Role of scaffold proteins in tyrosine kinase signal transduction pathways

PhD thesis outline

Dr. Szabolcs Pesti

Doctoral School of Molecular Medicine

Semmelweis University

Supervisor: Dr. László Buday DSc., member of HAS

Official reviewers: Dr. Csilla Csortos CSc.
Dr. Tamás Németh Ph.D.

Head of the Final Examination Committee:
Dr. Zoltán Benyó DSc.

Members of the Final Examination Committee:
Dr. Gábor Pál Ph.D.
Dr. András Balla Ph.D.

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Introduction

Scaffold and adaptor proteins may play an important role in tyrosine kinase signalling pathways.

Tyrosine kinases

Tyrosine kinases are enzymes that can transfer the gamma phosphate group from ATP to tyrosine amino acid residues of proteins. There are two classes of tyrosine kinases: receptor tyrosine kinases and non-receptor tyrosine kinases.

The Eph family is the largest family of receptor tyrosine kinases. In mammals, 14 different Eph kinases have been identified so far. These receptors can be divided into two subclasses (EphB and EphA). Ligands that interact with EphA receptors are generally attached to the cell surface via glycosylphosphatidylinositol (EphrinA ligands), while those that activate EphB receptors are trans-membrane proteins (EphrinB ligands). Receptors have highly conserved extra- and intracellular domains. Eph receptors can undergo homo- as well as heterodimerisation. Following ligand interaction, Eph receptors cluster together and become signalling centers. After ligand binding tyrosine residues in the juxtamembrane region become phosphorylated allowing the intracellular tyrosine kinase to convert into its active form. This is called forward signalling. However, Ephrin ligands can also signal into their host cell, which is referred to as reverse

signalling. For example, in reverse signalling, the cytoplasmic tail of EphrinB is subject to modifications.

Eph receptor kinases and their ligands play a critical role, *inter alia*, in spine formation, maintenance and axon guiding. It has been shown earlier that EphB1/B2 double and B1/B2/B3 triple knockout mice have reduced excitatory synapse density.

Scaffold and adaptor proteins

They are defined as proteins organising signaling complexes by binding at least two signalling enzymes together and promoting their communication by proximity. When proteins are bound to scaffold proteins, their local concentration is increased; thus, their interactions are re-enforced. The term ‘adaptor protein’ is generally used for low molecular mass molecules that serve to link two functional members of a catalytic pathway.

The Cask-interactive protein (Caskin) belongs to the group of scaffold proteins. It has some well-defined protein-protein interaction domains. Caskin can interact with LAR receptor tyrosine phosphatase, Nck/Dock, neurexin2 and synaptotagmin. Our workgroup showed that it also interacts with the adaptor protein Abi2.

Tks4 and Tks5 proteins are also scaffold proteins. Tks5 was found to be expressed in podosomes in invasive cancer cells. Tks4 has emerged as a candidate scaffold molecule that has the

capability to regulate the actin cytoskeleton via Src and EGFR. Mice lacking Tks4 and patients with Frank-ter Haar syndrome (FTHS) showed similar defects. These findings establish a role for TKS4 in FTHS and embryonic development.

Nck is a typical representative of adaptor proteins. The 47-kDa cytosolic protein is devoid of intrinsic catalytic activity and contains one Src homology 2 and three Src homology 3 (SH2 and SH3) domains. The SH2 domain binds specific phosphotyrosine residues on activated receptors or their substrates, whereas the SH3 domains bind proline-rich motifs on downstream target proteins. The Nck family of adaptors are involved in the organisation of actin cytoskeleton, cell movement, and axon guidance.

SH3 domain

SH3-mediated signalling processes are mostly driven by the recognition of proline-rich sequences of other proteins. The SH3 domain is one of the best characterized protein interaction modules.

