledge of the theoretical and even historical background. This is especially remarkable since the work involved very sophisticated techniques like Second Harmonic Atomic Force Microscopy, SMFS, Molecular Dynamics Simulations and more. The third part summarizes and discusses the results that are more detailed described in the appended publications (manuscripts).

The whole thesis is written in a concise and clear language.

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With the present thesis Alexander Dulebo has clearly proven that he is able to conduct independent scientific research and that he is also able to communicate his results in a concise and understandable way.

I therefore recommend without any reservation the acceptance of his PhD thesis.

For the discussion, I'd suggest to include the following questions/topics:

Involvement of ENaC channels in the TIP-peptide triggered events: As pointed out in the thesis, it seems that ENaC cation channels are directly or indirectly targets of the TIP-peptide. I'd be interested to learn which experiments would be possible to (conclusively) distinguish between these possibilities. Would it e.g. be possible to use modeling approaches to identify possible TIP-peptide/ENaC interaction domains and then experimentally verify/falsify them?

Identification of direct TIP-peptide interaction partners: How could the used approach be extended and which alternative approaches could be possibly used to find proteins that directly interact with the TIP-peptide?

Yours sincerely

(Jost Ludwig)

Opponent's Review of the Ph.D. Thesis

Alexander V. Dulebo:
Mechanisms involved in sodium uptake activation by the Tumor Necrosis Factor-derived TIP peptide

The aim of the work of Alexander Dulebo was to study Tumor Necrosis Factor-derived TIP peptide, its interaction with carbohydrate ligands, (like N,N'-diacetylchitobiose), interaction with protein receptors and binding to whole cells. These interactions seem to be important for therapeutic effect of TIP peptide during treatment of pulmonary edema, because TIP peptide activates sodium uptake in type II alveolar epithelial cells from lungs. The candidate also tried to characterize the structure of this TIP peptide, using X-ray crystallography and computer modeling.

Ph.D. thesis of Alexander Dulebo has 137 pages, and is divided into following parts: Preface, Materials and Methods, Results and Discussion, Conclusion, References and Papers. The last part (Papers) is represented by 4 scientific papers of the candidate. Two of them have been already accepted and published in scientific journals, (one is review and one methodical paper), one paper is just submitted, and one is in status of preparation. Alexander Dulebo is mentioned as the first author on three of these papers. The contribution of the candidate to each individual paper is specified at the beginning of this Ph.D. thesis, which is very helpful for evaluation of his work. The thesis has a correct graphical form and does not include many typing errors. The part Preface includes also the subchapter Introduction, which offers a good overview about the problematic and can certainly serve even in future as introductory material for younger colleagues in his lab. Also the part Materials and Methods is very good and contains detailed explanation of used techniques.

I think that one of the most important things reported in this thesis is described microscopic technique, which represents the resolution improvement of atomic force microscopy of living and fixed mammalian cells using higher harmonic signals. Second important point of this thesis is computational study of TIP peptide which predicts binding sites for N,N'-diacetylchitobiose and trimannose-O-ethyl. Results of this study are supported by the good agreement with the experimental data from the literature.

Comments:

a) The division of the work into typical parts is not respected sometimes. For example, the majority of the text on pages 60 and 61, (which are parts of Results and Discussion) refers about the experiments from other labs and should be included in Introduction. On the other hand, the last subchapter of Preface named "Aims of the thesis" includes not only these aims, (quite well specified), but continues with description of experiments and contains even figures of original results.

b) It would be helpful to use the part "Conclusion" for summarization of results in several points, (in similar way which has been used for summarization of aims of the work). Such a short summarization is missing and sentences in this part are too general and vague.

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c) In my opinion, results of affinity chromatography are highly overinterpreted. In such a preliminary stage of investigation it is too soon to speculate about "first indirect evidence of the inability of the TIP peptide to interact with ENaC" and some "unknown receptors". This contrasts with well evaluated results of other methods used in this Ph.D. thesis.

I have these questions:

1. Are there any antibodies available against TIP peptide, which could be used for immunoprecipitation of possible ligand-receptor complex?

2. What about to try washing with N,N'-diacetylchitobiose as the step, which would help to distinguish lectin-like interactions in affinity chromatography?

3. What is the origin of the name of TIP? I wasn't able to find it in the text of the thesis.

4. Could you please describe more details about the crystallization of TIP peptide and conditions which have been tested? How many experiments did you set?

Previous comments do not want to diminish the value and quality of this work. I think that it fulfills the requirements for Ph.D. thesis and I recommend to accept it for the defense.

Prague, 10th January 2012

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