# Identification of Possible Inhibitory Reactive Centers in Thrombospondin 1 That May Bind Cathepsin G and Neutrophil Elastase†

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*Received February 4, 1994*®

ABSTRACT: Thrombospondin 1 is a multidomain trimeric glycoprotein from platelets and a variety of normal and transformed cells of both mesenchymal and epithelial origin, which functions in cell adhesion and cell–cell interactions. We have recently shown that human thrombospondin 1 binds and inhibits the neutrophil enzymes, neutrophil elastase [Hogg, P. J., Owensby, D. A., Mosher, D. F., Misenheimer, T. M., & Chesterman, C. N. (1993a) *J. Biol. Chem. 268*, 7139–7146] and cathepsin G [Hogg, P. J., Owensby, D. A., & Chesterman, C. N. (1993b) *J. Biol. Chem. 268*, 21811–21818]. One mole of thrombospondin 1 trimer binds 3 mol of neutrophil elastase or up to 6 mol of cathepsin G, with site-binding dissociation constants around the nanomolar range, and the enzymes have been shown to interact with thrombospondin 1 in the vicinity of the calcium-binding type 3 repeats. None of the protein modules in this region, or within the whole thrombospondin 1 molecule, have previously been implicated in the inhibition of proteinases. We noted that there are two stretches of eight amino acids each in the human thrombospondin 1 type 3 repeats, residues 735–742 and 794–801, that have striking similarity to a reactive-site consensus sequence derived from selected members of the Kazal and *Streptomyces* subtilisin inhibitor families. Synthetic peptides corresponding to the putative $P_5$ through $P_4'$ residues of both proposed reactive centers interacted efficiently with the active site of cathepsin G and were competitive inhibitors of the fibronectin-degrading and platelet-activating activities of this enzyme, while only the peptide corresponding to residues 793–801 efficiently interacted with the active site of neutrophil elastase and competitively inhibited its fibronectin-degrading activity. These findings are in accordance with the neutrophil enzyme:thrombospondin 1 molar stoichiometries measured kinetically. We suggest that residues 735–742 and 794–801 represent reactive centers in thrombospondin 1 and define a novel proteinase inhibitor.

Thrombospondin 1[1] (TSP1) was originally identified as a major protein in platelet $\alpha$-granules and has since been shown to be a transient component of extracellular matrices during embryogenesis, wound repair, and in neoplastic tissues (Frazier, 1987; Mosher, 1990). From studies of the tissue distribution of TSP1 and the effects of TSP1 in cell biological systems, a role for this protein in tissue development and remodeling and in the disease processes, atherogenesis and tumorigenesis, has been suggested. TSP1 is a trimer of three identical 150000-Da disulfide-bonded subunits. Each subunit contains a unique heparin-binding domain at its amino terminus, a connecting region that contains the cysteines that participate in interchain disulfide linkages, a procollagen module, three properdin or type 1 modules, three epidermal growth factor or type 2 modules, unique calcium-binding or type 3 repeats, and a unique carboxy-terminal globular domain (Lawler & Hynes, 1986). Distinct thrombospondins are encoded by at least four homologous genes in human and mouse (Bornstein et al., 1991a,b; Vos et al., 1992; Lawler et al., 1991, 1993). TSP1, the platelet thrombospondin, and TSP2 have the same domain structures. The TSP1 gene is a member of the "immediate early" cellular response genes, while the TSP2 gene is not serum-responsive. TSP3 and TSP4 lack the connecting region and the properdin modules of TSP1 and TSP2, but contain an extra epidermal growth factor module. The thrombospondins are most homologous in the carboxy-terminal calcium-binding repeats and the globular domain. TSP1 and TSP2 are widely distributed *in vivo*, whereas TSP3 is preferentially expressed in the lung and TSP4 in cardiac and skeletal muscles. Cartilage oligomeric matrix protein, or COMP, is a glycoprotein synthesized by chondrocytes in cartilage (Oldberg et al., 1992). The domain structure of COMP is very similar to the domain structure of TSP4, and Bornstein (1992) has proposed that it be designated TSP4.

Neutrophil elastase and cathepsin G are serine proteinases of the azurophilic granules of neutrophils and are released into the phagosome and the extracellular millieu upon neutrophil activation. Neutrophil elastase has been implicated in the pathogenesis of pulmonary emphysema, acute respiratory distress syndrome, glomerulonephritis, rheumatoid arthritis, and other inflammatory disorders (Beith, 1986). The role of cathepsin G, however, is less clear, and it has not yet been proved to be directly involved in disease development, although it cleaves most of the same substrates as neutrophil elastase, albeit with lesser efficiency, and has been shown to activate platelets (Selak et al., 1988). We have shown that 1 mol of TSP1 trimer binds 3 mol of neutrophil elastase or

† This work was supported by the National Health and Medical Research Council of Australia and the National Heart Foundation of Australia.

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® Abstract published in *Advance ACS Abstracts,* May 1, 1994.

[1] Abbreviations: TSP, thrombospondin; HEPES, *N*-(2-hydroxyethyl)-piperazine-*N*'-ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; PV-pNA, pyroglutamyl-Pro-Val-*p*-nitroanilide; AAPV-pNA, methoxy-succinyl-Ala-Ala-Pro-Val-*p*-nitroanilide; AAPF-pNA, succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide; COMP, cartilage oligomeric matrix protein; SSI, *Streptomyces* subtilisin inhibitor.

