MAP KINASE DOCKING INTERACTIONS:
the role of linear motifs in mediating specificity in protein kinase mediated signalling

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PhD thesis

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Budapest, 2015

INTRODUCTION

Cells react constantly to their environment through intracellular signal transduction pathways where Mitogen-activated protein kinases (MAPKs) often play a central role. The MAPK family of protein kinases constitute functionally different groups: ERK1/2 (*Extracellular signal-Regulated* Kinase 1/2), JNK1/2/3 (c-jun N-terminal Kinase), p38 isoforms (α, β, γ, δ) and ERK5. The activation loop of these kinases contains a Thr-X-Tyr motif phosphorylated by MAPK kinases (MAP2K). MAPKs are proline directed kinases because they phosphorylate Ser/Thr amino aicds followed by a proline in their substrates.

Cell functions can be affected through the phosphorylation of many substrates. In addition to MAPKAPK (MAPK activated protein kinase) family members (RSKs, MSKs, MNKs, MAPKAPKs) and different transcriptional factors (e.g. MEF2A, NFAT4), which are substrates, scaffold proteins (e.g. KSR1) and phosphatases (e.g. HePTP) may also interact with MAPKs.

In MAPK signalling pathways, substrate specificity requires so-called docking interactions. This type of interaction forms between a short linear motif and the MAPK's complementary surface groove. Docking motifs are 10-15 amino acid long protein fragments and are recognized by the MAPKs' docking groove located at the opposite side of the kinase relative to its active site. These motifs consist of one or more N-terminal basic side chains ($R/K_{(1-2)}$), a linker region ($X_{(1-5)}$) and C-terminal hydrophobic amino acids (ΦXΦ). The docking groove is comprised of an acidic common docking (CD) region and a hydrophobic groove. Docking peptides occur in many known MAPK substrates and they may also be found on activating MAPK kinases (MAP2K), deactivating phosphatases and other regulator molecules like scaffold proteins. D-motifs were discovered in the yeast MAP2K Ste7, MAPK Kss1 and Fus3 pathway first, which is analogue with the mammalian p38, JNK and ERK network's docking interactions.

Because of the lack of systematic and comparative studies, the structural basis of MAPK docking specificity is still unknown. Our purpose was to investigate the contribution of docking interactions in the formation of physiologically relevant connections within MAPK signalling networks. Here, we present a coherent structural model for MAPK D-motif specificity that explains how D-motifs can be specific or promiscuous and why previously characterized docking peptides bind to MAPKs with their reported specificity.

AIMS

My aim was to map the specificity of interactions between MAP kinases and the linear motifs of their partners. From literature it is known that there are many MAPK docking partners Because of the lack of systematic and comparative studies, the structural basis is still unknown and quantitative data on MAPK docking specificity is missing. In my PhD work I investigated the docking interactions of three MAPKs (ERK2, p38α, JNK1) and a number of peptides from previously known partner proteins. My main questions were the following:

- How can we group the peptides according to their MAPK binding modes?
- What kind of differences can be discovered in the docking groove of MAPKs?
- Can MAPK and docking peptide complex structures explain the specificity of binding profiles?

To answer these questions I proposed the followings:

- Quantitative analysis of the affinity of the 15 MAPK binding docking motifs selected from the literature by fluorescence polarisation measurements and determination of their biochemical specificity in promoting MAPK target phosphorylation.
- Determination of peptide-MAPK complexes crystal structures with peptides representing different MAPK binding specificity profiles in order to elucidate the structural basis of MAPK specific binding.
- Verification of peptide based specificity profiles with measurements by full-length proteins.

APPLIED METHODS

- The DNA constructs used for protein expression were prepared from cDNA prepared from HEK293 cells by standard biochemical techniques.
- Proteins were recombinantly expressed in Escherichia coli Rosetta (DE3) pLysS (Novagen) cells. Recombinant proteins were purified with affinity (Ni, GST) and ion exchange (Resource Q és S) chromatography techniques.

