Mesotrypsin Has Evolved Four Unique Residues to Cleave Trypsin Inhibitors as Substrates*

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Background: Canonical serine protease inhibitors normally behave as "uncleavable" substrates; mesotrypsin targets these inhibitors as substrates.
Results: Four spatially separated amino acid residues cooperate to facilitate inhibitor cleavage by mesotrypsin.
Conclusion: Inhibitor cleavage is a complex evolutionary adaptation.
Significance: Mesotrypsin may regulate a network of serine proteases through its ability to cleave and inactivate multiple protease inhibitors.

Human mesotrypsin is highly homologous to other mammalian trypsins, and yet it is functionally unique in possessing resistance to inhibition by canonical serine protease inhibitors and in cleaving these inhibitors as preferred substrates. Arg-193 and Ser-39 have been identified as contributors to the inhibitor resistance and cleavage capability of mesotrypsin, but it is not known whether these residues fully account for the unusual properties of mesotrypsin. Here, we use human cationic trypsin as a template for engineering a gain of catalytic function, assessing mutants containing mesotrypsin-like mutations for resistance to inhibition by bovine pancreatic trypsin inhibitor (BPTI) and amyloid precursor protein Kunitz protease inhibitor (APPI), and for the ability to hydrolyze these inhibitors as substrates. We find that Arg-193 and Ser-39 are sufficient to confer mesotrypsin-like resistance to inhibition; however, compared with mesotrypsin, the trypsin-Y39S/G193R double mutant remains 10-fold slower at hydrolyzing BPTI and 2.5-fold slower at hydrolyzing APPI. We identify two additional residues in mesotrypsin, Lys-74 and Asp-97, which in concert with Arg-193 and Ser-39 confer the full catalytic capability of mesotrypsin for hydrolyzing APPI. Novel crystal structures of trypsin mutants in complex with BPTI suggest that these four residues function cooperatively to favor conformational dynamics that assist in dissociation of cleaved inhibitors. Our results reveal that efficient inhibitor cleavage is a complex capability to which at least four spatially separated residues of mesotrypsin contribute. These findings suggest that inhibitor cleavage represents a functional adaptation of mesotrypsin that may have evolved in response to positive selection pressure.

Proteases are among the most abundant and diverse of enzymes, responsible not only for protein degradation but also for modulating the functions of a majority of proteins in the proteome via highly specific and regulated limited proteolysis events. Network analysis of known cleavage events reveals the existence of a highly interconnected "protease web," in which a complex interplay between proteases and their inhibitors regulates the state of the proteome in vivo (1). Importantly, protein protease inhibitors represent critical nodes connecting this web; they themselves are highly represented as protease substrates, and because they typically inhibit protease families rather than individual enzymes, the inactivating cleavage of an inhibitor can function as a key on/off switch for an entire subnetwork of proteases (1). According to this newly evolving understanding of the proteolytic landscape, a protease with the evolved capability to inactivate protein protease inhibitors by cleavage could function as a master regulator, standing poised to engage a regulatory switch.

The largest family of proteases in the human proteome is the S1 family of serine proteases (MEROPS Database designation (2)), with more than 100 enzymes, including digestive trypsins, chymotrypsins, and elastases, enzymes of the coagulation, fibrinolysis, kallikrein, and complement systems, and a variety of membrane-associated signaling peptidases. These proteases are regulated by several types of inhibitors; the serpins (MEROPS family I4) are large proteins that trap their protease targets in a unique covalent inhibitory mechanism (3), whereas the canonical serine protease inhibitors, in humans represented by the Kazal (I1), Kunitz-BPTI (I2), and elafin (I17) MEROPS families, are small proteins or domains within larger proteins and are tight-binding reversible inhibitors (4). Canonical inhibitors are in fact limited proteolysis substrates for the very enzymes that they inhibit; acting by the Laskowski mechanism, they bind in a substrate-like fashion, positioning a specific "reactive site" peptide bond for cleavage in the protease

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§ The abbreviations used are: BPTI, bovine pancreatic trypsin inhibitor; APPI, amyloid precursor protein Kunitz protease inhibitor domain; PDB, Protein Data Bank.
active site, but bind many orders of magnitude more tightly and are cleaved many orders of magnitude more slowly than an ordinary substrate (4, 5). Another characteristic of these inhibitors is that the cleavage of the reactive site bond is reversible; the cleaved two-chain form of the inhibitor remains stably folded, can reassociate with a protease, and can undergo peptide bond resynthesis, resulting in a thermodynamic equilibrium. However, as binding of the cleaved form of the inhibitor is often very slow (5), cleavage at the reactive site bond can effectively impair protease inhibitory function. For example, the Kunitz protease inhibitor domain of the amyloid precursor protein, also known as protease nexin 2, inhibits target enzymes trypsin and factor Xa more than 100-fold more weakly after cleavage at the reactive site bond (6).

In 1980, Laskowski and co-workers (7, 8) reported that an unusual trypsin-like enzyme from the starfish Dermasterias imbricata was able to cleave the reactive site bonds of several canonical serine protease inhibitors at highly accelerated rates. Unfortunately, this discovery came a few years too early to benefit from the genomic revolution, i.e. the gene and amino acid sequences of the enzyme were not determined and the molecular adaptations responsible for its catalytic capability remain a mystery. More recently, the human trypsin isoform mesotrypsin, long known to possess peculiar resistance to inhibition by canonical inhibitors (9, 10), was found to cleave some canonical inhibitors with vastly accelerated rates (11, 12). We have recently reported that mesotrypsin targets multiple endogenous human canonical inhibitors for cleavage with substrate-like kinetics (6, 13), identifying a spectrum of likely physiological substrates that may enable mesotrypsin to function as a gatekeeper in the protease web. At present, it is not clear how prevalent such enzymes are in nature nor how complex the evolutionary adaptation involved in achieving this unusual gain-of-function.

