Renalase Secreted by Human Kidney HEK293T Cells Lacks its N-Terminal Peptide: Implications for Putative Mechanisms of Renalase Action

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Renalase • HEK293T cells • Secretion • N-terminal peptide

Abstract
Background/Aims: Renalase is a recently discovered flavoprotein involved in regulation of blood pressure. Altered renalase levels have been found in blood of patients with end stage renal disease. The antihypertensive effect of circulating renalase is attributed to putative FAD-dependent monoamine oxidase activity demonstrated by some authors. Being synthesized as an intracellular flavoprotein renalase requires the presence of its N-terminal peptide for FAD accommodation. However, conventional routes of export of secretory proteins outside the cell usually include cleavage of their N-terminal peptide. The aim of this study was to investigate whether renalase is secreted by HEK293T cells as a full length protein (via proposed nonconventional pathway) or its export is accompanied by the loss of its N-terminal peptide.

Methods: We have expressed human recombinant renalase-1 in human kidney HEK293T cells and analyzed this protein inside the cells and in the extracellular medium for the presence of the N-terminal peptide by using high resolution targeted MS/MS.

Results: Intracellular renalase contained clearly detectable N-terminal peptide, which was absent in extracellular renalase.

Conclusions: Lack of the N-terminal peptide, the structural precondition for FAD binding, suggests that extracellular (circulating) renalase acts in a FAD-independent manner and mechanisms of its action are not associated with FAD.

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Introduction

Renalase is a recently discovered secretory protein involved in regulation of blood pressure [1-6]. Possible mechanisms of renalase action that would account for the decrease of blood pressure remain elusive [4-6]. Nevertheless, administration of recombinant human renalase lowered arterial pressure in Sprague-Dawley rats [1, 7]. Recently it has been also demonstrated that in Sprague-Dawley rats with chronic kidney disease (CKD), modeled by subtotal nephrectomy (5/6), renalase gene delivery attenuated hypertension [8]. Clinical studies on representative groups of patients revealed altered levels of blood renalase in patients with hypertension. For example, Maciorkowska et al. reported that blood renalase levels were higher in hypertensive patients (n=121) than in normotensive volunteers (n=27) [9]. Another study demonstrated that plasma renalase levels were lower in hypertensive patients after surgical repair of coarctation of aorta (n= 50) than in controls (n=50) [10]. Monitoring of plasma and urine renalase levels in 26 patients with end stage renal disease (ESRD) before and after transplantation of cadaver kidneys revealed gradual normalization of increased renalase levels both in blood and urine [11]. All these data point to a clear need for better understanding of structural and functional characteristics of this protein.

All known renalases synthesized in eukaryotic and prokaryotic cells contain cleavable N-terminal signal peptide (residues 1-17) [3-7, 12-14] required for extracellular secretion of renalase (Fig. 1). It overlaps with the minimal Rossman fold (residues 2-32) responsible for accommodation of the adenine nucleotide moiety of FAD [5, 7, 12]. The presence of non-covalently bound FAD was originally demonstrated by the Aliverti’s group in purified recombinant renalase [7, 12] and further confirmed by other laboratories [6, 13, 15].

Based on some renalase sequence homology with amino acid sequences of monoamine oxidases, it was originally proposed that the hypotensive effect of renalase may be attributed to FAD-dependent degradation of circulating catecholamines [1, 16, 17]. In this context, some authors reported that serum and especially urinary samples (containing renalase protein) generated hydrogen peroxide in the presence of amine substrates [11, 17]. However, others [5-7, 12, 18] failed to detect any amine oxidase activity measured by independent methods. Subsequent studies have demonstrated that renalase acts as FAD-dependent α-NAD(P)H oxidase/anomerase converting isomeric forms of β-NAD(P)H, which could be inhibitory to intracellular metabolic processes [6, 13, 19]. It was also shown that human recombinant renalase-1 contained FAD and catalyzed adrenaline oxidation in a NAD(P)H dependent manner; its administration to animals lowered not only arterial pressure but also plasma adrenaline concentration [17]. However, this observation was not confirmed in another laboratory [6, 18].

Fig. 1. The scheme of human renalase with its N-terminal peptide. The question mark indicates lack of precise information on the amino acid residues involved in the catalytic domain.
Recently, endogenous NADH dependent renalase activity was detected both in plasma and urinary samples of kidney transplant recipients [11]. All these reactions catalyzed by or attributed to renalase require the presence of the FAD cofactor, which can be accommodated by full-length renalase (including the N-terminal peptide). Whether this FAD molecule is preserved in extracellular renalase(s) remains unknown.

