Maspin is a member of the serpin family with a reactive center loop that is incompatible with proteinase inhibition by the serpin conformational change mechanism. Despite this there are reports that maspin might regulate uPA-dependent processes in vivo. Using exogenous and endogenous fluorescence, we demonstrate here that maspin can bind uPA and tPA in both single-chain and double-chain forms, with $K_d$ values between 300 and 600 nM. Binding is at an exosite on maspin close to, but outside of, the reactive center loop and is therefore insensitive to mutation of Arg340 within the reactive center loop. The binding site on tPA does not involve the proteinase active site, with the result that maspin can bind to S195A tPA that is already complexed to plasminogen activator inhibitor-1. The ability of maspin to bind these proteinases without involvement of the reactive center loop leaves the latter free to engage in additional, as yet unidentified, maspin-protein interactions that may serve to regulate the properties of the exosite-bound proteinase. This may help to reconcile apparently conflicting studies that demonstrate the importance of the reactive center loop in certain maspin functions, despite the inability of maspin to directly inhibit tPA or uPA catalytic activity in vitro assays through engagement between its reactive center loop and the active site of the proteinase.

Maspin is a 375-residue globular protein that possesses potent anti-cancer and anti-angiogenic properties (1–4). It was first identified as a potent inhibitor of tumor invasion by subtractive hybridization of normal and cancerous breast tissue (5). As a result there has been an accelerating interest in maspin as a possible cancer therapeutic, and as a diagnostic indicator (6–8). In addition, it is known from the embryonically lethal effect of knocking out the maspin gene in mice that maspin must play a critical role in early development (9).

Upon sequencing its gene, it was realized that maspin is a member of the serpin superfamily of proteins (5), most members of which are suicide substrate inhibitors of serine and/or cysteine proteinases (10). This led to speculations that maspin might exert its biological properties through inhibition of one or more proteinases (11). The serpin mechanism involves initial recognition of an exposed reactive center loop (RCL) by the target proteinase, followed by initiation of substrate-like cleavage. Upon formation of the acyl intermediate, the RCL rapidly inserts into the major $\beta$-sheet (sheet A) of the serpin as an additional strand and thereby translocates the covalently attached proteinase to the distal end of the serpin, where the resulting tight contact between the serpin body and the proteinase active site distorts the latter and renders the proteinase non-functional (12–16). In maspin, however, the critical features that permit serpins to inhibit target proteinases by this conformational change-based mechanism are absent. Thus, inhibitory serpins have a run of four or more alanines, or similar small uncharged side chain, that are compatible with rapid integration into $\beta$-sheet A, close to the point of initial insertion (10). In maspin the relevant sequence of SIEVP contains at least two residues (Glu and Pro) that would be incompatible with such facile insertion, and one (Ile) that would probably be unfavorable (Fig. 1). Consistent, with this, it was found that cleavage of the RCL of maspin does not result in loop insertion (17). In addition, the position of the P1-P1’ scissile bond within the RCL is directly linked to the length of $\beta$-sheet A, such that loop insertion into this $\beta$-sheet can fully translocate the proteinase and permit inhibition by physical distortion (18, 19). In maspin, the arginine (Arg340) that would be the obvious P1 target residue for suggested cognate proteinases is four residues closer to the hinge point than required for this mechanism to operate, whereas the residue that would be in the appropriate place is a histidine, which would not be favored by most proteinases as a potential cleavage site (Fig. 1). Despite the unlikelihood that maspin could act as a proteinase inhibitor, at least by the conventional serpin mechanism, there have been a number of reports claiming such involvement, even to the extent of reporting a covalent maspin-proteinase intermediate (20–22). Countering this there have been studies showing that maspin appears incapable of directly inhibiting proteinases of the plasminogen activation system, uPA and tPA (23). Nevertheless, a very recent report suggested...
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FIGURE 1. Alignment of RCL sequences of maspin and PA-inhibitory serpins. The designation of P1, etc. for the three inhibitory serpins is based on the position of the scissile bond in the inhibitory serpin (37). For three serpins that inhibit tPA and uPA by the serpin-conformational change mechanism, neuroserpin, PAI-1, and protease nexin 1, the P1 arginine is shown in bold. The equivalent residue for maspin, based upon alignment is histidine, is also shown in bold. The arginine in maspin that would be the most likely target for proteinases such as trypsin, tPA, and uPA (residue 340) is shown in bold and underlined. The maspin residue changed to cysteine for labeling with fluorophores (residue 337) is shown in italics.

