INTERACTION OF RAT KIDNEY PROTEINS WITH THE RENALASE PEPTIDE RP220 AND ITS POTENTIAL PROTEOLYTIC FRAGMENT RP224-232: A COMPARATIVE PROTEOMIC ANALYSIS

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Renalase (RNLS) is a protein playing different roles inside and outside cells. A 20-mer synthetic peptide corresponding to the human RNLS amino acid sequence 220–239 (RP220) exhibits a number of pharmacologically attractive activities *in vitro* and *in vivo* and can bind to many renal intracellular proteins. The RP220 sequence contains several cleavage sites for extracellular and circulating proteases. Here, we investigated the interaction of model proteins with the renalase peptide RP220 and a synthetic peptide corresponding to the amino acid sequence of RNLS 224–232, named RP224-232. We also performed affinity-based proteomic profiling of normotensive rat kidney samples with these peptides as affinity ligands. The obtained results indicate that both peptides exhibit almost the same affinity for model proteins (pyruvate kinase and lactate dehydrogenase), and the kidney proteomic profiles differ slightly. At the same time, the relative content of a number of kidney proteins bound to the RP224-232 peptide was even higher than in the case of using RP220. This suggests that proteolytic processing of RP220 does not inactivate this peptide; moreover, it could contribute to the formation of shorter peptides with additional pharmacological activities.

Keywords: renalase; proteolytic processing; renalase peptides RP220 and RP224-232; SPR biosensor; proteomic profiling of rat kidney

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INTRODUCTION

Renalase (RNLS), discovered twenty years ago as a protein involved in the regulation of blood pressure, has various functions inside and outside cells [1–5]. Intracellular RNLS is a FAD-dependent oxidoreductase (EC 1.6.3.5) involved in metabolic repair or detoxification by oxidizing isomeric forms of β-NAD(P)H reduced at positions 2 or 6 of the nicotinamide ring instead of the metabolically active position 4 [5, 6]. Extracellular RNLS, lacking its N-terminal signal peptide during the secretion of this protein into the extracellular space, exhibits regulatory effects through non-catalytic (and FAD-independent) mechanisms [7–11]. Taking into account the known data on the absence of intact RNLS (lacking the N-terminal peptide) in the blood [12] and on the proteolytic cleavage of recombinant RNLS during incubation with the blood plasma of healthy volunteers [13], it becomes increasingly clear that the effects of extracellular RNLS are realized by peptides formed during proteolytic processing. One of the most interesting RNLS peptides attracting much attention of researchers is the RP220 peptide, a 20-mer peptide corresponding to the RNLS sequence including residues 220–239 [10, 11]. The fact that this peptide reproduces a number of the effects of extracellular RNLS [10] underlies its critically important regulatory role.

The comparison of kidney proteomes of normotensive (WKY) and hypertensive (SHR) rats, performed by using the RP220 peptide as an affinity ligand, revealed changes in the relative content of about 200 bound to the affinity sorbent proteins of the kidneys of hypertensive rats [14]. RP220-binding proteins, for which the most pronounced increase in the relative content has been found in hypertensive animals compared to normotensive ones, play a certain role in the development of cardiovascular pathology [14]. The latter may have a certain importance in the regulatory effects of this peptide. At the same time, according to the data of the RNLS sequence analysis [13], there are potential protease cleavage sites in the region of the amino acid residues 220–239. In this regard, a reasonable question arises, whether the RP220 peptide modification (shortening) by proteases changes RP220 interaction with its target proteins.

In this study, we have investigated the interaction of model proteins with the RP220 peptide and a synthetic peptide corresponding to the RNLS amino acid sequence 224–232 and named RP224-232. We have also performed proteomic profiling of kidney proteins from normotensive rats with these peptides as affinity ligands.

