

Frank-ter Haar syndrome protein Tks4 regulates EGF-dependent cell migration

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Background: The mechanism by which Tks4 regulates action cytoskeleton is largely unknown.

Results: In response to EGF treatment, Tks4 is tyrosine phosphorylated and associated with the EGF receptor.

Conclusion: The results provide a new mechanism of regulating cell migration.

Significance: Tks4 can be targeted in regulation of tumor cell migration.

SUMMARY

Mutations in the *SH3PXD2B* gene coding for the Tks4 protein are responsible for the autosomal-recessive Frank-ter Haar syndrome. Tks4, a substrate of Src tyrosine kinase is implicated in the regulation of podosome formation. Here, we report a novel role for Tks4 in the EGF signalling pathway. In EGF-treated cells, Tks4 is tyrosine phosphorylated and associated with the activated EGF receptor. This association is not direct but requires the presence of Src tyrosine kinase. In addition, treatment of

cells with LY294002, an inhibitor of PI 3-kinase, or mutations of the PX domain reduces tyrosine phosphorylation and membrane translocation of Tks4. Furthermore, a PX domain mutant (R43W) Tks4 carrying a reported point mutation in a Frank-ter Haar syndrome patient showed aberrant intracellular expression and reduced phosphoinositide binding. Finally, silencing of Tks4 was shown to markedly inhibit HeLa cell migration in a Boyden chamber assay in response to EGF or serum. Our results therefore reveal a new function for Tks4 in the regulation of growth factor-dependent cell migration.

Epidermal growth factor receptor (EGFR) is involved in diverse cellular processes, including proliferation and motility; however, it is also implicated in the development of various human cancers (1). Binding of EGF to its receptor at the plasma membrane induces dimerization of EGFR, which results in the autophosphorylation and activation of EGFR (2) A number of signaling pathways have been

identified through which EGFR may regulate rearrangement of the actin cytoskeleton, such as activation of phospholipase C γ 1 (3) and Rho GTPases (4,5). Tyrosine kinases of the Src family are also involved in transmitting signals downstream of EGFR and other receptors, and a variety of Src substrates are known to regulate actin cytoskeleton (6).

Tks5/FISH was first identified as a Src substrate possessing one phosphotyrosine (PX) domain on its N-terminus and five Src homology (SH3) domains (7). Tks5 was shown to be localized at the podosomes of Src-transformed cells and associated with some members of the ADAM metalloprotease family (8). Later, Tks5 was found to be expressed in podosomes in invasive cancer cells. In addition, Tks5 expression was required for protease-driven matrigel invasion in human cancer cells (9). In this process Nck adaptor proteins, Nck1 and Nck2, seem to link Tks5 to invadopodia actin regulation and extracellular matrix degradation (10). Finally, Tks5 has been shown to be required for migration of neural crest cell during development of zebrafish embryos (11).

Recently, a homolog of Tks5, Tks4, has been identified and shown to also influence podosomes in cells (12). Tks4 has similar domain architecture as that of Tks5, containing one PX domain and four SH3 domains (12). Tks4 was also implicated in the production of reactive oxygen species (ROS) by tumor cells (13-15), and in the differentiation of white adipose tissue (16). Intriguingly, in two independent mouse models, the absence of Tks4 resulted in abnormal development causing runted growth, craniofacial and skeletal abnormalities, hearing impairment, glaucoma and the virtual absence of white adipose tissue (17,18). In humans, the Tks4-deficiency is responsible for the development of Frank-ter Haar syndrome (18). Recently, we have shown that Tks4 is instrumental for the development of EGF-induced membrane ruffles and lamellipodia as well as for efficient cellular attachment and spreading of HeLa cells (19). In addition, it has been demonstrated that Tks4 associates with Src tyrosine kinase and cortactin, a well-established activator of Arp2/3 complex (19).

In this study we have investigated the mechanism by which Tks4 contributes to EGF signaling. We demonstrate that Tks4 is tyrosine phosphorylated and associated with

the activated EGF receptor upon EGF stimulation of cells. This association is not direct but requires the presence of Src tyrosine kinase. In addition, the PX domain is instrumental for the proper translocation of Tks4 to membrane ruffles in EGF-treated cells. A PX domain mutant (R43W) Tks4 carrying a reported point mutation in a Frank-ter Haar syndrome patient showed aberrant intracellular expression and reduced phosphoinositide binding. Finally, silencing of Tks4 was shown to markedly inhibit HeLa cell migration in response to EGF or serum. Our results therefore reveal a new function for Tks4 in the regulation of growth factor-dependent cell migration.

EXPERIMENTAL PROCEDURES

Antibodies, constructs and reagents. Antibodies against the EGF receptor (06-847) and phosphotyrosine residues (clone 4G10, 05-321) were obtained from Millipore (Billerica, MA). Antibodies against the V5 epitope (R96025 and A7345) were purchased from Invitrogen (Carlsbad, CA) and Sigma-Aldrich (St. Louis, MO), respectively. Antibodies against GST (sc-459) and Src (2109) were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) and Cell Signaling Technologies (Beverly, MA), respectively. Antibody against β -actin (A5316) was obtained from Sigma-Aldrich (St. Louis, MO). Alexa Fluor 488 goat anti-rabbit (A11008) and Alexa Fluor 488 rabbit anti-mouse (A11059) antibodies were purchased from Invitrogen (Carlsbad, CA). Generation of polyclonal anti-Tks4 antibody was described earlier (19). The V5 epitope-tagged Tks4 and PX domain of Tks4 expressed as GST fusion protein were described previously (19). V5-Tks4^{Y25,373,508F}, V5-Tks4^{R71,94L}, V5-Tks4^{R43Q}, V5-Tks4^{R43W}, as well as GST-PX^{R43W} mutants were generated using the QuickChange Site-directed Mutagenesis Kit (Stratagene, La Jolla, CA). cDNA of full length Src in mammalian expression vector pCMV6 was purchased from OriGene Technologies, Inc. Chicken Δ SH2 Src and Δ SH3 Src constructs subcloned into the mammalian expression vector pSGT were obtained from the laboratory of Giulio Superti-Furga (Vienna, Austria). Stock solutions of epidermal growth factor (EGF, Sigma-Aldrich), PP1 (Biomol, Hamburg, Germany), and LY294002 (Merck, Darmstadt, Germany)

were prepared according to the manufacturer's instructions.

