Fluorescent Substrates Useful as High Throughput Screening Tools for ADAM9

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Abstract: Fluorescence resonance energy transfer substrates were designed and tested as substrates for ADAM9. The donor/quencher pair used were 5-carboxyfluorescein (Fam) and 4-(4-dimethyl-aminophenylazo)benzoyl (Dabcyl) since they have been well studied sensitive fluorescent probes. The peptides based on precursor TNF-alpha, Dabcyl-Ser-Pro-Leu-Ala-Gln-Ala-Val-Arg-Ser-Ser-Lys(Fam)-NH_2 and Dabcyl-Leu-Ala-Gln-Ala-HomoPhe-Arg-Ser-Lys(Fam)-NH_2, and C-terminal TGF-alpha, Dabcyl-Glu-His-Ala-Asp-Leu-Leu-Ala-Val-Val-Ala-Ala-Lys(Fam)-NH_2 cleavage sites were effectively processed by ADAM9 with turnover numbers of $100 \pm 20 \times 10^{-2}$ min^{-1}, $20 \pm 10 \times 10^{-2}$ min^{-1}, and $10 \pm 3 \times 10^{-2}$ min^{-1}. In addition, a peptide based on the 33kDa cleavage site of the low affinity receptor for IgE, CD23, Dabcyl-Leu-Arg-Ala-Glu-Gln-Gln-Arg-Leu-Lys-Ser-Lys(Fam)- NH_2 was processed as well but with less efficiency. A more selective substrate for ADAM9 was found based on the betacellulin cleavage site. However, the valine containing precursor TNF-alpha based substrate was used to measure IC_{50} values of metalloproteinase inhibitors against ADAM9 since it was processed most efficiently. The tightest binding inhibitor was the Wyeth Aerst compound, TMI-1, with an IC_{50} of 2.1 ± 0.3 nM. In addition, GI254023, previously identified as a selective inhibitor of ADAM10, also inhibited ADAM9 with an IC_{50} of 280 ± 110 nM. These results demonstrate that sensitive substrates for ADAM9 can be developed that are useful in high throughput screening assays for ADAM9.

Keywords: ADAM8, ADAM9, ADAM10, ADAM12, ADAM17, TACE, fluorescent, substrate, inhibitor, TMI-1, GI254023, GM6001, disintegrin, metalloproteinase, FRET, CD23, TGF-alpha.

INTRODUCTION

ADAM9, also referred to as MDC9 [1], is a member of the ADAM (a disintegrin and metalloproteinase) family [2]. ADAM family members possess several domains including a pro, catalytic, disintegrin, cysteine rich, and transmembrane domain, and a cytoplasmic tail. The catalytic domain contains a typical zinc-binding motif, HEXXHXXGXXH, which is necessary to carry out proteolytic events. These proteolytic events, when dysregulated, result in the release or "shedding" of type I and II membrane proteins, yielding soluble forms. The soluble proteins are involved in disease states such as cancer [3-5], inflammation [6-10], cardiac hypertrophy [11], and obesity [12-14].

Researchers are actively seeking inhibitors of many ADAM family members for therapeutic use because they are sheddases for cytokines, growth factors, chemokines, and receptors. For example, in cancer proliferation, ADAM17, ADAM10, ADAM12, and ADAM9 process epidermal growth factor (EGF) ligand family members such as epidermal growth factor, heparin-binding epidermal growth factor, amphiregulin, transforming growth factor-alpha, betacellulin and epiregulin [11, 15-21]. Releases of EGF ligands promote cellular proliferation by binding to the EGF receptor and causing its dimerization and subsequent phosphorylation.

For inflammatory diseases such as asthma, it is well known that some ADAM family members process the cytokine, precursor TNF-alpha, to yield a soluble form that is pro-inflammatory [6]. But more recently, the ADAMs were found to play a role in the shedding of CD23, the low affinity receptor for IgE, to yield soluble forms of 37, 33 and 29 kDa [7-8]. The soluble CD23 proteins are able to mediate allergic responses by regulating IgE levels [22]. Therefore the enzyme(s) that release CD23 from the membrane are considered pharmaceutical targets of great importance. While ADAM10 is believed to be a principal sheddase for CD23, other family members exist that are also able to process CD23. Human ADAM8, for example, cleaves a CD23 peptide substrate from the 37kDa cleavage site at the same position as ADAM10 (unpublished observation), and ADAM8 is capable of processing CD23 in cell based assays [7-9]. In addition, when a metalloproteinase inactive E to A mutant ADAM8 is transfected into a transformed monocytic cell line, it acts to sequester factors that are necessary for CD23 release and reduces shedding by 30% [8].