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MATERIALS AND METHODS

Reagents

The following reagents were used in the study: cyanogen bromide-activated Sepharose 4B, ammonium bicarbonate, dithiothreitol, urea, guanidine hydrochloride, sodium chloride, Triton X-100, 4-vinylpyridine, Coomassie Brilliant Blue G-250 (Merck, USA); formic acid, sodium acetate, boric acid, sodium tetraborate, sodium hydroxide (Acros Organics, USA), acetonitrile (Fisher Chemical, UK); isopropanol, trifluoroacetic acid (Fluka, USA); tris-(2-carboxyethyl)-phosphine (Pierce, USA); modified trypsin (mass spectrometry grade, Promega, USA). The RNLS peptide RP220 and its fragment RP224-232 were synthesized at OOO Belkiantitela (Russia). HBS-EP+ (150 mM NaCl, 3 mM EDTA, 0.05% P20, 10 mM HEPES (pH 7.4)); 10 mM acetate buffers (pH 4.0, pH 5.0); reagents for covalent immobilization of proteins by primary amino groups (1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide-HCl (EDC), N-hydroxysuccinimide (NHS), 1 M ethanolamine-HCl (pH 8.5)) were obtained from Cytiva (USA). For the biosensor study of the interaction of intracellular proteins with RNLS peptides, electrophoretically homogeneous preparations of pyruvate kinase (PK) and lactate dehydrogenase (LDH) isolated from rabbit skeletal muscles were used [15, 16]. The specific activities of PK and LDH were 280 μmol/min per 1 mg protein and 495 μmol/min per 1 mg protein, respectively. The enzymes were stored at 4°C in ammonium sulfate suspension.

Other reagents of the highest purity available were from local suppliers.

Experimental Animals

Male 14-week-old WKY rats were from the Nursery of the laboratory animals (Pushchino branch of the M.M. Shemyakin and Yu.A. Ovchinnikov Institute of Bioorganic Chemistry RAS). The animals were decapitated under light ether anesthesia; the kidneys were quickly removed, immediately frozen, and stored at -70°C until the study.

Preparation of Lysates of Kidney Homogenates

Kidney tissue was homogenized using a Heidolph SilentCrusher homogenizer (50,000 rpm) in 0.05 M potassium phosphate buffer, pH 7.4, and diluted in it to a final protein concentration of 30 mg/ml. In order to evaluate relative quantitative changes in renal proteins, the same amount of total protein was used during sample preparation; protein content was controlled by the Bradford method [8]. After incubation in the presence of 3% Triton X-100 (4°C, 1 h), the lysates were diluted 3 times with the same buffer and centrifuged at 16,000 *g* for 30 min to obtain a cleared supernatant.

Affinity Chromatography of Rat Kidney Proteins using Renalase Peptide RP220 and its Proteolytic Fragment RP224-232 Immobilized on Cyanogen Bromide-Activated Sepharose

The preparation of the affinity sorbent and the affinity chromatography procedure were carried as described in [14].

Mass Spectrometric Analysis

The conditions of mass spectrometric analysis are described in detail in [14, 18]. Mass spectrometric analysis was carried out using the equipment of the "Human Proteome" Core Facility at the Institute of Biomedical Chemistry (IBMC).

Biosensor Study of Protein Interaction with Immobilized Peptides

The analysis of the interaction of the studied proteins with immobilized peptides was performed on a Biacore X-100 optical biosensor (Cytiva). All the measurements were performed at 25°C using CM5 optical chips (Cytiva) coated with a carboxymethylated dextran layer. The signal of the Biacore X-100 biosensor has been recorded in resonance units RU (1 RU corresponds to the binding of 1 pg of protein on the surface of the optical chip).

Peptide immobilization was carried out by forming covalent bonds between the carboxyl groups on the surface of the CM5 optical chip and the amino groups of the peptide. The carboxyl groups of the chip were activated with a mixture of 0.2 M EDC/0.05 M NHS for 7 min at a flow rate of 5 μl/min. Next, solutions of RNLS peptides (200 μg/ml) in 10 mM acetate buffer (pH 5.0 for RP220, pH 4.0 for RP224-232) were injected for 15 min at a flow rate of 5 μl/min. Unreacted carboxyl groups were blocked by injection of 1 M ethanolamine-HCl (pH 8.5) for 3 min at a flow rate of 5 μl/min. The interaction of proteins with immobilized peptides was studied in 50 mM phosphate buffer, pH 7.4; the injection time was 5 min at a flow rate of 10 μl/min. Between protein injections, the chip surface with immobilized peptides was regenerated by washing with 1 M NaCl in 50 mM phosphate buffer, pH 7.4, for 0.5 min at a flow rate of 30 μl/min. The resulting sensorgrams were analyzed using the BIAevaluation Version 4.1.1 program (Cytiva).

