Identification of Amino Acid Residues of the Matrix Metalloproteinase-2 Essential for Its Selective Inhibition by β-Amyloid Precursor Protein-derived Inhibitor*5

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The extracellular domain of β-amyloid precursor protein (APP) contains an inhibitor against matrix metalloproteinase-2 (MMP-2, gelatinase A). Our previous study (Higashi, S. and Miyazaki, K. (2003) J Biol Chem 278, 14020–14028) demonstrated that the inhibitor is localized within the ISYGN-DALMP sequence of APP, and a synthetic decapeptide containing this sequence (named APP-derived inhibitory peptide, APP-IP) selectively inhibits the activity of MMP-2. To determine the region of interaction that correlates with the selective inhibition, we constructed various MMP-2 mutants. An MMP-2 mutant, which had the hemopexin-like domain and three fibronectin-like type II domains of MMP-2 deleted, and native MMP-2 showed similar affinities for APP-IP, suggesting that only the catalytic domain of MMP-2 is essential for the interaction. Studies of chimeric proteases, consisting of various parts of the MMP-2 catalytic domain and those of MMP-7 (matrilysin) or MMP-9 (gelatinase B), further revealed that Ala88 and Gly94 in the non-prime side and Tyr145 and Thr146 in the prime side of the substrate-binding cleft of MMP-2 contribute separately to the selective inhibition. Replacement of the amino acid residue at position 94 of a chimeric MMP mutant affected its interaction with the C-terminal Pro10 of APP-IP, whereas that of residues 145–148 affected the interaction with Tyr3 of the inhibitor, suggesting that the N to C direction of APP-IP relative to the substrate-binding cleft of MMP is analogous to that of propeptide in proMMP, and opposite to that of substrate. When the APP-IP sequence was added to the N terminus of the catalytic domain of MMP-2, the activity of the protease was intramolecularly inhibited. We speculate that the direction of interaction makes the active site-bound APP-IP resistant to cleavage, thereby supporting the inhibitory action of the peptide inhibitor.

Matrix metalloproteinases (MMPs)2 are a family of zinc-dependent endopeptidases that degrade components of the extracellular matrix (ECM) and are believed to play pivotal roles in tissue remodeling under physiological and pathological conditions such as morphogenesis, angiogenesis, tissue repair, and tumor invasion (1, 2). The association of MMPs with cancer cell invasion and metastasis has suggested that these proteases represent attractive targets for the development of novel anti-tumor therapies. However, to date, no MMP inhibitor has been developed successfully as anti-tumor drugs mainly because of deleterious side effects. Recently, inhibition of some MMPs is considered to have protumorigenic effects (3). These effects may also partially account for the failure of the inhibitors in clinical trials. In both cases, the broad specificity of the MMP inhibitors must be a stiff obstacle for developing safe and effective drugs. Most MMPs are secreted as proMMP, an inactive zymogen that has an autoinhibitory propeptide in its N terminus and is proteolytically activated by serine proteases or some activated MMPs. The activities of activated MMPs are regulated by a family of inhibitors known as tissue inhibitors of metalloproteinases (TIMPs). These physiological inhibitors also have broad specificity against MMPs; the activities of almost all MMPs are susceptible to TIMP (TIMP-1 to TIMP-4) inhibition, and some members of a disintegrin and a metalloproteinase family are also inhibited by these inhibitors (4–6). To develop drugs for the treatment of diseases in which MMPs are involved, many hydroxamate-based inhibitors or other synthetic MMP inhibitors have been designed (7–10). Unfortunately, none of them is a specific inhibitor for individual MMPs. A common architecture of catalytic sites of MMPs probably relates to the broad specificity of the inhibitors.

Among the MMP family, MMP-2 (gelatinase A) and MMP-9 (gelatinase B) are critical in the invasion of tumor cells across basement membranes, because of their strong activity against type IV collagen, a major component of basement membranes (11–13). Although they have a common substrate, MMP-2 is categorized as a good target for antican-
cer drugs, whereas MMP-9 is considered not to be a safe target for the drugs (3). Unlike other zymogens of MMPs, activation of proMMP-2 is catalyzed specifically by a novel type of MMP that has a transmembrane domain and is thus localized on the cell surface (14). So far, six members of the membrane-type MMP have been identified, and membrane type 1-MMP (MT1-MMP)-catalyzed proMMP-2 activation has been well characterized (15–17). Upon the activation of proMMP-2, the protease zymogen is first recruited on the cell surface via the MT1-MMP complexed with TIMP-2, and then a TIMP-2-free, non-inhibited form of MT1-MMP (also present on the cell surface) cleaves the propeptide of proMMP-2 to initiate the activation (14, 18, 19). It is thought that both the cell-associated active MMP-2 and MT1-MMP are recruited into invadopodia, thus limiting proteolysis to the site of cell invasion (20). Therefore, the exertion of MMP-2 activity is strictly regulated on its zymogen activation level. Recently, we further proposed that the ECM-degrading activity of MMP-2 is regulated by β-amyloid precursor protein (APP) that contains its selective inhibitor (21, 22).

