

Intra- and intermolecular interactions mediated by adaptor protein Ruk/CIN85/SETA

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Ruk/CIN85/SETA is a member of a separate and evolutionary conserved family of adaptor/scaffold proteins implicated in apoptic and receptor tyrosine kinases signalling, rearrangement of actin cytoskeleton and cell adhesion, podocyte and T cell functions. Self-regulation through intra- and intercellular interactions can be supposed for Ruk/CIN85/SETA as this protein contains SH3 domains and proline-rich sequence, localized within one polypeptide chain, as well as C-terminal coiled-coil region. The ability of Ruk proline-rich motifs to interact with its own SH3 domains in an intramolecular fashion and coiled-coil region to mediate oligomerization between different isoforms was assessed in GST pull down experiments. It was shown that both Ruk SH3A and to a less extent SH3B domains can interact with its own proline-rich sequences in a cooperative manner, while coiled-coil region provide for isoforms oligomerization. SH3C domain appear exerts conformational constraints, imposed on coiled-coil region, restricting the level of oligomerization. We also demonstrated that the ability of exogenous ligands to interact with Ruk polyproline motifs is changing during the course of TNF α -induced apoptosis of human myelomonocytic U937 cells.

Key words: adaptor protein, SH3 domain, proline-rich region, coiled-coil region, protein-protein interaction.

Introduction. Up to date, it is well recognized and widely accepted that protein-protein interactions mediated by modular recognition domains represent structural and regulatory framework for the arrangement of functional signalling networks [1]. Src-homology 3 (SH3) domain, is one of the most widespread protein recognition modules in the proteome critically involved in the assembly of many intracellular signalling complexes and pathways [2]. SH3 domains represent independently folding modules (of about 60 amino acids residues in length)

able to establish intermolecular associations among signalling molecules through binding to prolinerich sequences which usually adopt left-handed polyproline type II (PPII) helices [2].

Ruk/CIN85/SETA, a member of a separate and evolutionary conserved family of SH3-containing adaptor/scaffold proteins, was cloned and characterized in the end of 2000 by three independent scientific groups [3–5]. Distinctive features of these proteins are three SH3 domains (A, B and C) located at the N-termini, followed by proline- and serine-rich sequences and C-terminal coiled-coil region [6]. Due to modular organization, Ruk/CIN85/SETA and its splice variants (see Fig. 1) play complex and varied roles in important biological processes such as prolifera-

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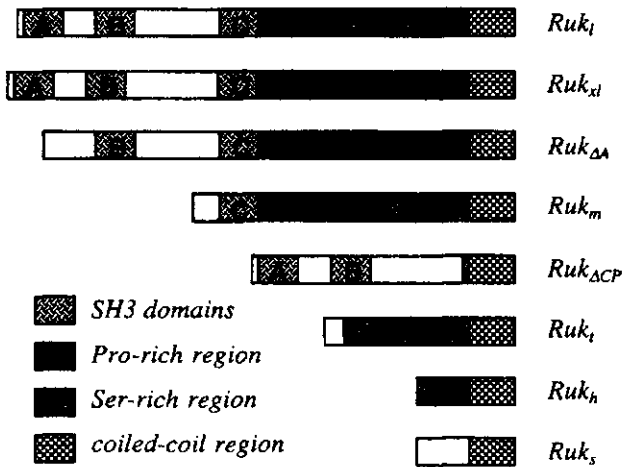