Labeling of Maspin—Maspin, either P337C or S75C (5–10 μM), was dialyzed in degassed 20 mM Tris, 50 mM NaCl, pH 8.0, overnight at 4 °C and then reacted with 20 μM dithiothreitol for 15 min at room temperature to ensure that the cysteine was in the reduced state. 100 μM of either 5-(((2-iodoacetoyl)-amino)ethyl)amino)-naphthalene-1-sulfonic acid or 5-iodoacetamidofluorescein were then added at room temperature in the dark and the reaction allowed to proceed for 2–3 h. The reaction was quenched with 1 mM (final concentration) β-mercaptoethanol, followed by addition of 2 mM iodoacetamide (final concentration) and reaction for 30 min at room temperature to acetylate any unlabeled cysteine on maspin. 5 mM dithiothreitol was added to quench the excess iodoacetamide. The resultant mixture was dialyzed with the same buffer above overnight at 4 °C and then passed over a desalting column to separate the labeled maspin from free fluorophore or fluorophore-small molecule conjugates. The degree of labeling of maspin was calculated from the relative absorptions at 280 and 340 nm, for dansyl, and at 280 and 498 nm for fluorescein, with corrections at 280 nm for absorption by each kind of fluorophore. The extinction coefficients used were 19,940 M⁻¹ cm⁻¹ for maspin at 280 nm, 75,000 M⁻¹ cm⁻¹ for fluorescein at 498 nm, and 5,700 M⁻¹ cm⁻¹ for dansyl at 340 nm. The efficiency of labeling for both fluorophores was ~95%.

Kinetic Measurements—For rates of substrate reaction, maspin (2.2 μM) was incubated with trypsin and thrombin at 1:2000 and 1:100 molar ratios of enzyme to protein, respectively. The reactions were run at 37 °C for times up to 30 min, then stopped by addition of 50 μM Phe-Pro Arg chloromethyl ketone. The samples were heated for 2 min, with the addition of SDS loading buffer, then run on 8% Tris glycine gels (Invitrogen). Following staining with Coomassie Blue, bands were quantitated using the program Quantity One (Bio-Rad). The upper band corresponded to unreacted maspin, whereas the lower band corresponded to maspin cleaved in the reactive center loop. The gel system used for these measurements gave clear separation of native and cleaved bands, with space between them that was about three times the width of the individual protein bands. The pseudo-first order rate constant for cleavage was obtained from a semi-log plot of unreacted maspin against time, fitted using Sigma Plot, and converted to a second-order rate constant by dividing by the concentration of proteinase used.

Spectroscopic assays of tPA and uPA activity as a function of added maspin were performed on a Shimadzu UV-2101PC spectrophotometer, using polyethylene glycol-coated cuvettes. The activity of tPA and uPA toward their chromogenic substrates, spectrozyme tPA, and spectrozyme UK, in the absence or presence of different concentrations of maspin, was monitored as the increase in absorption at 405 nm. For tPA the reactions were run for 5 min at room temperature, with final concentrations of 10 nM for tPA, 100 μM for substrate, and maspin concentrations ranging from 10 nM to 1 μM. uPA reactions were performed at 37 °C, and the reaction was monitored for 30 min at 405 nm, with final concentrations of 50 nM substrate, 100 μM for uPA, and maspin concentrations ranging from 10 nM to 1 μM.

MATERIALS AND METHODS

Proteins and Reagents—5-Iodoacetamidofluorescein and 5-(((2-iodoacetoyl)-amino)ethyl)amino)-naphthalene-1-sulfonic acid were purchased from Invitrogen and Molecular Probes. Low molecular weight uPA and scuPA were a gift from Abbott Research Laboratories. scuPA was treated with diospropyl fluorophosphate to inactive any traces of tucPA present (25). P1'-NBD-labeled PAI-1 and S195A tctPA were kindly provided by Dr. Steven Olson. The P1'-NBD-labeled PAI-1 was prepared by reaction of a P1’ cysteine variant of PAI-1, as previously described (26). sctPA (Activase) was a generous gift from Genentech, and was confirmed by SDS-PAGE to be fully in the single chain form. Human maspin, with all cysteines mutated to serine, or alanine (Cys337), was expressed and purified as described previously (27). The maspin variants S337C, S75C, and R340A were produced by site-directed mutagenesis using a kit (Stratagene). The inactive S195A variant of bovine cationic trypsinogen was expressed, refolded, and purified according to the protocol of Peterson et al. (28), with a yield of 7 mg/liter. S195A trypsinogen was transformed into S195A trypsin by incubating with enterokinase at room temperature for 4 h.

a direct binding of maspin to scuPA that involves Arg340 in the reactive center loop of maspin, with the implication that the active site of the arginine-specific proteinase is involved in this interaction (24), which in turn would imply the ability of maspin to act as an inhibitor of scuPA.

