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Structural Requirements For Bone Sialoprotein Binding And Modulation Of Matrix Metalloproteinase-2

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Abstract

Bone sialoprotein (BSP) has been shown to induce limited gelatinase activity in latent matrix metalloproteinase-2 (MMP-2) without removal of the propeptide and to restore enzymatic activity to MMP-2 previously inhibited by tissue inhibitor of matrix metalloproteinase-2 (TIMP2). The current study identifies structural domains in human BSP and MMP-2 that contribute to these interactions. The 26 amino acid domain encoded by exon 4 of BSP is shown by a series of binding and activity assays to be involved in the displacement of MMP-2′s propeptide from the active site and thereby inducing the protease activity. Binding assays in conjunction with enzyme activity assays demonstrate that both amino- and carboxy-terminal domains of BSP contribute to restoration of activity to TIMP2-inhibited MMP-2, while the MMP-2 hemopexin domain is not required for reactivation.

A family of secreted proteins, termed SIBLINGs[1] (for *s*mall *i*ntegrin-*b*inding *li*gand, *N*-linked *g*lycoproteins), share many exon-related motifs including the integrin-binding tripeptide, Arg-Gly-Asp (RGD), as well as several conserved phosphorylation and glycosylation sites (1,2). Family members include bone sialoprotein (BSP), osteopontin (OPN), dentin matrix protein 1 (DMP1), matrix extracellular phosphoglycoprotein (MEPE), and dentin sialophosphoprotein (DSPP) (1). All of the genes are in a tandem cluster on human chromosome 4 and were first observed to be expressed in bones and teeth but have since been reported to all be expressed in active ductal epithelial cells (3). SIBLINGs are also induced in certain neoplasms (4–7). SIBLINGs can propagate biological signals by acting as integrin ligands initiating integrin signaling intracellularly and by binding and sequestering other proteins to the cell surface altering extracellular interactions (4). For example, BSP is a cell surface-associated protein through its binding of αvβ3 integrin (8,9). BSP can bind complement factor H and tether it to the cell surface where factor H prevents alternate complement-mediated cell lysis (8,10). In addition, BSP binds to and modulates the activity of matrix metalloproteinase- 2 (MMP-2) (11).

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[1]The abbreviations used are: SIBLING, Small, Integrin-Binding LIgand, N-linked Glycoprotein; RGD, arginine-lysine-aspartate; BSP, bone sialoprotein; OPN, osteopontin; DMP1, dentin matrix protein-1; MMP, matrix metalloproteinase; TIMP, tissue inhibitor of metalloproteinase; mMMP2, mutant hemopexin-free matrix metalloproteinase; DTNB, 5,5′-dithio-bis-2-nitrobenzoic acid; HRP, horseradish peroxidase; BSPΔx4, bone sialoprotein with exon 4 deleted.

MMP-2 is a member of a family of structurally and functionally related endoproteinases involved in development and tissue repair as well as cancer angiogenesis and metastasis. We have shown that latent proMMP-2 can be at least partially activated through the binding of BSP (11). More recently, we have shown that BSP binding to MMP-2 significantly reduced the affinity of both small molecular weight synthetic inhibitors (ilomastat and oleoyl-*N*-hydroxylamide) and the large molecular weight tissue inhibitor of metalloproteinase-2 (TIMP2) (12). The current study was undertaken to determine sequences in both BSP and MMP-2 involved in the binding and modulation of MMP activity.

MATERIALS AND METHODS

Reagents

ProMMP-2 and active human MMP-2 were obtained from Oncogene Research Products (Boston, MA) and Research Diagnostic Systems, Inc. (Minneapolis, MN). Recombinant human MMP-2 lacking the hemopexin domain was purchased from Biomol Research Laboratories, Inc. (Plymouth Meeting, PA). Small molecular weight MMP-2 substrate Ac-PLG-[2-mercapto-4-methylpentanoyl]-LG-OC_2H_5 and 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) were obtained from Calbiochem (La Jolla, CA). TIMP2 was a generous gift of Dr. H. Birkedal-Hansen, NIDCR, NIH. Human serum-adsorbed goat anti-rabbit IgG conjugated to horseradish peroxidase (HRP) was obtained from Kirkegaard and Perry (Gaithersburg, MD). Recombinant human BSP that included posttranslational modifications was made using an adenovirus construct and eukaryotic cells and purified (>95% purity as defined by acrylamide gel electrophoresis) as previously described (13). The synthesized and purified polypeptide VFKYRPRYYLYKHAYFYPHLKRFPVQ, corresponding to exon 4 of BSP, was obtained from the Center for Biologics Evaluation and Research, Food and Drug Administration.

Recombinant BSP-SIBLING Chimera/Mutants in Replication- Deficient AdenoVirus Constructs

BSP chimera were made by separately amplifying the cDNA encoding the amino terminus (exons 2–5) of human BSP, human OPN, or bovine DMP1 and selectively combining them to PCR products containing the remaining coding sequences for each SIBLING (Figure 1). The 5′ PCR products' sense strand oligos also incorporated an Asp718 restriction site 5′ to the Kozak/start Met sequences. The antisense strand oligos for the 5′ PCR constructs at the end of exon 5 included blunt end-producing restriction sites (*Bsr*BI for BSP and OPN and *PVu*II for DMP1). The sense strand oligos for the carboxyterminal- encoding 3′ PCR products all incorporated blunt end-producing restriction sites (*Sca*I for BSP and OPN and *Fsp*I for DMP1) for ligation to the 5′ PCR products, and all contained *Bam*HI restriction sites 3′ to their stop codons. All PCR reactions were done with high fidelity enzyme (Turbo Pfu; Stratagene). All base changes required to introduce the blunt cutter sites always maintained the same amino acid (e.g., Leu -> Leu). The two PCR fragments for each SIBLING were first treated with alkaline phosphatase (to make the ends unable to ligate), purified, and then digested with their respective blunt-cutting restriction enzymes to create new, ligation-competent blunt ends. The 5′ PCR product of BSP was ligated with the 3′ PCR product of OPN (denoted BSP/OPN) or DMP1 (BSP/DMP1). Similarly, the 3′ PCR product of BSP was ligated to the 5′ end of OPN (OPN/BSP) or DMP1 (DMP1/BSP). The ligation product was subjected to PCR using the appropriate 5′-most (containing the Asp718 site) and 3′-most (containing the *Bam*HI site) oligo for each chimera. Bands of the correct size were gel purified, digested with Asp718 and *Bam*HI, ligated into Asp718/*Bam*HI-digested vector (within the multipurpose cloning site between the CMV promoter and the SV40 polyA/message stabilization 3′ flanking mRNA domain of the pACCMV.pLpA adenovirus shuttle plasmid), and transformed into competent *E. coli* cells. Successful plasmids were sequenced and used in a standard recombination protocol to make adenovirus particles in HEK293 cells. Chimeric proteins were purified from

the serum-free media of the adenovirus-infected human marrow stromal fibroblast using standard ion-exchange chromatography procedures previously described for individual SIBLING proteins (10).

The BSP construct lacking exon 4 (BSPΔx4) was made using the Stratagene in situ mutagenesis kit and complementary oligonucleotides whose 5′ end encoded bases from the end of exon 3 and whose 3′ end encoded the beginning bases of exon 5 as per the manufacturer's instructions. Limited PCR amplification of the BSP insert in pBluescript was performed, and the original template plasmids were destroyed by *Dpn*I. After transformation, single *Escherichia coli* colonies on ampicillin plates were selected and grown in LB-amp media, the plasmid was purified, and the loss of exon 4 sequences in the SIBLING sequence was verified by DNA sequencing. The modified SIBLING insert was shuttled into the adenovirus plasmid, and the virus was prepared, with the protein made and purified as previously described (10).

