Investigation of Caskin1 Scaffold Protein

Ph.D. Thesis

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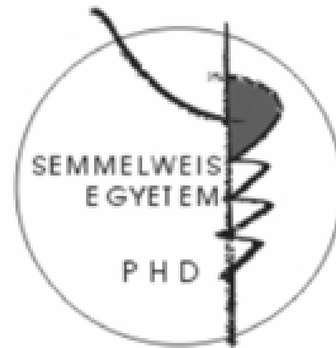

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Introduction

Most of the signalling proteins are built up of modular domains, which are able to form protein-protein interactions or have catalytic activity. My PhD study also focused on protein-protein interaction domains which I discuss briefly in my dissertation. Interactions between proteins are important for numerous biological functions. Associations result in different changes, for example translocation of a signalling protein to the site of activation, or conformational changes. The interaction of proteins may also have pathological consequence. Some protein mutations manifest in forming abnormal interactions that can reprogramme the cell.

During my PhD work I examined Caskin1 scaffold protein. Caskin1 was cloned in 2002 in Thomas Südhof's laboratory, it was found as a *Cask-in*teracting protein.

There are two isoforms of this protein, Caskin1 and Caskin2. Caskin proteins are present only in vertebrates and their structure is conserved across the species.

Caskins resemble Shank, a scaffold protein, expressed in the postsynaptic density. It seems that this kind of similarity suggests only functional similarity but not evolutionary connection. *In situ* experiments show that Caskin1 is present only in the brain, mainly in the postsynaptic density detected by immunocytochemistry.

Caskin1 contains protein-protein interaction domains. There are six ankyrin repeats at the N-terminus of the protein. The role of these repeats in formation of protein- protein interactions is unclear. There is an SH3 domain following repeats, that can bind proteins with proline rich regions. SAM domains of Caskin1 are able to form homo- or heterodimers. The C-terminal proline rich region binds SH3 domains.

Caskin1 binds to CaM kinase domain of Cask, in competition with Mint1. It was shown, that both Cask-Caskin1-Velis and Cask-Mint1-Velis complexes are present in the brain and bound to neurexins and syndecans. In contrast to ubiquitously expressed Mint1, Caskin1 is expressed only in the brain, it may regulate the formation of Cask-Mint1 complex in the brain.

Our group wished to examine the function of Caskin1. The structure of the protein suggests to be a scaffold protein, may have several interacting partners and has a role in the organization of the proteins in the PSD. We identified Caskin1-binding proteins in yeast two-hybrid test and examined the interaction with Abi2 in detail.

Abi2 was found as an Abl tyrosine kinase-binding protein and may have a role in the regulation of Abl. Abi2 is present in a WAVE inhibitor, actin cytoskeleton regulator complex with Nap1/Nap125, PIR121 and HSPC300. In 2002 Abi2 knock out mice were generated. Several abnormalities were detected in the brain and eyes. Mice are blind and have serious problems in short- and long-term memory.

We also examined the stucture of the C-terminal proline-rich region in collaboration with Peter Tompa.

Goals

Caskin1 is expressed in the postsynaptic density which is the most abundant in proteins in the organism. Being a scaffold protein, Caskin1 may have a role in the organization of the protein network in PSD. Our goal were to identify new interacting partners and lighten the role of Caskin1 in this way.

The structure of the C-terminal part of the Caskin1 is not well characterised. This half of the protein contains a long proline-rich strech. It is known, that proline brakes the protein chain and is quite rare in the well-known secondary protein structure but often present in unstuctured proteins. We proposed to examine the structure of that part of the protein using *in silico* and experimental methods.

Methods

Generation of antibodies:

There are no available Caskin1 antibody, so we had to produce it. We made two polyclonal antibodies in rabbits.

We generated human monoclonal antibody in collaboration with AbDSerotec using a recombinant method.

Anti-phospho Caskin1 antibodies were generated in collaboration with GenScript company. One of them recognizes the pThr 1065. and the another one is specific for pSer 1067.

Yeast two-hybrid test:

We identified binding partners using yeast two-hybrid method in collaboration with Hybrigenics S.A. We cloned amino acids 280-970 into B27 vector and used this construct as a bait. That fragment contains the SH3 domain, SAM domains and a part of the proline-rich region. Caskin1 was tested in a human embrional brain cDNA library.

GST-pulldown assay, immunprecipitation and Western blot:

We confirmed the interactions using GST-pulldown assay and immunprecipitation. GST tagged proteins were expressed in *E. coli* bacteria and were affinity purified. Lysates of rat brain and transfected COS7 cells were precipitated with GST tagged proteins and antibodies. Precipitated proteins were separeted by SDS-PAGE and the results were detcted by Western blot.