In the present study we have directly examined the interaction between maspin and the arginine-specific proteinases trypsin, thrombin, uPA, and tPA. By using both wild type and an R340A maspin variant, as well as thezymogen forms of trypsin, tPA, and uPA we have demonstrated a specific interaction between one or more exosites on tPA and uPA with one or more exosites on maspin, whereas trypsin and thrombin interact through their active site with the arginine in the RCL. Neither the active sites of tPA or uPA, nor the reactive center loop of maspin are involved in these interactions with plasminogen activators, although the bound proteinase appears to be reasonably close to the RCL, based on fluorescence resonance energy transfer (FRET) measurements. These findings provide a possible means whereby maspin could modulate the in vivo activity of tPA and uPA, and so account for its involvement in various biological processes, without direct competitive inhibition. These findings may thus also help to reconcile the otherwise conflicting reports in the literature.
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run for 100 s at room temperature, with the final concentrations of 50 nM for uPA, 100 μM for spectrozyme UK, and maspin concentrations ranging from 50 nM to 10 μM.

**Clot Lysis Assay**—Clot formation was induced by addition of thrombin (10 nM) to plasma (George King Biomedical Inc., Overton Park, KS) at room temperature and monitored on a Shimadzu UV2101 PC spectrophotometer by the increase in OD at 350 nm resulting from scattering. tPA (50 nM) was added to promote clot dissolution in the absence and presence of different concentrations of maspin. Reactions were performed in polystyrene non-coated cuvettes in 20 mM sodium phosphate buffer, pH 7.4, containing 100 mM NaCl.

**Isothermal Titration Calorimetry**—Binding studies were performed on a MicroCal VP isothermal titrational calorimeter at 25°C. Proteins were dialyzed overnight in the same 20 mM Tris, 20 mM NaCl, pH 8.0, buffer to minimize mixing effects. S195A trypsin and S195A tryspinogen were at a concentration of 7 μM in the cell and were titrated with 20-μl additions of maspin (80 μM). Binding isotherms, corrected for heat of dilution of maspin into the same buffer obtained from a separate control titration, were analyzed using Origin software provided by the manufacturer. Data were fitted to a single binding site model.

**Fluorescence Spectroscopy**—Binding experiments were performed at room temperature on a PTI Quantamaster instrument equipped with double monochromators on both the excitation and emission sides. The experiments were performed in 20 mM Tris buffer, either pH 8.0 or 7.4, as indicated, containing 50 mM NaCl and 0.1% polyethylene glycol 8,000. Excitation was at 498 nm for fluorescein-labeled maspin and 340 nm for dansyl-labeled maspin, with detection at the emission maxima of 516 and 482 nm, respectively. Excitation slits of 0.5 and 1 nm were used for fluorescein and dansyl-labeled samples, respectively, with emission slits at 8 nm in each case. The fluorescence signals were the average of 40–60 readings (each corresponding to 1 s) taken in a time-based mode, and were corrected for dilution and background emissions of the added enzymes at the corresponding excitation wavelength. Dissociation constants were determined by fitting fluorescence titration data to a single binding site model by non-linear least squares fitting using the program Scientist (MicroMath, Salt Lake City, UT).

FRET measurements were carried out over endogenous tryptophan as the donor fluorophore and dansyl as the acceptor. Excitation was at 295 nm to avoid excitation of tyrosine and energy transfer was monitored from increase in dansyl emission intensity at 482 nm. Experiments were carried out as titrations that were fitted to a single binding process to obtain the maximum fluorescence enhancement and to demonstrate that a specific, saturable binding process was being monitored by the fluorescence changes. Excitation slits of 1 and 8 nm were used for excitation and emission, respectively.

Fluorescence data for the titration of NBD-labeled PAI-1-S195A tctPA non-covalent complex with maspin, were recorded in 0.1 mM Hepes, 50 mM NaCl, 1 mM EDTA, 0.1% polyethylene glycol 8000, pH 7.4, buffer at 25°C on an SLM 8000 spectrofluorometer, with excitation at 480 nm, emission at 540 nm, and band-passes of 4 nm for both excitation and emission beams.

**RESULTS**

**Maspin Has an Exposed, Accessible RCL**—In the maspin crystal there are close interactions between the RCLS of pairs of maspin monomers through hydrophobic residues isoleucine (residue 341) and leucine (residue 342) that follow arginine at position 340 (27). Furthermore, whereas most of the residues preceding Arg340 are in extended β-conformation, residues 340–342 form a small right-handed helical turn (27). To determine whether this helical conformation that is present in the crystal translates into poor accessibility of Arg340 to arginine-specific proteinases, we examined the interaction with trypsin and thrombin. As has been shown previously, both of these proteinases cleave maspin as a substrate. By comparing the behavior of trypsin and thrombin with wild-type and R340A maspin, we showed that the cleavage depends on the presence of the arginine, with no cleavage of the R340A variant occurring (Fig. 2). The kinetics of these substrate cleavages was examined by SDS-PAGE of maspin incubated with low ratios of trypsin or thrombin for various periods. The resulting second-order rate constants (Table 1) indicate that Arg340 is cleaved at rates comparable with the reaction rate of several inhibitory serpins with their cognate proteinases, suggesting that the RCL of maspin is readily accessible in solution.