Cell lines, transfection, and stimulation. A431, COS7, and HeLa cells were purchased from American Type Culture Collection and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (Invitrogen), penicillin (100 units/ml), and streptomycin (50 µg/ml). All cell lines were transiently transfected with Lipofectamine (Invitrogen) according to the manufacturer's instructions. For stimulation, cells were serum-starved overnight and stimulated with EGF at 50 ng/ml for 10 min. Alternatively, cells were pre-treated with the PI 3-kinase inhibitor LY294002 at 20 µM or the Src inhibitor PP1 at 20 µM for 60 min and then stimulated with EGF as above.

Chemoinvasion assay. Boyden chamber invasion assays were carried out on HeLa cells 48 h after Stealth siRNA transfection. The Millipore QCM Cell Migration Assay (ECM508) was used according to the manufacturer's instructions with the modification that the membrane surface of each chamber was coated with 10 µg of collagen type I (Sigma-Aldrich, C8919) for 1 h in a 37 °C incubator, washed briefly in PBS and air dried. 1.5×10^5 cells in DMEM medium were added to the upper chamber and allowed to penetrate the porous membrane (8 µm) to the bottom chamber containing EGF (100 ng/ml) or 10% FBS. Cells on the top surface of the membrane were removed after 24 h incubation, and the cells on the bottom surface were quantified.

Confocal microscopy. COS7 cells plated on glass cover slips were transiently transfected with different Tks4 constructs as indicated and then serum starved overnight. Cells were pretreated with 20µM LY294002 for 60 minutes and then treated with 50 ng/mL EGF for 10 minutes. After treatment cells were fixed in 4% paraformaldehyde-PBS for 15 minutes, permeabilized in 0.2% Triton X-100 in PBS for 5 minutes, and blocked with 1% BSA in PBS for 20 minutes. Anti-Tks4 rabbit serum was applied in 1:1000 dilution for 30 minutes. After washing with PBS the samples were incubated with Alexa Fluor 488 labelled anti-mouse secondary antibody or anti-rabbit antibody (for staining endogenous Tks4) for 30 minutes. After 40 minutes of washing with PBS cover slips were mounted onto slides in a

100 mM Tris-HCl puffer, pH 8.5, containing 10% Mowiol 4-88 (Calbiochem), 25% glycerol, and 2.5% 1,4-diazobicyclo-[2.2.2]octane (DABCO, Sigma-Aldrich). Tks4 membrane-localization was quantified by counting at least 100 cells/sample. Microscopy was performed on a Nikon Eclipse E400 fluorescence microscope or Zeiss LSM 710 confocal microscope.

Lipid binding assay. PIP strips membranes were obtained from Invitrogen (#P23750). Protein-lipid overlay experiments were performed using GST-PX (aa 1-130) and GST-PX^{R43W} fusion proteins following the manufacturer's protocol.

siRNA transfection and siRNA-resistant mutant of Tks4. Silencing of Tks4 was achieved by transfecting HeLa cells (50% confluent) with specific stealth siRNA duplex (GCCUGAUACCAAUUGAUGAAUACUG) or non-targeting (GCCGGAUACCAAUUGAUUAACAUG) stealth siRNA duplex in a final concentration of 40 nM using Lipofectamine RNAiMAX Reagent (Invitrogen). Two days after transfection, the cells were used for experiments and the knockdown was verified by western blotting of equal protein amounts of lysates from control and Tks4-silenced cells. To rescue the phenotype of Tks4 siRNA, an siRNA-resistant mutant of V5-Tks4 was created by substituting five nucleotides in the Tks4 siRNA targeting region (GCCTGATCCCCATCGATGAGTATTG).

Statistics. All quantitative results are presented as the mean and s.d. of (at least 3) independent experiments. Statistical differences between two groups of data were analyzed by Student's t-test.

RESULTS

EGF induces tyrosine phosphorylation of Tks4 and its interaction with the EGFR. We have investigated if Tks4 is involved directly in the EGF signaling pathway. Serum-starved A431 cells were stimulated with EGF for 10 min or left untreated and then endogenous Tks4 was immunoprecipitated with a polyclonal anti-Tks4 antibody. As seen in Fig 1a, Tks4 is subject of tyrosine phosphorylation in response to EGF stimulation. In addition, the activated EGFR was coimmunoprecipitated with the scaffold protein. Interestingly, a 150 kDa phosphotyrosine protein was also consistently observed in the

immunoprecipitates, although the association of this unknown protein seemed to be independent of growth factor treatment. To confirm the involvement of Tks4 in the EGF signaling pathway in another cell system, V5 epitope-tagged Tks4 was transiently overexpressed in COS7 cells, then after EGF stimulation Tks4 was immunoprecipitated with an anti-V5 antibody. Fig 1b demonstrates that in this system Tks4 is also tyrosine phosphorylated upon EGF treatment and associates with the autophosphorylated EGFR. These results establish a novel interaction between Tks4 and the EGFR, implicating Tks4 in growth factor signaling pathways.