Some ADAM family members are also required for normal development, suggesting that treating with therapeutics against these metalloproteinases could lead to serious side effects. For example, the ADAM17 and

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ADAM10 knock-out mice are embryonic lethal [16, 23]. ADAM17 deficient embryonic mice die between day 17.5 and the first day of birth while ADAM10 knockouts die by day 9.5. On the other hand, transgenic animals that are deficient in ADAM9 have no overt phenotype [24]. However, when ADAM9 knockouts were used in a prostate carcinoma model, and compared to wild type littermates, there was a difference in phenotypes. In mice lacking ADAM9, the tumors were mostly well differentiated. In contrast, in mice having wild type ADAM9, the tumors were very advanced, and were larger and predominantly poorly-differentiated [21]. This finding indicates that ADAM9 may be required for tumor development *in vivo*.

Because ADAM9 is an attractive pharmaceutical target, researchers want to design inhibitors against it. In order to screen inhibitor libraries, a source of the enzyme and a robust assay are needed. The enzyme was previously expressed in COS-7 cells, and purified to near homogeneity [1], but can now be purchased from R&D Systems. For a robust assay, we want a substrate that has a large turnover number and high sensitivity so that we can rapidly and accurately detect substrate cleavage while using small amounts of enzyme, thereby reducing assay time and cost. Currently, there is no assay for ADAM9 that meets these criteria. For example, Mca-Pro-Cha-Gly-Cys(methyl)-His-Ala-lys(Nma)-NH_2 was described as useful for inhibitor screening [1]. However, the specificity constant for this substrate was fairly low, 1.3 10^4 M^{-1} s^{-1}, and the MCA/Nma fluorophore pair was not very sensitive.

Here, we describe a fluorescent substrate that has better turnover and is more sensitive for ADAM9 than the standard Mca/Nma peptide since a simple conversion of the MCA/Nma substrate to Dabcyl-Pro-Cha-Gly-Cys(methyl)-His-Ala-Lys(Fam)-NH_2 did not result in an efficient substrate (unpublished observation). The new substrate has Dabcyl and 5-carboxyfluorescein as quencher and donor groups respectively and contains the precursor TNF-alpha cleavage site, Ser-Pro-Leu-Ala-Gln-Ala-Val-Arg-Ser-Ser. The Dabcyl/Fluorescein pair has literature precedence for use in FRET-based procedures and possesses adequate spectral overlap that allows almost complete quenching of the donors' fluorescence [25-28]. This new ADAM9 substrate has proven to be the best reported to date, with a 1000 fold increase in fluorescence from the start of the reaction to complete hydrolysis.

We have also developed a more selective substrate for ADAM9 based on the cleavage site of betacellulin, Dabcyl-Val-Asp-Leu-Phe-Tyr-Leu-Gln-Gln-Pro-Lys(Fam)-NH_2. This substrate is efficiently processed by ADAM family members, but not by matrix metalloproteinases. This substrate could prove to be useful in cell based assays and to measure soluble ADAM concentrations in biological fluids.

MATERIALS AND METHODS

Recombinant human ADAM8, ADAM9, ADAM10, ADAM12, and ADAM17 were obtained from R & D Systems. MMPs were provided by Gillian Murphy, Cambridge Research Institute, London, and Hideaki Nagase, Imperial College of London. Fluorescent substrates were obtained from BioZyme, Inc. Both GM6001 and the MMP9/13 inhibitor (Cat. No. 444252) were purchased from Calbiochem. Marimastat and the TMI-1 inhibitor were synthesized as described [29, 30]. GI254023 was provided by GlaxoSmithKline. Purity of inhibitors was greater than 98%.

