Endostatin binds to the catalytic domain of matrix metalloproteinase-2

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Abstract We previously reported that endostatin inhibits endothelial and tumor cellular invasion by blocking activation and catalytic activity of matrix metalloproteinase (MMP)-2. Here we have examined the domain of proMMP-2 responsible for the binding of endostatin using surface plasmon resonance. ProMMP-2 and proMMP-2ΔHP lacking the hinge and hemopexin-like (HP) domains bound little to the immobilized endostatin. The active MMP-2 and MMP-2ΔHP, but not the HP domain of MMP-2, bound to endostatin at similar levels. In addition, preincubation of MMP-2 and MMP-2ΔHP with the MMP inhibitor actinonin, which binds to the active site of MMP-2, abolished their bindings to endostatin. These results indicate that endostatin binds to neither the latent proMMP-2 nor the HP domain but to the catalytic domain of MMP-2.

Key words: Active site; Catalytic domain; Endostatin; MMP-2; Surface plasmon resonance

1. Introduction

Endostatin, the 20-kDa C-terminal proteolytic fragment of collagen XVIII, was first identified in the conditioned media of hemangioendothelioma cells as an anti-angiogenic molecule [1]. Administration of the recombinant endostatin or endostatin gene therapy has been shown to suppress tumor growth and angiogenesis in mice of various tumor models [2,3]. On the cellular level, it was reported that endostatin inhibits endothelial cell proliferation [1] and migration [4,5] and induces endothelial cell apoptosis [6] and cell cycle arrest [7]. However, molecular targets and action mechanisms of endostatin have not been clearly elucidated.

Matrix metalloproteinases (MMPs), a family of zinc-dependent endopeptidases, are involved in angiogenesis and tumor metastasis [8]. MMP-2 can degrade various components of the extracellular matrix including type IV collagen, a major component of basement membranes. Like other MMPs except membrane types, MMP-2 is produced in a latent form (proMMP-2) requiring activation. The catalytic activity of MMP-2 is largely inhibited by tissue inhibitor of metalloproteinase (TIMP)-2 that binds to the active MMP-2 as well as proMMP-2 at 1:1 stoichiometric ratio [9], although TIMP-2 at a low concentration enhances activation of proMMP-2 at the cell surface [10]. It has been recently shown that tumor progression and angiogenesis were reduced in MMP-2-deficient mice [11] and that MMP-2 was required for the switch to the angiogenic phenotype in a tumor model [12].

We previously reported that endostatin inhibits endothelial and tumor cellular invasion by blocking the activation and catalytic activity of MMP-2 [13]. Here we have determined which domain of proMMP-2 interacts with endostatin by surface plasmon resonance (SPR) analysis.

2. Materials and methods

2.1. Preparation of the recombinant mouse endostatin and human TIMP-2

The recombinant mouse endostatin corresponding to the C-terminal 184 amino acids of mouse collagen XVIII was expressed in HEK293 cells and purified with heparin-Sepharose CL-6B column (Amersham Biosciences) and Superdex 75 column (Amersham Biosciences) chromatography as described previously [13]. The purified endostatin was dialyzed in phosphate-buffered saline (PBS) and stored at −70°C.

The recombinant human TIMP-2 was expressed in Sf9 cells with infection of the TIMP-2 baculovirus [14]. Purification of the recombinant TIMP-2 was followed by a method described [15].