Fig. 1. Domain organization of Ruk isoforms (schematic representation) [7]

ration and apoptosis [3, 8], ligand-induced endocytosis of receptor tyrosine kinases [9–11], rearrangement of actin cytoskeleton [12, 13], cell cytokinesis, adhesion and motility of T cells during antigen presentation [13, 14], and possibly in nuclear events [15, 16]. This means that biological activity of Ruk/CIN85/SETA has to be a subject of tight regulation depending on cellular context. The simultaneous presence of three SH3 domains and four blocks of proline-rich motifs in Ruk/CIN85/SETA structure, suggests the possibility of intra- and homophilic intermolecular interactions, which in turn may account for autoregulation of the protein. Besides, C-terminal coiled-coil region is supposed to be involved in the formation of oligomers between Ruk isoforms, which possess different biological significance. In attempt to further characterize interactions mediated by domains and motifs of Ruk/CIN85/SETA we used GST *in vitro* binding assay and specific polyclonal antibodies. It was shown that both Ruk SH3A and to a less extent SH3B domains can interact with its own proline-rich sequences, while coiled-coil region mediates oligomerization between different isoforms. We also demonstrated that the ability of exogenous ligands to interact with Ruk polyproline motifs changes in the course of TNF α -induced apoptosis of human myelomonocytic U937 cells.

Materials and Methods. *pCMV5* expression plasmids encoding various Ruk isoforms and mutants with C-terminal FLAG-tag (DYDDDDK) and *pGEX-2T* expression plasmids encoding GST-fusion SH3A, SH3B, SH3C domains and Ruk₁ isoform were des-

cribed earlier [17]. An expression plasmid encoding GST-3SH3 CIN85 fragment was provided by I. Dikic (Institute of Biochemistry II, Frankfurt, Germany). Anti-Ruk₁ polyclonal antibodies were raised in rabbit using standard protocol. Specific polyclonal antibodies were purified from serum of an immunized rabbit using GST-Ruk₁-Sepharose. An anti-FLAG-tag antibody was from «Sigma» (USA), and secondary horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG antibodies were purchased from «Promega» (USA). All reagents for cell culture were from GibcoBRL.

Cell culture and transfection. Human embryonic kidney (HEK) 293T cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % fetal calf serum (FCS), 2 mM L-glutamine, 50 IU/ml penicillin, and 50 μ g/ml streptomycin in humidified atmosphere containing 5 % CO₂ at 37 °C. Human myelomonocytic leukemia U937 cells were grown in RPMI-1640 medium containing 10 % FCS. HEK293 cells were plated on 10 cm diameter dishes and transfected with 10 μ g of plasmid DNA by a modified calcium phosphate method [18]. 24 hours after transfection, cells were washed twice with cold phosphate buffered saline (PBS) and lysed in the lysis buffer (LB) containing 10 mM Tris, pH 7.5, 150 mM NaCl, 1 % Triton X-100, 5 mM EDTA, 20 mM NaF, 1 mM Na-orthovanadate, 1 mM PMSF, and a cocktail of protease inhibitors from Roche at 4 °C for 20 min. Lysates were cleared by centrifugation at 17000 g for 20 min at 4 °C and supernatants were used for further experiments. Before induction, U937 cells were cultured in RPMI 1640 medium supplemented with 2 % FCS for 24 hours and then plated at $1 \cdot 10^6$ cells/ml in RPMI 1640 with 10 % FCS. Cells were induced with TNF α at the final concentration of 10 ng/ml. Cell lysates were prepared at the 2nd, 4th and 6th hour after induction.

Glutathione S-transferase (GST) binding assay. GST-fusion constructs were expressed in IPTG-induced *Escherichia coli* BL21 at 30 °C for 4 hrs. Cells were pelleted at 3000 g for 15 min at 4 °C. Pellets were washed in PBS and resuspended in the buffer containing 50 mM Tris, pH 7.5, 150 mM NaCl, 1 % Triton X-100, 1 mg/ml lysozyme, and protease inhibitors. After one freeze-thaw cycle, the bacteria were sonicated on ice and cleared from cell debris by centrifugation at 13000 g for 30 min at 4 °C. The efficiency of the bacterial lysis was checked by

SDS-PAGE. The GST-fusion proteins were isolated from the supernatants using glutathione-Sepharose beads, as recommended by the manufacturer («Amersham Pharmacia Biotech», UK).