Unless otherwise noted, all experiments were run using a Finstruments Fluoroskan II top reading fluorometer. The instrument's light source is a 40 watt xenon lamp with wavelength range of 300 to 1000 nm, and its detector is a photomultiplier tube with wavelength range 280 to 700 nm. The excitation and emission filter wavelengths are 485 and 530 nm respectively. The software used was Spectrosoft version 6.1 by MTX Lab Systems, Inc. Grenier Fluotrac 200 black polystyrene flat-bottom 96-well plates were used to minimize background fluorescence and bleed through between adjacent wells. Reactions were run using total volumes of 70 to 100 µl, with 10 µM substrate concentration, at room temperature, and 2 to 3 minute sample intervals. Background wells, where the enzyme was replaced with assay buffer, or the enzyme activity was eliminated using a strong inhibitor, were run side by side with the active wells. The fluorescence readings of the background wells were subtracted from the active wells to eliminate errors due to non-specific substrate cleavage or light intensity variations. Assays were performed minimally in duplicate and standard errors were calculated from at least two separate experiments. Where required, error propagation calculations were performed.

Determination of Dissociation Constants, K_s and Maximal Velocities, V_{max}

The dissociation constants against ADAM9 were determined by reacting the catalytic/disintegrin construct with the fluorescent substrates Dabcyl-Leu-Ala-Gln-Ala-Homophe-Arg-Ser-Lys(Fam)-NH_2, Dabcyl-Ser-Pro-Leu-Ala-Gln-Ala-Val-Arg-Ser-Ser-Lys(Fam)-NH_2, and Dabcyl-Glu-His-Ala-Asp-Leu-Leu-Ala-Val-Val-Ala-Ala-Lys(Fam)-NH_2. Substrate concentrations, S, varied from 12.5 to 200 µM, for the precursor TNF-alpha substrates, and 8 to 128 µM for the TGF-alpha peptide. Enzyme, 0.01-30 nM was added to initiate the reactions. Control wells containing substrate only were used to determine background levels of fluorescence. Data was collected with a BMG Lab Technologies Fluostar Optima, or Cambridge Scientific 7630 top to bottom reader microplate fluorometer at room temperature and two-three minute intervals.

Initial velocities (v) were determined from the slopes of the fluorescence units *vs* time graph. Then, the sigmoidal curves of v *vs* S were graphed with Sigma Plot software, and fit to the Hill equation (1). From the Hill equation we determined the Hill coefficient, n, the maximum velocity, V_{max}, and the dissociation constant, K_s. The slopes were converted to nmoles/min by multiplying the V_{max} by the moles of product per FU (Eq. 1).

$$v / V_{\max} = \frac{S^n}{(S^n + K_s)} \quad (1)$$

MALDI-MS of Treated Peptides

Samples were spotted, 0.3 µl, onto a 384 sample stainless steel MALDI plate and mixed on target with 0.3 µl of 33%

saturated a-cyano-hydroxycinnamic acid. Mass spectrometric analyses were then performed on an Applied Biosystems 4800 Proteomics Analyzer in the positive ion and reflector modes. The MS was externally calibrated using standard peptides. A focus mass of m/z 1500 was used for the MS acquisition. Cleavage sites were determined by comparing observed masses to theoretical masses at each possible cut site.

Determination of Turnover Number, V_{max}/E_t

Turnover numbers, V_{max}/E_t, were calculated by taking the maximal velocities, and dividing by the enzyme concentration. ADAM9 enzyme concentration was obtained by active site titration with TMI-1 followed by a fit to the Morrison equation [31]. ADAM8, 10, 12, and 17 and MMP1, 2, 3, 8, 9, 13, and 14 enzyme concentrations were determined previously by active site titration using tight binding inhibitors [32]. The amount of active enzyme for each ADAM was corrected for in each experiment by measuring the specificity value or constant for a known substrate and then back-calculation to determine active enzyme concentration.