2.2. Preparation of proMMP-2, proMMP-2ΔHP, GST-fused MMP-2 HP domain, and GST

The recombinant human proMMP-2 was expressed in Sf9 cells with infection of the 72Gel baculovirus and purified with gelatin–agarose column chromatography as described previously [16]. The human proMMP-2 lacking the hinge and hemopexin-like (HP) domains (proMMP-2ΔHP), which was processed in the medium of the Sf9 cells infected with the 72Gel baculovirus, was enriched by gelatin–agarose column chromatography. To purify proMMP-2ΔHP, the eluted proteins were subjected to gel filtration chromatography using a Superdex 75 column equilibrated with an MMP assay buffer (20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 5 mM $CaCl_2$, 100 μM $ZnCl_2$, and 0.025% Brij 35). pGEX-4T-1-MMP2-HP was constructed by the introduction of a cDNA fragment encoding the MMP-2 HP domain (Cys469–Cys660) into *Eco*RI–*Xho*I sites of pGEX-4T-1. The GST-fused MMP-2 HP domain and GST were expressed in *E. coli* containing pGEX-4T-1-MMP-2-HP and pGEX-4T-1, respectively, and purified by Glutathione-Sepharose 4B (Amersham Biosciences) column chromatography following the manufacturer's manual.

2.3. SDS–PAGE and gelatin zymography

Proteins were separated by SDS–PAGE and visualized by staining

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Abbreviations: APMA, 4-aminophenylmercuric acetate; HP, hemopexin-like; MMP, matrix metalloproteinase; PBS, phosphate-buffered saline; RU, response unit; SPR, surface plasmon resonance; TIMP, tissue inhibitor of metalloproteinase

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with Coomassie brilliant blue R-250. Gelatin zymography was performed in 10% SDS–PAGE containing 0.1% gelatin as described [16].

2.4. Catalytic activity of MMP-2 and MMP-2ΔHP

The purified proMMP-2 and proMMP-2ΔHP were activated in the presence of a final concentration of 1 mM 4-aminophenylmercuric acetate (APMA) at 37°C for 30 min. The APMA-activated enzymes (10 nM) were incubated in 50 μl of the MMP assay buffer containing 1 μM of a quenched fluorescent peptide, Mca-Pro-Leu-Gly-Leu-Dap(Dnp)-Ala-Arg-NH_2 (M1895, Bachem), as a substrate at 37°C for 30 min [17]. The reaction was stopped by the addition of 0.1 M sodium acetate (pH 4.0) at final concentration. The fluorescence was measured at excitation wavelength 328 nm and emission 393 nm.

2.5. SPR analysis using BIAcore

Bindings between endostatin or TIMP-2 and various MMP-2 species were analyzed by SPR technology using BIACORE 3000 (BIAcore) as described [18] with a minor modification. A sensor chip CM5, which consists of carboxymethylated dextran on a gold thin layer, was used for all experiments. The surface was activated by injecting a mixture of 0.2 M N-ethyl-N′-dimethylaminopropyl-carbodiimide and 0.05 M N-hydroxysuccinimide. After activation, endostatin or TIMP-2 in a coupling buffer (10 mM sodium acetate, pH 5.0) was injected for immobilization to the chip surface. The activated surface that was not immobilized with a ligand was blocked by 1 M ethanolamine–HCl (pH 8.5). PBS and the MMP assay buffer were used as running buffers during immobilization and binding analysis, respectively. In typical experiments, each immobilization level of endostatin and TIMP-2 was about 2200 response units (RU).

One hundred nM of analyte (latent or active MMP-2, latent or active MMP-2ΔHP, GST-fused MMP-2 HP domain, or GST) was injected to pass through the unmodified reference flow cell, the flow cell immobilized with endostatin, and then the flow cell immobilized with TIMP-2, for 2 min at a flow rate of 5 μl/min. For regeneration, proteins bound to ligands were removed by 1-min pulse injections of 3.3 mM NaOH and 133 mM NaCl for the endostatin flow cell and 20 mM HCl for the TIMP-2 flow cell. These regeneration conditions allowed for the retention of greater than 95% of the original capacity for binding to the active MMP-2. To correct for bulk shift, data in the reference flow cells were subtracted from experimental data.