0006-2960/94/0433-6531$04.50/0 

up to 6 mol of cathepsin G with site-binding dissociation constants around the nanomolar range and that the presentation of the TSP1-binding sites is regulated by calcium ions (Hogg et al., 1993a,b). The neutrophil enzymes bind to TSP1 tightly, reversibly, and close enough to the active site to block the interactions of small tripeptidyl *p*-nitroanilide and macromolecular protein substrates. Because TSP1 is distributed extensively in the extracellular matrix and is also released by platelets and neutrophils (Kreis et al., 1989) at sites of inflammation, we hypothesized that binding of neutrophil elastase and cathepsin G to TSP1 within the extracellular matrix might contribute to the regulation of the activity of these enzymes.

Several pieces of evidence localized the binding sites for neutrophil elastase and cathepsin G to the calcium-binding type 3 repeats of TSP1 (Hogg et al., 1993a,b). In this report, we identify two stretches of eight amino acids each in the type 3 repeats that have striking similarity to the reactive centers of selected Kazal and *Streptomyces* subtilisin inhibitors (Read & James, 1986). We have investigated these potential reactive centers by making peptides corresponding to the putative $P_5$ through $P_4'$ amino acids (Barrett, 1986) and testing them for interaction with the active sites of cathepsin G and neutrophil elastase.

## MATERIALS AND METHODS

*Chemicals.* *N*-(2-Hydroxyethyl)piperazine-*N'*-ethanesulfonic acid (HEPES), tris(hydroxymethyl)aminomethane (Tris), dithiothreitol, and iodoacetic acid were obtained from Sigma Chemical Co. (St. Louis, MO), and pyroglutamyl-Pro-Val-*p*-nitroanilide (PV-pNA), methoxysuccinyl-Ala-Ala-Pro-Val-*p*-nitroanilide (AAPV-pNA), and succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide (AAPF-pNA) were from Calbiochem (San Diego, CA). All other chemicals were of reagent grade.

*Proteins.* Neutrophil elastase and cathepsin G were purified from the leukocytes of patients with chronic myeloid leukemia (Saklatvala & Barrett, 1980). The concentrations of neutrophil elastase and cathepsin G were determined with AAPV-pNA and AAPF-pNA, respectively, using the kinetic constants and reaction conditions that were based on active-site-titrated enzymes (Nakajima et al., 1979; Hogg et al., 1993a,b). Fibronectin was a gift from Professor Deane Mosher, Departments of Medicine and Biomolecular Chemistry, University of Wisconsin, Madison, WI, and its concentration was determined using an $A^{1\%}_{1cm}$ value at 280 nm of 12.8 and a molecular weight of 440 000 (Mosher & Johnson, 1983). *N*-Acetyleglin C was from Sigma. All proteins were aliquoted and stored at −70 °C until use.

*Peptide Synthesis and Purification.* Peptides were synthesized in the solid phase on phenylacetamidomethyl resin using an Applied Biosystems peptide synthesizer (Model 430A) and *tert*-butyloxycarbonyl chemistry. Hydrogen fluoride cleavage of the fully protected peptides was performed by Auspep Pty. Ltd. (Melbourne, Australia). Lyophilized crude peptides were solubilized in 0.5 M Tris-HCl, 6 M guanidine hydrochloride, 2 mM ethylenediaminetetraacetic acid, and 0.1 M dithiothreitol (pH 8.2) and purified by $C_{18}$ reverse-phase chromatography (Delta-Pak 15 μm $C_{18}$-100A, Millipore Australia Pty. Ltd., Sydney, Australia) using a 0–70% linear acetonitrile gradient containing 0.1% trifluoroacetic acid. Peptides eluted between 30 and 36% acetonitrile. Purified peptides were lyophilized and stored dry at −20 °C. Peptide purity was >95% on the basis of sequence and amino acid analysis.

*Amino Acid Analysis.* Peptides were hydrolyzed in 6 M HCl for 16 h at 115 °C, and the phenylthiohydantoin-derivatized amino acids (Bidlingmeyer et al., 1984) were analyzed using Waters amino acid analysis equipment (W600, TCM, PicoTag column, WISP 710B, M441, Waters 745 data module).

*Reduction and Carboxymethylation of Peptides.* Purified peptides were dissolved in 0.25 M Tris-HCl (pH 8.0), reduced with 0.05 M dithiothreitol for 1 h at 20 °C, and carboxymethylated by adding 0.1 M iodoacetic acid and incubating for 1 h at 20 °C in the dark. The extent of carboxymethylation of the peptides was monitored by reaction with 5,5'-dithio-(2-nitrobenzoic acid) in a 0.1 M HEPES, 0.3 M NaCl, and 1 mM EDTA (pH 7.0) buffer according to Riddles et al. (1979).

*Purification of Disulfide-Bonded Peptide Dimers.* Peptides dissolved in 0.25 M Tris-HCl (pH 8.0) slowly formed disulfide-bonded dimers. Following incubation of the peptides for 24 h at 20 °C, the peptide dimers of DNCPFHYNP and DNCQYVYNV were separated from unreacted monomers by chromatography on a 3.9- × 150-mm Novo-Pak $C_{18}$ column (Millipore Australia Pty. Ltd.) using a 0–70% linear acetonitrile gradient containing 0.1% trifluoroacetic acid. The monomer and dimer of DNCPFHYNP eluted at 36% and 39% acetonitrile, respectively, while the monomer and dimer of DNCQYVYNV eluted at 35% and 40% acetonitrile, respectively. Purified dimers were lyophilized and stored dry at −20 °C.