Fluorescent Binding Studies

Intrinsic tryptophan fluorescence binding studies of wild-type and as well as the noted chimeric BSP constructs with both proMMP-2 and active MMP-2, and hemopexin-deleted MMP-2 mutant, were carried out as previously described (11). BSP contains no tryptophan groups while the chimeric BSP constructs (BSP/OPN, BSP/DMP1, OPN/BSP, and DMP1/BSP) have 1 tryptophan, MMP-2 contains 15 tryptophan residues, and hemopexin-deleted MMP-s contains 8 tryptophan residues. Briefly, the relative change in fluorescence in the area under the emission curve (300 to 500 at 295 nm excitation) was used to determine fractional acceptor saturation (f_a) as a function of nanomolar BSP added by calculating $f_a = (y - y_f)/(y_b - y_f)$, where y_f and y_b are the area under the curve of the fluorescent emission profile of free and fully bound MMP-2.

Solid-Phase Binding Assays

The binding of BSP to immobilized proMMP-2, active MMP-2, or hemopexin-deleted MMP-2 was measured by an indirect sandwich assay. Plates were coated with the different forms of MMP-2 by adding 0.1 mL of 3.5 nM recombinant purified MMP-2 in 50 mM $NaHCO_3$, pH 9.0, to each well of a Greiner High- Binding 96-well microtiter plate and incubated overnight at 4 °C. The plates were blocked with 5% (w/v) nonfat dry milk in Tris-buffered saline (TBS) containing 50 mM Tris, pH 7.4, and 150 mM NaCl for 60 min and then rinsed three times with TBS containing 0.05% Tween 20. BSP was added in nanomolar equivalents in TBS-Tween and incubated for 120 min at room temperature. After a second round of three washes, bound ligand was quantified by the addition of a 1:50000 dilution of specific rabbit anti-BSP antibody, LF100 (14), followed by a 60 min incubation. After three washes, secondary antibody (1:2000 goat anti-rabbit HRP-conjugated antibody) was added and incubated for a further 60 min. Color was developed using 3,3,5,5-tetramethylbenzidene substrate, and the absorbance at 405 nm was measured. Nonspecific binding was measured by determining the ligand binding to wells coated with 1% bovine serum albumin during the overnight coating step, and these values were subtracted from the corresponding values for MMP-coated wells.

High Molecular Weight Substrate Studies

Fluorescein-conjugated gelatin (Molecular Probes, Inc., Eugene, OR) substrate was used to follow gelatinase activity as previously described (11). This substrate is highly substituted with fluorescein moieties such that the fluorescent signal is self-quenched until proteolytic cleavage liberates fragments and a robust fluorescent emission is measured. For studies of proMMP-2 activation, 5 μg/mL large fluorescein-conjugated substrate was incubated with 10 nM proMMP-2 ± 10 nM BSP (or BSP chimera). For inhibition studies, typical reaction mixtures consisted of the 10 μg/mL fluorescein-substrate conjugate with 1.4 nM mutant hemopexin-free MMP-2 reacted with either 10 nM TIMP2, 10 nM TIMP2 + 10 nM BSP (or BSP chimera), or

buffer alone (50 mM Tris, pH 7.6, 150 mM NaCl, 5 mM $CaCl_2$). Relative velocity plots were determined by titrating the substrate concentration from 0.025 to 15 μg/mL and determining the change in fluorescence over the first hour of reaction. Inhibitor titrations were carried out by titrating TIMP2 concentration from 1.6 to 1600 nM. Fluorescent data were acquired on a Perkin-Elmer Victor 2 multilabel plate reader with excitation at 485 nm and emission at 535 nm. Reactions were run in duplicate in fluornunc microtiter plates.

Low Molecular Weight Substrate Studies

The activities of mutant hemopexin-free MMP-2 in the presence and absence of inhibitor (TIMP2) and BSP were measured using a small molecular weight thiopeptide substrate (Ac-PLG- [2-mercapto-4-methylpentanoyl]-LG-OC_2H_5). Substrate was incubated in assay buffer (50 mM HEPES, 10 mM $CaCl_2$, 0.05% Brij-35, 1 mM DTNB, pH 7.5) with 10 nM mutant MMP-2 + different concentrations of inhibitor or MMP2 + inhibitor + BSP added simultaneously. Data from the first 6 min were used to calculate initial velocity (pmol/s) values. Substrate cleavage was monitored using a Perkin-Elmer Victor 2 multilabel plate reader, and absorbance was measured at 412 nm.

RESULTS

Cysteine Switch Activation

MMPs are normally activated through disruption of a propeptide cysteine interaction with Zn^{2+} within the catalytic site. When recombinant human (latent) proMMP-2 alone is incubated with a large fluorescein-conjugated substrate, little enzyme activity is seen over a 4 h period, presumably because the propeptide is occupying the active site (Figure 2A). When recombinant human BSP was added to proMMP-2 and then incubated with substrate, significantly increased enzyme activity was observed, while the incubation of BSP alone with substrate led to no increase in fluorescence. Varying the substrate concentration and determining the relative velocity of the enzyme reaction in the absence and presence of BSP revealed that BSP conferred enzymatic activity to proMMP-2 over a large range of substrate concentrations (Figure 2B). If binding of latent MMP-2 leads to catalytic activity, then the binding should be associated with the breaking of the propeptide-associated zinc-cysteine bond. A cysteine trap assay, where free cysteine residues react with DTNB to stoichiometrically yield 2-nitro-5-thiobenzoic acid as a measure of free cysteine residues (15), was performed. BSP does not contain any cysteine. ProMMP-2 alone exhibited basal reactive cysteines. When BSP was added to proMMP-2, however, the amount of accessible cysteine increased (Figure 1C). This was consistent with zinc-binding cysteine residues becoming fully exposed and quantitatively labeled. MMP-2 has 14 cysteine residues in the mature active protein that are believed to be disulfide bonded, while the remaining 3 cysteines are present in the propeptide domain.

BSP's MMP-2 Binding and Activation Sequences

In order to determine the sequences in BSP that are involved in binding and modulating proMMP-2 activity, chimeras were made by swapping amino-terminal and carboxy-terminal domains between BSP and either OPN or DMP1. When binding was investigated by intrinsic fluorescence binding studies between proMMP-2 and wild-type BSP, the BSP binding was saturable with a K_d in the nanomolar range (Figure 3A,B, Table 1), while the chimera including only the first four coding exons of BSP (BSP/OPN and BSP/DMP1) exhibited two log order weaker but still saturable binding curves (Figure 3C,D). In contrast, OPN/BSP and DMP1/BSP chimera showed no saturable binding (inset, Figure 3C). Thus, important proMMP2-binding sequences lie within the first five small exons of BSP. Exon 2 contains the leader sequence and the first two amino acids of the mature protein. Exons 3 and 5 are short exons containing casein kinase II-type phosphorylation motifs as their major conserved sequences. Exon 4 is a well-conserved 26 amino acid exon that contains 6 tyrosines, 3 lysines, 3 arginines, and 2

histidines but no acidic residues. Unlike the tyrosines found flanking the RGD motif, the tyrosine groups in exon 4 are not thought to be sulfated due to the presence of these positively charged amino acids (16). When a BSP construct lacking exon 4 (BSPΔx4) was measured for binding activity, it did show saturable binding, though the K_d was a log order weaker than that of wild-type BSP (Figure 3E,F).

The activation of proMMP-2 by the same chimeric constructs was studied using the large molecular weight fluorescein-gelatin assay. Wild-type BSP added to proMMP- 2 resulted in the expected significant increase in enzymatic activity (Figure 4A). The BSP/OPN and BSP/DMP1 chimera, however, also conferred enzymatic activity to the latent MMP-2 (Figure 4B). In contrast, the OPN/BSP and DMP1/BSP chimera had, at best, minimal effects on enzymatic activity compared to the background autoactivation occurring for proMMP-2 alone (Figure 4C). These data suggest that sequences in the first four coding exons are involved in BSP conferring activity to latent MMP-2. In contrast, the BSPΔx4 protein added to proMMP-2 caused little change in basal activity (Figure 4D).

