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ORIGINAL CONTRIBUTIONS

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EXPRESSION OF ADAPTOR PROTEIN RUK/CIN85 ISOFORMS IN CELL LINES OF VARIOUS TISSUE ORIGINS AND HUMAN MELANOMA

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Aim: **Development of monoclonal and polyclonal antibodies against recombinant GST-fused proteins including correspondingly N- and C-terminal parts of Ruk/CIN85 adaptor protein. Analysis of Ruk/CIN85 expression patterns in cell lines of various tissue origins and human melanoma.** ***Methods:*** **Recombinant GST-fused fragments of Ruk/CIN85 were expressed in bacterial system and affinity purified. Monoclonal antibodies against SH3A domain of Ruk/CIN85 were produced using hybridoma technique. The specificity of generated antibodies was examined by ELISA. Polyclonal antibodies against C-terminal coiled-coil region of Ruk/CIN85 were affinity purified from serum of immunized rabbit. Expression patterns of Ruk/CIN85 isoforms and their subcellular localization in cell lines of various tissue origins and human melanoma samples were analyzed by immunoblotting, immunoprecipitation and immunofluorescence microcopy.** ***Results:*** **Ruk/CIN85 is ubiquitously expressed SH3-containing adaptor/scaffold protein which plays important roles in signalling processes. N-terminal half of Ruk/CIN85 molecule, including three SH3 domains, and its C-terminal coiled-coil region were used as antigens to produce monoclonal and polyclonal antibodies, respectively. Hybridoma cell lines secreting monoclonal antibodies (mAbs) to SH3 fragment of Ruk/CIN85 were established. One of the mAbs was extensively characterized and designated as MISh-A1. It was shown that this mAb recognizes an epitope, which resides within first SH3A domain. Polyclonal anti-Ruk_s Abs affinity purified from serum of immunized rabbit specifically recognized main Ruk/CIN85 isoforms, both endogenous and recombinant, in lysates of HEK293 cells. Notably, produced Abs did not cross-react with CD2AP, the member of the same family of adaptor/scaffold proteins. Multiple molecular forms of Ruk/CIN85 with apparent molecular weights of 130, 80–85, 70–75, 50–56, 34–40 and 29 kD were detected in cell lyzates of NIH3T3, Cos1, L1210, HEK293, Ramos, HeLa S3, MDCK, C6, A549 and U937 using anti-Ruk antibodies. Oligomerization between p85 and p50–56 forms of Ruk/CIN85 was revealed in C6 and NIH3T3 cells, but not in HeLa S3 and HEK293 cells by immunoprecipitation using MISh-A1 antibody following anti-Ruk Western-blot analysis. Using immunofluorescent microscopy and anti-Ruk antibodies, endogenous Ruk-variates were found mostly in cytoplasm of C6, NIH3T3, HEK293 cells and at lower level — in nuclei.** ***Conclusion:*** **Patterns of Ruk/CIN85 molecular forms expression are cell-specific and determined by cellular context. Assembly of oligomeric complexes between p85 and p50–56 Ruk/CIN85 isoforms in C6 and NIH3T3 cells but not in HeLa S3 and HEK293 cells may reflect their specific biological roles in different cell lines. High level of full-length Ruk_l/CIN85 form expression was revealed in extracts of human melanoma samples. Abs described in this paper may prove useful in future studies of Ruk/CIN85 expression and function in normal and transformed cells.**

Key Words: **adaptor proteins, Ruk/CIN85, isoforms, antibodies, cell lines, melanoma, expression patterns.**

Widely expressed Ruk_l/CIN85/SETA, also identified as SH3KBP1 and CD2BP3 is an adaptor protein composed of three SH3 domains, Pro- and Ser-rich sequences, and C-terminal coiled-coil region. Sequence identity between rat (Ruk and SETA), mouse (SH3KBP1) and human (CIN85 and CD2BP3) orthologues varies between 92 and 97% [1–7]. *Ruk/cin85* gene gives more then one protein product. Northern hybridization, direct cDNA cloning, analysis of EST clones and organization of genomic loci in the data banks found multiple *ruk/cin85* transcripts encoding different Ruk/CIN85 isoforms [6, 7]. Some of them reveal tissue specific or developmentally regulated expression patterns [1, 2, 6, 7]. cDNAs encoding isoforms without the first N-terminal SH3 domain (SETA and CD2BP3), two N-terminal SH3 domains (Ruk_m and SH3KBP1), and a shortest Ruk_s isoform containing only coiled-coil region have been cloned until now [3–5, 7]. However results of Finnis et al. suggest that additional splice variants remain to be discovered [8].