*Calculation of Michaelis Constants for the Cleavage of Peptides by Cathepsin G and Neutrophil Elastase Using Alternate Substrate Assays.* Progress curves for competitive inhibition of the amidolytic activity of cathepsin G and neutrophil elastase by peptides were obtained as follows. Peptide (0–4 mM) and AAPF-pNA (427 μM) or PV-pNA (230 μM) in 50 mM HEPES, 0.125 M NaCl, and 1 mg/mL PEG 6000 (pH 7.4) buffer were incubated at 25 °C in Linbro/Titertek E.I.A. microtitration plate wells for approximately 2 min, and the reactions were started by the addition of cathepsin G (46 or 137 nM) or neutrophil elastase (10 or 29 nM), respectively, to a final volume of 200 μL. The reactants were mixed by shaking on a rotary shaker (Titertek) at maximum speed for 5 s. The formation of *p*-nitroaniline as a function of time was monitored continuously by measuring the absorbance at 405 nm using a Molecular Devices Thermomax kinetic microplate reader. Stock concentrations of AAPF-pNA and PV-pNA were made using dimethyl sulfoxide as the solvent. Substrate concentration was determined spectrophotometrically at 342 nm in distilled water using an absorption coefficient of 8210 $M^{-1}$ $cm^{-1}$ (Lottenburg & Jackson, 1983). Dimethyl sulfoxide at concentrations up to 10% had no statistically significant effect on the amidolytic activity of cathepsin G or neutrophil elastase.

The initial velocity data were fit to eq 1a, which describes the competitive inhibition of chromogenic substrate hydrolysis by an alternate nonreporting substrate (Segel, 1975):

$$v_i = k_c[E]_T[S]/(K_m(1 + [P]/K_p) + [S]) \qquad (1a)$$

$v_i$ is the measured initial velocity expressed as M $s^{-1}$, $k_c$ and $K_m$ are the catalytic and Michaelis constants for hydrolysis of the chromogenic substrate, respectively, $[E]_T$ and [S] are the total and free concentrations of enzyme and chromogenic substrate, respectively, and [P] and $K_p$ are the free concentration of peptide and Michaelis constant for the peptide, respectively. Assays were performed under conditions where $[S]_T$, $[P]_T \gg [E]_T$; therefore, the total concentrations of S and P were good approximations of their free concentrations. Rapid equilibrium conditions are assumed. The kinetic

Table 1: Comparison of the Putative Inhibitory Reactive Centers in Human TSP1 with the Reactive-Site Residues of Selected Members of the Pancreatic Secretory Trypsin Inhibitor (PSTI or Kazal) and *Streptomyces* Subtilisin Inhibitor (SSI) Families[a]

| inhibitor or domain | reactive-site residues | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | $P_4$ | $P_3$ | $P_2$ | $P_1$ | $P_1'$ | $P_2'$ | $P_3'$ | $P_4'$ |
| PSTI (Kazal) family | | | | | | | | |
| silver pheasant ovomucoid, domain 3 | Ala | Cys | Thr | Met | Glu | Tyr | Arg | Pro |
| turkey ovomucoid, domain 3 | Ala | Cys | Thr | Leu | Glu | Tyr | Arg | Pro |
| Japanese quail ovoinhibitor, domain 5 | Ala | Cys | Thr | Met | Ile | Tyr | Asp | Pro |
| Japanese quail ovomucoid, domain 3 | Ala | Cys | Pro | Lys | Asp | Tyr | Arg | Pro |
| porcine pancreatic secretory trypsin inhibitor | Gly | Cys | Pro | Lys | Ile | Tyr | Asn | Pro |
| human acrosin–trypsin inhibitor | Gly | Cys | Pro | Arg | His | Phe | Asn | Pro |
| SSI family | | | | | | | | |
| *Streptomyces* subtilisin inhibitor | Met | Cys | Pro | Met | Val | Tyr | Asp | Pro |
| plasminostreptin | Ala | Cys | Thr | Lys | Gln | Phe | Asp | Pro |
| human TSP1 | | | | | | | | |
| human TSP1, residues 735–742 | Asn | Cys | Pro | Phe | His | Tyr | Asn | Pro |
| human TSP1, residues 794–801 | Asn | Cys | Gln | Tyr | Val | Tyr | Asn | Val |

[a] The first three Kazal inhibitors and SSI inhibit chymotrypsin-like enzymes, while the latter three Kazal inhibitors and plasminostreptin inhibit trypsin-like enzymes. The molecular structures of the avian ovomucoid third domains (Read et al., 1983; Bode et al., 1985; Papamokos et al., 1982) and *Streptomyces* subtilisin inhibitor (Hirono et al., 1984) have been determined both free and in complex with their cognate enzymes. The molecular structure of pancreatic secretory trypsin inhibitor in complex with trypsinogen (Bolognesi et al., 1982) and the free structure of plasminostreptin (Kamiya et al., 1984) have also been determined. The reactive-site residues of domain 5 of Japanese quail ovoinhibitor and human acrosin–trypsin inhibitor are from Kato and Kohr (1978) and Fink et al. (1990), respectively. The human TSP1 sequences are from Lawler and Hynes (1986).

parameters for hydrolysis of AAPF-pNA by cathepsin G ($k_c$ = 2.1 $s^{-1}$, $K_m$ = 1.08 mM) and of PV-pNA by neutrophil elastase ($k_c$ = 11.5 $s^{-1}$, $K_m$ = 455 $\mu$M) were determined previously (Hogg et al., 1993a,b).

To facilitate presentation of the results using different enzyme concentrations, the data have been normalized by dividing the initial velocity measured in the presence of peptide, $v_i$, by the velocity measured in the absence of peptide, $v_0$. Division of eq 1a by $v_0$ gives

$$v_i/v_0 = (K_m + [S])/(K_m(1 + [P]/K_p) + [S]) \quad (1b)$$

The data have been fit to eq 1b by nonlinear least-squares regression (Duggleby, 1984), with $K_p$ as the unknown parameter.

