Latent cytokines: development of novel cleavage sites and kinetic analysis of their differential sensitivity to MMP-1 and MMP-3

Sandrine Vessillier, Gill Adams and Yuti Chernajovsky

Bone and Joint Research Unit, William Harvey Research Institute, Barts and the London, Queen Mary’s School of Medicine and Dentistry, University of London, Charterhouse Square, London EC1M 6BQ, UK

1To whom correspondence should be addressed.
E-mail: y.chernajovsky@qmul.ac.uk

We have engineered a latent mouse interferon β (mIFNβ) using the latency associated peptide (LAP) of transforming growth factor β1 (TGF-β1) to protect the cytokine and avoid its interaction with its receptors. This approach improves the pharmacokinetic properties and reduces the pleiotropic effects limiting the current therapeutic use of cytokines. IFNβ was fused to the LAP using two flexible linkers flanking a matrix metalloproteinase (MMP) cleavage site for the specific release of IFNβ at disease sites. In order to improve the hydrolysis rate of the cleavage site, 15 different cleavable linkers were introduced in the LAP–mIFNβ construct. The kinetic parameters relative to the linker cleavage by MMP-1 and MMP-3 were measured by an ELISA method. Among the modifications done, one of the constructs bearing the activation site of pro-MMPs was the best substrate for both enzymes. The introduction of a hydrophilic sequence derived from the furin cleavage site of the anthrax toxin protective antigen increased the sensitivity to MMP-3 to up to 29-fold. These data suggest that this strategy could be useful for improving the effectiveness of the delivery and targeting of protein therapeutics.

Keywords: cytokine targeting/inflammatory disease/kinetic parameters/matrix metalloproteinase/specific activation

Introduction

Cytokines, local mediators of cellular signalling, are actually used as therapeutic drugs, but their clinical use poses some problems owing to their short half-life and pleiotropic effects (Aulitzky et al., 1994; Guterman, 1994). They have to be administered subcutaneously to prevent side-effects associated with systemic administration and frequently at high doses to achieve biologically active concentrations at sites of disease (Atkins et al., 1999; Golab and Zagozdzon, 1999; Margolin, 2000).

In order to overcome the pharmacokinetic and pleiotropic effects which limit the therapeutic application of cytokines, one approach was to engineer a latent cytokine that could be released by enzymatic cleavage specifically at the disease site (Adams et al., 2003). We have demonstrated that interferon (IFN) β can be made ‘latent’ by its fusion with the latency associated peptide (LAP) of transforming growth factor (TGF) β (Wakefield et al., 1990). The active cytokine mIFNβ is fused to the LAP using a matrix metalloproteinase (MMP) specific cleavage site as linker. The release of the cytokine occurs only through the cleavage by MMPs usually overexpressed at the disease site during a variety of pathological conditions such as arthritic diseases (Martel-Pelletier et al., 2001; Murphy et al., 2002), cancer (Lampert et al., 1998; Yoon et al., 2003) and inflammation (Yong et al., 1998; Leppert et al., 2001; Rosenberg, 2002). The latent cytokine could not interact with its cellular receptors until it was released from the shell structure provided by the LAP and in consequence had a long half-life of 55 h in vivo. Latent IFNβ has a half-life 37 times longer than native IFNβ (Pepinsky et al., 2001). The strategy for cytokine release could be further improved by optimizing the cleavage of the peptide linker because of the flexibility of the substrate specificity shown by MMPs.

Matrix metalloproteinases are a family of neutral zinc- endopeptidases able to degrade all the components of the extracellular matrix (ECM). This activity is essential for embryonic development, morphogenesis, reproduction and tissue resorption and modelling, but is also responsible for cancer metastasis and cartilage degradation. MMPs in vivo activity is regulated by different factors but TIMPs production is the relevant endogenous regulator in tissue (Woessner and Nagase, 2000). Two members of the MMP family, namely interstitial collagenase (MMP-1) and stromelysin 1 (MMP-3), have an increased expression and activity in patients with rheumatoid arthritis (Yoshihara et al., 2000; Klimiuk et al., 2002; Green et al., 2003; Hegemann et al., 2003; Tchetverikov et al., 2004). This overexpression of MMP-1 and MMP-3 is mainly associated with cartilage degradation in rheumatoid joint diseases. These MMPs are able to cleave fibrillar collagens and/or Type I, II and III collagens at a single site. Based on the cleavage site Gly-Leu reported for both enzymes, 15 different cleavage sites were designed and fusion proteins bearing those sequences were analysed for their relative sensitivity to cleavage by MMP-1 and MMP-3.