During the past two decades, tyrosine phosphorylation within the SH3 domains of several signalling proteins was discovered. Results of these studies brought substantial evidence for a significant role of phosphorylation on the well conserved tyrosines within SH3 domain hydrophobic pockets in regulating

the binding capacity of the SH3 domain and the intramolecular regulation of signalling proteins. In most cases, tyrosine phosphorylation interferes with binding of SH3 domain to its interacting partners, therefore leading to dissociation.

Objectives

In the past few years our workgroup have been intensively examining scaffold protein Caskin1. Caskin1 has been found in a yeast two-hybrid screen to bind many partners. Previously, Annamária Balázs showed that Caskin1 interacts with adaptor protein Abi2. We targeted to verify their interaction with other proteins besides Abi2.

- Hence, our first goal was to determine the interaction between Caskin1 and the adapter protein Nck.

- As a next step, we wished to map the possible interaction, and to determine the binding domains.

- Our further aim was to explore the signalling pathway. We looked for possible partners to the potential Caskin1/Nck complex. Previous results drew our attention to the EphB1 receptor tyrosine kinase Nck interaction.

- Therefore, our final goal was to detect and identify the function of the hypothetical Caskin1/Nck/EphB1 complex.

Besides Caskin1 we wished to analyse the intracellular expression and localisation of the scaffold protein Tks4 mutants causing Frank-ter Haar syndrome.

Methods

DNA constructs

GST tagged proteins were expressed in *E. coli* bacteria and were affinity purified. Fusion proteins were incubated with lysates of rat brain and transfected COS7 cells to identify binding proteins. Protein expression and purification was monitored on Coomassie blue-stained SDS–PAGE gels.

Biotinylated phosphopeptides and corresponding phosphospecific antibodies were produced by Life Technologies. Point mutants were generated using the QuickChange Mutagenesis Kit from Agilent Technologies.

Antibodies

Most of the antibodies we used were obtained from commercial sources. Bespoke monoclonal anti-Caskin1 antibody was custom made by AbDSerotec, whilst phosphospecific polyclonal antibodies were custom-synthesized by Genscript.

Cell lines, transfection and stimulation

Most of the experiments were performed on COS7 cells. Cells were cultured in Dulbecco’s Modified Eagle’s Medium containing 10% foetal bovine serum and antibiotics.

Transient transfections were carried out using the Lipofectamine reagent from Life Technologies.

For stimulation, cells were serum-starved overnight and stimulated with ephrin B1-Fc for 20 min.

Immunoprecipitation and Western Blot

Cells and rat brains were harvested using a buffer containing Triton and protease inhibitors. Immunoprecipitations were done using different Sepharose with the appropriate antibody. Lysates and immunoprecipitates were separated by SDS-PAGE and then blotted to nitrocellulose membranes. Western blot detection was carried out with HRP-conjugated secondary antibodies and an ECL detection kit.

Purification of Caskin1 SH3 domain

For structural characterisation, the SH3 protein was expressed in *E. coli*. After lysis and sonication the protein was purified in AKTA Explorer Protein Purification System (GE Healthcare). The purity of the protein was monitored by SDS-PAGE.

In vitro phosphorylation

The phosphorylation was initiated by ice-cold ATP addition to the kinase reaction mixture. The control reaction mixture did not include receptor. The incubation was carried out at 30°C for 1 h. For native gel electrophoresis, the phosphorylation of Caskin1

was performed as above, with the exception that the incubation was carried out at 15°C for the indicated times.

CD spectroscopy

Far and near UV circular dichroism (CD) spectra were measured with a Jasco J-720 spectropolarimeter using a quartz cuvette with 1 mm or 1cm path length in continuous mode with a bandwidth of 1 nm, response time of 8 s and scan speed of 20 nm/min. Background spectra without protein were collected and subtracted from spectra taken in the presence of proteins.

Modelling the 3D structure of the rat Caskin1 SH3 domain

The 3D structure of the rat Caskin1 SH3 domain was modelled using the public I-TASSER structure prediction server.

Native gel electrophoresis

Native gel electrophoresis was run in the absence of SDS and any reducing agent. The estimated isoelectric point of Caskin SH3 domain by ExPASy Bioinformatics Portal is 6.67. Consequently, the native protein is negatively charged and migrates to the positive pole.