Diverse protein binding domains of Ruk_l/CIN85 allow this protein to assemble various multiprotein complexes involved in several important intracellular processes [6]. For example, in complex with Cbl and endophilin, CIN85 regulates clathrin-mediated endocytosis of activated receptor tyrosine kinases [9, 10]. Role of Ruk_l/CIN85 in apoptosis has also been demonstrated. Firstly, it was found that overexpression of Ruk_l in cultured sympathetic and sensory neurons leads to apoptic death, but co-expression of activated forms of catalytic subunit of PI 3-kinase or its downstream effector PKB/Akt rescue this effect [1]. It was shown that complex set of intra- and intermolecular interactions between Ruk_l and p85α regulatory subunits of

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Abbreviations used: BSA – bovine serum albumin; CD2BP3 – CD2 binding protein 3; CIN85 – c-Cbl-interacting protein of 85 kDa; EGFR – epidermal growth factor receptor; FCS – fetal calf serum; GST – glutathione-S-tranferase; mAbs – monoclonal antibodies; pAbs – polyclonal antibodies; PBS – phosphate buffered saline; Ruk – Regulator for ubiquitous kinase; SETA – SH3 domain-containing expressed in tumorigenic astrocytes; SH3 – Src-homology type 3 domain; SH3KBP1 – SH3 domain kinase binding protein 1; TNFR1 – tumor necrosis factor receptor 1.

PI 3-kinase results in inhibition of this enzyme *in vitro* [1, 11]. This makes Ruk_l functionally similar to PTEN, an acknowledged tumor suppressor [12]. Important feature of the functional interplay between Ruk isoforms is the ability of Ruk_m (the form with only one SH3 domain) to act as dominant-negative regulator of a pro-apoptic function of Ruk_l in cultured neurons [1]. It has also been demonstrated that SETA protein, which retains two SH3 domains, is often overexpressed in gliomas and able to sensitize astrocytes to apoptosis in response to DNA damage [13]. As it was suggested, interaction between SETA, AIP1/Alix and ALG-2 proteins might comprise a mechanism of pro-apoptic function of SETA in glial cells. CIN85 involvement in the signalling complex with TNFR1, which lacks a tyrosine kinase domain, was described by Narita et al. [14]. Authors showed that CIN85 associates with the cytoplasmic tail of TNFR1 indirectly through Src and modulates TNF-α-induced apoptosis. In addition, CIN85 and CD2BP3 have been shown to regulate B- and T-cell receptor signalling, rearrangement of actin cytoskeleton and cell adhesion, play an important role in HSV-1 infection and biology of infected cells [5, 15–17]. Taken together, available functional data suggest that *ruk/cin85/seta* gene and encoded proteins play important roles in the regulation of homeostasis in normal cells and are also may be involved in oncogenic transformation.

Further comprehensive analysis of expression and function of Ruk/CIN85 proteins in both normal and tumor cells requires highly specific antibodies. Moreover, to study protein-protein interactions mediated by native Ruk/CIN85 proteins, antibodies, which work well and reliably not only in Western blotting but also in immunoprecipitation and immunohistochemistry as well as in immunofluorescence analysis, are required. Here we report development of N-terminal monoclonal and C-terminal polyclonal antibodies against Ruk/CIN85 and analysis of Ruk/CIN85 expression patterns in cell lines of various tissue origins, as well as in melanoma samples using these antibodies.

MATERIALS AND METHODS

Expression plasmids. 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 Ruk_s isoform were described previously [11]. Expression plasmid encoding GST-3SH3 CIN85 fragment was provided by I. Dikic (Institute of Biochemistry II, Germany). pcDNA 3.1 plasmid encoding myc-tagged CD2AP was provided by A. Shaw (Washington University School of Medicine, USA). An anti-FLAG-tag, anti-myc-tag antibodies and anti-β-actin antibodies were from (Sigma, USA), polyclonal anti-CD2AP were from (Santa Cruz Biotech, 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. Samples of non-transformed human skin and melanoma were obtained from Lviv Regional Oncological Centre.

Cell culture and transfection. Mouse fibroblast NIH3T3, African green monkey kidney Cos 1, human embryonic kidney HEK293, human cervix adenocarcinoma HeLa S3, Madin-Darby canine kidney MDCK, rat glioma C6, and human lung adenocarcinoma A549 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) while mouse leukemic L1210, human Caucasian Burkitt's lymphoma Ramos, and human myelomonocytic leukemia U937 cells were grown in RPMI-1640 medium supplemented with 10% FCS, 2 mM L-glutamine, 50 IU/ml penicillin, and 50 μg/ml streptomycin in humidified atmosphere containing 5% CO_2 at 37 °C. In transient transfection procedure, HEK293 cells were plated on 10 cm diameter dishes and transfected with 10 μg of plasmid DNA by a modified calcium phosphate method [18]. Subconfluent cultures of cells used and HEK293 cells, 24 hrs after transfection, were washed twice with cold 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 17,000 g for 20 min at 4 °C and supernatants were used for further experiments. Normal and tumor tissues were disrupted with liquid nitrogen an then lysed in LB.

