UDC 577.22

New partners of TKS4 scaffold protein

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TKS4 scaffold protein is involved in formation of invadopodia, production of reactive oxygen species (ROS) by tumor cells and other cellular processes. **Aim.** To identify new TKS4 partners involved in the actin cytoskeleton rearrangements and endo-/exocytosis. **Methods.** The GST pull-down assay was used to identify the interaction. **Results.** We revealed that TKS4 SH3 domains interacted with the actin cytoskeleton reorganization proteins N-WASP and CR16, as well as with DNM2, SYNJ1 and OPHN1 which are involved in endo-/exocytosis. We also tested the WIP, WIRE, SHIP2, RhoU, RhoV and NUMB proteins, but their interaction with the SH3 domains of TKS4 was not found. **Conclusions.** The SH3 domains of TKS4 interact with N-WASP, DNM2, SYNJ1, OPHN1 and weakly with CR16 *in vitro*.

K e y w o r d s: TKS4, adaptor/scaffold proteins, actin cytoskeleton, endo-/exocytosis.

Introduction

Adaptor/scaffold proteins play an important role in the functioning of cells. Adaptor proteins are composed of domains and motifs that mediate molecular interactions, and can thereby link signaling proteins, e.g. activated cell-surface receptors to downstream effectors. The recent data support the notion that adaptors are not simply coupling devices that hardwire the successive components of signaling pathways [1]. Scaffold proteins usually consist of multiple domains that bind different cell components and are believed to be responsible for anchoring scaffold proteins to various microdomains of the cell. Adaptors/scaffolds often contain Src-homology 2 and 3 (SH2 and SH3) domains that bind to phosphotyrosine residues (pY) and proline-rich motifs (PxxP) respectively [2]. Usually adaptor/scaffold proteins consist of domains which can bind different membrane phospholipids such as Phox homology (PX) or pleckstrin-homology (PH) domains [3].

TKS4 (Tyrosine kinase substrate with four SH3 domains) scaffold protein, known also as SH3PXD2B contain one PX domain, four SH3 domains, proline-rich motifs, mostly concentrated in the linker between 3rd and 4th SH3 domains; and three Src phosphorylation sites (Fig. 1) [4]. TKS4 was shown to play an important role in the formation of functional invadopodia [4] and lamellipodia [5] – cellular invasive and motion structures, the production of ROS by tumor cells [6, 7] and in the differentiation of white adipose tissue [8]. The *TKS4* gene mutations are linked to such pathologies as Frank-Ter Haar syndrome [9], Borrone dermato-cardio-skeletal syndrome [10] and glaucoma [11]. It was shown that together with the other family member, TKS5 (Tyrosine kinase substrate with five SH3 domains), TKS4 plays a critical role in the cancer cell invasiveness. For instance, after the TKS4 knockdown the cancer cells lose their ability to form invadopodia [4]. The detailed mechanisms of TKS4 participation in these processes are unknown, so the search and
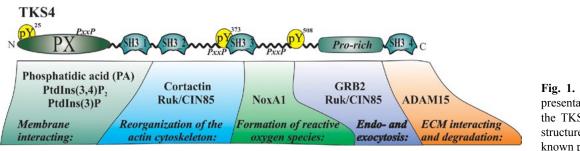
Fig. 1. Schematic presentation of the TKS4 domain structure and its known partners

characterization of the new partners are an important area of research.

To date only five TKS4 protein partners are known. One of them is cortactin, a regulator of actin cytoskeleton [5]. Gianni and co-authors have shown that TKS4 participates in the ROS production by the interaction with NoxA1 (NOX activating protein 1) [7]. Another TKS4 partner is metalloprotease ADAM15 (a disintegrin and metalloprotease 15) that takes part in the extracellular matrix degradation during the cell movement and invasion. The interaction was confirmed by GST pull-down assay [12]. Mass spectrometry revealed two more partners: scaffold Ruk/CIN85 (regulator of ubiquitous kinase/Cbl-interacting protein of 85 kDa) and adaptor GRB2 (growth factor receptor-bound protein 2) [13, 14]. Through the PX domain TKS4 interacts with the membrane phosphatidylinositol phosphates (such as PtdIns(3,4)P_2, PtdIns(3)P and PA) which probably allow it to recognize endosomes and invadopodia formation sites (Fig. 1) [4, 15].

In this work we searched for new TKS4 partners containing proline-rich motifs which could interact with the TKS4 SH3 domains. The proteins we have chosen for GST pull-down assay are involved in the actin cytoskeleton rearrangement, endo-/exocytosis and formation of invadopodia.

