

The neuronal scaffold protein Shank3 mediates signaling and biological function of the receptor tyrosine kinase Ret in epithelial cells

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Shank proteins, initially also described as ProSAP proteins, are scaffolding adaptors that have been previously shown to integrate neurotransmitter receptors into the cortical cytoskeleton at postsynaptic densities. We show here that Shank proteins are also crucial in receptor tyrosine kinase signaling. The PDZ domain-containing Shank3 protein was found to represent a novel interaction partner of the receptor tyrosine kinase Ret, which binds specifically to a PDZ-binding motif present in the Ret9 but not in the Ret51 isoform. Furthermore, we show that Ret9 but not Ret51 induces epithelial

cells to form branched tubular structures in three-dimensional cultures in a Shank3-dependent manner. Ret9 but not Ret51 has been previously shown to be required for kidney development. Shank3 protein mediates sustained Erk-MAPK and PI3K signaling, which is crucial for tubule formation, through recruitment of the adaptor protein Grb2. These results demonstrate that the Shank3 adaptor protein can mediate cellular signaling, and provide a molecular mechanism for the biological divergence between the Ret9 and Ret51 isoform.

Introduction

The Shank family of neuronal scaffolding proteins consist of three family members, Shank1 to 3, which harbor multiple protein-protein interaction sites such as ankyrin repeats, SH3, PDZ, and SAM motifs, and multiple proline-rich regions (Boeckers et al., 1999, 2002; Naisbitt et al., 1999; Tu et al., 1999; Yao et al., 1999; Sheng and Kim, 2000). Shank proteins are cytoplasmic and have been shown to function in the formation and maintenance of postsynaptic densities by integrating neurotransmitter receptors, like AMPA, NMDA and glutamate receptors, into the cortical cytoskeleton. They bind effector proteins such as PIX, IRSp53, and cortactin, which modulate signaling of small G proteins and actin assembly (Du et al., 1998; Naisbitt et al., 1999; Bockmann et al., 2002; Soltau et al., 2002; Park et al., 2003), but they are also connected to the actin cytoskeleton by interaction with α -fodrin (Boeckers et al., 2001) and the actin-binding protein Abp1, which can be recruited to dendritic spines in a Shank-dependent manner (Qualmann et

al., 2004). Shank proteins also link cell surface receptors to intracellular calcium stores by interaction with the scaffolding protein Homer (Tu et al., 1999; Sala et al., 2001). The so far-described protein-protein interactions suggest that Shanks act mainly as dynamic cytoskeletal adaptors in neurons and do not play an active role in signal transduction. A function of Shank proteins in nonneuronal cells has not been described.

The Ret receptor tyrosine kinase is crucial for development of enteric nervous system and mammalian kidney, as targeted deletion of Ret in mice leads to loss of enteric ganglia and severe kidney hypodysplasia or aplasia caused by a failure of ureteric bud outgrowth (Romeo et al., 1994; Schuchardt et al., 1994; Smith et al., 1994). Tubular outgrowth of the ureteric bud epithelium is regulated by signals emanating from surrounding metanephric mesenchyme (Saxen and Sariola, 1987; Sariola and Sainio, 1997), e.g., glial cell line-derived neurotrophic factor (GDNF). GDNF activates Ret at the tips of ureteric bud epithelia (Sanchez et al., 1996; Enomoto et al., 1998; Baloh et al., 2000; Vainio and Lin, 2002), where Ret is expressed in two major splice variants, Ret9 and Ret51, which differ only in their COOH-terminal amino acids (Tahira et al., 1990). Ret9 is of particular importance for both kidney and enteric nervous system development, as severe kidney agenesis and loss of

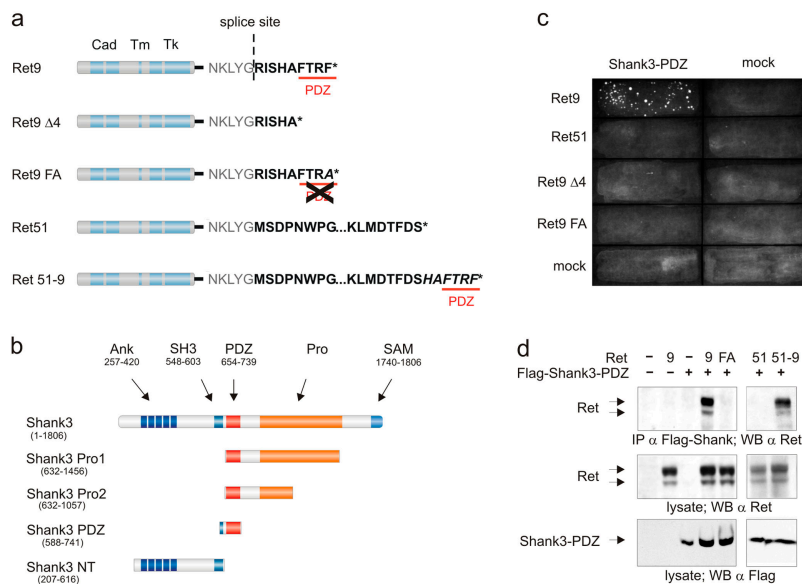
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Abbreviations used in this paper: GDNF, glial cell line-derived neurotrophic factor; HGF/SF, hepatocyte growth factor/scatter factor; MEN, multiple endocrine neoplasia; PI3K, phosphatidylinositol-3-kinase; sGFR α 1, soluble coreceptor GFR α 1.