Materials and methods

Synthesis of the sequences encoding the new MMPs cleavage sites

Oligonucleotides for the new cleavage sites were designed (see Table I) and synthesized by Invitrogen (Paisley, UK). The annealing of the different primers was performed by mixing 0.2 μg of the appropriate primers in ligase buffer (95 μl total volume). After boiling for 5 min, the mix was left to cool slowly to room temperature and 400 U of T4 DNA ligase (New England Biolabs, Hitchin, UK) were added to the solution to complete the reaction. All primers were 5'-phosphorylated with the exception of the two primers at either end used for cloning.

Cloning and screening

The annealed oligonucleotides were cloned into LAP–mIFNβ plasmid (Adams et al., 2003) after removal of the original MMP site (OM) with EcoR1 and Not1. DH5α recombinant
Table I. Primers used for the synthesis of new cleavage sites

<table>
<thead>
<tr>
<th>No.</th>
<th>Primer</th>
<th>No.</th>
<th>Primer</th>
<th>No.</th>
<th>Primer</th>
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</tr>
<tr>
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<td>23</td>
<td>5' P- GGT GGT CAT TTT TTA TTT CTT TTT TTT TTT CTT</td>
<td>35</td>
<td>5' P- GGT GGT CAT TAT TCT TTA TTT CTT</td>
<td>47</td>
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<td>12</td>
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<td>48</td>
<td>5' P- CCC TCC AGG AAA TAA AGA ATTA ATC AAC ATC AGG</td>
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</table>

Synthesis of the sequences encoding for the new MMPs cleavage sites (OM0–OM14) was performed by annealing different primers with overlapping sequences. Primers used for the OMk synthesis are indicated in parentheses: OM0 (1 + 2 + 3 + 4 + 11 + 12); OM1 (1 + 2 + 3 + 4 + 13 + 14); OM2: 5 + 6 + 15 + 16 + 9 + 10 + 3 + 4; OM3: 7 + 8 + 17 + 18 + 3 + 4; OM4: 5 + 6 + 25 + 26 + 27 + 28 + 3 + 4; OM5: 7 + 8 + 29 + 30 + 3 + 4; OM6: 7 + 8 + 31 + 32 + 3 + 4; OM7: 7 + 8 + 33 + 34 + 3 + 4; OM8: (7 + 8 + 35 + 36 + 3 + 4); OM9: (7 + 8 + 37 + 38 + 3 + 4); OM10: (7 + 8 + 39 + 40 + 3 + 4); OM11: (7 + 8 + 37 + 38 + 3 + 4); OM12: (7 + 8 + 39 + 40 + 3 + 4); OM13: (7 + 6 + 41 + 42 + 43 + 44 + 3 + 4); OM14: (7 + 8 + 45 + 46 + 3 + 4).
clones were first assessed by PCR with the 5’ primer annealed for each site and the 3’ mIFNβ primer used for the original cloning. Following cloning, DNA of positive clones was sequenced and the expected sequence was confirmed. Large preparations of recombinant DNA LAP–OMx–mIFNβ plasmids were obtained, using QIAfilter Mega columns (Qiagen, Crawley, UK).

**Transient transfection of 293T cells**

OMx plasmids (60 μg) and a luciferase reporter plasmid (3 μg) were co-transfected into 293T cells (2 × 10^6 cells per 150 cm² tissue culture dish) by the calcium phosphate coprecipitation method (Chernajovsky and Kirby-Sanders, 1990). After 24 h, the cells were osmotically shocked with 10% glycerol and left to recover for an additional 48 h in DMEM medium supplemented with 10% fetal bovine serum (Invitrogen). Supernatants were collected, aliquoted and stored at −70°C until kinetic analysis. 293T cells were lysed and their luciferase activity was assessed and normalized for cell protein content.