Expression and affinity purification of GST-fusion fragments of Ruk/CIN85 protein. GST-fusion constructs were expressed in IPTG-induced *Escherichia coli BL21* at 30 °C for 4 hr. Cells were pelleted at 3,000 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, bacteria were sonicated on ice and cleared from cell debris by centrifugation at 13,000 g for 30 min at 4 °C. The efficiency of 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).

Generation of polyclonal and monoclonal antibodies to Ruk/CIN85 protein. Polyclonal anti-Ruk antibodies were produced in rabbit against recombinant GST-fused the shortest Ruk isoform, Ruk_s, which contains common for all isoforms C-terminal coiled-coil region. The anti-Ruk_s antibodies were affinity purified from immune serum stepwise on *E. coli* proteins-4B-Sepharose, GST-4B-Sepharose, and, finally, on Ruk_s-4B-Sepharose. All proteins used in purification procedure were covalently conjugated to CNBr 4B-Separose as recommended by the manufacturer (Sigma-Aldrich). Monoclonal antibodies against GST-fusion protein, which includes three SH3 domains of CIN85, were generated essentially as described [19]. Specific IgGs were purifed from hybridoma medium of exponentially growing clones or ascitic fluid produced in mice [20].

Western blotting and immunoprecipitation. Cell lysates and tissue extracts were boiled for 5 min with 2 x Laemmli's sample buffer and electrophoresed

on gradient (5–18%) SDS-polyacrylamide gel [21]. Then proteins were electrophoretically transferred to Immobilon-P membrane (Millipore Corp., USA) in buffer containing 25 mM Tris, 192.5 mM Glycine, 20% methanol at 250 mA for 2 hrs [22]. After blocking with PBST (PBS/0.05% Tween-20) containing 5% dried skim milk, the membrane was incubated with the appropriate primary antibody followed by incubation with secondary anti-mouse or anti-rabbit IgGs conjugated with horseradish peroxidase. The filter was washed three times with PBST and two times with PBS and subjected to ECL detection system (Amersham Biosciences, USA). For Ruk/CIN85 immunoprecipitation, precleared cell lysates (1.0 mg of total protein) were mixed with 20 µl of anti-SH3A mAB-immobilized beads followed by incubation for 3 h at 4 °C and constant mixing. Anti-SH3A mAb was coupled to protein G-Sepharose beads (Amersham Biosciences, USA) using dimethylpimelimidate (Sigma) according to manufactures' protocol. Myc-tagged CD2AP from transiently transfected HEK293 cells was immunoprecipitated using monoclonal anti-myc antibodies followed by incubation with protein G-Sepharose beads. Thereafter, beads were washed 5–6 times with PBST and boiled in SDS-loading buffer. Obtained samples were analyzed by SDS-PAGE and Western blotting as described above.

Immunofluorescence microscopy. For immunofluorescence analyses cells were stained with polyclonal anti-Ruk_s or monoclonal anti-SH3A antibodies. In brief, cells were fixed with cold methanol for 5 min at –20 °C. Fixed cells on coverslips were blocked for 30 min with 2% BSA in PBS, incubated with primary antibodies for 2h, and then with secondary FITC-conjugated antibodies for 1h at room temperature. Afterwards cells were stained with 300 nM DAPI solution for 5 min and mounted on glass slides with Vectashield mounting medium (Vector Laboratories, USA). In case of polyclonal anti-Ruk_s, perincubation of antibody with 10-fold molar excess of Ruk_s was used to confirm immunofluorescence specificity (see supplementary data). Imaging was performed under Nikon Diaphot microscope (Nikon Instruments Inc., USA) with Retiga 1300 CCD camera (QImaging Inc., USA) under 100 x objective lens.

RESULTS AND DISCUSSION

The lack of knowledge regarding the biological significance both expression levels and ratios of different Ruk/CIN85 isoforms complicates investigation of their roles in cancer cells. The availability of highly specific antibodies recognizing different epitopes of adaptor protein is a prerequisite to perform a detailed analysis of the expression patterns of Ruk/CIN85 isoforms at protein level.

N-terminal half of Ruk/CIN85 molecule including three SH3 domains, and its C-terminal coiled-coil region were used as antigens to produce monoclonal and polyclonal antibodies, respectively. GST-fusion forms of these recombinant proteins were expressed in bacterial system. Using one-step affinity purification procedure on glutathione-Sepharose, we obtained milligram quantities of highly pure (approx. 95% purity) preparations of GST-3SH3 and GST-Ruk_s (see supplementary data).