Materials and Methods

RT-PCR and expression constructs

cDNA was synthesized from 1 to 5 µg of total RNA using oligo(dT$_{15}$) primer and Expand Reverse Transcriptase from Roche. Five percent of the cDNA

Table 1. **Primers used for cloning**

Constructs[a]	Primers Sequence[b]	Sites	Shifts
pCMVmyc-CR16 (aa 2-483)	f_tgac[gaattc]cagtgccaccgcca r_gac[ctcgag]ttcatctgtcttctc	EcoRI XhoI	G956 to A; C1039(Ala) to T(Val)
pRK5myc-WIRE (aa 2-440)	f_ca[ggatcc]ccaattcctcctcccccgcc r_cc[tctaga]acgggaccaagccaggcttc	BamHI XbaI	T228 to C
pcDNA4maxB-N-WASP (aa 3-505)	f_at[gaattc]cgtccagcagcagccg r_ta[gatatc]agtcttcccactcatc	EcoRI EcoRV	C394 to T
pGEX4T3-SH3_1(TKS4) (aa 152-212)	f_agt[gaattc]catggtcctggagca r_ctc[ctcgag]caccccatcctggcc	EcoRI XhoI	
pGEX4T3-SH3_2(TKS4) (aa 221-282)	f_tct[gaattc]tgaagaagaggagaa r_ggg[ctcgag]ggctccccactgttc	EcoRI XhoI	A845(Lys) to T(Met)
pGEX4T3-SH3_3(TKS4) (aa 368-426)	f_atc[ggatcc]caagtggaggaaga r_tgc[ctcgag]tcttgtacttgtcaa	BamHI XhoI	
pGEX4T3-SH3_4(TKS4) (aa 850-911)	f_tgc[gaattc]cctgaaggactcttt r_gaa[ctcgag]tcggctacggcttct	EcoRI XhoI	

a aa, amino acids;

b f, forward; r, reverse; brackets indicate restriction enzyme sites into the primers

obtained was used as a template for PCR as described previously [16]. The coding sequence of *CR16* (NM_001080529 nucleotides [nt] 186 to 1647), *WIRE* (NM_133264; nt 244 to 1581), *N-WASP* (NM_003941.3; nt 344 to 1853), and four SH3 domains of *TKS4* (NM_001017995; nt 624 to 807, 831 to 1016, 1273 to 1450 and 2718 to 2911 respectively), were PCR amplified from human cDNA using the primers listed in Table 1.

The plasmids encoding human GST-fused SH3A domain of ITSN1, omni-SYNJ1-PRD isoform d [17], HA-WIP [18] and HA-OPHN1 [19] were described previously. Flag-NUMB was a kind gift of Dr. P. P. Di Fiore [20], flag-SHIP2 was received from Dr. C. A. Mitchell [21], myc-RhoU, myc-RhoV were obtained from Dr. P. Aspenström [22, 23] and GFP-DNM2 was from Dr. S. Gasman [24].

Antibodies

Monoclonal anti-HA antibodies were purchased from Covance, USA; monoclonal anti-flag (clone M2) antibodies were purchased from Sigma, USA; monoclonal anti-omni (D-8) sc-7270 and polyclonal anti-OPHN C-19 (sc-8374) antibodies were purchased from Santa Cruz Biotechnology, USA; monoclonal anti-myc 7E10 antibodies were purchased from LifeSpan Biosciences, USA; polyclonal

anti-GFP antibodies were a kind gift of Dr. V. V. Filonenko. Secondary horseradish peroxidase-labeled anti-rabbit, anti-mouse and anti-goat antibodies were purchased from Promega, USA.

Cell culture and transfection

MDA-MB-231 or 293 cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % fetal bovine serum, 50 U/ml penicillin and 100 μg/ml streptomycin. The cells were transiently transfected with PEI (polyethyleneimine) according to the manufacturer's instructions and processed 24 h after transfection.