RESULTS

The study of the interaction of model intracellular proteins (PK and LDH), previously identified during proteomic profiling of rat kidney preparations, with the RP220 peptide [14] immobilized on a CM5 chip showed that this peptide exhibited moderate affinity for the studied proteins (Fig. 1, Table 1). The RP224-232 peptide, corresponding to a potential

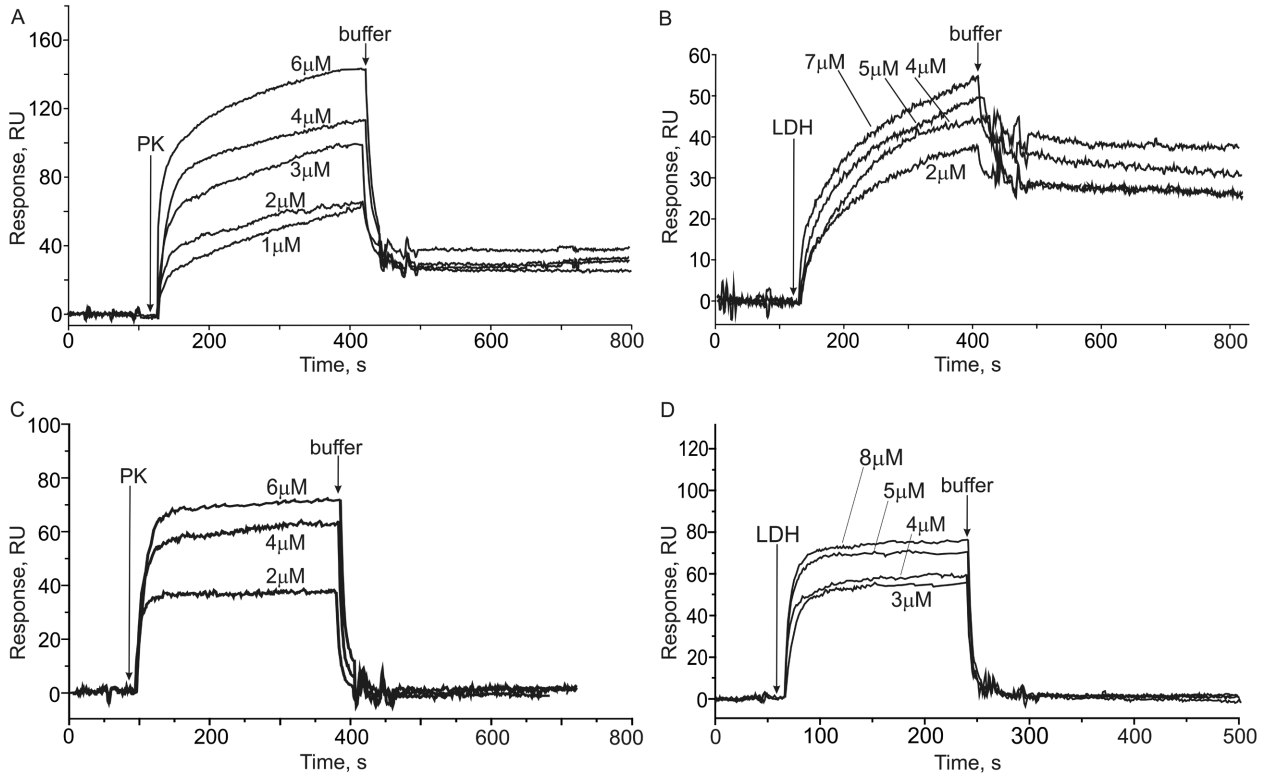

Figure 1. Interaction of the renalase peptides RP220 (**A**, **B**) and RP224-232 (**C**, **D**), immobilized on a Biacore optical biosensor chip, with rabbit muscle PK and LDH.

Table 1. The K_d values for the complexes of RP220 and RP224-232 peptides with PK and LDH

Peptide-enzyme complex	K_d, M	Peptide sequence and its position in the primary structure of human RNLS*
RP220/PK	$1.94 \cdot 10^{-6}$	220-CIRF**VSIDNKKRN**IESSEIG-239
RP220/LDH	$9.24 \cdot 10^{-7}$	220-CIRF**VSIDNKKRN**IESSEIG-239
RP224-232/PK	$3.04 \cdot 10^{-6}$	224-**VSIDNKKRN**-232
RP224-232/LDH	$1.39 \cdot 10^{-6}$	224-**VSIDNKKRN**-232

* – Residues common for both peptides are shown in bold and underlined.

proteolytic fragment of the RP220 peptide, also interacted with the studied proteins, demonstrating an affinity comparable to that of RP220 (Table 1). The latter indicates that the most important amino acid sequences in the context of the interaction of RP220 with its (at least model) targets include residues 224-VSIDNKKRN-232.