Immunoblot analysis revealed that affinity purified polyclonal anti-Ruk_s antibodies recognize equally effectively the main Flag-tagged Ruk isoforms in lysates of transiently transfected HEK293 cells (Fig. 1, *b*). Besides the endogenous full-length p85 Ruk_l/CIN85 form, a set of proteins with lower molecular weight was detected as well (Fig. 1, *a*). For more careful examination of the specificity of polyclonal antibodies, we performed an antigen competition experiment. For this purpose, anti-Ruk_s antibodies were preincubated with purified His-tagged Ruk_m, and then this mixture was used for Western blotting of Flag-tagged Ruk isoforms. We found that detection of all immunoreactive bands was abrogated by antigen competition (data not shown). In the second type of study, we first immunoprecipitated L1210 cell lysate with anti-Ruk_s antibodies and then Western blotted the immunoprecipitate with the same antibodies. As can be seen from Fig. 1, *c*, the main endogenous molecular forms of Ruk_l/CIN85 are detected in obtained immunoprecipitate comparing to whole L1210 cell lysate. Low level of Ruk_l/CIN85 recovering in immunoprecipitate may be explained by restricted accessibility of coiled-coil domain for anti-Ruk_s recognition in non-denatured conditions that is connected with the involvement of this region into homo- and heterotypic protein-protein interactions.

Immunization of male Balb/c mice with GST-3SH3 and generation of hybridoma cell lines were performed essentially as described by Savinska et al. [19]. According to ELISA results, 12 hybridoma clones of 263 analyzed produced antibodies recognizing GST-3SH3 but not GST alone. One of the hybridoma clones (designated MISh-A1), producing the highest titer of anti-3SH3 antibody, was recloned through two rounds of dilution cloning procedure. Epitope specificity of this mAb was further defined by Western blotting using lysates of HEK293 cells transiently transfected with constructs encoding Ruk_l, $Ruk_{\Delta A}$ and Ruk_m isoforms as well as the affinity purified preparations of GST-SH3A, GST-SH3B and GST-SH3C domains of Ruk. As shown in Fig 1,*d*, MISh-A1 antibody recognized full-length form of Ruk and SH3A domain only indicating its specificity for SH3A domain.

Taking into account high level of homology (39% identity and 54% similarity in amino acid sequence) between Ruk/CIN85 and CD2AP/CMS, which belongs to the same family of adaptor/scaffold proteins, it was important to test cross-reactivity of produced monoclonal and polyclonal antibodies. According to Western blot analysis (Fig. 2, lane 1), endogenous Ruk/CIN85 and CD2AP/CMS possess identical electrophoretic mobility in SDS-PAGE. For this reason, we at first performed immunoprecipitation of endogenous Ruk/CIN85 and myc-tagged CD2AP from lysates of HEK293 cells, transiently transfected with pcDNA-myc-CD2AP, followed by immunoblotting (see Fig. 2). In immunoprecipitates obtained with monoclonal anti-SH3A and anti-myc antibodies prominent specific bands with apparent molecular weight of 85 kDa corresponding