The affinity of trypsin for the RCL of maspin was determined by titrating inactive S195A trypsin into a P337C maspin variant derivatized with either fluorescein or dansyl. The placement of the fluorophore was chosen to be close to the proteinase without interfering with its interaction with the RCL. Both probes gave large, saturable, fluorescence changes that reported a binding interaction with K_d of about 110 nM (116 ± 6 and 114 ± 9 nm for Fig. 3, A and B, respectively). As expected from the proteolysis results, use of the fluorescently labeled R340A variant showed no measurable interaction (Fig. 3). A further demonstration of the normal substrate-like interaction between trypsin and the RCL of maspin was provided by ITC, which showed an exothermic interaction with S195A trypsin.
require the arginine at position 340, because the same strength of interaction was seen with the R340A variant ($K_d = 2.4 \pm 0.29 \mu M$) (Fig. 5B). This suggested that the interaction does not involve the RCL of maspin. To explore this possibility, we also examined the zymogen form, scuPA, and found that complex formation still occurred and that it was still independent of Arg340 (Fig. 6). Here the affinity was even stronger ($K_d = 0.64 \pm 0.07 \mu M$ for wild type and $0.41 \pm 0.05 \mu M$ for R340A variant), which may simply reflect the use of full-length scuPA, i.e. containing the kringle and epidermal growth factor domains as well as the protease domain that is the only domain present in low molecular weight uPA.

Given the tight binding of scuPA to maspin in a manner independent of the the arginine of the RCL, we next examined whether scuPA could also bind in the same way. Using the same fluorescence reporter group at residue 337, it was found that scuPA also bound to maspin without the need for Arg340 ($K_d = 320 \pm 33 \mu M$ for wild type and $300 \pm 25 \mu M$ for R340A) (Fig. 7). With dansyl instead of fluorescein as the reporter at position 337, both scuPA and scuPA gave saturable increases in intensity ($\sim 12\%$) for binding to Arg340 maspin, at pH 7.4, and $K_d$ values comparable with those obtained from fitting the fluorescence data (data not shown). It should be noted that the dependence of the sign of the fluorescence change on both the nature of the fluorophore and of the protease that binds is not unusual, and presumably reflects the different sensitivities of fluorescein and dansyl to environment (30).

**tPA Binding Does Not Involve the Protease Active Site**—The above findings that the zymogen form of uPA binds the same or tighter to maspin than the active enzyme suggests that maspin might not directly engage the protease active site. To address this more directly we examined the binding of maspin to a pre-formed non-covalent complex of the serpin PAI-1, labeled at the P1′ position with the sensitive fluorophore NBD, with S195A tctPA. By analogy with other serpin-protease complexes of known structure, such a complex is expected to involve direct engagement of the protease active site by the serpin RCL, with the P1 arginine occupying the S1 pocket of the protease (26).

Whereas addition of maspin led to a decrease in the NBD fluorescence in a saturable manner (Fig. 8), the limiting intensity was still much higher than in free NBD-labeled PAI-1 (64% higher), suggesting that maspin had not displaced the PAI-1, but had bound simultaneously to the protease, through an exosite on tPA. The fact that the NBD fluorescence is, nevertheless, perturbed by binding of maspin suggests that the

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**TABLE 1**

Second-order rate constants (M$^{-1}$S$^{-1}$) for selected serpin-proteinase interactions

| Protein | Trypsin | Thrombin | tctPA | sctPA | tcuPA |
|---------|---------|----------|-------|-------|-------|
| Neuroserpin | $5.9 \times 10^5$ | $2.1 \times 10^7$ | $6.2 \times 10^2$ | $8 \times 10^5$ | $4.7 \times 10^4$ |
| PAI-1 | $8 \times 10^5$ | $6.8 \times 10^2$ | $2.3 \times 10^4$ | $1.5 \times 10^4$ | $1.5 \times 10^4$ |
| Protease nexin 1 | $7.5 \pm 0.2 \times 10^5$ | $2.8 \pm 0.3 \times 10^4$ | ND | ND | ND |
| Maspin | $3 \times 10^5$ | $3 \times 10^5$ | $3 \times 10^5$ | $3 \times 10^5$ | $3 \times 10^5$ |

* Data from Ref. 38.
* Data from Refs. 39 and 40.
* Data from Refs. 41 and 42.
* ND, not detected.
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FIGURE 4. Trypsin binding requires the active proteinase conformation. S195A trypsin (left), but not S195A trypsinogen (right), bind exothermically to maspin, as monitored by isothermal titration calorimetry. The raw data are shown for titration of maspin into 7 μM S195A trypsin and S195A trypsinogen at pH 8.0. The raw data for S195A trypsin were corrected for a control titration of maspin into buffer prior to fitting to a single site binding model.