Proteomic profiling of rat kidney proteins bound to the RP220 peptide and its proteolytic fragment RP224-232 has shown that both model proteins belong to a common pool of peptide-bound proteins. This common pool (2233 proteins) significantly exceeded the groups of proteins demonstrating exclusive binding to the RP220 peptide and to the RP224-232 peptide (16 and 61, respectively) (Fig. 2, Table 2, and Supplementary Material Tables S1 and S2). Table 2 shows the functional distribution of proteins demonstrating exclusive binding to RP220 and RP224-232.

The relative content of 29 proteins bound to the RP224-232 peptide was two or more times higher as compared to their binding to the RP220 peptide. The highest increase in relative content was found for creatine kinase1 (more than 34 times), dihydropyrimidinase-like protein 2 (more than 26 times) and mitochondrial heat shock protein 60 kDa (almost 15 times). There was also a significant increase in the relative content (6–9 times) of the T-complex proteins, malate dehydrogenase, transketolase, 10 kDa and 70 kDa heat shock proteins, cytosolic nonspecific dipeptidase, ribosomal proteins eL30 and uS10, peroxiredoxin 6, and hemoglobin beta subunit bound to the RP224-232 fragment (compared to those bound to the full-length RP220 peptide) (Fig. 3, Supplementary Materials, Table S3). The relative content of histones H2A, H2B, H1.2, and H4, as well as vimentin, increased approximately 5-fold, and the relative content

Table 2. Functional distribution of proteins specifically bound to RP220 and its fragment RP224-232

Function	Proteins exclusively bound to RP220 peptide	Proteins exclusively bound to RP224-232 peptide	Increase in the relative content of proteins bound to RP224-232, versus RP220	Decrease in the relative content of proteins bound to RP224-232 versus RP220
Energy generation and carbohydrate metabolism	0	2	5	0
Cytoskeleton formation, transport and exocytosis	7	10	5	1
Signal transduction and regulation of enzyme activity	7	15	1	2
Antioxidant and protective proteins/enzymes	1	1	8	0
Regulation of gene expression, cell division, and differentiation	0	14	5	1
Metabolism of proteins, amino acids and other nitrogenous compounds	2	16	5	0
Lipid metabolism	0	3	0	0
Total	17	61	29	4

of the alpha and beta subunits of ATP synthase increased more than 4- and 3-fold, respectively; the relative abundance of ribosomal proteins S4 and uL6 and heterogeneous nuclear ribonucleoprotein A3 increased more than threefold. The relative abundance of cytoskeletal proteins (tubulin beta, filamin, clathrin heavy chain) and heat shock protein 1B from the HSP70 family increased more than twofold (Supplementary Materials, Table S3).

A decrease in the relative content (compared to RP220 binding) was found for only four proteins bound to RP224-232. These included annexin 1 (*Anxa1*, 10-fold), myosin heavy chain 9 (*Myh 9*, 5-fold), Ras-related protein Rab-11A (*Rab11a*, 2.3-fold), and elongation factor 2 (*Eef2*, 1.5-fold) (Fig. 4, Supplementary Materials, Table S3).

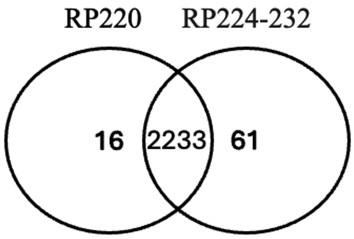

Figure 2. Venn diagram describing the number of rat kidney proteins bound to RP220 and RP224-232 peptides.

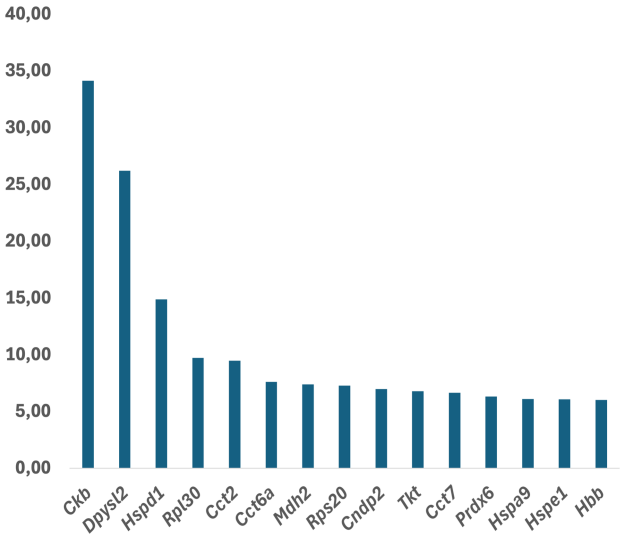

Figure 3. Normotensive rat RP224-232-binding kidney proteins with increased relative content versus the content of RP220-binding proteins. Here and in Figure 4 protein coding genes are indicated. More detailed information is available in Table S3 of Supplementary Materials.