GST pull-down assay and Western blot analysis

The recombinant GST-fused proteins were obtained in *Escherichia coli Top10A* cells and affinity purified using glutathione-Sepharose 4B (GE Healthcare) according to the manufacturer's instructions. Lysates of transiently transfected MDA-MB-231 or 293 cells were prepared in extraction buffer containing 20 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.5 % Nonidet P40, 1 mM EDTA, 1 mM phenylmethylsulfonylfluoride (PMSF) and protease inhibitor cocktail from Roche and centrifuged for 10 min at 12.000 g at 4°C. For pull-down experiments, 5–10 μg of GST or GST-fused proteins were bound
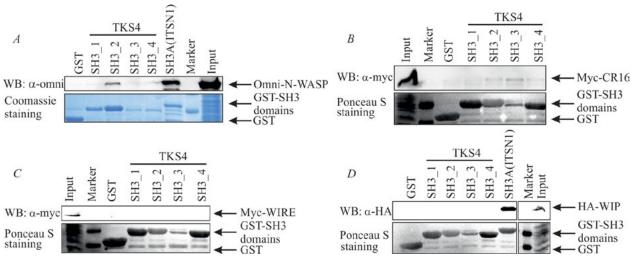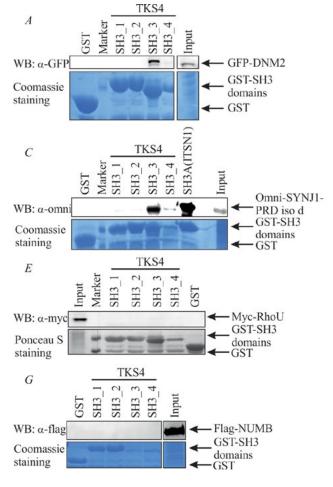

Fig. 2. The SH3 domains of TKS4 interact with the proteins involved in the actin cytoskeleton reorganization N-WASP (*A*), CR16 (*B*) but do not interact with WIRE (*C*) and WIP (*D*)
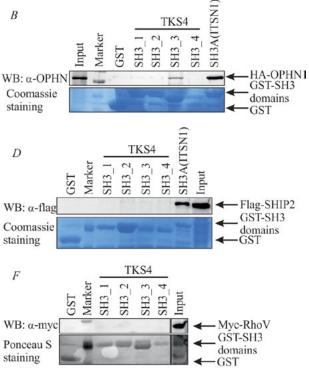
to 30 µl of 50 % glutathione-Sepharose 4B beads and incubated with the cell lysates for 1 h at 4°C. After extensive washing, the beads were boiled in Laemmli sample buffer. The proteins were resolved in SDS-PAGE, transferred to nitrocellulose membranes (Bio-Rad) and blocked in 5 % nonfat milk. The membranes were incubated with primary antibodies for 1 h followed by incubation with peroxidase-conjugated secondary antibodies for 40 min. Immunoreactive bands were detected using ECL reagents. Chemiluminescence was captured with Molecular Imager ChemiDoc™ XRS+ (Bio-Rad).

Results and Discussion

A GST pull-down assay was performed to test the interaction of TKS4 with the chosen protein partners

in vitro. For analysis we chose the actin cytoskeleton reorganization proteins such as N-WASP (neural Wiskott-Aldrich syndrome protein), CR16 (corticosteroids and regional expression 16), WIP (WASP-interacting protein) and WIRE (WIP-related); proteins involved in endo-/exocytosis such as DNM2 (dynamin 2), SYNJ1 (synaptojanin 1), OPHN1 (oligophrenin 1), SHIP2 (SH2 domain-containing inositol phosphate 5-phosphatase 2), NUMB (protein numb homolog) and two Rho GTPases: RhoU (Ras homolog gene family, member U) and RhoV (Ras homolog gene family, member V). Breifly, the recombinant GST-fused SH3 domains of TKS4 were immobilized on the glutathione sepharose and incubated with the lysates of MDA-MB-231 or 293 cell lines overexpressing one of the target proteins. GST

Fig. 3. The SH3 domains of TKS4 interact with proteins DNM2 (*A*), OPHN1 (*B*) and SYNJ1-PRD isoform d (*C*) but do not interact with SHIP2 (*D*), RhoU (*E*), RhoV (*F*) and NUMB (*G*), involved in endo-/exocytosis processes

alone was used as a control. The amount of the GST-fused SH3 domains was detected with Coomassie or Ponceau staining. Precipitated proteins were detected by Western blot analysis. For the previously described ITSN1 partners, GST-fused ITSN1 SH3A domain was used as a positive control [17–19, 25].

First, we have tested the interaction of the TKS4 SH3 domains with the proteins involved in the actin cytoskeleton reorganization such as N-WASP and the verprolin family (WIP, WIRE and CR16) [26]. We have shown that N-WASP binds to the second TKS4 SH3 domain (Fig. 2*A*). CR16 demonstrated a weak interaction with the 2nd, 3rd and 4th TKS4 SH3 domains (Fig. 2*B*), whereas no interaction was observed for WIRE and WIP (Fig. 2*C, D*).

