THERMODYNAMIC, KINETIC AND STRUCTURAL STUDIES OF THE INTERACTIONS OF DYNEIN LIGHT CHAIN, A EUKARYOTIC HUB PROTEIN

PhD Thesis

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Introduction

LC8 dynein light chain (DYNLL) is a conserved eukaryotic hub protein with high number of experimentally verified binding partners. By binding to these proteins DYNLL plays different roles in various intracellular events, including apoptosis, intracellular transport, viral infection, cancer development and transcription regulation.

DYNLL has a symmetric, homodimeric structure, with two deep grooves formed at the dimerization interface. Linear binding motifs of partners located usually in unstructured protein regions bind into these identical grooves as β-strands.

It has been widely assumed that DYNLL could function as a cargo adapter on dynein and myosin Va (myoVa) motors. This role would require the simultaneous binding of the motor and the partner by one DYNLL dimer. However, most of the DYNLL partners are also dimers, or contain sequences responsible for dimerization (coiled-coils), and DYNLL-partner complexes have „dimer to dimer" structure, including myoVa and dynein intermediate chain (DIC), which is the direct interaction partner of DYNLL in the dynein complex. Taking into consideration all these facts the cargo adapter function of DYNLL seems to be unlikely. Instead, it has been suggested, based on the effect of DYNLL on its partner proteins, that one of the major roles of DYNLL dimers could be their ability to promote dimerization and stabilization of their interaction partners.

Formerly, the binding motifs were divided into three classes based on sequence similarities: $K_{-3}X_{-2}T_{-1}Q_0T_1X_2$-type sequences, $X_{-3}G_{-2}I/V_{-1}Q_0V_1D_2$-type sequences and the so called non-canonical sequences. Interestingly, members of the first and second class show only a few similarities. Non-canonical binding motifs differ significantly from the main classes, as well as from each other. It is not yet clear whether the differences between sequences can be related to different functions or not.

DYNLL has two mammalian isoforms: DYNLL1 and DYNLL2, which differ from each other only in 6 residues, localized outside of the ligand binding grooves. DYNLL1 and DYNLL2 seem to discriminate some binding partners *in vivo*, although *in vitro* studies do not support this finding. Bmf, a pro-apoptotic protein, containing KXTQTX-type binding motif, was found to be selectively bound to DYNLL2 *in vivo*, while nNOS, containing XGI/VQTD-type binding motif, is a known binding partner of DYNLL1 only.

Phosphorylation of Ser88 of DYNLL interferes with partner binding by shifting the monomer-dimer equilibrium of DYNLL strongly to the monomer state, thus eliminating the binding grooves. (This equilibrium is shifted strongly to the dimer state if DYNLL is not phosphorylated.) Pak1 kinase has previously been shown to phosphorylate DYNLL; however recent studies did not support its direct regulatory role. According to our current knowledge, DYNLL isoforms are interacting partners, but not substrates of Pak1. It is not clear yet which protein kinase is involved in this regulation.

We have previously localized the binding site of DYNLL on myosin Va, an intracellular, processive motor, involved in short-range transport of vesicles along actin filaments. The binding motif is flanked by two coiled-coil domains of the tail of the motor protein. Three residues coded by the alternatively spliced exon B of myosin Va are absolutely essential for binding. Interestingly, this non-canonical DYNLL binding motif shows a central „$T_{-1}M_0T_1$" sequence instead of the typical „$T_{-1}Q_0T_1$", or „$I_{-1}Q_0V_1$" sequences.

We have identified EML3 (or EMAP-3); a microtubule-binding protein involved in mitosis as a potential DYNLL binding partner and confirmed the interaction *in vitro*. In these studies we used phage display, an *in vitro* evolution based approach to characterize the binding motif preference of DYNLL. Using the obtained information we have also predicted novel DYNLL binding partners in the human proteome. The selection during phage display utilizes the binding affinity of the motifs. Consequently, the binding motif of EML3, which is an exact match of the phage-evolved consensus sequence ($V_{-5}S_{-4}R_{-3}G_{-2}T_{-1}Q_0T_1E_2$) binds to DYNLL with nanomolar affinity (80 nM). This is the highest affinity ever observed among DYNLL binding motifs. (For comparison, affinities usually fall into the micromolar range.) The phage-selected consensus sequence resembles the natural one ($[D/S]_{-4}K_{-3}X_{-2}[T/V/I]_{-1}Q_0[T/V]_1[D/E]_2$) with some differences. Position -5 shows probably the most important difference: while in the natural consensus no amino acid preference was observed, in the phage-selected consensus most frequently apolar side chains (or polar side chains with long aliphatic groups) were found in this position. Valine was the most characteristic residue here.

