A Residue in the $S_2$ Subsite Controls Substrate Selectivity of Matrix Metalloproteinase-2 and Matrix Metalloproteinase-9

Matrix metalloproteinase (MMP)-2 and MMP-9 are closely related metalloproteinases that are implicated in angiogenesis. The two proteins have a similar domain structure and highly homologous catalytic domains, making them an excellent comparative model for understanding the structural basis of substrate recognition by the MMP family. Although the two MMPs exhibit some overlap in substrate recognition, our recent work showed that MMP-2 can cleave a set of peptide substrates that are only poorly recognized by MMP-9 (Chen, E. I., Kridel, S. J., Howard, E. W., Li, W., Godzik, A., and Smith, J. W. (2002) J. Biol. Chem. 277, 4485–4491). Mutation of the $P_2$ position of these peptide substrates dramatically reduced their selectivity for MMP-2. Inspection of the corresponding $S_2$ pocket of the substrate-binding cleft of the protease reveals that MMP-9 contains an Asp, whereas MMP-2 contains Glu. Here, we test the hypothesis that this conservative substitution has a role in substrate selectivity. Mutation of Glu$^{412}$ in MMP-2 to Asp significantly reduced the hydrolysis of selective substrates, with only a minor effect on hydrolysis of non-selective substrates. The predominant effect of the mutation is at the level of $k_{cat}$, or turnover rate, with reductions reaching as high as 37-fold. The residues that occupy this position in other MMPs are highly variable, providing a potential structural basis for substrate recognition across the MMP family.

The matrix metalloproteinase (MMPs)$^1$ family consists of over 25 secreted and cell surface proteases (1). The MMPs are involved in a wide range of normal biologic processes and have been linked to a number of pathologic events (2, 3). Of the associations with disease, a number of synthetic MMP antagonists have been developed and tested in human clinical trials (2, 4–7). Unfortunately, however, these trials did not succeed (8). One reason for the lack of success probably relates to the relatively broad spectrum of inhibition by the compounds that were tested. For example, the first compound to enter the clinic, Marimastat, has a similar affinity for at least five MMPs. The lack of selectivity of most of the small molecule MMP inhibitors can be traced to the fact that they were designed to exploit features common to all MMPs. These include a zinc ion and the catalytic glutamic acid (9), and especially, the deep $S_2$ pocket (10–15). With these ideas in mind, one of the objectives of the present study was to identify regions of the MMP catalytic pockets that contribute to substrate distinction and that could be exploited for the future design of highly selective MMP antagonists.

Information on substrate selection by the MMPs could also help explain the unique biological roles of these proteases. MMPs are no longer looked upon as proteases whose action is limited to the destruction of collagen and gelatin in basement membranes. Rather, the MMPs are involved in the control of a number of events where proteolysis must be exerted in a precise manner. For example, MMPs can activate other MMP zymogens (proMMPs) by hydrolyzing propeptide bonds (16). They also influence the activity of the serine proteases by selectively degrading their macromolecular inhibitors such as serpin $\alpha$1-proteinase inhibitor (17). MMPs can also influence that action of growth factors by activating and releasing them for their functions (18). Some MMPs potentiate the action of growth factors by selectively degrading growth factor-binding proteins (19). All of these functions are presumed to involve a relatively high degree of selectivity among the MMPs for their physiologic substrates. There are also circumstances in which closely related MMPs are present in the same locale but have different roles. For example, both MMP-2 and MMP-9 are expressed within tumors, but only one participates in the angiogenic switch (20). Similar distinctions in the role of these MMPs have also been observed in platelet function (21) and in cell migration (22). These findings strongly suggest that MMP-2 and MMP-9 operate by cleaving distinct substrates.

Despite the expanding awareness of selective hydrolysis by the MMPs, the structural basis for such selectivity is still not well understood. In a prior report, we used substrate phage display to gain an in-depth understanding of the substrate recognition properties of MMP-2. We identified a surprising number of highly selective substrates for MMP-2 (23). Interestingly, the selective substrates could be segregated into three subfamilies based on their sequences. However, within each family, the $P_2$ residue of substrate was key to selectivity for MMP-2 over MMP-9. The $S_2$ pocket, which interacts with the $P_2$ residue in substrate, is remarkably similar in both enzymes (14), save for the presence of Glu$^{412}$ in MMP-2, which is replaced by an Asp in MMP-9. In fact, our modeling work suggested that Glu$^{412}$ of MMP-2 forms a hydrogen bond with the backbone of the selective substrates, a bond that cannot be formed by the shorter Asp in MMP-9.