*Calculation of Catalytic Constants for the Cleavage of Peptides by Cathepsin G and Neutrophil Elastase and the Peptide Bond Cleavage by These Enzymes.* Reactions containing peptide at concentrations approximately 0.5$K_p$, 1$K_p$, and 2–7 $K_p$ in 50 mM HEPES, 0.125 M NaCl, and 1 mg/mL PEG 6000 (pH 7.4) buffer at 25 °C were initiated by the addition of 100–200 nM cathepsin G or neutrophil elastase. Reactions were quenched at discrete times with a 2-fold molar excess of *N*-acetyleglin C over enzyme concentration. The products of cleavage were separated and quantified by chromatography on a Novo-Pak $C_{18}$ column (3.9 × 150 mm) using a 0–70% linear acetonitrile gradient containing 0.1% trifluoroacetic acid. The products of cleavage of DNCPFHYNP by cathepsin G eluted at 25% and 32% acetonitrile. The products of cleavage of DNCQYVYNV by cathepsin G eluted at 25% and 29% acetonitrile, whereas cleavage by neutrophil elastase resulted in a doublet at 26–27% acetonitrile. On one occasion, the products were collected and their sequences determined. Initial rates of peptide cleavage were determined from reactions in which less than 20% of the peptide was cleaved. The initial rate data were fit to the Michaelis–Menten equation by nonlinear regression analysis to obtain $k_p$, the catalytic constant for peptide cleavage, and $K_p$. No statistically significant differences in the $K_p$ values measured as described here versus the $K_p$ values measured using the alternate substrate assays described above were observed.

*Fibronectin Degradation Assays.* Fibronectin (0.9 $\mu$M) and cathepsin G (23 nM) or neutrophil elastase (5 nM) were mixed in the absence and presence of 2 mM each of the seven peptides for 10 min at 37 °C in 50 mM HEPES, 0.125 M NaCl, and 1 mg/mL PEG 6000 (pH 7.4) buffer, after which 50-$\mu$L aliquots were sampled into SDS treatment buffer. The samples were subjected to SDS–PAGE on a 10% gel under reducing conditions, as described previously (Hogg et al., 1993a,b).

## RESULTS

*Identification of Possible Inhibitory Reactive Centers in TSP1.* The conserved reactive-site sequence, XXX-Cys-(Pro, Thr)-$P_1$-XXX-(Tyr,Phe)-(Arg,Asn,Asp)-Pro, is derived from the Kazal and SSI inhibitors in Table 1. The $P_1$ residue defines the enzyme specificity of the inhibitor: it is Lys or Arg for trypsin-like serine proteinases and usually Met or Leu for chymotrypsin-like enzymes (Laskowski & Kato, 1980). The two homologous sequences in human TSP1 (Lawler & Hynes, 1986), residues 735–742 and 794–801, are aligned with the Kazal and SSI reactive-site sequences in Table 1. Note the conservation of Cys at $P_3$, Pro at $P_2$ (at TSP1 residue 737), Tyr at $P_2'$, Asn at $P_3'$, and Pro (at TSP1 residue 742) at $P_4'$. The $P_1$ residues are Pro and Tyr, which for substrates and peptide chloromethyl ketone inhibitors of cathepsin G are the preferred amino acids at $S_1$ and $P_1$ (Tanaka et al., 1985), respectively, while Leu or Met is usually the preferred $P_1$ amino acid in inhibitors of neutrophil elastase. The sequence similarity between TSP1 residues 735–742 and 794–801 and the Kazal and SSI reactive centers does not extend to any other parts of the TSP1 or Kazal and SSI molecules.

The type 3 repeats of human TSP1 are aligned in Figure 1 according to homologies in the 12 calcium-binding loops. Sun et al. (1992) noticed that the Asp-XXX-Asp-XXX-Asp-Gly-XXX-XXX-Asp-XXX-XXX-Asp-XXX loop sequences are followed six times by a 10 amino acid extension they termed the break sequence. The two potential reactive centers are in the break sequences following loops 2 and 5.

*Interaction of the Break Sequence Peptides with the Active Sites of Cathepsin G and Neutrophil Elastase.* To examine the ability of the proposed reactive-center amino acids to interact with the active sites of the neutrophil enzymes, small linear peptides corresponding to the putative $P_5$ through $P_4'$ amino acids and five control peptides from the other homologous break sequences in the type 3 domains were

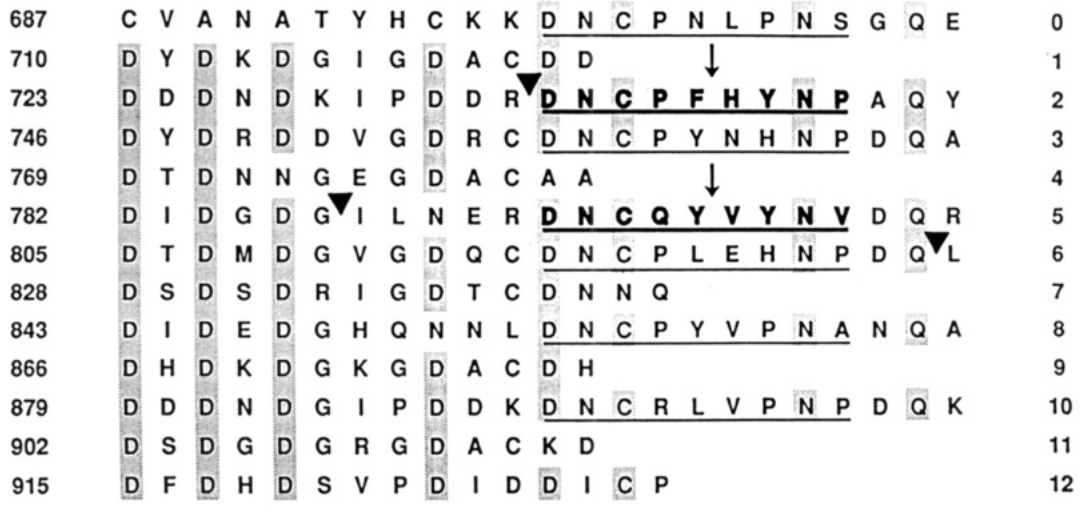


FIGURE 1: Alignment of the type 3 repeats of human TSP1 according to homologies in the 12 calcium-binding loops (Sun et al., 1992). Conserved Asp residues in the calcium-binding loops and Cys, Asn, and Gln residues in the break sequences are shaded. The seven break sequence peptides that were synthesized are underlined. The putative reactive-center sequences are in boldface type. The arrows indicate the proposed reactive-site peptide bonds. The triangles indicate where introns have been excised from exons 15 and 16.