It should be noted that extracellular renalases have been detected in blood, urine and also in supernatants of cell cultures by means of Western blot [1, 20-22] or ELISA tests [11, 20, 23-26].

Evaluation of the intracellular renalase protein is also based on the use of antibodies [27-29]. All these approaches do not detect either the N-terminal sequence (involved in cofactor accommodation) or the cofactor itself. Although Desir et al. [3] mentioned in their review that human renalase purified from blood contains the N-terminal signal peptide, no experimental evidence supporting this statement was presented to validate this statement. Thus, identification of the N-terminal signal peptide in extracellular renalases is very important due to proposed speculations on FAD-dependent mechanisms of renalase activity both in serum and urine [1, 3, 11, 17] and contradictory reports on catecholamine-metabolizing activity in this protein [1, 4-6, 18, 20].

Recently, using the full-length recombinant renalase as control of tryptic patterns, we found that human urinary renalase isolated by means of immunoaffinity chromatography lacked the N-terminal peptide, while in the recombinant protein this peptide was clearly detected [15]. As shown by molecular simulations performed using the renalase crystal structure obtained by the Aliverti's group [12], the absence of the N-terminal signal peptide excludes the possibility of FAD binding by the truncated protein [15].

Where cleavage of the N-terminal peptide occurs still remains to be elucidated. Although possible formation of the truncated renalase during urine storage may be ruled out [15], cleavage of the N-terminal peptide may occur: (a) during secretion of intracellular renalase from the cell; (b) in blood circulation; (c) during urinary excretion of proteins. Studying adrenaline induced renalase secretion by human renal proximal tubular epithelial cells (HK2) Wang et al. [22] detected this protein in the extracellular medium. However, Western blot analysis used for its detection was not aimed at investigation of the presence or absence of its N-terminal peptide.

In order to evaluate the first and most likely possibility (of N-terminal peptide cleavage of renalase) we have investigated human recombinant renalase in lysates of HEK293T cells and in the extracellular cultivation medium for the presence of its N-terminal peptide. Earlier these cells were already used for transfection with renalase gene and elaboration of the protein product [29].

Materials and Methods

Reagents

Trifluoracetic acid (TFA) was purchased from Fluka, Germany; triethylammonium bicarbonate (TEAB) was purchased from Sigma, St. Louis, MO, USA; heptafluorobutyric acid (HFBA) was purchased from Acros, Geel, Belgium; formic acid from Merck, Germany; acetonitrile from Fisher Chemical, Leicestershire, UK. Trypsin modified sequencing grade was obtained from Promega, Madison, WI, USA; flavin adenine dinucleotide disodium salt hydrate (FAD) was purchased from Sigma, St. Louis, MO, USA. A custom polyclonal sheep antibody was raised against human recombinant renalase1 and purified by Pocard Ltd. (Moscow, Russia). A monoclonal anti-rabbit/sheep IgG antibody conjugated with horseradish peroxidase was from IMTEK (Moscow, Russia) (see also [14]).

Cell cultures, plasmids and preparation of the eukaryotic vector pcDNA-hRen1

Human embryonic kidney 293 transformed cells (HEK 293T cells) were purchased from the American Type Culture Collection.
The pcDNA-hRenI vector was prepared using a commercially available vector pcDNA4 myc-His B (Invitrogen) and a DNA fragment containing the full-length coding region of the renalase-1 gene. The latter was obtained by amplification of the corresponding nucleotide sequence of the plasmid pET-hRenI, in which the renalase-1 gene contained an additional sequence coding a hexahistidine tag [14]. The renalase gene coding sequence was inserted by BamHI/XhoI restriction sites. Amplification of the DNA fragment was performed using the forward primer 5’-gagctcggatccgaaatggaagcgcaggtgctgatcgtgggc, including a BamHI restriction site and a Kozak sequence and the reverse primer 5’-tctagaccgagcgcagatgggaaatccaatcgcc, including the renalase stop codon and a XhoI restriction site (Fig. 2). Subsequent cloning in E. coli cells resulted in selection of a cell clone carrying the pcDNA-hRenI vector. Its sequencing has shown that the coding region of the renalase-1 gene did not contain any additional nucleotides (that would code additional amino acid residues).

Expression and isolation of renalase

The constructed plasmid vector pcDNA4 hRen1 was transfected into eukaryotic HEK293T cells, which were plated into 10 cm-Petri dishes (7 million cells per dish) 5 h before transfection and cultivated in DMEM medium (PanEco, Russia) containing 10% FCS (PAA Laboratories), 4 mM glutamine (PanEco,), and antibiotics penicillin and streptomycin (50 units/mL and 50 µg/mL, respectively; PanEco). After the incubation cells were transfected by means of a mixture containing the transfection reagent FuGene 6 (Promega, USA) and the plasmid vector pcDNA4 hRen1 in DMEM medium (5 µg plasmid DNA and 15 µL of FuGene 6 per each plate).