Objectives

1. **Direct, quantitative comparison of the partner binding properties of DYNLL isoforms based on thermodynamic and kinetic parameters using typical members of the KXTQTX and XGI/VQVD classes (Bmf and nNOS, respectively).** These proteins were chosen for this analysis because formerly in both cases selective binding to one DYNLL isoform was observed *in vivo*. Methods: isothermal titration calorimetry (ITC), stopped-flow spectroscopy.
2. **Structural investigation of the myosin Va-DYNLL interaction** by X-ray crystallography. We aimed to shed light on the details of this interaction: whether the non-canonical binding motif of myosin Va lays into the binding grooves of DYNLL or interacts with another molecular surface.
3. **Direct, quantitative comparison of the binding of different motifs to DYNLL based on thermodynamic and kinetic parameters by using two canonical (Bmf, nNOS) and two non-canonical (myoVa, Pak1) partners.** To discover the relationships between the motif sequence, the affinity to DYNLL and the kinetics of binding. Methods: ITC, stopped-flow spectroscopy.
4. **Investigation of the effect of partner dimerization and bivalency on DYNLL binding in terms of thermodynamics, kinetics and structure.** A dimeric fragment of myosin Va was chosen as a model system. We designed two similar, artificial model systems using a leucine-zipper as a dimerization motif fused to the DYNLL binding motif of Bmf and EML3. The former was used for thermodynamic and kinetic measurements, while the latter was necessary for X-ray crystallography. Methods: ITC, stopped-flow, surface plasmon resonance (SPR), X-ray crystallography.
5. **Investigation of the effects of phosphorylation of DYNLL Ser88 on the thermodynamics and kinetics of partner binding: is this modification an efficient way of regulation?** To mimic the phosphorylated state of DYNLL we mutated Ser88 to glutamate (S88E mutant). Bmf was used as a monomeric, while myosin Va was chosen as a dimeric model system. Methods: fluorescence anisotropy (Bmf), SPR (myoVa).
6. **Structural investigation of the EML3-DYNLL interaction** by X-ray crystallography. To reveal the structural bases behind the extremely strong interaction between DYNLL and this newly discovered motif compared to other motifs.

Methods

DNA constructs were created by general methods of gene technology. Recombinant proteins were produced using *E. coli* heterologous expression system. Proteins and synthetic peptides were purified by standard chromatographic techniques.

Thermodynamic parameters of DYNLL-partner interactions were determined by **Isothermal Titration Calorimetry (ITC)**, using a Microcal VP-ITC instrument. Model fitting was performed by Origin for ITC 5.0 software, assuming that the reaction occurs according to the „*one site binding*" model.

Surface Plasmon Resonance (SPR) experiments were carried out on a Biacore 3000 instrument. Partners of DYNLL were covalently attached to the surface, while solutions of DYNLL2 or DYNLL S88E mutant were injected in different concentrations. Data analysis was performed by BIAevaluation 3.1 software, using the most simple, 1:1 binding model.

During some titrations of Bmf, complex formation was followed by **fluorescence anisotropy** signal. Anisotropy was measured by a FLS920 spectrofluorometer (Edinburgh Instruments). Fluorescein-labelled Bmf peptide was titrated by concentration series of DYNLL2, or DYNLL2 S88E mutant. Determination of the apparent dissociation constant was performed by Origin 7.0 software, assuming a 1:1 binding model.

During transient kinetic **(stopped-flow)** measurements, the fluorescence intensity signal of Trp54, a single tryptophan residue in both isoforms of DYNLL was followed. Experiments were performed on a KinTek SF-2004 instrument. Model building, global fitting and kinetic parameter determination was carried out by KinTek Global Kinetic Explorer 2.2.563 software. Two minimal (most simple) models were built: Induced Fit (1) assumes a reversible conformational change of the DYNLL-partner complex, while Conformational Selection (2) assumes a conformational equilibrium between a binding competent and a binding incompetent state of DYNLL.

For **X-ray crystallography** only DYNLL2 was used. Crystals were grown by the hanging drop vapor diffusion method. Molecular replacement was used for structure determination of all DYNLL-partner complexes. The structures were deposited to the PDB under reference codes 2XQQ, 3P8M, and 4AEG.