synthesized and tested for interaction with the active sites of cathepsin G and neutrophil elastase. The Michaelis constants for peptide binding to the neutrophil enzymes were determined from the ability of the peptides to competitively inhibit the hydrolysis of a specific tripeptidyl *p*-nitroanilide substrate (Figure 2). The results were independent of the enzyme concentration used in the assays, which is predicted by the alternate substrate model proposed to account for the data, eq 1. The kinetic parameters for hydrolysis of the break sequence peptides by the neutrophil enzymes are shown in Table 2. TSP1 peptide 734–742, DNCPFHYNP, was bound and cleaved with high efficiency by cathepsin G, while peptide 793–801, DNCQYVYNV, was bound and cleaved by cathepsin G and neutrophil elastase with about 30-fold less efficiency. Peptide DNCPFHYNP was cleaved by cathepsin G between F and H, while peptide DNCQYVYNV was cleaved by cathepsin G between Y and V and by neutrophil elastase between V and Y. The peptide bond cleaved by cathepsin G in both peptides is that between the proposed $P_1$ and $P_1'$ amino acids, while peptide DNCQYVYNV is cleaved by neutrophil elastase one residue farther downstream, that is, between the proposed $P_1'$ and $P_2'$ amino acids.

The cysteine residues of all peptides were carboxymethylated to prevent intermolecular disulfide bond formation. To test the effect of disulfide bond formation on the interaction of the proposed reactive-site break sequence peptides with the neutrophil enzymes, disulfide-bonded dimers were purified free of monomers. As shown in Table 2, there was little difference in the binding of the dimeric peptides, compared to the monomeric peptides, to either cathepsin G or neutrophil elastase.

The control peptides from the other break sequences differed from the reactive-center peptides at residue $P_1$, $P_2$, or $P_4'$ and did not interact with the neutrophil enzymes. The only exception was the break sequence peptide following calcium-binding loop 3, DNCPYNHNP, which interacted weakly with both cathepsin G and neutrophil elastase but was not cleaved, or cleaved only very slowly.

*Break Sequence Peptides as Competitive Inhibitors of the Fibronectin-Degrading Activity of Cathepsin G and Neutrophil Elastase.* A qualitative demonstration of the ability of the reactive-center peptides to competitively inhibit hydrolysis of a macromolecular substrate by the neutrophil enzymes is shown in Figure 3. The proposed TSP1 reactive-center peptides were effective in inhibiting fibronectin degradation by cathepsin G and neutrophil elastase, whereas the control break sequence peptides were without significant effect. Both reactive-center peptides also competitively inhibited cathepsin G-mediated platelet aggregation (data not shown).

Table 2: Kinetic Constants for Hydrolysis of the Seven Break Sequence Peptides by Cathepsin G and Neutrophil Elastase

| peptide[a] | associated $Ca^{2+}$-binding domain | $K_p$ (mM)[b] | $k_p$ ($s^{-1}$) | $k_p/K_p$ ($M^{-1}$ $s^{-1}$) |
|---|---|---|---|---|
| | Cathepsin G | | | |
| DNCPNLPNS | 0 | NE[c] | | |
| DNCPF*HYNP | 2 | 0.28 ± 0.04 | 32 | $1.1 \times 10^5$ |
| $(DNCPFHYNP)_2$ | | 0.46 ± 0.04 | | |
| DNCPYNHNP | 3 | 1.7 ± 0.3 | ND[d] | |
| DNCQY*VYNV | 5 | 0.67 ± 0.05 | 2.4 | $3.6 \times 10^3$ |
| $(DNCQYVYNV)_2$ | | 0.46 ± 0.01 | | |
| DNCPLEHNP | 6 | NE | | |
| DNCPYVPNA | 8 | NE | | |
| DNCRLVPNP | 10 | NE | | |
| | Neutrophil Elastase | | | |
| DNCPNLPNS | 0 | NE | | |
| DNCPFHYNP | 2 | 2.5 ± 0.2 | ND | |
| $(DNCPFHYNP)_2$ | | 5.8 ± 0.5 | | |
| DNCPYNHNP | 3 | 4.7 ± 0.9 | ND | |
| DNCQYV*YNV | 5 | 1.5 ± 0.1 | 4.9 | $3.3 \times 10^3$ |
| $(DNCQYVYNV)_2$ | | 1.4 ± 0.2 | | |
| DNCPLEHNP | 6 | NE | | |
| DNCPYVPNA | 8 | NE | | |
| DNCRLVPNP | 10 | NE | | |

[a] The asterisks indicate the peptide bonds cleaved by the enzyme. The peptides in parentheses indicate disulfide-bonded dimers. The $K_p$ for dimers is expressed in terms of monomer concentration units. The cysteine residues of all peptides have been carboxymethylated. [b] Errors are two standard deviations. [c] No effect (NE) of up to 4 mM peptide in alternate substrate assays. [d] $k_p$ not determined (ND) because of the very slow rate of cleavage. No cleavage was detected following incubation of 2 mM peptide with 200 nM enzyme for 1 h at 25 °C.