APP is a type I integral membrane protein that was initially identified as a precursor of β-amyloid peptide, the principal component of extracellular deposits in senile plaques observed in the brain of Alzheimer disease patients (23). In cultured cells, APP synthesized and matured through the constitutive secretory pathway is proteolytically cleaved at the cell surface within the β-amyloid sequence, and the extracellular domain of APP is released as a soluble APP into the culture medium (24, 25). Because the extracellular domain of APP has the abilities both to interact with components of ECM (26–30) and to inhibit MMP-2 activity (31), this molecule is assumed to protect ECM from MMP-2-catalyzed degradation. Our previous study (21) demonstrated that the inhibitor is localized within the ISYGNDALMP sequence corresponding to residues 586–595 of APP770, and that the inhibitor is localized within the ISYGNDALMP sequence. Clarification of the mode of interaction between MMP-2 and APP-derived inhibitor benefits for individual MMPs.

**EXPERIMENTAL PROCEDURES**

**Materials**—The sources of materials used are as follows: human thrombin, Brij 35, anti-FLAG M2 affinity gel, FLAG peptide, and pFLAG-CTC vector from Sigma; pGEM-T vector from Promega; pGEX-2TK from GE Healthcare UK Ltd. (Amersham Biosciences); cDNA of human proMMP-2 and that of human proMMP-9 cloned into pCMV6 vector from OriGene Technologies, Inc. (Rockville, MD); gelatin from Difco (Detroit, MI); p-aminophenyl mercuric acetate (APMA) from Tokyo Kasei (Tokyo, Japan); purified human proMMP-9 (progelatinase B) from Chemicon International Inc. (Temecula, CA); bovine pancreatic trypsin treated with N-tosyl-l-phenylalanine chloromethyl ketone from Worthington (Freehold, NJ); human recombinant matrilysin, p-amidinophenyl methanesulfonyl fluoride hydrochloride (APMSF) from Wako Pure Chemical Industries (Osaka, Japan); Affi-Gel 10 from Bio-Rad; the synthetic MMP inhibitor TAPI-1 ([N-(R)-(2-hydroxamino-phenyl)(methyl)-4-methylpentanoyl-L-naphthylalanyl-L-alanine-2-aminoethyl amide); and the synthetic substrate for MMPs, 3163v (7-methoxycoumarin-4-yl)-acetyl-Pro-Leu-Gly-alanine-2-aminoethyl amide); and the synthetic substrate for stromelysin, 3163s (7-methoxycoumarin-4-yl)-acetyl-Pro-Leu-Gly-Leu-[Nβ-(2,4-dinitrophenyl)-L-2,3-diaminopropionyl]-Ala-Arg amide) from Peptide Institute, Inc. (Osaka). The cDNA of human proMMP-7 cloned into the pAPR vector (34) was provided by Dr. T. Tanaka, (Nagahama Institute of Oriental Yeast Co., Shiga, Japan). All custom oligo-DNA primers were provided by Rikaken Co., Ltd. (Tokyo). All custom peptides were provided by Bex Co., Ltd. (Tokyo). All other chemicals were of analytical grade or the highest quality commercially available.

**Proteins**—The TIMP-2-free form of proMMP-2 was purified from the conditioned medium of the human glioblastoma cell line T98G, as described previously (32). ProMMP-3 (stromelysin) was purified from the conditioned medium of the Rous sarcoma virus-transformed rat liver cell line (RSV, Invitrogen) as described previously (33).

**Construction of Expression Vector for MMP Mutants**—Gene constructions carried out in this study are described under supplemental data.

**Expression and Purification of MMP Mutants**—The expression vectors of various MMP mutants were transfectioned separately into the Escherichia coli strain DH5α. The transformants were cultured in 2× YT medium (0.08% (w/v) tryptophane, 0.5% (w/v) yeast extract, and 0.25% (w/v) NaCl) at 37 °C, and the GST fusion proteins of MMP mutants or other MMP-2 mutants were induced by the addition of 1.0 mM isopropyl-β-D-thiogalactopyranoside. After a 5-h induction, E. coli cells were broken in 50 mM Tris-HCl, pH 8.0 containing 50 mM NaCl and 5 mM EDTA with a sonicator, and the resultant inclusion bodies were collected by centrifugation. Solubilization of the inclusion bodies with guandine-HCl followed by refolding by rapid dilution method was performed as described previously (35, 36). In the case of GST fusion proteins, the refolded proteins were incubated with thrombin to remove the N-terminal GST region. The resultant pro-form of MMP mutants or the MMP-2 catalytic domain N-terminally fused with APP-IP-containing peptide was purified, using an anti-FLAG M2 monoclonal antibody-conjugated agarose column. The concentrations of the purified proteins were determined by the Bradford dye method with a Bio-Rad protein assay kit, using bovine serum albumin (BSA) as a standard.