For pull-down experiments, GST or GST-fusion proteins (7 μ g of protein) bound to glutathione-Sepharose (approximately 20 μ l of packed beads equilibrated in 50 mM Tris, pH 7.5, 150 mM NaCl, 1 % Triton X-100) were incubated for four hours with normalized cleared lysates (1.5 mg of total protein) of HEK293 cells transiently transfected with Ruk plasmids. The cleared lysates were prepared as described above. The beads were washed three times with PBS/1 % Triton X-100, 3 times with PBS and boiled in 50 ml of SDS-loading buffer. Eluted proteins were separated by SDS-PAGE [19] and transferred onto PVDF membrane for Western blot analysis with anti-FLAG or anti-Ruk_i antibodies. All Western blots were performed according to a standard protocol [20]. The final detection was done with ECL system («Amersham Pharmacia Biotech», UK).

Results and Discussion. Protein-protein interactions are ubiquitous regulatory events in cell biology. Therefore, it is not surprising that nature has evolved and coordinated multiple mechanisms to exquisitely control the biological activity of adaptor/scaffold proteins which function as the main organizing centers of signalling networks. These mechanisms usually include different types of post-translational modification and intermolecular or intramolecular associations with inhibitory molecules or domains [1]. These inhibitory interactions may be disrupted in response to appropriate stimuli allowing the adaptor/scaffold protein to adopt a biologically active conformation.

Since adaptor protein Ruk/CIN85/SETA contains four blocks of proline-rich motifs, we were interested whether its SH3 domains can bind to these motifs in an autoregulatory fashion.

To evaluate the possibility of intramolecular interactions between different Ruk SH3 domains and its own C-terminally located proline-rich stretches, GST *in vitro* binding assay was used. For this purpose, the GST-fused A, B, and C SH3 domains, 3SH3 (ABC) fragment, and a shortest Ruk_i isoform which includes only the C-terminal coiled-coil region were incubated with lysates of HEK293 cells transiently transfected with constructs encoding different Ruk isoforms and mutants. Proteins bound to glutathione-Sepharose beads were analysed by Western blotting using anti-

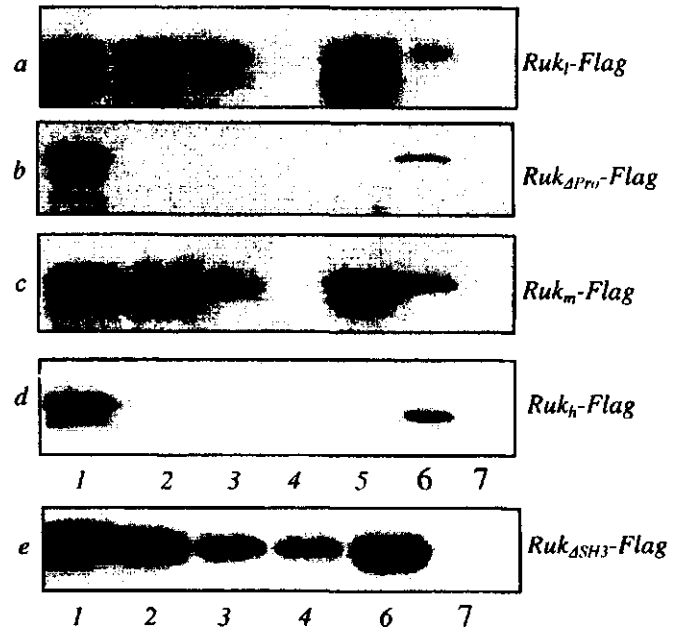


Fig. 2. Interaction of separate domains of Ruk with Ruk isoforms and mutants *in vitro*. Lysates of HEK293 cells, transiently transfected with *pCMV5* vectors encoding FLAG-tag Ruk isoforms and mutants, were incubated with glutathione-Sepharose-immobilised GST-SH3 domains or GST-Ruk_i. The presence of Ruk proteins in precipitates was assessed by Western blotting using anti-FLAG antibody: 1 — whole cell lysates; 2 — GST-SH3A; 3 — GST-SH3B; 4 — GST-SH3C; 5 — GST-3SH3; 6 — GST-Ruk_i; 7 — GST