## DISCUSSION

Several pieces of experimental evidence strongly suggest that the binding sites on TSP1 for cathepsin G and neutrophil elastase are in the calcium-binding type 3 repeats (Hogg et al., 1993a,b). We have now identified two stretches of eight amino acids in the type 3 repeats of human TSP1 that have

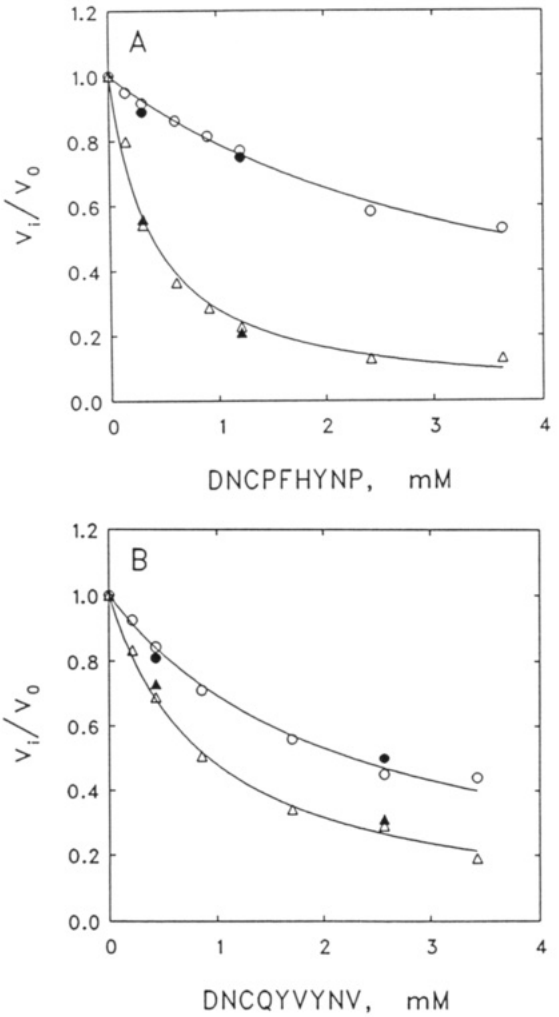


FIGURE 2: Calculation of the Michaelis constants for the cleavage of peptides by cathepsin G and neutrophil elastase using alternate substrate assays. (A) Effect of DNCPFHYNP on the initial rate of hydrolysis of AAPF-pNA and PV-pNA by cathepsin G (△, ▲) and neutrophil elastase (○, ●), respectively (see Materials and Methods). The open symbols are the results of experiments using 46 nM cathepsin G (△) and 10 nM neutrophil elastase (○), while the filled symbols are the results of experiments using 137 nM cathepsin G (▲) and 29 nM neutrophil elastase (●). The solid lines represent the best fits (Duggleby, 1984) of the data to eq 1 with $K_p$ = 0.28 ± 0.04 mM (△) and 2.5 ± 0.2 mM (○). (B) Effect of DNCQYVYNV on the initial rate of hydrolysis of AAPF-pNA and PV-pNA by cathepsin G (△, ▲) and neutrophil elastase (○, ●), respectively (see Materials and Methods). The open symbols are the results of experiments using 46 nM cathepsin (△) and 10 nM neutrophil elastase (○), while the filled symbols are the results of experiments using 137 nM cathepsin G (▲) and 29 nM neutrophil elastase (●). The solid lines represent the best fits (Duggleby, 1984) of the data to eq 1 with $K_p$ = 0.67 ± 0.05 mM (△) and 1.5 ± 0.1 mM (○).