**Assay of Inhibitory Activity of APP-IP or Its Variants toward MMP Mutants**—The TIMP-2-free form of MMP-2 (1 μM) or pro-forms of the MMP mutants (1 μM) were activated by incu-
bation with 1 mM APMA and rat proMMP-3 (0.2 μM) at 37 °C for 2 h as described previously (31). Activated MMP-2 and the MMP mutants were first measured for their activities toward a synthetic substrate 3163v as described previously (21). The peptidolytic activities of the mutants are listed in supplemental Table SII. As MMP-3 used for the activation had almost no activity toward 3163v, this protease did not interfere with the activity assay. MMP-2 (0.61 nM) and the mutants, of which concentrations were adjusted to give the activity equivalent to that of 3163v hydrolyzed by each enzyme, were incubated with 50 μM 3163v at 37 °C for 30 min in the presence of the indicated concentrations of APP-IP. All reaction mixtures contained 50 mM Tris-HCl, 150 mM NaCl, 10 mM CaCl₂, 0.01% Brij 35, and 0.01% BSA. The amount of 3163v hydrolyzed by each enzyme in the absence of APP-IP was taken as 100%. The enzyme activity on the ordinate is shown as the relative amount of 3163v hydrolyzed by the enzyme.

FIGURE 1. Effect of the deletion of the hemopexin-like domain and three fibronectin-like type II domains of MMP-2 on its affinity for APP-IP. A, the construction and the preparation of the catalytic domain of MMP-2 bearing the FLAG tag, named MMP-2-cat-FLAG described under "Experimental Procedures" are schematically represented. The thrombin arrow represents the site of the GST fusion protein susceptible for the thrombin cleavage. The APMA, MMP-3 arrow represents the site of the pro-form of MMP-2-cat-FLAG susceptible for both the autocatalytic and MMP-3-catalyzed cleavages upon treatment with APMA and MMP-3. PRO, the propeptide region; CAT, the catalytic domain; NF, fibronectin-like type II domain. B, active forms of MMP-2-cat-FLAG (0.58 nM, ○), native MMP-2 (0.58 nM, ●), and MMP-7 (3.6 nM, ▲) were incubated with 50 μM 3163v at 37 °C for 30 min in the presence of the indicated concentrations of APP-IP. All reaction mixtures contained 50 mM Tris-HCl, 150 mM NaCl, 10 mM CaCl₂, 0.01% Brij 35, and 0.01% BSA. The amount of 3163v hydrolyzed by each enzyme in the absence of APP-IP was taken as 100%. The enzyme activity on the ordinate is shown as the relative amount of 3163v hydrolyzed by the enzyme.

were measured for their peptidolytic activity using 3163v as a substrate.

RESULTS

The Effect of Deletion of the Hemopexin-like Domain and Three Fibronectin-like Type II Domains of MMP-2 on Its Affinity for APP-IP—It has been reported that APP-IP is likely an active site-directed inhibitor that has high selectivity toward MMP-2 (21). To examine whether the catalytic domain of MMP-2 is sufficient for its selective interaction with APP-IP, we constructed an MMP-2 mutant, named MMP-2-cat-FLAG, that had the hemopexin-like domain and three fibronectin-like type II domains of MMP-2 deleted (Fig. 1A), and susceptibilities of the activities of the mutant, native MMP-2, and MMP-7 to APP-IP inhibition were compared.

APP-IP Inhibition of Peptidolytic Activities of Chimeric MMP Mutants Consisting of Various Parts of MMP-2 and Those of MMP-7—The data shown in Fig. 1 strongly suggest that the structural difference between the catalytic domain of MMP-2 and that of MMP-7 correlates with about a 300-fold difference in their affinities for APP-IP. To explore the regions of the catalytic domain that correlate with the affinity difference, we constructed chimeric MMP mutants that consist of various N-terminal parts of the catalytic domain of MMP-2 and the remaining C-terminal parts of MMP-7 (Fig. 2A), and susceptibilities of their activities to APP-IP inhibition were compared. To facilitate understanding, we use active MMP-7 numbering for the amino acid residue numbers of the catalytic domain of MMPs and the chimeric mutants. As shown in Fig. 2B, the peptidolytic activities of native MMP-2 (IC₅₀ = 32 nm) and MMP-2-cat-FLAG (IC₅₀ = 65 nm) were inhibited by APP-IP with similar IC₅₀ values, whereas the activity of MMP-7 (IC₅₀ = 20 μM) was inhibited with a much higher IC₅₀ value. Therefore, only the catalytic domain of MMP-2 is likely sufficient for the selective interaction with APP-IP.
affinity for APP-IP (IC$_{50}$ = 1.5 μM), whereas M2n-153-M7c showed much higher affinity (IC$_{50}$ = 100 nM) that was comparable to the affinity between MMP-2-cat-FLAG and APP-IP (Fig. 2C). Therefore, it is likely that the region corresponding to residues 137–153 of MMP-2 also contributes to the selective interaction. To explore the amino acid residues responsible for the selective interaction, the residues 110–122 of M2n-122-M7c were further replaced with the corresponding residues of MMP-7 as shown in Fig. 3A. We found that the replacement of residues 110–114 or that of the individual residue Phe$^{116}$, Leu$^{117}$, or Ala$^{120}$ did not significantly affect the affinity for APP-IP, whereas replacement of Leu$^{115}$ and Val$^{118}$ led to an 8.0- and 2.5-fold increase in the IC$_{50}$ values, respectively (Fig. 3A). We also replaced the residues 137–153 of M2n-137-M7c with the corresponding residues of MMP-2 as shown in Fig. 3B, and found that the replacement of residues 137–144, that of residues Tyr$^{145}$ and Thr$^{146}$, and deletion of two extra residues Asp$^{147}$ and Pro$^{148}$ led to 2.6-, 4.1-, and 1.9-fold reductions in the IC$_{50}$ values, respectively. Collectively, the difference of amino acid residues at positions 115 and 145–148 between the catalytic domains of MMP-2 and MMP-7 mainly correlated with the difference in their affinities for APP-IP.

