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# Interaction between adaptor proteins Ruk/CIN85 and Tks4 in normal and tumor cells of different tissue origins

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**Aim.** To study the interaction of adaptor protein Ruk/CIN85 SH3 domains with endogenous adaptor protein Tks4 in normal and tumor cells of different tissue origins. **Methods.** GST *in vitro* pull-down assay was performed using total cell lysates of cell lines of different origins. **Results.** Using GST *in vitro* pull-down assay, we have determined that SH3A domain of adaptor protein Ruk/CIN85 precipitated full-length form of adaptor protein Tks4 (M, 120 kDa) from lysates of human breast (MCF-7, MDA-MB-231), melanoma (MM-4), colon (HT-29, DLD-1) tumor cells as well as from lysates of mouse Lewis lung carcinoma cells (LLC) and fibroblasts (NIH 3T3). It has been also revealed that all Ruk/CIN85 SH3 domains (A, B and C) with high efficiency precipitate the additional forms of Tks4 with M, 75, 90 and 160 kDa from lysates of human colon carcinoma cells and mouse fibroblasts. The molecular nature of new multiple forms of Tks4 has not been determined to date. **Conclusions.** New multiple molecular forms of adaptor protein Tks4 with M, 75, 90 and 160 kDa, able to interact with high affinity with SH3 domains of Ruk/CIN85, were identified using GST *in vitro* pull-down assay. The data obtained suggest that interaction between Ruk/CIN85 SH3 domains with Tks4 endogenous forms is determined by cellular context while a level of this interaction can be regulated in the course of physiological cellular responses.

**Keywords:** adaptor proteins, Ruk/CIN85, Tks4, SH3 domains, GST *in vitro* pull-down assay.

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**Introduction.** Adaptor proteins consist of domains and motifs, which mediate intermolecular interactions and are able to form intracellular protein complexes in a regular and selective manner. It determines their important role in the maintenance of specificity and efficiency of all known signal transduction pathways [1]. In particular, adaptor proteins Ruk/CIN85 (Regulator for ubiquitous kinase/Cbl-interacting protein of 85 kDa) and Tks4 (Tyrosine kinase substrate with 4 SH3 domains) contain multiple SH3 domains and Pro-rich regions in their structure [2, 3]. Of note, these adaptor proteins are involved in the control of fundamental cellular and sig-

naling events as well as in carcinogenesis [4, 5]. More than 100 protein partners of Ruk/CIN85 SH3 domains were identified in HeLa cells *in vitro* using the high-throughput technique combining affinity purification of interacting partners from cell lysates with liquid chromatography-coupled tandem mass spectrometry (LS-MS/MS). Adaptor protein Tks4 was revealed as one of the main protein partners according to the number of unique peptides matching to protein sequence and percentage of protein sequence covered by matching peptides. In general, 21 peptides, which overlap 27 % of 911 amino acids of Tks4, were identified [6]. However, no data regarding the peculiarities of interaction of the individual Ruk/CIN85 SH3 domains with the endogenous

Tks4 protein in cell context dependent manner have been reported so far.

**Materials and methods.** *Cell culture.* Cell lines MCF-7 (human breast adenocarcinoma) and its subline G4 (with stable overexpression of adaptor protein Ruk/CIN85) [7], MDA-MB-231 (human breast adenocarcinoma), LLC (mouse Lewis lung carcinoma), MM-4 (human melanoma), DLD-1 (human colonic adenocarcinoma), HT-29 (human colonic adenocarcinoma), NIH 3T3 (mouse fibroblasts) were maintained in DMEM («Gibco®», USA), containing 10 % fetal bovine serum («HyClone», USA), 2 mM glutamine, 50 U/ml penicillin, 50 µg/ml streptomycin («Gibco®») at 37 °C and 5 % CO<sub>2</sub> in a humidified atmosphere. Cells were split 1:3–1:5 every 2–3 days at 70–80 % confluence.