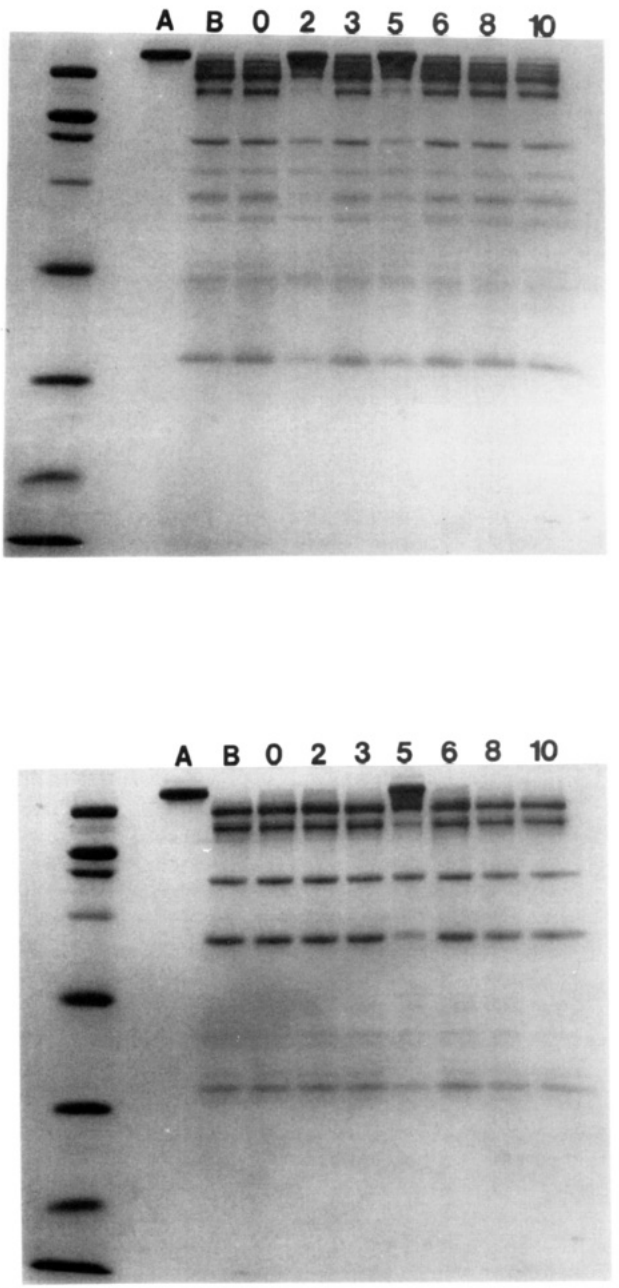


FIGURE 3: Effect of the seven break sequence peptides on the fibronectin-degrading activity of cathepsin G (A, top) and neutrophil elastase (B, bottom). Fibronectin (0.9 μM) and cathepsin G (23 nM, A) or neutrophil elastase (5 nM, B) were mixed in the absence (lane B) and presence (lanes 0–10) of 2 mM each of the seven peptides for 10 min at 37 °C as described under Materials and Methods. The samples were subjected to SDS–PAGE on a 10% gel under reducing conditions. Lane A is intact fibronectin subunit that has an $M_r$ of 220 000. The numbers correspond to the calcium-binding domains that immediately precede the break sequence peptides (see Table 2). The molecular weight markers are in the left-hand lanes and are myosin (200 000), β-galactosidase (116 250), phosphorylase B (97 400), bovine serum albumin (66 200), ovalbumin (45 000), carbonic anhydrase (31 000), and soybean trypsin inhibitor (21 500).

striking homology with the reactive centers of selected members of the Kazal and *Streptomyces* subtilisin inhibitor families. We suggest that these sequences represent the reactive centers in TSP1 that bind the neutrophil enzymes.

An essential feature of the reactive-center residues of Kazal inhibitors is their ability to bind with high affinity to the active sites of their target enzymes. Cleavage of the reactive center at the $P_1$–$P_1'$ peptide bond usually occurs only slowly or not at all, and even if the $P_1$–$P_1'$ peptide bond is cleaved, the enzyme often remains tightly bound. This is a consequence of the reactive center being stabilized by a disulfide-bonded loop, which is forced into a rigid canonical conformation by the protein core of the inhibitor (Laskowski & Kato, 1980; Read & James, 1986; Bode & Huber, 1992). In contrast, synthetic peptides based on reactive-center sequences are usually efficiently cleaved by the target enzymes, and the products of the cleavage no longer interact or interact only weakly with the enzymes. This is most likely because the reactive-center peptides do not share the rigid conformation imposed on the intact reactive center by the protein core of the inhibitor, which probably also accounts for their invariably weaker binding to the target enzymes. Despite these limita-

tions, an investigation of the ability of putative reactive-center peptides to interact with the active sites of target enzymes does provide information about the likelihood of these sequences interacting with the target enzyme in the intact inhibitor.

We have applied the above reasoning by making peptides corresponding to the putative $P_5$ through $P_4'$ amino acids of the proposed TSP1 reactive centers and five control peptides from the other homologous break sequences in the TSP1 type 3 domains and have tested them for interaction with the active sites of cathepsin G and neutrophil elastase. Both proposed reactive-center peptides, DNCPFHYNP and DNCQYVYNV, were bound and cleaved with high efficiency by cathepsin G, while only the reactive-center peptide DNCQYVYNV was bound and cleaved by neutrophil elastase. Peptides were cleaved by cathepsin G at the proposed $P_1$–$P_1'$ peptide bond. Peptide DNCQYVYNV was cleaved by neutrophil elastase at the proposed $P_1'$–$P_2'$ peptide bond, suggesting that the reactive site for neutrophil elastase may be offset one residue downstream. The possibility of separate but overlapping reactive sites in TSP1 has a precedent in the serpin, $\alpha_2$-antiplasmin, which has separate reactive sites for plasmin or trypsin and chymotrypsin. The reactive site in $\alpha_2$-antiplasmin for chymotrypsin occurs one residue downstream from the reactive site for plasmin or trypsin (Potempa et al., 1988). The control peptides from the other break sequences did not interact, or interacted only poorly, with the neutrophil enzymes.

Several other observations support the TSP1 sequences proposed as reactive centers for the neutrophil enzymes. In the absence of calcium ions, 6 mol of cathepsin G or 3 mol of neutrophil elastase bind 1 mol of TSP1 trimer with site-binding dissociation constants of 2 or 35 nM, respectively (Hogg et al., 1993a,b). The finding that both reactive-center peptides interact efficiently with cathepsin G but only one interacts efficiently with neutrophil elastase is consistent with the enzyme:TSP1 molar stoichiometries measured kinetically. The relative efficiency with which the reactive-center peptides interact with the neutrophil enzymes also parallels the relative affinity of the enzymes for TSP1, that is, cathepsin G interacts with both reactive-center peptides more efficiently than does neutrophil elastase and also binds with greater affinity to TSP1. In particular, peptide DNCPFHYNP is cleaved approximately 10 times more efficiently than the best peptide substrate for cathepsin G hitherto reported, angiotensin I (Klickstein et al., 1982).

Comparison of the seven peptides examined in this study indicates that Tyr at $P_2'$ is important for efficient binding of the peptides to the active sites of the neutrophil enzymes and for their subsequent cleavage. This observation is supported by X-ray crystallography studies of complexes between Kazal inhibitors and their target enzymes. For example, the $P_2'$ Tyr of the third domain of the turkey ovomucoid inhibitor is second only to the $P_1$ Leu residue in number of intermolecular contacts with *Streptomyces griseus* protease B (Read et al., 1983).