To further investigate the role of exon 4 in proMMP-2 activation, the 26 amino acid sequence coded for by exon 4 was synthesized and purified. The action of exon 4 peptide on proMMP-2 activation by wild-type BSP was studied using the large molecular weight gelatin-substrate assay. In the absence of any exon 4 peptide, full-length BSP conferred activity of proMMP-2, as exhibited by the linear increase in the fluorescent signal (Figure 5A). Titrating the levels of exon 4 peptide into reaction mixtures containing wild-type BSP and proMMP-2 led to a decrease in the evolution of the fluorescent signal. The rate of reaction was calculated by determining the change in fluorescence over the first 60 min (Figure 5B). Exon 4 peptide at 17 nM (with BSP and proMMP-2 at 10 nM each) decreased the reaction rate by 10%. Increasing the concentration of exon 4 peptide up to 1.7 μM decreased the reaction rate by 68%. The observation that the activity of proMMP-2 + wild-type BSP was reduced by the addition of exon 4 peptide to the enzyme reaction mixture suggests that this conserved exon likely plays a role in the activation process.

BSP Binding to MMP-2 Does Not Require the Hemopexin Domain

MMP-2 possesses a hemopexin-like domain believed to be involved in the binding of many natural substrates (17). Whether BSP interactions with MMP-2 require the hemopexin domain was investigated by studying the binding characteristic of BSP to recombinant human MMP-2 that lacks the hemopexin domain. When the intrinsic tryptophan fluorescence of the mutant MMP-2 (mMMP-2) was followed during titration with BSP, quenching of the signal similar to that previously reported for the intact MMP-2 was observed (Figure 6A). BSP binding was saturable, and its affinity for the mMMP-2 was actually slightly higher than that for intact MMP-2 (Figure 6B,C and Table 1). The accuracy of the determinations of the molarities of the three MMP proteins from the information originally supplied by the manufacturer may contribute to the apparent minor differences in calculated binding constants.

An alternative method to confirm BSP and mMMP-2 binding was also employed. Solid-phase binding assays confirmed that binding of BSP to MMP variants was saturable (Figure 6D) and K_d values were determined (Table 1). The K_d values for the two binding systems (solution versus solid phase) were both found to be in the nanomolar range. The minor difference in values may reflect differences in solid-phase binding conformation, orientation, or better accessibility of BSP-MMP contact points in the solution phase.

BSP Modulation of MMP-2 Activity Does Not Require the Hemopexin Domain

The effect of BSP on the activity of the hemopexin-free mMMP-2 was investigated using the gelatin-fluorescein large molecular weight substrate assay. The change in substrate

fluorescence caused by mMMP-2 alone compared to a complex of equimolar mMMP-2 + BSP was not significantly different (Figure 7A). As expected, the addition of equimolar TIMP2 to mMMP-2 caused a significant decrease in the rate of fluorescence change. However, addition of equimolar BSP to mMMP-2 + TIMP2 complexes restored the rate of fluorescence change to that of mMMP-2 alone, showing that the TIMP2 became ineffective in the presence of bound BSP (Figure 7A). For complexes of equimolar mMMP-2 + TIMP2, the rate of the reaction was decreased to 67%, and the addition of equimolar BSP restored activity to 97%. Titration of mMMP-2 with TIMP2 using the large molecular weight substrate assay revealed that over a 100-fold excess of TIMP2 was required to inhibit activity to 20% of that seen by mMMP-2 alone (Figure 7B). Increasing the concentration of BSP added to fixed amounts of equimolar TIMP2 and mMMP-2 increased the reaction rate in a dose-dependent fashion (Figure 7C).

The interaction of the hemopexin-free mMMP-2 with TIMP2, BSP, and the fluorescein-gelatin substrate may be reflecting steric effects between these large macromolecules. Potential steric effects of substrate, enzyme, inhibitor, and BSP interactions were studied by using a low molecular weight, freely diffusible peptide substrate to follow enzyme activity. Similar to results with the large molecular weight substrate, the addition of BSP alone did not significantly alter mMMP-2 enzyme product evolution (Figure 7D). Furthermore, TIMP2 inhibited product evolution, as expected, while the addition of BSP to mMMP-2/TIMP2 complexes increased the digestion (Figure 7D). Titration of mMMP-2 with various concentrations of TIMP2 indicated that, at 10-fold excess, TIMP2 inhibited mMMP-2 activity on the small molecular weight substrate to 20%, with equimolar TIMP2 inhibiting the mMMP-2 to 34% (Figure 7E). As before, the addition of equimolar BSP was able to restore mMMP-2 treated with TIMP2 to 85% of normal activity. Increasing the concentration of BSP in reaction mixtures of equimolar TIMP2 and mMMP-2 restored activity further (Figure 7F). These data suggest that BSP reactivation of TIMP2- inhibited MMP-2 does not require the hemopexin domain of MMP-2.

Restoration of TIMP2-Inhibited MMP-2 Activity Requires both Amino-Terminal and Carboxy-Terminal BSP Sequences

The BSP chimeras were tested for their ability to restore activity to TIMP2-inhibited wild-type MMP-2 and mMMP-2 using the small, freely diffusible substrate assay. The hemopexin-free mMMP-2 exhibited a reaction velocity of 0.10 ± 0.02 pmol/s over the first 6 min of the reaction (Figure 8a). The addition of 10 nM TIMP2 reduced activity to 12 ± 5% that of mMMP-2 alone, while the inclusion of 10 nM BSP with the TIMP2 restored activity to 79 ± 12% of mMMP-2 alone (a 6.5-fold increase relative to mMMP-2+inhibitor). For mMMP-2, addition of equimolar chimera proteins exhibited various abilities in restoring TIMP2- inhibited activity. The percentage of the activity of mMMP-2 was 39 ± 11% for BSP/OPN (a 3-fold increase relative to mMMP-2 + inhibitor), 30 ± 16% for BSP/DMP1 (2.5-fold relative to mMMP-2 + inhibitor), 32 ± 12% for DMP1/ BSP (2.7-fold relative to mMMP-2 + inhibitor), and 17 ± 12% for OPN/ BSP chimera. When wild-type active MMP-2 was substituted for the hemopexin-free MMP-2, a reaction velocity of 0.94 ± 0.10 pmol/s was observed over the first 6 min of the reaction (Figure 8b). The addition of 10 nM TIMP2 reduced activity to 19 ± 3% that of MMP-2 alone, while the inclusion of 10 nM BSP with the TIMP2 restored activity to 93 ± 7% of MMP-2 alone (an <5-fold increase relative to MMP-2 + inhibitor). The chimera exhibited a similar pattern in restoring TIMP2 inhibited activity to wild type MMP-2. The percentage of the activity of MMP-2 was 53 ± 20% for BSP/OPN (2.8-fold relative to MMP-2 + inhibitor), 47 ± 13% for BSP/DMP1 (2.5-fold relative to MMP-2 + inhibitor), 58 ± 12% for DMP1/BSP (3-fold relative to MMP-2 + inhibitor), and 19 ± 6% for OPN/ BSP chimera.

DISCUSSION

The MMP gene family consists of at least 25 members of zinc-containing, calcium-dependent extracellular endoproteinases (18). MMPs may be cell surface-associated or secreted into the extracellular space where they are involved in degrading extracellular matrix components as well as processing of non-matrix components such as cytokines, adhesion molecules, and receptors. MMPs often share functional domains such as the propeptide, the catalytic domain containing the active site, a hinge or linker region, and a hemopexin-like domain. The propeptide domain contains a zinc-ligating free thiol group that contributes to enzyme latency. This has been termed the "cysteine switch" (15,19) or "velcro" (20) mechanism and involves the cysteine residue in the propeptide binding to the catalytic Zn^{2+} of the enzyme. The hemopexin-like domain is thought to be essential for the binding of many natural substrates, contains a secondary TIMP-binding site, and is involved in membrane activation and possibly some proteolytic activities (21–23). MMP-2 and other gelatinases contain cysteine-rich repeats within the catalytic domain that resemble the collagen- binding type II repeats of fibronectin and are required to bind and cleave collagen and elastin (22).