Figure 1. **Ret9 interacts with the Shank3 PDZ domain.**

(a) Schematic representation of Ret9, Ret51, and of COOH-terminal mutants. Cad, cadherin domain; Tm, transmembrane domain; Tk, tyrosine kinase domain; Ret9 $\Delta 4$, deletion of the PDZ-binding motif; Ret9 FA point mutation in the PDZ-binding motif; Ret51-9, Ret9 PDZ-binding motif attached to Ret51. (b) Domain structure of Shank3 and Shank3 deletion mutants. Ank, ankyrin repeats; SH3, src homology 3 domain; Pro, proline-rich region; SAM, sterile alpha motif. (c) Interaction of Ret9 and Shank3 in a yeast two-hybrid analysis. Binding of the GAL4-binding domain–Shank3 PDZ domain fusion protein (or of the GAL4-binding domain alone) to GAL4-activation domain fusion proteins of the cytoplasmic domains of Ret9, Ret51, Ret9 $\Delta 4$, and Ret9 FA was tested. Growth of yeast on selective medium is shown. (d) Interaction of Ret9 and Shank3 in mammalian cells. HEK293 cells were cotransfected with the indicated Ret mutants and Flag-tagged Shank3–PDZ. Coimmunoprecipitation with Flag–M2 Sepharose was performed followed by SDS-PAGE and Western blotting with the indicated antibodies.



enteric ganglia of Ret-null mutant mice can be rescued through reexpression of Ret9, but not of Ret51 (Srinivas et al., 1999; de Graaff et al., 2001). Gain-of-function mutations of Ret in human patients are associated with various inherited cancer syndromes leading to neuroendocrine tumor formation, such as multiple endocrine neoplasia (MEN 2A and MEN 2B) and familial medullary thyroid carcinoma (Jhiang, 2000). Patients with MEN 2A and MEN 2B mutations also show renal dysplasia (Lore et al., 2000, 2001; McIntyre et al., 2003).

Activation of Ret by GDNF in the presence of its cognate coreceptor, GFR α 1, leads to autophosphorylation of tyrosine residues in the cytoplasmic domain of Ret and subsequent recruitment of signaling mediators such as PLC γ and the adaptor proteins Grb2, Shc, FRS-2, and dok1-5 (Borrello et al., 1996; Arighi et al., 1997; Alberti et al., 1998; Grimm et al., 2001; Kurokawa et al., 2001). Grb2 can recruit Sos and signaling complexes mediated by the Gab adaptor proteins, containing the tyrosine phosphatase SHP-2 and phosphatidylinositol-3-kinase (PI3K; Hayashi et al., 2000). These adaptors interact with either of the two Ret isoforms. Binding of adaptor proteins to Ret9 and Ret51 leads to activation of the Erk–MAPK and PI3K pathways (van Weering and Bos, 1998) as well as to cytoskeletal reorganization through activation of Rac (Fukuda et al., 2002). However, the signaling mechanisms that underlie the functional differences between Ret9 and Ret51 during embryonic development remained unclear.

We have here identified a new signaling complex of the Ret tyrosine kinase, involving the Shank family of neuronal adaptors. Complex formation depends on the direct interaction of the Shank3 PDZ domain with a novel PDZ-binding motif in the Ret9 isoform. This complex mediates tubulogenesis of kidney epithelial cells in vitro, which mimics ureteric bud branching during kidney development (Sachs et al., 1996; Pollack et al., 1998). By recruiting further signaling mediators, Shank3 induces sustained signaling by the Erk–MAPK and PI3K pathways. Our findings reveal a novel function for Shank proteins in signal transduction of tyrosine kinase receptors, and provide a molecular mechanism for the divergent functions of Ret9 and Ret51.

Results

Identification of Shank proteins as Ret interaction partners

The protooncogene Ret is expressed in two splice variants, Ret9 and Ret51, which differ only in carboxyl-terminal residues. 9 additional amino acids are present in Ret9; 51 entirely different amino acids in Ret51 (Fig. 1 a). To identify signaling effectors downstream of the functionally essential Ret9 isoform, we performed yeast two-hybrid screens with the entire cytoplasmic domain of Ret9 as a bait against a mouse embryonic cDNA library (Weidner et al., 1996). From these screens, we isolated cDNA clones encoding the PDZ domain of Shank3, e.g., amino acids 588–741 (Fig. 1 b). Shank proteins are scaffolding adaptors, which contain several domains such as ankyrin repeats, SH3, PDZ, and SAM domains, and multiple proline-rich regions (Boeckers et al., 1999; Naisbitt et al., 1999; Tu et al., 1999; Yao et al., 1999). The Shank3 PDZ domain binds exclusively to Ret9 and not to Ret51, as shown by yeast two-hybrid analysis (Fig. 1 c). Upon sequence analysis, we observed that Ret9, but not Ret51, harbors a putative PDZ-binding motif (FTRF) at the very COOH terminus (Fig. 1 a). Deletion of this motif (Ret9 $\Delta 4$) or mutation of the crucial COOH-terminal phenylalanine to alanine (Ret9 FA; Borg et al., 2000) prevents interaction with Shank3 (Fig. 1, a and c), demonstrating that Shank3 interacts with Ret9 through the newly identified PDZ-binding motif.