**Enzymes**

The catalytic domains of collagenase 1 [MMP-1 (ΔC)] (EC 3.4.24.7) and stromelysin 1 [MMP-3 (ΔC)] (EC 3.4.24.17) (kindly provided by H. Nagase, Kennedy Institute of Rheumatology, Imperial College, London) expressed in *Escherichia coli* were used for the hydrolysis studies (Suzuki et al., 1998). The specific activities of the [MMP-1 (ΔC)] and [MMP-3 (ΔC)] were 10 400 and 18 300 s⁻¹ M⁻¹, respectively, against the synthetic substrate Mca-Pro-Leu-Glu-Lys-Dpa-Ala-Arg-HN2 where Mca is (7-methoxycoumarin-4-yl)acetyl and Dpa is 3-(2,4-dinitrophenyl)-1,2,3-diaminopropaniopropyl.

**Determination of LAP–OMx–mIFNβ concentration by competitive ELISA**

The LAP–OMx–mIFNβ concentration in 293T cell supernatants was determined by competitive ELISA using serial dilution of 40 ng of LAP protein (Sigma-Aldrich, Poole, UK) added together with supernatant samples diluted 4-fold; 96-well microtitre plates were coated with 20 ng of goat anti-human LAP antibody (R&D Systems, Oxford, UK) in 0.5 M carbonate buffer pH 9.6, overnight at 4°C. After coating, the wells were blocked for 1 h at room temperature with PBS–0.05% Tween 20. In order to detect intact LAP–OMx–mIFNβ fusion proteins, anti-mouse IFNβ was added to each well and incubated at room temperature for 1 h. Binding of anti-IFNβ antibody to intact LAP–OMx–mIFNβ was then detected with a goat anti-rat IgG(Fab)2–HRP antibody followed by incubation with TMB–H₂O₂ and a reading at 450 nm.

**Substrate hydrolysis**

Each set of hydrolysis experiments was performed at the optimal pH for each enzyme (Johnson et al., 2000, Lauer-Fields et al., 2000). Hydrolysis with MMP-1 (ΔC) was performed in buffer 1 (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 10 mM CaCl₂, 50 μM ZnCl₂, 0.05% Brij-35) and buffer 2 (50 mM PIPES, pH 6, 150 mM NaCl, 10 mM CaCl₂, 50 μM ZnCl₂, 0.05% Brij-35) was used for MMP-3 (ΔC) activity. Supernatants containing the fusion proteins LAP–OMx–mIFNβ were diluted 8-fold with the hydrolysis buffer in order to be in the linear response range for ELISA assay. Five different substrate concentrations were used for each assay. Substrate hydrolysis was performed at 37°C under low agitation using enzyme concentrations ranging from 20 to 130 nM. At time zero, hydrolysis was started by adding the respective MMPs in a total volume of 1440 μl of substrate solution. Aliquots (120 μl) of the mixture were removed at various incubation periods and the reaction was stopped by adding EDTA to a final concentration of 25 mM.

**Hydrolysis analysis by ELISA**

Hydrolysis of individual substrates was analysed using ELISA as described above. LAP–OMx–mIFNβ hydrolysates (100 μl) from the time course analysis were added to each well of a 96-well microtitre plate coated with anti-LAP antibody and incubated overnight at 4°C. Unbound samples were removed with five washes in PBS–0.05% Tween 20. In order to detect intact LAP–OMx–mIFNβ fusion proteins, rat anti-mouse IFNβ was added to each well and incubated at room temperature for 1 h. Binding of anti-IFNβ antibody to intact LAP–OMx–mIFNβ was then detected with a goat anti-rat IgG(Fab)2–HRP antibody followed by incubation with TMB–H₂O₂ and a reading at 450 nm.