Determination of Specificity Value, k_p/E_t

For ADAM family members all reactions were carried out in buffer containing 25mM Tris, pH8 and 6 x 10^{-4}% Brij-35. For ADAM8 and ADAM12, 10mM $CaCl_2$ was used in the buffer. MMP specificity values were measured by incubation of MMPs in buffer containing 50mM Tris, pH8, 200mM NaCl, 10mM $CaCl_2$, 5µM $ZnCl_2$, and 1 x10^{-2}% Brij-35. The rate constant, k_p, represents: (Slope of Fluorescence Units *vs* Time)/ (total fluorescence at the endpoint * 60 (to convert minutes to seconds)) measured at 10µM substrate concentration. The enzyme concentrations were calculated as described above.

Determination of IC_{50} Values

The ADAM9 catalytic/disintegrin construct was reacted with the fluorescent substrate Dabcyl-Ser-Pro-Leu-Ala-Gln-Ala-Val-Arg-Ser-Ser-Lys(Fam)-NH_2, with either inhibitor (4 nM–1 µM) in 2% DMSO, or a vehicle control. Background wells contained substrate, 2% DMSO, and lacked enzyme, and were subtracted from all other wells. Endpoints were determined in wells containing substrate and an excess of ADAM17 to ensure complete turnover. Concentrations of enzyme ranged from 0.01–2.0 nM for ADAM9, with 2.0- 4 nM being used for the enzyme titration experiment.

The IC_{50} was calculated by running the inhibition assays at varying inhibitor concentrations. Next, fractional inhibition, I_f, was calculated by dividing the substrate turnover initial velocities in wells containing inhibitor, v_i, to the velocities in control wells without inhibitor, v_o. Finally, the fractional inhibitions were graphed against inhibitor concentration using Sigma Plot software, and the data was fit to equation (2). From this equation, the unknown IC_{50} can be calculated from the known fractional inhibition, I_f, and the known inhibitor concentration.

$$v_i / v_o = I_f = \frac{1}{1 + (I / IC_{50})^n} \quad (2)$$

RESULTS

Determination of K_s and V_{max} from Sigmoidal Plots of v *vs* S

When trying to determine a K_m from a Michaelis-Menten plot with Dabcyl-Ser-Pro-Leu-Ala-Gln-Ala-Val-Arg-Ser-Ser-Lys(Fam)- NH_2 (Val sub), we discovered that the plot was sigmoidal indicating cooperative behavior (Fig. **1**). The data was fit to the Hill equation, where the Hill coefficient was approximately 2 (2.2 ± 0.6; Table **1**). This cooperative binding was not solely a property of the valine containing substrate as Dabcyl-Leu-Ala-Gln-Ala-HomoPhe-Arg-Ser-Lys(Fam)- NH_2 (HomoPhe sub), and Dabcyl-Glu-His-Ala-Asp-Leu-Leu-Ala-Val-Val-Ala-Ala-Lys(Fam)-NH_2, (TGF sub) also exhibited sigmoidal plots with Hill coefficients of 1.9± 0.3 and 1.8± 0.3 respectively. From equation (1), the dissociation constants (Table **1**), K_s, were 46 ± 20 µM (Val sub), 47 ± 12 µM (HomoPhe sub), and 16 ± 6 µM (TGF sub). The maximal velocities for the substrates were also determined from the Hill plot and were 70 ± 1 x 10^{-2}, 40 ± 10 x 10^{-2}, and 40 ± 10 x 10^{-4} nmole/min for the Val, HomoPhe, and TGF subs, respectively.

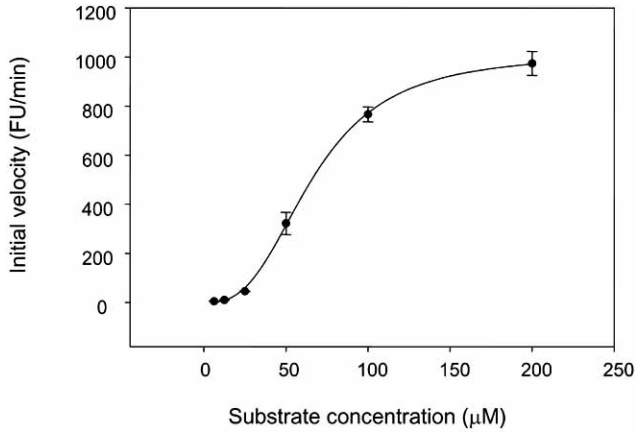

Fig. (1). Sigmoidal Plot of initial velocity vs. Val sub concentration. The initial velocity calculated from the slopes of the fluorescence units *vs* time graphs, were plotted vs. substrate concentration, for ADAM9 reacted with Dabcyl-Ser-Pro-Leu-Ala-Gln-Ala-Val-Arg-Ser-Ser-Lys(Fam)-NH_2. The plot is sigmoidal indicating cooperative binding. The data were fit to the Hill equation (Materials and Methods), and K_s, the dissociation constant was determined.

