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Characterization of Mca-Lys-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH_2, a fluorogenic substrate with increased specificity constants for collagenases and tumor necrosis factor converting enzyme

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Abstract

Matrix metalloproteinases (MMPs) and the related tumor necrosis factor converting enzyme (TACE) are involved in tissue remodeling, cell migration, and processing of signaling molecules, such as cytokines and adhesion molecules. Fluorescence-quenched peptide substrates have been widely used to quantitate the actual enzymatic activity of MMPs. However, the various MMPs have very different specific activities toward these substrates. This restricts their value for the determination of composite proteolytic activity of mixtures of metalloproteinases in biological fluids. The N-terminal elongation of the most widely used MMP substrate (FS-1) with a Lys to the sequence Mca-**Lys**-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH_2 (FS-6) yields a fluorogenic peptide with improved substrate properties. As compared to FS-1, the specificity constant (k_{cat}/K_m) of FS-6 for collagenases (MMP-1, MMP-8, MMP-13) and MT1-MMP (MMP-14) is increased two- to ninefold and threefold, respectively, while those for gelatinases and matrilysin remain equally high. Using high-performance liquid chromatography-fluorescence detection, MMP activity can be quantitated in the picomolar range. FS-6 shows up to twofold higher specificity constants (k_{cat}/K_m of $0.8 \times 10^6\,M^{-1}\,s^{-1}$) for TACE, as compared to standard substrates Mca-PLAQAV-Dpa-RSSSAR-NH_2 and Dabcyl-LAQAVRSSSAR-EDANS. FS-6 is fully water soluble and thus allows measurement of metalloproteinase activity in tissue culture conditions, e.g., on the surface of viable cells in situ.

Keywords: Fluorogenic enzyme substrate; MMP; TACE; Kinetics

Matrix metalloproteinases (MMPs)[1] are Zn-containing, neutral endoproteinases that degrade extracellular matrix molecules such as collagen, aggrecan, laminin, and others in physiological processes, like organogenesis and wound repair [1,2]. The activity of MMPs is tightly regulated by endogenous inhibitors, but pathogenic conditions, e.g., tumor metastasis and multiple sclerosis, have been associated with excess MMP activity [3,4]. Fluorescence-quenched peptide substrates are widely used for the quantification of enzymatic activity of various endoproteinases, in particular for the measurement of MMPs [5–7]. Since its introduction, the fluorescence-quenched MMP substrate Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH_2 (FS-1) [8] was extensively used for monitoring MMP activity in biological samples, including crude cell culture supernatants [9], and for screening of MMP inhibitors. Because of its high k_{cat}/K_m value, this peptide is very useful for the measurement of enzymatic activity of gelatinases (MMP-2, MMP-9) and, to a lesser extent,

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[1] *Abbreviations used:* MMP, matrix metalloproteinase; Mca, (7-methoxycoumarin-4-yl)acetyl; Dpa, *N*-3-(2,4-dinitrophenyl)-L-2,3-diamino propionyl; FS-1, Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH_2; FS-6, Mca-Lys-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH_2; TACE, tumor necrosis factor converting enzyme; ADAM, a disintegrin and metalloprotease; APMA, 4-aminophenylmercuric acetate; AEBSF, 4-(2-aminoethyl)-benzenesulfonylfluoride, hydrochloride; TFA, trifluoroacetic acid; RFU, relative fluorescence unit; DMSO, dimethyl sulfoxide; DMEM, Dulbecco's modified Eagle's medium; FCS, Fetal calf serum.

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of collagenase-3 (MMP-13), but it is a poor substrate for other metalloproteinases such as collagenase-1 (MMP-1), matrilysin (MMP-7), or collagenase-2 (MMP-8) [8,10,11]. While zymography with gelatin, casein, or other proteins offers a sensitive alternative measurement system for some of these enzymes, the high detection threshold for collagenase-1 and -2 has made their quantification difficult with this method [13]. Moreover, the solubilization of many oligopeptide substrates like FS-1 requires organic solvents, such as DMSO, that could interfere with the biological properties of assay samples and inhibit the enzymatic activity of metalloproteinases [12].

We hypothesized that in FS-1 steric hindrance by the relatively large Mca molecule may decrease substrate affinity for some MMPs and hence that increasing the distance between fluorophore and scissile bond will improve substrate properties. Here we describe that N-terminal elongation of the recognition sequence by a P4 lysyl residue (Mca-Lys-Pro-Gly-Leu-…) creates a novel substrate (FS-6) with markedly improved kinetic properties for the hydrolysis by MMPs, specifically for collagenase-1 and -2. We further demonstrate that FS-6 is an excellent substrate for TACE (ADAM-17), a Zn-metalloproteinase that is structurally and functionally closely related to MMPs [14]. FS-6 was used to investigate the contribution of cell-associated metalloproteinase activity, as compared to that in supernatants, of cultured glioma cells.