The mixture preincubated at room temperature for 30 min was then added to the cell culture, which was cultivated at 37°C for 16 h. After this incubation the transfection medium was removed and replaced with fresh DMEM medium containing 4 mM glutamine, and antibiotics penicillin and streptomycin (50 units/mL and 50 µg/mL respectively). Under these conditions, transfected HEK293T cells were incubated for 30 h. After incubation, the cultivation medium was harvested, cleared by low speed centrifugation (1000 rpm for 5 min; Heraeus Megafuge 40 centrifuge; Thermo Scientific USA) and filtered through a sterile PVDF-Filter with a pore diameter of 0.45 m (Millipore). Based on renalase isolation from human urine samples [14] the protein concentrated by 75% ammonium sulfate precipitation was then dissolved in 50 mM Tris-HCl buffer, containing 5 mM EDTA, pH 8.0, and stored at -20°C. The cell monolayer was mechanically scraped off from the substrate, washed with saline, and sedimented at 5000 rpm for 10 min using the Eppendorf centrifuge 5415R. The resultant cell pellet was suspended in 50 mM Tris-HCl buffer, containing 5 mM EDTA, pH 8.0, sonicated in an ice bath and after removal of cell debris by centrifugation (15000 rpm for 20 min, Eppendorf centrifuge 5415R) stored at -20°C.

Transfection efficiency was controlled using the plasmid vector pTagGFP2-N (Evrogen). According to TagGFP2 fluorescence the transfection efficiency was about 65%.

SDS-electrophoresis of proteins isolated from in the conditioned medium and cell lysates was performed in 15% polyacrylamide gel [30]. Immunoblotting was performed by the method of Gallagner et al. [31] as described in Fedchenko et al. [14] using sheep antibody to renalase 1 and horseradish peroxidase conjugate with rabbit anti-sheep immunoglobulin.

Gel slices corresponding to the range of molecular masses from 45 kDa to ~25 kDa evaluated by both protein molecular mass markers and purified recombinant renalase 1 as a reference were used for
subsequent in-gel digestion with trypsin (see below). The range of molecular masses covered by gel slices roughly corresponded to full-length renalase and processed renalase (lacking its N-terminal peptide).

**In-gel digestion with trypsin**

Gel slices with putative renalase protein were cut into small pieces. After washing and destaining gel was dried under vacuum and rehydrated in 10 µL of trypsin 100 ng/µL in the supplied 1 mM hydrochloric acid for 5 min at 4°C following addition of 20 µL of 75 mM TEAB. The reaction of trypsic digestion was performed overnight at 37°C under stirring at 1200 rpm for 5 min every 45 min [15]. Peptides were extracted from gel pieces via three changes by 0.5% TFA. For each change 100 µL of 0.5% TFA was added to the gel pieces, which were subsequently incubated for 20 min at gradient temperature from 40°C to 65°C. The collected fractions were dried under vacuum at 30°C for 60 min and reconstituted in 10 µL of 0.1% formic acid for LC-MS analysis.

**Liquid chromatography-Mass spectrometry analysis**

Proteomic profiling of samples exposed to tryptic digestion was performed using a high resolution Q-Exactive mass spectrometer (Thermo Scientific, Waltham, MA USA). The full proteome experiment data-dependent MS/MS method was used for proteomic profiling and a targeted-MS/MS method (t-MS2) was designed to detect the N-terminal peptide of renalase. In both cases the instrument was operated in positive ionization mode and equipped with an Easy-Spray® ion source. The capillary voltage was set at 1.9 kV and transfer capillary temperature was 280°C, the 5-sens RP level was 60%. Internal calibration was enabled during the whole analysis using user-defined lock masses (m/z=371.10124, 445.12002 and 741.1952). In the data-dependent MS/MS analysis the MS scan was performed at 70K resolution with an AGC target value of 1e6 ions for 128 ms in range of 400 – 1500 m/z following top N10 MS/MS scans at 35K resolution with automatic gain control (AGC) target of 1e5 for 48 ms, mass isolation window of 2 Th and fixed mass of 150 m/z. The normalized collision energy was 35 eV and applied in the stepwise manner with ±25% shift. Apex trigger mode and dynamic exclusion were used. Ions with charge states z=1+, z>4+ and with unassigned charge were excluded from analysis.