The evolutionary divergence of mesotrypsin from the major inhibitor-sensitive trypsin isoforms is relatively recent, having occurred after the divergence of Old World monkeys and hominids (14), and mesotrypsin differs from human cationic trypsin (hereafter referred to as “trypsin”) at only 28 of its 224 residues. Although previous structural and mutagenesis studies have identified two amino acid substitutions that contribute to mesotrypsin’s inhibitor resistance and inhibitor-targeted catalytic activity, Gly-1934 to Arg (11, 12, 15) and Tyr-39 to Ser (16), it has not been established whether these mutations are sufficient to confer full gain-of-function, or whether trypsin-like enzymes that have evolved resistance to inhibition via other substitutions may also possess enhanced catalytic activity toward canonical inhibitors. Here, we have probed the contributions to functional specialization of a series of residues that differ between mesotrypsin and trypsin by identifying the minimal set of substitutions required to confer mesotrypsin-like inhibitor cleaving capability on trypsin. We find that the ability to cleave canonical trypsin inhibitors is a complex capability to which at least four spatially separated residue substitutions make significant contributions. We also examine rat peptidase p23, another trypsin-like protease that is resistant to inhibition by canonical inhibitors, finding that the alternative set of residue substitutions that confer inhibitor resistance in this enzyme do not confer comparable gain-of-function for cleaving trypsin inhibitors. Our results demonstrate that the canonical inhibitor-cleaving capability is a complex catalytic activity that is not automatically conferred upon enzymes with reduced inhibitor binding affinity, and our results suggest that in mesotrypsin the ability to cleave and inactivate trypsin inhibitors is a functional adaptation that may have evolved in response to positive selection pressure.

Experimental Procedures

Protein Expression and Purification—Expression plasmids derived from pTrap-T7, harboring genes for human cationic trypsinogen (PRSS1) (17) and human mesotrypsinogen (PRSS3) (11), were generous gifts from Dr. Miklós Sahin-Toth (Boston University). Mutations were introduced using the QuikChange kit (Agilent Technologies) according to the manufacturer’s protocols, and mutant plasmids were verified by DNA sequencing. The rat p23 (trypsin IV) peptidase coding sequence (NP_775423.1) was obtained as a synthetic gene codon optimized for Escherichia coli (Mr. Gene GmbH, Regensburg, Germany) and was subcloned into the pTrap-T7 expression vector (17) using Ncol and SacI restriction sites. Trypsin, mesotrypsin, mutant trypsins, and p23 were expressed recombinantly aszymogen forms in E. coli as described previously (12). Inclusion bodies were isolated and washed with 2 M urea, 2% Triton X-100, 50 mM Tris, pH 8.0, 10 mM EDTA, and 5 mM DTT, and then trypsinogenes were solubilized in 4 M guanidine HCl, 0.1 M Tris, pH 8.0, and 30 mM DTT. Trypsinogens were diluted to a final concentration of 0.25 mg/ml and refolded overnight in 0.9 M guanidine HCl, 0.1 M Tris, pH 8.0, 1 mM l-cysteine, and 2 mM l-cysteine in a nitrogen atmosphere devoid of oxygen. The next day, refolded trypsinogenes was purified on an ecotin affinity column using a gradient from 50 mM Tris, pH 8.0, 0.2 mM NaCl to 50 mM HCl as described previously (18). Trypsinogens were activated by proteolytic cleavage with bovine enteroproteidase in 100 mM Tris, pH 8.0, 1 mM CaCl$_2$. The ratio of enteropeptidase to trypsinogen was 1:300 (w/w) for mesotrypsin and p23, and 1:1000 for trypsin and trypsin mutants; incubation times at 37°C were 3 h for mesotrypsin, 2 h for p23, and 1 h for trypsin and trypsin mutants. The activated enzymes were purified by benzamidine affinity chromatography as described previously (12). The concentration of active trypsins was quantified by titration with 4-nitrophenyl 4-guanidinobenzoate (19).

Amyloid precursor protein Kunitz protease inhibitor domain (APPi) was expressed in Pichia pastoris and purified by ammonium sulfate precipitation, Q-Sepharose chromatography, and trypsin affinity chromatography as described previously (6). BPTI was purchased from Sigma (T0256). Concentrations of both inhibitors were determined by titration with bovine trypsin (Sigma) as described previously (12).

Enzyme Inhibition Studies—$K_i$ values of BPTI and APPi toward trypsin variants possessing the G193R mutation, alone or in combination with other mutations, were found to be in the

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**Notes:**

4 Trypsin amino acid numbering used throughout this study is standard chymotrypsin family numbering derived by homology with bovine chymotrypsin.
micromolar or nanomolar range and were determined by competitive inhibition studies using methods described previously (12). Initial enzyme rates were measured using the chromogenic substrate benzoxycarbonyl-Gly-Pro-Arg-4-nitroanilide spanning a range of concentrations (25–250 μM), in the absence or presence of inhibitor (BPTI or APPI) at a range of concentrations bracketing the $K_i$ value. Assay buffer (100 mM Tris, pH 8.0, 5 mM CaCl$_2$), inhibitor, and substrate were mixed and equilibrated at 37°C, and the reactions were then started by addition of trypsin at a final concentration of 0.1–0.25 nM. Reactions were followed over a period of 3 min, and initial rates were determined from the absorbance increase caused by the release of $p$-nitroaniline ($ε_{410} = 8480$ M$^{-1}$ cm$^{-1}$) (20). Data were globally fitted by multiple regression to Equation 1, the classic competitive inhibition equation, using Prism 5 (GraphPad Software, San Diego).

$$v = \frac{k_{cat}[E][S]}{k_{m}(1 + [I]/K_i) + [S]} \quad \text{(Eq. 1)}$$

Reported $K_i$ values represent the average and standard deviation obtained from two independent experiments.

**Inhibitor Hydrolysis Studies**—The $k_{cat}$ values for the hydrolysis of inhibitors by trypsin mutants were determined by time course experiments in which time-dependent depletion of intact inhibitors was monitored by HPLC, using methods described previously (6, 12). The inhibitor (final concentration 50 μM) was mixed with the enzyme (final concentration 5 or 0.5 μM) in 100 mM Tris, pH 8.0, 5 mM CaCl$_2$, and incubated at 37°C. This inhibitor concentration is expected to saturate the enzyme, and thus rates of inhibitor hydrolysis are anticipated to approximate true $k_{cat}$ values (6, 12). Aliquots were taken from the reaction at predetermined time points, quenched, and then analyzed by HPLC on a 50×2.0–33 Jupiter 4μ 90 Å C$_{18}$ column (Phenomenex) with a gradient of 0–100% acetonitrile in 0.1% trifluoroacetic acid. For APPI studies, aliquots (30 μl) were quenched in a solution composed of 10 μl of 8 M HCl and 60 μl of water. For BPTI studies, aliquots (30 μl) were quenched in a denaturing mixture containing 59 μl of 10 M urea and 1 μl of fresh 1 M DTT and then incubated at 37°C for 10 min to achieve reduction of disulfide bonds, after which 10 μl of 8 M HCl was added to the mixture. Each experiment included 10 time points in duplicate. A standard curve related known quantities of inhibitors to areas under the curve corresponding to the inhibitor HPLC peak. The amount of unreacted inhibitor was plotted against reaction time, and the absolute value of the slope ($|a|$) was obtained by linear regression. The $k_{cat}$ values were calculated from Equation 2,

$$k_{cat} = |a|/M_i \times ([\text{enzyme}] \times \text{volume}) \quad \text{(Eq. 2)}$$

where $M_i$, inhibitor is the molecular weight of BPTI or APPI, [enzyme] is the trypsin concentration in the hydrolysis reaction, and volume reflects the 30-μl volume of the aliquot withdrawn per time point. Experimental results are reported as average and standard deviation from multiple independent experiments.