**Kinetic measurements**

Initial rates of hydrolysis were quantified as OD/min by ELISA and then converted to μM/min using the linear relation existing between OD and molar concentration (1 OD = 52 nM). The molar concentration of each construct was determined by correcting the quantity of protein (ng) with its molecular mass (ranging from 104.8 to 107.9 kDa). The molecular weight was obtained from the amino acid sequence of each LAP–OMx–mIFNβ without taking into account post-translational modifications. Catalytic parameters were obtained by determining initial rates at various substrate concentrations and fitting the data directly to the Michaelis–Menten equation using Kaleidagraph software (Synergy Software, Reading, MA). When the individual kinetic parameters $K_m$ and $k_{cat}$ could not be determined owing to large $K_m$, the specificity constant $k_{cat}/K_m$ was derived using the equation with the substrate concentration below the $K_m$ value: $k_{cat}/K_m = \frac{v}{[E_0][S_0]}$ (Segel, 1975) where $v$, $[S_0]$ and $[E_0]$ correspond to the initial velocity of hydrolysis, initial substrate concentration and enzyme concentration, respectively. Experiments were performed in duplicate. Data that exceeded 15% SD were discarded. In certain cases the experiments were repeated independently.

**Results**

**Development of new cleavage sites**

Three different types of modification were introduced. The original MMP cleavage site (Pro-Leu-Gly-Trp-Ala),...
derived from a fluorogenic substrate for MMPs (Stack and Gray, 1989; Ye et al., 1995; Peng et al., 1997), was extended by addition of one or two amino acids (Gly and Gln), constructs designated as OM0 and OM1 (see Table II). This choice is based on the observation that synthetic peptides containing these two amino acid residues showed a higher sensitivity to cleavage by MMPs (Netzel-Arnett et al., 1991, 1993; Nagase and Fields, 1996). The second strategy was based on the observation that MMPs do not function unless they are activated by proteolysis and usually this final activation step is conducted mainly by other members of the MMP family (Nagase, 1997). We therefore reasoned that using the ‘activation’ cleavage site of MMPs themselves will give further specificity to the ‘latent’ compound. For this purpose, we designed a series of novel cleavage sites derived from the propeptide region of MMPs (see Tables II and III). The alignment of sequences around the activation sites of different MMPs, immediately after the Cys switch propeptide sequence whose removal leads to enzyme activation, allowed us to define a consensus sequence as target (see Table III). We avoided the presence of the Cys in the design of the site, because peptides containing the Cys and homologous to the switch area have been reported as good MMPs inhibitors (Fotouhi et al., 1994). Eleven new constructs bearing the cleavage sites (OM3 to OM14) were made. The cleavage site of the construct OM3 corresponds to the pro-MMP-9 sequence that is activated by MMP-3 (Ogata et al., 1992). The last approach was the addition, inside the MMPs cleavage site, of the surface-exposed flexible loop corresponding to the amino acids 162–175 around the furin cleavage site of the anthrax toxin protective antigen (Petosa et al., 1992). The last approach was the addition, inside the MMPs cleavage site, of the surface-exposed flexible loop corresponding to the amino acids 162–175 around the furin cleavage site of the anthrax toxin protective antigen (Petosa et al., 1992; Liu et al., 2000). This sequence should increase the accessibility of MMPs. These constructs are designated OM2, OM6 and OM13 (see Table II).

**Transient transfection and determination of LAP–OMx–IFNβ concentration**

The efficiency of the transfection was evaluated by the luciferase activity per total protein present inside the cells. The data (not shown) indicated equivalent luciferase expression for each transfection. The ELISA determination of LAP–OMx–mIFNβ concentration in cell supernatants also gave comparable values ranging between 50 and 67 μM (Figure 1).