We confirmed this interaction by coimmunoprecipitation of Ret9 with the PDZ domain of Shank3 in HEK293 cells. Indeed, only Ret9 containing an intact PDZ-binding motif, and not Ret9 FA or Ret51, could be coimmunoprecipitated with the Shank3 PDZ domain (Fig. 1 d). Furthermore, Shank3 binding could be reconstituted by fusion of the Ret9 PDZ-binding motif to the COOH terminus of Ret51 (Ret51-9; Fig. 1, a and d). As expected, this interaction is not dependent on tyrosine phosphorylation of Ret, as Shank still binds to a kinase-dead Ret9 mutant (unpublished results). These results demonstrate that

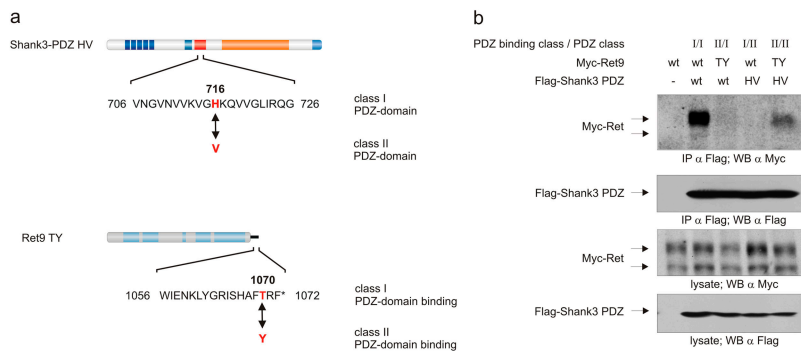
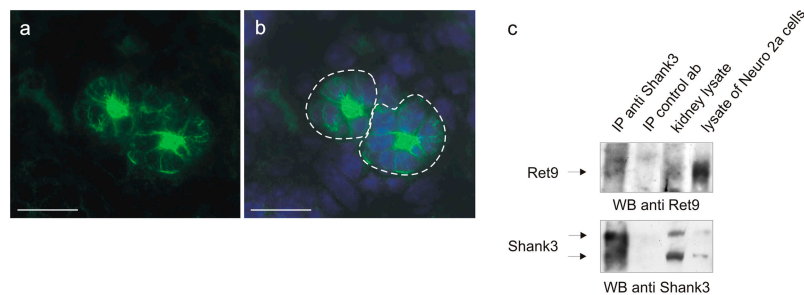


Figure 2. PDZ class-switch experiment. (a) Schematic representation of the PDZ class-switch mutants in Ret9 and Shank3. (b) Coimmunoprecipitation of Ret9 and Shank3 and their mutants. Flag-tagged Shank3-PDZ and Myc-tagged Ret9 were coexpressed in HEK293 cells, and Shank3-PDZ was precipitated using Flag-M2 Sepharose followed by SDS-PAGE and Western blotting with anti-Myc or anti-Flag tag antibodies. The Ret PDZ-binding class and the Shank3 PDZ domain class are indicated.

the Shank3 protein forms a specific complex with the Ret9 isoform through a novel Ret9 PDZ-binding motif.

To confirm that the Shank–Ret9 interaction is direct, we performed a PDZ class switch experiment (Kaech et al., 1998) by introducing compensatory mutations in Shank3 and Ret9. PDZ domains can be subdivided by their ability to bind to different receptor tails (Songyang et al., 1997; Vaccaro and Dente, 2002). Shank3 harbors a type I PDZ domain characterized by the presence of a histidine residue at position 716 (Fig. 2 a). Ret9 contains a type I PDZ-binding motif characterized by a threonine residue at position 1070. Mutation of the critical histidine residue to valine converts the Shank3 PDZ domain to a class II domain (Shank3–PDZ HV), which no longer binds to Ret9 (Fig. 2 b). Introduction of a compensatory mutation in Ret9 (threonine 1070 to tyrosine, Ret9 TY) converts the PDZ-binding motif into a type II-binding motif and reconstitutes the Shank3–Ret9 interaction (Fig. 2 b). These data demonstrate that Ret9 and Shank3 interact in a PDZ domain-mediated fashion, and that this interaction is direct.

Both Ret and Shank2 have been reported to localize to the renal tubular system (Pachnis et al., 1993; Redecker et al., 2001). Using immunohistochemistry with a specific antibody, we could show that Shank3 localizes to basolateral cell membranes in epithelial tubules of the developing kidney in E16.5 mouse embryos (Fig. 3, a and b). No expression of Shank was observed in surrounding mesenchymal cells. Strong staining was also observed at apical sites of renal tubules, which may indicate a further scaffolding function of Shank proteins. We could also demonstrate interaction of endogenous Ret9 with Shank3 in lysates of mouse kidneys by coimmunoprecipitation (Fig. 3 c). Taken together, our data indicate that Ret and Shank3 interact in the kidney in vivo, and that Shank3 is expressed at sites of Ret function.



