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Adaptor Protein Ruk_1 Forms Protein–Protein Complexes with Endonuclease Activity in HEK293 Cells

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Abstract—The structural and functional organization of the adaptor protein Ruk_1 is characterized by the presence of three SH3-domains at the N-terminus followed by Pro- and Ser-rich sequences and a C-terminal coiled-coil region. Multiple modules in the Ruk_1 structure involved in protein–protein interactions can provide for formation of ligand clusters with varied properties and subcellular location. To study the nature and biological role of such complexes, the recombinant protein Ruk_1 with a Glu-epitope at the C-terminus (Ruk_1 Glu-tagged) was purified from transfected HEK293 cells by affinity chromatography on protein G-Sepharose with covalently conjugated anti-Glu-tag antibodies. By SDS polyacrylamide gel electrophoresis with subsequent staining with silver, a set of minor bands in addition to the 85-kD Ruk_1 Glu-tagged was detected in the purified preparation of the recombinant protein. Proteins with affinity for nucleic acids were also revealed in the Ruk_1 Glu-tagged preparation by retardation of electrophoretic mobility of ^{32}P-labeled oligodeoxyribonucleotides in gel. The Ruk_1 Glu-tagged preparation was also shown to hydrolyze both deoxyribonucleotides and plasmid DNA. $ZnCl_2$ and heparin inhibited the DNAse activity. These findings suggest the presence of DNases associated with the Ruk_1 protein in HEK293 cells. Such complexes were isolated from lysates of HEK293 cells by chromatography on heparin-Sepharose. By elution with 0.5 and 1.0 M NaCl, two fractions with DNase activity and containing proteins with molecular weights of 83, 80 and 72 kD were obtained. The reaction was inhibited by $ZnCl_2$ and heparin, and previous precipitation of Ruk-related proteins with anti-Ruk antibodies resulted in the exhaustion of nuclease activity. By immunoblotting with anti-Ruk antibodies, 83-kD protein immunologically related to the Ruk_1 protein was identified in the fractions. It was concluded that the adaptor protein Ruk_1 forms complexes with endonucleases in HEK293 cells.

Key words: adaptor proteins, SH3-domain, Ruk_1, CIN85, SETA, protein–protein interactions, heparin-Sepharose, DNAse activity

Ruk_1 protein (regulator for ubiquitous kinase), also known as CIN85 and SETA, belongs to the recently identified subfamily of adaptor/scaffold proteins which play an integrating role in the regulation of such fundamental cellular processes and systems as survival and apoptosis [1, 2], endocytosis mediated by receptor tyrosine protein kinases [3, 4], and organization of the actin cytoskeleton [5, 6]. Ruk_1 contains three SH3-domains located in the N-terminal moiety of the polypeptide chain followed by Pro- and Ser-rich regions and the C-terminal coiled-coil domain [1]. Due to the modular organization Ruk_1/CIN85/SETA interact *in vitro* with such signaling proteins as Cbl, Grb2, CrkI, CrkII, Sos, Cas, BLNK, SB1, Src-family protein tyrosine kinases, and endophilins [1-4, 7-9]. Interaction of Ruk_1 with p85a regulatory subunit of IA class PI 3-kinases, is accompanied by inhibition of the lipid kinase activity of the holoenzyme, whereas overexpression of Ruk_1 in culture of primary mouse and rat neurons results in their apoptotic death, which can be abrogated by overexpression of constitutively activated forms of PI 3-kinase or PKB-Akt kinase [1, 2]. The assembly of a supramolecular Cbl–CIN85–endophylin complex mediated by a ligand-

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dependent activation of EGF and Met receptors is accompanied by their internalization and degradation [3, 4]. CIN85 is suggested to regulate different stages of endocytosis of receptor tyrosine protein kinases by binding multiple adaptor proteins (Crk, p130Cas, p85α) involved in the cross-talk between endosomes and the actin cytoskeleton. Considering these data, as well as wide distribution of Ruk_1 in various tissues and cell lines, the existence of multiple Ruk isoforms generated by different promoter usage and alternative splicing [10], this signaling protein is suggested to play an important regulatory role in many yet unknown cellular processes.