3. Results and discussion

3.1. Preparation and characterization of the human proMMP-2 and its domains

In an attempt to determine which domain of MMP-2 inter-

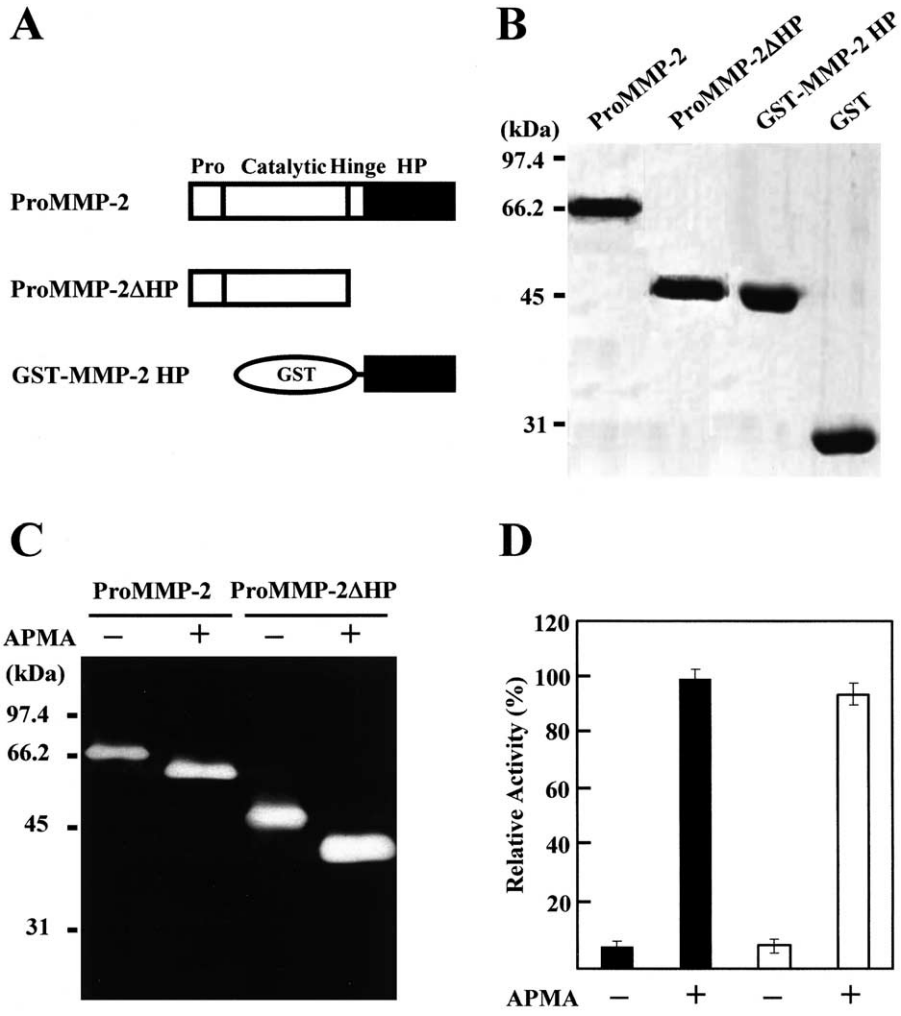

Fig. 1. Characterization of the purified proMMP-2, proMMP-2ΔHP, and GST-fused MMP-2 HP domain. ProMMP-2, proMMP-2ΔHP, and GST-fused MMP-2 HP domain (GST-MMP-2 HP) are schematically represented (A). The purified proMMP-2, proMMP-2ΔHP, GST-fused MMP-2 HP domain, and GST were analyzed by 10% SDS–PAGE under reducing condition (B). The purified proMMP-2 and proMMP-2ΔHP without (−) or with (+) APMA activation were analyzed in gelatin zymography (C). Catalytic activities of the purified proMMP-2 (black bar) and proMMP-2ΔHP (white bar) without (−) or with (+) APMA were measured by fluorogenic peptide cleavage assay (D). The catalytic activity of each enzyme (10 nM) was expressed as a relative activity to that of the active MMP-2. Each value is the mean ± standard deviation of three independent determinations of duplicate experiments.

