

**Mapping of the protein-protein interaction network of LC8  
dynein light chain by characterizing its binding motif using *in  
vitro* directed evolution and biophysical approaches**

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PhD thesis



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## Introduction

Practically all biological processes manifest through protein-protein interactions. During my PhD thesis I studied a ubiquitous eukaryotic hub protein, the LC8 dynein light chain.

LC8 dynein light chain is a highly conserved, 10 kDa protein possessing no enzymatic activity. LC8 was first identified as a component of both the dynein and the myosin 5a motor proteins (Hodi, Nemeth et al. 2006). Since then, dozens of LC8 partners have been identified and this number is still increasing. Although LC8 was primarily thought to be involved in the cargo binding function of dynein and myosin 5a, the known partners are in fact implicated in diverse cellular events – unrelated from cytoskeletal motors –, such as nuclear transport (NUP159), mitosis (EML3), apoptosis (Bim and Bmf) and signal transduction (nNOS) (Rapali, Szenes et al. 2011).

LC8 protein forms homodimers by  $\beta$ -swapping mechanism (Benison, Karplus et al. 2008). Two symmetric grooves are formed at the edges of the dimerization interface. These grooves recognize a short linear motif, which has a loose consensus sequence. The LC8 binding motifs are located mainly in the disordered region of the interacting proteins, frequently in the vicinity of a coiled coil domains. The most conserved position of the motif, referred to as position 0, contains mainly glutamine. Based on sequence homology, the motifs are traditionally classified into three groups, namely  $K_3X_2T_1Q_0T_1$ ,  $I/V_{-1}Q_0V_1D_2$  and non-canonical motif families (where X means any amino acids) (Lo, Naisbitt et al. 2001; Rodriguez-Crespo, Yelamos et al. 2001). However, it has not been studied yet whether the three motif families differ in function or binding behavior (Hodi, Nemeth et al. 2006; Wagner, Fodor et al. 2006; Hall, Hall et al. 2008; Lightcap, Sun et al. 2008; Song, Wen et al. 2008).

Vertebrates contain two LC8 paralogs, DYNLL1 and DYNLL2. The two isoforms share 93% sequence identity at the protein level. All differences (6 amino acid residues) are located outside the binding grooves. Interestingly, despite the sequential and structural similarity, partner specificity was observed *in vivo* between the two paralogs (Day, Puthalakath et al. 2004). Moreover, in some cases the partners are considered as only DYNLL1 or only DYNLL2 binders (Jaffrey and Snyder 1996; Puthalakath, Villunger et al. 2001).

Very often LC8 forms dimer-dimer complexes with its interacting partners via coiled coil or other domains (Williams, Roulhac et al. 2007). However, it is not established how it affects complex formation.

LC8 binding can be regulated by various post-translational modifications. Phosphorylation of LC8 at Ser88 is thought to shift the monomer-dimer equilibrium of LC8 towards the

monomeric state. Monomeric LC8 is not able to bind partners (Song, Benison et al. 2007; Song, Wen et al. 2008).

## **Aims**

During my PhD studies, my primary goal was to characterize the complex formation between LC8 and partners; furthermore, I intended to further map the protein interaction network of LC8.

The specific objectives of the study were:

- To compare the binding properties and motif specificity of the human paralogs (DYNLL1 and DYNLL2) of LC8 *in vitro*;
- To characterize thermodynamic and kinetic aspects of LC8 binding, focusing on different motif families;
- To investigate how partner dimerization state affects the binding affinity and kinetics, and whether it has a role in LC8 function or regulation;
- To analyze LC8 linear binding motifs using a high-throughput, *in vitro* directed evolution approach, phage display;
- To design a high affinity peptide based on the binding sequence pattern obtained from phage display. This peptide could be used as a competitive inhibitor for subsequent studies;
- To predict novel LC8 partners from the human proteome based on binding sequence pattern obtained from phage display;
- To experimentally validate a subset of newly predicted partners (EML3, ATMIN)

## **Materials and methods**

The investigated proteins and protein fragments were obtained by heterolog expression in *E. coli* cells. They were purified by affinity, ionic exchange and RP-HPLC chromatography. Binding parameters between LC8 and partners were determined by isothermal titration calorimetry (ITC), fluorescence anisotropy, surface plasmon resonance (SPR) and stopped-flow fluorescence measurements. LC8 binding sequence was characterized by phage display using a bivalent naïve peptide library. Atomic structures of LC8-SRGTQTE and LC8-dimeric VSRGTQTE complexes were solved by crystallization and X-ray diffraction.