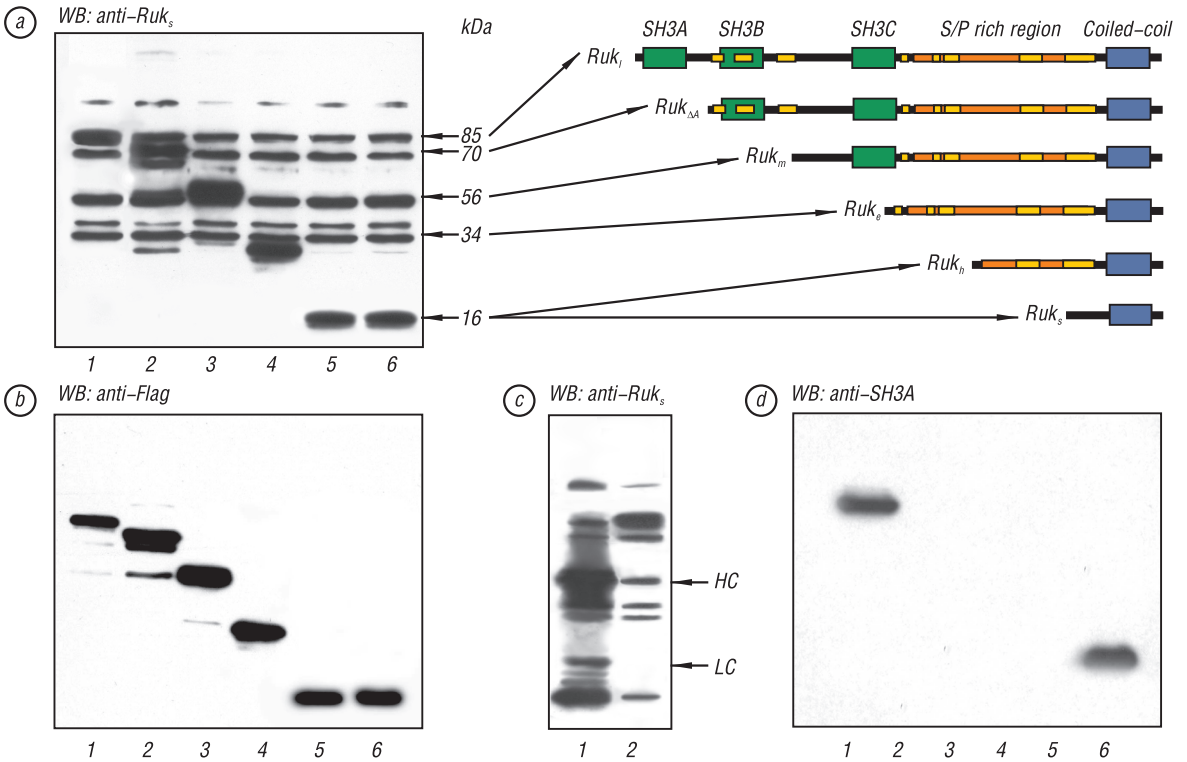

Fig. 1. Specificity of produced C-terminal anti-Ruk$_s$ polyclonal antibodies (*a–c*) and N-terminal monoclonal anti-SH3A antibody (*d*). *a, b*) immunoblot analysis of lysates of HEK293 cells transiently transfected with constructs encoding Flag-tagged isoforms of Ruk: 1 — Ruk$_l$; 2 — Ruk$_{\Delta A}$; 3 — Ruk$_m$; 4 — Ruk$_e$; 5 — Ruk$_h$; 6 — Ruk$_s$; *c*) immunoprecipitation of endogenous Ruk isoforms from lysate of L1210 cells using anti-Ruk$_s$ antibodies: 1 — immunoprecipitate; 2 — whole cell lysate; *d*) epitope specificity of MISh-A1 hybridoma clone. Immunoblot analysis of lysates of HEK293 cells transiently transfected with constructs encoding Flag-tagged Ruk$_l$ (*1*), Ruk$_{\Delta A}$ (*2*) and Ruk$_m$ (*3*) isoforms, and the affinity purified preparations of GST-SH3C (*4*), GST-SH3B (*5*) and GST-SH3A (*6*) domains of Ruk

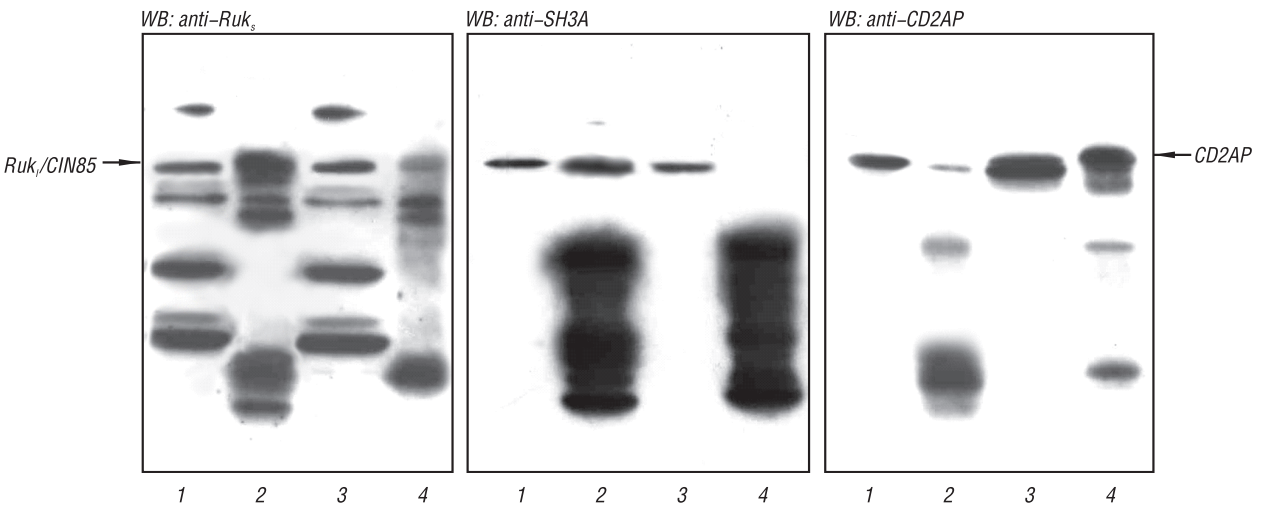

Fig. 2. Analysis of the crossreactivity of anti-Ruk$_s$ polyclonal and MISh-1 monoclonal antibodies to CD2AP: total cell lysates of non-transfected HEK293 cells (*1*) and HEK293 cells transfected with pcDNA-myc-tag CD2AP (*3*), proteins immunoprecipitated from lysates of transfected HEK293 cells with monoclonal anti-SH3A (*2*) and anti-myc (*4*) antibodies

to Ruk$_l$/CIN85 (lane 2) and myc-tagged CD2AP (lane 4) were detected. Very faint Ruk/CIN85- and CD2AP-specific bands were revealed in anti-myc (WB with anti-Ruk$_s$ antibodies, lane 4) and anti-SH3A (WB with anti-CD2AP antibodies, lane 2) immunoprecipitates correspondingly. Obtained data are in agreement with previous results [15] indicating the existence of oligomerization between two proteins in living cells.