Materials and methods

Fluorescent peptide substrates

FS-6 (Mca-Lys-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH_2) was custom synthesized by Bachem AG (Bubendorf, Switzerland). Analytical data (amino acid analysis, mass spectroscopy) were in agreement with the proposed structure, the purity (HPLC) was $>96\%$. FS-1 (Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH_2; Cat. No. M-1895) and the TACE substrates Mca-Pro-Leu-Gln-Ala-Val-Dpa-Arg-Ser-Ser-Ser-Arg-NH_2 (Cat. No. M-2255) and Dabcyl-Leu-Gln-Ala-Val-Dpa-Arg-Ser-Ser-Ser-Arg-EDANS (Cat No. M-2155) were purchased from Bachem. Stock solutions were made in water (FS-6) or DMSO (all other substrates) at 20 mM. Concentrations of stocks were determined on a Cary 3 Bio photometer (Varian, Palo Alto, CA) by measuring the absorbance at 410 nm, using an absorbance coefficient of $7500\ M^{-1}\,cm^{-1}$.

Enzymes and enzyme inhibitors

Recombinant human MMP-1, MMP-3, MMP-9, MMP-13, and TACE were prepared in-house using the baculovirus expression system. MMP-2 was purchased from Roche Applied Science (Rotkreuz, Switzerland), and MMP-7 and MMP-8 was from Chemicon Intl. (Temecula, CA). The pro-MMPs were activated with APMA (1 mM, 37 °C, 30 min). MMP-14 (recombinant catalytic domain) was a gift from Professor H. Tschesche, University of Bielefeld, Germany. ADAM-10 was from R&D Systems Inc. (Abingdon, UK). Concentrations of active enzyme were determined using the published values for k_{cat}/K_m with FS-1 [8,10,11] or by active-site titration with a peptide–hydroxamate inhibitor. Inhibitor concentrations below and above the enzyme concentrations were used, and enzyme concentration was adjusted well above the dissociation constant, K_i. Enzyme and inhibitor were incubated for 1 h, FS-6 (final concentration 5 μM) was added, and residual enzyme activity, v, was measured. The enzyme concentration, E_o, was obtained by fitting the {inhibitor concentration; residual activity} data pairs to the tight-binding equation:

$$v = (v_o/2E_o)\{[(I_o + K_i - E_o)^2 + 4E_oK_i]^{0.5} - (K_i + I - E_o)\}.$$

Kinetic measurements were done with a PerkinElmer LS50B luminescence photometer (Rotkreuz, Switzerland) equipped with temperature-controlled cuvette holder or on a Spectramax Gemini microplate reader (Molecular Devices, Sunnyvale, CA).

The broad spectrum MMP inhibitor GM6001 [15] was a gift of Richard Galardy. The serine-protease inhibitor 4-(2-aminoethyl)-benzenesulfonylfluoride, hydrochloride (AEBSF, Pefabloc) was from Roche Applied Science. The TACE inhibitor PKF242-484 ((2*S*,3*R*-*N**4*-(*S*)-2,2-dimethyl-1-methylcarbamoyl-propyl)-*N**1*-hydroxy-2-hydroxymethyl-3-(4-methoxy-phenyl)-succinamide) [16] was produced in-house.

Enzyme assays

MMP assays were performed in 0.1 M Tris, 0.1 M NaCl, 10 mM $CaCl_2$, 0.05% (w/v) Brij35, 0.1% (w/v) PEG6000, pH 7.5. TACE and ADAM-10 were assayed in 0.01 M Hepes, pH 7.5. The final concentration of DMSO in the assay solution for FS-1 was <0.075%. First-order rate constants, k_{obs}, were determined from the progress curves of hydrolysis of 0.5 μM substrate by fitting the {fluorescence; time} data points to the equation

$$\text{fluorescence}(t) = \text{fluorescence}_{max}(1 - \exp(-k_{obs}t))$$

and used together with the active enzyme concentration (E_o) to calculate the second-order rate constants according to $k_{cat}/K_m = k_{obs}/[E_o]$.

Correction for inner-filter effects was done using a recently described method [17]. The fluorescence of various concentrations (0–25 μM) of intact FS-6 was measured alone and in presence of fixed amounts of hydrolyzed substrate (0.5 μM). The subtraction of the

resulting two curves allowed calculation of the net fluorescence of 0.5 μM split product.