*Preparation of total cell lysates.* For obtaining total cell lysates, monolayer cultures of cells were washed in ice-cold PBS and scraped into lysis buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 50 mM NaF, 1 % Triton X-100, 5 mM EDTA supplemented with complete protease inhibitor cocktail tablet («Roche», France), 1 mM sodium orthovanadate, and 1 mM phenylmethylsulfonyl fluoride (PMSF) («Fluka», Switzerland). The lysates were kept at 4 °C for 20 min and then were clarified by centrifugation at 12000 rpm at 4 °C. Protein content in supernatants was determined using bicinchoninic acid protein assay kit (Pierce® BCA Protein Assay Kit, «Thermo Scientific», USA) according to the manufacturer's instructions.

*GST in vitro pull-down assay.* Plasmids *pGEX-4T*, which encode adaptor protein Ruk/CIN85 fragments fused with the glutathione S-transferase (GST), are described in [8].

Single clone of BL21(DE3)pLysS cells («Stratagene», USA) transformed with appropriate plasmid was resuspended in LB supplemented with 75 µg/ml ampicillin and 34 µg/ml chloramphenicol. Cells were grown to OD<sub>600</sub> = 0.5–0.6 at 37 °C.

Expression of recombinant proteins was induced by 1 mM IPTG (isopropyl-β-D-thiogalactoside) with further incubation at 25–27 °C for 4 h. Cells were pelleted by centrifugation at 3000 rpm, washed with ice-cold PBS, lysed in appropriate amount of lysis buffer (see above) at the ratio 1 : 10 for 20 min on ice. Cell lysates were sonicated 3 times for 20 s and centrifuged at 12000 rpm at 4 °C for 30 min.

Supernatants were added to Glutathione-Sepharose beads 4B («Amersham Biosciences», USA) in lysis buffer. Samples were incubated for 2 h at 4 °C. Glutathione-Sepharose beads were washed by PBS supplemented with 0.05 % Tween-20 and pelleted by centrifugation at 2000 rpm for 1–1.5 min. Amount of recombinant proteins bound to Glutathione-Sepharose beads was verified using SDS-PAGE. For the pull-down experiments, equal amounts of fusion proteins (about 15 µg at density 0.5 µg per 1 µl of Glutathione-Sepharose beads 4B) were used.

Cell lysates (about 1.5 mg of total protein) were incubated with 60 µl of 50 % Glutathione-Sepharose beads conjugated with corresponding recombinant proteins. Protein complexes were pelleted by centrifugation at 3000 rpm. Unbound proteins were removed by washing the beads 5 times with lysis buffer, whereas retained proteins were resolved by SDS-PAGE [9] and analyzed by Western blotting [10]. For detection of Tks4 on blots, the affinity purified polyclonal rabbit antibodies against SH3AB fragment of Tks4 were used. These antibodies were obtained and characterized in our laboratory. Anti-rabbit secondary antibodies (peroxidase-conjugated IgG from «Sigma», USA) were used. The enhanced chemiluminescence kit («Amersham Pharmacia Biotech», USA) was used for detection of immunoreactive protein bands.

**Results and discussion.** Cell lines of different tissue origins, in particular, MCF-7, MDA-MB 231, HT-29, DLD-1, MM-4, NIH 3T3 and LLC were investigated. According to Fig. 1, the obtained antibodies to SH3AB fragment of Tks4 (dilution 1:5000) effectively recognize the endogenous full-length form of Tks4 (*M<sub>r</sub>* 120 kDa) in all analyzed cell lysates. The highest levels of Tks4 were identified in MDA-MB-231, LLC, MM-4 and DLD-1 cells, while lower levels – in MCF-7 and HT-29 cells.

Based on these results we can conclude that the level of Tks4 expression is specific for each cell line and does not depend on tissue origin. As can be seen in Fig. 1, the additional form of Tks4 (*M<sub>r</sub>* 160 kDa) is detected in MDA-MB-231, LLC, MM-4, DLD-1 and NIH 3T3 cell lysates.