Immunofluorescence staining

COS7 cells plated on glass cover slips were transiently transfected with different constructs. The cells were fixed,

permeabilized and blocked. Anti-V5 polyclonal rabbit and anti-α-tubulin antibody was applied. After washing, the samples were incubated with Alexa Fluor 488 (or 546) labeled anti-mouse or anti-rabbit secondary antibody. The pictures of fixed samples were acquired on an inverted confocal microscope.

Results

To confirm and characterise the interaction between Caskin1 and Nck, endogenous Caskin1 was immunoprecipitated from rat brain lysate with a monoclonal anti-Caskin1 antibody. We showed that Caskin1 interacts stably with Nck *in vivo*. To study the interaction in another system, V5 epitope-tagged Caskin1 and/or GFP-tagged Nck were transiently expressed in COS7 cells. When both proteins were co-expressed in cells, Caskin1 was capable of associating with Nck.

Secondly, we wanted to determine the required SH3 domains for the Caskin1/Nck interaction. We performed a GST pull- down assay, and we found that only all three SH3 domains containing constructs were able to pull down Caskin1.

It has been well documented that EphB1 can bind to Nck through its phosphorylated Tyr-594 residue. After we proved the Caskin1/Nck interaction, we hypothesised that Nck could mediate an interaction between EphB1 and Caskin1 and could promote their communication by proximity.

To address this question, first, EphB1 transiently expressing COS7 cells were stimulated with the receptor activating ephrin B1 ligand. We found that EphB1 alone in these cells induces activation of the kinase. Interestingly, addition of the ligand did not further increase the receptor tyrosine phosphorylation. We

could also show the known EphB1-Nck interaction. Finally, we would have liked to prove the existence of the EphB1/Nck/Caskin1 complex. In the experiment COS7 cells were transiently transfected with HA-EphB1 or/and V5-Caskin1. Our results demonstrate that Caskin1 immunoprecipitates both Nck and EphB1 in those cells wherein both constructs were expressed. Next, we precipitated the complex of Nck/Caskin1 with biotinylated phosphopeptides immobilised on Streptavidin-agarose beads. These experiments strongly suggest that the activated and autophosphorylated EphB1 receptor may recruit the complex of Nck/Caskin1.

We hypothesised that Caskin1 recruited to the activated EphB1 receptor tyrosine kinases could be subject to tyrosine phosphorylation. We found that co-expression of Caskin1 with EphB1 resulted in a significant tyrosine phosphorylation of the scaffold protein.

We used mass spectrometry to determine the site(s) of Caskin1 phosphorylation. Two tyrosine residues were identified to be phosphorylated in Caskin1 in the presence of EphB1: Y296 and Y336. Interestingly, both tyrosine residues are located within the SH3 domain of the scaffold protein.

To determine the position of the phosphorylated tyrosine residues, the 3D structure of the rat Caskin1 SH3 domain was modelled. Tyr296 is localised in the flexible RT loop that links the first two

β-strands, while Tyr336 is present in the more compact fourth β-strand.

To show definitively that Y296 and Y336 are tyrosine phosphorylated, we performed again the phosphorylation experiment with mutant constructs. Point mutations were introduced into the SH3 domain of Caskin1, changing tyrosine 296 (Y296F) and tyrosine 336 (Y336F), respectively, or both tyrosine 296 and 336 (Y296/336F) to phenylalanines.

We demonstrated, as shown earlier, that wild type Caskin1 is tyrosine phoshorylated in the presence of EphB1. However, both the Caskin1 Y296F and Y336F constructs showed markedly reduced phosphorylation levels. The lowest level of phosphorylation was detected when both tyrosine residues were mutated.

We wanted to approach the examination of the phosphorylation sites by a different way. Therefore, we performed the previous experiment under similar conditions with phosphospecific antibodies (anti-PTyr296 and anti-PTyr336).