Substrates for ADAM9: Determination of Turnover Number

Because the plots of initial velocity *vs* substrate concentration were sigmoidal, we could not calculate an actual specificity constant, k_{cat}/K_m. However we could obtain maximal velocities from a fit of the data to the Hill equation. We therefore decided to determine the turnover number for each substrate which is the maximal velocity divided by the enzyme concentration. Listed for comparison in Table **1** are the turnover numbers for the substrates, Dabcyl-Ser-Pro-Leu-Ala-Gln-Ala-Val-Arg-Ser-Ser-Lys(Fam)-NH_2, Dabcyl-Leu-Ala-Gln-Ala-HomoPhe-Arg-Ser-Lys(Fam)-NH_2 and Dabcyl-Glu-His-Ala-Asp-Leu-Leu-Ala-Val-Val-Ala-Ala-Lys(Fam)-NH_2 tested against ADAM 9. Substitution of Val for

Table 1. Parameters Obtained from Hill Plots of Initial Velocity *vs* Substrate Concentration[a]

Substrate	K_s (μM)	Hill coef. (n)	V_{max}[b] (nmole/min)	V_{max}/E_t[c] (min^{-1})
Dabcyl-Ser-Pro-Leu-Ala-Gln-Ala-Val-Arg-Ser-Ser-Lys(Fam)-NH_2	46 ± 20	2.2 ± 0.6	70 ± 10 x 10^{-2}	100 ± 20 x 10^{-2}
Dabcyl-Leu-Ala-Gln-Ala-HomoPhe-Arg-Ser-Lys(Fam)-NH_2	47 ± 10	1.9 ± 0.3	40 ± 10 x 10^{-2}	20 ± 10 x 10^{-2}
Dabcyl-Glu-His-Ala-Asp-Leu-Leu-Ala-Val-Val-Ala-Ala-Lys(Fam)- NH_2	16 ± 6	1.8 ± 0.3	40 ± 10 x 10^{-4}	10 ± 3 x 10^{-2}

[a]Values were obtained by plotting initial velocities *vs* substrate concentration and fitting the curve to the Hill Equation as described in Materials and Methods.
[b]V_{max} values were converted from FU/min to nmole/min. Error propagation was used for this conversion.
[c]Error propagation was used for this measurement. Unless otherwise noted, all other errors represent the standard deviation from at least two separate experiments.

HomoPhe and lengthening of the peptide chain proved to be beneficial for ADAM9, with turnover numbers of 100 ± 20 x 10^{-2} min^{-1}, *vs* 20 ± 10 x 10^{-2} min^{-1}. The TGF sub is also processed efficiently even though the substrate sequence is quite different (10 ± 3 x 10^{-2} min^{-1}). The increase in V_{max} with the Val and HomopPhe subs and the sensitivity of the Dabcyl/Fluorescein pair compared to previous substrates allows for the use of 5 to 100-fold less ADAM9 in each enzymatic reaction. This becomes critical when potent inhibitors are used and also for cost purposes, which becomes important in high throughput screening.