**Protein Crystallization, Data Collection, Structure Determination, and Refinement**—Trypsin-G193R and trypsin-K97D were each mixed with BPTI in equimolar ratio at a total protein concentration of 3–4 mg/ml, mixed 1:1 (v/v) with reservoir solution, and crystallized by the hanging drop method over a reservoir containing 0.2 M ammonium sulfate, 0.1 M sodium cacodylate trihydrate, pH 6.5, and 30% PEG-8000, at room temperature. Trypsin-K97D used for crystallization contained an additional S195A mutation resulting in a catalytically inactive enzyme as a precaution to avoid autoproteolysis of the enzyme. Crystals were harvested, cryoprotected, and flash-cooled in liquid N$_2$. X-ray diffraction data were collected at beamline X29A of the National Synchrotron Light Source, Brookhaven National Laboratory. Data were collected at 100 K from one crystal of trypsin-G193R-BPTI that diffracted to 1.7 Å resolution but with weak reflection intensity and poor completeness in the highest resolution shells. Although all measured reflections were used in refinement, the effective resolution of this structure is considered to be ~1.83 Å; the 1.87–1.83 resolution shell had mean $I/\sigma$ of 2.3 and 82% completeness, whereas overall completeness to 1.83 Å was 97.5%. The trypsin-G193R-BPTI crystal belonged to the space group P2$_1$, with unit cell dimensions $a = 53.0$, $b = 63.2$, and $c = 90.5$, and it contained two copies of the complex in the asymmetric unit. X-ray data were merged and scaled using DENZO/SCALEPACK (21), and both structures were solved by molecular replacement using MOLREP in CCP4 (22), using as the search model the complex of human cationic trypsin with BPTI (Protein Data Bank (PDB) code 2RA3). Refinement employed alternating cycles of manual rebuilding in COOT (23) and automated refinement using Refmac5 (24). The final stage of refinement included addition of solvent molecules into peaks greater than 1σ and within acceptable H-bonding distance from neighboring protein atoms. The quality of the final models was analyzed using wwPDB validation tools (25). The coordinates and structure factors have been submitted to the Protein Data Bank under the accession codes 4WWY for trypsin-G193R-BPTI and 4WVV for trypsin-K97D-BPTI. Structure figures were generated using PyMOL (Schrödinger, LLC).

**Results**

G193R and Y39S Substitutions Confer Most of the Inhibitor Resistance of Mesotrypsin but Leave Substantial Inhibitor-Cleaving Activity Unexplained—Mesotrypsin and human cationic trypsin (hereafter designated trypsin) are 87.5% identical, differing at only 28 of 224 residues (Fig. 1). To identify the subset of substitutions at these positions that are both necessary and sufficient to account for mesotrypsin resistance to inhibition and for inhibitor-cleaving activity, we introduced mesotrypsin residues into the corresponding positions of trypsin by site-directed mutagenesis. We then assessed inhibitor resistance by measuring inhibition constants ($K_i$) for canonical inhibitors in competitive inhibition experiments versus a colorimetric short peptide substrate, and we directly measured enzymatic cleavage rates of the canonical inhibitors using HPLC-based assays. For these studies, we chose two representative canonical inhibitors as models. BPTI is an exceptionally stable.
inhibitor that is remarkably resistant even to cleavage by mesotrypsin (12, 26), and thus it serves as a rigorously challenging benchmark for the catalytic capability of the trypsin mutants, and the Kunitz protease inhibitor domain of the APPI is a likely physiological substrate that is cleaved within seconds by mesotrypsin (6), and it thus serves as a relevant benchmark of biological fitness toward an evolved enzymatic capability.

Our first candidate for mutation was Gly-193 within the active site cleft, chosen because in mesotrypsin Arg-193 is known to be essential for resistance to canonical inhibitors; the mesotrypsin-R193G mutant was previously shown to regain full sensitivity to the canonical inhibitors SPINK1 and soybean trypsin inhibitor (11). As anticipated, the trypsin-G193R mutant acquired considerable resistance to trypsin inhibitors BPTI and APPI (Table 1). Trypsin was inhibited by these inhibitors with inhibition constants in the picomolar range, whereas mesotrypsin was inhibited 3–6 orders of magnitude more weakly. The trypsin-G193R single mutant was still inhibited more strongly by BPTI and APPI than was mesotrypsin, but only by a factor of 4–5; thus, the single G193R substitution alone is capable of conferring 20–25% of the inhibitor resistance of mesotrypsin (Table 1).

Next, we examined the impact of the G193R substitution on the rates at which trypsin cleaves the inhibitors BPTI and APPI (Fig. 2). Highly proteolytically stable BPTI was cleaved by trypsin with a turnover time of 31 days (Fig. 2A) and by mesotrypsin with a turnover time of 2 h (Fig. 2C); the trypsin-G193R mutant, with an intermediate turnover time of 3.2 days (Fig. 2B), showed a 10-fold enhancement in activity over trypsin, but it still pos-
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FIGURE 2. Kinetics of proteolysis of BPTI and APPI by trypsin, trypsin-G193R, and mesotrypsin. Depletion of intact inhibitors in reaction time courses was monitored by reversed phase HPLC and quantified by peak integration. Reactions with BPTI (A–C) contained 50 μM BPTI and 5 μM enzyme. The large plots compare hydrolysis by trypsin (A), trypsin-G193R (B), and mesotrypsin (C) on a similar time scale; small inset plots show a longer time scale for the much slower hydrolysis by trypsin (A) and a shorter time scale for the much faster hydrolysis by mesotrypsin (C). Reactions with APPI (D–F) contained 50 μM APPI and 0.5 μM enzyme. The large plots compare hydrolysis by trypsin (D), trypsin-G193R (E), and mesotrypsin (F) on a similar time scale; small inset plots show a longer time scale for the much slower hydrolysis by trypsin (D) and a shorter time scale for the much faster hydrolysis by mesotrypsin (F).