Structure analysis:

We analised the C-terminal proline-rich region using *in silico* and experimental methods. We used IUPred prediction programme, limited proteolysis, CD and NMR spectroscopy.

Analysis of phosphorylation:

We detected phosphorilytaion by in vitro and in vivo methods. In *in vitro* kinase assay we phosphorylated Caskin1, precipitated from transfected COS7 cells, by PKA and PKC. Phosphorylation was detected by anti-phospho antibody. For in vivo assay transfected cells were treated with eighter PKC activator TPA or PKA activator dbcAMP for 10 minutes. After treatment Caskin1 was precipitated and phosphorylation was detected also by anti-phospho-Caskin1 antibody.

Results:

We started expression and functional analysis of Caskin1, using different antibodies. Caskin1 specific antibody is not available in the market, so we had to produce that. Our polyclonal antibodies were convenient for immunoprecipitation and Western blot detection, but not for immuncytochemistry. In cooperation with AbDSerotec we generated a monoclonal antibody suitable for immunocytochemistry. To examine the phosphorylation of Caskin1 we produced two phospho-specific antibodies in cooperation with Genescript company. One of them is specific for phospho-Thr1065, and the another one is for phospho-Ser1067.

Later on we wanted to examine the interacting partners of Caskin1. The reason why we studied interacting partners for Caskin1 is that, Caskin1 is a scaffold protein havig several protein-protein interaction domains. It is expressed in the postsynaptic density of neurons and may have a role in the organizaton of that huge protein network. We choose yest two-hybrid test out of the suitable methods and cooperated with Hybrigenics company. We identified several partners and investigated the interaction with Abi2 out of them. Binding of the proteins was demonstrated using GST-precipitation and immunoprecipitation. We showed that Abi2 binds proline-rich region of Caskin1 through its SH3 domain. We managed to detect the particular interacting site in Caskin1.

During our work it has been suggested that the C-terminal part of Caskin1 is disordered. It means that, this part of the protein either does not have or partly has a secondary structure and does not have tertiary protein structure. In cooperation with the laboratory of Peter Tompa we proved this hypothesis. We further investigated whether disordered structure is a general characteristic of scaffold proteins, so we tested several docking-, adapter- and scaffold proteins using in silico method and found that disordered structure occures in high amount of scaffold proteins.

Further we examined that Caskin1 may have any posttranslational modification so we studied its phosphorylation in details. Our results show that, both protein kinase-A and protein kinase-C can phosphorylate the threonin 1065. of Caskin1.

Conclusions

Our results suggest that Caskin1 belongs to scaffold family of proteins. Our finding is also supported by following observations: 1, Caskin1 contains some protein-protein interaction domains and proline-rich region; 2, Caskin1 has no catalytic activity; 3, we have identified several Caskin1 interacting partner sin yeast two-hybrid test, for example Abi2.

We have shown that Caskin1 binds Abi2 through its proline-rich region, and proved the interaction both *in vitro* and *in vivo*. It may suggests that Caskin1 participates in the signalling pathway of Abl tyrosine kinases and in the other hand, proline-rich region takes part in the formation of protein-protein interactions. Caskin1/Abi2 complex may have a role in the formation and remodeling of dendritic spines.

Caskin1 contains well-defined protein-protein interaction domains and a structurally uncharacterised proline-rich region. We have shown that this region is intrinsically unstructured. That means, it has no secondary and tertiary protein structure. Disordered structure has some advantages for example extended interaction forming capability with other proteins, but it may have also some disadvantages, such as oncogen fusion proteins or amyloid aggregates may appear.

We examined phosphorylation of Caskin1, and showed that both PKA and PKC phosphorylate the proline-rich region of the protein. This phosphorylation may influence localization and/or interactions of the protein.

Publications

Articles related to the thesis

1, Balazs A, Csizmok V, Buday L, Rakacs M, Kiss R, Bokor M, Udupa R, Tompa K, Tompa P.

High levels of structural disorder in scaffold protein as exemplified by a novel neuronal protein, Caskin1.

FEBS J. In press (IF: 3,39)

2, Illés A, Enyedi B, Tamás P, Balázs A, Bogel G, Lukács M, Buday L.

Cortactin is required for integrin-mediated cell spreading.

Immunol Lett. 2006 Apr 15;104(1-2):124-30. Epub 2005 Dec 7.

Articles indirectly related to the thesis

1, Illés A, Enyedi B, Tamás P, Balázs A, Bogel G, Buday L.

Inducible phosphorylation of cortactin is not necessary for cortactin-mediated actin polymerisation.

Cell Signal 2006 Jun;18(6):830-40. Epub 2005 Aug 16.