HPLC measurements were done on a Thermo Separation Products system (ThermoQuest, San Jose, CA), consisting of P2000 pump, A3000 autoinjector, and UV-1000 and Fl-3000 detectors running under the PC-1000 software, using a Vydac C18 50 × 2.1-mm column. Eluent A was water/0.1% (v/v) TFA and eluent B was water/acetonitrile/TFA, 30/70/0.085% (v/v/v). A gradient from 20 to 55% of eluent B in 20 min at a flow rate of 0.2 ml/min was used. Detection wavelengths were 324 nm (excitation) and 400 nm (emission).

Cell culture

The human glioblastoma cell line T98G (American Type Culture Collection, Rockville, MD) was maintained in DMEM (Gibco, Invitrogen AG, Basel, Switzerland) supplemented with 10% heat-inactivated fetal calf serum (Gibco), 2 mM *N*-acetyl-L-alanyl-L-glutamine (Biochrom, Berlin, Germany), 1 mM sodium pyruvate (ICN Biomedicals, Birsfelden, Switzerland), and 20 μg/ml gentamycin (Gibco) at 37 °C in a humidified atmosphere containing 5% CO_2. For assays, 1×10^4 glioma cells were cultured in 100 μl phenol-red-free medium (DMEM/F12; Gibco) with the same additives as above with the exception that FCS had been replaced by 1% Nutridoma HU (Roche Applied Science), for 2 days to reach confluency in 96-well F-plates (BD Biosciences, Basel, Switzerland). After washing once with phosphate-buffered saline, 100 μl medium containing the MMP inhibitor GM6001 (final conc. 10 μM; Calbiochem, JURO Supply AG, Lucerne, Switzerland), the serine protease inhibitor AEBSF (final conc. 1 mM) (Roche Applied Science) or solvent control (medium containing 0.1% DMSO) was added and kept for 4 h at 37 °C. Supernatants were transferred to black 96-well plates (Corning, INTEGRA Biosciences, Wallisellen, Switzerland) and 10 μl of substrate FS-6 (final conc. 5 μM) was added to each well. After incubation for 30, 60, 120, and 240 min at 37 °C, reactions were stopped with 90 μl of 3% (w/v) sodium acetate, pH 4.0, and fluorescence of the samples (200 μl) was measured in a microplate fluorimeter as described above. To measure cell membrane-associated MMP activity, FS-6 was dissolved in fresh medium and added directly to cell cultures. For measurement of MMP activity samples of conditioned media (200 μl) were removed from tissue culture plates and, after addition of 90 μl of 3% (w/v) sodium acetate, transferred into 96-well black plates to measure the fluorescence. Medium without cells in the presence or absence of GM6001 or AEBSF were incubated for background correction. Each experiment was performed in triplicate and the mean value was subtracted by the background fluorescence at each time point.

Results and discussion

Peptide substrates for MMPs mimic the sequence around the cleavage sites of native substrates, in particular collagen. The substrate Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH_2 [8] spans the cleavage site from the P3 to the P4′ subsite of collagens (the scissile bond is Gly-Leu, nomenclature according to Schechter and Berger [18]). It is known for many endoproteinases that the length of the peptide substrate in both the P and the P′ directions from the scissile bond determines the efficiency of the hydrolytic step. Additional enzyme–substrate interactions at the extended subsites can increase k_{cat} and/or decrease K_m values [19]. Moreover, steric hindrance between the fluorophore and the proline ring in position P3 of FS-1 could decrease fluorescence yield [7].

Solubility

The introduction of an additional Lys at the N terminus of FS-1 makes the resulting substrate FS-6 water soluble. Stock solutions were routinely prepared at 20 mM in water and stored at −20 °C; precipitation was never observed. Although the final concentration of DMSO (0.075–0.0025% v/v) in the assay solution for FS-1 might not interfere with its performance [12], the use of a water-soluble substrate is obviously more convenient and excludes the possibility of unknown nonphysiologic effects due to organic solvents, specifically for measurements of enzymatic activity on the surface of living cells (see below).

Spectral properties and quenching efficiency

The spectral properties of FS-6 ($excitation_{max}$ 325 nm, $emission_{max}$ 400 nm) are almost identical to those of FS-1 ($excitation_{max}$ 328 nm; $emission_{max}$ 393 nm) [8]. The quenching efficiency was calculated by comparing the fluorescence of solutions (0.1 μM) of intact and completely cleaved substrate obtained by incubation with 0.1 μM MMP-9 for 24 h. Upon complete cleavage, the fluorescence of FS-6 increased 62.5-fold, corresponding to a quenching efficiency of 98.4%. This is slightly lower than the value reported for FS-1 (99.5%) [8], probably due to the increased distance between fluorophore and quencher [20] or to the slight differences in excitation/emission wavelength settings.