**APP-IP Inhibition of Peptidolytic Activities of Chimeric MMP Mutants Consisting of Various Parts of MMP-2 and Those of MMP-9**—Our previous study (21) demonstrated that APP-IP is a poor inhibitor for MMP-9, although MMP-2 and MMP-9 have similarity in their structures and substrate specificities. To examine whether the low-affinity interaction between MMP-9 and APP-IP is caused by the structural elements in the catalytic domain of MMP-9, we constructed an MMP-9 mutant, named MMP-9-cat-FLAG, that had the hemopexin-like domain and three fibronectin-like type II domains of MMP-9 deleted (Fig. 4A), and susceptibility of the activity of the mutant to APP-IP inhibition was tested. As shown in Fig. 4B, the activity of MMP-9-cat-FLAG was inhibited by APP-IP with a very high IC$_{50}$ value (250 μM), suggesting that the catalytic domain contains structural elements unfavorable for its interaction with APP-IP. To explore the region that contains the unfavorable elements,
we constructed chimeric mutants that consisted of various N-terminal parts of the catalytic domain of MMP-2 and the remaining C-terminal parts of that of MMP-9 (Fig. 4A), and susceptibilities of their activities to APP-IP inhibition were compared. As shown in Fig. 4B, the activities of the mutants, named $M_{2n}$-57-M9c and $M_{2n}$-84-M9c, which, respectively, consist of residues 1–56 and 1–83 of MMP-2, and residues 57–162 and 84–162 of MMP-9, were inhibited by APP-IP with almost the same IC$_{50}$ value as that for the inhibition of MMP-9-cat-FLAG. Compared with these mutants, $M_{2n}$-95-M9c showed about a 660-fold greater affinity for APP-IP (IC$_{50}$ = 380 nM), suggesting that the region corresponding to residues 84–95 of the catalytic domain of MMP-9 contains the unfavorable elements. In this region, only four amino acid residues at positions 88, 91, 93, and 94 are different between MMP-2 and MMP-9 (Fig. 5A). To examine which residues are responsible for the unfavorable elements, we replaced these residues of MMP-9-cat-FLAG with the corresponding residues of MMP-2 as shown in Fig. 5A. When these four residues of MMP-2 were grafted into MMP-9-cat-FLAG, the resultant mutant, named MMP-9-cat(84–95M2), showed 195-fold enhanced affinity for APP-IP (IC$_{50}$ = 1.3 μM) compared with that of MMP-9-cat-FLAG (Fig. 5B). On the other hand, replacement of the individual residue Pro88 with Ala or Gln94 with Gly led to an 8.9- or 22-fold reduction in the IC$_{50}$ value, respectively, whereas replacement of Pro91 with Thr did not affect the affinity. When both the Pro88 and Gln94 of MMP-9-cat-FLAG were replaced with Ala and Gly, respectively, the resultant mutant named MMP-9-cat(P88A/Q94G) and MMP-9-cat(84–95M2) showed almost the same affinity for APP-IP (Fig. 5B). Therefore, among the four residues, only Pro88 and Gln94 of the catalytic domain of MMP-9 are likely responsible for the unfavorable elements.

Inhibition of Peptidolytic Activities of Chimeric MMP Mutants by Variants of APP-IP—The data shown in Figs. 3 and 5 strongly suggest that amino acid residues at positions 88, 91, 93, and 94 play a critical role in the selectivity of their interaction with APP-IP. Based on the crystal structures of the catalytic domain of MMP-2 and MMP-9, it is proposed that these residues interact with the inhibitor in a specific manner that is different from the interaction with the substrate.

FIGURE 3. The effects of replacements of amino acid residues at positions 110–122 or 137–152 of chimeric MMP mutants on their affinities for APP-IP. Two series of chimeric mutants were constructed. One of the series consisted of residues 1–109 of MMP-2, 122–174 of MMP-7, and various sequences between residues 110 and 121 (A). The other series consisted of residues 1–136 of MMP-2, 153–162 of MMP-7, and various sequences between residues 137 and 152 (B). The sequences of MMP-2, MMP-7, and the chimeric mutants corresponding to residues 110–121 or 137–152 are shown on the left. The bold letters in the sequence represent the residues that are different between MMP-2 and MMP-7, and the non-bold letters represent the common residues. Among the different residues, the residues identical to those of MMP-2 and MMP-7 are shown in black and gray letters, respectively. Active forms of the chimeric mutants indicated on the left were incubated with 50 μM 3163v at 37 °C for 30 min in the presence of the indicated concentrations of APP-IP as described under “Experimental Procedures.” The amount of 3163v hydrolyzed by each enzyme in the absence of APP-IP was taken as 100%. The enzyme activity on the ordinate is shown as the relative amount of 3163v hydrolyzed by the enzyme (right). The broken and gray lines represent the corresponding activities of MMP-2-cat-FLAG and MMP-7, respectively, which were obtained from the results in Fig. 1. The arrows represent the change in IC$_{50}$ values for inhibition upon replacement of the indicated amino acid residues (star) in the chimeric mutants.
MMP-2, MMP-7, and MMP-9 (37–39), the hydrophobic residues of the MMPs at position 115 are buried into the molecules and interact with internal hydrophobic residues. On the other hand, amino acid residues of the catalytic domain at positions 88 and 94 are located in the non-prime side of the substrate-binding cleft, through which the protease interacts with the residues of the substrate on the N-terminal side of the scissile bond, whereas residues 145–148 are located in the prime side, through which the protease interacts with the residues on the C-terminal side of the scissile bond. Therefore, the residues at positions 88, 94, and 145–148 may interact separately with certain residues of APP-IP. To examine this possibility, we compared the affinities between two pairs of chimeric mutants and variants of APP-IP. One of the pair of mutants compared was MMP-9-cat(P88A/Q94G) and MMP-9-cat(P88A), of which the difference was the residue at position 94 (Fig. 5A), and the other one was M2n-145-M7c and M2n-145-M7n, of which the difference was the residues at positions 145–148 (Fig. 3B). The names and structures of variants of APP-IP tested and their IC₅₀ values for inhibition of the activity of each mutant are listed in Table 1. The effects of modifications of inhibitor on the differences in the IC₅₀ values for inhibition of the activity of each protease before and after replacement of the residue(s) were then compared. As shown in Fig. 6A, deletions