DISCUSSION

The obtained results indicate that possible proteolytic cleavage of the twenty-membered RP220 peptide to form the RP224-232 nanopeptide has a minor effect on the qualitative composition of the protein fractions bound to these peptides. Taking into consideration the biosensor analysis data on the interaction of model proteins (PK and LDH) with immobilized peptides, it can be assumed that such shortening of RP220 preserves the key amino acid residues (VSIDNKKRN) that determine the interaction of the studied peptides with proteins. It should be noted that the relative content of proteins bound to the immobilized nanopeptide was higher for most proteins than in the case of immobilized RP220.

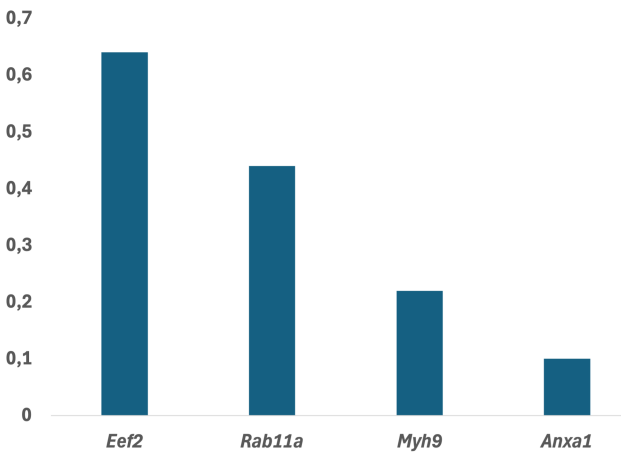

Figure 4. Normotensive rat RP224-232-binding kidney proteins with decreased relative content versus the content of RP220-binding proteins. More detailed information is available in Table S3 of Supplementary Materials.

This suggests that further proteolytic processing will not lead to the inactivation of this peptide; moreover, the processing may contribute to the emergence of additional biological activities that can possibly find pharmacological application.

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COMPLIANCE WITH ETHICAL STANDARDS

The experiments were carried out in compliance with generally accepted norms of humane treatment of laboratory animals, in accordance with the Order of the Ministry of Health of the Russian Federation No. 199n of April 1, 2016 "On Approval of the Rules for Good Laboratory Practice".

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

Supplementary materials are available in the electronic version at the journal site (pbmc.ibmc.msk.ru).

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ВЗАИМОДЕЙСТВИЕ БЕЛКОВ ПОЧЕК КРЫС С ПЕПТИДОМ РЕНАЛАЗЫ RP220 И ЕГО ПОТЕНЦИАЛЬНЫМ ПРОТЕОЛИТИЧЕСКИМ ФРАГМЕНТОМ RP224-232: СРАВНИТЕЛЬНЫЙ АНАЛИЗ ПРОТЕОМОВ

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Реналаза (RNLS) — белок, которому свойственны различные функции внутри и снаружи клеток. Двадцатичленный синтетический пептид, соответствующий аминокислотной последовательности RNLS человека 220–239 (RP220), проявляет ряд фармакологически привлекательных активностей *in vitro* и *in vivo* и может связываться со многими внутриклеточными белками почек. При этом последовательность RP220 содержит несколько участков расщепления внеклеточными и циркулирующими в крови протеазами. В данной работе мы исследовали взаимодействие модельных белков с пептидом реналазы RP220 и синтетическим пептидом, соответствующим аминокислотной последовательности RNLS 224–232 и названным RP224-232. Мы также выполнили протеомное профилирование белков почек нормотензивных крыс с этими пептидами в качестве аффинных лигандов. Полученные результаты свидетельствуют о том, что оба пептида проявляют практически одинаковое сродство к модельным белкам (пируваткиназе и лактатдегидрогеназе), а протеомные профили почек незначительно отличаются. При этом относительное содержание ряда связавшихся с пептидом RP224-232 белков почек было даже выше, чем в случае использования RP220. Это свидетельствует о том, что протеолитический процессинг RP220 не только не будет приводить к инактивации этого пептида, но будет способствовать появлению дополнительных свойств, которые могут найти фармакологическое применение.

Полный текст статьи на русском языке доступен на сайте журнала (http://pbmc.ibmc.msk.ru).

Ключевые слова: реналаза; протеолитический процессинг; реналазные пептиды RP220 и RP224-232; SPR-биосенсор; протеомное профилирование почки крысы

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