Src is required for EGF-dependent Tks4 phosphorylation. Considering the structure of Tks4, it seemed unlikely that it could associate directly with the tyrosine kinase receptor. Since both Tks4 and its close kin Tks5 are prominent substrates of the Src tyrosine kinase implicated in podosome formation (7-9,12), we proposed that Src might function as a linker between Tks4 and the EGFR as described for many other protein complexes (20). To challenge our hypothesis, first, COS7 cells were transiently transfected with V5-Tks4 construct and then pre-treated with PP1, a specific inhibitor of Src. Following EGF treatment V5-Tks4 was immunoprecipitated and subjected to anti-phosphotyrosine immunoblot. The inhibitor markedly decreased tyrosine phosphorylation of Tks4 (Fig 2a). A previous study has shown that Src kinase phosphorylates Tks4 on three tyrosine residues (12). Therefore, we introduced point mutations into Tks4, changing tyrosines 25, 373, and 508 to phenylalanines, respectively. V5-Tks4^{Y25,373,508F} was transiently expressed in COS7 cells, then after serum starvation cells were treated with EGF. Tks4 was immunoprecipitated from lysates with anti-V5 antibody. The anti-phosphotyrosine immunoblot demonstrated that phosphorylation of the triple mutant protein is significantly decreased upon EGF treatment (Fig 2b). It is worth noting that the expression level of the triple Tks4 mutant is somewhat reduced compared to that of the wild type, nevertheless it is clearly not able to be phosphorylated, as indicated by the densitometry of the appropriate bands (Fig. 2b). Next, we examined if Src tyrosine kinase could associate with Tks4. To this end, COS7 cells were transiently transfected with V5-Tks4

construct and then stimulated with EGF or left untreated. Tks4 proteins were immunoprecipitated with anti-V5 antibody and subjected to anti-Src immunoblot. Fig 2c demonstrates that EGF treatment of the cells induced the association of Tks4 with Src. To prove that Src functions as an adaptor molecule linking EGFR to Tks4, wild type Src and Src mutants lacking either the SH2 (Δ SH2 Src) or the SH3 (Δ SH3 Src) domains were co-expressed with V5-Tks4 in COS7 cells. As Fig 2d demonstrates, expression of either Δ SH2 Src or Δ SH3 Src inhibited the association of Tks4 with EGFR, while expression of wild type Src did not interfere with the interaction. We have to note that when overexpressed all Src constructs seem to be co-immunoprecipitated with Tks4 independent of cell stimulation. These results collectively suggest that Src kinase serves as a linker between the receptor and the scaffold protein and is responsible for Tks4 phosphorylation in response to EGF stimulation.

PX domain is instrumental for Tks4 function. The family of Tks proteins possesses a Phox homology (PX) domain which can bind specific membrane lipids and is implicated in the appropriate cellular localization of Tks4 and Tks5 (7-9,12). Therefore, we asked if the PX domain is required for tyrosine phosphorylation and proper subcellular localization of Tks4. First, V5 epitope-tagged wild type Tks4 was transiently expressed in COS7 cells, and then cells expressing V5-Tks4 were stimulated with EGF or left untreated. As shown in Fig 3a, EGF-dependent phosphorylation of the scaffold protein could be inhibited by addition of the specific PI 3-kinase inhibitor LY294002. To confirm that the intact PX domain is instrumental for the adequate tyrosine phosphorylation, point mutations were introduced into the PX domain of Tks4 changing the conserved arginines 71 and 94 to leucines, as described earlier for other PX domains (21-23). Fig 3b demonstrates that the mutations introduced reduced the tyrosine phosphorylation of Tks4 despite the fact that the expression level of the mutant Tks4 in COS7 cells was somewhat lower. To further examine the roles of PX domain in the function of Tks4, subcellular rearrangement of endogenous Tks4 was studied using immunofluorescence microscopy. In quiescent COS7 cells, Tks4 showed a uniform cytoplasmic distribution,

with membrane localization in only a low proportion of cells (approx. 20%). In response to EGF, Tks4 was seen to be translocated to membrane ruffles in approximately 60% of cells (Fig 3c and d). However, when cells were pre-treated with LY294002, membrane translocation of Tks4 was strongly inhibited. These findings suggest that the functional PX domain as well as the activation of PI 3-kinase is important for the proper EGF-dependent tyrosine phosphorylation and membrane localization of Tks4.

The PX domain mutant of Tks4 is unstable intracellularly. Very recently, Iqbal and colleagues have identified a family with Frank-ter Haar syndrome whose Tks4 gene (*SH3PXD2B*) contains a substitution mutation which results in the change of the conserved arginine 43 to tryptophane in the PX domain (18). This mutation was predicted to abolish binding to phospholipids (18). We were interested to discover if the above mutation really impaired the expression and/or the function of the protein. The R43W mutant of V5 epitope-tagged Tks4 was therefore generated and expressed in COS7 cells. As shown in Fig 4a, the expression level of the mutant protein was significantly decreased compared to the wild type form. Intriguingly, upon EGF treatment, despite the low expression the mutant protein was capable of associating with the EGF receptor. It has been well established that the PX domain is required for lipid binding (24-26). To test directly whether the R43W mutation functionally inactivates the PX domain, both wild type and R43W PX domains were expressed as GST fusion proteins and examined in a protein-lipid overlay assay, as described earlier (19). Fig 4b demonstrates that while the wild type PX domain could bind to a selection of different phosphoinositides, the R43W mutant was practically unable to recognize any of those lipids. We have to note that a weak interaction of the wild type PX domain was detected with phosphatidylserine, which was not seen in our previous work (19). Finally, we analyzed the intracellular localization of mutant Tks4 proteins. In a previous study we showed that Tks4^{R43Q} mutant is able to be recruited to membrane ruffles induced by EGF (19). However, quantification of subcellular localization of this mutant protein was not performed. To compare the subcellular localization of the mutant Tks4 proteins in the

same cell type, both Tks4^{R43Q} and Tks4^{R43W} were transiently expressed in COS7 cells stimulated with EGF or left untreated. As shown in Fig. 4c and 4d, membrane localization of both mutants was detected in approximately 20% of the cells. However, in contrast to wild type Tks4, the mutant proteins were not capable of translocating to membrane ruffles induced by EGF. Intriguingly, when mutant Tks4 proteins were expressed in cells both proteins formed aggregates and accumulated around the nucleus. It has been documented that misfolded and aggregated proteins could be sequestered into specialized structures named aggresomes (27,28). Therefore, it is very likely that both Tks4 mutants are unstable intracellularly and sequestered into aggresomes. Taken together, our data suggests that the R43W mutation found in Frank-ter Haar syndrome seriously impairs the folding and the function of Tks4 leading to accelerated degradation of the protein.