TABLE 2
Impact of G193R and Y39S substitutions on rates of inhibitor proteolysis by trypsin (Tr) mutants

| Enzyme           | Inhibitor |  \( k_{cat} \) | Turnover time | Catalytic activity relative to mesotrypsin |
|------------------|-----------|---------------|---------------|------------------------------------------|
| Tr               | BPTI      | 3.70 ± 0.40 × 10^{-7} \( a \) | 31 days       | 0.27                                      |
| Tr-G193R         |           | 3.60 ± 0.78 × 10^{-6} \( a \) | 3.2 days      | 2.6                                       |
| Tr-Y39S          |           | 6.85 ± 2.74 × 10^{-7} \( a \) | 17 days       | 0.50                                      |
| Tr-Y39S-G193R    |           | 1.31 ± 0.17 × 10^{-6} \( a \) | 21 h          | 9.5                                       |
| Mesotrypsin      |           | 1.37 ± 0.05 × 10^{-4} \( a \) | 2 h           | 100                                       |
| Tr               | APPI      | 2.96 ± 0.21 × 10^{-5} \( a \) | 9.4 h         | 0.032                                    |
| Tr-G193R         |           | 7.60 ± 1.38 × 10^{-3} \( a \) | 2.2 min       | 8.2                                       |
| Tr-Y39S          |           | 3.33 ± 0.15 × 10^{-5} \( a \) | 8.3 h         | 0.036                                    |
| Tr-Y39S-G193R    |           | 3.67 ± 0.63 × 10^{-3} \( a \) | 27 s          | 40                                        |
| Mesotrypsin      |           | 9.57 ± 0.39 × 10^{-2} \( a \) | 11 s          | 100                                       |

\( a \) Data were previously reported in Ref. 12.

The large plots compare hydrolysis by trypsin with a 9.4-h turnover time (Fig. 2). We next examined the substitution of Tyr-39 to Ser, alone and in combination with G193R. This candidate residue was selected because Tyr-39 forms a direct H-bond to a bound canonical inhibitor, and we previously found that the absence of this H-bond in mesotrypsin contributes to both inhibitor resistance and cleavage capability (16). Consistent with our previous studies, the Y39S substitution weakened affinity toward both canonical inhibitors by ∼5-fold. In combination with G193R, the Y39S mutation resulted in resistance to inhibition even slightly enhanced beyond that of mesotrypsin (Table 1).

Mutagenesis of Tyr-39 to Ser alone in trypsin had little impact on rates of inhibitor cleavage; however, in concert with G193R, Y39S conferred an addition 4–5-fold enhancement in cleavage rates for both BPTI and APPI (Table 2). The studies with the trypsin-Y39S/G193R mutant show that these two residues together are sufficient to completely account for mesotrypsin's resistance to inhibition by BPTI and APPI. However, cleavage of both BPTI and APPI by the double mutant was still substantially slower than that of mesotrypsin, suggesting that mesotrypsin possesses additional substitutions that assist in optimizing catalytic capability for cleavage of canonical trypsin inhibitors.

Addition of K97D as a Third Substitution Enhances Catalysis in a Substrate-specific Fashion—To identify additional candidate substitutions that may contribute to mesotrypsin's catalytic capability for cleavage of canonical inhibitors, we next examined sequence alignments of mammalian trypsins, looking for positions at which mesotrypsin possesses atypical residues. We included in our alignment a number of well studied trypsins known to be sensitive to inhibition by canonical inhibitors, along with three trypsins, rat peptidase p23 and mouse trypsins 4 and 5, that previously have been reported to be resistant to inhibition (27–29). One candidate residue to emerge from this analysis was an acidic mesotrypsin Asp-97, which is also found in rat p23 and mouse trypsins 4 and 5, where other trypsins possess a basic or neutral amino acid (Fig. 3). Residue 97 is located on the periphery of the trypsin substrate-binding cleft and comes into contact with a bound Kunitz domain inhibitor (Fig. 1B). We introduced the K97D mutation into trypsin, in isolation and in combination with the G193R and Y39S/G193R mutations, and we measured the rates of hydrolysis of BPTI and APPI. Although the K97D substitution had minimal impact on the rate of APPI cleavage, for the more cleavage-resistant inhibitor BPTI, addition of the K97D mutation in the context of pre-existing G193R and Y39S trypsin mutations resulted in a 7.6-fold enhancement in BPTI cleavage rate (Table 3).

Addition of E74K as a Fourth Substitution Confers the Full Catalytic Activity of Mesotrypsin for Cleavage of Trypsin Inhibitors—Because the trypsin-Y39S/K97D/G193R triple mutant was still less active than mesotrypsin for cleavage of...
both BPTI and APPI, we further examined sequence alignments to identify additional candidate residues potentially responsible for the remaining gap in activity. Residues 74, 217, and 221 were selected for mutagenesis studies because the residues found at these positions in mesotrypsin do not match the properties of residues more typically found at these positions in other trypsins. Additionally, none of these residues directly contact a bound Kunitz inhibitor molecule, each is located on the periphery of the trypsin substrate-binding cleft and within 10 Å of a bound inhibitor molecule. Mesotrypsin possesses Lys-74, where Asp, Glu, or Asn is more commonly found in other trypsins (Fig. 4A). His-217 where Tyr or Ser are more common, and Trp-221 where most mammalian trypsins have Gln (Fig. 4B). To test the potential contribution of these residues to inhibitor-cleaving activity, we generated the quadruple mutants trypsin-Y39S/K97D/G193R/D217H, trypsin-Y39S/K97D/G193R/Q221W, and trypsin-Y39S/K97D/G193R/E74K and measured rates of BPTI and APPI hydrolysis. Among these mutations, D217H and Q221W had little impact, whereas E74K enhanced the rates of hydrolysis of both BPTI and APPI ∼2-fold (Table 4). Comparing the catalytic activity of the single, double, triple, and E74K quadruple trypsin mutants to that of mesotrypsin, the combined effect of the four function-enhancing mutations approximates the catalytic capability of mesotrypsin (Fig. 5); notably, the quadruple mutant shows 127 and 78% activity relative to mesotrypsin for cleavage of BPTI and APPI, respectively (Table 4 and Fig. 5).

