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A substrate-optimized electrophoretic mobility shift assay for ADAM12

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ABSTRACT

ADAM12 belongs to the A disintegrin and metalloprotease (ADAM) family of secreted sheddases activating extracellular growth factors such as epidermal growth factor receptor (EGFR) ligands and tumor necrosis factor-alpha (TNF-α). ADAM proteases, most notably ADAM17 (TNF-α-converting enzyme), have long been investigated as pharmaceutical drug targets; however, due to lack of potency and in vivo side effects, none of the small-molecule inhibitors discovered so far has made it beyond clinical testing. Ongoing research on novel selective inhibitors of ADAMs requires reliable biochemical assays to validate molecular probes from large-scale screening efforts. Here we describe an electrophoretic mobility shift assay for ADAM12 based on the identification of an optimized peptide substrate that is characterized by excellent performance and reproducibility.

A disintegrin and metalloproteases (ADAMs)[4] are disease-relevant secreted human proteases that form a subgroup within the metzincin clan of zinc-dependent metalloproteases closely related to A disintegrin and metalloprotease with thrombospondin motif (ADAMTS), snake venom metalloproteases (SVMPs), and matrix metalloproteases (MMPs) [1]. ADAMs are single-pass type I transmembrane proteins mediating proteolytic cleavage of hormone and cytokine pro-proteins, cell surface receptors, and other secreted proteins, thereby modulating protein–protein, cell–cell, and cell–matrix interactions. The human ADAM family consists of 21 members exerting roles in diverse pathological conditions such as osteoarthritis, cancer, allergic disease, neurological disorders, and Alzheimer disease [1]. The role of ADAMs in activating ligands of the epidermal growth factor (EGF) family and tumor necrosis factor-alpha (TNF-α), molecules that are often involved in tumorigenesis and inflammation, has led to increased efforts in developing potent and selective small-molecule inhibitors [2,3].

Although some ADAM proteases such as ADAM8, ADAM9, and particularly ADAM17 (TNF-α-converting enzyme) have been thoroughly studied in terms of small-molecule inhibition, such data are largely lacking for ADAM12. ADAM12 is a proteolytically active enzyme involved in the proliferation, migration, and invasion of tumor cells [4,5] and has been reported to be a key molecule in the transactivation of EGF receptor (EGFR) by heparin binding EGF (HB–EGF) in cardiomyocytes [6], thereby representing an interesting target for structure-based drug discovery [7]. Alternative splicing results in two isoforms of ADAM12 that are suggested to act on different physiological substrates and, thus, exert distinct physiological functions [5]. Membrane-bound ADAM12-L acts as a sheddase for cell adhesion molecules and receptors [8] as well as other membrane-anchored proteins such as the pro- forms of EGF, HB–EGF and betacellulin, which are all ligands of the EGFR [9–11]. ADAM12-S, which in contrast to ADAM12-L lacks a transmembrane domain and which therefore is not associated with the cell membrane, mediates cleavage of insulin growth factor binding proteins IGFBP-3 and IGFBP-5 [12]. IGFBP-3 and IGFBP-5 are part of the IGF signaling system whose dysregulation contributes to the progression of cancer [13].

The screening of small-molecule libraries for inhibitors of ADAMs, but also MMP and ADAMTS proteases, is often carried out using fluorimetric assays employing quenched peptides [14–

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[4] *Abbreviations used:* ADAM, A disintegrin and metalloprotease; ADAMTS, A disintegrin and metalloprotease with thrombospondin motif; MMP, matrix metalloprotease; EGF, epidermal growth factor; TNF-α, tumor necrosis factor–alpha; EGFR, EGF receptor; HB–EGF, heparin binding EGF; IGFBP, insulin growth factor binding protein; CE, capillary electrophoresis; DMSO, dimethyl sulfoxide; FRET, Förster resonance energy transfer; TBS-T, Tris-buffered saline with Tween 20; HRP, horseradish peroxidase; hF, homophenylalanine; 5-FAM, 5-carboxyfluorescin.

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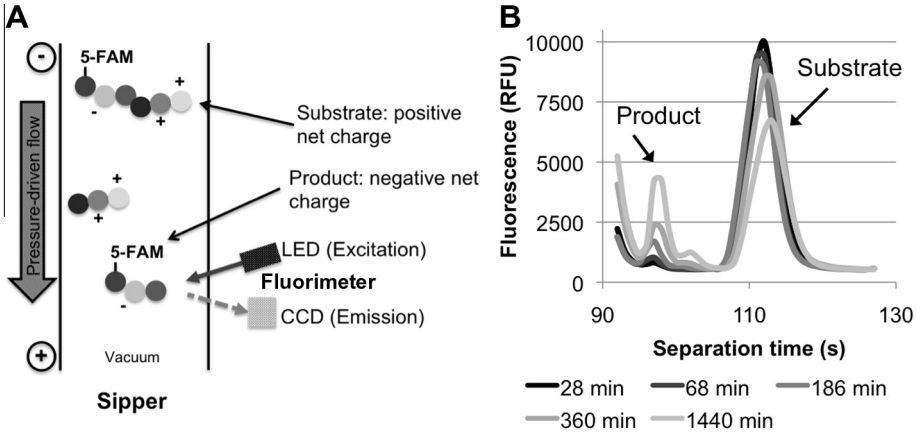

Fig.1. Principle of the mobility shift assay for ADAM12. (A) A mixture of substrate and product peptides is separated in one of the sippers of a 12-sipper chip commonly used with the EZ Reader II. (B) Electropherogram of a cleavage assay for ADAM12. Enzyme and peptide were incubated at 37 °C, and at different time points the reaction was analyzed on the EZ Reader II. As the height of substrate and product peaks is altered in an inversely proportional manner, a percentage conversion value (C) can be calculated from the peak heights (H) using the formula $C = H_{Product}/(H_{Product} + H_{Substrate})$. The conversion rate can be used directly to determine the amount of product formed. RFU, relative fluorescence units.