Tks4 is required for EGF-dependent cell migration in HeLa cells. We have reported recently that Tks4 could associate with cortactin, an important protein implicated in cell migration (29,30), and regulate cell spreading (19). This raised the intriguing possibility that Tks4 might contribute to EGF-dependent cell migration. To analyze the role of Tks4 in cell migration, Tks4 was knocked down in HeLa cells by means of RNA interference, which resulted in a considerable degree of reduction of the protein level (Fig. 5a). Cells were then serum-starved and placed in Boyden chambers to perform the chemo-invasion assay according to the manufacturer's instructions. After challenging the cells with EGF or fetal calf serum, cell migration was quantified. As seen in Fig 5b, although EGF induced detectable cell migration, the effect of serum, possibly due to the number of growth factors present, was more prominent. Silencing of Tks4 in HeLa cells resulted in a remarkable inhibition of cell migration in both EGF- and serum-treated cells. To confirm that the inhibitory effect of Tks4 was specifically due to Tks4 silencing rather than to stress responses or other off-target effects Tks4 siRNA expressing cells were transfected with a Tks4 'rescue' expression plasmid mutated within the siRNA-targeted sequence (Fig. 5a). Expression of the mutant Tks4 cDNA restored

the ability of EGF or serum to induce cell migration (Fig. 5b).

DISCUSSION

In this paper we report a novel role for Tks4 in the EGF signaling pathway. In our previous work we showed that Tks4 was instrumental for lamellipodium and membrane ruffle formation upon EGF stimulation as well as efficient cellular attachment and spreading of HeLa cells. In addition, we have demonstrated that Tks4/HOFI could associate with cortactin, an important protein implicated in cell migration (19). Here we completed our investigation and established that Tks4 protein forms a complex with the EGF receptor and becomes tyrosine phosphorylated upon EGF stimulation. Tks4 does not contain any typical phosphotyrosine binding domain (e.g. SH2 or PTB domains) and EGF receptor does not possess also any typical proline-rich sequences suitable for interaction with any of the SH3 domains of Tks4 (<http://www.uniprot.org/uniprot/P00533>), therefore it is highly likely that the interaction between the two proteins is indirect. Furthermore, when we used EGFR purified from A431 cells for an in vitro kinase assay, as described previously (31), we could not detect EGFR-dependent tyrosine phosphorylation of Tks4 and also could not observe any direct interaction between EGFR and Tks4 (data not shown). On the other hand, upon EGF treatment of COS7 cells inducible interaction between Src tyrosine kinase and Tks4 was seen. Since the interaction of Src kinase with the EGF receptor has been well established (20), we propose that Src serves as an adaptor molecule which bridges between the EGFR and Tks4. This model is further supported by our several experiments in which the crucial role of Src kinase in the tyrosine phosphorylation of Tks4 upon EGF treatment was underlined: a, the specific Src kinase inhibitor PP1 prevented Tks4 phosphorylation; b, the triple phosphorylation mutant of Tks4, based on the phosphorylation sites of Src on Tks4 (12), was not capable of tyrosine phosphorylation in response to EGF; c, finally, expression of either Δ SH2 Src or Δ SH3 Src inhibited the association of Tks4 with EGFR. These results collectively demonstrate that Tks4 is implicated in the EGF signaling pathway by forming a complex with the EGFR. The interaction of the receptor and the scaffold

protein is not direct; Src tyrosine kinase may serve as a bridging adaptor. In addition, upon EGF treatment Src kinase can phosphorylate Tks4 on three tyrosine residues; the importance of this phosphorylation is not yet clear.

Protein-lipid interaction is a well-underlined mechanism by which eukaryotic cells regulate membrane recruitment and activation of proteins (26). The family of Tks proteins possesses a Phox homology (PX) domain which can bind specific membrane lipids and is implicated in the appropriate cellular localization of Tks4 and Tks5 (7-9,12). The PX domain of both Tks4 and Tks5 shows a very similar binding affinity, the preferred lipids are the lipid products of the PI 3-kinase (12). Here we show that the PX domain is instrumental for Tks4 to participate properly in the EGF signaling pathway. Point mutations were introduced into the PX domain of Tks4 changing the conserved arginines 71 and 94 to leucines, as described earlier for other PX domains (21-23). Intriguingly, this mutant was not able to be phosphorylated on tyrosine residues upon EGF treatment. Moreover, when cells were pretreated with the specific inhibitor of PI 3-kinase, LY294002, EGF-dependent tyrosine phosphorylation of Tks4 was also markedly inhibited. When subcellular localization of endogenous Tks4 was monitored by confocal microscopy in EGF treated cell, a significant portion of Tks4 was seen to be translocated from the cytoplasm to the plasma membrane. This effect was prevented by addition of PI 3-kinase inhibitor LY294002. Based on our findings the following model could be proposed: in quiescent cells Tks4 is predominantly localized in the cytoplasm. EGF stimulation through EGFR activates Src tyrosine kinase (20) which recruits Tks4 to the plasma membrane and phosphorylates it on three tyrosine residues. Interestingly, for proper membrane localization Tks4 requires its PX domain which can bind to the lipid products of PI 3-kinase (Fig 6). It is not unique that a regulatory protein requires two independent sites for membrane translocation. For example, the guanine nucleotide exchange factor Sos is recruited to the membrane through interactions with the SH3 domains of adaptor protein Grb2, while its PH domain binds certain phospholipids, such as lipid products of PI 3-kinase or phosphatidic acid (32,33).

Our data show that Tks4 is involved in the regulation of cell migration. Silencing of Tks4 in HeLa cells resulted in a remarkable inhibition of cell migration in both EGF- and serum-treated cells. This finding is in agreement with the previously suggested role of Tks4 in the remodeling of actin cytoskeleton, such as cell spreading (19). However, currently it is not clear how membrane-bound and tyrosine phosphorylated Tks4 regulates actin polymerization leading to membrane ruffle formation or cell migration. One possible downstream candidate is cortactin which interacts with Tks4 via its SH3 domain (19). Cortactin is a well established

activator of Arp2/3 complex which is the key regulator of the branch-chained actin polymerization (29,34,35). Interestingly, in addition to Tks4 and Tks5 scaffold proteins, cortactin is also implicated in the regulation of podosome formation (36). Therefore it is likely that cortactin recruited to the membrane via Tks4 may contribute either to the formation of branched-chain actin polymerization or to the development of podosomes. However, further experiments will be required to clarify how Tks proteins, in concert with cortactin, regulate the actin cytoskeleton.