Progress in the field of functional proteomics during the last decades is determined by the development and further improvement of methodological approach

ches for decoding intermolecular interactions networks. It is known that these methods are divided into two groups, synthetic and analytical. GST *in vitro* pull-down assay is one of the analytical methods, which allow identification of the complexes usually purified by affinity chromatography. The components of purified supramolecular complexes can be recognized by specific antibodies or mass-spectrometry. The aim of analytical methods is the identification of protein partners *in cellulo* under physiological conditions. As a result, the data on protein composition of purified complexes provide an immediate success on the way to the determination of their functional significance.

GST-fused SH3A, SH3B, SH3C, SH3AB, SH3BC, SH3ABC domains, Pro-rich, Ser-rich, coiled-coil regions, which overlapped all amino acid sequence of Ruk<sub>p</sub>, were used for GST *in vitro* pull-down assay. According to the results obtained (Fig. 2), only SH3A domain precipitates Tks4 from lysates of breast adenocarcinoma (MCF-7 and MDA-MB-231) and melanoma (MM-4) cells. In mouse Lewis lung carcinoma cells (LLC) Tks4 is preferentially precipitated by SH3A domain, whereas weak immunoreactive bands are detected in SH3C- and SH3BC-precipitates. Tks4 is not detected in SH3B- and SH3AB-precipitates. However, a significant reduction of Tks4 binding in SH3ABC-precipitate in comparison with SH3A domain is observed. The performed analysis allows suggesting that at the level of three-dimensional structure of Ruk/CIN85 SH3ABC fragment it is SH3B domain that can screen the binding surface of SH3A domain for Pro-rich sequences of Tks4 in LLC cells. We also suggest that phosphorylation of specific Ser/Thr residues localized in SH3AB domains as well as C-terminally from Pro-rich region in endogenous Ruk/CIN85 mediated by input signal may lead to destabilization of its auto-inhibitory conformation and provides the capacity to form complexes with specific binding partners. In mouse fibroblasts, the full-length form of Tks4 is mainly precipitated by SH3A and weakly by SH3BC and SH3ABC domains.

Interesting results were obtained using pull down assay for colonic cell lines such as DLD-1 and HT-29. As can be seen in Fig. 2, the main Tks4 form (M<sub>r</sub> 120 kDa) is detected only in SH3A-precipitate. Simultaneously, all used GST-SH3 fragments of Ruk/CIN85 (A, B, C,

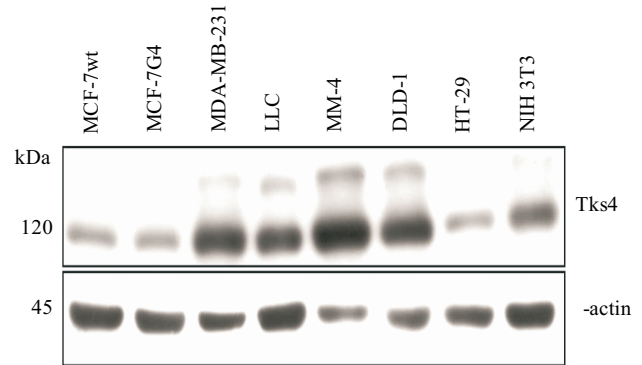


Fig. 1. Expression of adaptor protein Tks4 in cell lines of different tissue origins. Immunoblot analysis of cell lysates was performed using the affinity purified rabbit polyclonal anti-Tks4 antibodies produced against Tks4 SH3AB fragment

