The Role of the Scaffolding Protein Tks4 in EGF Signaling

PhD Theses

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1. Introduction

1.1. Structure of the Scaffolding Proteins Tks5 and Tks4

The Tks5 scaffolding protein was first identified in 1998 as a novel substrate of the non-receptor tyrosine kinase Src. It has an N-terminal PX (phox-homology) domain capable of binding membrane lipids and five SH3 domains which are well-known protein-protein interaction modules. The linker regions between the SH3 domains contain several proline rich regions which may also participate in the interaction of Tks5 with other proteins. This structure suggests that the protein may play the role of a scaffold in signaling pathways.

Tks4 was recently identified as a homolog of Tks5 with a somewhat broader expression pattern. Its structure is very similar to that of Tks5 but it has only four SH3 domains.

1.1.1. The PX domain

The PX domain was first identified in the $p40^{phox}$ and $p47^{phox}$ proteins as a lipid-binding conserved domain. Now, at least 49 PX domain-containing proteins are known in mammals. Most of these proteins are involved in the regulation of endocytic trafficking.

According to structural and functional data the most important binding partners of PX domains seem to be phosphatidylinositol 3-phosphate (PI3P), a product of PI3-kinases. PX domains have several conserved basic amino acid residues – mostly arginines – participated in

this interaction. Nevertheless, some PX domains were also shown to bind other phosphorylated inositol lipids and there are also data suggesting a role of PX domains in protein-protein interactions.

1.1.2. The SH3 domains

SH3 domains are widespread protein-protein interaction modules among signaling proteins. Although in most of the cases they bind proteins with proline-rich sequences it has also been shown that some of them bind to partners lacking such an element. An interesting possibility is the regulation SH3 domain function by phosphorylation of binding partners or the domain itself. In most of the cases this leads to a decreased binding affinity.

Biochemical data suggest that the first two SH3 domains of the Tks4 and Tks5 proteins may function together as a 'super-SH3' domain, that is they might be able to bind proteins with a common interface.

1.2. The Physiological and Pathological Roles of Tks5 and Tks4

1.2.1. Podosomes and invadopodia

Both Tks5 and Tks4 are currently best known for their roles played in actin-rich ventral extensions called podosomes and invadopodia. These structures were first observed in Src-transformed cells as subcellular structures capable of binding and degrading the extracellular matrix. Now, it is known that they are not only present in transformed cells but also in some specialized cell types, such as macrophages or

osteoclasts. Although recent reviews on the subject suggest that that the name “invadopodium” should only be used for transformed or cancerous cells while the word “podosome” should refer to their physiological counterparts, the two designations are still often used interchangeably in the literature.

Both Tks4 and Tks5 were shown to be localized to invadopodia and according to currently available data both of the proteins are also required for the formation of these invasive structures. Tks4 and Tks5 were also shown to bind several proteins present in invadopodia, such as members of the ADAM family of proteases, the cell adhesion molecule dystoglycan and the actin regulatory protein N-WASP.

It has to be noted that although invadopodia are thought to play a role in tumor invasion it has been shown experimentally that knocking down Tks5 in Src transformed cells does not effectively inhibit their capability to form metastases. However, it does lead to a significant reduction in the size of the metastases due to reduced vascularization.

1.2.2. Regulation of NADPH oxidases

As the closest relatives of Tks4 and Tks5 are proteins regulating the activity of the NADPH oxidase family of enzymes it has been investigated if these scaffolding proteins also play a role in this process. Although there are data in the literature supporting this possibility it is still not clear if these scaffolding proteins indeed contribute to the production of reactive oxygen species under physiological or pathological conditions.

1.2.3. Ontogenesis

Investigation of animal models and a rare human genetic disease suggest a role for Tks4 and Tks5 in vertebrate ontogenesis.

Mice lacking a functional Tks4 gene show a characteristic phenotype. Leading symptoms are reduced body size, cranial and other skeletal deformities, mineralization defects, glaucoma and infertility.

Very similar symptoms were observed in patients with a rare genetic disease called the Frank – ter Haar syndrome. Genetic analysis showed that 7 out of 13 families carrying the diseases had mutations in the gene coding for Tks4. Interestingly, while most of the mutations lead to the complete loss of a functional Tks4 protein, in one of the families Tks4 was only affected by a point mutation. This mutation causes the replacement of a conserved arginine in the PX domain of the protein to tryptophan.