**FIGURE 4.** APP-IP inhibition of peptidolytic activities of chimeric MMP mutants consisting of various parts of MMP-2 and those of MMP-9. A, active forms of the chimeric mutants consisting of various parts of MMP-2 (white columns) and those of MMP-9 (gray columns) are schematically represented. The positions of amino acid residues corresponding to the restriction enzyme sites used for the construction of the chimeric mutants are shown on the top of the scheme of MMP-9-cat-FLAG. The numbers shown at the bottom of the scheme represent the amino acid residue numbers in active MMP-7 numbering. B, active forms of MMP-9-cat-FLAG (●), M2n-57-M9c (○), M2n-84-M9c (△), M2n-95-M9c (□), and M2n-110-M9c (×) were incubated with 50 μM 3163v at 37 °C for 30 min in the presence of indicated concentrations of APP-IP as described under “Experimental Procedures.” The amount of 3163v hydrolyzed by each enzyme in the absence of APP-IP was taken as 100%. The enzyme activity on the ordinate is shown as the relative amount of 3163v hydrolyzed by the enzyme. The broken line represents the corresponding activity of MMP-2-cat-FLAG, which was obtained from the results in Fig. 1. The arrow in B represents the change in IC₅₀ values for the inhibition upon replacement of the indicated amino acid residues in the chimeric mutants that also correspond to the structural difference represented by the arrow in A.

**FIGURE 5.** The effects of replacements of amino acid residues at positions 84–95 of chimeric mutants on their affinities for APP-IP. A series of chimeric mutants consisting of residues 1–83 and 95–162 of MMP-9 and various sequences between residues 84 and 94 were constructed. A, sequences of MMP-2, MMP-9 and the chimeric mutants corresponding to residues 84–95 are shown. The bold letters in the sequence represent the residues that are different between MMP-2 and MMP-9 and the non-bolded letters represent the common residues. Among the different residues, residues identical with those of MMP-2 and MMP-9 are shown in black and gray letters, respectively. B, active forms of the chimeric mutants indicated in A were incubated with 50 μM 3163v at 37 °C for 30 min in the presence of indicated concentrations of APP-IP as described under “Experimental Procedures.” The amount of 3163v hydrolyzed by each enzyme in the absence of APP-IP was taken as 100%. The enzyme activity on the ordinate is shown as the relative amount of 3163v hydrolyzed by the enzyme. The broken line represents the corresponding activity of MMP-2-cat-FLAG, which was obtained from the results in Fig. 1. The arrows represent the changes in IC₅₀ values for the inhibition upon replacement of the indicated amino acid residues (star) in the chimeric mutants.
of N-terminal residues Ile\(^1\) and Ser\(^2\) of APP-IP or replacement of its residue Asn\(^5\) with Ala did not affect significantly the difference in the IC\(_{50}\) values before and after the replacement of residue Gly\(^94\) of MMP-9-cat(P88A/Q94G) with Gln, whereas deletion of the C-terminal Pro\(^{10}\) of the inhibitor led to a significant reduction of the difference, suggesting that the C-terminal residue of APP-IP interacts directly or indirectly with the residue at position 94 of the protease. On the other hand, deletions of the N-terminal residues Ile\(^1\) and Ser\(^2\) or that of the C-terminal Pro\(^{10}\) of APP-IP did not affect significantly the difference in the IC\(_{50}\) values before and after the replacement of residues Tyr\(^{145}\) and Thr\(^{146}\) of M2n-149-M7c with the NGDP sequence. The replacement of residue Asn\(^5\) of APP-IP with Ala slightly reduced the difference in the IC\(_{50}\) values, whereas deletion of Tyr\(^3\) of the inhibitor abolished the difference (Fig. 6B), suggesting that Tyr\(^3\) of APP-IP interacts with the residues at positions 145–148 of the protease.