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FOOTNOTES

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FIGURE LEGENDS

Figure 1. Tks4 is tyrosine phosphorylated and associated with the EGF receptor upon EGF treatment of cells. (a) Serum-starved A431 cells were stimulated with EGF (50 ng/ml) for 10 min, and then endogenous Tks4 was immunoprecipitated (IP) with a polyclonal anti-Tks4 antibody. After SDS-PAGE and transfer to nitrocellulose, samples were analysed by anti-phosphotyrosine, anti-EGFR, and anti-Tks4 antibodies. (b) Wild type, V5 epitope-tagged Tks4 was transiently expressed in COS7 cells and then serum-starved cells were stimulated with EGF or left untreated. Cell lysates were immunoprecipitated with anti-V5 antibody and the immunoprecipitates were immunoblotted with anti-phosphotyrosine, anti-EGFR, and anti-V5 antibodies. These results are representative of three experiments.

Figure 2. Src tyrosine kinase interacts with and phosphorylates Tks4 in response to EGF stimulation. (a) COS7 cells were transiently transfected with V5-Tks4 construct and after overnight serum-starvation cells were stimulated with EGF or left untreated. Prior to stimulation the cells were treated with the Src kinase inhibitor PP1. Tks4 was immunoprecipitated with anti-V5 antibody and subjected to anti-phosphotyrosine and anti-V5 immunoblots. (b) COS7 cells were transiently transfected with wild type V5-Tks4 or triple phosphorylation mutant (3xF) V5-Tks4^{Y25,373,508F} constructs. Lysates of serum-starved cells were then subjected to immunoprecipitation with anti-V5 antibody. Bound proteins were separated by SDS-PAGE, transferred to nitrocellulose, and probed with anti-phosphotyrosine and anti-V5 antibodies. Results of densitometry of the corresponding bands are also indicated. (c) COS7 cells were transiently transfected with V5-Tks4. Serum-starved cells were treated with EGF and Tks4 was immunoprecipitated with anti-V5 antibody. Immunoprecipitates were then immunoblotted with anti-Src and V5 antibodies. Immunoblot of lysates used in this experiment shows expression levels of Src (bottom panel). These results are typical of at least three experiments. (d) Wild type Src, Src mutants lacking either the SH2 (Δ SH2 Src) or the SH3 (Δ SH3 Src) domains were co-expressed with V5-Tks4 in COS7 cells. Serum-starved cells were challenged with EGF for 10 min

or left untreated. Tks4 was then immunoprecipitated from cell lysates and immunoprecipitates were analyzed by anti-EGFR, anti-V5, and anti-Src antibodies.

Figure 3. EGF induces PI 3-kinase-dependent plasma membrane translocation of Tks4. **(a)** COS7 cells were transiently transfected with V5-Tks4 and after serum-starvation cells were stimulated with EGF or left untreated. Prior to stimulation the cells were treated with the PI 3-kinase inhibitor LY294002. Tks4 proteins were immunoprecipitated with anti-V5 antibody and subjected to anti-phosphotyrosine and anti-V5 immunoblots. **(b)** COS7 cells were transiently transfected with wild type V5-Tks4 and V5-Tks4^{R71,94L} constructs and challenged with EGF. Cell lysates were then subjected to immunoprecipitation with anti-V5 antibody. Bound proteins were separated by SDS-PAGE, transferred to nitrocellulose, and probed with anti-phosphotyrosine and anti-V5 antibodies. Results of densitometry of the corresponding bands are also indicated. **(c)** Serum-starved COS7 cells were stimulated with EGF (B) or left untreated (A). Prior to stimulation with EGF, cells were treated with the PI 3-kinase inhibitor LY294002 (C). Cells were then fixed and processed for immunofluorescence. Endogenous expression and subcellular localization of Tks4 were detected using a Tks4-specific polyclonal antibody. Arrows indicate Tks4 present at the plasma membrane. The scale bar represents 20 μ m. **(d)** The percentage of cells with Tks4-immunoreactivity in membrane ruffles under serum-starved condition or after EGF or EGF+LY294002 treatments was quantified by an observer who was blinded to cell treatment status (n = 100 cells for each group per experiment).

Figure 4. Tks4^{R43W} mutant shows aberrant intracellular expression and reduced phosphoinositide binding. **(a)** COS7 cells were transiently transfected with wild type V5-Tks4 or V5-Tks4^{R43W} constructs and challenged with EGF. Cell lysates were then subjected to immunoprecipitation with anti-V5 antibody. Bound proteins were separated by SDS-PAGE, transferred to nitrocellulose, and probed with anti-phosphotyrosine and anti-V5 antibodies. Results of densitometry of the corresponding bands are also indicated. **(b)** The lipid-binding ability of GST-PX (left panel) and GST-PX carrying a mutation (R43W, right panel) of Tks4 was tested in protein-lipid overlay assay. Layout of the PIP strip was the following: 1. Lysophosphatidic acid 2. Lysophosphatidylcholine 3. Phosphatidylinositol (PtdIns) 4. PtdIns(3)P 5. PtdIns(4)P 6. PtdIns(5)P 7. Phosphatidylethanolamine 8. Phosphatidylcholine 9. Sphingosine 1-phosphate 10. PtdIns(3,4)P₂ 11. PtdIns(3,5)P₂ 12. PtdIns(4,5)P₂ 13. PtdIns(3,4,5)P₃ 14. Phosphatidic acid 15. Phosphatidylserine 16. Blank. **(c)** COS7 cells were transiently transfected with V5-Tks4, V5-Tks4^{R43Q} or V5-Tks4^{R43W}. After 48 h, cells were fixed and stained for Tks4 with anti-V5 antibody. Cell nuclei were visualized by DAPI staining. Arrows indicate Tks4 aggregates close to the nucleus. The scale bar represents 20 μ m. **(d)** The percentage of cells with Tks4-immunoreactivity in membrane ruffles under serum-starved condition or after EGF treatment was quantified by an observer who was blinded to cell treatment status (n = 100 cells for each group per experiment). Asterisk indicates P < 0.005.