AB, BC and ABC) with high efficiency precipitate the additional form of 90 kDa, the content of which in cells is extremely low. This form is revealed in Western blotting only in the case of increased protein addition into the pockets of PAA gel. It is possible that the form with M<sub>r</sub> 160 kDa, which is detected in precipitates, could be a result of posttranslational modification of Tks4 by ubiquitination or related protein modifications. New additional form in NIH 3T3 cells is characterized by higher mobility (M<sub>r</sub> about 75 kDa). It is precipitated by all GST-SH3 fragments of Ruk/CIN85 (A, B, C, AB, BC and ABC). It should be noted that the high molecular weight form of Tks4 is detected in NIH 3T3 cell lysates but it is not precipitated by SH3 domains of Ruk/CIN85. For more careful examination of the specificity of the obtained anti-Tks4 polyclonal antibodies, we performed an antigen competition experiment. For this purpose, anti-Tks4 antibodies were preincubated with purified GST-Tks4/SH3AB fragment, and then this mixture was used for Western blotting of Tks4 endogenous forms. We found that detection of all immunoreactive bands was abrogated by antigen competition (data not shown).

The molecular nature of new multiple Tks4 forms with M<sub>r</sub> 75, 90 and 160 kDa identified in our study is unknown. At present there are no data about posttranslational modifications of Tks4 and additional mRNAs, which could be formed as a result of alternative splicing or alternative promoter usage in *TKS4* gene. The database analysis of expressed sequence tags (ESTs) allows identifying some ESTs, which could encode the

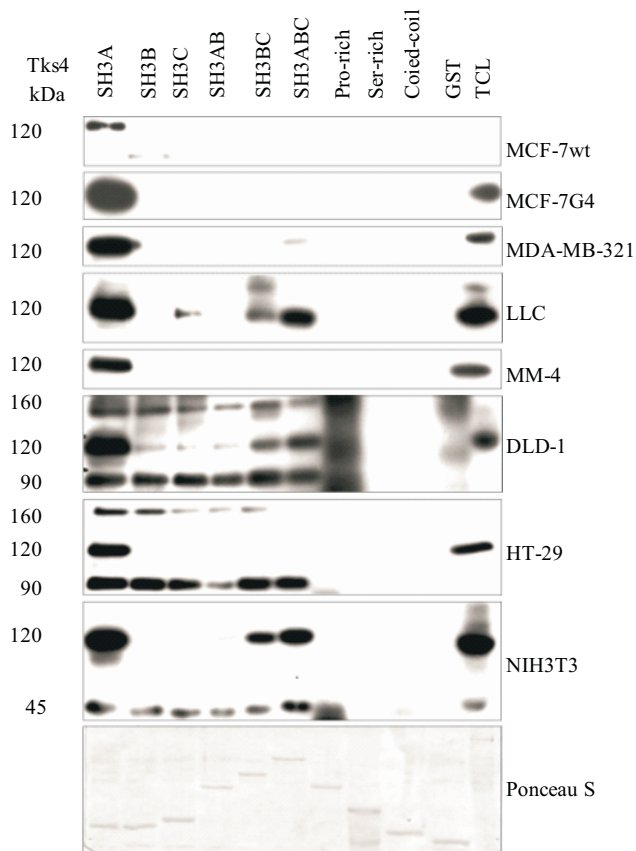


Fig. 2. SH3 domains of Ruk/CIN85 precipitate multiple forms of endogenous protein Tks4 from cell lysates of different tissue origins. Western blotting was performed using the polyclonal anti-Tks4 antibodies (1:5000). TCL – total cell lysate; GST – Glutathione-S-Transferase

isoforms of Tks4 [11]. However, ESTs are just segments of corresponding cDNAs. So, further investigations are necessary to elucidate the mechanisms of generation and molecular nature of new Tks4 multiple forms.

**Conclusions.** New multiple molecular forms of adaptor protein Tks4 with  $M_r$  75, 90 and 160 kDa, which interact with SH3 domains of Ruk/CIN85, were identified using GST *in vitro* pull-down assay following by Western blotting. The capacity of Ruk/CIN85 SH3 domains to interact with multiple endogenous forms of Tks4 is determined by the cellular context while the level of their interaction can be regulated in the course of physiological cellular responses.