FLAG antibodies. The results in Fig. 2 show that Ruk SH3A and, to a lesser extent, SH3B domains were able to bind to proline-rich motif-bearing Ruk_i (full length) and Ruk_m (without two of three N-terminal SH3 domains) isoforms and Ruk_{ΔSH3} mutant (Fig. 2, a, c, e) but not to Ruk_{ΔPro} or Ruk_n (heart specific Ruk isoform) (Fig. 2, b, d) which don't contain SH3 recognition sequences.

The increase of binding was detected in precipitates using GST-3SH3 fragment in comparison with using GST-SH3A and B domains as baits, separately. It was also shown that Ruk SH3C domain does not bind to Ruk_i and Ruk_m isoforms (Fig. 2, a, c) but pulls down Ruk_{ΔSH3} mutant quite effectively (Fig. 2, e). In similar experiments using human orthologues of Ruk, CIN85 [21] and CD2BP3 [14], binding of GST-SH3B to their own proline-rich stretches was not detected. These partially differing results may be explained by certain peculiarities of carrying out GST pull-down experiments. As it can be seen from Fig 2, all the five studied Ruk proteins were pulled down effectively by GST-Ruk_i, confirming the results of previous studies, which sug-

gested the role of coiled-coil region in the di(oligo)merisation of Ruk proteins [22, 23]. One should note a significant increase of Ruk_{ΔSH3} binding to GST-Ruk₄ compared to the extent of Ruk₁, Ruk_m, Ruk_h and Ruk_{ΔPro} interactions.

The above-mentioned observations allow us to draw the following conclusions. First, intramolecular interactions mediated by Ruk SH3A and B domains are characterized by a cooperativity. Second, the SH3C domain, although not involved in intramolecular interactions, may play a regulatory role at the level of whole conformation of Ruk protein providing correct SH3A/B — proline-rich motifs recognition. Possibly, the SH3C domain also exerts conformational constraints imposed on coiled-coil region, restricting the level of oligomerisation.

The obtained data suggest that Ruk may be self-regulated by a physical interaction between its SH3 domains and the SH3 binding sites the exact nature of which is to be determined. Besides, we can conclude that SH3 domains of Ruk serve both as the regulatory domains and as the recruiting units.

While Ruk/CIN85/SETA represents an example of SH3-mediated autoregulation of adaptor protein, the well studied tyrosine kinases of Src family [24—27] and a serine/threonine mixed lineage kinase 3 (MLK3) [28] as well as NADPH oxidase [29] offer some interesting parallels. It was shown that the SH3 binding sequence in Src, and also in its relative Fyn, as well as in MLK3 harbors only a single proline residue, although their SH3's preferred ligands were found to be classical proline-rich sequences [24, 25, 28]. These data suggest that the SH3-binding sequences in Src kinases and MLK3 represent the suboptimal ligands for their SH3 domains. Although the GAPPR sequence in p47^{phox} cytosolic component of the NADPH oxidase is not the canonical SH3 binding sequence, it can adopt PPII helix conformation that was determined from the X-ray crystal structure data [29]. There are no PXXP motifs or hydrophobic residues that confer specificity toward the SH3 domains. It is interesting to note that this PPII helix is recognized by the tandem SH3 domains of NADPH oxidase using different sides of a triangular prism as the basal planes, with the plus and minus orientations, respectively. Thus, the authors proposed that in this respect GAPPR does not bind tightly to each SH3 domain, but at the same time its specificity is still maintained by simultaneous binding to both SH3 domains [29].