acts with endostatin, we have purified the human proMMP-2, proMMP-2ΔHP, GST-fused HP domain, and GST (Fig. 1A). The 68-kDa proMMP-2 was purified from the supernatant of the Sf9 insect cells infected with the 72Gel baculovirus (Fig. 1B). A 48-kDa product of proMMP-2 generated during expression of proMMP-2 in the Sf9 cells [16] was purified (Fig. 1B). The N-terminal sequence of the 48-kDa product was the same as that of proMMP-2 (Ala-Pro-Ser-Pro-Ile...). The molecular mass of the 48-kDa product which was determined using MALDI-TOF mass spectrometry was *m/z* 47,163 Da [$M+H^+$]. From these mass and N-terminal sequencing data, the 48-kDa product was expected to be the Ala30–Asp450 of proMMP-2. Since this region contains the pro- and catalytic domains of proMMP-2, it was named proMMP-2ΔHP. The 47-kDa GST-fused MMP-2 HP domain (Cys469–Cys660) and the 26-kDa GST were expressed as soluble proteins in *E. coli* and purified (Fig. 1B).

Upon APMA activation, the 48-kDa proMMP-2ΔHP was converted to the 42-kDa gelatinolytic form, analogous to conversion of the 68-kDa proMMP-2 to the 62-kDa active MMP-2 (Fig. 1C). As expected from the presence of pro-domain, the catalytic activities of both proMMP-2 and proMMP-2ΔHP, without APMA activation, were at background levels in fluorogenic peptide cleavage assay (Fig. 1D). In addition, the catalytic activity of the APMA-activated proMMP-2ΔHP was comparable to that of the APMA-activated proMMP-2, that is, the active MMP-2. These results imply that the 42-kDa gelatinolytic form processed from proMMP-2ΔHP is the active MMP-2ΔHP.

3.2. Interaction of endostatin with the latent proMMP-2 and proMMP-2ΔHP

It has been shown that proMMP-2 binds to TIMP-2 with high affinity through electrostatic interactions between the HP domain of MMP-2 and the C-terminal domain of TIMP-2 [19]. In addition, a truncated form of proMMP-2 lacking the C-terminal domain (Ala447–Cys660), similar to our proMMP-2ΔHP, was found to bind poorly to TIMP-2 [20]. To examine whether endostatin interacts with proMMP-2 or proMMP-2ΔHP, these proteins were allowed to bind to the immobilized endostatin and examined by SPR. In addition, these proMMP-2 species were also applied to the immobilized TIMP-2 as a control. As shown in Fig. 2B, D, show that the binding of proMMP-2 to TIMP-2 was strong (2,000 RU at the end of the association phase) but the binding of proMMP-2ΔHP to TIMP-2 was very weak (50 RU). In the same condition, bindings of both proMMP-2 and proMMP-2ΔHP to endostatin were barely detectable (only 15 RU and 10 RU, respectively; Fig. 2A, C). These results indicate that only a trace amount of the proMMP-2 species, regardless of the presence of the HP domain, binds to endostatin.