**Kinetic analysis**

Hydrolysis of the different recombinant proteins by MMP-1 (ΔC) and MMP-3 (ΔC) was followed as a function of time using the ELISA technique. Initial rates of hydrolysis were

**Fig. 1.** Determination of recombinant protein quantity by competitive ELISA. The quantity of LAP–OMx–mIFNβ (ng) present in cell supernatant was determined as defined in Materials and methods. A representative experiment showing the quantification of OM2, OM3 and OM5 is presented.
quantified in OD/min (Figure 2) and then converted to μM/min (1 OD = 52 nM). To estimate the values of the individual kinetic parameters for the different recombinant proteins, initial rates were measured at different substrate concentrations. The individual parameters $K_m$ and $k_{cat}$ were determined by fitting the data to the Michaelis–Menten equation. For some substrates (OM3, OM5, OM9, OM10 and OM11), $K_m$ and $k_{cat}$ could not be determined individually but the specificity constant $k_{cat}/K_m$ was derived by the simplified equation where $[S] << K_m$. The kinetic parameter values are indicated in Table IV.

The three constructs (OM, OM0 and OM1) containing the sequence Pro-Leu-Gly-Leu-Trp-Ala present the same profile of hydrolysis. In each case, MMP-3 (ΔC) hydrolysed more efficiently than MMP-1 (ΔC); $k_{cat}/K_m$ values for MMP-3 (ΔC) (0.26–0.54 μM$^{-1}$ h$^{-1}$) were 2- to 4-fold higher than the value for MMP-1 (ΔC) (0.12–0.16 μM$^{-1}$ h$^{-1}$). The two constructs OM0 and OM1 present a catalytic constant for MMP-1 (ΔC) of 2.4 and 1.85 h$^{-1}$, respectively, which is about 4- to 5-fold higher than that estimated for OM (0.48 h$^{-1}$). These constructs are hydrolysed rather fast, but as the $K_m$ is relatively higher (20 and 12.8 μM, respectively, in comparison with 2.9 μM for OM), the catalytic efficiency is maintained in the same range as for OM. Hence the introduction of Gly and Gln did not really modify the sensitivity of the cleavage site to MMPs.

The overall analysis of the new constructs (OM3 to OM14), containing the consensuses sequence corresponding to the activation site of the pro-MMPs into their active forms, did not show any improvement of catalytic efficiency with respect to OM. A surprising result was obtained for OM3, which corresponds to the pro-sequence of MMP-9 usually cleaved by MMP-3. The construct OM3 presents a catalytic efficiency about 12-fold lower than OM when hydrolysed by MMP-1 (ΔC) (0.013 compared with 0.16 μM$^{-1}$ h$^{-1}$) and 24-fold lower than OM when hydrolysed by MMP-3 (ΔC) (0.022 compared with 0.54 μM$^{-1}$ h$^{-1}$). The same result was found for OM5 with $k_{cat}/K_m$ for MMP-1 (ΔC) and MMP-3 (ΔC) of 0.005 and 0.019 μM$^{-1}$ h$^{-1}$, respectively. OM12 is also a construct with low catalytic efficiency [$k_{cat}/K_m$ for MMP-1 (ΔC) and MMP-3 (ΔC) of 0.007 and 0.014 μM$^{-1}$ h$^{-1}$, respectively]. It could be that the presence of the consensus tripeptide Asn-Tyr-Ser in the cleavage site is susceptible to N-glycosylation (Shakin-Eshleman et al., 1996; Mellquist et al., 1998) and therefore could affect the enzyme accessibility and the hydrolysis.

A further attempt to improve the sensitivity of the cleavage site to MMP activity was by the introduction of a surface-exposed flexible loop, a sequence that should increase the site accessibility. The introduction of this hydrophilic sequence increases the sensitivity to MMP-3 (ΔC) by 3-fold in the case of OM2 ($k_{cat}/K_m = 0.75$ μM$^{-1}$ h$^{-1}$) compared with OM1 ($k_{cat}/K_m = 0.26$ μM$^{-1}$ h$^{-1}$) and by 29-fold in the case of OM6 ($k_{cat}/K_m = 0.56$ μM$^{-1}$ h$^{-1}$) compared with OM5 ($k_{cat}/K_m = 0.019$ μM$^{-1}$ h$^{-1}$). However, no significant improvement was observed for MMP-1 (ΔC) hydrolysis.