Tyrosine phosphorylation of Caskin1 was unambiguously detected with the anti-PTyr296 antibody which signal was completely abolished when Y296F or Y296/336F constructs were used. The phosphospecific anti-PTyr336 antibody also clearly detected the phosphorylation of wild type Caskin1. This

phosphorylation level was decreased when either the Y336F or the Y296/336F constructs were applied.

Taken together, these results suggest that Caskin1, when recruited to EphB1 tyrosine kinase, is phosphorylated on tyrosine residues 296 and 336.

Lastly, we determined structural changes caused by phosphorylation. Therefore, we expressed and purified the Caskin1 SH3 domain and phosphorylated it *in vitro* by a recombinant active EphB1. In this experiment we demonstrated that EphB1 was directly responsible for the phoshorylation of Caskin1.

Next, far-UV CD spectrum was measured which was expected to reveal important characteristics of the secondary structure of a protein. No major difference was detected between the spectra of unphosphorylated and phosphorylated SH3 domains suggesting that phosphorylation of tyrosine residues does not cause significant changes in the secondary structure. Interestingly, when near-UV CD spectra were taken noticeable changes were detected upon tyrosine phosphorylation of the SH3 domain. It seems that the chemical environment of these residues is clearly sensitive to phosphorylation suggesting that tertiary structure of the SH3 domain is likely altered around the phosphorylated tyrosines.

However, it is also possible that there are associated quaternary structural changes, e.g. dimerisation that could alter the spectrum.

To exclude this possibility, time-course of Caskin1 phosphorylation was followed by using native gel electrophoresis. We demonstrated that tyrosine phosphorylated Caskin1 SH3 domain migrated faster than the unphosphorylated protein, possibly due to the incorporated negative charges. Nevertheless, the native gel electrophoresis did not show any sign of protein dimerisation or aggregation induced by EphB1-dependent phosphorylation. Therefore, we suggested that the tertiary structure of the SH3 domain is likely altered around the phosphorylated tyrosines.

We wished to check the expression and the intracellular localization of the scaffold protein Tks4 mutants described by Iqbal et al (R43W, $Tks4^{1-48}$, $Tks4^{1-341}$). We demonstrated that the shorter Tks4 mutant, $Tks4^{1-48}$ shows no expression at all. Finally, we found that a portion of $Tks4^{R43W}$ localizes at the juxtanuclear region in the aggresome, and $Tks4^{1-341}$ shows an interesting picture accumulating in the nuclei of the cells. Taken together, a lack of expression of $Tks4^{1-48}$ or aberrant intracellular expressions of $Tks4^{R43W}$ and $Tks4^{1-341}$ strongly suggest that these mutations result in dysfunctional proteins which are not capable of operating properly, leading to the development of Frank-ter Haar syndrome.

Conclusions

From our research data we came to the following major conclusions:

1. We have shown in our experiments that Caskin1 binds Nck.
2. We have determined that all three SH3 domains of Nck are necessary for the interaction with Caskin1.
3. We have shown the EphB1/Nck/Caskin1 complex.
4. Complex formation resulted in the tyrosine phosphorylation of Caskin1.
5. We have shown that Y296 and Y336 are phoshorylated and both tyrosines are localised within the SH3 domain.
6. SH3 domain is likely altered around the phosphorylated tyrosines.
7. We have demonstrated that the Tks4 mutants show abnormal expression and intracellular localizations.

Proposed model of the signalling pathway: Ligand-activated EphB1 recruits the adaptor protein Nck through the phosphorylated Y594 residue. In turn, Nck forms a complex with Caskin1 via the SH3 domains of Nck. Recruitment of Caskin1 in

the vicinity of the receptor leads to the phosphorylation of the Caskin1 SH3 domain on tyrosine residues 296 and 336.

EphB receptor kinases contribute to the dynamic reorganisation of the action cytoskeleton leading to spine morphological features. Altogether, these and our data suggest that EphB receptors recruiting the complex of Nck and Caskin1 to the plasma membrane may also contribute to the cytoskeletal organisation of dendritic spines.

Bibliography

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