Thus, produced C-terminal polyclonal and N-terminal monoclonal antibodies possess Ruk/CIN85-specificity and may be used further to study expression of Ruk/CIN85 isoforms in cell lines of different species and tissue origins as well as tumour samples using different approaches. The main immunoreactive bands in total cell lysates detected with anti-Ruk$_s$ antibodies are presented by proteins with apparent molecular weights of 130, 80–85, 70–75, 50–56, 34–40 and 19 kDa (Fig. 3). According to the current experimental data, multiple molecular forms of Ruk/CIN85, identified by Western-blot analysis with C-terminal antibodies, may result from alternative splicing of pre-mRNA (85 kDa full-length form, 70 kDa form without first SH3A domain, 56 kDa form without two SH3 domain, and 16 kDa Ruk$_s$ form containing C-terminal coiled-coil

region) [7]; post-translational modification through ubiquitination (130 kDa) [23] and limited proteolysis caused by the presence of PEST-motifs in C-terminal half of polypeptide chain (p40) [1, 24]. The existence of multiple sites for Ser/Thr-specific protein kinases in Ruk/CIN85 structure allow us to suppose that subforms around indicated molecular weights are mainly the result of post-translational modification of Ruk/CIN85 molecular forms through phosphorylation. The highest levels of full-length form of Ruk/CIN85 are observed in L1210, Ramos, HeLa S3, C6 and U937 cells while lower expression is revealed in NIH3T3 cells and low to undetectable – in A549 cells. As can been seen in Western blot p85 generally migrates as doublet with more prominent upper band for exception of Cos1 and MDCK cells for which the lower band is stoichiometrically greater. These results generally agree with Western-blot analysis using monoclonal anti-SHA antibody (Fig. 3). However, in cases of Cos1 and MDCK cells anti-SH3A antibody recognizes only upper faint band. It is possible that specific features of p85 post-translational modification in different cell lines can determine differential recognition of these subforms by MISh-A1 antibody. The obtained data also show that expression of p70 is high in HEK293, Ramos and MDCK cells, while p50–56 — in NIH3T3, MDCK and C6 cells. P16 is detected in Cos1 and L1210 cells only. Thus, the patterns of multiple Ruk/CIN85 molecular forms expression are characteristic for each cell line studied that may be determined by cellular context.

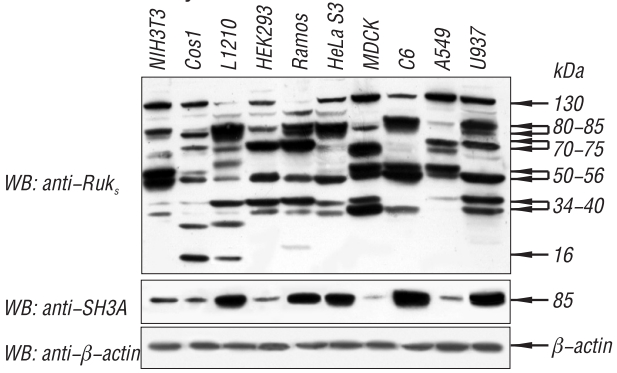

Fig. 3. Patterns of Ruk/CIN85 isoforms expression in cell lines of various tissue origins

As it is discussed in Introduction, the formation of oligomers between Ruk isoforms can result in different biological consequences [1]. The availability of specific Ruk/CIN85 N-terminal and C-terminal antibodies allowed precise investigation of oligomerization between different isoforms *in vivo*. For this purpose, we first immunoprecipitated full-length form with monoclonal MISh-A1 antibody, and then performed Western blot analysis using both polyclonal anti-Ruk$_s$ antibodies and MISh-A1 antibody. We used MISh-A1 antibody covalently conjugated to protein G-Sepharose for immunoprecipitation. The results in Fig. 4 show that anti-SH3A antibody effectively precipitates p85 from lysates of all cell lines studied: HeLa S3, HEK293, C6 and NIH3T3. Co-precipitation of proteins around molecular weight of 50–56 kDa, which might represent a form without two N-terminal SH3 domains, is observed for C6 and NIH3T3 cells but not for HeLa S3 and HEK293 cells. Please note specific ratios between these subforms in immunoprecipitates in comparison to total cell lysates. Possibly, differences in the state and composition of isoforms in Ruk/CIN85-mediated oligomeric complexes define their different biological role in these cell lines.