Fig. 2. Kinetic analysis of LAP–OM–mIFNβ hydrolysis with MMP-1. The plot corresponds to the variation of OD as function of time obtained during the hydrolysis of five different concentrations of LAP–OM–mIFNβ. 20 nM MMP-1 was used in each digestion test.

### Table IV. Kinetic parameters for the hydrolysis of LAP–OMx–mIFNβ by MMP-1 (ΔC) and MMP-3 (ΔC)

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<th></th>
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<tr>
<td></td>
<td>$K_m$ (μM)</td>
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Kinetic parameters were determined as defined in Materials and methods.

$^a$Relative rate is the % of hydrolysis compared with the hydrolysis of OM.
Discussion

The development of latent cytokines protected by the shell provided by the LAP of TGFβ could be a new approach to the systemic administration of molecules that are usually toxic. The fusion protein composed of LAP linked to mIFNβ by a small peptide acting as substrate for MMPs allows the targeting of activation of the cytokine only at sites with high MMPs levels. This strategy previously reported (Adams et al., 2003) also increases the in vivo half-life of the LAP–mIFNβ complex with respect to chemical stabilization reported for pegylated IFNβ (Pepinsky et al., 2001). These findings prompted us to investigate how to improve the hydrolysis rate of the linker peptide at the specific site of disease. Based on the flexibility of kinetic parameters, by an ELISA method, of 15 new constructs with different cleavage sites obtained on the basis of considerations reported below. Determination of the specificity constant $k_{cat}/K_m$ has usually been measured by the activity of an MMP towards a synthetic peptide substrate. In the case of natural substrates comparison is rather difficult as interactions outside the catalytic cleft can affect the activity. The amino acid positions at either end of the scissile peptide bond are designed $P_1$ through to $P'_4$ since it is known that an octapeptide can accommodate in the active site of the enzyme. Hexapeptide and octapeptide are usually hydrolysed more slowly than natural substrate but their structures give the possibility of elucidating the role of each residue (P) interacting with the sub-sites (S) present in the active site of the enzyme. Thus, in order to improve the substrate sensitivity to MMP-1 and MMP-3, Gly in $P_4$ or the combination Gly and Gln in $P_4$ and $P'_4$ were introduced in the reference sequence Pro-Leu-Gly-Leu-Trp-Ala. As reported also by Liu et al. (2000), this modification should increase the rate of hydrolysis and also give a broad specificity to different members of MMPs. This sequence should increase mainly the activity of MMP-1 since these two amino acid residues (Gly and Gln) are present at the same positions in the peptide model (Gly-Pro-Gly-Gln-Gly-Ile-Ala) present in the $\alpha_1(1)$ chain of calf and chick skin collagens (Netzel-Arnett et al., 1991, 1993).

The constructs named OM0 and OM1 showed increased values of $K_m$ (4- to 6-fold) and $k_{cat}$ (3- to 5-fold) with MMP-1 (ΔC). The slight increase in the turnover rate was compensated by a decrease in affinity leading to moderate loss of catalytic efficiency. The introduction of Gly in $P_4$ did not affect the kinetic parameters for MMP-3 (ΔC) (site OM0) but, in contrast, the introduction of Gln in $P'_4$ led to a marked decrease in turnover rate and a slight increase in affinity (site OM1). The introduction of Gly and Gln showed a different effect with MMP-1 (ΔC) and MMP-3 (ΔC). It seems possible that the introduction of Gln instead of Ser in $P_4$ (present in the linker Gly-Gly-Gly-Gly-Ser) leads to the loss of a hydrogen bonding interaction that could account for the decreased affinity observed in the case of MMP-1 (ΔC), but this modification showed no effect on MMP-3 (ΔC) hydrolysis. The introduction of Gln (large hydrophilic residue) instead of Gly in $P'_4$ could contribute to the enzyme–substrate destabilization in the case of MMP-3 (ΔC).