**APP-IP Sequence Added to the N Terminus of the Catalytic Domain of MMP-2 Works as an Intramolecular Inhibitor**

When one assumes that APP-IP binds to the substrate-binding cleft of the catalytic domain of MMP-2 to make its residues Pro\(^{10}\) and Tyr\(^3\) close to Gly\(^94\) and Tyr\(^{145}\) of the protease, respective

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**FIGURE 6. Effects of modification on the interaction between chimeric MMP mutants and APP-IP variants.**

A, IC\(_{50}\) values for inhibition of the activities of MMP-9-cat(P88A/Q94G) and MMP-9-cat(P88A) by each variant of APP-IP were determined and are listed in Table 1, and the difference in the IC\(_{50}\) values before and after the replacement of the residue Gly\(^94\) with Gln are shown on the ordinate. \(\Delta\), \(\Delta S\), N/A, and \(\Delta P\) represent APP-IP-\(\Delta\), APP-IP-\(\Delta S\), APP-IP-N/A, and APP-IP-\(\Delta P\), respectively, whose structures are also listed in Table 1. B, IC\(_{50}\) values for inhibition of the activities of M2n-149-M7c and M2n-145-M7c by each the variants of APP-IP were determined (listed in Table 1), and the difference in IC\(_{50}\) values before and after the replacement of residues Tyr\(^{145}\) and Thr\(^{146}\) with the NGDP sequence is shown on the ordinate. \(\Delta SY\) represents APP-IP-\(\Delta SY\). C, the crystal structure of the catalytic domain of MMP-2 cited from the Protein Data Bank code 1Q1B is shown in magenta. The residues Gly\(^94\), Tyr\(^{145}\), and Thr\(^{146}\) of the protease are colored yellow. The structure of APP-IP in the extended conformation, shown in orange sticks, is placed to make its residues Pro\(^{10}\) and Tyr\(^3\) close to Gly\(^94\) and Tyr\(^{145}\) of MMP-2, respectively. The residues Tyr\(^3\) and Pro\(^{10}\) of APP-IP are colored light blue, and its residue Asp\(^{6}\) is shown in red. A white arrow with broken line represents the N to C direction of substrate bound to the substrate-binding cleft, and a white arrow with solid line represents that of the placed APP-IP.
tively, the C- and N-terminal sides of APP-IP interact with the non-prime and prime sides of the substrate-binding cleft of MMP-2, respectively (Fig. 6C). Therefore, in the protease-inhibitor complex, the N to C direction of APP-IP relative to the substrate-binding cleft is likely opposite to that of the substrate. Based on the crystal structure of proMMP-2 (37), the C-terminal region of the propeptide, containing the PRCGNPDVAN sequence that precedes the residue Tyr1 of active MMP-2, interacts with its substrate-binding cleft in the N to C direction, also opposite to that of substrate. Considering that the propeptide inhibits the activity of MMP-2 intramolecularly and the C-terminal region of the propeptide and APP-IP interact with the substrate-binding cleft probably in the same N to C direction, it is possible that APP-IP works as the intramolecular inhibitor if it is added to the N terminus of active MMP-2. To test this possibility, we constructed four mutants of MMP-2 as shown in Fig. 7A. The mutants named N-APP-IP-MMP-2-cat-FLAG and N-ins-dProMMP-2-cat-C-APP-IP had no activity. To test this possibility, we constructed four mutants of MMP-2 as shown in Fig. 7A. The mutants named N-APP-IP-MMP-2-cat-FLAG and N-ins-dProMMP-2-cat-C-APP-IP had no activity.

FIGURE 7. The effect of the addition of the APP-IP sequence to the N or C terminus of the catalytic domain of MMP-2 on its activity. A, the construct of the catalytic domain of MMP-2, of which the N or C terminus was fused with APP-IP-containing peptides, is schematically represented. The amino acid sequence preceding the N-terminal Tyr1 of the mature protease or that added to the C terminus instead of the FLAG tag is shown at the bottom of each scheme. The dark gray and light gray columns represent the added regions with and without the APP-IP sequence, respectively. Trp is the site of the proteins susceptible for the trypsin cleavage. *i, the first methionine residue corresponding to the initiation codon; *, the termination codon. B, each of the lysates of E. coli cells expressing N-APP-IP-MMP-2-cat-FLAG (lane 1), N-APP-IP(D/N)-MMP-2-cat-FLAG (lane 2), N-ins-dProMMP-2-cat-C-APP-IP (lane 4) was subjected to SDS-PAGE followed by Coomassie Brilliant Blue R-250 staining (right) and zymographic analysis (left) as described under "Experimental Procedures." Ordinate, molecular mass in kDa. C, the purified N-APP-IP-MMP-2-cat-FLAG (8.7 μM) was digested with trypsin at 37 °C for the indicated length of time. Each sample taken from the reaction mixture was treated with APMSF as described under "Experimental Procedures." The resultant digests equivalent to 0.58 nM N-APP-IP-MMP-2-cat-FLAG and the active form of MMP-2-cat-FLAG (0.58 nM) were separately incubated with 5 μM 3163v at 37 °C for 30 min. The amount of 3163v hydrolyzed by MMP-2-cat-FLAG was taken as 100%. The enzyme activity on the ordinate is shown as the relative amount of 3163v hydrolyzed by each digest. The digests equivalent to 1.4 μg of N-APP-IP-MMP-2-cat-FLAG were also subjected to SDS-PAGE (inset). An arrow at 23 kDa and an arrowhead at 20 kDa in the inset indicate the bands of N-APP-IP-MMP-2-cat-FLAG and its APP-IP-less form, respectively.
APP-IP sequence works as an effective intramolecular inhibitor. However, there is another possibility that the N-terminally added APP-IP sequence interfered with the refolding of the catalytic domain of MMP-2 during the zymographic analysis. To rule out this possibility, we purified the refolded N-APP-IP-MMP-2-cat-FLAG, and its activities before and after trypsin treatment were measured. As shown in Fig. 7C, N-APP-IP-MMP-2-cat-FLAG (23 kDa) was converted to its APP-IP-less form (20 kDa) during incubation with trypsin. Before the incubation, the mutant had almost no peptidolytic activity, whereas the mutant gained the activity together with the conversion. Based on the pattern of SDS-PAGE, the conversion was completed at 80 min of incubation, and the activity of the mutant reached the level comparable to that of MMP-2-cat-FLAG. These data are consistent with the view that the catalytic domain of N-APP-IP-MMP-2-cat-FLAG is refolded correctly, and the N-terminal APP-IP region of the mutant intramolecularly inhibits the activity.