Inhibitor-resistant Rat Peptidase p23 Possesses Relatively Little Activity for Cleavage of Canonical Trypsin Inhibitors—In addition to mesotrypsin, several other mammalian trypsins have been found to be resistant to inhibition by canonical serine protease inhibitors, and they have been suggested to be functional homologs of mesotrypsin. These include rat peptidase p23 (27), mouse trypsin 4 (28), and the closely related mouse trypsin 5 (29). From sequence comparisons of these enzymes to mesotrypsin, it is apparent that the amino acid substitutions responsible for inhibitor resistance of the rodent trypsins are distinct from those found in mesotrypsin, but likely involve similar regions of the enzyme. Whereas Arg-193 in mesotrypsin plays the single most important role in conferring inhibitor resistance (11), rat p23 and mouse trypsins 4 and 5 feature the conserved Gly at this position. However, all three of these rodent trypsins feature Asp instead of the conserved Gln at the adjacent position 192 (Fig. 6A); this substitution has been proposed to interfere with inhibitor binding based on molecular modeling studies (27). We have found that Ser-39 also contributes to inhibitor resistance of mesotrypsin (16); Ser-39 is present in the inhibitor-resistant rodent trypsins as well, here in the context of a loop featuring an amino acid insertion and additional distinctive sequence elements that may be functionally significant (Fig. 6B). To our knowledge, it has not been previously reported whether the inhibitor-resistant rodent trypsins are capable of cleaving canonical protein protease inhibitors with kinetics similar to mesotrypsin. To answer this question, we recombiantly expressed and purified rat p23 peptidase, assessed its inhibition by BPTI and APPI, and measured its catalytic activity for cleavage of these inhibitors. In competitive inhibition studies, we found that p23 was inhibited several orders of magnitude more weakly than cationic trypsin by both Kunitz domain inhibitors, as anticipated (Table 5). However, in studies directly measuring cleavage rates of BPTI and APPI by p23, p23 showed only modest gains in activity compared with trypsin, by con-
Inhibitor-resistant rodent trypsins possess the standard Gly-193 but feature an unusual Asp-192. In contrast with mesotrypsin (Table 6), Rat p23 cleaved BPTI less than 2-fold more rapidly than trypsin, and although it cleaved APPI 15-fold more rapidly than trypsin, it was still about 200-fold slower than mesotrypsin for cleavage of this inhibitor. We conclude that the specific evolutionary adaptations of p23 have optimized this enzyme for resistance to inhibition, but less so for inhibitor cleavage.

**Crystal Structures of Trypsin-G193R and Trypsin-K97D in Complex with BPTI Provide Insights into Catalytic Enhancements**—To gain structural insights into the impact of amino acid substitutions on the catalytic activity of trypsin toward canonical inhibitors, we co-crystallized trypsin mutants in complex with BPTI and solved crystal structures of the complexes. Data collection and refinement statistics are summarized in Table 7. The trypsin-G193R-BPTI structure was solved by molecular replacement from a crystal that diffracted with effective resolution of ∼1.83 Å (see under “Experimental Procedures” for further information). The structure contains two nearly identical copies of the heterodimeric complex in the asymmetric unit which superimpose with an r.m.s.d. of 0.14 Å. The region surrounding the Arg-193 substitution, which lies at the molecular interface between trypsin and BPTI, is very well defined by electron density (Fig. 7A). The positioning of the Arg-193 side chain in the trypsin-G193R-BPTI structure is identical to that observed previously in the structure of mesotrypsin bound to BPTI (12), with the Arg-193 side chain forming hydrogen bonds with the carbonyl oxygen of Trp-141, the carbonyl oxygen of Pro-152, and a water-bridged H-bond with the side chain of BPTI Arg-17 (Fig. 7A). By comparing this structure to our previously solved structure of WT trypsin bound to BPTI (PDB code 2RA3), we find that, as is the case for mesotrypsin (12), the presence of Arg-193 results in a less complementary interface with the primed side of the BPTI-binding loop and loss of several intra- and intermolecular H-bonds (Fig. 7B). Our previous structural and biochemical studies of mesotrypsin have implicated this suboptimal molecular interface, involving unfavorable steric and electrostatic interaction between Arg-193 and the Arg-17 Primed residue5 of BPTI, in both reduced affinity toward and accelerated cleavage of BPTI (12, 16, 30). By finding these features precisely recapitulated in the trypsin-G193R-BPTI structure, we are able to confirm that the G193R mutation in trypsin successfully mimics the role of this key residue in mesotrypsin both structurally and functionally, lending confidence in our interpretation of the kinetic studies.

**TABLE 6** Relative catalytic capability of trypsin (Tr), rat p23, and mesotrypsin for inhibitor proteolysis

| Enzyme   | Inhibitor | $k_{cat}$ | Fold increased catalytic activity compared to trypsin |
|----------|-----------|-----------|------------------------------------------------------|
| Trypsin  | BPTI      | $3.70 \pm 0.40 \times 10^{-7}$ | 1                                                   |
| Rat p23  | BPTI      | $6.58 \pm 0.82 \times 10^{-7}$ | 1.78                                                |
| Mesotrypsin |          | $1.37 \pm 0.05 \times 10^{-6}$ | 370                                                 |
| Trypsin  | APPI      | $2.96 \pm 0.21 \times 10^{-5}$ | 1                                                   |
| Rat p23  | APPI      | $4.50 \pm 0.24 \times 10^{-5}$ | 15                                                  |
| Mesotrypsin |          | $9.27 \pm 0.39 \times 10^{-5}$ | 3132                                                |

* Data were previously reported in Ref. 12.

A sequence alignment of trypsins spanning residues Ala-188–198 shows unique mesotrypsin residue Arg-193 highlighted in gold. Inhibitor-resistant rodent trypsins possess the standard Gly-193 but feature an unusual Asp-192. B, sequence alignment of trypsins spanning residues 33–44 shows Ser-39 as found in mesotrypsin highlighted in gold. Additional surrounding residues that are unique to the rodent inhibitor resistant trypsins are also highlighted in gold.