Although the mechanism underlying these symptoms is not clear it is very likely that podosome-dependent or -independent cell migration plays an important role in the pathomechanism. It is also interesting that Tks4 was shown to be required for *in vitro* adipocyte differentiation, a function obviously independent of podosomes.

2. Major Research Goals

The cDNA of Tks4 was first cloned and its sequence deposited to the EMBL databank by Miklós Geiszt and Árpád Lányi. During their investigations they showed that Tks4 is localized to actin-rich membrane ruffles in EGF-treated mammalian cells and knocking down the gene inhibits the formation of these structures. Since the major research area of our laboratory is the investigation of EGF signaling to the actin cytoskeleton, we continued to investigate the function of Tks4 in collaboration with Miklós Geiszt's and Árpád Lányi's laboratories. The major questions we asked were the following:

1. Does Tks4 become phosphorylated upon EGF treatment of cells?
2. If yes, which kinases can be responsible for this covalent modification and which side chains can be phosphorylated?
3. What is the role of the PX domain of Tks4?
4. Does Tks4 play a role in the regulation of the actin cytoskeleton?

3. Materials and Methods

3.1. Plasmids and Constructs

The vector encoding V5-Tks4 was a kind gift of Dr. Miklós Geiszt. pGEX plasmids with the N (aa 1..334) and C terminal (aa 336..542) parts of cortactin were created using PCR based cloning. Point mutations were introduced using the QuickChange kit from Agilent Technologies.

3.2. Cell Lines and Transfection

All cell lines used were cultured in Dulbecco's Modified Eagle's Medium containing 10% fetal bovine serum and antibiotics. This was replaced with serum free mediums one night before treating cells with EGF.

Transient transfections were carried out using the Lipofectamine reagent from Invitrogen.

3.3. RNA Interference

Tks4 specific siRNA was introduced to the cells in 40 nM final concentration using the Lipofectamine RNAiMAX reagent. The control siRNA contained four point mutations relative to the effective RNA used. Knockdown of Tks4 was controlled by Western blotting 48 hours after transfection.

The siRNA resistant version of Tks4 containing five silent point mutations in the sequence recognized by the siRNA was also used.

3.4. Antibodies

Most of the antibodies used were obtained from commercial sources. The Tks4 specific polyclonal antiserum were custom made for our laboratory by immunizing rabbits with a part of Tks4 expressed as a GST fusion protein.

3.5. Immunoprecipitation and Western Blotting

Cells were harvested using a buffer containing Triton and protease inhibitors. Immunoprecipitations were done using anti-V5 Sepharose or Protein A Sepharose with the appropriate antibody.

Cell 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.

3.6. Immunofluorescent Staining

Cells were cultured in low density on glass coverslips. After transfection and appropriate treatment cells were fixed with paraformaldehyde and stained with unlabeled primary and fluorescently-labeled secondary antibodies. DAPI was used for staining cell nuclei.

Images were taken with the confocal microscope Zeiss LSM 710.

3.7. GST Fusion Proteins

GST fusion proteins were produced in *E.coli* and affinity purified with glutathione-agarose beads. The

beads were incubated with cell lysates in order to identify binding proteins.

For the *in vitro* lipid binding assay, GST fusion proteins were eluted from the glutathione-agarose beads using a buffer containing reduced glutathione.

The expression and purification of proteins were verified by SDS-PAGE and Coomassie staining.

3.8. *In Vitro* Lipid Binding

The binding of purified GST-PX domains to membrane lipids was assayed using LipidStrips from Invitrogen. The membranes were blocked with fatty acid free BSA and then incubated with the appropriate protein. Bound proteins were detected by anti-GST antibodies.

3.9. Cell Migration Assay

For our experiments we used the "QCM 24-Well Colorimetric Cell Migation Assay" kit from Millipore according to the manufacturer's instructions. 10% FBS or 200 ng/mL EGF were used as chemoattractants. Migrated cells were quantified by staining and photometry.

4. Results

4.1. Tks4 Becomes Phosphorylated and Associates with the EGF Receptor in EGF-Treated Cells

In order to assess Tks4 tyrosine phosphorylation in response to EGF treatment V5-Tks4 transfected COS7 cells were serum-starved overnight and then treated with 100ng/mL EGF for 10 minutes. V5-Tks4 was immunoprecipitated and phosphorylation was assessed by anti-phosphotyrosine Western blotting.

