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Biochemical characterization of human tissue kallikrein 15 and examination of its potential role in cancer

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ABSTRACT

Objective: Human tissue kallikrein 15 (KLK15) is the last cloned member of the KLK-related gene family. Despite being implicated in multiple cancers, its pathophysiological role remains unknown. We aimed to biochemically characterize KLK15 and preliminarily study its role in cancer.
Design & methods: Recombinant KLK15 protein was produced, purified to homogeneity and quantified by mass spectrometry (parallel reaction monitoring analysis). We profiled the enzymatic activity of KLK15 using fluorogenic peptide substrates, and performed kinetic analysis to discover the cleavage sites. As KLK15 has mainly been associated with prostate cancer, we used a degradomic approach and subsequent KEGG pathway analysis to identify a number of putative protein substrates in the KLK15-treated prostate cancer cell line PC3.
Results: We discovered trypsin-like activity in KLK15, finding that it cleaves preferentially after arginine (R). The enzymatic activity of KLK15 was regulated by different factors such as pH, cations and serine protease inhibitors. Notably, we revealed that KLK15 most likely interacts with the extracellular matrix (ECM) receptor group.
Conclusion: To our knowledge, this is the first study that experimentally verifies the trypsin-like activity of KLK15. We show here for the first time that KLK15 may be able to cleave many ECM components, similar to several members of the KLK family. Thus the protease could potentially be linked to tumorigenesis by promoting metastasis via this mechanism.

1. Introduction

Representing the largest cluster of serine proteases in the human genome, human tissue kallikreins (KLKs) have numerous physiological functions and are also useful for the diagnosis and treatment of many diseases, including cancer [1, 2]. Among the 15 members of the KLK-family, the newest cloned member is KLK15, whose pathophysiological roles remain largely unknown [3]. KLK15 is located adjacent to the KLK3 gene [encodes for prostate specific antigen (PSA)], and the encoded protein shares structural similarity with KLK3 [3, 4]. Limited studies to date have speculated that KLK15 functions alongside other KLKs via regulatory cascades in the prostate [5]. Following translation, KLKs are secreted in an inactive pro-form, awaiting activation by a regulatory mechanism that involves the proteolytic removal of the pro-peptide. This process likely includes a KLK-initiating activation cascade [6–8], and it is noteworthy that KLK proteolytic cascade pathways have been implicated in normal physiology and cancer [8]. In the prostate, KLK2 is thought to cleave pro-PSA [9]. It was proposed that KLK15 may also cleave pro-PSA [10], thus possibly taking part in the enzymatic cascade within the prostate [5]. The role that KLK15 may play in the prostate is highlighted by its aberrant expression in prostate cancer. KLK15 mRNA splice variants were found to be differentially expressed in prostate tissue specimens [11], and the mRNA levels were associated with aggressive prostate cancer [12]. Besides being upregulated in prostate cancer [11], KLK15 mRNA levels are also elevated in ovarian cancer [13, 14] and have diagnostic value for breast cancer [15].

Although KLK15 holds promise as a biomarker for many cancers, we have yet to elucidate its putative pathophysiological role. In the present study, we aimed to biochemically characterize KLK15 and delineate its enzymatic activity and substrate specificity. We also searched for native endogenous substrates using degradomic and substrate specificity analysis [16]. Our findings offer insights into the role of KLK15 in human pathobiology.

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2. Materials and methods

2.1. Reagents

Fluorogenic 7-amino-4-methylcoumarin (AMC) peptides were purchased from Bachem Bioscience (King of Prussia, PA) and were dissolved in DMSO. KLK15 heavy isotope-labeled peptide with a quantitation tag was purchased from SpikeTides™_TQL, JPT Peptide Technologies GmbH, Berlin, Germany [17]. For inhibition studies, serine protease inhibitors (serpins) were used (R&D Systems, Minneapolis, MN, USA). Phenylmethylsulfonyl fluoride (PMSF) (Sigma Aldrich) was freshly prepared in isopropanol.

2.2. Cell lines

Human kidney embryonic cells Expi293F™ were grown according to the manufacturer's protocol (ThermoFisher Scientific, Invitrogen, Waltham, MA, USA). Human prostate cancer PC3 cells (ATCC® CRL-1435™,ATCC, Manassas, VA) were grown in RPMI 1640 medium supplemented with 10% (*v*/v) fetal bovine serum (FBS) and incubated at 37 °C in 5% CO_2 atmosphere.

2.3. Recombinant KLK15 mammalian protein production and purification

The full length KLK15 protein (amino acids 1–256) (UniProtKB-Q9H2R5 (KLK15_HUMAN) was produced in a transient mammalian expression system (Expi293 system, ThermoFisher Scientific, Carlsbad, CA, USA) [18]. Verification of KLK15 production and quantification were performed using parallel reaction monitoring (PRM)-based targeted mass spectrometry (MS) analysis (Supplementary Material-1, Table S1).

KLK15 protein was purified using a two-step protocol (Fig. 1) with the first step being anion-exchange chromatography (Akta FPLC system, Amersham Pharmacia Biotech) using a HiTrap Q-HP column (GE Healthcare, Life Sciences) as described elsewhere [18, 19]. In the second step, reverse-phase liquid chromatography (HPLC) was used [19], and KLK15 was eluted at approximately 45% acetonitrile. From the eluted fractions, KLK15 quantity was determined by PRM and purity was verified by silver and Coomassie staining (Fig. 1). In detail, the major band ~40 kDa and the weakest band ~30 kDa were excised from the Coomassie-stained gel and analyzed by mass spectrometry. The results confirmed the identity of the KLK15 protein in both bands (data not shown). It is already known that KLK15 (UniProtKB - Q9H2R5 (KLK15_HUMAN)) has two glycosylated sites within the active protease domain. Consequently, it is possible for the weakest band ~ 30 kDa to be the deglycosylated form of KLK15.