We have previously shown that the recombinant protein Ruk_1 Glu-tagged is located not only in the cytoplasm but also in the nucleus of transfected HEK293 cells and that the affinity-purified preparation of the recombinant protein can interact with DNA *in vitro* [11]. Data presented here show that the affinity-purified recombinant Ruk_1 Glu-tagged protein and endogenous Ruk_1 protein from HEK293 cells are associated with endonucleases.

MATERIALS AND METHODS

Cell cultures and preparation of cell lysates. Human embryonic kidney 293 cells were cultured in DMEM supplemented with 10% fetal calf serum (GibcoBRL, USA), 2 mM L-glutamine, 50 IU/ml penicillin, and 50 μg/ml streptomycin in humidified atmosphere of 5% CO_2 at 37°C. To prepare total lysates, cells were washed twice with ice-cold phosphate buffered saline (PBS) containing 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.7 mM KH_2PO_4 (pH 7.3) and lysed for 20 min on ice bath in lysis buffer of the following composition: 10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Triton X-100, 5 mM EDTA, 50 mM NaF, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride (PMSF) (Fluka, Switzerland), 5 mM benzamidine (Sigma, USA), aprotinin (25 μg/ml) (Sigma), leupeptin (10 μg/ml) (Sigma), pepstatin (1 μg/ml) (Fluka). The detergent-insoluble fraction was precipitated by centrifugation at 12,000 rpm and 4°C for 15 min. The protein concentration in supernatants was determined as described in [12]. Aliquots of the lysates were heated for 5 min at 95°C in loading buffer and separated by 5-17% SDS-PAGE [13].

Construction of the pRc/CMV2/Ruk_1-Glu-tag vector, which additionally encoded the Glu-epitope (MEFMPME) at the C-terminus, is described in [11].

Transient transfection of HEK293 line cells. Before transfection, HEK293 cells were cultured in Petri dishes of 10 cm in diameter to 70% maximal plating density. The cells were transfected with plasmid DNA (10 μg per dish) using a LipofectAMINE reagent (GibcoBRL) according to the manufacturer's instructions.

Immunoblotting of proteins. Proteins were separated by 5-17% SDS-PAGE [13] and transferred onto a polyvinyldifluoride membrane (Whatman, Great Britain) for 2 h at 250 mA in buffer containing 20 mM Tris, 192.5 mM glycine (pH 8.3), 0.1% SDS, and 20% methanol. Free binding sites on the membrane were blocked for 1 h with 5% dry skimmed milk in PBS with 0.1% Tween-20. The Ruk_1 Glu-tagged protein was detected with monoclonal anti-Glu-tag antibodies, which were kindly provided by L. Steffens (AFRC Babraham Institute, Cambridge). Endogenous forms of the Ruk protein on blots were detected with polyclonal monospecific antibodies to the unique C-terminal 17 amino acid sequence of the Ruk_1 protein [1]. Anti-mouse or anti-rabbit IgG conjugated with horseradish peroxidase (Sigma) were used as second antibodies respectively. Immunoreactive bands were detected by enhanced chemiluminescence (ECL) (Amersham, Great Britain).

Purification of the recombinant Ruk_1 Glu-tagged and endogenous Ruk_1 proteins from HEK293 cells. The recombinant Ruk_1 Glu-tagged protein was purified from lysates of transfected HEK293 cells by affinity chromatography on protein G-Sepharose covalently conjugated to monoclonal anti-Glu-tag antibodies [14]. The cell lysate (15 mg of protein) was incubated with 200 μl of affinity matrix for 2 h at 4°C with constant stirring. The matrix was washed five times in a 10-fold volume of lysis buffer with subsequent centrifugation at 500*g* for 5 min. Specifically bound proteins were eluted in the presence of a synthetic Glu-tag peptide (100 μg/ml) in 50 mM Tris-HCl buffer (pH 7.5) containing 300 mM NaCl. The resulting eluate was dialyzed against 50 mM Tris-HCl (pH 7.5) supplemented with 150 mM NaCl, 1 mM dithiothreitol (DTT), 50% glycerol.