The N-terminal peptide of renalase was detected by a targeted MS/MS method as described by Fedchenko et al. [32]. Taking into consideration that newly synthesized proteins often undergo N-terminal methionine excision (see for example, [33]) we were looking for two the following sequences with and without the first methionine residue: MAQVLIVGAGMTGSLCAALLR (residues 1-21) and AQVLIVGAGMTGSLCAALLR (residues 2-21), respectively.

Due to uncertainty of charge states and the possible dehydration of the precursor ion we created a preliminary inclusion list comprised of 6 available precursor ions: [M+2H]^{2+}, [M+3H]^{3+} and [M+2H-H2O]^{2+} for peptide MAQVLIVGAGMTGSLCAALLR (m/z = 1038.0623, m/z = 692.3773 and m/z = 1029.0570) and for peptide AQVLIVGAGMTGSLCAALLR (m/z = 972.5421, m/z = 648.6972 and m/z = 963.5368). The MS2 scan was performed with 2 microscans at 140K resolution with AGC target of 600 ms and mass isolation window of 1 Th (±0.5 amu). The collision energy was optimized for each precursor ion and defined in the inclusion list at 27 eV for m/z = 648.6972 ([M+3H]^{3+}), 31 eV for m/z = 972.5421 ([M+2H]^2+ and 29 eV for m/z = 963.5368 ([M+2H-H2O]^{2+}). The different charge state of the defined precursor had to be detected at the same retention time to confirm identification.

**Results**

**Renalase expression in HEK-293T cells**

Transfection of HEK-293T cells with the vector pcDNA4-hRenI carrying renalase gene coding full length renalase (without any additions such as a hexahistidine tag, etc) resulted in appearance of a protein, which was not detected in non-transfected cells (Fig. 3). Its molecular mass well corresponded to the calculated molecular mass (37.5 kDa) of human renalase-1. According to the electrophoresis of cell lysates, followed by immunoblot analysis with anti-renalase antibodies, maximal protein expression was observed after cell incubation for two days. Longer incubation of cells (up to four days) was accompanied by
a decreased content of this protein in cell lysates. In the extracellular medium immunoblot analysis with anti-renalase antibodies revealed appearance of a protein with molecular mass of about 36.0 kDa (Fig. 2). Its highest content in the extracellular (cultivation) medium was observed after four days of cell cultivation.

Detection of renalases in lysates of HEK cells and the cultivation medium

Renalase was confidently detected in both the lysate of HEK cells and the cultivation medium (Fig. 4). The coverage of amino acid sequence of renalase extracted from HEK cells was basically the same as in the case of purified preparation of human recombinant renalase-1 expressed in Escherichia coli cells and used as a reference standard (Table 1). Although the coverage of amino acid sequence of renalase, detectable in the cultivation medium, was somewhat lower than in the case of the purified recombinant renalase1 and renalase detected in the cell lysate, such coverage is usually considered as reasonable for reliable identification of a protein of interest.
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In accordance with our previous study, the N-terminal peptide was detected in human renalase expressed in E. coli cells [15]. Under our experimental conditions, the N-terminal peptide was detected only in human renalase extracted from HEK cells, a polypeptide containing residues 2-21 (Fig. 4, Table 1). Lack of the first methionine residue deduced from corresponding nucleotide gene sequence may be attributed to the well-known process of N-terminal methionine excision observed commonly with newly synthesized proteins [33]. In contrast to intracellular renalases (expressed in E. coli and HEK cells) we did not find any convincing evidence for the presence of the N-terminal peptide in renalase confidently identified in the extracellular medium. The absence of this peptide in renalase isolated from the conditioned medium (as well as the presence of this peptide in other renalase preparations) was confirmed by targeted MS2 (see details in the Materials and Methods Section). In this context it is especially important that in all renalase preparations studied we were able to identify next tryptic peptide (residues 23-35) adjacent to the N-terminal peptide as well as several other peptides of the N-terminal part of renalase (Fig. 4, Table 1). This suggests that lack of the peptide 2-21 in extracellular renalase is not an experimental artifact and the proteolytic cleavage involves only the N-terminal peptide.

Thus taken together these results obviously mean that extracellular renalase lacks an important region required for accommodation of its FAD cofactor.

**Discussion**

HEK293(T) cells are widely used for production of recombinant proteins and their subsequent export into the extracellular space [34]. Taking into consideration that they do not express detectable amounts of own renalase they do represent a very convenient tool for analysis of export of intracellular (recombinant) renalase outside the cell. The latter is especially important as kidneys represent an important source of circulating renalase [3, 21, 29], which is significantly altered at (end stage) renal disease [21]. It also should be noted that HEK293(T) cells were already used for transfection with the renalase gene (containing one His Tag gene after the coding sequence) [29]. However, the cited study was focused on other problems unrelated to renalase transport outside the cell [29].