Kinetic data of hydrolysis of FS-6 with MMPs

Two sets of kinetic data were determined for the novel peptide substrate. The second-order rate constant, k_{cat}/K_m, was used to compare the specificity of different substrates for a given enzymatic reaction for FS-1 and FS-6 under identical conditions. To determine the concentration of active enzyme of MMP-2, -7, -8, -9, and

-13, the known values for k_{cat}/K_m for FS-1 under the conditions were used as reference [8,10,11]. Concentrations of active MMP-1 and MMP-14 were determined by active-site titration using the tight-binding, reversible hydroxamate inhibitor PKF242-484 [16]. Progress curves for complete enzymatic hydrolysis of substrates FS-1 and FS-6 were run side-by-side using identical stocks for enzymes and substrate under pseudo first-order conditions ($[S_o] \ll K_m$). In this direct comparison, the novel substrate FS-6 showed considerably increased hydrolysis rates for MMP-1 (Fig. 1), MMP-8, and MMP-14 (not shown), compared to the shorter FS-1. The cleavage at the scissile bond Gly-Leu was confirmed by HPLC/MS which showed the generation of the hydrolysis products Mca-Lys-Pro-Leu-Gly-OH and Leu-Dap-Ala-Arg-NH_2 (data not shown).

Table 1 shows that at 25 °C, k_{cat}/K_m values for MMP-1, -8, -13, and -14 were two- to ninefold higher with FS-6, whereas those for MMP-2 and -9 were similar for both substrates. Moreover, the first-order rate constants for MMP-3 and -7, measured at 25 °C, were equal or considerably higher with FS-6, respectively, as compared to reference values using FS-1 at 37 °C. For all MMPs shown in Table 1, the specificity constants increased uniformly by a factor of about two, when the hydrolysis was done at 37 °C (data not shown). The initial turnover rates for all MMPs tested were sufficiently high to allow the use of enzyme at a concentration of 0.1 nM in 96-well plate inhibitor screening assays. FS-6 was slowly hydrolyzed by high concentrations (> 10 μg/ml) of human leukocyte elastase, but was resistant to cleavage by trypsin at concentrations up to 50 μg/ml (data not shown). The mechanistic origin of the increased specificity constants of FS-6 with some of the MMPs is currently unknown. Systematic investigations on the influence of substrate length and variation of amino acids beyond P3 have not been done. However, it was noted in a study on substrate specificity of MMP-13 that an elongated peptide substrate (with Gly in P4) has a higher specificity constant, compared to known fluorescence-quenched substrates [21].

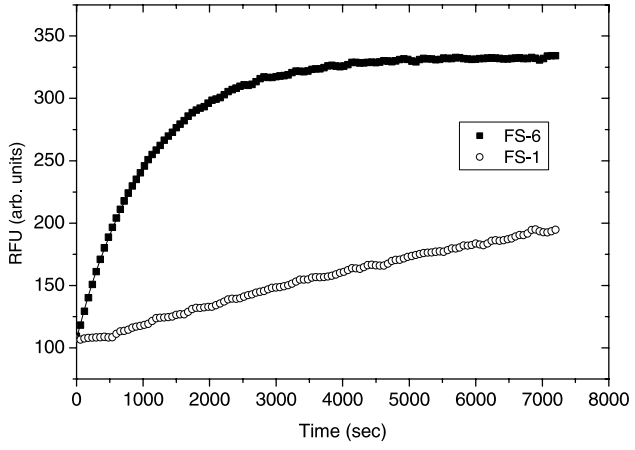

Fig. 1. Hydrolysis of FS-1 (○) and FS-6 (■) by MMP-1. ($[E_0] = 10$ nM in 0.1 M Tris, pH 7.5, at 25 °C, (S_o) = 0.94 μM. First-order rate constants, k_{obs}, were obtained by fitting to the equation(product) = limit$(1 - \exp(-k_{obs}t))$ +offset, second-order rate constants, k_{cat}/K_m, were calculated from $k_{obs}/[E_o]$.

Table 1
Second-order rate constants for the hydrolysis of substrates Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH_2 (FS-1) and Mca-Lys-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH_2 (FS-6) by MMPs at 25 °C, measured in 0.1 M Tris, pH 7.5

Enzyme	Second-order rate constant k_{cat}/K_m (M^{-1} s^{-1})	
	FS-1	FS-6
MMP-1	10,300	92,200
MMP-2	629,000[a,*]	687,000
MMP-3	23,000[b]	20,400 (48,500)[e]
MMP-7	nd[x]	295,000
MMP-8	135,000[c]	418,000
MMP-9	895,000[d]	623,000
MMP-13	757,000[c]	1,135,000
MMP-14	393,000	1,253,000

[*] k_{cat}/K_m values from references ([a][8], [b][8] (at 37 °C), [c][10], [d][11]) were used to determine the concentrations of enzymes in assays and to extrapolate for k_{cat}/K_m values for FS-6.
[e] At pH 6.0.
[x] Not done; reported value at 37 °C is 169,000 M^{-1} s^{-1} [8].