The endogenous Ruk_1 protein from lysates of HEK293 cells transfected with the insertion-free pRc/CMV2 vector was partially purified by chromatography on heparin-Sepharose. The cell lysate (30 mg of protein) was loaded onto a column with heparin-Sepharose (2 ml) preliminarily equilibrated with lysis buffer without proteinase and phosphatase inhibitors. The column was washed with the same buffer, and the proteins bound were eluted with 0.5 and 1 M NaCl in 20 mM Tris-HCl (pH 7.5) supplemented with 0.1% Triton X-100. The obtained fractions were dialyzed against 50 mM Tris-HCl (pH 7.5) containing 150 mM NaCl, 1 mM DTT, and 50% glycerol.

DNA–protein binding assay. DNA-binding proteins in the Ruk_1 Glu-tagged preparation were detected by gel retardation assay [15]. For DNA–protein binding, 0.5 μg of protein was incubated in 20 μl of buffer containing 20 mM Tris-HCl (pH 7.6), 150 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT, 1 mM EDTA, 5% glycerol, and ^{32}P-labeled 150-mer fragment of the 5S rRNA gene of *Lytechinus variegatus* (30 μCi) (^{32}P-labeled oligonucleotide, [^{32}P]ON) for 30 min on ice. Calf thymus DNA was used as a nonspecific competitor. The incubation mixture was separated by 6% PAGE in Tris-borate-EDTA (TBE)

buffer (pH 8.3) [16]. The gels were dried and autoradiographed.

DNase activity assay. DNA-hydrolyzing activity was studied with [^{32}P]ON and the pRc/CMV2 plasmid DNA as substrates. In the first case, [^{32}P]ON was incubated with 1 μg Ruk_1 Glu-tagged protein under the same conditions as for DNA–protein binding. Hydrolysis was performed for 30 min at 37°C. The hydrolysis products were separated by 15% PAGE in presence of 7 M urea in TBE buffer (pH 8.3) [15]. The gels were dried and autoradiographed. In the second case, 2 μg of pRc/CMV2 plasmid was incubated with Ruk_1 Glu-tagged protein (0.5 μg) or with protein fractions eluted from heparin-Sepharose (3 μg) in 20 μl of buffer containing 20 mM Tris-HCl (pH 7.5), 50 mM NaCl, 5 mM $MgCl_2$ for 1 h at 37°C. To inhibit the DNAse activity, the reaction medium was supplemented with $ZnCl_2$ or heparin to the final concentration of 1 mM or 0.2 mg/ml, respectively. The hydrolysis products were separated by electrophoresis in 1% agarose gel in TBE buffer (pH 8.3) in the presence of 0.05% ethidium bromide. To remove proteins immunologically related to the Ruk protein, the fractions eluted from heparin-Sepharose were incubated for 30 min with anti-Ruk antibodies and then for 30 min with protein G-Sepharose. The DNAse activity in the supernatants was determined by hydrolysis of pRc/CMV2 plasmid as described above.

RESULTS AND DISCUSSION

The structural and functional organization of the adaptor protein Ruk_1 is characterized by the presence of three SH3-domains at the N-terminus followed by Pro- and Ser-rich sequences and a C-terminal coiled-coil region. Multiple modules in Ruk_1 structure involved in protein–protein interactions can mediate formation of ligand clusters with varied biological properties and subcellular location. Thus, we have shown previously that the recombinant Ruk_1 Glu-tagged protein is located not only in the cytoplasm but also in the nuclei of transfected HEK293 cells and interacts with DNA from calf thymus but not with DNA of *Escherichia coli* immunodot blots [11]. To study the nature and possible biological role of supramolecular complexes formed by Ruk_1, we purified recombinant Ruk_1 Glu-tagged protein from the lysates of transfected HEK293 cells by affinity chromatography on protein G-Sepharose with covalently conjugated anti-Glu-tag antibodies. Proteins specifically bound to the matrix were eluted with Glu-tag peptide and analyzed by SDS-PAGE followed by immunoblotting. Eluate from protein G-Sepharose incubated with lysates of HEK293 cells transfected with the insertion-free pRc/CMV2 vector was used as control. On staining the gels with silver, besides the 85-kD protein with affinity for anti-Glu-tag antibodies (Fig. 1b, lane *2*), a set of minor peptides was detected in the eluate. In addition to 85-kD protein with affinity to anti-Glu-tag antibodies, a set of minor bands was detected on silver-stained gels (Fig. 1a, lane *2*). Most likely, these proteins had the affinity to the Ruk_1 Glu-tagged protein and isolated in complexes with it.