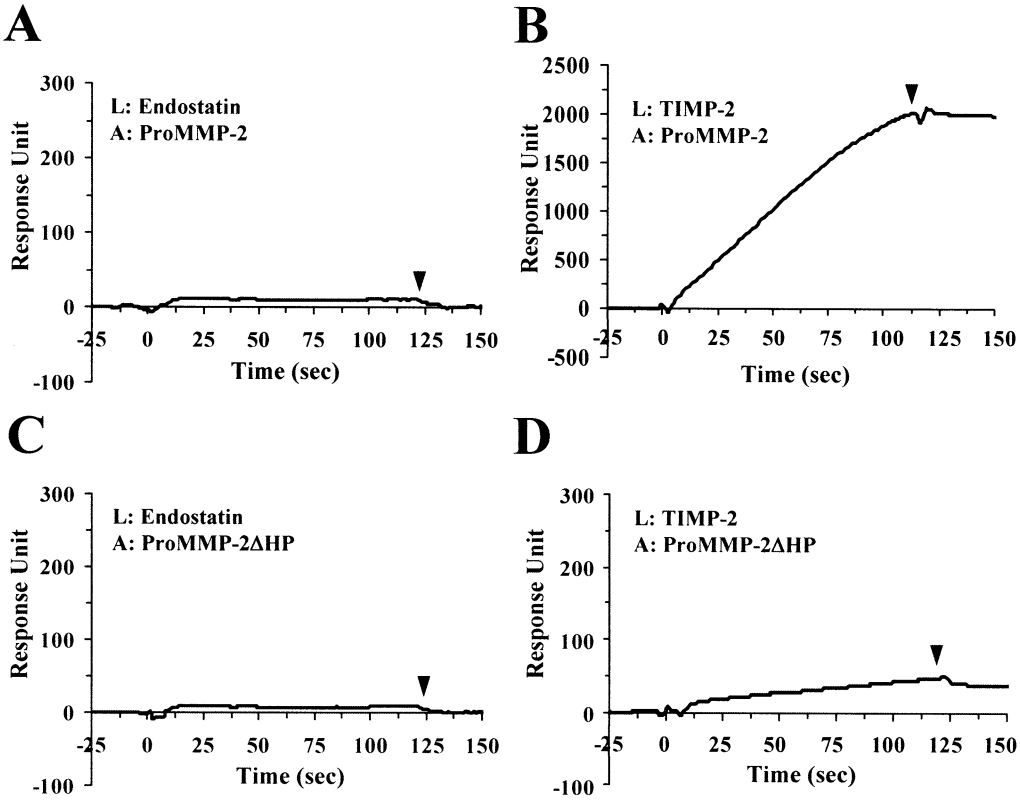

Fig. 2. Interaction between endostatin and either proMMP-2 or proMMP-2ΔHP. Sensorgrams of proMMP-2 (A, B) and proMMP-2ΔHP (C, D) binding to the immobilized endostatin or TIMP-2 as a control were shown. The enzymes (100 nM) were allowed to bind endostatin (A, C) or TIMP-2 (B, D) and their bindings were examined by SPR analysis as described in Sec. 2. Each enzyme was run in duplicate, the data were corrected for bulk shift, and the average of the duplicate experiments was plotted. The arrowhead indicates the end of the association phase. L, ligand; A, analyte.

3.3. Interaction of endostatin with active MMP-2, active MMP-2ΔHP, and the HP domain of MMP-2

To examine which domain of MMP-2 is responsible for binding to endostatin, the active MMP-2, the active MMP-2ΔHP (catalytic domain), and the GST-fused MMP-2 HP domain were allowed to bind to the immobilized endostatin and examined by SPR. TIMP-2, which is known to bind to the catalytic and HP domains with low and high affinities, respectively [19,21], was used as a positive control. As expected, the active MMP-2 as well as the GST-fused MMP-2 HP domain bound strongly to TIMP-2 (Fig. 3A, C), but this binding was significantly reduced by the deletion of its HP domain (active MMP-2ΔHP) (Fig. 3B). Unlike the latent forms of MMP-2, the active MMP-2 apparently bound to endostatin although its binding was much weaker compared to TIMP-2 (Fig. 3A). The active MMP-2ΔHP also bound to endostatin (Fig. 3B). The RU for the active MMP-2 at the end of the association phase (215 RU) was slightly higher than that for the active MMP-2ΔHP (175 RU). Considering that the active MMP-2 and the active MMP-2ΔHP are 62 kDa and 42 kDa, respectively, their binding affinities to endostatin are likely to be similar. Moreover, neither the GST-fused MMP-2 HP domain (Fig. 3C) nor GST (data not shown) bound to endostatin. These results indicate that endostatin binds to the catalytic domain of MMP-2 but not to the HP domain of MMP-2. Unfortunately, we could not determine the dissociation equilibrium constant for the interaction of endostatin with the active MMP-2 species in SPR analysis, mainly due to rapid autodegradation of MMP-2 species at higher concentrations.

