The role of the scaffolding protein Tks5 in EGF signaling

PhD Thesis

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1. Introduction

The signal transduction processes between cells and within cells are long and widely researched fields of the biological sciences. The importance of the area can be easily seen, because with the understanding the complicated processes, the curability of many diseases might get into close proximity.

Signal transduction generally means a process, when the stimulus – which can be a hormone or growth factor or other - coming from the extracellular compartment activates one of the cell receptors and the cell gives a response in some sort. Changes in metabolism or gene expression, as well as actin cytoskeleton rearrangement might happen. The scaffold proteins are important participants of the intracellular signaling initiated by some activated receptors. They play a role in binding and keeping together signaling enzymes, receptors. These complexes are the functional components of signal transduction processes. This group includes the Tks protein family, whose two known members are Tks5 and Tks4.

The proteins identified as Src tyrosine kinase substrates play a role primarily in actin cytoskeleton rearrangement, podosomes/invadopodia formation. These large, multidomain proteins contain five or four SH3 domains which are well-known protein-protein interaction modules and an N-terminal PX (phox-homology) domain capable of binding membrane lipids. The Tks proteins showing diffuse localization in resting cells are phosphorylated by Src kinase and translocated to the membrane. Through binding certain actin-binding and actin polymerization initiating proteins they are involved in podosome formation necessary to the movement/migration of cells. The exact mechanism and the functional difference between Tks5 and Tks4 is remain to be elucidated.

EGFR may regulate the rearrangement of actin cytoskeleton through many signal transduction pathways such as the activation of phospholipase Cγ1 or Rho GTPases. Since the regulation through Src tyrosine kinase was also detected, therefore raised the possibility that Tks proteins may regulate the actin skeleton even via the EGF pathway. Previously, we have shown that Tks4 can modulate the actin cytoskeleton through Src and EGFR. In my dissertation I present the results which identify Tks5 as a member of the EGF signaling pathway, as well.

The mutations occurred in Tks4 gene could lead to a rare autosomal recessive disorder called Frank-ter Haar syndrome. In most cases, the mutations cause the complete loss of the protein, but in one affected family a point mutation have been described which results in the change of the conserved arginine 43 to tryptophan in the PX domain of Tks4. The symptoms
of these particular patients were indistinguishable of those who had more severe mutations leading to complete loss of Tks4 protein synthesis. I also present the results which demonstrate the isolation of mutant Tks4\textsuperscript{R43W} protein to so-called aggresomes in the proximity of the nucleus, which may explain the aforementioned phenomenon.

2. Aims

The role of Tks5 in podosome formation has been described in several publications, but their role in growth factor-induced cell motility has not yet been examined. The major research area of our laboratory is the investigation of EGF signaling pathway and the actin cytoskeleton regulation of EGF. Previously, we have shown that the Tks4 is part of the EGF signaling pathway and plays a role in the EGF-dependent reorganization of the actin cytoskeleton. Therefore we extended our investigations to Tks5 protein as well. In the first round the following questions we aimed to answer:

1. Does Tks5 become phosphorylated upon EGF treatment of cells?
2. Which kinases can be responsible for this phosphorylation?
3. Is the PX domain and the membrane contact through it necessary for the phosphorylation?
4. Where is the protein localized within the cell upon EGF treatment? Is there any translocation?

According to our preliminary experiments the related Tks4 PX domain mutant (R43W) show abnormal subcellular localization. To further examine this phenomenon our aim was to answer the following questions:

5. Where is the R43W mutant Tks4 protein localized within the cell?
6. Is the formed protein aggregate considered to be aggresome?
3. Methods

Plasmids and Constructs, Site-Directed Mutagenesis

The pcDNA3.1/V5-Topo vectors encoding V5-Tks5 and V5-Tks4 were a kind gift of Dr. Miklós Geiszt. R42A and R43W mutants were introduced using the QuickChange Site-Directed Mutagenesis Kit (from Agilent Technologies) according to the manufacturer’s instructions.

Cell Lines and Transfections

COS7 and A431 cell lines were maintained in Dulbecco’s Modified Eagle’s Medium supplemented with 10% Foetal Bovine Serum, penicillin (100 units/ml), streptomycin (100 µg/ml) and L-Glutamine (2 mM). Transient transfections of each plasmid were performed in COS7 cells using Lipofectamine reagent (from Life Technologies).

Treatment of cells with inhibitors and EGF stimulation

For stimulation, cells were serum-starved overnight and stimulated with EGF at 50 ng/ml for 10 minutes. Alternatively, cells were pre-treated with the PI3-kinase inhibitors (LY294002 at 20 µM and BKM120 at 5 µM) or the Src inhibitors (PP1 at 10 µM, PP2 at 10 µM and Src Kinase Inhibitor I at 5 µM) for 60 minutes and then stimulated with EGF as above.