**TABLE 5** Relative inhibitor resistance of trypsin (Tr), rat p23, and mesotrypsin

| Enzyme   | Inhibitor | $K_i$     | Fold enhanced inhibitor resistance relative to trypsin |
|----------|-----------|-----------|------------------------------------------------------|
| Trypsin  | BPTI      | $2.00 \pm 0.30 \times 10^{-11}$ | 1                                                   |
| Rat p23  | BPTI      | $6.16 \pm 0.10 \times 10^{-9}$ | 3080                                                |
| Mesotrypsin |          | $1.40 \pm 0.30 \times 10^{-5}$ | 700,000                                             |
| Trypsin  | APPI      | $1.70 \pm 0.30 \times 10^{-10}$ | 1                                                   |
| Rat p23  | APPI      | $6.97 \pm 0.53 \times 10^{-9}$ | 410                                                  |
| Mesotrypsin |          | $1.40 \pm 0.20 \times 10^{-7}$ | 824                                                  |

* Data were previously reported in Ref. 12.

$^5$ Substrate residues surrounding the cleavage site are designated by the nomenclature of Schechter and Berger (60). Starting from the scissile bond, substrate residues are numbered P1, P1', P2, etc. in the direction of the N terminus (collectively the nonprimed residues) and P1, P1', P2, etc. in the direction of the C terminus (collectively the primed residues).
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We also obtained high quality crystals and solved the structure of the trypsin-K97D/BPTI complex. This structure was solved by molecular replacement and refined against data extending to 2.1 Å resolution. The structure possesses two copies of the complex in the asymmetrical unit, both of which are well ordered and show reasonably strong electron density in the vicinity of the K97D substitution (Fig. 8). In considering how the K97D substitution at this position may function to selectively accelerate the cleavage of BPTI but not APPI, we note that trypsin Asp-97 makes close contact with the oppositely charged BPTI residue Arg-39, although the corresponding residue at this position in APPI is uncharged Gly-39. The BPTI Arg-39 contacts, forming a salt bridge with the N-terminal amine of an adjacent BPTI molecule (Fig. 8). Although the specific side chain does not form a direct salt bridge with the side chain of the binding loop maintains cleaved inhibitors in a conformational state allowing crystal packing forces, the proximity of Asp-97, but instead it forms an H-bond with the backbone carbonyl oxygen of Asp-97 (Fig. 8). Further examination of the structure reveals that the Asp-97 side chain mediates crystal contacts, forming a salt bridge with the N-terminal amine of an adjacent BPTI molecule (Fig. 8). Although the specific side chain conformations of these residues in our structure are likely to be influenced by crystal packing forces, the proximity of Asp-97 to BPTI Arg-39 and their potential to form attractive interactions may influence the rate of BPTI cleavage by favoring conformational dynamics that bring these residues into closer proximity, as described further under “Discussion.”

Discussion

In this study, using trypsin as a template, we successfully engineered a gain-of-function mutant possessing the ability to cleave canonical serine protease inhibitors at accelerated rates, an ability found in the natural trypsin isofrom mesotrypsin. Using our approach, we were able to evaluate the contributions to inhibitor cleavage of four unique residues found in mesotrypsin. We confirmed important roles for Arg-193 and Ser-39, residues previously implicated in cleavage of trypsin inhibitors by mesotrypsin, and we also identified for the first time two additional residues, Asp-97 and Lys-74, that significantly enhance the function of the engineered enzyme for cleaving trypsin inhibitors. The four mutated residues, separated in the sequence and dispersed across the catalytic cleft in the protein structure, appear to work cooperatively to confer gain-of-function for cleavage of canonical protease inhibitors. Residues Arg-193 and Ser-39, closest to the catalytic site, revealed the highest propensity for modulating mesotrypsin interactions with binding partners and catalytic activity, as one would expect. Surprisingly, however, Asp-97 and Lys-74, residues relatively far away from the catalytic site, also proved to be essential to achieve full catalytic activity toward inhibitors. Consideration of the structural context of these four key residues in light of the serine protease mechanism offers clues as to how they may function.

Like all serine proteases, trypsin catalyzes peptide bond hydrolysis using a two-step mechanism in which the nucleophilic serine first attacks the scissile bond to generate an acyl-enzyme intermediate, and subsequently, a water molecule hydrolyzes the acylenzyme. High resolution crystal structures of trypsin acyl-enzymes reveal that the hydrolytic water approaches the acyl bond via the space vacated by the P1 substrate residue, which is the leaving group in the acylation reaction (31). In previous work, we found that during proteolysis of a Laskowski mechanism canonical inhibitor the acylation step may proceed rapidly, but failure of the leaving group to dissociate from the enzyme can block acyl-enzyme hydrolysis and favor peptide bond religation (32). This model is consistent with studies from the Goldenberg laboratory on the pH dependence of trypsin cleavage of BPTI mutants (33), and with a crystal structure of cleaved BPTI bound to trypsin that reveals how the P1’ Ala-16 residue is retained in the active site blocking entry to the hydrolytic water (34). A structural study of insect metalloproteinase inhibitor, from another family of Laskowski mechanism inhibitors but unique in targeting metalloproteinases rather than serine proteases, likewise reveals how structural stabilization of the binding loop maintains cleaved inhibitors in a conforma-

### TABLE 7

| Crystal data collection and structure refinement statistics | Trypsin-G193R/BPTI | Trypsin-K97D/BPTI |
|-----------------------------------------------------------|------------------|------------------|
| PDB code                                                 | 4WWY             | 4WXV             |
| Complexes per asymmetric unit                            | 2                | 2                |
| Space group                                              | P2               | P2,2,2,2         |
| Unit cell, Å                                             | 52.95, 63.23, 90.53, 90, 94.74, 90 | 42.85, 56.58, 228.58 90,90,90 |
| Resolution, Å                                            | 1.70             | 2.1              |
| Unique reflections                                       | 56150            | 31212            |
| Completeness, %                                          | 89.9 (45.7)a,b    | 97.8 (85.3)b     |
| Multiplicity                                             | 4.7 (2.2)b       | 5.5 (4.1) b      |
| Mean I/σ                                                 | 17.0 (1.21)      | 15.9 (4.24)      |
| Rmerge                                                   | 0.068 (0.845)b   | 0.077 (0.256) b  |
| Rfree                                                   | 0.096 (0.625)b   | 0.110 (0.410) b  |
| Rcryst/Rfree (%)(b)                                     | 18.2/21.7        | 22.2/28.1        |
| Average B-factor, Å                                      | 17.8             | 25               |
| Protein atoms                                            | 4789             | 4345             |
| Water molecules                                          | 390              | 75               |
| r.m.s.d. bonds, Å                                        | 0.0198           | 0.0167           |
| r.m.s.d. angles, (°)                                     | 1.956            | 1.799            |
| Favored regions (%)                                      | 96               | 98               |
| Allowed regions (%)                                      | 4                | 2                |
| Outliers (%)                                             | 0                | 0                |

a For the trypsin-G193R/BPTI data, imposing a resolution cutoff of 1.83 Å results in 97.5% overall completeness (82.4% in the 1.87 to 1.83 Å shell).
b Values in parentheses are for the highest resolution shell.