Results and discussions

We compared quantitatively the binding properties of DYNLL1 and DYNLL2 isoforms to two different partners, Bmf and nNOS *in vitro*. The binding motifs of these partners can be classified into the KXTQTX and XGI/VQVD families, respectively. **DYNLL1 and DYNLL2 showed highly similar behavior in terms of the thermodynamic and kinetic constants** in the case of both partners. Interestingly, results from the literature indicated isoform specific binding *in vivo*. As the reason behind the partner preference *in vivo* is not the difference between the binding motif sequences, **we suppose the existence of an unknown „specificity determining factor" (e.g. a protein)**. **This factor could somehow modify the partner preference of DYNLL isoforms *in vivo*** (e.g. by binding selectively to one isoform).

Comparison of the binding properties of different partners was carried out by using synthetic peptides corresponding to the binding motifs of four partners (Bmf, nNOS, myoVa, Pak1) and DYNLL2. Thermodynamic and kinetic parameters were determined in each case. Sequences of these motifs belonging to different classes showed significant differences, however, **binding affinities were quite similar ($K_{d,eq}$: ~10^{-6} - 10^{-5} M)**. Interestingly, binding enthalpy, entropy and kinetic constants indicated **different binding mechanisms and interaction patterns** between DYNLL2 and the investigated partners. It is not yet clear whether there is any biological relevance of these different binding mechanisms.

Our thermodynamic and kinetic studies using a dimeric (myoVa) and two artificially dimerized (Bmf, EML3) partners showed that the **dimer state of partners increases the apparent affinity by several orders of magnitude**, compared to the respective values of the corresponding monomeric peptides. According to ITC measurements, **the stoichiometry of complex formation was 1:1** (dimer to dimer interaction). Moreover, **the kinetic base of the affinity enhancement was the decrease of the off-rate constant**, while the on-rate constant was almost unchanged. Therefore, **we concluded that bivalency causes avidity** in the DYNLL interactions by decreasing the dissociation rate constant. In general, avidity refers to the affinity enhancement phenomenon caused by multivalency; the synergistic, additive effect of several individual

interactions. **Crystal structures of the monomeric and the artificially dimerized EML3 motif in complex with DYNLL2 presented in this work demonstrate, that partner dimerization by a leucine-zipper is an adequate strategy for modeling bivalency and avidity.** This is probably true for natural dimeric partners as well, where in many cases coiled-coil sequences near the DYNLL binding motifs are responsible for dimerization (e.g. myoVa). **Slow dissociation of bivalent partners** and hence the longer lifetime of these complexes **could be ideal for DYNLL to function as a dimerization hub involved in formation of supramolecular structures and sequestration of other proteins.** If there was a competition among different proteins for interaction with DYNLL *in vivo*, the differences in motif affinities would probably be not enough to confine DYNLL binding to only some of these partners. Our results suggest that bivalency and avidity is more important in this respect. **Differences in binding motifs may thus be a "fine-tuning mechanism" in the interaction network of DYNLL.**

In order **to mimic the phosphorylation of DYNLL2**, we mutated the Ser88 residue to glutamate (DYNLL2 S88E mutant). We compared the binding properties of a monomeric (Bmf) and a dimeric (myoVa) partner to DYNLL2 S88E and wild type DYNLL2. **The mutation caused ~30 and ~50-fold weaker interaction in the case of the monomeric and dimeric partner, respectively. The affinity decrease in the case of the dimeric partner could be attributed to the decrease of the apparent on-rate constant**, while the apparent off-rate constant was almost unchanged. The low apparent k_{on} value most probably results from a very low degree of dimer formation at the applied concentration of S88E in these experiments. The monomeric form of the S88E mutant is unable to bind its partners because binding groove formation is coupled to dimerization. At the beginning of binding reactions, complex formation depletes the small amount of S88E dimers quickly. As the reaction proceeds, new dimers are formed from monomers at a slower rate. **Thus partner binding pulls the monomer-dimer equilibrium towards dimer formation.** This is also true for monomeric partners, but for sufficient stability of the resulting complex the dimeric state of the partner seems to be important, as bivalency causes avidity in the case of S88E as well. We conclude that **affecting the monomer-dimer equilibrium of DYNLL by phosphorylation of Ser88 could provide an effective way of regulation: under physiological conditions monomeric DYNLL2 could bind,**

though with diminished apparent affinity, to dimeric partners that locally have high concentration, however binding to monomeric partners is rather unlikely.