Ret9, but not Ret51, induces epithelial tube formation

In vivo experiments have indicated that only Ret9 is crucial for kidney development (Srinivas et al., 1999; de Graaff et al., 2001). Here we have used an in vitro tubulogenesis assay with MDCK kidney epithelial cells to mimic the formation of kidney tubules (Montesano et al., 1991; Santos and Nigam, 1993; Weidner et al., 1993). MDCK cells seeded in a three-dimensional collagen matrix form cysts, consisting of a single polarized epithelial cell layer enclosing a fluid-filled lumen. Stimulation with growth factors such as hepatocyte growth factor/scatter factor (HGF/SF) induces the reorganization and growth of these cysts into branched, hollow tubes, which invade the surrounding matrix. We have generated MDCK cell lines that express various forms of Ret and stimulated these with the Ret ligand GDNF and the essential soluble coreceptor GFR α 1 (sGFR α 1; Paratcha et al., 2001). We found that cells expressing Ret9, but not Ret51, respond to ligand stimulation and form tubules (Fig. 4, a, b, d, and e). Remarkably, mutation of the PDZ-binding motif in Ret9 (Ret9 FA) prevented tubule formation (Fig. 4, g and h). In contrast, cells expressing the Ret51-9 protein, where the Ret9 PDZ-binding motif is attached, are capable of undergoing tubule formation (Fig. 4, j and k). Parental MDCK cells, which do not express endogenous Ret, do not respond to stimulation with GDNF–sGFR α 1 (Fig. 4, m and n). As a further control, addition of HGF/SF leads to tubulogenesis in all cell lines (Fig 4, HGF/SF column). The expression of the various Ret constructs and of endogenous Shank3 in the MDCK cell lines is shown by RT-PCR (Fig. 4, p and q). These data demonstrate that signaling by the Ret9 isoform is sufficient to induce epithelial tube formation, and that this activity depends on an intact PDZ-binding motif on Ret.

Figure 3. Ret9 interacts with Shank3 in vivo. (a and b) Immunofluorescence for Shank3 of embryonic kidney sections. Frozen sections of E16.5 mouse embryos were stained for Shank3 in green and DNA in blue. The dashed lines in b outline epithelial tubules. (c) Coimmunoprecipitation of Ret9 with Shank3 from lysates of mouse kidneys. Immunoprecipitations were subjected to SDS-PAGE and Western blotting. The Western blot was developed using antibodies against Ret9 and Shank3 as indicated. Controls were an immunoprecipitation with a control antibody, whole cell lysate from mouse kidneys, and whole cell lysate from Neuro-2A cells (mouse neuroblastoma 2A), which are rich in Ret9 and Shank3. Bars: 50 μ m.

Shank3 binding is crucial for Ret9-induced tube formation

The PDZ-binding motif of Ret9 is also required for Shank3 binding. To analyze whether Shank proteins indeed mediate tube formation, we fused distinct fragments of Shank3 to Ret9 and examined the hybrids in the biological assays. It should be noted that fusing any sequence to the COOH terminus of Ret9 abrogates PDZ binding (Songyang et al., 1997) and tubule formation (unpublished results). However, by attachment of the proline-rich domain of Shank3 to Ret9 (Ret9–Shank3–Pro1), we could reconstitute tube formation (Fig. 5, d and e). A fusion construct of Ret with a shorter fragment of the proline-rich region of Shank3 (Shank3–Pro2, aa 632–1057; Fig. 1 b) did also induce tubule formation (see Fig. 8, a and b). Fusion of other domains of Shank3 to Ret9, e.g., the ankyrin repeat and the SH3 domains, are not sufficient to induce tubulogenesis (Ret9–Shank3–NT; Fig. 5, a and b). Expression of Ret9–Shank3–NT and Ret9–Shank3–Pro1 is shown by RT-PCR (Fig. 5 g). Again, all cell lines were responsive to HGF/SF (Fig. 5, c and f). We could not obtain expression of a fusion construct between Ret and full-size Shank3. These results indicate that Shank3 can indeed act downstream of Ret9 to stimulate the formation of epithelial tubes. This activity is mediated by the proline-rich domain of Shank3.

The Ret9–Shank3 interaction facilitates sustained Erk–MAPK and PI3K signaling

Tubulogenesis by HGF/SF and Met has been shown to require sustained activation of the Erk–MAPK and PI3K pathways (Khwaja et al., 1998; Maroun et al., 2000; Schaeper et al., 2000). Previous investigations have shown that these pathways are also activated by Ret9 and Ret51 (van Puijenbroek et al., 1997; Besset et al., 2000; Hayashi et al., 2000; Melillo et al., 2001; Pelicci et al., 2002). Here we have addressed the question whether activation of the Erk–MAPK and PI3K pathways is modulated by the Ret9 PDZ-binding motif and Shank3 interaction, using the MDCK cell lines described above. Activation of Erk1/2, as measured by phosphorylation, is induced 10 min after GDNF–sGFR α 1 stimulation of Ret9-expressing cells, and remains sustained for at least 8 h (Fig. 6 a). Maximal phosphorylation of PKB–Akt, as indicator of activation of the PI3K pathway, in Ret9-expressing cells occurs at 30 min after stimulation, and also remains sustained for at least 8 h (Fig. 6e). However, mutation of the Ret9 PDZ-binding motif (Ret9 FA) prevents sustained but not transient activation of Erk1/2 and activation of PKB–Akt (Fig. 6, b and f). Stimulation of Ret51-expressing cells also fail to induce sustained activation of Erk–MAPK and cannot activate PKB–Akt (Fig. 6, c and g). MDCK

Figure 5. The Shank3 proline-rich domain is crucial for Ret-induced tubule formation. MDCK cell lines expressing fusion proteins of the full-length Ret9 receptor with either the NH₂ terminus of Shank3 (Ret–Shank3 NT; a–c) or the proline-rich domain of Shank3 (Ret9–Shank3–Pro1; d–f) were examined for tubulogenesis in three-dimensional collagen matrices. After stimulation with GDNF–sGFR α 1 or HGF/SF, cell cultures were fixed and photographed. Bar in f: 200 μ m (applies to a–f). (g) Expression of the indicated cDNA constructs shown by RT-PCR.