The group of endo-/exocytosis proteins included OPHN1, GTPase activating protein for the Rho family small GTPases [27], GTPase DNM2, phosphatidylinositol phosphatases SYNJ1 and SHIP2, and scaffold protein NUMB [28]. We have also chosen two atypical members of the Rho family GTPases: RhoU and RhoV. These two atypical GTPases are highly conserved across vertebrates and involved in the adhesion and migration of many cell types [29].

After a series of GST pull-down experiments we have demonstrated the interaction of the third TKS4 SH3 domain with DNM2 and OPHN1 (Fig. *3A, B*). The proline-rich domain of SYNJ1 isoform d described in 2008 in our lab [17] binds to the third and weakly to the fourth TKS4 SH3 domains (Fig. 3*C*). No interaction was shown for SHIP2, RhoU, RhoV, and NUMB (Fig. 3*D–G*).

The obtained results on the TKS4 binding to N-WASP and CR16 indicate the TKS4 potential involvement in the actin cytoskeleton reorganization, which is in line with the previously demonstrated TKS4 interaction with the nucleation promoting factor cortactin [5]. TKS4 may regulate the interaction between N-WASP and CR16 which highly cooperate in the regulation of the actin cytoskeleton rearrangement [26].

The TKS4 interaction with such endo-/exocytosis components as DNM2, SYNJ1 and OPHN1 [28, 27] may bring new evidence for its participation in these membrane transport processes, which has been demonstrated for the first time. Besides, our data suggest that TKS4 is likely involved in the invadopodia formation: N-WASP and DNM2 are the key components of these invasive structures, and phosphotidylinositol phosphates (PtdInsP) that bind to the TKS4 PX domain are the markers for the invadopodia initiation sites [15]. This process, in turn, may be regulated by the TKS4 interaction with the SYNJ1 phosphatidylinositol phosphatase which dephosphorylates phosphatidylinositol bis- or trisphosphates localized in plasma membranes [30].

Conclusions

The SH3 domains of TKS4 interact with N-WASP, DNM2, SYNJ1-PRD isoform d, OPHN1 and weakly with CR16 *in vitro*. The interaction of the TKS4 SH3 domains with WIP, WIRE, SHIP2, RhoU, RhoV and NUMB has not been found. These results suggest the TKS4 participation in the invadopodia formation including the actin cytoskeleton rearrangements and indicate its potential involvement in endo-/exocytosis. The analysis of their co-localization in a cell and the functions of TKS4/partners complexes to confirm the identified interaction are in progress.

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Нові партнери скафолдного білка TKS4

С. В. Кропивко

Скафолд TKS4 приймає участь у формуванні інвадоподій, продукції активних форм кисню (ROS) в ракових клітинах та інших клітинних процесах. **Мета.** Ідентифікувати нових партнерів TKS4, задіяних в перебудовах актинового цитоскелету та ендо-/екзоцитозі. **Методи.** *In vitro* GST pull-down assay. **Результати.** Показано, що SH3 домени TKS4 взаємодіють з білками-реорганізаторами актинового цитоскелету N-WASP та CR16, а також з DNM2, SYNJ1 та OPHN1, які задіяні в ендо-/екзоцитозі. Проте взаємодії WIP, WIRE, SHIP2, RhoU, RhoV та NUMB, з SH3 доменами TKS4 не знайдено. **Висновки.** SH3 домени TKS4 взаємодіють з білками N-WASP, DNM2, SYNJ1, OPHN1 та слабко з CR16 *in vitro*.

К л ю ч о в і с л о в а: TKS4, адаптерні/скафолдні білки, актиновий цитоскелет, ендо-/екзоцитоз

Новые партнеры скаффолдного белка TKS4

С. В. Кропивко

Скаффолд TKS4 принимает участие в формировании инвадоподий, продукции активных форм кислорода (ROS) в раковых клетках и других клеточных процессах. **Цель.** Идентификация новых партнеров TKS4, участвующих в перестройках актинового цитоскелета и эндо-/экзоцитозе. **Методы.** *In vitro* GST pull-down assay. **Результаты.** Показано, что SH3 домены TKS4 взаимодействуют с белками-реорганизаторами актинового цитоскелета N-WASP и CR16, а также с DNM2, SYNJ1 и OPHN1, которые задействованы в эндо-/экзоцитозе. Взаимодействия WIP, WIRE, SHIP2, RhoU, RhoV и NUMB, с SH3 доменами TKS4 не выявлено. **Выводы.** SH3 домены TKS4 взаимодействуют с белками N-WASP, DNM2, SYNJ1, OPHN1 и слабо с CR16 *in vitro*.

К л ю ч е в ы е с л о в а: TKS4; адапторные/скаффолдные белки; актиновий цитоскелет ; эндо-/экзоцитоз