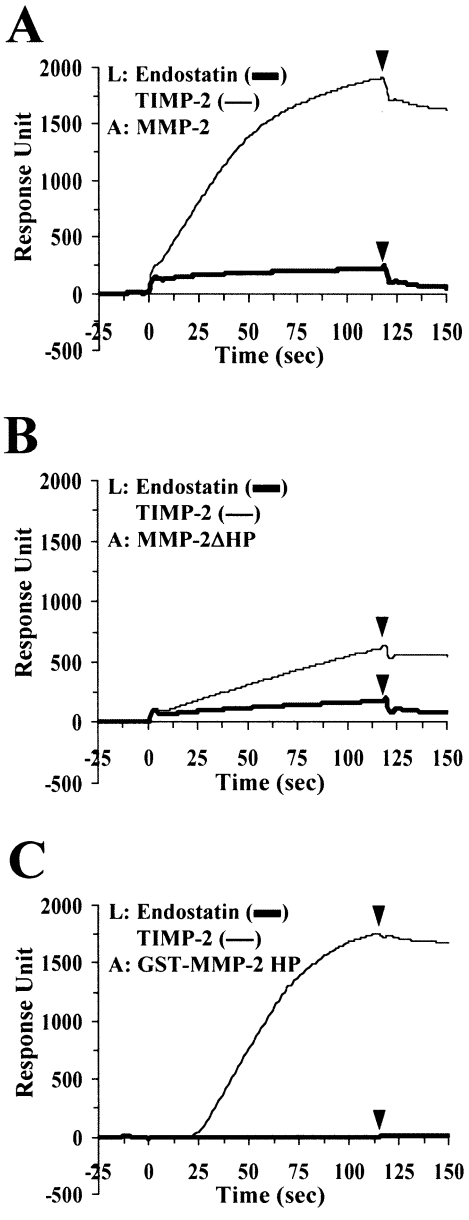

Fig. 3. Interaction of endostatin with the active MMP-2, the active MMP-2ΔHP, and the GST-fused MMP-2 HP domain. Sensorgrams of the active MMP-2 (A), the active MMP-2ΔHP (B), and the GST-fused MMP-2 HP domain (C) binding to the immobilized endostatin (thick line) or TIMP-2 as a control (thin line) were shown. Experimental procedures are described in Fig. 2.

3.4. Effect of a hydroxamate peptide analog inhibitor of MMPs in interaction between Endostatin and MMP-2

To examine whether the interaction between endostatin and the catalytic domain of MMP-2 occurs at the active site of MMP-2, the active MMP-2 and MMP-2ΔHP preincubated with a natural hydroxamate–pseudopeptide MMP inhibitor, actinonin, were tested for their interaction with endostatin. Actinonin is known to bind to the active site of MMPs by the hydroxamate group chelating the catalytic zinc ion and pseudopeptide side chains interacting with substrate-binding subsites [22]. As shown in Fig. 4A, B, preincubation of the active MMP-2 and MMP-2ΔHP with actinonin completely prevented their binding to endostatin. However, the binding of the active MMP-2 to TIMP-2 was slightly decreased by preincubation of actinonin (Fig. 4C). The binding of the active MMP-2ΔHP to TIMP-2 was also partially reduced by actinonin (Fig. 4D). Although a complex structure of MMP-2 and TIMP-2 has not been reported, it is known that TIMP-2 interacts with the catalytic domain of MMP-14 through six separate segments, including interaction of the N-terminal five TIMP-2 residues with the P1–P4′ subsites of the MMP-14 active site [23]. Thus, only a partial decrease of the interaction of the MMP-2ΔHP and TIMP-2 in the presence of actinonin reflects that TIMP-2 is still able to interact with the actinonin-bound MMP-2ΔHP at sites other than the occupied active site. However, since actinonin completely blocked the interactions of endostatin with the active MMP-2 species (Fig. 4A, B), it is most likely that endostatin binds to the active site of MMP-2.