FIGURE 5. uPA binds to maspin independently of Arg340. Fluorescence detection of binding of low molecular weight (LMW) scuPA to maspin at pH 8.0. Panel A, wild-type maspin; panel B, R340A maspin. In each case fluorescein was attached to an engineered cysteine four residues N-terminal from Arg(Ala)340. Maspin concentration was 0.5 μM.

FIGURE 6. Maspin binds the zymogen form of uPA independently of Arg340. Fluorescence detection of binding of scuPA to maspin at pH 8.0. Panel A, wild-type maspin; panel B, R340A maspin. In each case fluorescein was attached to an engineered cysteine four residues N-terminal from Arg(Ala)340. Maspin concentration was 0.4 μM.

FIGURE 7. Maspin binds the zymogen form of tPA independently of Arg340. Fluorescence detection of binding of sctPA to maspin at pH 8.0. Panel A, wild-type maspin; panel B, R340A maspin. In each case fluorescein was attached to an engineered cysteine four residues N-terminal from Arg(Ala)340. Maspin concentration was 0.5 μM.

Exosite on tPA used by maspin may be the one that tPA uses to enhance binding of PAI-1 (31). The KD obtained from the fit (210 ± 20 nm) for binding to this two-chain tPA is similar to that obtained above for the direct binding of sctPA to maspin. Consistent with the lack of direct involvement of the proteinase active site in either tPA or uPA in binding to maspin, measurements of enzymatic activity, using the small chromogenic substrates Spectrozyme tPA and Spectrozyme UK, respectively, showed no evidence for inhibition even in the presence of high levels of maspin.

sctPA and scuPA Bind Close to the RCL of Maspin—In an attempt to localize the exosite on maspin used by sctPA, we carried out FRET measurements on the sctPA-maspin complex. sctPA was titrated into Cys337-dansyl-labeled maspin and tryptophans excited selectively at 295 nm. A saturable enhancement of dansyl fluorescence at 482 nm of about 12% was seen (fitted to a KD of 120 ± 10 nm) (Fig. 9A). Whereas it is not possible to unambiguously interpret this enhancement in terms of a specific separation between sctPA and the dansyl in the RCL, given that there are 5 well separated tryptophans in sctPA, and nothing is known of the relative fluorescence of each, the 12% value can be compared with a negative control titration of sctPA into Cys337-dansyl-labeled maspin (a position on the opposite side of the serpin, well removed from the RCL), which gave only 2% FRET (not shown). Conversely, a positive control of FRET resulting from binding of S195A trypsin to the Cys337-dansyl maspin, where the label is expected to be almost in direct contact with the proteinase, gave an enhancement of 250% (fitted to a KD of 114 ± 13 nm) (Fig. 9B).

The large FRET measured for the S195A trypsin-maspin complex was further used to examine whether the binding of scuPA and sctPA was sufficiently close to the RCL to interfere with binding of S195A trypsin in the RCL. Titrations of S195A trypsin into Cys337-dansyl-labeled maspin complexed either with scuPA (five titrations with 0.5 μM maspin and scuPA ranging from 0.5 to 3 μM) or with sctPA (two titrations with 0.5 μM maspin and sctPA of 0.5 and 1.0 μM) resulted in the same end point FRET enhancement as the titrations in the absence of scuPA or sctPA, respectively, and an unperturbed KD for the maspin-S195A trypsin interaction. These data suggest that both scuPA and sctPA bind at a site that, whereas close to the RCL, is not close enough to perturb the interaction between S195A trypsin and the P4-P4′ region of the RCL of maspin.

Effect of Maspin on tPA-mediated Clot Lysis—The effect of maspin on the ability of tPA to promote clot lysis was carried out in vitro in a cell-free system, using whole plasma. Addition of maspin (0.1, 0.5, and 1.0 μM) failed to have any detectable effect on the rate of lysis by 50 nM tPA of a clot formed by addition of 10 nM thrombin.

DISCUSSION

Our previously published x-ray structure showed that maspin has the expected serpin fold, together with an exposed reactive center loop that is mostly in extended conformation, although with a small helical region around Arg340 (27). Results presented here on the second order rate constants for reaction of maspin with trypsin and with the more selective proteinase thrombin (Table 1) establish that the reactive center loop around Arg340 is readily accessible as a site of cleavage by these
proteinas in. The requirement for arginine at this position, demonstrated by the failure of either proteinase to cleave an R340A variant, is in keeping with the specificity of these proteinases. In addition, the failure to detect binding of S195A trypsinogen to maspin by ITC, whereas binding of S195A trypsin is readily detected as an exothermic event, is analogous to the behavior with the reactive center proteinases trypsin and thrombin.