To further illustrate the improved substrate properties of FS-6, the initial rates for the hydrolysis of FS-1 and FS-6 (5 μM, respectively) by two MMPs (1 nM), with low (MMP-1) and high (MMP-14) second-order constant were measured side-by-side (Fig. 2). Hydrolysis of FS-6 (35.9 RFU/min) by MMP-1 was nine times more efficient than that of FS-1 (4.0 RFU/min). Similarly, for MMP-14 the initial rate increased by a factor of three with FS-6 (302 RFU/min) compared to FS-1 (113 RFU/min).

Knowledge about the Michaelis–Menten constant (K_m) of a substrate is important when true binding affinities of ligands to an enzyme are to be calculated. We therefore determined the values for k_{cat} and K_m for the hydrolysis of FS-6 by MMPs (Table 2). For MMP-13 and MMP-14, K_m values are in the range of 5–10 μM. In contrast, K_m values for MMP-1, MMP-2, and MMP-9 are considerably higher, and substrate saturation was not achieved. However, a K_m value could be calculated for MMP-1 with the use of HPLC detection (Fig. 3, see below).

Inner-filter effect correction

Inner-filter effects typically occur when using fluorescence-quenched substrates. They originate from the overlap of the absorption spectra of fluorophore and quencher and reduce the practically useful substrate concentration range. A method to determine correction factors for the inner-filter effect at different substrate concentrations was recently described [17] and was applied here for the determination of Michaelis–Menten

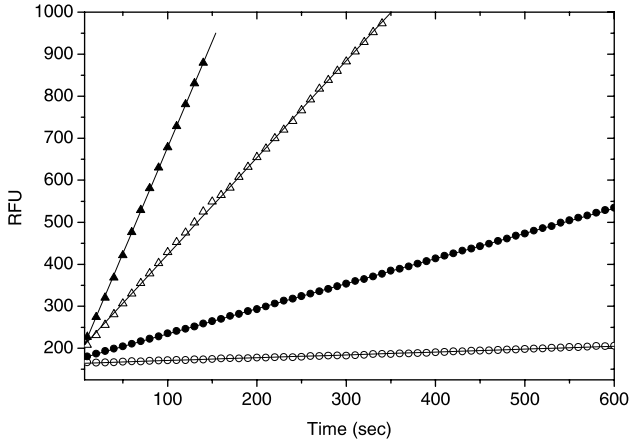

Fig. 2. Initial rates for hydrolysis of FS-1 (open symbols) and FS-6 (closed symbols) (5 μM, respectively) by MMP-1 (●, ○) and MMP-14 (▲, △) (1 nM, respectively) in 0.1 M Tris, pH 7.5, and 25 °C.

Table 2
Michaelis–Menten parameters for hydrolysis of FS-6 by MMPs (measured in 0.1 M Tris buffer, pH 7.5, 25 °C, initial rates corrected for inner-filter effects)

Enzyme	k_{cat} (s^{-1})	K_m (μM)
MMP-1	2.5	27.5[a]
MMP-2	n.d.	>30
MMP-9	n.d.	>30
MMP-13	5.9	5.2
MMP-14	9.9	7.9

[a] Done by HPLC.

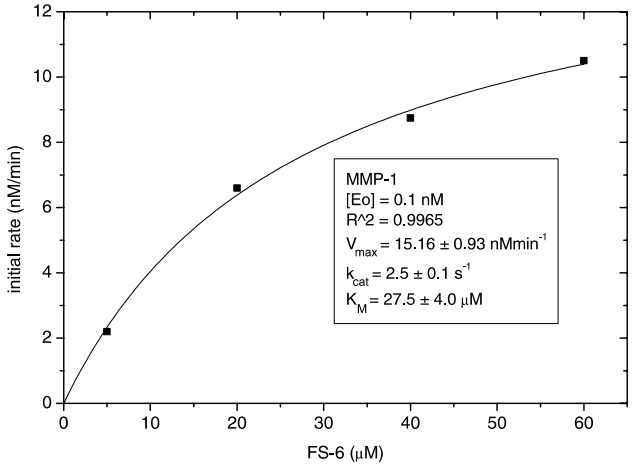

Fig. 3. Determination of kinetic parameters k_{cat} and K_m for the hydrolysis of FS-6 by MMP-1 in 0.1 M Tris, pH 7.5, at 25 °C. MMP-1 ($[E_o] = 0.1$ nM) was incubated with FS-6 (5–60 μM) and product concentration after 30 min was determined by HPLC. Data were fitted to the Michaelis–Menten equation.

constants. Product fluorescence was quenched 10% at a substrate concentration of 5 μM and 40% in presence of 25 μM FS-6. Respective correction factors for each substrate concentration were used to compensate these errors in the calculation of initial rates for the determination of K_m and k_{cat} values of MMP-13 and -14 (see above, Table 2).