Pro-MMPs can be activated either by proteases that remove the propeptide or by chemical compounds that disrupt or destabilize the zinc-cysteine interaction (24). In the current study, BSP binding to latent proMMP-2 leads to catalytic activity as well as the appearance of free cysteine residues. The presence of exons 2 through 5 of BSP in chimeric constructs with OPN and DMP1 led to proMMP-2 activation, while exons 2 through 5 of DMP1 and OPN linked to the 3′ exons of BSP did not enable proMMP-2 enzymatic activity, indicating that latent MMP-2 activation was specific to exons 2–5 of BSP. Indeed, the 26 amino acid sequence encoded for by exon 4 was required for the highest degree of BSP-mediated activity. Exon 4 alone did not activate the MMP although it could act as a competitive inhibitor and interfere with proMMP-2 activation by intact wild-type BSP. BSP is generally acidic and hydrophilic in nature (p*I* of 3.9 (25)), but exon 4 encodes the protein's only positively charged domain of significant size, being comprised of 1/3 positively charged amino acids (Arg, His, and Lys, p*I* = 10.1). Exon 4 also contains 1/4 of BSP's tyrosine residues, none of which are thought to be sulfated.

Like osteopontin, purified BSP protein subjected to standard one-dimensional proton NMR revealed a lack of ordered structures over the NMR time scale, indicating that the protein was extended and flexible in solution (26). Protein flexibility and lack of ordered structure are shared by a number of proteins that have multiple binding partners (27). Unstructured proteins have multiple contact points across the molecule, and structure is conferred through their binding interactions. In addition to MMP-2, BSP binding partners include hydroxyapatite (25), collagen (28), complement factor H (8), and αvβ3 integrin (29). Thus, in addition to potential structural, mineralization-related, and receptor-mediated signaling roles for the BSP in the extracellular matrix, BSP may regulate matrix turnover based on its modification of TIMP2 inhibition of MMP-2 activation.

We have shown previously that BSP can bind to both proMMP-2 and active MMP-2 (11). The current solution and solid-phase binding studies confirm that the proMMP-2 and active MMP-2 as well as hemopexin-deleted forms of MMP-2 have K_d values in the biologically significant nanomolar range. Binding of BSP to recombinant human MMP-2 that lacked the hemopexin domain yielded K_d values similar to those for proMMP-2 and active MMP-2, suggesting that the hemopexin domain does not contribute significantly to the binding interaction. While the chimera with the amino-terminal portion of BSP (BSP/OPN and BSP/DMP1) exhibited saturable binding to proMMP-2, the K_d values were 20-fold weaker than that of wild-type BSP. In contrast, chimera retaining the carboxy-terminal portion of the BSP protein (78% of the total BSP sequence, with the amino-terminal sequences replaced with either OPN or DMP1 exons 2–5) showed much weaker and non-saturable binding to proMMP-2. BSP protein with exon 4

deleted had a K_d value that was 6-fold weaker than full-length BSP. However, both the chimera lacking the amino-terminal portion of BSP and the BSP construct lacking exon 4 were extremely ineffective at activating proMMP-2. This suggests that while the amino-terminal regions, especially exon 4, are essential in BSP-mediated proMMP-2 activation, more carboxy-terminal domains of BSP also contribute to the overall binding interaction.

TIMP2 has binding sites in both the hemopexin and catalytic domains (30). The ability of BSP to bind to and restore activity to TIMP2-inhibited mMMP-2 that lacked a hemopexin domain indicates that BSP interactions with the hemopexin domain are not required for BSP-dependent modulation of TIMP2 inhibition. Similar restoration of activity was measurable with both the large natural substrate (gelatin) and small, freely diffusible substrate. Thus, the changes in TIMP2 inhibition of mMMP-2 induced by BSP are not steric effects of exclusion of TIMP2 by the large macromolecular gelatin-MMP-2-BSP complex. The inability of chimera to restore full activity to TIMP2-inhibited wild type and mutant MMP-2 is consistent with distinct amino- and carboxy-terminal domains of BSP contributing to the enzyme reactivation.

In summary, these structure-function studies reveal for the first time that amino-terminal portions of BSP, particularly exon 4, are required for MMP-2 propeptide displacement and enzyme activation. On the other hand, domains spanning the entire BSP sequence contributed to binding affinity and reactivation of TIMP2-inhibited MMP-2. Finally, the hemopexin-like domain of MMP-2 is not required for BSP-mediated enzyme modulation. These observations are consistent with the model of BSP as a flexible, intrinsically unstructured protein that utilizes distinct regions or overlapping interaction surfaces for more than one function.

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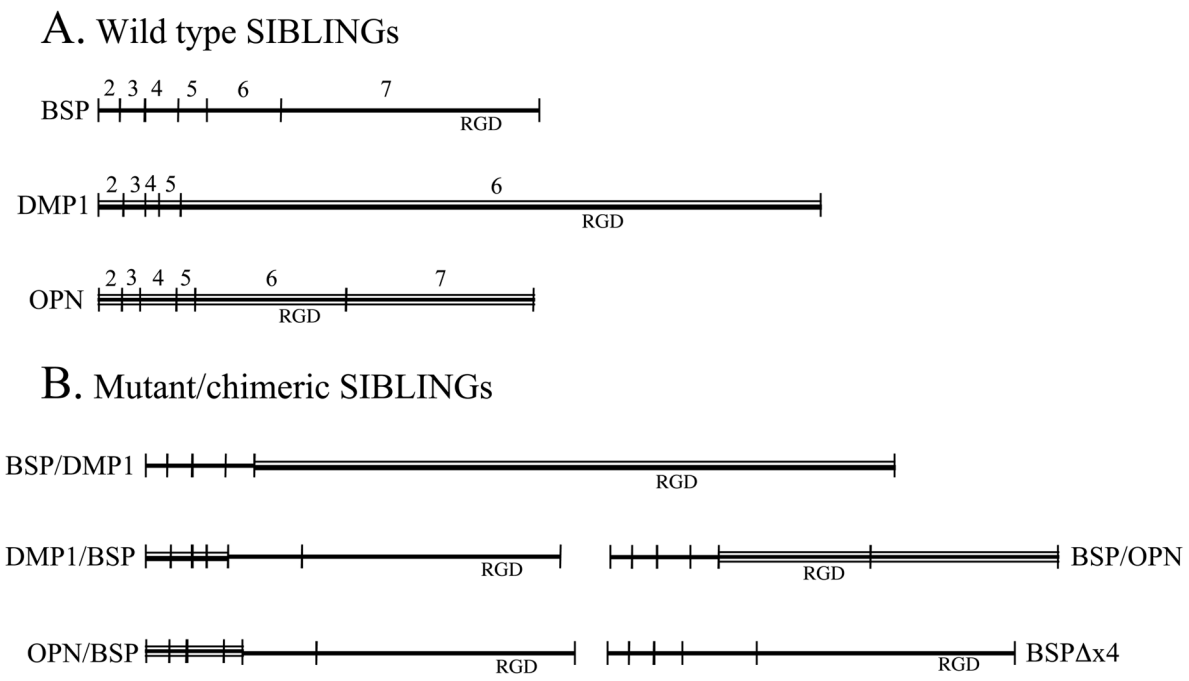

FIGURE 1.

Wild-type, chimeric, and exon 4-deleted BSP recombinant protein constructs. (A) The relative protein domain sizes encoded by the corresponding exons are depicted for BSP, DMP1, and OPN. The first exon is noncoding and not depicted. The second exon contains the start codon, the hydrophobic signal peptide, and the first two amino acids of the mature protein. Exons 3 and 5 contain consensus sequences for serine phosphorylation. (B) SIBLING chimera were constructed whereby amino-terminal portions of one protein were ligated to carboxy-terminal sequences of another. Exons 2 to 5 of BSP were linked to exon 6 of DMP1 (BSP/DMP1) or exons 6 and 7 of OPN (BSP/OPN). Exons 6 to 7 of BSP were also linked to exons 2 to 5 of DMP1 (DMP1/BSP) or OPN (BSP/OPN). An additional BSP species was made by deleting exon 4 (BSPΔx4). The relative location of the integrin-binding tripeptide, Arg-Gly-Asp (RGD), is noted for each protein and mutant/chimera.