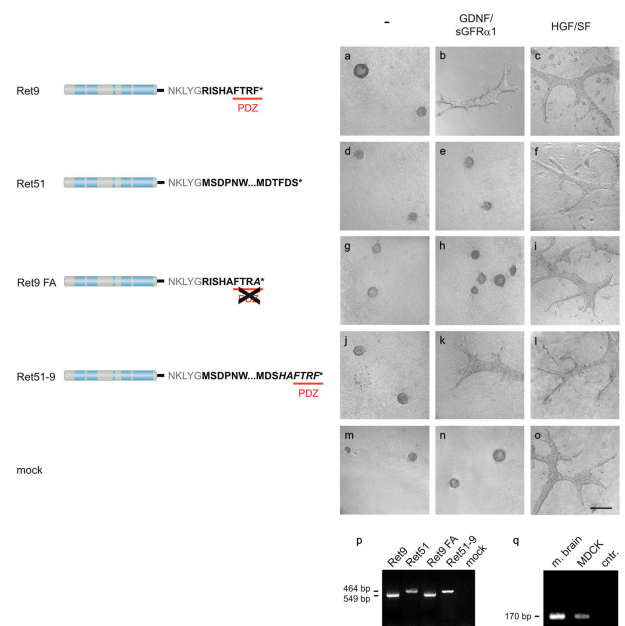
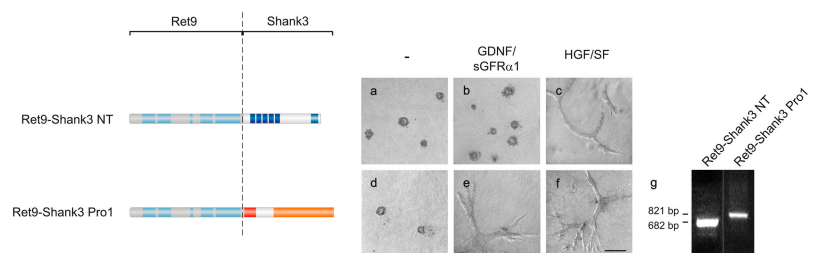


Figure 4. Induction of tubular structures by Ret depends on the PDZ-binding motif. MDCK cell lines expressing Ret9 (a–c), Ret51 (d–f), Ret9 FA (g–i), Ret51-9 (j–l), or control cells without Ret (m–o) were seeded as single cell suspensions into three-dimensional collagen matrices and stimulated with GDNF–sGFR α 1 or HGF/SF. Photographs were taken after fixation. Bar in o: 200 μ m (applies to a–o). (p) Expression of the Ret cDNA constructs in the various MDCK cell lines is shown by RT-PCR. (q) Expression of endogenous Shank3 in mouse brain and MDCK cells is shown by RT-PCR.

cells expressing the proline-rich domain of Shank3 fused to Ret (Ret–Shank3–Pro1), but not other Ret–Shank3 fusion proteins, exhibit sustained Erk–MAPK and PI3K activation similar to Ret9 (unpublished results).

Taken together, our results indicate that Shank3 functions downstream of Ret9 to enable sustained Erk–MAPK and PI3K signaling and the formation of epithelial tubules. These effects are mediated by the central proline-rich domain of Shank3.

Shank3 interacts with the adaptor protein Grb2, which mediates sustained Erk–MAPK and PI3K signaling and epithelial tube formation

Several adaptor molecules such as Shc, Grb2, FRS2, and dok4/5 are known to be involved in stimulation of the Erk–MAPK pathway downstream of Ret and are recruited to the plasma membrane (Borrello et al., 1996; Arighi et al., 1997; Alberti et al., 1998; Grimm et al., 2001; Kurokawa et al., 2001). Analysis of the Shank3–Pro2 sequence revealed a potential binding site