Thus, the 12% FRET enhancement between sctPA and a dansyl reporter at the much more distant residue 75, reinforce this conclusion. Whereas the exosite on maspin must be close to its RCL, we have, however, shown that binding of either scuPA or sctPA to maspin still permits binding of S195A trypsin to the RCL without alteration of its 

Based on these conclusions, a model for the uPA(tPA)-maspin interaction can be proposed that accommodates the present findings and that can also help explain some otherwise contradictory findings in the literature (Fig 10). In this model single-chain or two-chain PAs bind close to the RCL of maspin, such that a fluorophore in the RCL is close enough to sense binding either directly or through FRET from the proteinase, although not so close that binding interferes with engagement of the RCL by trypsin. The active site of the proteinase, however, must be oriented away from maspin, such that an inhibitory serpin, such as PAI-1, can engage the active site without interference from the bound maspin, but still be close enough that a fluorophore attached to the PAI-1 RCL at P1’ can sense the binding of maspin. Finally the exosite on maspin must be far removed from helix C, because a reporter fluorophore placed there (Cys337) shows minimal FRET from tryptophans in bound PA.

Although the affinities reported here between maspin and the various forms of uPA and tPA lie in the 300 – 600 nM range, and so cannot be considered tight, they are comparable with the concentrations of maspin reported by others to manifest its various physiological properties in in vivo experiments. Thus, the demonstration that maspin can block the response of endo-

FIGURE 8. Maspin forms a ternary complex with PAI-1-S195A tPA. Titration of maspin into PAI-1-S195A tctPA complex at pH 7.4, monitored by fluorescence of NBD fluorophore attached to PAI-1 P1', position 340. Although the binding is saturable, with $K_d$ of 210 nM, the final NBD fluorescence is still 86% of initial and 64% higher than in uncomplexed PAI-1-NBD.

FIGURE 9. SctPA binds close to the RCL of maspin. FRET measurements on maspin-proteinase complexes at pH 8.0. Panel A, saturable FRET enhancement of dansyl attached to Cys337 of the RCL of maspin as a function of added sctPA; panel B, much larger FRET when S195A trypsin binds directly to Arg340 in the RCL.

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FIGURE 8. Maspin forms a ternary complex with PAI-1-S195A tPA. Titration of maspin into PAI-1-S195A tctPA complex at pH 7.4, monitored by fluorescence of NBD fluorophore attached to PAI-1 P1' position 340. Although the binding is saturable, with $K_d$ of 210 nM, the final NBD fluorescence is still 86% of initial and 64% higher than in uncomplexed PAI-1-NBD.

FIGURE 9. SctPA binds close to the RCL of maspin. FRET measurements on maspin-proteinase complexes at pH 8.0. Panel A, saturable FRET enhancement of dansyl attached to Cys337 of the RCL of maspin as a function of added sctPA; panel B, much larger FRET when S195A trypsin binds directly to Arg340 in the RCL.

Just as it appears that maspin uses an exosite to engage tPA and uPA, so it appears that these proteinases use their own exosites, rather than their active sites, to bind to maspin. Thus, maspin can bind to S195A tPA already complexed through its active site with the serpin PAI-1 without displacing the PAI-1. The perturbation of the NBD fluorophore attached to the RCL of the tPA-bound PAI-1 suggests that the tPA exosite is close to the active site and may even be the one used by tPA to enhance binding to PAI-1 (31). Also in keeping with the lack of involvement of the proteinase active sites is the ability of both zymogen and active forms of tPA and uPA to bind to maspin, and the failure of maspin to diminish the rate of clot lysis promoted by addition of tPA, which also rules out the kringle region of tPA as the site of interaction. This is again in marked contrast to binding of trypsinogen versus trypsin, for which the interaction with maspin is through the proteinase active site. It is also worth noting that sctPA and scuPA differ from one another in that sctPA has a well formed active site and acts similarly to mature proteinase, whereas scuPA has activity that is 1/1000th that of the active proteinase. The ability of both proteinases in both zymogen and activated forms to bind to maspin, whereas other proteinases do not, thus suggests an exosite interaction that is highly specific for the plasminogen activators.

