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An improved fluorescent substrate for assaying soluble and membrane-associated ADAM family member activities

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ABSTRACT

A fluorescent resonance energy transfer substrate with improved sensitivity for ADAM17, −10, and −9 (where ADAM represents a disintegrin and metalloproteinase) has been designed. The new substrate, Dabcyl-Pro-Arg-Ala-Ala-Ala-Homophe-Thr-Ser-Pro-Lys(FAM)-NH_2, has specificity constants of 6.3 (±0.3) × 10^4 M^{-1} s^{-1} and 2.4 (±0.3) × 10^3 M^{-1} s^{-1} for ADAM17 and ADAM10, respectively. The substrate is more sensitive than widely used peptides based on the precursor tumor necrosis factor-alpha (TNF-alpha) cleavage site, PEPDAB010 or Dabcyl-Ser-Pro-Leu-Ala-Gln-Ala-Val-Arg-Ser-Ser-Lys(FAM)-NH_2 and Mca-Pro-Leu-Ala-Gln-Ala-Val-Dpa-Arg-Ser-Ser-Arg-NH_2. ADAM9 also processes the new peptide more than 18-fold better than the TNF-alpha-based substrates. The new substrate has a unique selectivity profile because it is processed less efficiently by ADAM8 and MMP1, −2, −3, −8, −9, −12, and −14. This substrate provides a unique tool in which to assess ADAM17, −10, and −9 activities.

ADAMs (a disintegrin and metalloproteinases) are processing enzymes for many type I and II integral membrane proteins [1–3]. The ADAM family of proteinases is important for many disease states, including, cancer, inflammation, and neurological afflictions such as Alzheimer's disease [4–7]. ADAM17, also referred to as tumor necrosis factor-alpha (TNF-alpha)-converting enzyme or TACE, and ADAM10, −12, −9, and −8 are also considered to be biological markers for diseases such as cancer and inflammation where they are dysregulated [8–11]. Therefore, researchers are actively searching for ways in which to assess activity of these metalloproteinases via enzyme-linked immunosorbent assays (ELISAs), where total protein is determined, or with substrates, where only the active enzyme concentrations are quantified.

Research efforts have focused on using fluorescence resonance energy transfer (FRET) substrates to measure ADAM activity in biological samples and in cell-based assays [11–14]. A technique called proteolytic activity matrix analysis (PrAMA) was developed, where activities of individual ADAM and MMP (matrix metalloproteinase) family members can be determined using a combination of nonselective substrates and computational analyses [15]. With this technique, individuals have been able to quantify active concentrations of a number of ADAM and MMP enzymes in vitro and in vivo. PrAMA was used to show that ADAM9, for example, is a predictive marker for inflammatory endometriosis [11], and MMP2 and MMP14 activities are downregulated by a therapeutic agent that modulates ADAM8 activity in pancreatic cancer models [16].

Recently, researchers developed a biosensor for ADAM17 that is expressed in cells and was used to quantify the enzyme's activity in the presence of different therapeutic agents [17]. The substrate was based on the cleavage site in precursor TNF-alpha, the first protein determined to be processed by ADAM17. Although the biosensor proved to be effective, it cannot be used with biological samples because the cells need to produce the substrate in situ.

Another TNF-alpha-based substrate, PEPDAB010 or Dabcyl-Ser-Pro-Leu-Ala-Gln-Ala-Val-Arg-Ser-Ser-Lys(FAM)-NH_2 that was

Abbreviations used: ADAM, a disintegrin and metalloproteinase; TNF-alpha, tumor necrosis factor-alpha; TACE, TNF-alpha-converting enzyme; ELISA, enzyme-linked immunosorbent assay; FRET, fluorescence resonance energy transfer; PrAMA, proteolytic activity matrix analysis; MMP, matrix metalloproteinase; PMA, phorbol 12-myristate 13-acetate; DMSO, dimethyl sulfoxide; PBS, phosphate-buffered saline; EV, extracellular vesicle.

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reported previously, was also used to assess ADAM17 activity in cells after PMA (phorbol 12-myristate 13-acetate) stimulation [13]. PMA is known to activate ADAM17, and several mechanisms have been proposed for this activation. PEPDAB010 is also processed well by ADAM9 [18]. In fact, several other ADAM and MMP family members cleave PEPDAB010, thereby limiting its usefulness [15].

Several attempts have been made to design both a sensitive and selective substrate for ADAM family members. A substrate based on the cleavage site in precursor transforming growth factor-alpha was designed, PEPDAB014, and is highly selective for ADAM17 over most ADAM and MMP enzymes [18]. However, this substrate is not as sensitive as PEPDAB010. The substrate, Flsub13 or PEPDAB013, is highly selective for ADAM8 and was used in urine as a way to try to detect invasive and metastatic breast cancer [12,19]. Caescu and coworkers used substrate libraries to determine the optimal sequence and found that a substrate they called "TENtide" is sensitive and selective for ADAM10 [20]. The same group also found that "TACEtide", with the sequence Pro-Arg-Ala-Ala-Ala-Val-Lys-Ser-Pro, was efficiently cleaved by ADAM17 but not by ADAM10. Furthermore, the substrate was almost as sensitive as a substrate with the precursor TNF-alpha cleavage site sequence. The specificity against other ADAM and MMP family members was not reported. We decided to modify the sequence of TACEtide and have found a substrate that is highly sensitive for ADAM17. In addition, the new substrate is the most sensitive substrate for ADAM9 reported to date.