Determination of Specificity Values

Specificity values, defined as k_p/E_t, (see Materials and Methods) were determined to try to evaluate how effective and selective each substrate was for the ADAM and MMP family members at concentrations of 10μM. Five sequences were chosen: Val TNF sub, Dab-Ser-Pro-Leu-Ala-Gln-Ala-Val-Arg-Ser-Ser-Lys(Fam)-NH_2; HomoPhe sub, Dabcyl-Leu-Ala-Gln-Ala-HomoPhe-Arg-Ser-Lys(Fam)-NH_2; TGF sub, Dabcyl-Glu-His-Ala-Asp-Leu-Leu-Ala-Val-Val-Ala-Ala-Lys(Fam)- NH2; CD23 (33kDa) sub, Dabcyl-Leu-Arg-Ala-Glu-Gln-Gln-Arg-Leu-Lys-Ser-Lys(Fam)-NH_2; and beta-cellulin (BTC) sub, Dabcyl-Val-Asp-Leu-Phe-Tyr-Leu-Gln-Gln-Pro-Lys(Fam)-NH_2. Table **2** contains the specificity values. The Val, TGF, and HomoPhe subs had specificity values of 1.1 x 10^5 $M^{-1}s^{-1}$, 1.8 x 10^4 $M^{-1}s^{-1}$, and 8.3 x 10^3 $M^{-1}s^{-1}$ respectively for ADAM9. Against the other ADAM family members, CD23 sub was processed efficiently by ADAM17,10 and 8 while the BTC sub was approximately 50 fold less sensitive than Val sub (Table **2**) for ADAM9. The most selective substrate of all was the BTC sub, demonstrating no detectable turnover for most of the MMPs tested.

The CD23 and BTC subs have better specificity values for ADAM9 relative to those for ADAM10, a known physiological convertase. In addition, while ADAM8 and 10 were able to process efficiently a CD23 substrate based on the 37kDa cleavage site, all ADAMs tested were kinetically competent in proteolysis of the 33kDa CD23 peptide.

Mass spectral analysis of the reaction products showed that the site at which ADAM9 cleaved the precursor TNF-alpha based Val and HomoPhe subs was identical to the ADAM10 cleavage sites: between Ala-Val or Ala-Homophenyl (Table **3**). The CD23 substrate was processed between Gln-Gln for all the ADAM family members. ADAM10 and 17 cleaved the CD23 and TGF-alpha peptides at the reported physiological site: between Arg*Leu and Ala*Val respectively. The BTC sub was also processed at

Table 2. Specificity Values, k_p/E_t ($M^{-1}s^{-1}$), of Substrates Tested Against ADAMs and MMPs at 10μM Substrate Concentration[a]

	Val sub	HomoPhe sub[b]	CD23 sub (33kDa)	BTC sub	TGF sub
ADAM8	3.3 ± 0.6 x 10^4	1.0 x 10^5	1.2 ± 0.3 x 10^4	6.8 ± 1.2 x 10^3	1.0 ± 0.3 x 10^4
ADAM9	1.1 ± 0.6 x 10^5	8.3 ± 0.7 x 10^3	1.6 ± 0.2 x 10^3	2.1 ± 0.6 x 10^3	1.8 ± 0.5 x 10^4
ADAM10	3.6 ± 0.5 x 10^3	6.2 x 10^3	5.8 ± 1.2 x 10^2	6.9 ± 0.6 x 10^1	1.9 ± 0.6 x 10^3
ADAM12	3.8 ± 0.5 x 10^4	2.8 x 10^5	NA[c]	NA[c]	1.0 ± 0.2 x 10^4
ADAM17	9.6 ± 0.3 x 10^5	4.3 x 10^5	4.6 ± 1.1 x 10^4	9.4 ± 1.1 x 10^3	1.1 ± 0.2 x 10^5
MMP1	5.1 x 10^4	2.8 x 10^4	1.9 x 10^3	ND[d]	ND[d]
MMP2	1.7 x 10^5	3.2 x 10^5	2.4 x 10^3	ND[d]	6.3 x 10^3
MMP3	3.9 x 10^4	4.0 x 10^3	2.4 x 10^3	ND[d]	2.4 x 10^3
MMP8	2.6 x 10^4	1.4 x 10^5	7.7 x 10^3	1.2 x 10^3	4.8 x 10^3
MMP9	6.0 x 10^5	2.2 x 10^5	1.0 x 10^5	ND[d]	ND[d]
MMP13	1.7 x 10^6	4.6 x 10^5	2.2 x 10^4	ND[d]	ND[d]

[a]The rate constant, k_p, is described under Materials and Methods. Standard error was calculated from values obtained for at least two separate experiments for k_p. Active enzyme concentration, E_t, was measured for each experiment. Error propagation was used in the analysis to obtain k_p/E_t. Errors for MMP values are less than 30%.
[b]Except for ADAM9, values are reported in Moss and Rasmussen [32].
[c]NA, not attempted.
[d]ND, no cleavage detected.