The detailed kinetic analysis of complex formation revealed, that **ligand binding of DYNLL occurs according to a two step mechanism: besides the second order association with the ligand, there is also a first order reaction. The latter can represent the conformational change of either ligand-free or ligand-bound DYNLL (conformational isomerization).** Therefore, experimental data was analyzed by globally fitting two possible minimal models: conformational selection and induced fit. Structures of both apo-DYNLL and several DYNLL-partner peptide complexes have been determined by X-ray crystallography. Comparison of these structures revealed a ligand-dependent widening of the binding grooves. In other words: ligand binding is coupled to the conformational change of DYNLL. The order of the ligand binding and the conformational change was revealed by several NMR experiments: while apo-DYNLL shows dynamic transitions, constant exchanges between multiple conformational states, the flexibility of DYNLL-peptide complexes is significantly less pronounced. Therefore, **we assume that DYNLL-ligand binding occurs mainly via the conformational selection mechanism**.

We successfully crystallized DYNLL2 in complex with a peptide corresponding to the DYNLL binding motif of myosin Va and solved the structure by X-ray diffraction. **The non-canonical binding motif of myoVa lays into the binding grooves of DYNLL.** Although there is a methionine instead of the conserved, central glutamine in the sequence, **the conformation adopted by this peptide highly resembles to the conformation adopted by canonical motifs**. The side chain of methionine and the conserved glutamine in other motifs is localized in a very similar position in the complexes, giving rise to the occurrence of almost identical interactions, except one hydrogen bond between the methionine and DYNLL. The lower affinity of myosin Va to DYNLL compared to canonical motifs can be most probably explained by this missing hydrogen bond. **The fact that binding motifs of myosin Va lay into the grooves of DYNLL in a parallel fashion supports our former results: DYNLL binds and holds the two heavy chains of myoVa in close proximity, thereby stabilizing its coiled-coil tail region.** However, it seems to be unlikely that DYNLL could have a cargo adaptor role by binding with one groove to the motor and with the other to the cargo, because in this case the motor would

be in competition with the cargo. Avidity of bivalent partners could also be hard to reconcile with the cargo-adaptor hypothesis. The structure of the complex revealed the role of the three residue long exon B of myoVa in the interaction as well. Interestingly, a few residues flanking the strictly defined binding motif gain some structure and participate in binding to some extent. It is not yet clear, whether this interaction represents a novel way of DYNLL binding, a real secondary binding site, or just an artifact.

We successfully solved the atomic resolution structures of DYNLL2 in complex with monomeric and artificially dimerized versions of a peptide corresponding to the DYNLL binding motif of EML3 by X-ray crystallography. In our models, the conformation of DYNLL highly resembles to the conformation in other known structures. Moreover, EML3 peptides are laying in the binding grooves of DYNLL as expected, while adopting similar conformations to other binding motifs. The monomeric and dimeric motifs are also highly similar. **The structural model of the dimerized EML3 motif-DYNLL complex is important evidence supporting our avidity hypothesis. The Val_{-5} residue of the binding motif contributes to the increased binding affinity (and complex stability) of EML3 by forming two backbone hydrogen-bonds with DYNLL and by forming Van-der Waals interactions with the imidazole ring of His_{68}.** The most probable explanation why nature rarely utilizes the full binding potential of position -5 can be the common bivalency and avidity among DYNLL partners. Duplicating the binding motif through dimerization causes highly enhanced complex stabilities. However, enhanced stability means less selective pressure on individual binding motifs during evolution, and this allows the appearance of lower affinity sequences on a long timescale, which are still able to reach the required complex stability, but only in a bivalent form. **The ligand binding-dependent, partially β-sheet mediated dynamic polymerization of DYNLL2-EML3 complex observed during crystallographic experiments may place the cargo adaptor hypothesis in a new context.** We suppose that the binding of individual DYNLL-partner complexes mediated by the protruding β-sheet edges on the surfaces instead of the "old" cargo adaptor hypothesis, assuming the simultaneous binding of one DYNLL dimer to the motor and the cargo. Yet the only evidence supporting this kind of polymer formation is the crystal structure of the dimeric EML3 peptide-DYNLL complex. Demonstration of the roles and *in vivo* existence of this interaction requires further research and experiments.