In order to rule out any possible effects of additional amino acid stretches (e.g. hexahistidine tags, fused peptides/proteins) we have constructed a vector containing the
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The presence of a protein product corresponding to human renalase in HEK293T was demonstrated by Western blot analysis with the anti-renalase antibodies (Fig. 3) successfully used earlier for detection of protein products expressed in a prokaryotic system [14, 15]. Mass spectrometry analysis of intracellular renalase revealed the presence of an intact N-terminal terminus (see Table 1). Since we analyzed renalase preparations initially subjected to electrophoretic separation, lack of FAD may be well explained by its non-covalent interaction with the renalase protein. Earlier we found [15] that electrophoretic procedure caused a loss of FAD from purified renalases. Mass spectrometry analysis confidently detected recombinant renalase in the cultivation medium of transfected HEK293T cells. However, in contrast to recombinant renalase expressed in the prokaryotic system and in the eukaryotic system (HEK293T cells), renalase presented in the cultivation medium of HEK293T cells did not contain the N-terminal peptide.

Thus, results of the present study provide first convincing evidence that human recombinant renalase expressed in HEK293T cells and secreted into extracellular medium lacks its N-terminal peptide. This argues against possibility of renalase secretion from these cells via a nonconventional pathway as a full-length protein [3]. Since the cells are immediate source of extracellular renalase, the absence of the N-terminal peptide suggests that renalases detected in biological body fluids cannot contain their N-terminal peptide (Fig. 5). This is supported by our recent observation that urinary renalase lacks its N-terminal peptide and formation of this truncated renalase cannot be attributed to urinary/blood proteases [15]. Thus, considering all possible scenarios of cleavage of the renalase N-terminal peptide it appears that the cleavage occurs during secretion of intracellular renalase from the cell, rather than in blood circulation or during urinary excretion of this protein.

Results of our study strengthen the current viewpoint on distinct physiological roles of intracellular and extracellular renals [5, 15]. Full length intracellular renalase is a flavoprotein that catalyzes FAD-dependent oxidative conversion of α-NAD(P)H to NAD(P)⁺.

Fig. 5. The scheme accumulating current information about synthesis and excretion of kidney renalase. Renalase is synthesized by kidney cells as a protein containing the N-terminal peptide (shown as a hatched block). Transmembrane translocation of synthesized renalase into the extracellular space (circulation) is accompanied by loss of the N-terminal peptide. Urinary renalase is excreted as a truncated protein lacking its N-terminal peptide. Asterisks show experimentally confirmed steps in the renalase metabolism (see details in the text).

The scheme accumulating current information about synthesis and excretion of kidney renalase. Renalase is synthesized by kidney cells as a protein containing the N-terminal peptide (shown as a hatched block). Transmembrane translocation of synthesized renalase into the extracellular space (circulation) is accompanied by loss of the N-terminal peptide. Urinary renalase is excreted as a truncated protein lacking its N-terminal peptide. Asterisks show experimentally confirmed steps in the renalase metabolism (see details in the text).
and/or oxidative conversion of β-NADH isomers (inhibiting activity of NAD-dependent dehydrogenases) to β-NAD\(^+\) [6, 19]. Extracellular renalase lacks the N-terminal peptide, it cannot accommodate FAD and therefore cannot catalyze FAD-dependent reactions in blood and urine described in the literature [11, 17], which are obviously unrelated to the renalase protein. In this context it is especially important that the Desir’s group has recently described some effects exhibited by renalase-derived peptide(s) acting in a FAD-independent manner [35] and identified a renalase receptor [36]. Implementation of these “extracellular” effects clearly involves principally different (FAD-independent) mechanisms. These may include (non-enzymatic) regulatory cytokine-like effects [37] of renalase and/or renalase-derived peptides [35]. Such effects could be realized via interaction with the renalase receptor(s) described in the literature [36] and interfered with protein kinase B and the mitogen activated protein kinase (MAPK) signaling [37]. In this context it is interesting that p38 MAPK inhibition ameliorates kidney damage in hypertensive rats [38].

Conclusions

Secretion of human recombinant renalase by human kidney HEK293T cells is accompanied by cleavage of its N-terminal peptide. Lack of the N-terminal peptide, the structural precondition for FAD binding, suggests that extracellular (circulating) renalase acts in a FAD-independent manner and mechanisms of action of extracellular renalase are not associated with FAD.

Disclosure Statement

Authors declare that they have no conflict of interest.

Acknowledgments

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