- Protein-protein binding assays for in vitro experiments:

- Pull-down experiments: Immobilized GST fusion peptides or fusion proteins (bait) were incubated with the prey proteins then the reaction compound was subjected to SDS-PAGE. GST pull-downs with MAPKs from HEK293T cell lysates were detected by Western blot.
- The Fluorescenc Polarisation (FP) measurements served to quantitatively measure the binding affinity between MAPKs and chemically synthesized peptides. For this method, reporter peptides were labelled at the N-terminus with carboxyfluorescein (CF) or tetramethylrhodamine (TAMRA) fluorescent dyes. An increase in the FP signal, which indicated complex formation between the MAPK and the labelled peptide, was monitored as a function of increasing concentration of purified MAPKs with Synergy H4 (BioTek Instruments) plate reader in 384-well plates (FC reporter: excitation/emission: 515/535nm; TAMRA reporter: 550/590nm).
- Radioactive kinase assay: Different activated kinases – constitutively activated MAP2Ks or phosphorylated MAPKs – were used in the presence of ^{32}P-γATP. The samples were subjected to SDS-PAGE, then gels phosphorimaged by Typhoon Trio scanner (GE, Amersham Biosciences) to detect the phophorylation rate of the substrates.
- Surface Plasmon resonance (SPR): The measurements were carried out on a Biacore 3000 instrument. GST fusion proteins (bait) were immobilized to the surface by anti-GST capture, while solutions of MAPKs (prey) were injected in different concentrations.
- Western-blot: This technique was used after pull-down assay from HEK293T lysate to detect preys by different antibodies. Anti-phospho MAPK, anti-GST (Cell Signaling) and anti-FLAG (Sigma) were primary and anti-rabbit was the secondary (Cell Signaling) antibodies.

– Protein-protein binding assays for cell-based experiments:
  - Bimolecular fluorescence complementation (BiFC): The two fragments of yellow fluorescence protein (YFP) was built into pcDNA3.1 vector and the transgenes were inserted and the proteins were coexpressed in HEK293T. Fluorescence intensity measurements were carried out using a Synergy H4 (BioTek Instruments) (excitation/emission: 515/535nm). Cells were imaged by confocal laser scanning microscope (Olympus IX81; Olympus FluoView 500) (excitation/emission: 514/535-560nm).

- X-ray crystallography**:** Ion exchanged proteins were used (10-15 mg/ml) for crystallization. Crystals were grown by the hanging drop vapour diffusion method. The structures were deposited to the PDB under reference codes: JNK1-pepNFAT4 (*PDB: 2XRW*); p38α-pepMKK6 (*PDB: 2Y8O*); ERK2-pepMNK1 (*PDB: 2Y9Q*), ERK2-pepRSK1 (*PDB: 3TEI*), ERK2-pepSzint_revD (*PDB: 4FMQ*).

RESULTS (THESIS)

- First we determined the binding affinity of 11 synthetically produced D-peptides to three MAPKs (ERK2, p38α, JNK1). Then we grouped them to three different classes: specific, selective and promiscuous.
- MEF2A, NFAT4 and NFAT1 transcription factor fragment phophorylation profiles were determined by in vitro kinase activity measurements, which matched the binding specificity of docking peptides determined above.
- We revealed that the binding specificity of 4 docking peptides from the C-terminus of MAPKAP kinases (MAPKAPK) corresponds to MAPK-MAPKAPK in vivo signalling specificity. These peptides bound in a reversed N- to C-terminal orientation compared to classical D-motif peptides.
- We solved the crystal structure of four MAPK-docking peptide complexes: JNK1-pepNFAT4; p38α-pepMKK6; ERK2-pepMNK1, ERK2-pepRSK1. This revealed the structural basis of MAPK-docking peptide binding specificity.
- We showed that intra-peptide hydrogen-bond staples play a pivotal role in MAPK - docking peptide specificity.
- After studying the crystal structures we planned peptides with directional selectivity, to verify the importance of certain peptide regions.
- We classified the docking peptides according to their binding modes: classical JIP1-, NFAT4-, MKK6-, HePTP-type and reverse MAPKAPK type motifs.
- Experiments with full-length proteins showed that docking peptides determine the specificity in signal transduction. However, in some cases other mechanisms may play a role to limit signalling between non-cognate interaction partners (e.g. ERK2-MKK6 and p38α-MKK1).
- We revealed the main factors that contribute to specific MAPK→MAPKAPK signalling.