Figure 5. Tks4 silencing inhibits EGF- and serum-dependent cell migration. **(a)** HeLa cells were transfected with control and Tks4-specific stealth siRNA duplexes as well as with siRNA-resistant V5-Tks4 (Tks4 rescue) constructs. 48 h later cells were harvested and lysates were immunoblotted with anti-Tks4 antibody. Actin was used as a loading control for cell lysates. Results of densitometry of the corresponding bands are also indicated. **(b)** HeLa cells were transfected with control, Tks4-specific stealth siRNA duplexes, and Tks4-specific siRNA duplexes together with a siRNA-resistant Tks4 construct (Tks4 rescue). Cells were then harvested and tested using a modified Boyden chamber assay as described in “Methods”. After 24 h, migrated cells were quantified according to the manufacturer’s protocol. Error bars represent the standard error of the mean, SEM, of three independent experiments. Asterisk indicates P < 0.005.

Figure 6. Proposed model of Tks4 activation upon EGF stimulation. In quiescent cells, Tks4 is basically present in the cytoplasm. In response to growth factor treatment, Tks4 is translocated to the plasma membrane through at least two independent sites: an Src-binding site and its lipid binding PX domain. The Tks4 PX domain may recognize lipid products of PI 3-kinase in the membrane. At the plasma membrane, Tks4 is tyrosine phosphorylated by Src kinase. The role of Tks4 tyrosine

phosphorylation is currently not known. Finally, Tks4 may signal toward podosome formation or the actin cytoskeleton. One of the known binding partners of Tks4 is cortactin, an important activator of the Arp2/3 complex.

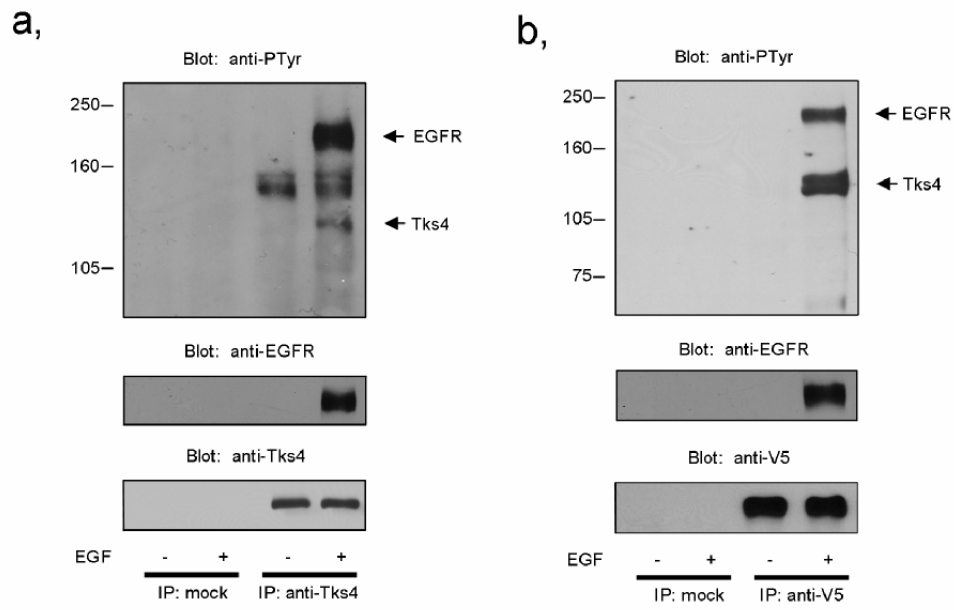
Figure 1.

Figure 2.