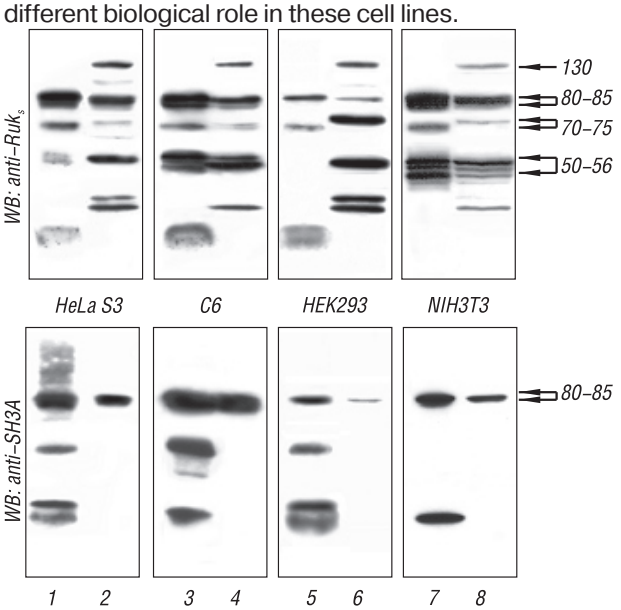

Fig 4. Oligomerization between different Ruk/CIN85 isoforms *in vivo*. Total cell lysates of HeLa S3, HEK293, C6 and NIH3T3 cells were immunoprecipitated with MISh-A1 antibody followed by Western blot analysis using anti-Ruk$_s$ and MISh-A1 antibodies: 1, 3, 5, 7 — immunoprecipitates; 2, 4, 6, 8 — total cell lysates

To obtain detailed picture of the intracellular distribution of Ruk/CIN85 we performed further immunofluorescence analysis using C6, NIH3T3 and HEK293 cells as a model. In all these cells stained with polyclonal anti-Ruk$_s$ antibodies endogenous Ruk variants were localized predominantly in the cytoplasm and to a lesser extent in the nuclei (Fig. 5). The cytoplasmic localization revealed both diffuse and punctate pattern. Unfortunately results of immunofluorescent staining with monoclonal antibody were not reproducible.

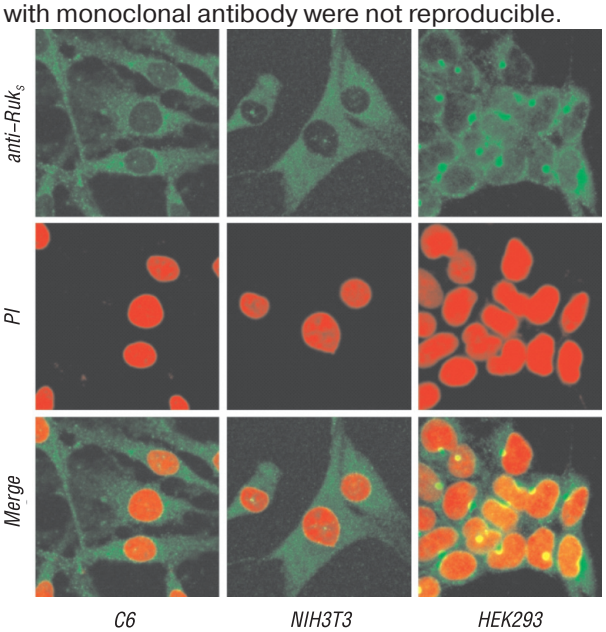

Fig. 5. Immunofluorescence microscopy of C6, NIH3T3 and HEK293 cells stained with polyclonal anti-Ruk$_s$ antibodies. Nuclei were stained with propidium iodide

In the last series of experiments we checked convenience of the produced Ruk/CIN85-specific antibodies for studies of Ruk/CIN85 isoforms expression across a large panel of samples representing diverse human tissues and tumor types. For this purpose we used human non-transformed skin and melanoma samples, obtained under surgical procedures in Lviv Regional Oncological Center, as a model. According to data of anti-Ruk_S Western-blot analysis, presented at Fig. 6, immunoreactive bands with apparent molecular weight around 70 and 50–56 kDa are the main in extracts of normal skin tissue. In comparison to the adjacent normal tissue, pattern of CIN85/Ruk molecular forms in melanoma samples is characterized by the substantial increase in the expression level of full-length p85 form, that presumably can lead to the loss of coordinated control of apoptosis and proliferation in the transformed melanocytes [25].

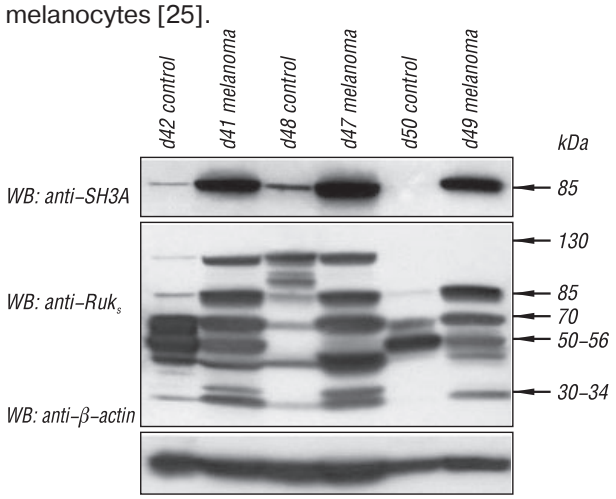

Fig. 6. Patterns of Ruk/CIN85 isoforms expression in human non-transformed skin and melanoma samples

The obtained results demonstrate that produced polyclonal and monoclonal antibodies work well and reliably not only in Western blotting but also in immunoprecipitation. This allows us to continue investigations in the field of functional proteomics including studies of expression and biological role of adaptor/scaffold protein Ruk/CIN85 in human tumors as well as to carry out search for Ruk_I/CIN85 binding partners.