## Results and discussion

- **Comparison of the binding properties and motif specificity of the human paralogs (DYNLL1 and DYNLL2) of LC8 dynein light chain *in vitro***

We determined the binding parameters of both LC8 isoforms (DYNLL1 and DYNLL2) with two different motifs (nNOS: T<sub>-3</sub>G<sub>-2</sub>L<sub>1</sub>Q<sub>0</sub>V<sub>1</sub>D<sub>2</sub>; Bmf: K<sub>-3</sub>A<sub>-2</sub>T<sub>-1</sub>Q<sub>0</sub>T<sub>1</sub>D<sub>2</sub>), which have been previously referred to as isoform-specific binders. Although we showed that DYNLL1 binds both motifs slightly weaker and with a lower association rate compared to DYNLL2, it is doubtful that such small differences could cause the partner specificity observed *in vivo*. Taken together, these results suggest that the sequence difference between the two paralogs does not significantly alter the “function” of the binding grooves.

- **Thermodynamic and kinetic characterization of LC8 dynein light chain binding, focusing on different motif families**

We determined the thermodynamic and kinetic binding parameters of complex formation with peptides belonging to the three putative motif families, namely Bmf (K<sub>-3</sub>A<sub>-2</sub>T<sub>-1</sub>Q<sub>0</sub>T<sub>1</sub>D<sub>2</sub>), TP53bp1 (A<sub>-3</sub>A<sub>-2</sub>T<sub>-1</sub>Q<sub>0</sub>T<sub>1</sub>I<sub>2</sub>), nNOS (T<sub>-3</sub>G<sub>-2</sub>L<sub>1</sub>Q<sub>0</sub>V<sub>1</sub>D<sub>2</sub>), GKAP (V<sub>-3</sub>G<sub>-2</sub>V<sub>-1</sub>Q<sub>0</sub>V<sub>1</sub>E<sub>2</sub>), myosin 5a (K<sub>-3</sub>N<sub>-2</sub>T<sub>-1</sub>M<sub>0</sub>T<sub>1</sub>D<sub>2</sub>) and Pak1 (V<sub>-3</sub>A<sub>-2</sub>T<sub>-1</sub>S<sub>0</sub>P<sub>1</sub>I<sub>2</sub>). Although the members of K<sub>-3</sub>X<sub>-2</sub>T<sub>-1</sub>Q<sub>0</sub>T<sub>1</sub> family were slightly closer to the thermodynamic binding optimum compared to members of L<sub>1</sub>Q<sub>0</sub>V<sub>1</sub>D<sub>2</sub> family, the dissociation constant of all the examined motifs was around the micromolar range. The weakest binders were the non-canonical motifs (myosin 5a and Pak1). Interestingly, members of the different motif families achieve this micromolar affinity by different binding mechanisms. While binding of nNOS, GKAP, myosin 5a and Pak1 peptides were enthalpy- as well as entropy-driven, members of K<sub>-3</sub>X<sub>-2</sub>T<sub>-1</sub>Q<sub>0</sub>T<sub>1</sub> family – Bmf and TP53bp1 peptides – were enthalpy-driven with unfavorable entropy change. The kinetic on-rate constant ranged from 6 to 58 mM<sup>-1</sup>s<sup>-1</sup>. The kinetic off-rate constant of nNOS, myosin 5a and Pak1 peptides were from 0.1 to 0.6 s<sup>-1</sup>, while in case of Bmf peptide it was about 100 times slower (k<sub>off</sub><sup>C</sup> = 0.00185 s<sup>-1</sup>).

It is unlikely that the observed small differences of dissociation constants explain how LC8 selects among various partners *in vivo*; it is rather important as a “fine-tuning mechanism” in the interaction network of DYNLL.