The two break sequences in the TSP1 type 3 repeats that contain the putative reactive centers are preceded by a calcium-binding loop and followed by a loop plus a break sequence. This loop–break sequence–loop–break sequence arrangement occurs only twice in the type 3 repeats, in accordance with the number of binding sites for cathepsin G, and this suggests that the loop–reactive center–loop–break sequence combination may be important for the tertiary structure of the inhibitory domains.

It is timely to speculate whether other thrombospondins might also interact with and inhibit the neutrophil enzymes.

Table 3: Comparison of the Putative Reactive-Site Sequences Identified in Human TSP1 with the Corresponding Amino Acid Sequences in Mouse TSP1, TSP2, and TSP3, Chicken TSP2, Frog TSP4, and Rat and Bovine COMP

| | putative reactive-site residues | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | $P_4$ | $P_3$ | $P_2$ | $P_1$ | $P_1'$ | $P_2'$ | $P_3'$ | $P_4'$ |
| human TSP1 (735–742) | Asn | Cys | Pro | Phe | His | Tyr | Asn | Pro |
| mouse TSP1 | Asn | Cys | Pro | Phe | His | Tyr | Asn | Pro |
| mouse TSP2 | Asn | Cys | Gln | Leu | Leu | Phe | Asn | Pro |
| chicken TSP2 | Asn | Cys | Pro | Leu | Leu | Phe | Asn | Pro |
| mouse TSP3 | Asn | Cys | Arg | Leu | Phe | Pro | Asn | Lys |
| frog TSP4 | Asn | Cys | Val | Leu | Ala | Ala | Asn | Ile |
| rat COMP | Asn | Cys | Pro | Leu | Val | Arg | Asn | Pro |
| bovine COMP | Asn | Cys | Pro | Leu | Val | Arg | Asn | Pro |
| human TSP1 (794–801) | Asn | Cys | Gln | Tyr | Val | Tyr | Asn | Val |
| mouse TSP1 | Asn | Cys | Gln | Tyr | Val | Tyr | Asn | Val |
| mouse TSP2 | Asn | Cys | Pro | Tyr | Val | Tyr | Asn | Thr |
| chicken TSP2 | Asn | Cys | Pro | Tyr | Val | Tyr | Asn | Thr |
| mouse TSP3 | Asn | Cys | Pro | Lys | Val | Pro | Asn | Pro |
| frog TSP4 | Asn | Cys | Gln | Arg | Val | Pro | Asn | Val |
| rat COMP | Asn | Cys | Pro | Arg | Val | Pro | Asn | Phe |
| bovine COMP | Asn | Cys | Pro | Lys | Val | Pro | Asn | Ser |

There are at least four distinct thrombospondins in human and mouse. TSP1 and TSP2 have the same domain structure, whereas TSP3 and TSP4 lack the connecting region and the properdin modules of TSP1 and TSP2, but contain an extra epidermal growth factor module. The thrombospondins are most homologous in the carboxy-terminal calcium-binding repeats and the globular domain, which is of particular relevance to these studies as this is the region of human TSP1 that contains the reactive sites for the neutrophil enzymes. Comparison of the reactive-center amino acid sequences in human TSP1 with the corresponding translated sequences in mouse TSP1, TSP2, and TSP3 (Vos et al., 1992), chicken TSP2 (Lawler et al., 1991), frog TSP4 (Lawler et al., 1992), and rat and bovine COMP (Oldberg et al., 1992) is shown in Table 3. Cartilage oligomeric matrix protein, or COMP, has a domain structure very similar to that of TSP4. Comparison of the human or mouse TSP1 and mouse or chicken TSP2 sequences indicates that TSP2 probably is also an effective inhibitor of cathepsin G and neutrophil elastase. This is of particular interest because mouse TSP1 and TSP2 recently have been shown to be expressed as both homo- and heterotrimers (O'Rourke et al., 1992). Note that the $P_4$, $P_3$, $P_2$, $P_1$, $P_2'$, $P_3'$, and $P_4'$ residues match the consensus sequence derived from Table 1 or the human TSP1 sequences. Indeed, preliminary results indicate that recombinant mouse TSP2 expressed in the baculovirus system is a potent competitive inhibitor of cathepsin G activity and a weak competitive inhibitor of neutrophil elastase activity (P. J. Hogg, C. Hui, and D. F. Mosher, unpublished observations). The corresponding sequences in mouse TSP3, frog TSP4, and rat and bovine COMP are less likely to interact with the active sites of serine proteinases because Tyr has been replaced by Pro, Ala, or Arg at $P_2'$, although other members of the Kazal family of inhibitors have Pro, His, or another amino acid at $P_2'$, indicating that Tyr at this position is not a prerequisite for inhibitory activity (Laskowski & Kato, 1980). Also, one of the $P_1$ residues in mouse TSP3, frog TSP4, and rat and bovine COMP is Lys or Arg (bottom panel of Table 3), which usually confers specificity for the inhibition of trypsin-like serine proteinases (see Table 1). Despite the absence of Tyr at $P_2'$, it will be of interest to determine whether TSP3, TSP4, and COMP can bind and inhibit trypsin-like serine proteinases.

Unlike the properdin and epidermal growth factor repeats of human TSP1, each of which are encoded by separate exons,

the calcium-binding type 3 repeats overlap exon/intron boundaries (Bornstein, 1992). In contrast, the two reactive-center peptides are coded by exons 15 and 16, respectively (Figure 1). This suggests the possibility that exon/intron boundaries may be organized around intact inhibitory domains rather than intact calcium-binding structures.

The diversity of structure and expression of the thrombospondins implies a multifunctional role for this family of proteins in biology. The findings in this study indicate that future studies of the functional properties of thrombospondins should consider their enzyme regulatory potential.

## ACKNOWLEDGMENT

We thank Albert Tseng for help with peptide synthesis and sequence analysis.

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