16]. Such assays are easily adaptable to high-throughput settings, are inexpensive, and are also adaptable to cross-reactivity studies. However, there are disadvantages to using such fluorimetric assays, most notably their susceptibility to autofluorescent compounds but also buffer components or impurities interfering with the fluorescence of the product peptide resulting from proteolytic cleavage of a quenched peptide [17,18]. Moreover, as the absolute fluorescence is measured, correct endpoint values, which are required to calculate accurate kinetic and inhibitory constants, are not easily obtained. Electrophoretic mobility shift assays, which similarly to fluorimetric assays rely on the cleavage of a peptide substrate, have the advantage that the assay mixture is analyzed by means of capillary electrophoresis (CE), allowing the separation of molecules interfering with peptide fluorescence but also a direct analysis of both substrate and product of the enzymatic reaction (Fig. 1). Assays based on microfluidic CE devices are often characterized by a high degree of assay consistency and reproducibility as well as low sample consumption (nanoliter range). Such assays have successfully been applied in screening campaigns to identify inhibitors of acetyl transferases [19], protein kinases [20,21], methyltransferases [22], and a range of other enzymes.

Searching for alternatives to fluorimetric assays for ADAM12 reported in the literature [23,24], we aimed at using the microfluidic microcapillary electrophoresis technology implemented in the EZ Reader II (Caliper, Hopkinton, MA, USA) for assessing the catalytic activity of ADAM12 in compound screenings. In this article, we describe the development of improved peptide substrates for ADAM12 using the SPOT technology, which offers a cost-effective way of screening a large range of peptide substrates for cleavage by enzymes such as ADAM12. An optimized substrate was then characterized and employed in a mobility shift assay for ADAM12. Dose–response experiments with hydroxamic acid-based small-molecule inhibitors of metalloproteases demonstrate that the newly created assay provides reproducible and accurate data for ADAM12.

Materials and methods

Peptides

All peptides were purchased from Biosyntan (Berlin, Germany) with greater than 95% purity and verified by mass spectrometry. Lyophilized peptides were dissolved in dimethyl sulfoxide (DMSO; Sigma–Aldrich, Denmark) at concentrations of 10 mM and 150 μM and were stored in aliquots at −20 °C.

Purification of ADAM12

ADAM12 was purified as described previously [12]. Briefly, HEK293 EBNA cells were transfected with a plasmid harboring the gene for human ADAM12-S (Uniprot ID O43184-2; plasmid kindly provided by U. Wewer, University of Copenhagen). Transfected cells were grown in suspension under serum-free conditions for 5 days at 37 °C, 70% humidity, and 5% CO_2. The cell culture supernatants were filtered and used in a two-step purification procedure comprising anion exchange and concanavalin A chromatography steps. The recombinant purified ADAM12 was dialyzed against 30 mM Hepes, 300 mM NaCl, 10% (v/v) glycerol, 0.005% Brij-35, 1 μM $ZnCl_2$, and 1 mM $CaCl_2$ (pH 7.5), concentrated and characterized by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), a Förster resonance energy transfer (FRET)-based activity assay, and mass spectrometry. ADAM12 was purified as a noncovalent complex of pro-domain and the mature part consisting of catalytic, disintegrin, Cys-rich, and EGF-like domains.

ADAM12 electrophoretic mobility shift assays

Assays were performed in 20 mM Tris (pH 8.0) supplemented with $CaCl_2$, Brij-35, and Tween 20 as described in the online supplementary material. Peptide stocks (10 mM in DMSO) were diluted in reaction buffer at the required concentration. ADAM12 stock solution (7.9 μM) was mixed with ice-cold reaction buffer just prior to the experiment and kept on ice until it was added to the assay plate. All other reagents were handled at room temperature. After the cleavage experiment, the reactions were transferred to 384-well plates (Corning, NY, USA). The 384-well plates were centrifuged (3200*g*, 1 min) and immediately analyzed on a LabChip EZ Reader II (Caliper) equipped with a LabChip EZ Reader 12-Sipper Chip (Caliper) and equilibrated with ProfilerPro separation buffer (100 mM Hepes [pH 7.3], 0.015% Brij-35, 5% DMSO, and 1 mM ethylenediaminetetraacetic acid [EDTA] supplemented with 0.5% Coating Reagent 8) (Caliper). The run conditions were adjusted depending on the peptide. Electropherograms were analyzed using instrument-specific software (Caliper). The resulting data were analyzed using XLfit (ID Business Solutions, UK).