### • Effect of the partner dimerization state on the binding affinity and kinetics

We studied how the partner dimerization state affects binding affinity and kinetics. We found that both the dimeric Bmf and the dimeric myosin 5a construct bound to LC8 with an approximately 200-250 times higher affinity compared to their monomeric counterparts (monomeric myosin 5a peptide:  $K_{d, eq}=9 \mu\text{M}$ ; dimeric myosin 5a:  $K_{d, eq}= 0.037 \mu\text{M}$ , monomeric Bmf peptide:  $K_d= 0.7 \mu\text{M}$ ; dimeric Bmf:  $K_d= 0.003 \mu\text{M}$ ). Comparing binding kinetics of monomeric and dimeric myosin 5a suggests that the enhanced binding affinity of bivalent ligands is mostly due to the lower off rate constant (monomeric myosin 5a:  $k_{on}^C=6590 \text{ M}^{-1}\text{s}^{-1}$ ,  $k_{off}^C=0.1 \text{ s}^{-1}$ ; dimeric myosin 5a:  $k_{on}^C=4020 \text{ M}^{-1} \text{ s}^{-1}$ ,  $k_{off}^C=0.0007\text{s}^{-1}$ ). The difference seen in the binding affinity and kinetics between monovalent and bivalent could mostly be explained by an avidity effect. LC8 binds to both the monomeric and the dimeric constructs at a lower kinetic on rate constant compared to other eukaryotic linear motifs which are implicated in signal transduction events (Seet, Dikic et al. 2006). Moreover, due to the avidity effect, the dimeric peptides dissociate extremely slowly from LC8. In conclusion, LC8 binds its partners slowly and releases them slowly, as well. The long-lifetime of LC8 complexes suggests that LC8 functions most likely as a dimerization subunit of partners. In other words it may be regarded as a “molecular velcro”.

### • Role of avidity on the LC8 regulation by phosphorylation at Ser88

We examined how avidity affects LC8 regulation. Firstly, we determined the affinity of a strong LC8 binder (Bmf,  $K_d= 3.5 \mu\text{M}$ ) to LC8 Ser88Glu mutant that mimics LC8 phosphorylation at Ser88. We found that it interacted with a physiologically irrelevant dissociation constant of  $>100 \mu\text{M}$ . Although the mutant LC8 binds to the dimeric myosin 5a fragment 50-times weaker than wild-type LC8 (wt LC8  $K_d= 50 \text{ nM}$ , LC8 Ser88Glu  $K_d= 2.7 \mu\text{M}$ ), this complex with micromolar affinity could exist in the cell. The lowered affinity was clearly due to a reduction of the on-rate constant (wtDYNLL2  $k_{on}= 4840 \text{ M}^{-1}\text{s}^{-1}$ , DYNLL2 Ser88Glu  $k_{on}= 147 \text{ M}^{-1}\text{s}^{-1}$ ), while the off-rate constants did not change (wtDYNLL2  $k_{off} = 2.24 \times 10^{-4} \text{ s}^{-1}$ , DYNLL2 Ser88Glu  $k_{off} = 3.41 \times 10^{-4} \text{ s}^{-1}$ ).

Although it is not yet known which protein kinase phosphorylates LC8 at Ser88, this post-translational modification could selectively regulate binding of LC8 to monomeric or dimeric partners.

- **Comparative analysis of the LC8 linear binding motifs using a high-throughput, *in vitro* directed evolution approach, phage display**

I performed a comparative analysis of the binding sequence pattern of human LC8 (DYNLL1) using a directed protein evolution approach, phage display. I determined the conservation state and amino acid frequency of each position of the linear binding motif. A naive peptide library was displayed in bivalent manner using a GCN4 leucine-zipper. In case of the first peptide library, position 0 was fixed to glutamine while all of the 20 natural amino acids were allowed at the 7 other positions (XXXXXQXX). Based on the results of the first library, a second library was designed (XXVSRGXXXEX) that refined the binding sequence pattern and allowed characterization of position 0. In summary, the evolved binding pattern was characteristically similar to the one determined by the hitherto known natural binding motifs. Although members of the  $K_{-3}X_{-2}T_{-1}Q_0T_1$ , the  $L_1Q_0V_1D_2$  as well as the non-canonical motif families were all identified among the evolved phage clones, no novel motif type was selected. The largest difference from the known sequences was at position -5 whose conservation state was increased. Apolar amino acids were most frequently selected here and the most frequent amino acid was valine.

- **Design of a high-affinity peptide based on the binding sequence pattern obtained from phage display and the structural basis of the enhanced affinity**

I successfully designed a high-affinity peptide based on the sequence pattern obtained from the first peptide library. A consensus sequence was made by combining the most frequent amino acids at each position. The corresponding peptide –  $V_{-5}S_{-4}R_{-3}G_{-2}T_{-1}Q_0T_1E_2$ ,  $K_d = 84$  nM – had an affinity one order of magnitude higher than the previously known strongest binder of LC8, Bmf.

Dimerization of the  $V_{-5}S_{-4}R_{-3}G_{-2}T_{-1}Q_0T_1E_2$  peptide through a leucine-zipper further increases the affinity into the subnanomolar range. Moreover, using various strategically designed modified peptide variants, the source of the enhanced affinity was identified to be the valine at position -5. The role of valine was explained by determining the crystal structure LC8-selected peptide complexes with and without this residue.

The high-affinity monomeric as well as the dimeric peptides could be utilized as competitive inhibitors of LC8 in further *in vivo* investigation of LC8 function.