Additionally we could draw a parallel connected with the presence of the cluster of four arginine residues in the cleft between two SH3 domains in NADPH oxidase, which appear to constitute the interaction core [29]. Similar arginine clusters are also present in Ruk/CIN85/SETA structure [4—6]. Thus, the evolutionary selection may have given rise to relatively low affinity intramolecular ligands for SH3 domains so that they might be outcompeted by the presentation, in response to an appropriate physiological signal, of a high affinity proline-rich ligand on another signalling molecule [30, 31]. Signalling molecules that overcome SH3-mediated autoregulation of Ruk/CIN85/SETA have yet to be identified. One potential candidate is the regulatory subunit of PI 3-kinase that contains SH3 domain and proline-rich sequences through which it interacts with Ruk in a concerted manner [17]. Another example represents members of Cbl ubiquitin ligase family which interact with CIN85 via their SH3 domains, and this association is enhanced by growth factor-induced tyrosine phosphorylation of Cbl [21].

There are data consistent with the idea that phosphorylation of specific serine residues could destabilize additionally the autoinhibited conformation of signalling proteins to form the unmasked state as it was shown for MLK3 and NADPH oxidase [28, 29]. Potential serine phosphorylation sites that reside C-terminally to the proline-rich region in Ruk/CIN85/SETA structure might play such functions.

An unresolved issue is also a possibility that intramolecular interactions mediated by domains and motifs within Ruk molecule take place in a homodimeric manner and have a more complex character. For example, studies on Bruton's tyrosine kinase (Btk) demonstrated that both proline-rich sequences in the Tec homology (TH) region located immediately N-terminally to the SH3 domain stabilize intermolecular interactions, whereas the related Itk kinase containing one proline-rich motif can fold onto and bind its SH3 domain in an intramolecular association [32].

The next question which we decided to elucidate is whether the system of Ruk-dependent intramolecular interactions undergoes changes during the biological responses of cells. We used leukemic U937 cells induced to apoptosis with TNF α as a model. Lysates of control and induced U937 cells were subjected to GST *in vitro* binding assay using as bait GST-3SH3 fragment. DNA ladder assay was perfor-