**DISCUSSION**

We explored the amino acid residues of MMP-2 that correlate with the selective inhibition by APP-IP and found that residues Ala^{88}, Gly^{94}, Leu^{115}, Tyr^{145}, and Thr^{146} in the catalytic domain of MMP-2 were involved in the selectivity at least in comparison with the corresponding residues of MMP-7 and those of MMP-9. Among them, Ala^{88} and Gly^{94} are located in the non-prime side of the substrate-binding cleft of MMP-2. In the three-dimensional structures of the catalytic domain of MMPs (37–41), the residues at positions 88 and 94 are close to each other. As APP-IP preferred small residues at these positions to inhibit the activities of the chimeric mutants, the small residues may contribute to provide a space to accommodate the residue Pro^{10} of APP-IP. The catalytic domain of MMP-7 bearing Pro and Gln residues at positions 88 and 94, respectively, showed very low affinity for APP-IP. These bulky residues may hamper the interaction between MMP-7 and APP-IP by steric hindrance. Based on the crystal structure of the catalytic domain of MMP-9 (39), the N-terminal region corresponding to residues 4–7 of the protease partially occupies the non-prime side of the substrate-binding cleft, and the N terminus is also localized close to the residues Pro^{88} and Gln^{94}. In contrast, the N terminus of the catalytic domain of MMP-2 is positioned apart from the residues Ala^{88} and Gln^{94} (Fig. 6C). Therefore, there is another possibility that Pro^{88} and Gln^{94} of MMP-9 stabilize the interaction between its N terminus and the part of the substrate-binding cleft, thereby obstructing the binding of APP-IP. We previously tested the susceptibilities of the activities of humans MMP-2, MMP-7, MMP-9, and MT1-MMP, and rat MMP-3 to APP-IP inhibition and found only the activity of MMP-2 was highly susceptible (21). Among the MMPs tested, MMP-9 and MT1-MMP have Pro and Phe residues at position 88, respectively, whereas other MMPs have Ala at this position. On the other hand, MMP-3 and MMP-9 have Asn and Gln at position 94, respectively, whereas other MMPs have Gly at this position. As is the case of MMP-9, the non-alanine residue at position 88 of MT1-MMP and the non-glycine residue of at position 94 of MMP-3 may also be the structural elements unfavorable for their interaction with APP-IP. Among MMPs, only MMP-7 has aromatic residue at position 115, whereas other MMPs have aliphatic ones (Leu or Ile). In the three-dimensional structures of the catalytic domain of MMPs (37–41), the hydrophobic residues at position 115 are buried into the molecules and interact with other internal hydrophobic residues. Therefore, it is unlikely that the Leu^{115} of MMP-2 interacts directly with APP-IP. We found that the replacement of the residue Phe^{115} of MMP-7 with Leu did not affect the affinity for APP-IP (data not shown), whereas the same replacement of the corresponding residue of the chimeric mutant M2n-110-M7c led to an 8.0-fold enhancement of the affinity (Fig. 3A). Considering that all of the internal hydrophobic residues interacting with the Leu^{115} of MMP-2 is on the N-terminal side of the Leu^{115} and the chimeric mutant M2n-110-M7c contained the N-terminal part of MMP-2, both the residue Leu^{115} and the N-terminal part of MMP-2 are likely important for the high-affinity interaction with APP-IP. The residues at position 115 of MMPs are located at the N-terminal edge of the catalytic helix that contains two histidine residues chelating the catalytic zinc ion and a glutamate residue providing the general acid-base catalyst. The catalytic helix also forms the bottom of the substrate-binding cleft. The interaction between the Leu^{115} and the internal residues of the N-terminal part of MMP-2 may determine the orientation of the catalytic helix, thereby determining the shape of the substrate-binding cleft to which APP-IP preferentially binds. The residues Tyr^{145} and Thr^{146} are located in the prime side of the substrate-binding cleft of MMP-2. Because Tyr^{145} is directed inside of the cleft, whereas Thr^{146} is directed outside, Tyr^{145} is a candidate for the residue with which APP-IP interacts directly. The residue Tyr^{7} of APP-IP may interact with Tyr^{145} of MMP-2 by hydrophobic or ring-ring interaction. When Tyr^{145} and Thr^{146} of the chimeric mutant M2n-110-M7c were replaced with Asn and Gly, respectively, its affinity for APP-IP was indeed reduced significantly (Fig. 3B). MMP-3 and MMP-7, respectively, have Ser and Asn at position 145, whereas MMP-2, MMP-9, and MT1-MMP, respectively, have Tyr, Phe, and Trp at this position. As is the case of MMP-7, the non-aromatic residue at position 145 of MMP-3 may also be the unfavorable structural element for its interaction with APP-IP.