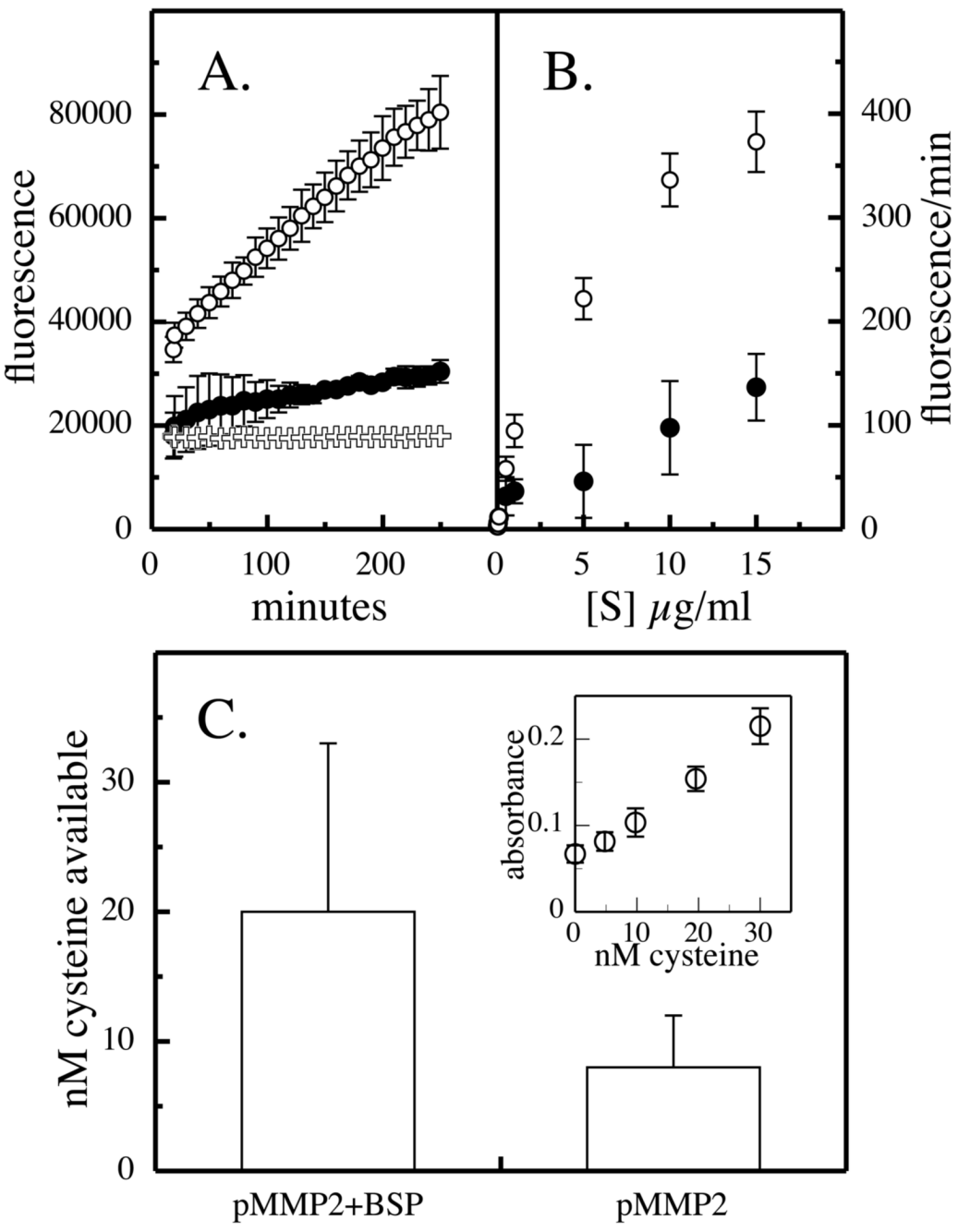

FIGURE 2.

BSP binding to proMMP-2 confers enzymatic activity and exposes the free cysteine residue. (A) The large fluorescein-gelatin substrate was incubated with pro-MMP-2 (closed circle), proMMP-2 + BSP (open circle), or BSP alone (plus). (B) Substrate velocity plots were generated by varying substrate concentration with fixed proMMP-2 levels in the presence (open circle) and absence (closed circle) of BSP. (C) ProMMP-2 at 14 nM final concentration was incubated ± 14 nM BSP in the presence of 180 μM DTNB in reaction buffer (0.1 M sodium phosphate, pH 8.0). The reactions were incubated for 15 min at room temperature, and absorbance was measured at 412 nM. A standard curve (inset) of absorbance versus nM

cysteine was made by incubating noted concentrations of free cysteine under the same reaction conditions. Errors bars represent standard deviation values based on triplicate analyses.

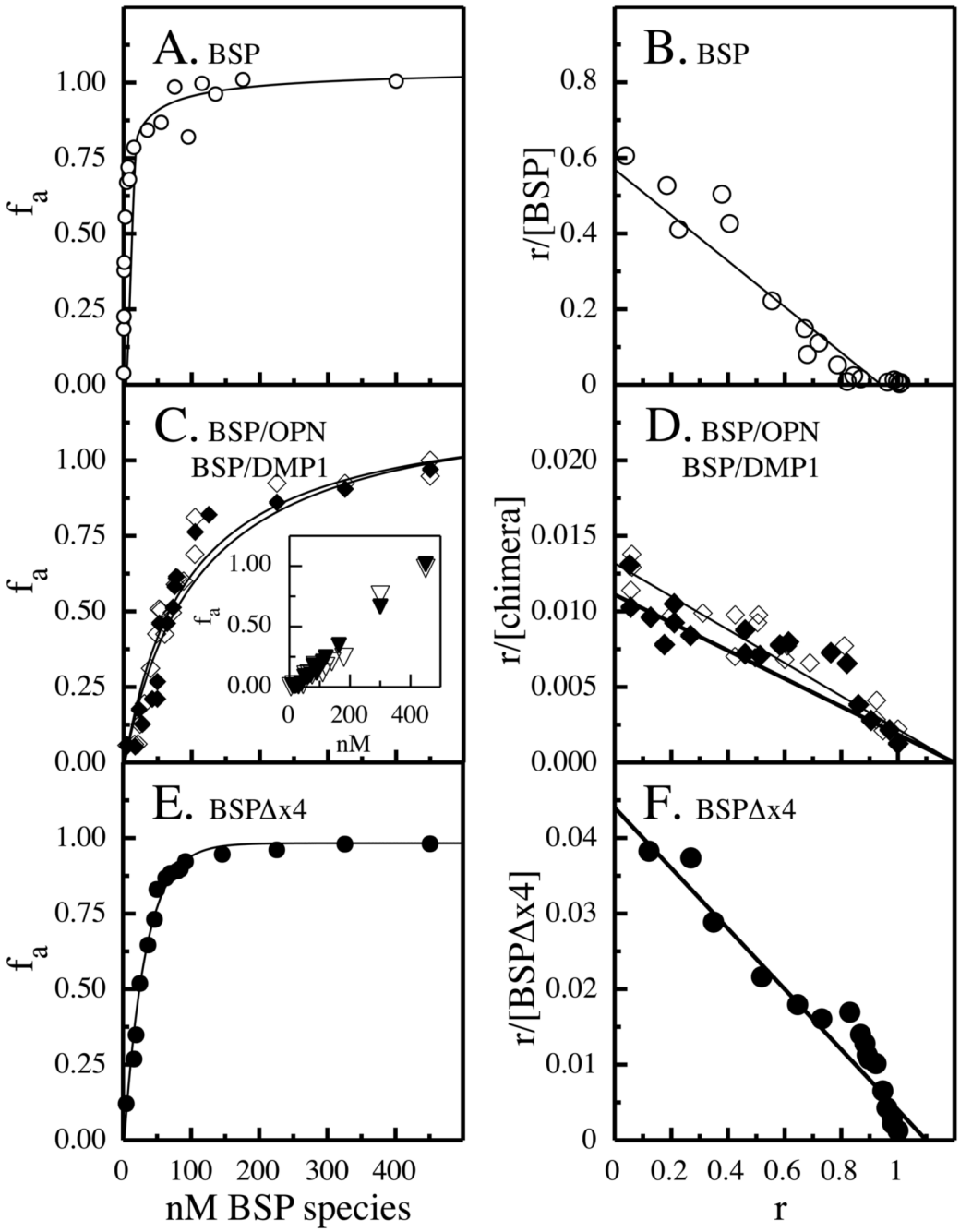

FIGURE 3.