In a previous study, we showed that endostatin was co-precipitated with proMMP-2 in a mixture of these proteins [13]. Therefore, it seems that binding of a trace amount of proMMP-2 to endostatin in SPR analysis would not be an artifact. It was reported that the purified proMMP-2 at a higher concentration is autoactivated in the absence of external activators [24]. This finding suggests that the 'active' proMMP-2 in which the pro-domain is dissociated from the active site should be present at least in a trace amount in a proMMP-2 population. In addition, we show here that the catalytic domain of MMP-2 binds to endostatin and that the unoccupied active site of MMP-2 is required for endostatin binding. It is thus likely that proMMP-2 complexed with endostatin that was detected by sensitive immunoprecipitation/Western blot analyses and SPR analysis would be the

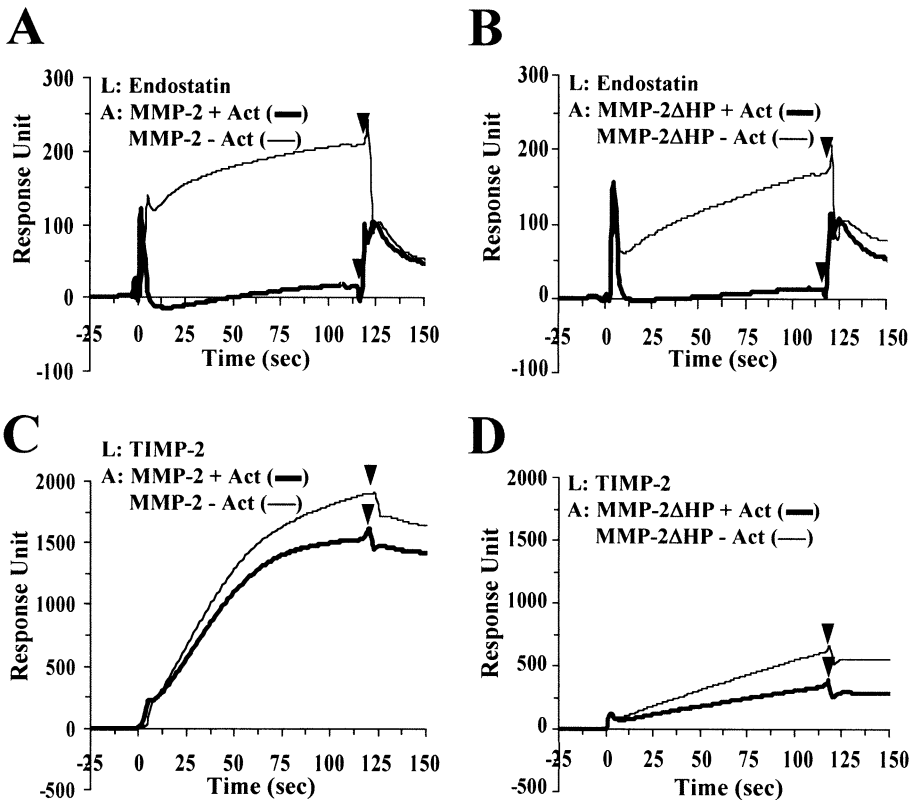

Fig. 4. Effect of the MMP inhibitor actinonin on interaction between endostatin and either the active MMP-2 or the active MMP-2ΔHP. The active MMP-2 (A, C) and MMP-2ΔHP (B, D) (100 nM) which were preincubated with (thick line) or without (thin line) 7 μM of actinonin (Act) were allowed to bind endostatin (A, B) and TIMP-2 (C, D). Experimental procedures are described in Fig. 2.

active proMMP-2 present in a trace amount. We therefore believe that the latent proMMP-2 does not bind to endostatin.

Here we have shown that endostatin binds only to the catalytic domain of MMP-2, while TIMP-2 binds to MMP-2 through interactions with its HP domain at high affinity and with its catalytic domain at low affinity. Although the interaction of the MMP-2 catalytic domain with endostatin was somewhat weaker than that with TIMP-2, it seems possible that endostatin inhibits MMPs in the stromal area surrounding tumors in which the concentration of MMPs overwhelms that of TIMPs. Therefore, our findings suggest that the potent anti-angiogenic and anti-tumor activities of endostatin are at least in part associated with its ability to inhibit the activities of MMP-2 and other MMPs through direct interaction with their catalytic domains.

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