Interestingly, we could not only detect the phosphorylation of Tks4 but we also observed a phsophorylated protein with a molecular mass of approximately 170kDa. This band was identified as the EGF receptor using specific antibodies.

The experiment was later repeated with endogenous Tks4 immunoprecipitated with specific antiserum and gave a similar result.

4.2. The Non-Receptor Tyrosine Kinase Src Phosphorylates Tks4 upon EGF Treatment

Tks4 is a well-known substrate of the non-receptor tyrosine kinase Src. It is also known from the literature that Src is activated downstream of the EGF receptor. Therefore, Src was the most probable candidate for phosphorylating Tks4 in the investigated system. To test this hypothesis, PP1 – a specific inhibitor of Src – was used.

In our experiments we showed that pretreating cells with PP1 for one hour nearly completely prevents tyrosine phosphorylation of Tks4 upon EGF treatment.

To further verify our hypothesis, we created a mutant V5-Tks4 construct by changing all three tyrosine residues known to be phosphorylated by Src to phenylalanine. We could not detect the phosphorylation of this protein after 10 minutes of EGF treatment, what further supports our presumption that Src phosphorylates Tks4 in this setting.

4.3. Src Associates with Tks4 in EGF-stimulated Cells

In many cases Src establishes a long-term contact with its substrates. To test this possibility V5-Tks4 was immunoprecipitated from serum-starved and EGF-treated cells. After resolving the samples by SDS-PAGE we performed a Western blot using monoclonal anti-Src antibodies. Using this experimental procedure we could show that Src indeed associates with Tks4 in EGF treated but not in unstimulated cells.

4.4. Binding of the PX Domain to PI3K Lipid Products is Required for the Membrane Translocation and Phosphorylation of Tks4

According to the current literature the major binding partners of PX domains are lipid products of the PI 3-kinase. It was known from earlier experiments of Dr. Geiszt's laboratory that Tks4 is transolcated to membrane ruffles in EGF-treated cells and membrane translocation of the protein is also supported by our result concerning the association between Tks4 and the EGF receptor.

Since it is well known that PI 3-kinases are activated in EGF stimulated cells we tested if the PX domain of Tks4 contributes to its membrane translocation.

First we showed that in cells pretreated with a specific inhibitor of PI3-kinases (LY294002) Tks4 is hardly present at the plasma membrane of EGF treated cells. This suggests a role of the PX domain in membrane translocation.

As the phosphorylation of Tks4 most probably takes place close to the plasma membrane we also tested if Tks4 can be phosphorylated if PI 3-kinases are inhibited. Using immunoprecipitation and Western blotting we could show that the phosphorylation of Tks4 is inhibited by LY294002.

To further verify that an intact PX domain is required for the phosphorylation of Tks4 we showed that the mutation of two conservative arginine residues to leucine (R71,94L) prevents the phophorylation of Tks4 upon EGF treatment.

4.5. The R43W Mutant of Tks4 is not Able to Bind Membrane Lipids

The R43W mutation of Tks4 was identified in one of the investigated Frank – ter Haar syndrome families. As this point mutation lead to the presence of the same symptoms as the complete lack of the protein we decided to investigate the effects of this amino acid change.

First we expressed the wild type and R43W mutant forms of the Tks4 PX domain as GST fusion proteins. Proteins were eluted from a glutathione-agarose beads using a buffer containing reduced glutathione. PIPStrip

membranes were incubated with the proteins and than developed with anti-GST antibodies.

While the wild type form was found to be associated with several phosphorylated inositol lipids the R43W mutant showed no specific binding to any of the investigated membrane components. This result suggests that the mutation of the conserved arginine 43 leads to the loss of lipid binding by the PX domain.

4.6. The R43W Mutant Aggregates in Cells

In order to investigate the ability of the Tks4 R43W mutant to be translocated to the cell membrane where it can be phosphorylated we overexpressed the mutant protein in COS7 cells. By immunoprecipitation and Western blotting we could not detect the phosphorylation of Tks4. Interestingly, the expression of the mutant form was consequently and significantly lower than that of the wild type protein.