As described above, most MMPs, except for MMP-2, likely have the unfavorable elements in either the non-prime or prime side of the substrate-binding cleft or in both the sides. Although both sides of the substrate-binding cleft of MMPs provide structural variation, several MMPs have common or analogous structures in either side of the cleft. Therefore, inhibitors designed to occupy one side of the substrate-binding cleft of MMP may be the group-specific MMPs inhibitors, but may not be specific ones of individual proteases. The peptide mimetic small synthetic inhibitors such as the hydroxamate-, barbiturate-, sulfodiimine-, and thiadiazole-based inhibitors have been designed to be accommodated in one side of the substrate-binding cleft of MMP (7–10), and they are indeed not specific inhibitors of individual MMPs. The residues of MMP-2 found in the present study to correlate with selective interaction with APP-IP were located relatively far from the catalytic zinc ion (Fig. 6C). The residues Ala^{88} and Gly^{94} of MMP-2 probably contribute to form its S4 subsite, and Tyr^{145} is close to the S4’...
subsite of the protease. The thiazodazole-based inhibitors that have relatively high selectivity toward MMP-3 interact with the S4 subsite of the protease (10), whereas none of the other synthetic inhibitors interacts with the S4 or S4’ subsite of MMPs. This may be another reason why the small synthetic inhibitors have broad specificity toward MMPs. Protein engineering of TIMPs (42–44) may be an effective strategy to produce specific inhibitors for individual MMPs, because the protein inhibitors have wide range contact with MMPs to inhibit their activities; the contact regions of MMPs include both the non-prime and prime sides of the substrate-binding cleft and exosites. As APP-IP likely interacts with both sides of the substrate-binding cleft of MMP-2, substitutions of its amino acid residues may convert it into the inhibitors that have high selectivity toward other MMPs. This possibility is currently under investigation in our laboratory.

Studies of the relationship among effects of modifications on the interaction between chimeric MMP mutants and APP-IP variants (Fig. 6) suggest that the N to C direction of APP-IP relative to the substrate-binding cleft of the MMP-2 is analogous to that of the propeptide in proMMP-2 and opposite to that of substrate. When the sequence of APP-IP was added to the N terminus of the catalytic domain of MMP-2, the resultant mutant, named N-APP-IP-MMP-2-cat-FLAG, showed no activity (Fig. 7). The APP-IP region of the mutant probably works as an intramolecular inhibitor, similar to the propeptide. This inhibition is unlikely caused by the intermolecular interaction, because the concentration of the mutant bearing APP-IP (0.58 nm) in the assay system was about 100-fold lower than the IC50 value (65 nm) for APP-IP inhibition of the activity of MMP-2-cat-FLAG. At the low concentration of APP-IP, the activity of the protease will not be affected significantly. We also constructed a variant of N-APP-IP-MMP-2-cat-FLAG, which had the catalytically essential residue Glu122 replaced with Ala, and found that this APP-IP-bearing mutant had no inhibitory activity toward MMP-2-cat-FLAG (data not shown). The data were also consistent with the view that the N-terminally added APP-IP region interacts intramolecularly with the substrate-binding cleft of the mutant; therefore, cannot bind intramolecularly to the active site of MMP-2-cat-FLAG. As the addition of APP-IP to the N terminus of the protease restricts the N to C direction of the peptide inhibitor, the observed intramolecular inhibition also supports the proposal that the direction of interaction of APP-IP with the cleft of MMP-2 is opposite to that of substrate. As reported previously, APP-IP is resistant to MMP-2 cleavage (21). We speculate that the peptide bonds of APP-IP are not presented appropriately to the catalytic center of MMP-2 because of the direction of interaction; the mode of interaction, therefore, supports the inhibitory action of the peptide inhibitor. Unlike peptide-mimetic synthetic inhibitors, APP-IP does not have artificial zinc-chelating group like hydroxamate. The residue Asp6 of the inhibitor is accessible to the catalytic zinc ion (Fig. 6C) by the assumption that its residues Pro10 and Tyr3 are close to Gly94 and Tyr145 of MMP-2, respectively. As the residue Asp6 is the most important residue for the inhibitory activity of APP-IP (21), the residue may interact with the catalytic zinc of MMP-2. However, clarification of the detailed mechanism of the inhibition must await analysis of the crystal structure of the MMP-2-APP-IP complex. Considering that TIMPs and peptide-mimetic inhibitors as yet designed interact with the active site of MMPs in the direction analogous to substrate and have broad enzyme specificity, involvement of the inversely directed interaction of APP-IP in MMP-2-selective inhibition provides insights into designing specific inhibitors for individual MMPs.

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