Whereas we have not been able to accurately map the location of the exosite (or exosites) on maspin responsible for tPA and uPA binding, we have presented data that are consistent with proximity of the proteinase to the reactive center loop. Thus, the 12% FRET enhancement between sctPA and a dansyl in the reactive center loop of maspin at position 337 suggests reasonable proximity, given that FRET efficiency falls off as the inverse sixth power of the inter-fluorophore separation, and that a typical separation between tryptophan and dansyl to give 50% efficiency of transfer ($R_o$) is about 22 Å (14). Positive and negative controls, with FRET to S195A trypsin reported by the same Cys337-dansyl fluorophore and with scuPA with a dansyl reporter at the much more distant residue 75, reinforce this conclusion. Whereas the exosite on maspin must be close to its RCL, we have, however, shown that binding of either scuPA or sctPA to maspin still permits binding of S195A trypsin to the RCL without alteration of its $K_d$, which may have important functional consequences for modulating the activity of maspin-bound plasminogen activators.
that Arg340 is also necessary, even though the RCL is not needed for optimal inhibition of cell motility and cell invasion (11), or for promotion of stromal cell adhesion (34). Significantly, quantitative measurement of maspin concentration in vivo, in corneal stroma, gave a value (~2.5 μM) well in excess of the $K_d$ values reported here for the various maspin-PA interactions (34), suggesting physiological relevance of these interactions.

Because engagement of the RCL at Arg-340 by S195A trypsin still permits scuPA to bind to the exosite of maspin, one could imagine binding of a maspin-scuPA complex to a cell-surface receptor through the RCL of maspin, without displacing the scuPA. This may account for reports elsewhere that not only is the RCL of maspin needed for certain in vivo properties, but that Arg-340 is also necessary, even though the RCL is not needed for PA binding. Thus, it was shown that the RCL of maspin alone can stimulate adhesion of corneal stromal cells to type I collagen, fibronectin, and laminin, with the result that either a maspin RCL peptide, or a serpin chimera constructed from the body of ovalbumin and the RCL of maspin behaves qualitatively like maspin in this assay (35). That study also showed that replacement of Arg-340 with glutamine was tolerated, whereas replacement with alanine was not. A study on the anti-angiogenic properties of maspin also demonstrated a role for the RCL (4). Finally, it was recently shown that mutation of Arg-340 to alanine diminished the inhibitory effects of maspin on scuPA activation and on cell detachment (24) and separately that, whereas wild type maspin inhibits Rac1, an R340Q point mutant is ineffective in such inhibition (36). These separate interactions of the RCL of maspin with one or more unknown proteins and of a maspin exosite with tPA and uPA may thus provide the explanation for the modulation of the plasminogen activator system by maspin without a direct engagement of the active sites of the proteinases. Thus, in the clot lysis assay, where no possible cell-surface receptors for maspin were present, we found that maspin had no effect on PA activity. In contrast, in the presence of a cell surface, binding of maspin:(sc)tPA or maspin:(sc)uPA to the maspin receptor through the RCL could sterically hinder the activity of the proteinase or lead to other receptor-mediated processes of down-regulation, such as have been documented by others.