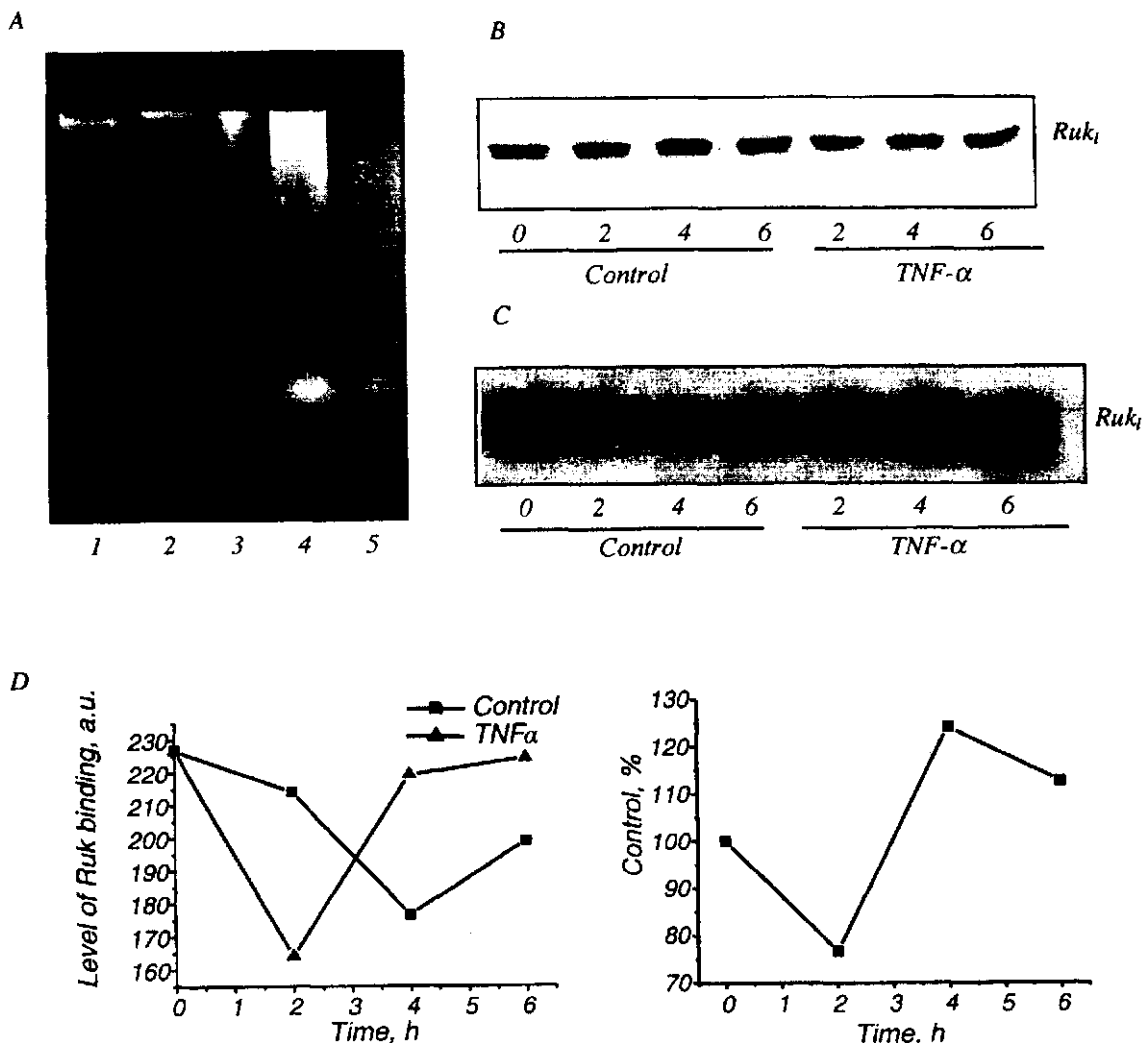


Fig. 3. The ability of exogenous ligands to interact with Ruk polyproline motifs is changing during the course of TNF α -induced apoptosis of human myelomonocytic U937 cells: *A* — DNA fragmentation by TNF α in U937 cells (*1* — DNA markers; *2* — control cells; *3–5* — TNF α -treated cells at 2nd, 4th and 6th hours of induction); *B* — expression of Ruk₁ protein in control and TNF α -treated U937 cells; *C*, *D* — interaction of endogenous Ruk₁ protein with GST-3SH3 fragment *in vitro* using lysates of control and TNF α -treated U937 cells

med to monitor the course of apoptosis. As can be seen from Fig. 3, *A*, incubation of U937 cells in the presence of TNF α (10 ng/ml) for 6 hours is followed by oligo- and mononucleosomal DNA fragmentation that is a characteristic feature of apoptosis. Under such conditions, the level of expression of endogenous full-length size Ruk₁ isoform with molecular weight of 85 kDa decreases insignificantly already after two hours of induction in comparison with control cells and does not change during the next studied time intervals up to the 6th hour (Fig. 3, *B*). Western blotting of GST precipitates using polyclonal anti-Ruk₁ antibodies had shown that the quantity of Ruk₁ bound to GST-3SH3 fragment is not supported at the

constant level in the lysates of non-induced cells. The decrease of binding is observed at the 4th hour followed by a small increase at the 6th hour of cell culturing (Fig. 3, *C*, and *D*). On the contrary, the dramatic decrease of Ruk₁ — GST-3SH3 interaction is detected in lysates of TNF α -treated U937 cells at the 2nd hour followed by a significant increase during the 4th — 6th hour period of induction, in comparison with intact cells, which coincides with the accumulation of nucleosomal DNA fragments (Fig. 3, *C*, *D*).