2.4. SDS PAGE analysis

SDS-PAGE was performed as described and gels were either Coomassie-stained (Biosafe Coomassie, Invitrogen), or silver-stained (PlusOne Silver staining protein kit, GE Healthcare) [19].

2.5. Activity assays using fluorogenic AMC substrates and enzymatic kinetic analysis

The purified KLK15 protein was used to test for enzymatic activity (Supplementary Material-1, Tables S2 and S3). KLK15 (25 nM) was incubated in the activity assay buffer (50 mM Tris, 0.15 M NaCl, 10 mM $CaCl_2$ pH 8.0) with each fluorogenic AMC peptide substrate separately (final concentration 0.5 mM at a final volume of 100 µl). Different peptide substrates were used and AMC fluorescence was measured at 0.5 min intervals for 30 min at 37 °C using a fluorometer (PerkinElmer Life Sciences, Waltham, MA, USA) with excitation/emission wavelengths at 380/460 nm.

For the KLK15 kinetic enzymatic analysis, the optimal AMC tryptic-like substrates (2 substrates cleaved after R; QAR-AMC and VPR-AMC) were used. A constant amount of KLK15 (25 nM) was incubated with increasing final concentrations of each substrate (0.015, 0.03, 0.06, 0.125, 0.25 mM and 0.5 mM). Enzyme Kinetics Module 1.1 (Sigma Plot, SPSS, Chicago) was employed to determine the enzyme kinetic parameters.

2.6. Effect of cations and pH on the KLK15 activity

To determine the optimal pH for KLK15 activity for the VPR-AMC substrate hydrolysis, enzyme (25 nM) and substrate (VPR-AMC, final concentration 0.5 mM) were diluted in the appropriate pH buffer (0.1 M sodium acetate/acetic acid buffer (pH 5.0), 0.1 M MES (pH 6.0), 0.1 M Tris-HCl (pH 7.0, 7.5, 8.0, 9.0), 0.1 M sodium hydroxide/sodium bicarbonate (pH 10–11). Fluorescence was measured as described above and % activity was estimated using the optimal pH as 100% [19].

The effect of different cations at various concentrations ($ZnCl_2$, $MgCl_2$ and $CaCl_2$) on the KLK15 enzymatic activity was also tested. Pure KLK15 (10 nM) was incubated without (control, 50 mM Tris pH 8.0) or with different cation salt concentrations at 37 °C for 15 min with agitation. VPR-AMC substrate (0.5 mM) was then added and fluorescence was measured. Reactions were performed in duplicates, and % KLK15 activity was estimated by defining the control sample (without metal ion added) as 100%.

2.7. Inhibition studies on KLK15 activity

Active KLK15 (22 nM) was incubated with each inhibitor separately (Serpins, A1, A3, A5, F2) (final concentrations 0.5, 1, 2 µM) for 15 min at 37 °C with gentle agitation at a final volume of 100 µl. PMSF was tested as a non-specific serine protease inhibitor (final concentration 0.5, 1 and 2.5 mM). As a positive control, active KLK15 was tested using the VPR-AMC substrate without added inhibitor. After adding VPR-AMC (final concentration: 0.25 mM), fluorescence was measured every minute for 30 min. Reactions were performed in duplicate, and % activity was estimated using the control as 100%.

2.8. In vitro stability of KLK15 protein

Equal amounts of recombinant KLK15 protein were incubated at 37 °C in optimal activity assay buffer (50 mM Tris, 0.15 M NaCl, 10 mM $CaCl_2$ pH 8.0) for 0, 6, 12, and 24 h time intervals. The protein aliquots were analyzed by Coomassie-stained SDS-PAGE, and their activity was tested against VPR-AMC substrate (12 nM KLK15; 0.5 mM final concentration).

2.9. KLK15 PRM analysis

KLK15 quantification in crude KLK15-secreting extracts and purified fractions was performed using MS analysis, as previously described [17]. Samples were trypsin-digested [17, 18], and PRM was performed on a Q-Exactive Plus hybrid quadrupole-Orbitrap mass spectrometer (Thermo Fisher Scientific).

Tryptic peptides were loaded onto a 3 cm long 5 µm particle C18 trap precolumn (i.d. 200 µm) via an EASY-nLC pump (Proxeon Biosystems, Odense, Denmark) at 8 µl/min. The mobile phase consisted of 0.1% formic acid (FA) in water (buffer A) and 0.1% of FA in acetonitrile (buffer B). Peptides were separated on a 15 cm long 3 µm particle C18 analytical column (i.d. 75 µm) with an 8 µm tip (New Objective, Woburn, MA, USA), using a 22 min gradient elution and at a flow rate of 300 nl/min. The gradient started with 1% buffer B, increasing to 14% over 1 min, followed by an increase to 40% over the next 11 min. The gradient was then further increased to 65% buffer B over 2 min, before it reached 100% within 1 min and maintained for 7 min.

The PRM settings were as follows: In-source collision induced