Ramachandran distributions are reported as defined by the PDB validation server/MolProbity.
tion poised for peptide bond resynthesis (35). In aggregate, these published data suggest that during cleavage of Kunitz domains by trypsins, displacement of the leaving group from the primed side of the enzyme active site is likely to be rate-limiting and to require substantial structural distortion of the enzyme, the Kunitz domain, or both.

Our two new structures of trypsin mutants along with previously solved structures lead us to hypothesize how Arg-193, Ser-39, Lys-74, and Asp-97 work in concert to accelerate the cleavage of Kunitz domain inhibitors. Superposition of multiple mesotrypsin/Kunitz domain structures reveals that each of these four cleavage-enhancing residues can assume a variety of side chain conformations, even showing conformational variability among copies of noncrystallographic symmetry-related molecules within a single crystal structure (Fig. 9A). This observation suggests that the role of these residues in general may be to influence the active site conformational dynamics of the enzyme during catalysis, and we hypothesize that these residues may facilitate dissociation of the primed side leaving group from the acyl-enzyme by enhancing mobility at the enzyme-inhibitor interface. Intriguingly, studies of dihydrofolate reductase with a series of ligands have provided evidence that internal protein motion can serve as a mechanical initiator of ligand dissociation (36), and studies with maltose-binding protein mutants have likewise demonstrated that protein dynamics can regulate ligand dissociation rates (37); a similar phenomenon may be at work in mesotrypsin.

Arg-193 is the most critical substitution; consistent with its major impact on inhibitor affinity (Table 1) and on hydrolysis rates (Table 2), its presence disrupts multiple H-bonds on the primed side of the enzyme-inhibitor interface and introduces deleterious steric and electrostatic interactions with the P1 inhibitor residue (Fig. 7B) (12, 30). The Y39S substitution likewise eliminates a stabilizing H-bond between the Tyr-39 side
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FIGURE 9. Superpositions of mesotrypsin structures suggest conformational mobility of functionally important residues. A, structures of mesotrypsin (gray schematic representations) bound to Kunitz domain inhibitors (white schematic representations) indicate the inhibitor reactive site bond targeted for proteolysis with a black arrow; inhibitor nonprimed side residues are to the right of the arrow, and primed side residues are to the left of the arrow. Catalytic triad residues of mesotrypsin are colored red. The four mesotrypsin residues identified in this study that confer inhibitor-cleaving functionality are colored according to crystal structure: fuchsia, complex with BPTI (PDB code 2R9P, four copies); dark green, complex with BPTI-K15R/R17G (PDB code 3P92, one copy); light green, complex with BPTI-K15R/R17D (PDB code 3P95, one copy); yellow, complex with APPI (PDB code 3L33, four copies); cyan, complex with APPI-R15K (PDB code 3L3T, four copies); purple, complex with bikunin-KD2 (PDB code 4U30, four copies); pink, complex with HAI2-KD1 (PDB code 4U32, one copy). B, mesotrypsin residues Arg-193, Ser-39, and Lys-74 form a cluster in close proximity to the P2′ residue 17 of bound Kunitz domain inhibitors APPI and BPTI. Structures shown include the following: complex with BPTI (PDB code 2R9P, four copies, fuchsia); complex with APPI (PDB code 3L33, four copies, yellow); complex with APPI-R15K (PDB code 3L3T, four copies, cyan). Black dotted line indicates closest contact (3.1 Å) between like-charged Lys-74 NZ and Arg-193 NH2 (in PDB code 3L3T chain D). Inhibitor cleavage site is indicated by the black arrow. C, mesotrypsin Asp-97 and BPTI Arg-39 form close contacts near the nonprimed side of the enzyme active site. Structures shown include the following: complex with BPTI (PDB code 2R9P, four copies, fuchsia); complex with BPTI-K15R/R17G (PDB code 3P92, one copy, dark green); complex with BPTI-K15R/R17D (PDB code 3P95, one copy, light green). Catalytic triad residues of mesotrypsin are colored red. Inhibitor cleavage site is indicated by the black arrow. Black dotted line indicates closest contact (2.5 Å) between oppositely charged side chains of mesotrypsin Asp-97 and BPTI Arg-39 (in PDB code 3P95).

Asp-97 contacts BPTI on the opposite side of the molecule from the Arg-193/Ser-39/Lys-74 cluster, closer to the nonprimed inhibitor residues, and offers the opportunity for a favorable electrostatic interaction with BPTI Arg-39. In our new structure of the trypsin-K97D mutant complex with BPTI, this residue is constrained by crystal contacts (Fig. 8), whereas structures of mesotrypsin-BPTI complexes show diverse side chain conformations, including some in which formation of a direct salt bridge of 2.5–2.6 Å between Asp-97 and BPTI Arg-39 is observed (PDB codes 3P92 and 3P95, mesotrypsin bound to BPTI variants) (Fig. 9C). Because of the interactions of the BPTI primed side residues with both the enzyme and the rigid inhibitor core, localized conformational fluctuations in the acyl-enzyme may be insufficient to enable dissociation of the primed side leaving group, and we speculate that that larger displacements of the BPTI molecule may be required. The potential for Asp-97 to form favorable electrostatic interactions with BPTI Arg-39 may favor larger rocking or twisting motions of BPTI relative to mesotrypsin in the acyl-enzyme that bring these residues into closer proximity, while enlarging the enzyme-inhibitor spacing on the primed side of the interface and assisting with leaving group dissociation. Such a mechanism, although speculative, would be consistent with the enhancements in BPTI cleavage rates observed for the double and triple mutants possessing the K97D mutation (Table 3).

The cooperative involvement of four noncontiguous residues in the inhibitor-cleaving capability of mesotrypsin demonstrates a functional specialization with a complex molecular basis and suggests that this specialized function may have evolved in response to positive selection pressure. Previously, statistical analysis of correlated evolution between amino acids has been used to identify coevolved “protein sectors” in the larger chymotrypsin family to which trypsin belongs; these sectors were shown to have distinct functional roles relating to catalytic power, substrate specificity, and thermal stability (38). None of the four residues identified by our study was previously identified as belonging to a coevolving functional protein sector. However, genetic analysis of selective pressures acting on human, monkey, and mouse trypsinogen genes previously identified residue 97 (identified as residue 100 in the cited publication) as one of two sites with very high probability of evolving under positive selection in these trypsinogens (14). This study reveals surprising new functional roles for previously unstudied residues in mesotrypsin; further analyses will be required to determine whether the four identified residue positions interact functionally in other evolutionary branches of the chymotrypsin family.