Determination of BSP domains required for binding to proMMP-2. Binding isotherms between proMMP-2 and different forms of BSP (A, C, E) were determined using intrinsic tryptophan fluorescence binding assays as described in Materials and Methods. The corresponding Scatchard plots for proMMP-2 and the different forms of BSP were calculated (B, D, F). The forms of BSP studied included (A, B) wild-type BSP (open circle), (C, D) BSP/OPN (closed diamond) or BSP/ DMP1 chimera (open diamond), OPN/BSP (inverted closed triangle) or DMP1/BSP chimera (inverted open triangle), and (E, F) BSP exon 4 deletion (BSPΔx4, closed circle). The inset figure in panel C depicts the binding isotherm for the OPN/BSP and DMP1/ BSP chimera where no saturation was observed and a corresponding Scatchard analysis could

not be performed. The values plotted represent the average of two separate experiments for each condition run in duplicate.

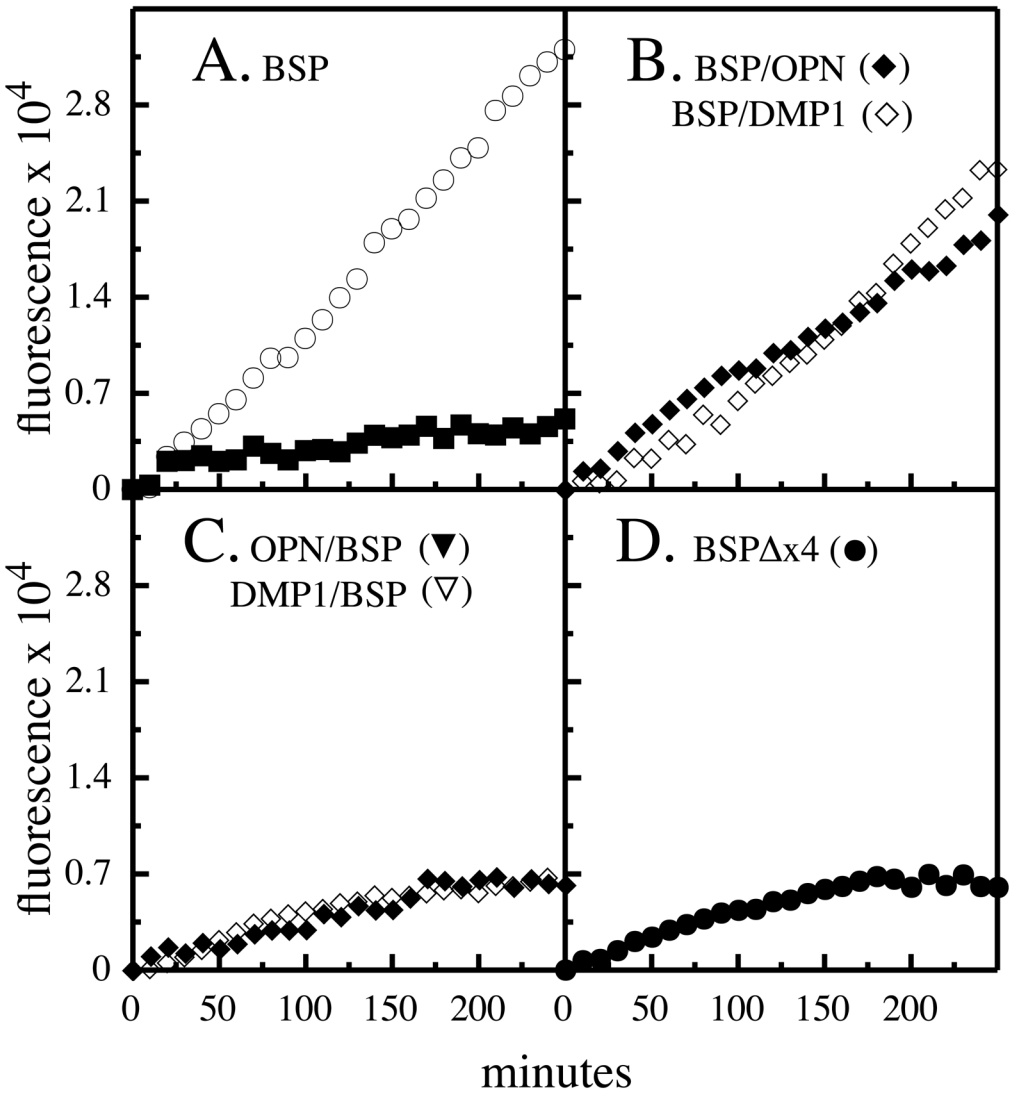

FIGURE 4.

Determination of BSP domains required for activation of proMMP-2. Changes in enzymatic activity for proMMP-2 treated with different forms of BSP were followed using the fluorescein-labeled gelatin substrate and proMMP-2 incubated with vehicle or BSP or noted constructs. The conditions employed were (A) vehicle (closed square) or BSP (open circle), (B) BSP/OPN (closed diamond) or BSP/DMP1 chimera (open diamond), (C) OPN/BSP (inverted closed triangle) or DMP1/BSP chimera (inverted open triangle), and (D) BSPΔx4 (closed circle). Note that the exons 2 to 5 and particularly exon 4 are required for inducing activity in proMMP-2. Enzyme activity assays were performed in triplicate.