The strategy adopted by introducing the surface-exposed flexible loop (amino acid residues 162–175) of the furin cleavage site from the anthrax toxin protective antigen as reported by Liu et al. (2000) gave different results when the flanking sequence was added to the reference peptide or to the peptide sequences based on the self-activation sites of MMPs. The OM2 kinetic analysis showed a different behaviour for MMP-1 (ΔC) and MMP-3 (ΔC). In fact, in the first case an improved increase in turnover rate was balanced by a decrease in affinity that meant a relative rate of 50% with respect to OM. In the case of MMP-3 (ΔC), a reduced turnover (4-fold) was measured but it was balanced by an interesting increase in affinity (5-fold) leading to a relative rate of 140%.

The complete analysis of the constructs bearing the flanking sequences of the furin cleavage site in combination with the peptide based on the self-activation sequence of MMPs, namely OM6 and OM13, confirmed that MMP-3 (ΔC) was the enzyme with the best activity. MMP-1 (ΔC) showed a marked decrease in turnover rate whereas the affinity did not change. Overall, these modifications lead to a dramatic fall in the relative hydrolysis rate (5% and 15% for OM13 and OM6, respectively). The analysis is more heterogeneous for MMP-3 (ΔC), OM6 had a 10-fold decrease in the turnover rate that was balanced from an increase in affinity of the same order that means a relative rate of 103%, but with OM13 the low turnover measured was not compensated by the slow increase in the affinity. This picture could suggest that the introduction of the surface-exposed flexible loop of the furin cleavage site can contribute to enhance the exposure of the linker substrate to enzyme action and at the same time increase enzyme specificity.

All the constructs containing an activation sequence of pro-MMPs showed a dramatic decrease in catalytic efficiency. The only exception was the construct OM14 that was among the best substrates for MMP-1 (ΔC) and MMP-3 (ΔC). It seems possible that the removal of the Cys residue is not sufficient by itself to suppress the inhibitory effect that some peptides derived by the Cys ‘switch’ area could exert on MMPs. It is unlikely that the presence of a polar Ser residue in the linker (Gly-Gly-Gly-Gly-Ser), able to bind to zinc, could be responsible for some inhibitory effect. Indeed, Fotouhi et al. showed that the substitution of the Cys by Ser resulted in complete loss of inhibitory activity with an $IC_{50} > 500 \mu M$ compared with 5 μM when the Cys was present (Fotouhi et al., 1994). The data assessed for the self-activation linker sequence reinforce the idea that the surface flexible loop of the furin cleavage-site could play a fundamental role to favour the interaction between substrate and enzyme (e.g. OM6 with respect to OM5).

The overall analysis of the kinetic data shows a heterogeneous and complex pattern sometimes very difficult to explain. It seems likely that this heterogeneity together with the low catalytic efficiency measured compared with data reported in the literature (for a review, see Nagase and Fields, 1996) could be due in part to the concentration of the enzyme used in the tests. Nevertheless, it is also evident that LAP could play a key role in modulating the interaction between the enzyme and the substrate, conditions not found in the case of small, unprotected peptides. As reported in a previous paper (Adams et al., 2003) it is evident that in vitro the cleavage occurs with both MMP-1 (ΔC) and MMP-3 (ΔC) as measured with IFNβ biological activity. In fact, after treatment of the LAP–OM–mIFNβ fusion protein with MMP-1 (ΔC) and MMP-3 (ΔC), an increase in biological activity of 21- and 32-fold, respectively, was measured. Furthermore, immunoprecipitated complexes of the fusion protein, digested with MMP-1 (ΔC) and MMP-3 (ΔC) and analysed by western blotting, showed the presence of some cross-reacting bands.
of two fragments (43 and 32 kDa) corresponding to LAP and mIFNβ, respectively. The higher hydrolytic activity on the complex obtained with MMP-3 (ΔC) is in good agreement with the kinetic parameters measured for LAP–OM–mIFNβ with the same enzyme.

The present ELISA methodology gave us good indications on the rate of cleavage of MMP-1 (ΔC) and MMP-3 (ΔC) and could be used to investigate the hydrolytic activity of other MMPs. The determination of a cleavage site specific to a particular member of the MMP family could be useful for applying this targetting strategy to other biological compounds for the treatment of diseases implicating MMPs overexpression.

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References