Based on current data, a substrate concentration of 5 μM in routine MMP assays was found to be most economical. Higher substrate concentrations may increase the initial turnover rates due to better saturation of the enzyme. However, for cuvette- or microplate-based assays the overall sensitivity deteriorates as inner-filter effects increase exponentially.

HPLC assay of MMP-1 activity

The interference of inner-filter effects of quenched fluorogenic substrates can be circumvented by the use of HPLC-based fluorescence detection. Here, the separation of fluorogenic split product from the quencher-containing fragment and from intact substrate allows the determination of the net fluorescence produced by enzymatic cleavage. This would allow detection of MMP activity with increased sensitivity. As MMP-1 has the lowest specificity constant of all enzymes tested and hence the lowest sensitivity with either FS-1 or FS-6, we chose to test for the detection threshold of an HPLC-based assay with MMP-1. Fig. 4 shows that the rates of product formation were 3.1 and 0.63 nM/min (5 μM FS-6, 37 °C) with 0.1 and 0.02 nM MMP-1, respectively, resulting in sharp product peaks in the chromatogram after 1-h incubation. Increasing the concentration of FS-6 to 20 μM gave a rate of product formation of 1.54 nM/min with 0.02 nM MMP-1. With HPLC/fluorescence, the detection of MMP-1 activity with FS-6 is two orders of magnitude more sensitive than that reported for a EDANS-DABCYL fluorescence-quenched substrate [22]. The increase in sensitivity results from both the improvement of the turnover number upon N-terminal elongation and the use of the Mca fluorophore, which

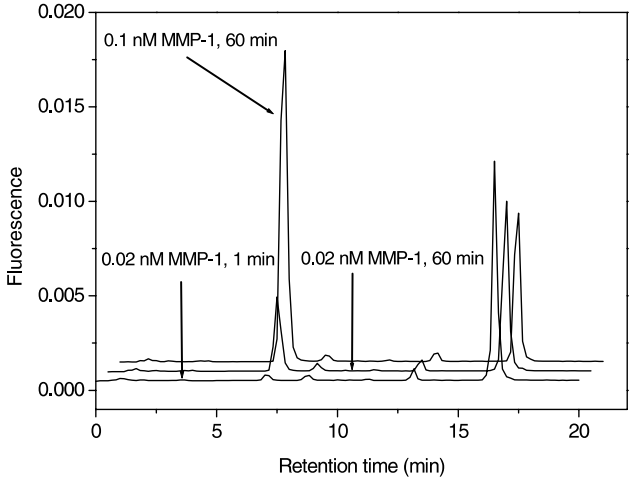

Fig. 4. Hydrolysis of FS-6 (5 μM) by MMP-1 using HPLC/fluorescence detection. Bottom trace, 0.02 nM MMP-1 (1 min incubation); middle trace, 0.02 nM MMP-1 (60 min incubation); top trace, 0.1 nM MMP-1 (60 min incubation). Peaks at 7 min, Mca-Lys-Pro-Leu-Gly, peak at 16.7 min, intact FS-6 substrate.

has a higher molar absorption and quantum yield, than the dansyl group [23].

FS-6 as a substrate for TACE and ADAM-10

The biological functions of MMPs and the metalloproteinases TACE (ADAM-17) and ADAM-10 are closely interconnected [14]. We therefore investigated their ability to cleave FS-6 as compared to proTNF-derived substrates, which are commonly used to determine their enzymatic activity. The active concentration of TACE was determined by titration with the synthetic inhibitor PKF242-484 [16] (Fig. 5). The MMP-substrate FS-1 was almost completely resistant to hydrolysis by TACE. In contrast, FS-6 was a particularly good TACE substrate. Its k_{cat}/K_m value was 66 and 140% higher than those of the standard TACE substrates Dabcyl-LAQAVRSSSAR-EDANS (M-2155) and Mca-PLAQAV-Dpa-RSSSAR-NH_2 (M-2255), respectively (Fig. 6). The latter two substrates base on the amino acid sequence in proTNF-α around the TACE cleavage site (Pro-Leu-Ala-Gln-Ala-Val, scissile bond is Ala-Val), but have different fluorophore–quencher pairs (Table 3). Determination of Michaelis–Menten parameters using HPLC gave $K_m = 26.3\,\mu M$ and $k_{cat} = 23\,s^{-1}$, the resulting k_{cat}/K_m was in good agreement with the one obtained from the progress curve under pseudo first-order conditions. Several data for the hydrolysis of peptide substrates by TACE have been published. Peptide sequences originated from the amino acid sequences around the known TACE cleavage sites of various proteins, and the best second-order rate constant of $170{,}000\,M^{-1}\,s^{-1}$ was obtained for a 12-mer peptide derived from proTNF-α [24]. In another study, aminobenzoyl-Leu-Ala-Gln-Ala-Val-Arg-Ser-Ser-Ser-Arg-Dpa was found to be cleaved by TACE with a $k_{cat} = 21.6\,s^{-1}$, but displayed non-