Then the DNA-binding activity of the Ruk_1 Glu-tagged preparation was analyzed by gel retardation assay. Figure 2a shows the presence in the preparation under study of at least one protein with affinity to DNA (lane *2*) that was not revealed in the control eluate (lane *5*). Formation of such complexes was competitively suppressed in the presence of DNA from calf thymus suggesting their specificity (lanes *3* and *4*). Electrophoresis under denaturing conditions showed that the incubation of Ruk_1 Glu-tagged with [^{32}P]ON at 37°C was accompanied by the generation of low-molecular-weight labeled fragments of the probe used (Fig. 2b).

Further, the DNAse activity of the Ruk_1 Glu-tagged preparation was analyzed by its ability to hydrolyze the plasmid DNA (pRc/CMV2) *in vitro*. The reaction prod-

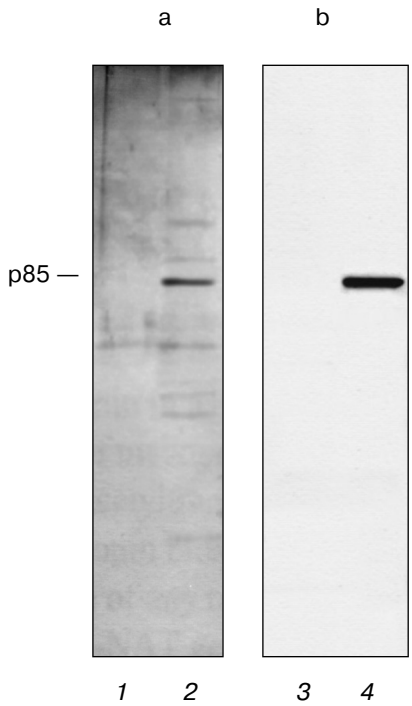

Fig. 1. Affinity purification of Ruk_1 Glu-tagged protein from transfected HEK293 cells on protein G-Sepharose with covalently conjugated anti-Glu-tag mouse monoclonal antibodies: a) 5-17% SDS-PAGE stained with silver; b) immunoblotting with mouse monoclonal anti-Glu-tag antibodies. *1*, *3*) Eluates from the affinity matrix incubated with lysates of control HEK293 cells transfected with the insertion-free pRc/CMV2 vector; *2*, *4*) Eluates from the affinity matrix incubated with lysates of HEK293 cells transfected with the pRc/CMV2/Ruk_1 Glu-tag vector.