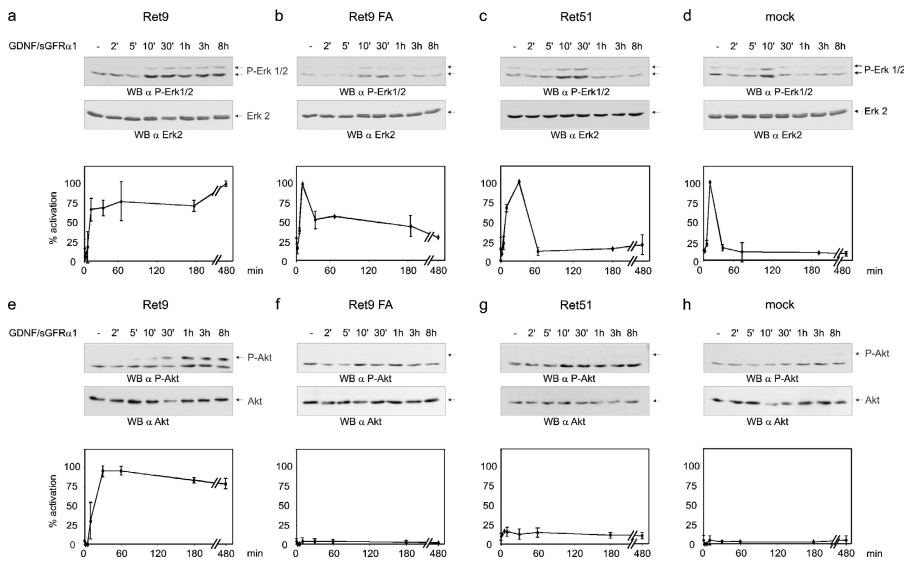


Figure 6. The Ret9 PDZ-binding motif mediates sustained Erk-MAPK and PI3K signaling. MDCK cells, expressing Ret9 (a and e), Ret9 FA (b and f), Ret51 (c and g), or control cells without Ret (d and h) were stimulated with GDNF and sGFR α 1 and examined for Erk-MAPK and Akt phosphorylation by SDS-PAGE. Phosphorylated Erk1/2 (P-Erk1/2) or Akt (P-Akt) proteins, total Erk2 and total Akt were detected by Western blotting with specific antibodies. The specific bands for phosphorylated and unphosphorylated proteins are indicated by arrows. Western blots were quantified and activation levels over time are shown. Error bars show standard deviation from three independent experiments.

for the Grb2 SH2 domain, with the consensus sequence pYXNX. Indeed, endogenous Grb2 was recruited to this binding site, as shown by coimmunoprecipitation of Shank3-Pro2 upon coexpression of Ret9 (Fig. 7 b). Coexpression of the PDZ-binding motif mutant Ret9 FA did not allow binding of Grb2 to Shank3-Pro2. Moreover, coexpression of Ret9 leads to significantly higher levels of Shank3 phosphorylation than Ret9 FA. We determined that Grb2 is not complexed indirectly with Ret9 through Shc, because Shank3-Pro2 still binds Grb2 when the Shc-binding-deficient Ret9 Y1062F mutant is coexpressed (Fig. 7, a and b). Finally, we mutated tyrosine 1006 to phenylalanine in the Grb2-binding site of Shank3 (Shank3-Pro1 Y1006F), which does not interact with Grb2 (Fig. 7, a and b).

Mutation of the Grb2-binding site of Shank3 in our Ret9 receptor hybrid prevented tubule formation (Fig. 8). These results show that Shank3 recruits Grb2 protein to Ret9 receptor

complexes in a phosphorylation-dependent manner, and that this interaction is functionally relevant. Two other signaling effectors are also known to bind to Shank3-Pro2: the Rho family GDP-GTP exchange factor PIX, which binds to the PDZ domain (aa 654-739; Park et al., 2003), and the actin polymerization regulator IRSp53, which binds to residues 908 to 918 (Soltau et al., 2002). Mutation of these sites in Shank3 does not interfere with tube formation (unpublished results). Taken together, our findings provide a molecular mechanism for the biological differences between Ret9 and Ret51 in epithelial tube formation: Ret9 recruits the multiadaptor Shank3, and binding of additional Grb2 protein is required for tubule formation.

Discussion

In this study we have identified the scaffolding adaptor Shank3 as novel Ret9-binding partner, which interacts with a newly identified PDZ-binding motif in the Ret9 isoform. We also demonstrate that the Ret9 isoform, and not Ret51, is able to induce the formation of tubular structures of kidney epithelial cells in three-dimensional culture, and that this ability is dependent on interaction with Shank3. Moreover, the interaction of Shank3 with Ret9 is crucial for the sustained activation of the Erk-MAPK and PI3K pathways downstream of Ret9, both of which are required for the induction of epithelial tubules.

Originally identified in neuronal cells (Boeckers et al., 1999; Naisbitt et al., 1999; Tu et al., 1999; Yao et al., 1999), Shank proteins are also expressed in other tissues such as in epithelial ducts of lung, pancreas, and kidney, and in hepatic bile ducts (Redecker et al., 2001). In neuronal cells, Shank proteins localize to postsynaptic densities and have been previously shown to regulate dendritic spine morphology by linking the postsynaptic signaling machinery to the cortical cytoskeleton (Naisbitt et al., 1999; Tu et al., 1999; Sheng and Kim, 2000; Sala et al., 2001; Boeckers et al., 2002). We demonstrate here that Shank3 also has a defined biological role outside of neuronal tissues, and that Shank3 actively participates in signal

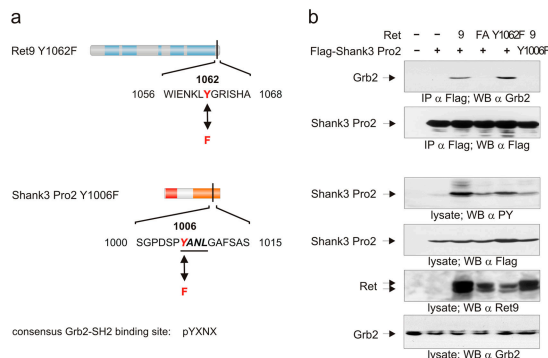
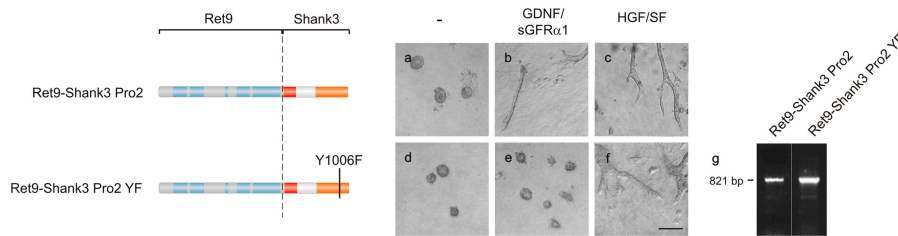