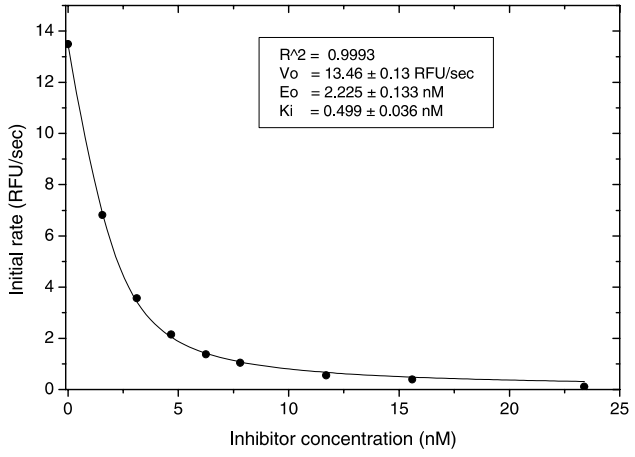

Fig. 5. Active-site titration of recombinant TACE using FS-6 and the synthetic inhibitor PKF242-484. Data were fit to the equation $v = (v_o/2E_o)\{1 - [(E_o + I_o + K_i) - (E_o + I_o + K_i)^2 - (4E_oI_o)^{0.5}\}$ and the solid line was drawn using the best-fit parameters.

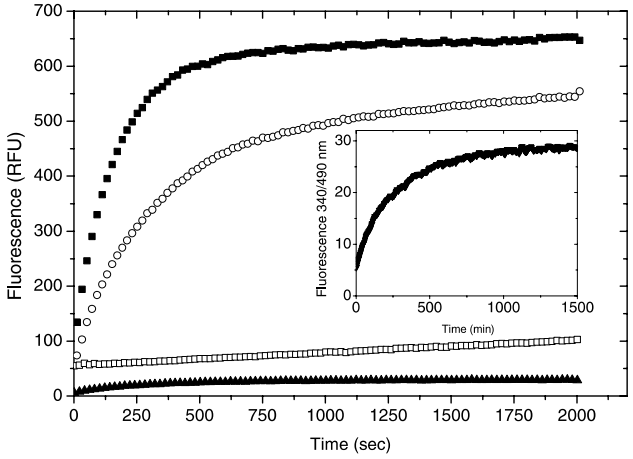

Fig. 6. First-order hydrolysis of different synthetic substrates (all at 0.5 μM) by recombinant TACE (7.1 nM) in 0.01 M Hepes, pH 7.5, at 25 °C. Mca/Dpa-substrates FS-1(□), FS-6 (■), and M-2255 (○) were measured using 325/400 nm and the DABCYL/EDANS substrate M-2155 (▲) was measured at 340/490 nm for excitation and emission wavelength, respectively. The insert is a magnification of the M-2155 curve.

Table 3
Second-order rate constants of hydrolysis of peptide substrates in 0.01 M Hepes (pH 7.5 at 25 °C) by TACE

Substrate	k_{cat}/K_m ($M^{-1}\,s^{-1}$)
Mca-KPLGL-Dpa-AR-NH_2 (FS-6)	778,400
Mca-PLAQAV-Dpa-RSSSAR-NH_2 (M-2255)	363,800
Dabcyl-LAQAVRSSSAR-EDANS (M-2155)	466,300
Mca-PLGL-Dpa-AR-NH_2 (FS-1, M-1895)	nd[a]

[a] Not determined, value too low for calculation, see Fig. 6.

Michaelis–Menten behavior [25]. The data presented here indicate that substrate specificities of TACE and MMPs overlap when N-terminally elongated substrates are used. More systematic investigations are needed to determine the preferred length and amino acid sequence in synthetic TACE substrates. In contrast, ADAM-10 hydrolyzed FS-6 only very slowly. Due to the low amounts of enzyme available, the exact concentration of active enzyme could not be determined. Using values of total protein amounts as declared by the supplier, a k_{cat}/K_m of approximately $1100\,M^{-1}\,s^{-1}$ was calculated. This value is close to values published for other fluorescence-quenched substrates of ADAM-10, and it was noted that a peptide substrate based on the proTNF-α sequence was cleaved 100-times faster by TACE, as compared to ADAM-10 [26].