Immunoprecipitation and Western Blot

COS7 and A431 cells subjected to various treatments were washed with ice-cold phosphate buffered saline (PBS) and then lysed in ice-cold lysis buffer containing Triton X-100, protease and phosphatase inhibitors. In case of transfected cells, immunoprecipitations were done using agarose-conjugated anti-V5 antibody, whereas the endogenous protein from A431 cells was collected with anti-Tks5 antibody bound to Protein A Sepharose beads. Cell lysates and precipitated complexes were separated by SDS-PAGE and then transferred to nitrocellulose membranes. Western blot detection was carried out with HRP-conjugated secondary antibodies and an ECL detection kit.
**Immunofluorescent Staining**

Cells were seeded in low density on glass coverslips. Next day cells were transfected with the appropriate constructs and subsequent day, after different treatments and washes with PBS they were fixed with paraformaldehyde. For the visualization of Tks5, cells were stained with anti-V5 primary and fluorescently-labeled secondary antibodies. Nuclei were visualized with DAPI, F-actin was stained with TRITC-phalloidin. The examination of intracellular localization of PX domain mutant Tks4 protein occurred similar as described above. Images were taken with the confocal microscope Zeiss LSM 710.
4. Results

1. **We have shown that Tks5 is tyrosine phosphorylated in response to EGF.**

   The phosphorylation of both overexpressed protein (in COS7 cells) and endogenous protein (in A431 cells) was detected. Cells were serum-starved overnight and then treated with EGF for 10 minutes. Tks5 was immunoprecipitated (with anti-V5 antibody from COS7 cells and with anti-Tks5 antibody from A431 cells) and phosphorylation was assessed by anti-phosphotyrosine Western blotting.

2. **We proved that the level of tyrosine phosphorylation of Tks5 reaches its maximum after 5 minutes of EGF treatment and its intensity is almost unchanged over a two hour time period.**

   According to the current literature, the kinetics of Tks5 phosphorylation caused by PDGF is quite slow, reaching maximal intensity two hours after stimulation. In contrast, our experiments demonstrated that the time course of protein phosphorylation after EGF treatment follows the general course of this signaling pathway. COS7 cells were transiently transfected with V5-Tks5 and stimulated with EGF for 5, 10, 30, 60 and 120-minute time periods. V5-Tks5 was immunoprecipitated and protein phosphorylation was analyzed by anti-phosphotyrosine Western blotting.

3. **We have shown that the Src tyrosine kinase is responsible for Tks5 phosphorylation upon EGF stimulation, but the interaction between the kinase and the substrate – in contrary to Tks4 – is not detectable with immunoprecipitation.**

   Tks5 is a well-known substrate for the Src tyrosine kinase. It is also known from literature that in the EGF signaling pathway members of the Src kinase family are activated. We therefore assumed that Tks5 phosphorylation upon EGF stimulus occurs via Src. To test this hypothesis, COS7 cells expressing V5-Tks5 and A431 cells were pre-treated with specific inhibitors of Src for one hour. All of the inhibitors (PP1, PP2 and Src Kinase Inhibitor I used in COS7 cells and PP1 used in A431 cells) markedly decreased the tyrosine phosphorylation of Tks5 upon EGF treatment.
Considering that Tks4 associates with Src upon EGF treatment, we also investigated if such a complex with Tks5 is also formed. V5-Tks5 was immunoprecipitated from EGF-treated COS7 cells and anti-Src Western blotting was performed. The Src tyrosine kinase was not detected in the EGF-treated sample.

4. **We confirmed that the PX domain of Tks5 protein mainly bound to the lipid products of PI3-kinase in EGF-treated cells; in the absence of these, the protein shows decreased phosphorylation.**

According to the current literature the PX domain prefers the phosphatidylinositol lipids phosphorylated in the D-3 position which are the lipid products of the PI3-kinase. Since it is well known that EGFR can activate the PI3-kinase, therefore raised the possibility that the activity of PI3-kinase is necessary for Tks5 phosphorylation. To test this hypothesis, COS7 cells expressing V5-Tks5 and A431 cells before EGF treatment were pre-treated with different inhibitors (LY294002 and BKM120) of the PI3-kinase. We found that inhibition of PI3-kinase reduced or abrogated the phosphorylation of Tks5 caused by EGF.

5. **We have demonstrated that mutation of a conserved arginine (R42A) in the PX domain leads to the loss of the lipid-binding ability of Tks5 protein, thereby prevents the phosphorylation of Tks5.**

In the PX domain of the protein the conserved arginine in position 42 was changed to alanine and this mutant protein was expressed in COS7 cells. Using immunoprecipitation and Western blot analysis we showed that in contrary to the wild type protein, the PX domain mutant Tks5 was not phosphorylated on tyrosine residues upon EGF treatment. This result confirms our findings about PI3-kinase inhibition: the membrane linkage via phosphatidylinositol lipids mediated by PX domain is necessary for the phosphorylation of Tks5 protein.
6. We have revealed that upon EGF treatment the Tks5 protein translocates to the plasma membrane, to the emerging actin-rich membrane ruffles. The pharmacological inhibition of PI3-kinase impedes this membrane recruitment only partially, while the membrane translocation of PX domain-mutant protein upon EGF-treatment fails completely.