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REFERENCES

1. Sheng, S., Pemberton, P. A., and Sager, R. (1994) J. Biol. Chem. 269, 30988–30993
2. Sager, R., Sheng, S., Pemberton, P., and Hendrix, M. J. C. (1996) Curr. Top. Microbiol. Immunol. 213, 51–64
3. Seftor, R. E. B., Seftor, E. A., Sheng, S. I., Pemberton, P. A., Sager, R., and Hendrix, M. I. C. (1998) Cancer Res. 58, 5681–5685
4. Zhang, M., Volpert, O., Shi, Y. H., and Bouck, N. (2000) Nat. Med. 6, 196–199
5. Zou, Z., Anisowicz, A., Hendrix, M. J. C., Thor, A., Neveu, M., Sheng, S., Rafidi, K., Seftor, E., and Sager, R. (1994) Science 263, 526–529
6. Khalkhal-Hillis, Z. (2006) Clin. Cancer Res. 12, 7279–7283
7. Smith, S. L., Watson, S. G., Ratshiller, D., Gugger, M., Betticher, D. C., and Heighway, J. (2003) Oncogene 22, 8677–8687
8. Chen, Z., Fan, Z., McNeal, J. E., Nolley, R., Caldwell, M. C., Mahadevappa, M., Zhang, Z., Warrington, J. A., and Stamey, T. A. (2003) J. Urol. 169, 1316–1319
9. Gao, F., Shi, H. Y., Daughty, C., Cellu, N., and Zhang, M. (2004) Development 131, 1479–1489
10. Gettins, P. G. W. (2002) Chem. Rev. 102, 4751–4804
11. Sheng, S., Carey, J., Seftor, E. A., Dias, L., Hendrix, M. J. C., and Sager, R. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 11669–11674
12. Lawrence, D. A., Ginsburg, D., Day, D. E., Berkenpas, M. B., Verhamme, I. M., Kvassman, J., and Shore, J. D. (1995) J. Biol. Chem. 270, 25309–25312
13. Stratikos, E., and Gettins, P. G. W. (1998) J. Biol. Chem. 273, 15582–15589
14. Stratikos, E., and Gettins, P. G. W. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 4808–4813
15. Huntington, J. A., Read, R. J., and Carrell, R. W. (2000) Nature 407, 923–926
16. Dementievs, A., Dobos, J., and Gettins, P. G. W. (2006) J. Biol. Chem. 281, 3425–3452
17. Pemberton, P. A., Wong, D. T., Gibson, H. L., Kiever, M. C., Fitzpatrick, P. A., Sager, R., and Barr, P. I. (1995) J. Biol. Chem. 270, 15832–15837
18. Zhou, A., Carrell, R. W., and Huntington, J. A. (2001) J. Biol. Chem. 276, 27541–27547
19. Tesch, L. D., Raghavendra, M. P., Bedsted-Faarvang, T., Gettins, P. G. W., and Olson, S. T. (2005) Protein Sci. 14, 533–542
20. Sheng, S., Truong, L., Frederickson, D., Wu, R., Pardee, A. B., and Sager, R. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 499–504
21. Biliran, H., and Sheng, S. (2001) Cancer Res. 61, 8676–8692
22. McGowen, R., Biliran, H. J., Sager, H., and Sheng, S. (2000) Cancer Res. 60, 4771–4778
23. Bass, R., Fernández, A.-M. M., and Ellis, V. (2002) J. Biol. Chem. 277, 46845–46848
24. Yin, S., Lockett, J., Meng, Y., Biliran, H. J., Blouse, G. E., Li, X., Reddy, N., Zhao, Z., Anagli, J. Y., Cher, M. L., and Sheng, S. (2006) Cancer Res. 66, 4173–4181
25. Schwartz, B. S., and España, F. (1999) J. Biol. Chem. 274, 15278–15283
26. Olson, S. T., Swanson, R., Day, D., Verhamme, I., Kvassman, J., and Shore, J. D. (2001) Biochemistry 40, 11742–11756
27. Al-Ayyoubi, M., Gettins, P. G. W., and Volz, K. (2004) J. Biol. Chem. 279, 19508 JOURNAL OF BIOLOGICAL CHEMISTRY VOLUME 282 • NUMBER 27 • JULY 6, 2007
28. Peterson, F. C., Gordon, N. C., and Gettins, P. G. W. (2001) Biochemistry 40, 6275–6293
29. Ke, S. H., Coombs, G. S., Tachias, K., Corey, D. R., and Madison, E. L. (1997) J. Biol. Chem. 272, 20456–20462
30. Lakowicz, J. R. (1999) Principles of Fluorescence Spectroscopy, 2nd Ed., Kluwer Academic/Plenum Publishers, New York
31. Ibarra, C. A., Blouse, G. E., Christian, T. D., and Shore, J. D. (2004) J. Biol. Chem. 279, 3643–3650
32. Pasternak, A., White, A., Jeffery, C. J., Medina, N., Cahoon, M., Ringe, D., and Hedstrom, L. (2001) Protein Sci. 10, 1331–1342
33. Filfil, R., Ratavosi, A., and Chalikian, T. V. (2004) Biochemistry 43, 1315–1322
34. Ngamkitidechakul, C., Burke, J. M., O’Brien, W. J., and Twining, S. S. (2001) Investig. Ophthalmol. Vis. Sci. 42, 3135–3141
35. Ngamkitidechakul, A., Warejcka, D. J., Burke, J. M., O’Brien, W. J., and Twining, S. S. (2003) J. Biol. Chem. 278, 31796–31806
36. Shi, H. Y., Stafford, I. J., Liu, Z., and Zhang, M. (2007) Cell Motil. Cytoskeleton 64, 338–346
37. Schechter, I., and Berger, A. (1967) Biochem. Biophys. Res. Commun. 27, 157–162
38. Krueger, S. R., Ghisu, G. P., Cinelli, P., Gschwend, T. P., Osterwalder, T., Wolfer, D. P., and Sonderegger, P. (1997) J. Neurosci. 17, 8984–8996
39. Lawrence, D. A., Strandberg, L., Ericson, J., and Ny, T. (1990) J. Biol. Chem. 265, 20293–20301
40. Naski, M. C., Lawrence, D. A., Mosher, D. F., Podor, T. J., and Ginsburg, D. (1993) J. Biol. Chem. 268, 12367–12372
41. Scott, R. W., Bergman, B. L., Bajpai, A., Hersh, R. T., Rodriguez, H., Jones, B. N., Barreda, C., Watts, S., and Baker, J. B. (1985) J. Biol. Chem. 260, 7029–7034
42. Filfil, R., Stone, S. R., Guidolin, A., Sommer, J., and Monard, D. (1992) Biochemistry 31, 3542–3549