Related publications:

1. **Radnai, L.**; Rapali, P.; Hódi, Z.; Süveges, D.; Molnár, T.; Kiss, B.; Bécsi, B.; Erdődi, F.; Buday L.; Kardos, J.; Kovács, M.; Nyitray, L. (2010). „Affinity, avidity and kinetics of target sequence binding to LC8 dynein light chain isoforms" *J Biol Chem. 2010 Dec 3;285(49):38649-57. Epub 2010 Oct 2.*
2. Rapali, P., **Radnai, L.**, Süveges, D., Harmat, V., Tölgyesi, F., Wahlgren, W.Y., Katona, G., Nyitray, L., Pál, G. (2011) „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 Apr 18;6(4):e18818.*
3. Rapali, P., Radnai, L., Süveges, D., Harmat V., Wahlgren, Y.W., Katona, G., Nyitray, L. és Pál, G. (2010) „Az LC8 Dinein könnyűlánc kötőmotívumának jellemzése és új kölcsönható partnerek jóslása irányított evolúció segítségével." *Biokémia, (2010) 34(4), 28-34*
4. Bodor, A., **Radnai, L.**, Chazal, R., Tichy-Rács, A., Wahlgren, W.Y., Rapali, P., Süveges, D., Láng, A., Kövér, E.K., Harmat, V., Katona, G., Perczel, A. Nyitray, L. (2012) Binding of an unstructured myosin Va fragment to the LC8 dynein light chain (DYNLL) *Kézirat előkészítése folyamatban*

Other publications:

1. Hodi, Z.; Nemeth, A. L.; **Radnai, L.**; Hetenyi, C.; Schlett, K.; Bodor, A.; Perczel, A.; Nyitray, L. (2006). "Alternatively spliced exon B of myosin Va is essential for binding the tail-associated light chain shared by dynein." *Biochemistry. 2006 Oct 17;45(41):12582-95.*
2. Rapali, P., Szenes, Á., **Radnai, L.**, Bakos, A., Pál, G., Nyitray, L. (2011). „DYNLL/LC8: a light chain subunit of the dynein motor complex and beyond." *FEBS J. 2011 Sep;278(17):2980-96. Epub 2011 Aug 8. Review.*

International conference abstracts:

1. Hodi, Z; Rapali, P; **Radnai, L**; Molnar, T; Szenes, A; Kardos, J; Buday, L; Stafford, WF; Nyitray, L (2007). "The LC8 family of dynein light chains: Multifunctional chaperon-like proteins." *32nd FEBS Congress (Vienna, Austria 7–12 July, 2007); FEBS JOURNAL 274: 106-106.*
2. Bodor, A.; Láng, A.; Rapali, P.; **Radnai, L.**; Nyitray, L.; Perczel A. (2008) „Structural and dynamical characterization of an unstructured myosin fragment (M) upon binding to the dimeric dynein light chain (DLC)" *4th Central European Conference: Chemistry towards Biology (Dobogókő, Hungary September 8-11, 2008)*
3. Rapali, P.; **Radnai, L.**; Kardos, J.; Süveges, D.; Molnár, T.; Kiss, B.; Hódi, Z.; Nyitray, L. (2008) „Mechanism of DLC binding to multiple protein partners" *4th Central European Conference: Chemistry towards Biology (Dobogókő, Hungary. September 8-11, 2008)*
4. Süveges, D.; Rapali, P.; **Radnai, L.**, Wahlgren, Y.W.; Katona, G.; Nyitray, L. (2010) „Echinoderm microtubule associated protein like 3 (EML3): a novel binding partner of the dynein light chain, DYNLL" *35th FEBS Congress (Göteborg, Sweeden, 26 June - 1 July, 2010); FEBS JOURNAL 277 (Suppl. 1) 301–302 (2010)*
5. Rapali, P.; **Radnai, L.**; Harmat, V.; Wahlgren, Y.W.; Katona, G.; Hetényi, C.; Pál, G.; Nyitray, L. (2010) „Affinity enhancement of linear peptide motifs by in vitro evolution: the case of dynein light chain (DYNLL) binding peptides" *5th Central European Conference: Chemistry towards Biology (Primošten, Croatia, September 8-11, 2010)*
6. **Radnai, L.**; Rapali, P.; Tichy-Rács, A.; Hetényi, C.; Harmat, V.; Wahlgren, Y.W.; Katona, G.; Nyitray, L. (2010) "Interaction of the LC8 dynein light chain with myosin Va and EML3: structural studies" *5th Central European Conference: Chemistry towards Biology (Primošten, Croatia, September 8-11, 2010)*
7. Rapali, P.; Szenes, Á.; **Radnai, L.**; Bakos, A.; Grolmusz, V.; Pál, G; Nyitray L. (2011) „DYNLL/LC8: a hub protein that recognizes linear peptide motifs localized in disordered segments of its interaction partners." „DYNLL/LC8: a light chain subunit of the dynein motor complex and beyond." *4th European Conference on Chemistry for Life Sciences (Budapest, Hungary, 3 August – 3 September, 2011)*