- **Prediction of novel LC8 partners from the human proteome based on the binding-selected sequence pattern obtained from phage display**

We predicted novel partners from the human proteome based on the binding sequence pattern obtained from phage display. A simple position-specific scoring matrix was used for the prediction and we screened only for LC8 binding motifs that are located in disordered region of intracellular partners. Finally, a statistical threshold level was determined. Using the two libraries together, 87 predicted LC8 partners were found above this threshold. Among them, 22 motifs had already been identified experimentally as LC8 binder.

- **Experimental validation of two predicted LC8 partners; EML3 and ATMIN**

Two candidates, namely the EML3 (*Echinoderm microtubule-associated protein-like 3*) and the ATMIN (*ATM kinase interactor*), were chosen from nearly one hundred potential human LC8 partners to be experimentally validated for binding.

The LC8 binding motif of EML3 (SLVSRGTQTET) is located in a disordered region of protein at the carboxyl-terminus of a coiled coil domain. The binding to LC8 to an EML3 fragment containing the motif and the flanking coiled coil domain was experimentally validated by ITC.

We have predicted five LC8 binding motifs that were juxtaposed in a disordered region of ATMIN located at the carboxyl-terminus. The interaction between LC8 and ATMIN was successfully validated using pepscan, Y2H and ITC approaches.

## Summary

In the first step, we determined and compared the kinetic and thermodynamic binding properties of various known DYNLL-binding sequences and motif families. We observed that the two paralogs (DYNLL1 and DYNLL2) do not discriminate among various motifs and motif families *in vitro*. The dissociation constants ranged from 750 nM to 50  $\mu$ M. Although the members of different motif families may possess similar binding constants, they interact through characteristically different mechanisms indicated by different thermodynamic and kinetic parameters. Moreover, we found that the affinity of dimeric motifs –due to avidity effect – can be increased by nearly three orders of magnitude.

I determined the thermodynamically optimal binding pattern of LC8 (DYNLL1) via directed evolution, namely phage display. The naive peptide library was displayed on M13 phage in a bivalent manner using a GCN4 leucine zipper, which utilizes the same avidity effect that occurs in nature. Based on the positional amino acid frequencies obtained from phage display, we predicted nearly one hundred proteins from the human proteome as potential LC8-partners. Based on this prediction, two novel partners – EML3 and ATMIN – were chosen to be validated *in vitro*.

A high affinity consensus sequence was designed based on the phage display results. The corresponding monomeric peptide has an affinity ( $K_d = 84$  nM) ten times higher than that of the hitherto known strongest binding motif identified in the Bmf protein. Dimerization through a leucine zipper further increases the affinity into the subnanomolar range. The monomeric as well as the dimeric peptides could be utilized as competitive inhibitors of LC8. The high affinity of the *in vitro* evolved motif was explained by a structural study using monomeric and dimeric peptide-LC8 complex crystals.

Our results suggest that LC8 could function as a universal dimerization hub protein forming stable complexes with proteins of pleiotropic functions. Moreover, these results significantly extend the scope of the human interactome around LC8 and will certainly shed more light on the biological functions and organizing role of LC8 in the human and other eukaryotic interactomes, as well.

## Publications

### The thesis was written based on the following articles:

Radnai L., **Rapali P.**, Hódi Z., Süveges D., Molnár T., Kiss B., Bécsi B., Erdódi F., Buday L., Kardos J., Kovács M. and Nyitray L. Affinity, avidity, and kinetics of target sequence binding to LC8 dynein light chain isoforms. *J Biol Chem*, (2010) 285, 38649-38657.

**Rapali P.**, Radnai L., Süveges D., Harmat V., Tölgyesi F., Wahlgren W.Y., Katona G., Nyitray L. and Pál G. Directed evolution reveals the binding motif preference of the LC8/DYNLL hub protein and predicts large numbers of novel binders in the human proteome. *PLoS ONE*, (2011) 6, e18818.

**Rapali P.**, García-Mayoral MF., Martínez-Moreno M., Tárnok K., Schlett K., Albar JP, Bruix M., Nyitray L., Rodriguez-Crespo I. LC8 dynein light chain (DYNLL1) binds to the C-terminal domain of ATM-interacting protein (ATMIN/ASCIZ) and regulates its subcellular localization. *BBRC*, (2011) 414(3):493-8.

### The following review was published related to the thesis:

**Rapali P.**, Szenes A., Radnai L., Bakos A., Pál G. and Nyitray L. DYNLL/LC8: A Light Chain Subunit of the Dynein Motor Complex and Beyond. *FEBS J.*, (2011) 278(17), 2980-96

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