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ScienceDirect
Analytical Biochemistry 366 (2007) 144–148
ANALYTICAL BIOCHEMISTRY
www.elsevier.com/locate/yabio

Fluorescent substrates for the proteinases ADAM17, ADAM10, ADAM8, and ADAM12 useful for high-throughput inhibitor screening

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Received 12 December 2006
Available online 3 May 2007

Abstract

In this paper we describe novel fluorescent substrates for the human ADAM family members ADAM17, ADAM10, ADAM8, and ADAM12 that have good specificity constants and are useful for high-throughput screening of inhibitors. The fluorescence resonance energy transfer substrates contain a 4-(4-dimethylaminophenylazo)benzoyl and 5-carboxyfluorescein (Dabcyl/Fam) pair and are based on known cleavage sequences in precursor tumor necrosis factor-alpha (TNF-alpha) and CD23. The precursor TNF-alpha-based substrate, Dabcyl-Leu-Ala-Gln-Ala-Homophe-Arg-Ser-Lys(Fam)-NH_2, is a good substrate for all the ADAMs tested, including ADAM12 for which there is no reported fluorescent substrate. The CD23-based substrate, Dabcyl-His-Gly-Asp-Gln-Met-Ala-Gln-Lys-Ser-Lys(Fam)-NH_2, is more selective, being hydrolyzed efficiently only by ADAM8 and ADAM10. The substrates were used to obtain inhibition constants for four inhibitors that are commonly used in shedding assays: TMI-1, GM6001, GW9471, and TAPI-2. The Wyeth Aerst compound, TMI-1, is a potent inhibitor against all of the ADAMs tested and is slow binding against ADAM17.

Keywords: Disintegrin; Metalloproteinase; ADAM; TACE; ADAM17; ADAM10; ADAM8; ADAM12; GM6001; TAPI-2; TMI-1; GW9471; Fluorescent; Substrate; Inhibitor; Slow binding; FRET

ADAM[1] (a disintegrin and metalloproteinase) family members process many membrane-bound proteins, yielding soluble forms [1–3]. ADAM substrates are varied and consist of growth factors, receptors, and cytokines, and they may be processed by one or more family members. For example, several ADAMs can cleave amyloid precursor protein [4–7], but ADAM17 (TACE) is the only known physiological ADAM to release soluble TNF-alpha from its membrane-bound form [8,9].

Fluorescent substrates based on the cleavage sequence of TNF-alpha have been used successfully to screen for TACE inhibitors. A substrate with a 10-amino-acid peptide, Leu-Ala-Gln-Ala-Val-Arg-Ser-Ser-Ser-Arg, capped on the amino terminus with *o*-aminobenzoyl and on the

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[1] *Abbreviations used:* ADAM, a disintegrin and metalloproteinase; CD23 sub, Dabcyl-His-Gly-Asp-Gln-Met-Ala-Gln-Lys-Ser-Lys(Fam)-NH_2; Cha, cyclohexylalanyl; Dabcyl, 4-(4-dimethylaminophenylazo)benzoyl; DMSO, dimethyl sulfoxide; Dnp, 2,4 dinitrophenyl; Dpa, *N*-3-(2,4 dinitrophenyl)-L-2,3-diaminopropionyl diaminopropionic amide; EDANS, 5-[(2′aminoethyl)-amino]napthalenesulfonic acid; E, enzyme; EI, enzyme–inhibitor complex; Fam, 5-carboxyfluorescein; Flu, fluorescein; FRET, fluorescence resonance energy transfer; Homophe, homophenylalanyl; I, inhibitor; k_3, forward rate constant for formation of the enzyme–inhibitor complex; k_4, reverse rate constant; k_{cat}, turnover number; k_{cat}/K_m, specificity constant; K_{iapp}, apparent inhibition constant; K_i, inhibition constant; K_m, Michaelis–Menten constant; k_{obs}, observed rate constant; Mca, 7-amido-4-methylcoumarin; MMP, matrix metalloproteinase; P, product; ProCha sub, Dabcyl-Pro-Cha-Gly-Cys(Me)-His-Ala-Lys(Fam)-NH_2; Sub, substrate; TACE, tumor necrosis factor-alpha converting enzyme; TIMP, tissue inhibitor of metalloproteinase; TNF-alpha, tumor necrosis factor-alpha; TNF-alpha sub, Dabcyl-Leu-Ala-Gln-Ala-Homophe-Arg-Ser-Lys(Fam)-NH_2; v_i, initial velocity; v_o, initial velocity at zero inhibitor concentration; v_s, velocity at steady state.

0003-2697/$ - see front matter
doi:10.1016/j.ab.2007.04.043

carboxy terminus with 2,3-diaminoproprionic dinitrophenyl, was one of the first TACE fluorescent substrates to be described [10]. This substrate was not very sensitive, providing only an 11-fold enhancement of fluorescence after complete hydrolysis, and it displayed non-Michaelis–Menten kinetics. Other published fluorescent substrates had slightly improved specificity constants. For example, Mca-Lys-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH_2, Mca-Pro-Leu-Ala-Gln-Ala-Val-Dpa-Arg-Ser-Ser-Ser-Ala-Arg- NH_2, and Dabcyl-Leu-Ala-Gln-Ala-Val-Arg-Ser-Ser-Ser-Ala-Arg-EDANS- NH_2 have been described and have specificity constants in the range of 10^5 $M^{-1}s^{-1}$ [11].