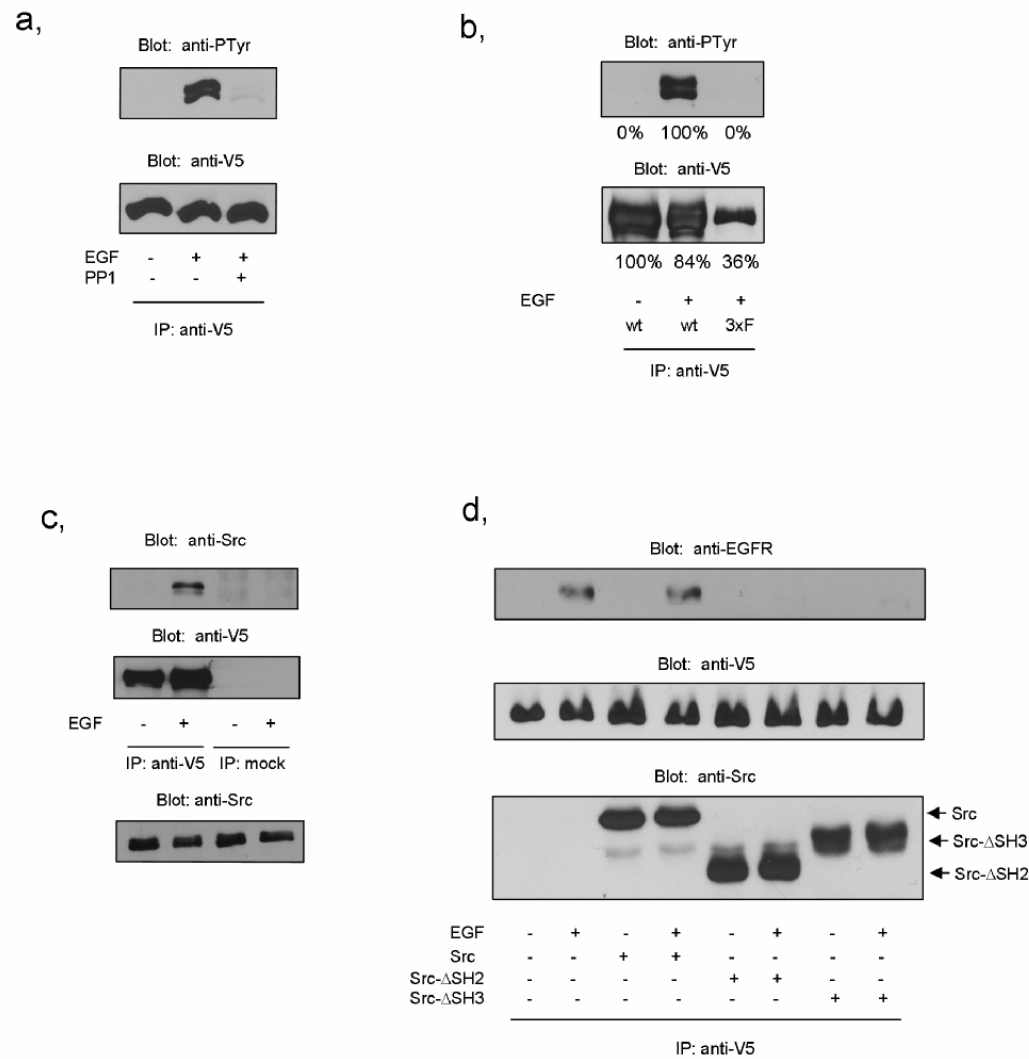


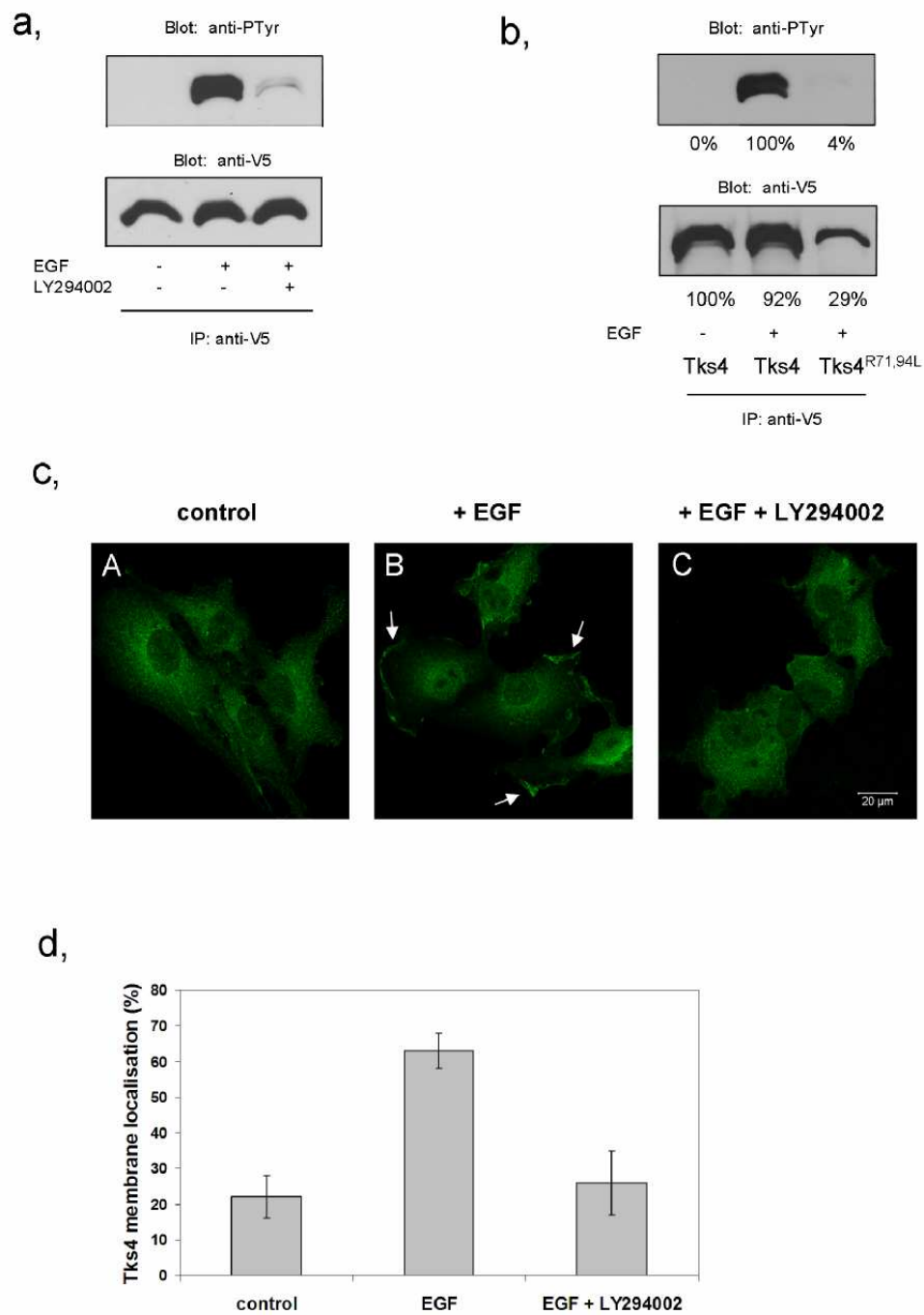
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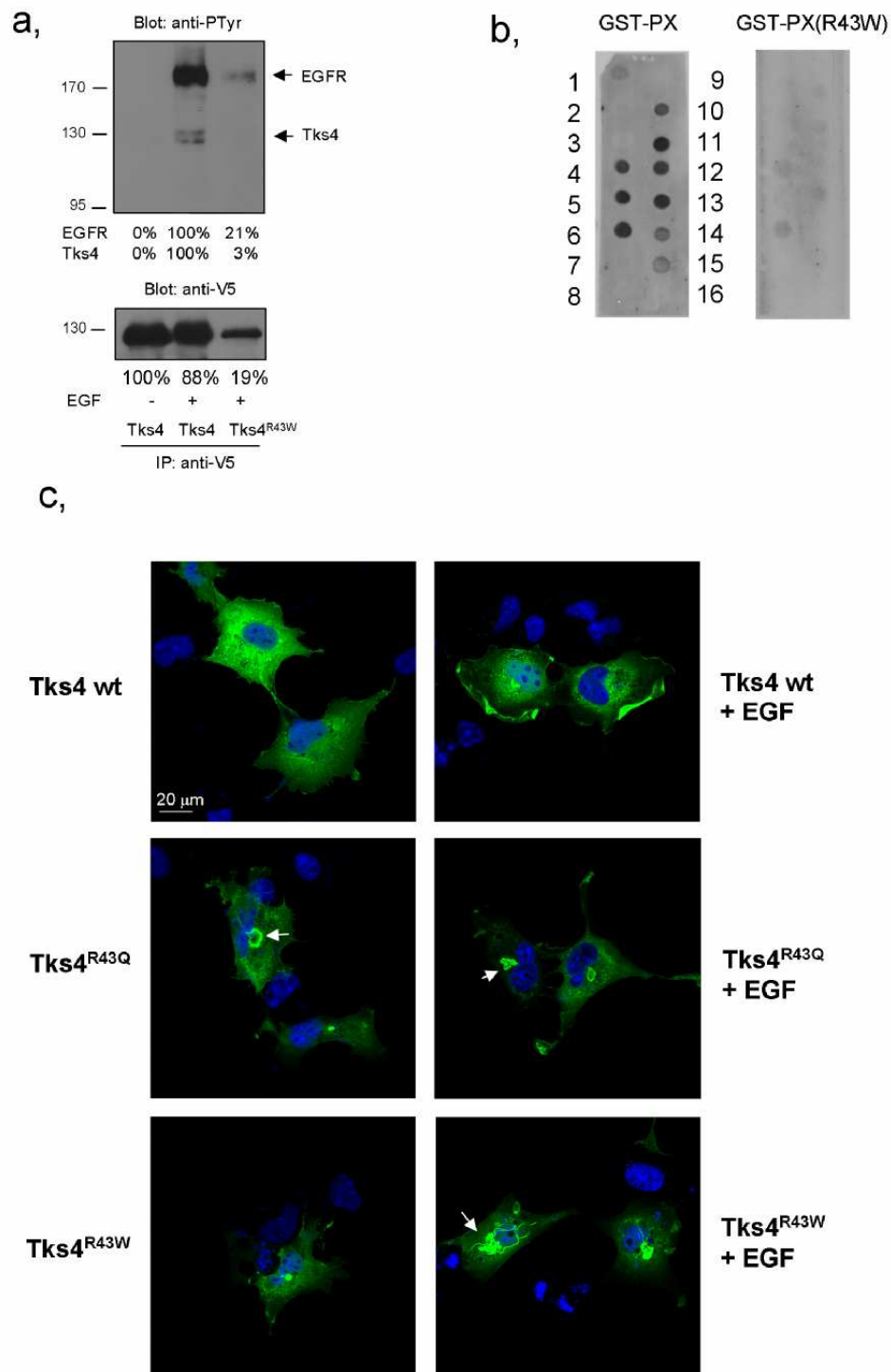
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Figure 4d.