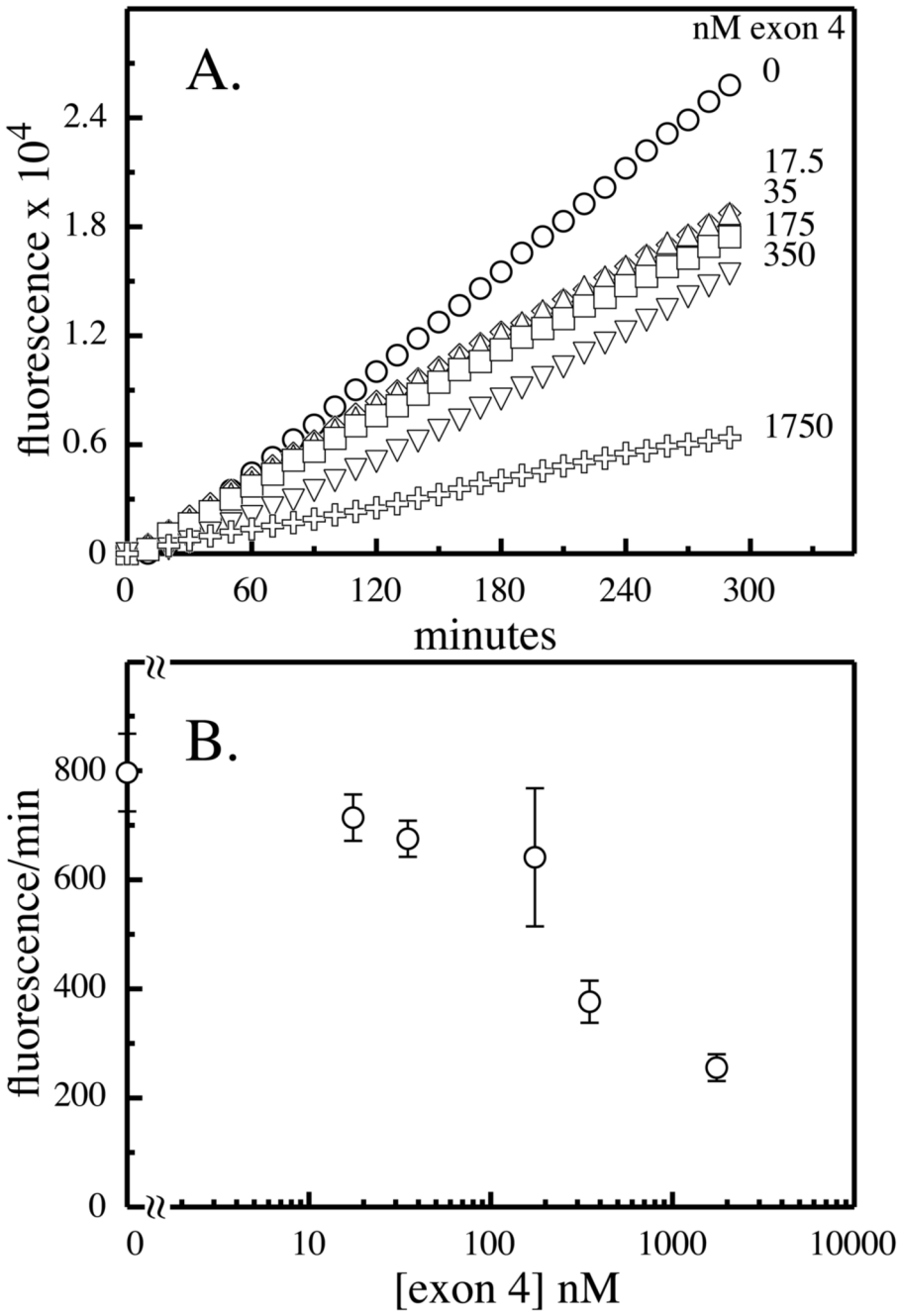

FIGURE 5.

BSP exon 4 peptide reduces intact BSP activation of proMMP-2. (A) Synthetic exon 4 peptide was added at noted concentrations to a mix of 10 nM wild-type intact BSP and 10 nM proMMP-2, and the changes in enzymatic activity were followed using the fluorescein-labeled gelatin substrate. The concentrations of exon 4 peptide were 0 (open circle), or 17.5 nM (open diamond), 35 nM (open triangle), 175 nM (open square), 350 nM (inverted open triangle), and 1750 nM (+). (B) The relative velocity of the reactions was determined as the change in fluorescence over the first 60 min. Enzyme activity assays were performed in triplicate, and error bars represent standard deviations for combined experiments.

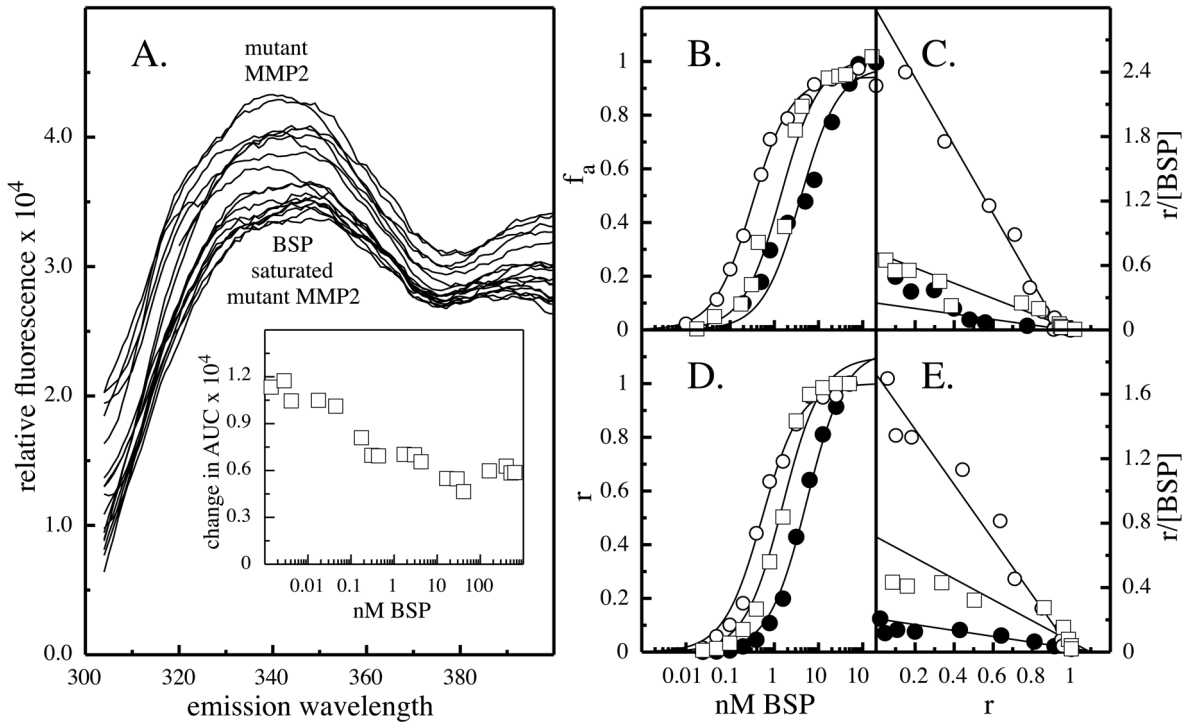

FIGURE 6.

BSP binding to MMP-2 does not require the hemopexin domain. (A) Binding interactions between mutant MMP-2 lacking the hemopexin domain (mMMP-2) and BSP were followed by intrinsic tryptophan fluorescence of the mMMP-2 protein. One nanomolar mMMP-2 was reacted with increasing concentration of BSP. Intrinsic tryptophan fluorescence was monitored by excitation at 295 nm and recording emission from 300 to 500 nm. Binding saturation was followed by monitoring the change in the area under the emission peak curve (inset). (B, C) The area under the emission peak curve was used to determine a binding curve by calculating fractional acceptor saturation versus nM BSP added and the corresponding Scatchard plot. (D) The binding interaction between BSP and proMMP-2 (closed circle), active MMP-2 (open circle), and mMMP-2 (open square) was investigated by solid-phase binding assays. (E) Scatchard plots derived from solid-phase binding assays of BSP and proMMP-2, active MMP-2, and mMMP-2 were determined. For both solution and solid-phase binding assays, the values plotted represent the average of two separate experiments for each condition run in duplicate.