The obtained data allow us to conclude that several Ruk conformational states with different binding capabilities may co-exist at equilibrium. Due to the concerted nature of Ruk-mediated intra- and

intermolecular interactions, disruption of any of them, to a greater or lesser extent, shifts the equilibrium from the self-regulated (closed) conformation to the biologically active (exposed) conformation that enables interaction with exogenous target sequences in other signalling molecules. To clarify all these issues, further structural studies are necessary.

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Внутрішньо- і міжмолекулярні взаємодії, опосередковані адаптерним білком Ruk/CIN85/SETA

Резюме

Ruk/CIN85/SETA є представником окремої еволюційно консервативної родини адаптерних/риштувальних білків, залучених до апоптичного сигналювання і сигналювання, залежного від рецепторних тирозинових протеїнкіназ, перебудови актинового цитоскелету і адгезії клітин, контролю функцій подоцитів і Т клітин. Оскільки *Ruk/CIN85/SETA* містить одночасно SH3 домени і багаті на пролін послідовності на рівні одного поліпептидного ланцюга та С-кінцевий суперспіралізований район, можна припустити, що його біологічна активність регулюється за рахунок внутрішньо- і міжмолекулярних взаємодій. Здатність багатих на пролін мотивів *Ruk* взаємодіяти з власними SH3 доменами та суперспіралізованою ділянкою — опосередковувати олігомеризацію різних ізоформ досліджували за допомогою методу зв'язування *in vitro* GST-злитих білків. Показано, що SH3A і, в меншій мірі, SH3B домени *Ruk* кооперативно взаємодіють із власними багатими на пролін послідовностями, у той час як суперспіралізований район забезпечує олігомеризацію ізоформ. Не виключено при цьому, що SH3C домен чинить конформаційний вплив на суперспіралізований район, обмежуючи, в такий спосіб, ступінь олігомеризації. Нами також встановлено, що здатність екзогенних лігандів взаємодіяти з багатими на пролін мотивами *Ruk* координовано змінюється протягом TNF α -індукованого апоптозу мієломоноцитарних клітин лінії U937.

Ключові слова: адаптерний білок, SH3 домен, багата на пролін ділянка, суперспіралізований район, білково-білкові взаємодії.

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Внутри- и межмолекулярные взаимодействия, опосредованные адаптерным белком Ruk/CIN85/SETA

Резюме

Ruk/CIN85/SETA является представителем отдельного эволюционно консервативного семейства адаптерных/стеллажных белков, вовлеченных в апоптотическую сигнализацию и сигнализацию, зависящую от рецепторных тирозиновых протеинкиназ, перестройку актинового цитоскелета и адгезию клеток, контроль функций подоцитов и Т клеток. Поскольку *Ruk/CIN85/SETA* содержит одновременно SH3 домены и богатые на пролин последовательности на уровне одной полипептидной цепи, а также С-концевой суперспирализованный район, можно предположить, что его биологическая активность

регулируется за счет внутри- и межмолекулярных взаимодействий. Способность богатых на пролин мотивов *Ruk* взаимодействовать с собственными SH3 доменами и суперспирализованного участка — опосредовать олигомеризацию различных изоформ исследовали с помощью метода связывания *in vitro* GST-конъюгированных белков. Показано, что SH3A и, несколько слабее, SH3B домены *Ruk* кооперативно взаимодействуют с собственными богатыми на пролин последовательностями, в то время как суперспирализованный район обеспечивает олигомеризацию изоформ. Не исключено при этом, что SH3C домен оказывает конформационное влияние на суперспирализованный район, ограничивая таким образом степень олигомеризации. Нами также установлено, что способность экзогенных лигандов взаимодействовать с богатыми на пролин мотивами *Ruk* координировано изменяется в процессе TNF α -индуцированного апоптоза миеломоноцитарных клеток линии U937.

Ключевые слова: адаптерный белок, SH3 домен, богатый на пролин участок, суперспирализованный район, белково-белковые взаимодействия.

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