For other ADAM family members such as ADAM10, ADAM8, and ADAM12, there are no reported substrates that can be used in high-throughput assays. With the emergence of ADAM family members as targets for therapeutic intervention, there is interest in finding specific inhibitors of these metalloproteinases. We have found fluorescent substrates for these ADAMs that can be used for inhibitor screening. These substrates were used to determine inhibition constants for TMI-1 [12], GM6001 [13], TAPI-2 [8], GW9471 [9], and a Calbiochem MMP inhibitor. We have discovered that TMI-1 is a slow binding inhibitor of TACE and the mechanism of inhibition is described. In addition, with the exception of the Calbiochem inhibitor, all of these compounds have been used in shedding assays to determine whether the processing events are metalloproteinase dependent [14–16].

Materials and methods

Recombinant human TACE, ADAM10, and ADAM8 were obtained from R & D Systems. Human ADAM12 was prepared as described [17]. The catalytic domain of human ADAM17 was the kind gift of Marcos Milla, Roche Bioscience. Matrix metalloproteinases (MMPs) were the kind gifts of Gillian Murphy, Cambridge University, Hideaki Nagase, Imperial College of London, Chris Overall, University of British Colombia, Canada, and William Stetler-Stevenson, National Cancer Institute. Fluorescent substrates were obtained from BioZyme Inc. Both GM6001 and the MMP9/13 inhibitor (Cat. No. 444252) were purchased from Calbiochem. The other inhibitors were synthesized as described [8,9,12].

Substrate assays with ADAMs

ADAM catalytic/disintegrin constructs were monitored at 1-to3- min intervals using the fluorescent substrate Dabcyl-Leu-Ala-Gln-Ala-Homophe-Arg-Ser-Lys(Fam)-NH_2, Dabcyl-His-Gly-Asp-Gln-Met-Ala-Gln-Lys-Ser-Lys (Fam)-NH_2, or Dabcyl-Pro-Cha-Gly-Cys(Me)-His-Ala-Lys(Fam)-NH_2 with excitation and emission wavelengths of 485 and 530 nm, respectively. The substrates were diluted from a 10 mM stock in DMSO to 10 μM in assay buffer containing 20 mM Tris, pH 8.0, and 6×10^{-4}% Brij-35. For experiments with ADAM8 only, 10 mM $CaCl_2$ was added to the buffer. Reactions were run in a 96-well plate with either inhibitor (1 nM–10 μM) in 1% DMSO or a 1% DMSO control. Background wells contained substrate and 1% DMSO and were subtracted from all other wells. Endpoints were determined in wells containing substrate and an excess of ADAM17 for the TNF-alpha substrate and ADAM8 for the CD23 substrate. Concentrations of enzyme ranged 0.1–2.0 nM for TACE, 1–5 nM for ADAM10, 2–10 nM for ADAM8, and 0.5–2 nM for ADAM12. For the enzyme titration experiments, the highest concentration indicated above was used.

Substrate assays with MMPs

The fluorescent substrates Dabcyl-Leu-Ala-Gln-Ala-Homophe-Arg-Ser-Lys(Fam)-NH_2, Dabcyl-His-Gly-Asp-Gln-Met-Ala-Gln-Lys-Ser-Lys(Fam)-NH_2, or Dabcyl-Pro-Cha-Gly-Cys(Me)-His-Ala-Lys(Fam)-NH_2 (10 μM) in assay buffer containing 50 mM Tris, pH 7.5, 150 mM NaCl, 2 mM $CaCl_2$, 5 μM $ZnSO_4$, and 0.01% Brij-35 were used to monitor enzyme activities. MMP1, 2, 3, 8, 9, 13, and 14 (0.1–20 nM) were incubated with substrates. Fluorescence was measured every 3–5 min using excitation and emission wavelengths of 485 and 530 nm, respectively. Endpoint wells contained substrate and an excess of MMP13 while background rates were determined with substrate only. Enzyme concentrations were back calculated by comparing reaction rates to those found for the standard substrate, Dabcyl-Gly-Pro-Leu-Gly-Met-Arg-Gly-Cys(Flu)-NH_2(10 μM), whose specificity constants for the MMPs are reported in Rasmussen et al. [18].

Slow binding inhibition of TACE by TMI-1

Human TACE, catalytic or catalytic and disintegrin domain, 10 μl of a 1.6 nM solution, was added to 90 μl of 10 μM substrate Dabcyl-Leu-Ala-Gln-Ala-Homophe-Arg-Ser-Lys(Fam)-NH_2 in assay buffer containing 20 mM Tris, pH 8.0, and 6.0×10^{-4}% Brij-35. Inhibitor concentration varied from 10 to 0.6 nM in twofold increments. Reaction progress was monitored at 27 °C in a BMG Fluostar fluorometer using excitation and emission wavelengths of 485 and 530 nm, respectively. Plots of fluorescence versus time were fit to Eq. (1),

$$P = v_s * t + ((v_i - v_s)/k_{obs})(1 - \exp^{-k_{obs} * t}), \quad (1)$$

where P is product, v_s is the steady state velocity, t is time, v_i is the initial velocity, and k_{obs} is the rate constant for slow binding inhibition.

Determination of inhibition constants

For all of the compounds studied, a competitive inhibition model was used. For experiments where the enzyme concentration approached the inhibition constant value, the Morrison equation was used to calculate active enzyme levels and K_i [19]. If the amount of active enzyme was