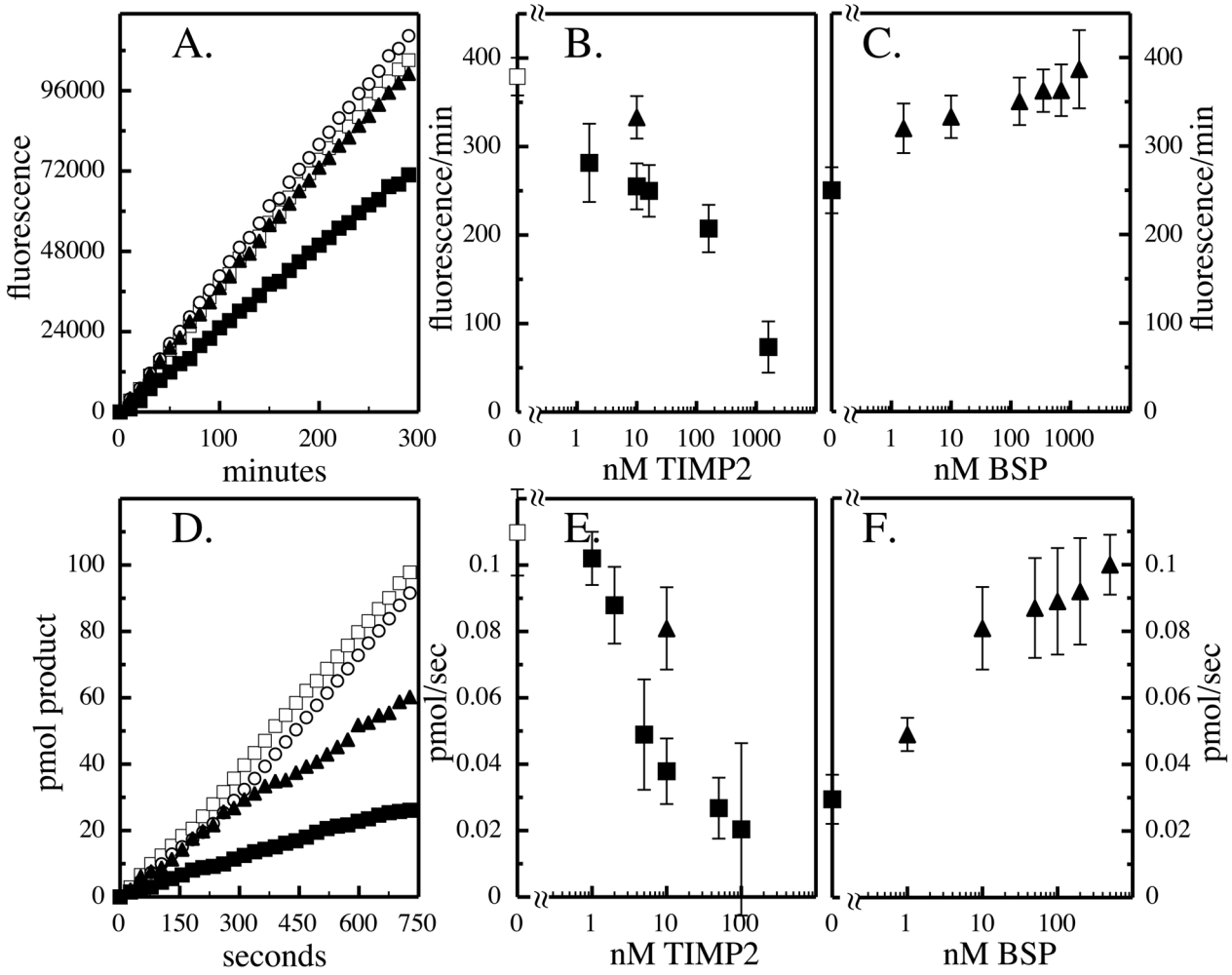

FIGURE 7.

BSP binding to hemopexin-deleted MMP-2 keeps TIMP2 from inhibiting the protease activity. (A) The effect of BSP on the activity of the mMMP-2 was profiled using the fluorescein-labeled large molecular weight (gelatin) substrate assay. Reaction conditions included mutant MMP-2 (open square), mutant MMP-2 + BSP (open circle), mutant MMP-2 + TIMP2 (closed square), and mutant MMP-2 + TIMP2 +BSP (closed triangle). (B) The effect of varying conditions on the relative velocity of the mutant enzyme was analyzed by linear regression analysis over the first hour and the slope determined. (C) The effect of BSP on TIMP2's inhibition of mMMP-2 was further studied by titrating a reaction mixture of equimolar mMMP-2 + TIMP2 with increasing concentrations of BSP. (D) The action of BSP on mMMP-2 activity using the small substrate Ac-PLG-[2-mercapto-4-methylpentanoyl]-LG-OC_2H_5 was determined by following picomoles of product evolution over time. (E) TIMP2 inhibition curves and (F) BSP dose response of mMMP-2 recovery from inhibition by TIMP2 were determined. For substrate titrations and TIMP2 dose response, three separate experiments were combined, and values plotted present the slope of the reaction over the first 6 min with error bars representing the standard deviation.

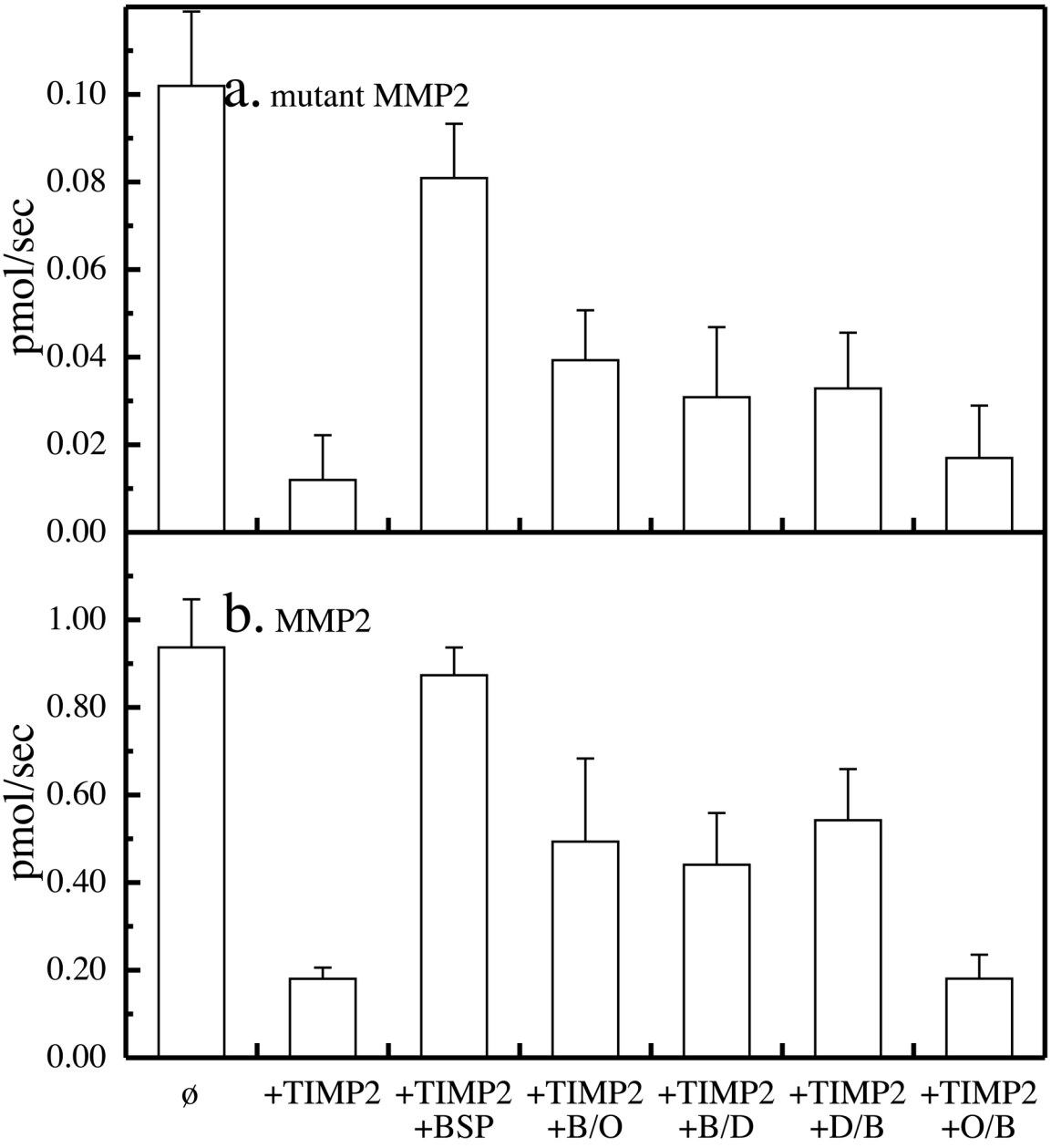

FIGURE 8.

TABLE 1

BSP and MMP-2 binding constants.

Ligand	receptor	K_d (nM)
BSP	mMMP-2[1]	1.40 ± 0.34 (1.54 ± 0.24)[2]
BSP	active MMP-2	0.32 ± 0.02 (0.58 ± 0.06)
BSP	proMMP-2	3.9 ± 0.9 (5.4 ± 0.5)
BSP/OPN	proMMP-2	108 ± 21
BSP/DMP1	proMMP-2	91 ± 13
BSPΔx4	proMMP-2	25 ± 8

[1] Mutant MMP-2 lacking the hemopexin domain.

[2] Values in parenthesis are from solid phase binding assays.