Figure 7. Shank3 interacts with Grb2. (a) Schematic representation of the used Ret9 and Shank3 mutants. The consensus Grb2-binding motif in Shank3 is underlined. (b) Interaction of Shank3 with endogenous Grb2. HEK293 cells were cotransfected with Flag-tagged Shank3-Pro2 or Shank3-Pro2 containing the Y1006F mutant and Ret9, Ret9FA, or Ret9Y1062F. Immunoprecipitation of Shank3 using Flag-M2 Sepharose beads was performed followed by SDS-PAGE and Western blotting. Western blots of immunoprecipitations and total lysates were developed with antibodies against Grb2, Flag, phospho-tyrosine (PY), and Ret9 as indicated.

Figure 8. Grb2 binding to Shank3 is required for tubule formation. MDCK cells expressing either Ret9–Shank3–Pro2 (a–c) or Ret–Shank3–Pro2 containing the Y1006F mutation (d–f) were examined for tubulogenesis in three-dimensional collagen matrices. After stimulation with GDNF–sGFR α 1 or HGF/SF, cell cultures were fixed and photographed. Bar in f: 200 μ m (applies to a–f). (g) Expression of the indicated cDNA constructs shown by RT-PCR.



transduction. Shank3 thus provides a platform for the regulated recruitment of additional effectors, such as Grb2, that are involved in stimulation of downstream signaling, although Shank3 may still be involved in integration of receptor complexes with the cytoskeleton. Other receptor tyrosine kinases, for instance ErbB2, have been shown to harbor PDZ-binding motifs, which interact with scaffold proteins (Maudsley et al., 2000; Shelly et al., 2003). The PDZ protein ERBIN was described initially to be important for the correct cellular localization of ErbB2, but is now also known to participate in downstream signaling (Borg et al., 2000; Huang et al., 2003).

The kinetics of activation of the Erk–MAPK and PI3K pathways is crucial for determining the biological outcome of receptor tyrosine kinase signaling, e.g., increased migration and differentiation of epithelial cells. From studies of the Met receptor, it is known that sustained activation of Erk–MAPK and PI3K signaling pathways is required for tubule formation, whereas transient activation of these pathways is insufficient to induce such morphological alterations (for review see Rosario and Birchmeier, 2003). We show here that Shank3 function is required for sustained activation of both Erk–MAPK and PI3K pathways downstream of Ret9. The central region of Shank3 (aa 632–1057) is sufficient to mediate sustained activation of the Erk–MAPK and PI3K pathways and epithelial tubule formation, and a newly identified Grb2-binding site on Shank3 is essential for this activity. Interaction between Shank3 and Grb2 is SH2 domain mediated and requires phosphorylation by Ret9. In contrast, binding of Grb2 to Ret9 through Shc or to Ret51 through an additional Grb2-binding site (Basset et al., 2000) does not induce sustained activation of the downstream pathways. Thus, Shank3 acts as a true scaffolding adaptor, which amplifies receptor tyrosine kinase signaling. Shank-associated Grb2 may recruit Gab1 and Shp-2, which are essential in tube formation (Maroun et al., 2000; Schaeper et al., 2000; Rosario and Birchmeier, 2003). Shank proteins may also serve to recruit additional signaling effectors through the proline-rich and other tyrosine-containing sequences in the central domain. The biological functions of these sites have not been determined. Shank3 is thus the first isoform-specific Ret signaling effector. The isoform specificity of the Ret–Shank interaction provides a molecular explanation for the crucial role of Ret9 during mammalian kidney development. Moreover, the Shank-binding site is conserved in the Ret–PTC2 fusion protein found in human patients with papillary thyroid carcinomas (Bongarzone et al., 1993), and Shank proteins may therefore also be involved in the development of Ret-induced tumors.