Here we test the importance of the Glu/Asp alteration in substrate recognition. Mutation of Glu$^{412}$ to Asp has a signifi-
The hydrolysis of synthetic peptides by each MMP was quantified using procedures outlined under “Experimental Procedures.” For each protease, hydrolysis was measured across a concentration range of peptide. Values for $k_{cat}$ and $K_m$ were derived from Lineweaver-Burk plots. Each measurement was repeated at least three times and the relative error of $k_{cat}/K_m$ in these measurements was less than 10%.

| Peptide-2 | Sequence | MMP-2 | MMP-E412D | MMP-9 |
|-----------|----------|-------|-----------|-------|
| m1A11     | SGKIPPRQ | 3.8E + 04 | 3.7E + 04 | 2.5E + 04 |
| C15       | SGKIPPRQ | 8.4E + 04 | 5.1E + 04 | 5.4E + 04 |

EXPERIMENTAL PROCEDURES

Source of Commercial Proteins and Reagents—Illomat was purchased from AMS Scientific (Concord, CA). Restriction enzymes were from New England Biolabs. Oligonucleotides were synthesized by Integrated DNA technologies, Inc. (Coralville, IA). Tissue culture media and reagents were from Irvine Scientific (Irvine, CA). All other reagents, chemicals, and plastic ware were from Sigma or Fisher.

Docking of the MMP-2 Peptide Substrates into the Catalytic Domains of MMP-2 and MMP-9—The complexes between MMP-2 and the peptide substrates were modeled using the Sybyl package from Tripos (www.tripos.com). The coordinates of MMP-2 are from the crystal structure (Protein Data Bank accession number 1jhb). For each donor, the initial structure of each peptide was built in an extended conformation within the catalytic cleft of the protease. Since the binding pocket of the enzyme is known, and some interactions between the protein and site around P$_1$ of the substrate are obvious, these interactions were used as restrictions in the docking procedure. The anchoring restrictions included a hydrogen bond between the NH group of P$_2$ and the oxygen atom of Ala$_{165}$, the hydrogen bond between the oxygen atom of P$_1$ and the NH group of Ala$_{165}$, the interaction between the side chain of P$_1$ and the hydrophobic patch of MMP-2 comprised of Val$_{179}$ and its surroundings.

The substrate was first placed at the binding cleft at a position favoring the formation of bonds making up these restrictions. Altogether, the findings of this study raise the interesting hypothesis that selectivity between MMP-2 and MMP-9 arises because MMP-9 is a “slower” protease than MMP-2.

Quantifying the Kinetic Parameters of Peptide Hydrolysis—The kinetic parameters of substrate hydrolysis were measured using a fluoroscence incorporation assay (24). The method for determining the $K_m$ and $k_{cat}$ of peptide hydrolysis was described previously (23).
hydrolysis of a panel of peptides that were found previously to be selective for MMP-2 (Table II). Each of the selective peptides has a higher $k_{cat}/K_m$ ratio for MMP-2 than for MMP-9. In all cases, the mutation of Glu to Asp caused a decrease in the $k_{cat}/K_m$ such that the ratio for the mutant falls between the value for MMP2 and MMP-9. This observation is consistent with the fact that the E412D mutation alters the structure of the catalytic pocket to more closely resemble that of MMP-9.

Interestingly, the primary effect of the mutation seemed to be at the level of $k_{cat}$, a parameter that was decreased for all of the selective peptides. Decreases in $k_{cat}$ ranged from 3 to 36-fold, in most cases approximating the values we have measured for MMP-9. In contrast, the effects of the E412D mutation on $K_m$ were less consistent. We observed this parameter to increase and decrease depending on the peptide being tested. Consequently, the E412D mutation in the S$_2$ pocket appears to primarily affect the rate of substrate turnover.

One peptide was identified that has an increased $k_{cat}/K_m$ ratio for the E412D mutant of MMP-2. This peptide, A13R, was originally synthesized and characterized in our prior report on substrates selective for MMP-2 (23). The parent peptide, A13, contains the RXS$_2$L motif that is selective for MMP-2, but A13R contains the RX$_2$L sequence and is cleaved better by MMP-9. Here, we compared the rate of hydrolysis of the A13R peptide with all three of the MMPs (Fig. 1). In three independent replications of this experiment, the rate of peptide hydrolysis was measured over a concentration range of peptide such that values for $k_{cat}$ ranged from 3 to 36-fold, in most cases approximating the values we have measured for MMP-9. In contrast, the effects of the E412D mutation on $K_m$ were less consistent. We observed this parameter to increase and decrease depending on the peptide being tested. Consequently, the E412D mutation in the S$_2$ pocket appears to primarily affect the rate of substrate turnover.