Using immunofluorescent staining of the overexpressed proteins we could show that the R43W mutant aggregates in most of the cells and is often found sequestered in so-called aggresomes in the proximity of the nucleus. This might also be an explanation for the seemingly lower expression of the mutant protein on Western blots.

4.7. The SH3 Domain of Cortactin is Able to Bind Tks4

As early experiments of Dr. Gieszt's group showed that Tks4 and the actin regulatory protein cortactin colocalize in EGF-induced membrane ruffles we tested the possibility of a direct interaction between the two proteins. In order to asses this we expressed N- and C-

terminal parts of cortactin as GST fusion proteins and tested their ability to bind Tks4 in cell lysates.

Using this method we could show that the C-terminal part of cortactin which posesses the SH3 domain of the protein could directly interact with Tks4. This interaction was abolished if a conserved tryptophan residue in the cortactin SH3 domain was mutated to lysine (W525K mutation).

4.8. Silencing of Tks4 Inhibits Cell Spreading

If cells in suspension are allowed to attach to a fibronectin-coated solid surface they begin to spread due to actin polymerization beneath the cell membrane, a process triggered by integrin engagement.

Together with our collaborative partners we made use of this method in order to investigate the possible role of Tks4 in the regulation of the actin cytoskeleton. HeLa cell clones expressing a Tks4 specific shRNA or ineffective scrambled shRNA were seeded to fibronectin-coated cover glasses and were allowed to spread for 30 minutes.

Measuring average cell surface areas showed that clones with reduced Tks4 levels were less spread than the control cell lines.

4.9. Silencing of Tks4 Inhibits Cell Migration

To further asses the role of Tks4 in regulating the actin cytoskeleton we tested the ability of Tks4-silenced cells to migrate in the direction of an EGF or serum gradient. Using a Boyden chamber based assay we showed that silencing of Tks4 by siRNA causes a marked reduction of

cell motility in the direction of both chemoattractants used.

To verify that this effect is indeed caused by reduced levels of Tks4 and not by off-target effects of the siRNA used we re-expressed an siRNA resistant form of Tks4 and showed that it nearly completely restored the ability of migration of the cells.

5. Conclusions

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

1. Upon EGF treatement of COS7 cells the scaffolding protein Tks4 becomes phosphorylated on tyrosine residues and associates with the EGF receptor.
2. The abovementioned tyrosine phosphorylation is very likely to be mediated by the non-receptor tyrosine kinase Src which is also associated with Tks4 in EGF-treated cells.
3. We have shown that PI 3-kinase activity is required for the membrane translocation and tyrosine phosphorylation of Tks4.
4. We conclude furthermore that the point mutation R43W which was found in a Frank – ter Haar syndrome family causes misfolding and aggregation of Tks4 in cells.
5. We have shown using RNA interference-based experiments that Tks4 is required for cell spreading and migration of HeLa cells in response to EGF and serum.

6. List of Publications

6.1. Publications Directly Concerning the Subject of the Dissertation

1. Lanyi A, Barath M, Peterfi Z, Bogel G, Orient A, Simon T, Petrovszki E, Kis-Toth K, Sirokmany G, Rajnavolgyi E, Terhorst C, Buday L, Geiszt M (2011) The homolog of the five SH3-domain protein (HOFI/SH3PXD2B) regulates lamellipodia formation and cell spreading. PLoS One 6: e23653.

2. Bogel G, Gujdar A, Geiszt M, Lanyi A, Fekete A, Sipeki S, Downward J, Buday L (2012) Frank-ter Haar syndrome protein Tks4 regulates epidermal growth factor-dependent cell migration. J Biol Chem 287: 31321-31329.

6.2. Other Publications

1. Illes A, Enyedi B, Tamas P, Balazs A, Bogel G, Buday L (2006) Inducible phosphorylation of cortactin is not necessary for cortactin-mediated actin polymerisation. Cell Signal 18: 830-840.

2. Illes A, Enyedi B, Tamas P, Balazs A, Bogel G, Melinda, Lukacs, Buday L (2006) Cortactin is required for integrin-mediated cell spreading. Immunol Lett 104: 124-130.

3. Pesti S, Balazs A, Udupa R, Szabo B, Fekete A, Bogel G, Buday L (2012) Complex formation of EphB1/Nck/Caskin1 leads to tyrosine phosphorylation and structural changes of the Caskin1 SH3 domain. Cell Commun Signal 10: 36.