Materials and methods

Reagents and biochemical analysis

Antibodies to Ret9 (C-19-G), Ret51 (C-20), and c-Myc (A14-G) were purchased from Santa Cruz Biotechnology Inc., antibodies to the extracellular domain of Ret from R&D Systems, antibodies to active Erk–MAPK and Flag tag from Sigma-Aldrich, antibodies to active Akt pS473 from Cell Signaling, and anti-Akt-PKB α and pan-Erk antibodies from BD Biosciences. Production of rabbit anti–guinea pig antiserum against Shank3 was described earlier (Bockmann et al., 2002). Antibodies were used at the following concentrations: Ret9, Ret51, and PKB α , 1:500; pS473-Akt, c-Myc, and Shank3, 1:1,000; pan-Erk, 1:2,000; and active Erk–MAPK, 1:5,000, for Western blotting and Ret and Shank3, 1:50, for immunofluorescence; secondary antibodies were donkey anti–goat, goat anti–guinea pig or donkey anti–rabbit IgG, which were conjugated with peroxidase, Cy2, or Cy3 (Jackson Laboratories). Erk–MAPK and PI3K Western blots were quantified using TINA image analysis software (raytest Isotopengeraete GmbH). Point mutations were introduced into Ret9 and Shank3 cDNAs using PCR or the QuickChange site directed mutagenesis kit (Stratagene). Primers were used as follows: Ret9 FA forward: CTCGCTATGTGAGCGGTGGAGGC, reverse: TTTTCAGCTGCTAGGCTCTAGTAAATGCATGTGAAATCTACC; Ret9 TY reverse: CATAGTCGACCTAGAAATCTATAAAATGCATGTG; Ret9 YF reverse: TTTTCAGCTGCTAGGCTCTAGTAAATGCATGTGAAATCTACCAGAGTITG; Ret51-9 reverse: GATTGTGACCTAGAAATCTAGTAAATGCATGGCTATCAAATGTGTCC; Shank3-PDZ HV forward: GTC-GTGAAGGTTGGAGTCAAG-CAAGTGGTGGGTCTC, reverse: GAGACC-CACCACITGCTTGACTCCA-ACCTTCACGAC; Shank3-Pro1 YF forward: GGCCCTGATAGTCCCTTTGCCAACCTGGGGCGCC, reverse: GGCGC-CCAGGTTGGCAAAGGGACTATCAGGGCC. Expression of Ret and Shank in MDCK cells was verified by RT-PCR using SuperScript II reverse transcriptase (Invitrogen). Primers were used as follows: Ret9 and Ret9 FA forward CAAGTGGATGGCAATTGAGTCC, reverse GTAAATGCATGTGAAATCTACC; Ret51 and Ret51-9 reverse GITAGCATATACACTAT-CATTTGC; Ret9-Shank3-NT reverse GCAGGGTCTGCAATGCTC; Ret9-Shank3-Pro1 reverse GCCGAGCACTATCCTCTG; Ret9-Shank3-Pro1 and Ret9-Shank3-Pro1 YF reverse TCCAGTAGGGATGCCAGC; Shank3 forward CCGAAGCGGAAACTTTAC, reverse ACCCTCCCTCCCA-GAAACC. Human recombinant GDNF (TEBU) and sGFR α 1 (R&D Systems) were used at 25 ng/ml and 0.5 μ g/ml, respectively. Yeast two-hybrid analysis was performed as described previously (Weidner et al., 1996). For coimmunoprecipitation, HEK293 cells were seeded at 3×10^6 cells per 10 cm dish and transfected using the calcium phosphate method. Cells were lysed in Triton X-100 lysis buffer (1% Triton X-100, 20 mM Hepes, pH 7.5, 150 mM sodium chloride, and 10% glycerol) for 15 min, centrifuged at 20,000 g for 15 min and precipitated using Flag-M2–coupled Sepharose (Sigma Aldrich) for 2 h at 4°C, followed by SDS-PAGE and Western blotting. For coimmunoprecipitation of endogenous Shank3 and Ret9, two adult mouse kidneys per immunoprecipitation were homogenized, lysed in CHAPS lysis buffer (1% CHAPS, 10 mM Tris, pH 7.5, 10 mM sodium chloride), and centrifuged for 20 min at 4°C. The supernatant was incubated with guinea pig anti-Shank3 antiserum for 2 h at 4°C before precipitation with protein G–Sepharose. For Western blots of active Erk–MAPK and active Akt, MDCK cells were seeded at 1.5×10^5 per well on six-well dishes, starved for 20 h in serum-free DMEM, stimulated with 50 ng/ml GDNF and 1 μ g/ml sGFR α 1, and lysed Triton X-100–containing lysis buffer.

Immunofluorescence and cell culture

Mouse embryos were dissected and fixed overnight in 4% formaldehyde. For frozen sections, fixed embryos were rinsed with PBS, and embedded in water soluble glycol's and resins compound (Tissue-Tek O.C.T. compound; Sakura Finetek). After acetone postfixation, sections were labeled using anti-Shank3 antibody. Pictures were taken with a Zeiss Axiophot

fluorescence microscope using Zeiss 40× F Fluor optics and a Diagnostic Instruments SPOT-RT-Monochrome CCD camera. Contrast was adjusted using Adobe Photoshop imaging software.

MDCK cell lines were maintained in DMEM supplemented with 10% FCS, 100 IU/ml penicillin, and 100 mg/ml streptomycin at 37°C in 5% CO₂. To generate stable MDCK cell lines, cells were transfected with Lipofectamine 2000 (Invitrogen) and selected with 120 μg/ml G418 or 1.25 μg/ml Puromycin. HEK293 (human embryonic kidney 293) cells and Neuro2A (mouse neuroblastoma 2A) cells were maintained in DMEM, 10% FCS as for the MDCK cells, and transfected using the calcium phosphate method. In the tubulogenesis assay, single MDCK cells were embedded in a collagen type I solution containing 1.5 mg/ml Vitrogen 100 (Nutacon BV), 10% 10× DMEM (Invitrogen), 10% FCS, 2.2 mg/ml NaHCO₃, and 20 mM HEPES, pH 7.6. After gelation, medium was added and cells were grown for 3–5 d until they formed cysts. Cells were stimulated with GDNF and sGFRα₁, or HGF/SF, and grown for 5–7 d further before fixation in 4% formaldehyde. Pictures were taken with a Zeiss Axiocvert 135 microscope, using Zeiss 10× Achromaplan optics and a Jenoptik ProgRes 3012mf camera. Pictures were deconvoluted manually by stacking several images of different focus layers and reducing nonfocused regions. Contrast was adjusted using Adobe Photoshop imaging software.

Submitted: 19 April 2004

Accepted: 25 October 2004

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