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**Comparing the Hydrolysis of EphB1 by Wild Type and Mutated MMP-2**—The EphB1 receptor tyrosine kinase contains the SX$_2$L motif and is selectively cleaved by MMP-2 (23). In fact, we found that this protein is generally resistant to hydrolysis by MMP-9. Here we tested the activity of MMP-2$^{E412D}$ to hydrolyze EphB1 (Fig. 2). The EphB1 fusion protein was incubated with equimolar amounts (280 nM) of wild type MMP-2, mutated MMP-2, and MMP-9 for 4 h. The quantity of each protease was measured by active site titration prior to initiating the experiment. The extent of hydrolysis of EphB1 was gauged by SDS-PAGE (Fig. 2). The EphB1-Fc fusion protein was almost quantitatively cleaved by MMP-2 (Fig. 2, lane 2). As expected, MMP-9 had no effect on the migration of the protein, indicating a lack of hydrolysis (Fig. 2, lane 4). Interestingly, the hydrolysis of the EphB1-Fc fusion protein by MMP-2$^{E412D}$ fell between that of wild type MMP-2 and MMP-9 (Fig. 2, lane 3). This result is consistent with the observations made with the panel of peptide substrates and corroborates the conclusion that the E412D mutation shifts the phenotype of the protease away from that of MMP-2 and toward that of MMP-9.

**DISCUSSION**

MMP-2 and MMP-9 are considered to be close homologs because of their unique domain structure and their high sequence similarity and because they both have gelatinase activ-
Control of Substrate Selectivity of MMP-2 and -9

Fig. 3. Arg at P_2 supports favorable docking to both MMP-2 and MMP-9. Peptide substrate B74R, which is not selectively cleaved, is shown in the cleft of MMP-2 (A) and MMP-9 (B). The extension of the guanidino group of the Arg into the S_2 pocket is the key feature of this substrate because it is positioned to interact favorably with the acidic side of chain of either Glu^{112} in MMP-2 or Asp^{110} in MMP-9 (circled).

ity. Nevertheless, the two proteases clearly have distinct biological functions. In many instances, the two proteases have different effects even when they are analyzed in the same biological system (21, 22). Consequently, it follows that MMP-2 and MMP-9 are likely to cleave different substrates.

We have recently begun to examine differences in the way that MMPs recognize peptide substrates. This analysis places focus on structural and functional distinctions at the catalytic cleft, a region that has generally been overlooked as a feature that can distinguish one MMP from another. In fact, early comparisons of peptide substrates for MMP-2 and MMP-9 showed that they each cleaved the same set of collagen-like peptide substrates (25), a finding that indicates structural and functional similarity at the catalytic pocket. Nevertheless, we recently made the observation that MMP-2 and MMP-9 recognize distinct sets of peptide substrates (23). We found that both MMP-2 and MMP-9 hydrolyze peptides with the canonical recognition motif PXXX_H, as originally described by Netzel-Arnett et al. (25), but we also found MMP-2 to uniquely recognize three additional sets of peptide substrates. Like the canonical motif, the three other families of substrates all contain a hydrophobic residue at the P_1 position, but they lack the characteristic proline at P_3. In an extension of this study, we found that the substrates selective for MMP-2 could be converted to substrates for MMP-9 by inserting Arg into the P_2 position (23). This finding underscored the significance of the P_2 position within substrate and strongly suggested that the corresponding S_2 subsite within the catalytic clefts of MMP-2 and MMP-9 must interact differently with substrate. Interestingly, the S_2 subsite contains one of the few differences between the catalytic clefts of the two MMPs; Glu^{112} of MMP-2 is replaced by Asp^{110} in MMP-9. The objective of the present study was to test the hypothesis that this conservative substitution accounts for the distinct substrate recognition profiles of the two enzymes.

We swapped this residue by mutating each protease. Although we were able to express the mutated MMP-2, the mutant MMP-9 could not be expressed (see below). Consequently, most of our discussion focuses on results obtained with the mutant of MMP-2. The mutated MMP-2 was expressed to high levels in HEK 293 cells, could be purified on gelatin-agarose affinity columns, and exhibited gelatinase activity in zymography gels. In addition, the E412D mutant hydrolyzed substrates with the canonical PXXX_{H}^1 recognition motif to essentially the same degree as wild type MMP-2. This is to be expected since MMP-9, which displays the Asp rather than Glu at this position, also hydrolyzes this subfamily of substrates with high efficiency. In conjunction with the fact that the mutation is conservative and represents a sequence found in the closest homolog, these observations allow us to exclude the possibility that substitution of Glu for Asp at position 412 causes a general diminution of catalysis by forcing improper folding.