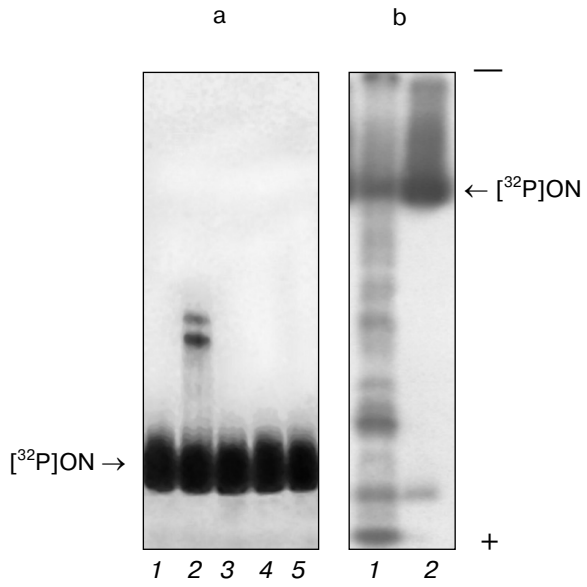

Fig. 2. Detection of the DNA-binding and DNA-hydrolyzing activity in the affinity-purified preparation of the recombinant Ruk_1 Glu-tagged protein: a) retardation of protein–nucleic acid complexes on gel electrophoresis. The 150-mer [^{32}P]ON of *L. variegatus* 5S rRNA gene was incubated with Ruk_1 Glu-tagged (0.5 μg) for 1 h at 0°C. The retardation of the complexes was analyzed by 6% PAGE in TBE buffer followed by autoradiography: *1*) [^{32}P]ON; *2*) [^{32}P]ON + Ruk_1 Glu-tagged; *3*) [^{32}P]ON + Ruk_1 Glu-tagged + calf thymus DNA (0.5 μg); *4*) [^{32}P]ON + Ruk_1 Glu-tagged + calf thymus DNA (1 μg); *5*) [^{32}P]ON + control eluate. b) DNase activity assay. The affinity-purified preparation of the Ruk_1 Glu-tagged protein was incubated with [^{32}P]ON at 37°C for 30 min. The reaction products were separated by 15% PAGE in the presence of 7 M urea: *1*) [^{32}P]ON + Ruk_1 Glu-tagged (1 μg); *2*) [^{32}P]ON + control eluate.

ucts were separated by electrophoresis in 1% agarose gel. The DNAse activity was detected (Fig. 3) only in the eluate from the affinity matrix incubated with lysates of HEK293 cells transfected with pRc/CMV2/Ruk_1 Glu-tag (lane *4*) but not in the control eluates (lane *2*). Both $ZnCl_2$ and heparin completely inhibited the hydrolysis (Fig. 3, lanes *5* and *6*). The findings suggest that the preparation of the recombinant protein Ruk_1 Glu-tagged purified by affinity chromatography possesses both DNA-binding and DNA-hydrolyzing activities.

It is also likely that the endogenous protein Ruk_1 can form complexes with DNAses in HEK293 cells similarly to the recombinant protein Ruk_1 Glu-tagged. Since heparin inhibited the activity of nucleases associated with the Ruk_1 Glu-tagged protein, we attempted to isolate such complexes by chromatography on heparin-Sepharose [17]. Two fractions (F1 and F2) containing proteins of 83, 80 and 72 kD and a set of minor bands were obtained by step-wise elution with buffers containing 0.5 and 1 M NaCl, respectively (Fig. 4a, lanes *2* and *3*). The retention of these proteins on heparin-Sepharose in the presence of 1% Triton X-100 provides evidence for their high affinity to the sorbent used. The p83 which showed affinity to anti-Ruk antibodies was revealed by immunoblotting in both total cell lysates and material of the F1 and F2 fractions (Fig. 4b, lanes *1-3*). The amount of this protein in F2 was significantly higher than in F1. Most likely, the protein identified is an endogenous form of the adaptor protein Ruk_1 in HEK293 cells. It should be noted also that compared to the total cell lysate the chromatographic fractions included an additional immunoreactive band with molecular weight of ~76 kD, which could be a product of degradation of Ruk_1 during fractionation. Similar to the Ruk_1 Glu-tagged preparation purified by affinity chromatography, the isolated fractions possessed DNase activity sensitive to $ZnCl_2$ and heparin (Fig. 5). To make sure that the nuclease activity is associated with the Ruk_1 protein, we studied the DNAse activity of the chromatographic fractions after their exhaustion by anti-Ruk antibodies. Figure 6 shows that immunoprecipitation of Ruk resulted in a partial (F1, lanes *2* and *3*) or complete (F2, lanes *4* and *6*) suppression of the DNAse activity. The specific profile of proteins detected in F1 and F2 with anti-Ruk antibodies, the degree of plasmid DNA hydrolysis, and the nature of DNase activity