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ЭКСПРЕССИЯ ИЗОФОРМ АДАПТЕРНОГО БЕЛКА RUK/CIN85 В КЛЕТОЧНЫХ ЛИНИЯХ РАЗЛИЧНОГО ТКАНЕВОГО ПРОИСХОЖДЕНИЯ И МЕЛАНОМЕ ЧЕЛОВЕКА

Цель: **получение моноклональных и поликлональных антител против рекомбинантных GST-конъюгированных форм белков, включающих соответственно N-концевую и C-концевую части адаптерного белка Ruk/CIN85. Анализ профилей экспрессии изоформ Ruk/CIN85 и их субклеточной локализации в клеточных линиях различного тканевого происхождения и образцах меланомы человека.** ***Методы:*** **рекомбинантные GST-конъюгированные фрагменты Ruk/CIN85 (GST-3SH3/CIN85 и GST-Ruk_s) экспрессировали в бактериальной системе с последующей аффинной очисткой на глутатион-сефарозе. Для получения моноклональных антител против 3SH3 фрагмента CIN85 использовали гибридомную технологию. Поликлональные антитела против С-концевого суперспирализованного района Ruk очищали из сыворотки иммунизированного кролика аффинной хроматографией на Ruk_s-сефарозе. Профили экспрессии изоформ Ruk/CIN85, их олигомеризацию и субклеточную локализацию изучали методами Вестерн-блот анализа, иммунопреципитации и иммунофлуоресцентной микроскопии.** ***Результаты:*** **Ruk/CIN85, SH3-содержащий адаптерный "стеллажный" белок, играет важную роль в сигнальных процессах клеток. N-концевую часть молекулы CIN85, включающую 3 SH3 домена, и С-концевой суперспирализованный район использовали в качестве антигенов для получения моноклональных и поликлональных антител, соответственно. Получены гибридомы, секретирующие моноклональные антитела к 3SH3-фрагменту. Показано, что одно из этих антител (MISh-A1) специфически узнает эпитоп, находящийся в первом SH3A домене адаптерного белка. Поликлональные анти-Ruk_s антитела, аффинно очищенные из сыворотки иммунизированного кроля, специфически узнавали основные изоформы Ruk/CIN85, как эндогенные, так и рекомбинантные, в лизатах клеток HEK293. Важно, что полученные антитела не реагировали перекрестно с CD2AP, представителем этого же семейства адаптерных белков. Множественные молекулярные формы Ruk/CIN85 с кажущейся молекулярной массой 130, 80–85, 70–75, 50–56, 34–40 and 29 кДа были детектированы в лизатах клеток NIH3T3, Cos1, L1210, HEK293, Ramos, HeLa S3, MDCK, C6, A549 и U937 с помощью анти-Ruk_s антител. Олигомеризацию между p85 и p50–56 формами Ruk/CIN85 выявили в клетках С6 и NIH3T3, но не в HeLa S3 и HEK293, иммунопреципитацией с помощью антитела MISh-A1 и последующего анти-Ruk_s Вестерн-блот анализа. Иммунофлуоресцентной микроскопией с использованием анти-Ruk_s антител эндогенные варианты Ruk выявляли преимущественно в цитоплазме клеток С6, NIH 3T3, HEK293 и в более низкой степени в ядре. Высокий уровень экспрессии полноразмерной формы Ruk_l/CIN85 выявлен в образцах меланомы человека.** ***Выводы:*** **профили экспрессии множественных эндогенных вариантов Ruk/CIN85 и способность к формированию олигомерных комплексов между различными изоформами являются характерными для каждой исследованной линии клеток. Не исключено, что выявленные особенности могут определять специфическую биологическую значимость изоформ Ruk/CIN85 в различных типах клеток в зависимости от клеточного контекста. Следствием высокого уровня экспрессии полноразмерной формы Ruk_l/CIN85 в образцах меланомы человека может быть нарушение согласованного контроля процессов пролиферации и апоптоза в трансформированных меланоцитах. Антитела, описанные в этой работе, могут быть использованы для изучения экспрессии и функций Ruk/CIN85 в опухолях человека.**

Ключевые слова: **Ruk/CIN85, адаптерные белки, антитела, олигомеризация, локализация**