CONCLUSION

Here, we probed the specificity of linear motifs binding to MAPK docking grooves in vitro to compare the biochemical specificity of these protein peptide interactions with the biological specificity of MAPKs and their signalling partners. We found that classical D-motif docking peptides efficiently distinguished only JNK from ERK and p38, and only motifs containing a reversed N- to C-terminal consensus sequence compared to classical D-motifs could discriminate between the latter two MAPKs.

Thus, peptides interacting with the MAPK docking groove conformed to two distinct consensus motif sequences: classical D-motifs and reverse D (revD)-motifs. We found that linear motifs contain consensus motif positions and variable regions. The consensus positions in the docking motifs served as anchor points that bound to common MAPK surface features and mostly contributed to docking in a non-discriminatory fashion, while the conformation of the intervening region between the anchor points mostly determined specificity.

Thus, linear motifs with different intervening region lengths and distinct compositions are specific and simple protein-protein interaction tools. Amino acids located in variable intervening regions form intra-motif hydrogen bonds or limit peptide main-chain flexibility through prolines (or both). Because of these properties, D motifs can stay short (<15 to 20 amino acids) but form specific binding with shallow protein-protein interaction surfaces. The coherent structural model of MAPK–D motif binding specificity may enable highly specific interference with various MAPK pathway–mediated physiological functions.

The specificity of artificially designed peptides can be fine-tuned, and thus, they may be used to ablate MAPK-specific signalling in vivo, even if they bind to the docking groove that is common to all MAPKs.

Our results show that small peptides can efficiently discriminate ERK, p38, and JNK docking grooves and mainly determine the signalling logic between MAPKs and their partners. These results can lead to the development of inhibitor molecules that can specifically inhibit or activate through the MAPK docking groove.

PUBLICATIONS CONCERNING THE THESIS:

Scientific publications:

- **Garai, Á.**, Zeke, A., Gógl, G., Törő, I., Fördös, F., Blankenburg, H., Bárkai, T., Varga, J., Alexa., A, Emig, D., Albrecht, M. and Reményi, A. „Specificity of Linear Motifs That Bind to a Common Mitogen-Activated Protein Kinase Docking Groove." *Science Signaling* (2012), 5 (245): ra74.
- Alexa, A., Gógl, G., Glatz, G., **Garai, Á.**, Zeke, A., Varga, J., Dudás, E., Jeszenői, N., Bodor, A., Hetényi, Cs. and Reményi, A. „Structural assembly of the signaling competent ERK2–RSK1 heterodimeric protein kinase complex." *Proc Natl Acad Sci U S A.*(2015), 112 (9): 2711-6.

International conference proceedings:

- **Garai, Á. Sz.**, Zeke, A., Gógl, G., Törő, I., Reményi, A. „Biochemical specificity of map kinase binding linear motifs." *FEBS 3+ Meeting* (Opatija, Croatia, 13-16th June, 2012)
- **Garai, Á. Sz.**, Zeke, A., Fördös, F., Gógl, G., Reményi, A. „MAP kinase – linear motif interactions restrain signaling specificity in paralogous pathways." *EMBO meeting* (Wien, Austria 10-12th September, 2011)

Hungarian conference proceedings:

- **Garai, Á**. „MAP-kinázok fehérje-fehérje kölcsönhatásainak specificitása."Scientific Students' Associations Conference (Budapest, Hungary, ELTE, TTK, 27th of november, 2009)
- Bárkai, T., **Garai, Á. Sz.**, Törő, I., Reményi, A. „MAP-kinázok specificitása - a dokkoló peptidek szerepe a jeltovábbításban." Hungarian Biochemical Society Migrant Assembly (Budapest, Hungary 23-26th August, 2009)
- Bárkai, T., **Garai, Á. Sz.**, Reményi, A. „MAPK-kaszkád elemeinek dokkoló kölcsönhatásai." Hungarian Biochemical Society Migrant Assembly (31st August -03rd September, 2008)