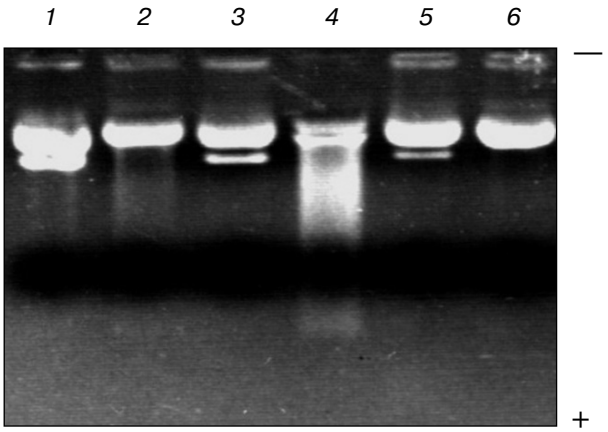

Fig. 3. Hydrolysis of plasmid DNA in the presence of affinity-purified preparation of the Ruk_1 Glu-tagged protein. The plasmid DNA (pRc/CMV2, 2 μg) was incubated with the preparation of the Ruk_1 Glu-tagged protein (0.5 μg) as described in "Materials and Methods". The reaction products were separated by electrophoresis in 1% agarose gel in TBE buffer: *1*) pRc/CMV2; *2*) pRc/CMV2 + control eluate; *3*) pRc/CMV2 + control eluate + $ZnCl_2$; *4*) pRc/CMV2 + Ruk_1 Glu-tagged; *5*) pRc/CMV2 + Ruk_1 Glu-tagged + $ZnCl_2$; *6*) pRc/CMV2 + Ruk_1 Glu-tagged + heparin.

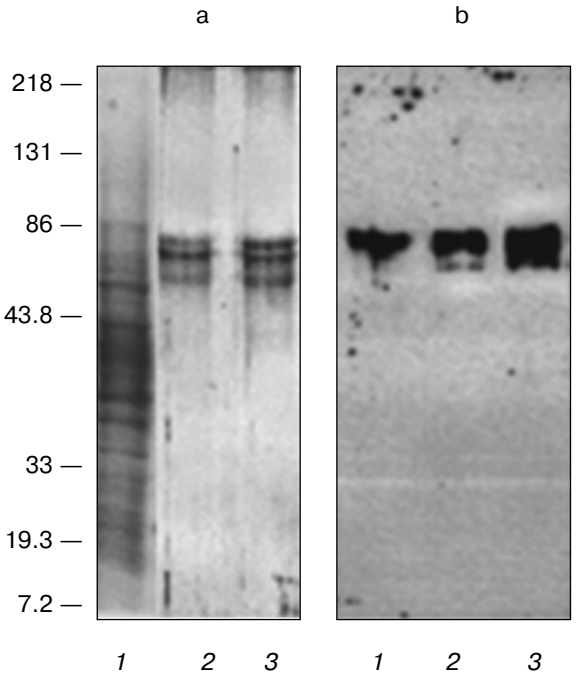

Fig. 4. Purification of the endogenous Ruk_1 protein from lysates of HEK293 cells by chromatography on heparin-Sepharose. a) 5-17% SDS-PAGE stained with Coomassie G-250; b) immunoblotting with anti-Ruk_1 antibodies. *1*) Total lysate of HEK293 cells; *2*, *3*) fractions eluted from the matrix with 0.5 (*2*) and 1 M NaCl (*3*). To the left the molecular weights of standard proteins (kD) are shown.