Use of FS-6 for assay of metalloproteinase activity in cell culture systems

The bulk of collagenases and gelatinases remain attached to the cell surface and only a fraction is released into soluble form. Moreover, the six-membrane-type MMPs (MMP-14, -15, -16, -17, -24, and -25) [2,14]

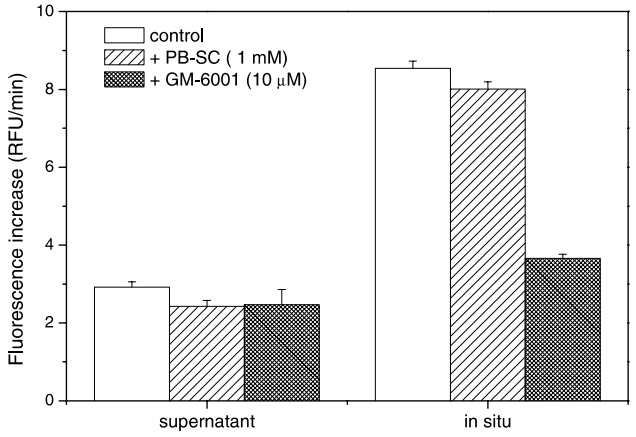

Fig. 7. Hydrolysis of FS-6 by T98G-cells in situ and by cell-free supernatant in presence and absence of metallo- (10 μM GM6001) and serine-protease (1 mM AEBSF) inhibitors were measured in triplicate. FS-6 was added at a final concentration of 5 μM to cell cultures in fresh culture medium and to the cell-free supernatants. Fluorescence increase was measured after 30, 60, 120, and 240 min. Rates of fluorescent increase per time were linear for the first three time points (all $R^2 > 0.90$). Bars represent mean and SD from triplicate experiments after deduction of background values from raw values.

which anchor with a transmembrane domain on the cell surface are not released at all and their enzymatic activity cannot be measured in biological fluids ex vivo or in cell culture supernatants. Hence, there is a need for substrates that can be used for measurements of MMP activity in cell culture conditions. To determine whether FS-6 can be used for this purpose, we compared the substrate hydrolysis on the cell surface (in situ) and in cell-free conditioned media from the glioma cell line T98G. This cell line is known to produce MMP-2 and the endogenous MMP inhibitor TIMP-1 [27]. Proteolysis of FS-6 was measured in supernatants and on the cell surface in the presence of the broad-spectrum MMP inhibitor GM6001 (10 μM) and the serine protease inhibitor AEBSF (1 mM) to determine the specificity of readings. In 4-h supernatants (Fig. 7) substrate hydrolysis was low and slightly decreased by both AEBSF (control, 2.9 RFU/min; with AEBSF, 2.4 RFU/min, −17%) and GM6001 (2.5 RFU/min, −12%). In contrast, cell-associated hydrolysis of FS-6 was three times higher than that observed in supernatants. Here, GM6001 inhibited substrate cleavage by 57% (control, 8.5 RFU/min; with GM6001, 3.7 RFU/min), whereas AEBSF had almost no effect (control, 8.5 RFU/min, with AEBSF, 8.0 RFU/min, −6%). The effect of GM6001 was dose-dependent in the concentration range from 10 nM to 10 μM (data not shown). These data suggest that the bulk amount of measurable metalloproteinase activity is membrane bound and hence operational at the cell–cell or cell–matrix interface. Serine- and metalloprotease activity in the supernatant may be blocked by endogenous inhibitors secreted by the cells (TIMP-1). However, T98G cells release a considerable amount of proteolytic activity into the supernatant that is not inhibited by the synthetic inhibitors used, either because it originates from other enzymes (e.g., cysteine- or aspartate-type proteases) or because GM60001 exerts only partial inhibition to one or more unknown metalloproteinases.

In summary, the new substrate FS-6 is considerably more sensitive than FS-1 for measuring activity of collagenases (MMP-1, -8, -13) and can be used to determine picomolar amounts of MMPs in biological fluids. FS-6 can be considered the most sensitive broad-spectrum MMP substrate presently available. In addition, the substrate can be used for the sensitive detection of TACE activity. The data presented in this study suggest that with FS-6 the detection threshold of MMP activity assays can be lowered and the assay time shortened. This, together with good water solubility, makes FS-6 also an attractive candidate for use in MMP assays involving viable cells. As this substrate, like many others, lacks specificity within the MMP family of metalloproteinases, it is not suitable for the quantification of specific proteases in crude biological samples, i.e., cell culture supernatants or body fluids. Possible solutions are the use of synthetic inhibitors specific for individual enzymes or the use of specific capture antibodies in 96-well based enzyme activity assays marketed by Amersham [28] and R&D Systems. Here, current assay protocols use FS-1 as substrate which results in long incubation times (17–20 h; instruction by manufacturer, R&D Systems) which may be shortened considerably by the use of FS-6.

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