Table 3. Cleavage Sites in Peptides by ADAM Family Members[a]

	Val TNF sub	HomoPhe sub	CD23 sub (33kDa)	BTC sub	TGF sub
ADAM8	Ala*Val	**Ala*Homophe** Ala*Gln	Gln*Gln	Phe*Tyr	Ala*Ala
ADAM9	Ala*Val	Ala*Homophe	**Gln*Gln** Glu*Gln Gln*Arg	NA[b]	Ala*Ala
ADAM10	Ala*Val	Ala*Homophe	**Gln*Gln** Glu*Gln Arg*Leu	Phe*Tyr	Ala*Val
ADAM17	Ala*Val	**Ala*Gln** Arg*Ser	**Arg*Leu** Arg*Ala Gln*Gln Lys*Ser	NA[b]	Ala*Val

[a]Cleavage sites were determine by Mass spectral analysis as described in the Materials and Methods section. Sequences in bold represent the major cleavage sites for the substrates listed.
[b]NA, not attempted.

the physiological site by ADAM8 and 10: Phe*Tyr. That ADAM9 processed substrates at the same site as the other physiological ADAM family members suggests that ADAM9 could act as a convertase *in vivo*, especially under conditions where the expression of these proteinases is dysregulated, as in cancer and inflammatory diseases.

Inhibition of ADAM Family Members by Marimastat, TMI-1, Calbiochem MMP9/13, GI254023 and GM6001

Marimastat was previously described as an inhibitor of ADAM9 with an inhibition constant of 270 nM [1]. Since we were using a different enzyme source, we decided to test the broad-spectrum inhibitor against R&D Systems' enzyme to confirm its inhibition constant and to provide confidence that the enzyme was functionally pure. If the two enzyme preparations were not similar, we reasoned that we would not get the same inhibition constant against Marimastat. Similar to the COS-7 cell derived ADAM9, R&D Systems' recombinant human enzyme exhibited an IC_{50} of 430 ± 130 nM. This value is also comparable to what was determined previously by Incyte [33]. IC_{50} values were also obtained for TMI-1 [34], a Calbiochem MMP9/13 inhibitor, GI254023 [10] and GM6001 [35, 36]. The results are shown in Table **4**.

Table 4. IC_{50} (nM) Values for Inhibitors of ADAM9[a]

Inhibitor	IC_{50}
Marimastat	430 ± 130
TMI-1	2.1 ± 0.3
MMP9/13 inhibitor	11 ± 3.0
GI254023	280 ± 110
GM6001	66% at 1000

[a]IC_{50} values were determined as described in the Materials and Methods section.

The most efficient inhibitor was TMI-1 with an IC_{50} of 2.1 ± 0.3 nM. The Calbiochem MMP9/13 inhibitor was less effective, with an IC_{50} of 11 ± 3.0 nM. GI254023, previously identified as a selective inhibitor of ADAM10 [10] was also inhibitory against ADAM9 with an IC_{50} of 280 ± 110 nM. The GM6001 was the least potent of all, with an IC_{50} greater than 500 nM.

CONCLUSION

A substrate useful for high throughput screening of ADAM8, 10, 12, and 17 was recently described [32]. This substrate was based on the precursor TNF-alpha cleavage site, but had homophenylalanyl in place of valine. In this report, this substrate was tested against ADAM9 for activity, and its turnover number was $20 \pm 10 \times 10^{-2}$ min^{-1}. However, the best substrate to date for ADAM9, is also a modified version of the precursor TNF-alpha substrate. The homophenylalanyl is replaced with the wild type valine residue, and the sequence is longer in length. This substrate gives a turnover number of $100 \pm 20 \times 10^{-2}$ min^{-1}.

A plot of initial velocity *vs* substrate concentration demonstrated that all substrates tested exhibit cooperativity. Mechanistically, this could mean that there are multiple binding sites for the substrate that may or may not have differing K_s values. A Hill plot was used to calculate K_s, so only one value for the dissociation constant is obtained.