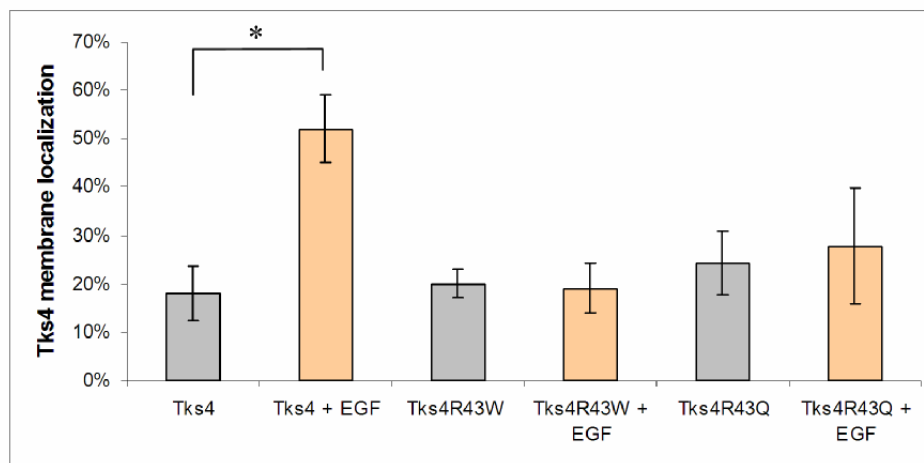


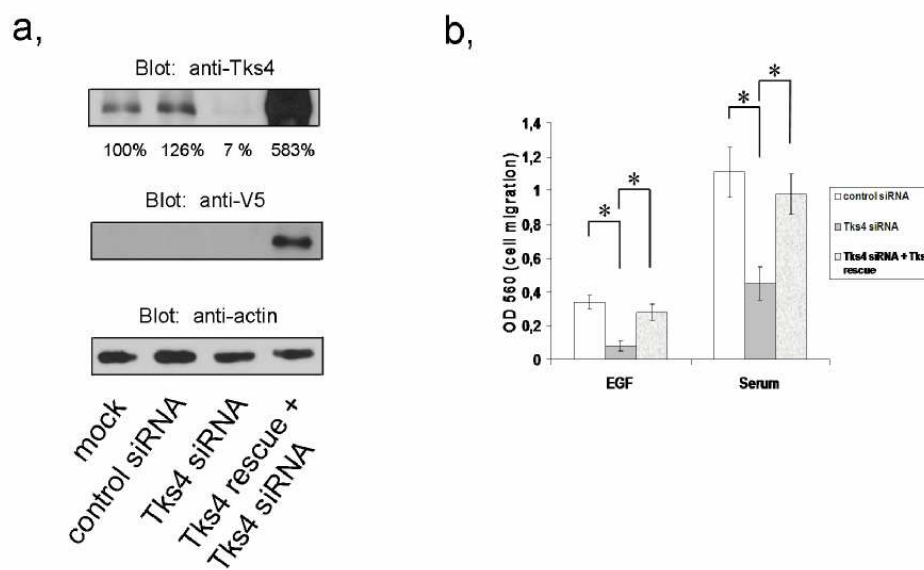
Figure 5.

Figure 6.