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Взаємодія між адаптерними білками Ruk/CIN85 і Tks4 у нормальних і пухлинних клітинах різного тканинного походження

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Резюме

**Мета.** З'ясувати особливості взаємодії окремих доменів SH3 адаптерного білка Ruk/CIN85 з ендogenous адаптерним білком Tks4 у нормальних і пухлинних клітинах різного тканинного походження. **Методи.** Аналіз GST *in vitro* pull-down проводили з використанням загальних клітинних лізатів клітин різного тканинного походження. **Результати.** Встановлено, що домен SH3A адаптерного білка Ruk/CIN85 преципітує повнорозмірну форму адаптерного білка Tks4 ( $M_r$  120 кДа) з лізатів пухлинних клітин молочної залози (MCF-7, MDA-MB-231), меланому (MM-4), ободової кишки (HT-29, DLD-1) людини, а також карциноми легені Льюїс (LLC) та фібробластів (NIH 3T3) миші. Виявлено також, що всі домени SH3 білка Ruk/CIN85 (A, B і C) з високою ефективністю преципітують додаткові форми Tks4 з  $M_r$  75, 90 і 160 кДа з лізатів клітин карциноми ободової кишки людини та фібробластів миші. Молекулярну природу нових множинних форм Tks4 на сьогодні не встановлено. **Висновки.** Ідентифіковано нові множинні молекулярні форми адаптерного білка Tks4 з  $M_r$  75, 90 і 160 кДа, здатні з високою афінністю взаємодіяти з доменами SH3 білка Ruk/CIN85. Отримані дані дозволяють припустити, що можливість зв'язування доменів SH3 Ruk/CIN85 з ендogenous формами білка Tks4 обумовлена клітинним контекстом, тоді як рівень взаємодії регулюється в процесі реалізації фізіологічних відповідей клітин.

**Ключові слова:** адаптерні білки, Ruk/CIN85, Tks4, домени SH3, аналіз GST *in vitro* pull-down.

Взаимодействие между адаптерными белками Ruk/CIN85 и Tks4 в нормальных и опухолевых клетках различного тканевого происхождения

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Резюме

**Цель.** Выяснить особенности взаимодействия отдельных доменов SH3 адаптерного белка Ruk/CIN85 с ендogenous адаптерным белком Tks4 в нормальных и опухолевых клетках различного тканевого происхождения. **Методы.** Анализ GST *in vitro* pull-down проводили с использованием общих клеточных лизатов линий клеток различного тканевого происхождения. **Результаты.** Установлено, что домен SH3A адаптерного белка Ruk/CIN85 преципитирует полноразмерную форму адаптерного белка Tks4 ( $M_r$  120 кДа) из лизатов опухолевых клеток молочной железы (MCF-7, MDA-MB-231), меланомы (MM-4), ободочной кишки (HT-29, DLD-1) человека, а также карциномы легкого Льюис (LLC) и фибробластов (NIH 3T3) мыши. Виявлено также, что все домены SH3 белка Ruk/CIN85 (A, B и C) с высокой эффективностью преципитируют дополнительные формы Tks4 с  $M_r$  75, 90 и 160 кДа из лизатов клеток карциномы ободочной кишки человека и фибробластов мыши. Молекулярная природа новых множественных форм Tks4 на сегодня не установлена. **Выводы.** При помощи анализа GST *in vitro* pull-down идентифицированы новые множественные молекулярные формы адаптерного белка Tks4 с  $M_r$  75–90 и 160 кДа, способные с высокой аффинностью взаимодействовать с домена-

ми SH3 белка Ruk/CIN85. Полученные данные позволяют предположить, что возможность взаимодействия доменов SH3 белка Ruk/CIN85 с эндогенными формами белка Tks4 определяется клеточным контекстом, в то время как уровень связывания регулируется в процессе реализации физиологических ответов клеток.

Ключевые слова: адаптерные белки, Ruk/CIN85, Tks4, домены SH3, анализ GST *in vitro* pull-down.

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