The E412D mutation had substantial effects on the hydrolysis of peptide substrates that are selective for MMP-2. The mutation significantly reduced the k_{cat}/K_{m} ratio for all of the MMP-2-selective substrates. Reductions to the k_{cat}/K_{m} ratio were primarily driven by reductions to the turnover rate, k_{cat}, which was reduced in every case. Changes in both directions were observed for K_{m}, so the E412D mutation has no uniform influence on binding affinity. The E412D mutation also extended to larger protein substrates. We recently demonstrated that the extracellular domain of the EphB1 receptor tyrosine kinase is cleaved by MMP-2, but not by MMP-9. The ability of the E412D mutant of MMP-2 to cleave EphB1 fell in between that of wild type MMP-2 and MMP-9, indicating the mutation shifted the function of the protease closer to that of MMP-9. This is taken as another indication that the Glu^{112}/Asp^{110} alteration has a role in substrate distinction.

Altogether the results of this study suggest a structural basis for the role of the S_2 subsite in substrate recognition and catalysis. In large part, our results support the idea that the S_2 subsite can engage in two types of interactions that depend on the composition of the substrate (Figs. 3 and 4). When Arg is present at P_2, substrates show selectivity for MMP-2 and MMP-9 over other MMPs (26). In this case, the Arg in the substrate extends far enough into the S_2 subsite to interact favorably with the side chains of both Asp in MMP-9 or the Glu in MMP-2 (Fig. 3). Because Arg at this position decreases K_{m} (26), we suggest that the salt bridge that Arg forms with the side chains of Glu or Asp increases binding affinity for the protease (Fig. 3). A second type of interaction at the S_2 subsite is likely with the peptides that lack Arg and are selectively cleaved by MMP-2. In the absence of Arg at P_2, only the Glu of MMP-2 extends far enough into the S_2 pocket to form a hydrogen bond with the backbone of bound substrate (Fig. 4A), a
concept that is generally supported by our mutational studies. The corresponding Asp in MMP-9 fails to extend far enough into the pocket to make a similar contact (Fig. 4B). Interestingly, our findings also suggest that a switch to this binding mode in MMP-2 changes the overall effect of the S2 subsite on substrate recognition. Rather than playing a role in binding affinity, the hydrogen bond between Glu412 and the backbone of bound substrate influences $k_{cat}$, indicating that it helps to properly position the substrate for optimal catalysis.

Although our findings strongly support the role of the Glu$^{412}$/Asp$^{410}$ substitution in determining substrate recognition by the two MMPs, this mutation alone was not sufficient to completely convert the recognition profile of MMP-2 to that of MMP-9. Consequently other factors must be involved. These are likely to include cooperativity among other subsites where subtle differences exist or a global difference in the shape of the catalytic pocket that is governed by sequence differences outside of the catalytic cleft. A resolution to this particular issue will require a three-dimensional structure for MMP-9.

The importance of the residue corresponding to Glu$^{412}$ in the S2 subsite may extend across the MMP family. Most of the residues surrounding the S2 subsite are strictly conserved among all MMPs (Fig. 5). The two histidines that flank the subsite and the glycine in the center of the subsite are conserved across all family members. In contrast, the position taken by Glu$^{412}$ in MMP-2 is highly variable. It is occupied by acidic residues, large hydrophobic residues, and in some cases, glycine (Fig. 5). The distinctions among these residues are likely to have an influence on substrate recognition by each MMP.

Certainly an analysis of the analogous substitution within MMP-9 would be informative. However, we were unable to express this mutant, although we tested a number of different host cell types. Although the inability to express this mutant may seem surprising given the extensive homology between MMP-2 and MMP-9, there may be a mechanistic basis for this observation. It is well established that the MMPs require the propeptide, which inserts into the catalytic cleft via a cysteine switch, for proper folding (27, 28). We suspect that this mutant of MMP-9 cannot be expressed because it interferes with the insertion of the propeptide into the catalytic cleft during synthesis. This hypothesis is based on inspection of the crystal structure of MMP-2 with the propeptide inserted into the cleft. In MMP-2, Glu$^{412}$ extends up from the floor of the cleft to within 3–4 angstroms of the side chain of Asn$^{109}$. In MMP-9, the corresponding residues are Arg (longer than Asn) and Asp (shorter than Glu). The Arg in the propeptide of MMP-9 would be expected to extend further into the catalytic cleft than Asn. Such positioning would likely be favorable with Asp in the S2 subsite. However, mutation of Asp$^{410}$ to Glu, which would extend further into the cleft, is likely to interfere with insertion of Arg in the MMP-9 propeptide. Such interference would be likely to prevent proper folding of the mutated MMP-9 and cause its degradation.

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