To investigate the subcellular localization of Tks5, wild-type and PX domain-mutant (R42A) proteins were expressed in COS7 cells. After EGF and PI3-kinase inhibitor treatments, cells were studied by immunofluorescent staining and confocal microscopy. We found that upon EGF treatment the hitherto cytoplasmic Tks5 protein translocated to the plasma membrane, to the actin-rich membrane ruffles. The use of PI3-kinase inhibitors prevented the translocation of Tks5 partially, while the inactivating point mutation (R42A) in the PX domain resulted in complete abrogation of its ability to translocate to the plasma membrane upon EGF challenge.

7. We proved that the PX domain-mutant (R43W) Tks4 protein identified in Frank-ter Haar syndrome patients localized to so-called aggresomes in the proximity of the nucleus, because the Tks4\textsuperscript{R43W} shows colocalization with the HDAC6 aggresome marker and their aggregate is encaged by vimentin.

Testing the subcellular localization of different Tks4-constructs, COS7 cells were transiently transfected with V5-tagged wild-type and mutant (R43W) Tks4 or V5-empty constructs. Using immunofluorescent staining and confocal microscopy we demonstrated that wild-type Tks4 shows normal, diffuse cytoplasmic distribution while the R43W mutant Tks4 forms aggregate at the juxtanuclear region. According to the current literature misfolded proteins accumulate in so-called aggresomes after the proteasome system is saturated. In this system, the polyubiquitinated protein aggregates are delivered \textit{via} HDAC6-dependent, microtubule-based transport toward the microtubule-organizing center (MTOC) where vimentin-cage enveloping them.

The intracellular localization of HDAC6 and vimentin proteins were studied in COS7 cells expressing wild-type and R43W mutant Tks4 by immunofluorescent staining and confocal microscopy. We demonstrated that HDAC6 aggresome marker shows colocalization with the Tks4\textsuperscript{R43W} protein aggregate and vimentin filaments form a ring-like structure around the aggregate.
5. Conclusions

Our results presented in my doctoral dissertation confirm that the Tks4 related scaffold protein Tks5 is a component of the EGF signaling pathway and participates in the EGF-induced actin cytoskeleton rearrangement.

We have shown that Tks5 is tyrosine phosphorylated upon EGF treatment. Studying the kinetics of phosphorylation the general scheme of EGF signal transduction has been found, because the level of tyrosine phosphorylation of Tks5 reaches its maximum after 5 minutes and its intensity is almost unchanged over the two hour time period. This is interesting because Tks5 was reported earlier to be phosphorylated upon PDGF stimulation, but the time course of phosphorylation was quite slow, reaching maximal intensity two hours after stimulation. We have shown that Src tyrosine kinase is responsible for Tks5 phosphorylation upon EGF stimulation, as well. In addition to the high degree of similarity, several differences were revealed in the regulation of Tks5 and Tks4, which may indicate the distinct function of the two proteins too. Number of experiments demonstrated that the PX domain is instrumental for Tks5 to participate properly in the EGF signaling pathway. Based on our results, it appears that prior to the Tks5-phosphorylation via Src, phosphatidylinositol lipids phosphorylated in the D-3 position are needed to appear in the membrane and the protein through its PX domain can associate with these lipids. In other words, membrane connection predates the phosphorylation. This result contradicts the published data, according to which the phosphorylation by Src is a requirement for Tks5 to open the inactive structure. It also raises the possibility of a new, hitherto unknown regulatory step as well.

We have shown that upon EGF treatment Tks5 protein translocates to the plasma membrane, to the emerging actin-rich membrane ruffles. We have confirmed that disruption of the lipid-binding of the Tks5 protein abrogated its translocation to the membrane ruffles. Our results reveal that the Tks5 (and Tks4) proteins can participate in the formation of other structures (different from podosomes and invadopodia) accompanied with actin rearrangement e.g. membrane ruffles.

We have also demonstrated by confocal microscopy that mutating the conserved amino acid residue Arg43 to tryptophan in the PX domain of Tks4 protein leads to the production of misfolded Tks4 which is transported to aggresomes within cells. This is probably explaining the observation why this mutation leads to the same symptoms in Frank-ter Haar syndrome patients as observed in the case of the complete loss of the protein.
6. List of Publications

6.1. Publications Directly Concerning the Subject of the Dissertation


6.2. Other Publications