The new valine-containing substrate was used to determine IC_{50} values of several popular inhibitors. The most potent of all was TMI-1, a non-specific ADAM and matrix metalloproteinase inhibitor [34]. Because a sensitive substrate was used, we were able to keep the ADAM9 concentration down to 0.01-0.2 nM in a typical assay. This is important since the enzyme concentration should be lower than the inhibitor concentration for an accurate determination. In this case, the enzyme concentration of 0.01-0.2 nM was well below the most potent IC_{50} of 2.1 nM for TMI-1. The increase in the specificity constant and sensitivity of the Dabcyl/Fluorescein pair make the substrate, Dab-Ser-Pro-Leu-Ala-Gln-Ala-Val-Arg-Ser-Ser-Lys(Fam)-NH_2 well suited for screening of inhibitors for ADAM9.

GI254023, previously reported as a selective inhibitor of ADAM10, also appears to inhibit ADAM9 in our assays.

GI254023 was used to identify ADAM10 as a CD23 shedding enzyme in human peripheral B cells [7]. While ADAM9 is unlikely to be a major CD23 convertase, contributions from this enzyme may exist. Caution should be used in the interpretation of experiments using GI254023 treatment for ADAM10 inhibition because it is not completely selective.

Since GI254023 was an inhibitor of ADAM9, we tested a CD23 substrate of the known cleavage site for the 33kDa form. We had found earlier, that both ADAM8 and ADAM10 could process the 37kDa CD23 peptide at the physiological processing site and that against ADAM9, it was ineffective. In this report, ADAM9 proteolyzed the 33kDa based substrate more efficiently and at the same Gln*Gln site as ADAM10. In addition, ADAM8 also generates the same product as ADAM9 and 10, but with the best specificity value. This data shows that like ADAM10, both ADAM9 and 8 are kinetically competent to process peptides based on CD23.

The TGF peptide may also be used for inhibitor screening and it is slightly more selective against MMPs compared to the two TNF subs. On the other hand, the MMPs don't efficiently process the BTC sub. However, the specificity values may be too low for the peptide to be useful. Increasing the substrate concentration may be an effective way to change the specificity value, as the plots of velocity *vs* substrate concentration are sigmoidal with ADAM9 whereas plots of Dabcyl substrates with MMPs are typically hyperbolic (data not shown).

In addition to using these substrates in high throughput inhibitor screening, the sequence of the peptides could also have potential with uses for *in vivo* imaging and assaying ADAMs in cell based assays. By using multiple substrates that target different metalloproteinase family members, one should be able to quantify the contribution from each enzyme [37]. This is especially important since soluble forms of ADAMs have been reported. For example, ADAM12 has been found in urine from cancer patients [38, 39] and soluble forms of ADAM8 [40], ADAM9 [41] and ADAM10 [42] also exist. Investigating the use of these substrates to measure activities in cell based assays and biological fluids are currently underway.

ABBREVIATIONS

ADAM = A disintegrin and metalloproteinase

BTC = Betacellulin

Cha = Cyclohexylalanyl

COS-7 = African green monkey kidney fibroblast-like cells transformed with SV40 T antigen

Dabcyl = 4-(4-dimethylaminophenylazo)benzoyl

DMSO = Dimethyl sulfoxide

Dnp = 2,4 Dinitrophenyl

E_t = Active enzyme concentration

Fam = 5-Carboxyfluorescein

FRET = Fluorescence resonance energy transfer

HomoPhe = Homophenylalanyl

I = Inhibitor concentration

IC_{50} = Inhibitor concentration at which there is 50% inhibition of enzyme activity

I_f = Fractional inhibition

k_p/E_t = Specificity value at 10μM substrate concentration

K_m = Michaelis–Menten constant

K_s = Dissociation constant

Mca = 7-Amido-4-methylcoumarin

n = Hill coefficient

Nma = N-methyl anthranilate

S = Substrate concentration

TACE = Tumor necrosis factor-alpha converting enzyme

TGF = Transforming growth factor-alpha

TNF-alpha = Tumor necrosis factor-alpha

v = Initial velocity

V_{max} = Maximum velocity

V_{max}/E_t = Turnover number.

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