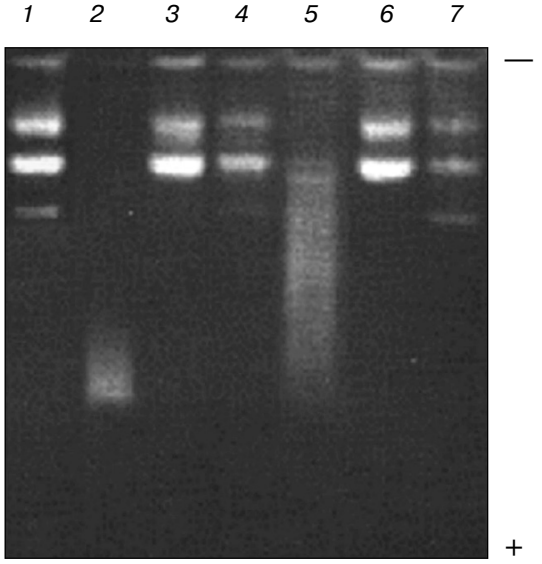

Fig. 5. DNase activity of the endogenous Ruk_1 preparations purified by chromatography on heparin-Sepharose using plasmid DNA as substrate. The plasmid DNA (pRc/CMV2, 2 μg) was incubated with fractions eluted from heparin-Sepharose with 0.5 and 1 M NaCl (F1 and F2, respectively) as described in "Materials and Methods". The reaction products were separated by electrophoresis in 1% agarose gel in TBE buffer: *1*) pRc/CMV2; *2*) pRc/CMV2 + F1; *3*) pRc/CMV2 + F1 + heparin; *4*) pRc/CMV2 + F1 + $ZnCl_2$; *5*) pRc/CMV2 + F2; *6*) pRc/CMV2 + F2 + heparin; *7*) pRc/CMV2 + F2 + $ZnCl_2$.

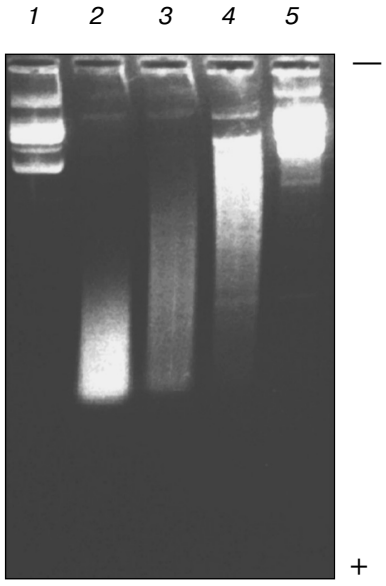

Fig. 6. DNase activity of the endogenous Ruk_1 preparations purified by chromatography on heparin-Sepharose before and after incubation with polyclonal anti-Ruk antibodies. The experimental conditions are described in "Materials and Methods" and in the legend to Fig. 5. The reaction products were separated by electrophoresis in 1% agarose gel in TBE buffer: *1*) pRc/CMV2; *2*) pRc/CMV2 + F1; *3*) pRc/CMV2 + F1 + anti-Ruk antibodies; *4*) pRc/CMV2 + F2; *5*) pRc/CMV2 + F2 + anti-Ruk antibodies.

exhaustion in the chromatographic fractions suggest that different pools of Ruk_1-mediated supramolecular complexes associated with endonucleases can exist in HEK293 cells.

At present, we cannot precisely say what endonucleases are associated with Ruk_1. Similarly, the biological role of the interaction found is unknown. Some recent publications report that SH3-containing proteins can be involved in various signaling functions in the cell nucleus. For instance, the SH3-domain of human arginine methyltransferase HRMT1L1 mediates the interaction with a recently identified member (E1B-AP5) of heterogenous nuclear ribonucleoprotein family functioning during different stages of RNA metabolism [18], and the nuclear SH3-binding protein SNP70 colocalizes with RNA splicing factors [19]. The adaptor protein Grb4, which contains three SH3 domains and one SH2 domain, acts as a nuclear repressor of the v-Abl-induced transcription from the c-jun/c-fos promotor elements [20]. It is interesting that the proline-rich region p53, which is able to interact with SH3-domains of signaling proteins, mediates the interaction of p53 with the nuclear matrix, and the level

of this binding increases as a result of genotoxic stress [21]. In this relation it is worth note that proteins involved in endocytosis (eps 15, epsin 1, CALM, α-adaptin) unexpectedly were found to accumulate in the nucleus under conditions of inhibited nuclear export and to modulate directly or indirectly the transcription of specific genes [22]. Further studies are needed to show the role of Ruk_1 in nuclear signaling and the nature of its interaction with nucleases.

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