Materials and methods

Buffer components were obtained from Sigma–Aldrich (St. Louis, MO, USA). The fluorescent substrate Mca-Pro-Leu-Ala-Gln-Ala-Val-Dpa-Arg-Ser-Ser-Arg-NH_2 and recombinant ADAM17, −10, and −9 were purchased from R&D Systems (Minneapolis, MN, USA). Recombinant ADAM8 was a gift from Joerg Bartsch (University of Marburg, Germany). MMP1 was obtained from Hideaki Nagase (University of Oxford, UK). MMP2 and MMP9 were obtained from William Stetler-Stevenson (National Institutes of Health, Bethesda, MD, USA). All other MMP enzymes were gifts from Gillian Murphy (Cambridge University, UK). The metalloproteinase inhibitor Ro32-7315 was provided by Roche Diagnostics (Penzberg, Germany).

Kinetic experiments with ADAM17 and ADAM10

Active enzyme concentrations were determined as described elsewhere [21]. Substrate stock solutions were prepared at various concentrations in R&D Systems' recommended assay buffer. Assays were conducted by incubating a range of substrate concentrations (2–50 μM) with various ADAM enzyme concentrations at 25 °C. Fluorescence was measured on a multimode microplate reader (Synergy Neo2, Biotek Instruments, Winooski, VT, USA) using $\lambda_{excitation} = 480$ nm and $\lambda_{emission} = 535$ nm. Rates of hydrolysis were obtained from plots of fluorescence versus time using data points from only the linear portion of the hydrolysis curve. The slope from these plots was divided by the fluorescence change corresponding to complete hydrolysis and then multiplied by the substrate concentration to obtain rates of hydrolysis in units of μM/s. Kinetic parameters were calculated by nonlinear regression analysis using the GraphPad Prism 6.07 suite of programs.

ADAM9 specificity value determination

PEPDAB010, PEPDAB064, or MCA-PLAQAV-Dpa-RSSR-NH_2 was dissolved in 10 mM dimethyl sulfoxide (DMSO) and then further diluted to 5–10 μM in buffer containing 25 mM Tris (pH 8.0), 100 mM NaCl, 10 mM $CaCl_2$, and 1e-3% Brij-35. ADAM9 (100 ng of R&D Systems' enzyme) was added to 150 μl of substrate in buffer to initiate the reaction. Fluorescence readings were taken in a TECAN Infinite 200 PRO Microplate Reader (BioExpress, Kaysville, UT, USA) at excitation and emission values of 485 and 530 nm, respectively, for the Dabcyl/FAM substrates and excitation and emission values of 323 and 390 nm, respectively, for the Mca/Dpa substrate. Data were fit using Microsoft Excel. Experiments were run in duplicate. The active enzyme concentration of ADAM9 was not determined as done previously for PEPDAB010 [18]. Rather, the enzyme concentration was based on a molecular weight as specified by the manufacturer.

Selectivity of PEPDAB064

PEPDAB010 or PEPDAB064 was diluted from 10-mM DMSO stocks to 5 and 10 μM in buffer containing 25 mM Tris (pH 8.0), 100 mM NaCl, 10 mM $CaCl_2$, and 1e-3% Brij-35. MMP or ADAM enzyme (2 μl) was added to 70 μl of buffer with substrate to start the reaction. Fluorescence readings were taken in a Cambridge FLUOstar fluorometer at excitation and emission wavelengths of 485 and 530 nm, respectively. The data represent experiments run in duplicate. Initial velocities were fit to straight lines using Microsoft Excel. Total fluorescence values were determined by adding trypsin (Sigma–Aldrich) to duplicate wells. The k_{cat}/K_m values for PEPDAB010 and PEPDAB064 were reported by dividing by the total enzyme concentrations that were not corrected for by determining active enzyme using tight binding inhibitors. Experiments were run at 5- and 10-μM substrate concentrations to ensure that kinetic constants were measured at values below the K_m for the enzymes.

Experiments with extracellular vesicles

L540 Hodgkin lymphoma cells (7×10^7, 5×10^6/ml) were cultivated for 2 h at 37 °C in serum-free RPMI 1640 medium. Cells were pelleted by 10 min of centrifugation at 200 *g* and discarded. The supernatant was cleared from traces of debris by a series of consecutive centrifugation steps, starting at 2000 *g*/10 min, followed by 2 × 3500 *g*/10 min and finally by 10,000 *g*/30 min. Extracellular vesicles from the supernatant were then pelleted by 2 h of ultracentrifugation at 100,000 *g*. The pellet was washed twice in phosphate-buffered saline (PBS) by ultracentrifugation and finally suspended in PBS. Aliquots (50 μl) of the extracellular vesicle (EV) suspension were suspended in triplicate in black 96-well microtiter plates (Nunc, Denmark), then 25 μl of 25 mM Tris–HCl (pH 8.0) containing 0.0006% Brij-35 ± 20 μM Ro32-7315 (5 μM final concentration), and subsequently another 25 μl of the buffer containing 40 μM PEPDAB064 (10 μM final concentration). The substrate was diluted from a 10-mM stock solution in DMSO. Samples with PBS without EVs served as background controls. Then, the 530-nm fluorescence (485-nm excitation) was determined every 5 min in an ELISA plate reader (SpectraMax M4, Molecular Devices, Sunnyvale, CA, USA).

Results

Two-dimensional substrate mapping experiments, using libraries of substrates with both unnatural and natural amino acids, determined that ADAM17 prefers homophenylalanyl at S1′ and threonine at S2′ over valine and arginine in an 8-mer substrate based on the precursor TNF-alpha cleavage sequence [22]. We decided to test the hypothesis that a sensitive and yet selective substrate for ADAM17, reported by Caescu and coworkers [20], with the sequence Pro-Arg-Ala-Ala-Ala-Val-Lys-Ser-Pro would be even