In this study, we chose human cationic trypsin to represent normal inhibitor-sensitive trypsins in part because of its close sequence homology with mesotrypsin. However, trypsins normally considered “inhibitor-sensitive” display a broad range of behaviors in terms of inhibitor affinity and rates of cleavage; along this spectrum, human cationic trypsin displays weaker inhibitor affinity and faster inhibitor cleavage rates than several chain and the P4′ inhibitor backbone, and it widens the spacing between the enzyme and inhibitor in the vicinity of the P2′ and P4′ residues (16). Lys-74 is located near Arg-193 and Ser-39 in the three-dimensional structure of mesotrypsin, and although it is found in diverse conformations in different structures, the distance between the Lys-74 ε-amine and Arg-193 NH2 is found to be as short as 3.1 Å (PDB code 3L3T chain D, mesotrypsin bound to APPI-R15K) (Fig. 9B). Relative to Glu-74, the presence of Lys-74 and corresponding potential for repulsive electrostatic interaction with Arg-193 may influence the dynamics of Arg-193, promoting movements that push Arg-193 downward toward the P2′ side chain of the inhibitor; in the context of the acyl-enzyme, such motions could assist in expulsion of the primed side leaving group. This scenario is consistent with the ~2-fold enhancement of inhibitor cleavage rates observed with addition of the E74K mutation (Table 4 and Fig. 5).
other major trypsins that have been studied. In early studies from the Laskowski laboratory (39), human trypsin was shown to cleave soybean trypsin inhibitor and bovine pancreatic secretory trypsin inhibitor (Kazal) more rapidly than did porcine, rat, or bovine trypsins. In our own studies with recombinant human trypsins, we likewise found that human cationic trypsin cleaved BPTI at a rate intermediate between those of bovine trypsin and mesotrypsin (12). These observations reveal that human cationic trypsin is already partially predisposed to inhibitor cleavage, suggesting that additional residues beyond the four identified in our study, conserved among human trypsins but differing from other mammalian trypsins, may also contribute to the inhibitor-cleaving phenotype. Elucidation of these additional functional determinants offers another intriguing avenue for future study.

The specific survival advantages that led to evolution of inhibitor cleavage as a novel function of mesotrypsin remain uncertain, but a plausible explanation could involve more efficient digestion of foods rich in natural trypsin inhibitors (11). Importantly, endogenous human trypsin inhibitors may also represent specific substrates for mesotrypsin. In the gut, mesotrypsin could possibly help to degrade the pancreatic trypsin inhibitor SPINK1, although work by Sahin-Toth and co-workers (11) suggests that gradual cleavage by the major trypsin isoforms probably represents the main mechanism for SPINK1 clearance, and our own unpublished studies show that cleavage of SPINK1 by mesotrypsin is relatively slow ($k_{\text{cat}} = 1.0 \times 10^{-3} \text{s}^{-1}$; data not shown). Outside of the digestive tract many other endogenous human canonical protease inhibitors represent potential physiological substrates of mesotrypsin. We have previously found that a number of these inhibitors are cleaved very rapidly by mesotrypsin, with kinetic profiles characteristic of biologically relevant substrates (6, 13), and that cleavage of an inhibitor’s reactive site bond by mesotrypsin can profoundly compromise inhibitory activity toward other serine proteases (6). These findings suggest the potential for a broader role for mesotrypsin in regulating the activities of multiple proteases.

Looking beyond normal physiology, ectopic expression of mesotrypsin by tumors is functionally linked to increased malignancy (40–44). In this context, there are multiple mechanisms whereby endogenous inhibitor cleavage by mesotrypsin may contribute to tumor growth and progression. Mesotrypsin cleavage of APP1 (6) and of the amyloid precursor-like protein 2 (APLP2) (13), both of which regulate factor Xla and other proteases of the coagulation cascade (45–47), could lead to increased thrombin generation and corresponding promotion of tumor growth and metastasis (48–50). Mesotrypsin cleavage of bikunin (13), an inhibitor of tumor cell surface-localized plasmin (51), is expected to promote tumor metastasis (51, 52). Mesotrypsin cleavage of hepatocyte growth factor (HGF) activator inhibitor type 2 (HAI2) (13), a physiological inhibitor of HGF activator (53), hepsin (54) and matriptase (55, 56) is expected to lead to greater production of active HGF and increased signaling through the MET receptor tyrosine kinase, leading to malignant growth and cancer progression (57). The

inhibitors thus far identified as good substrates for mesotrypsin may represent but a subset of the inhibitors regulated by mesotrypsin cleavage, through which mesotrypsin may widely impact protease activity in the tumor microenvironment.

Compared with the catalytic capability for cleaving trypsin inhibitors, the ability to resist inhibition by proteinaceous trypsin inhibitors is revealed here to be a simpler molecular phenotype, requiring only two of the four mutations that confer optimal inhibitor cleavage capability on mesotrypsin. Loss of inhibitor affinity can be acquired via multiple mutational pathways, as is apparent from the inhibitor-resistant rodent trypsins. However, our results with rat p23 peptidase reveal that its alternative mutational pathway has not conferred capability for efficient cleavage of canonical inhibitors. One caveat to this conclusion is that here we tested trypsins only for cleavage of BPTI and APPL as representative canonical inhibitors, although it remains possible that rat p23 might possess greater cleavage capability toward one or a few specific inhibitors, such as Spin k3, the major pancreatic trypsin inhibitor in rodents. Regardless, p23 does not appear to possess a generalized capability for inhibitor cleavage comparable with mesotrypsin. This finding suggests that the rodent inhibitor-resistant trypsins may not represent true functional homologs of mesotrypsin, but rather that mesotrypsin and p23 may have evolved to carry out distinct biological functions. Mesotrypsin, by cleaving trypsin inhibitors and rendering them less effective for inhibition of other proteases, may regulate the downstream activity of a spectrum of serine proteases, acting as a regulatory node in the protease web (1). Inhibitor-resistant trypsins lacking this capability may have instead evolved for digestion of noninhibitor proteins in an inhibitor-rich environment or for a signaling role targeting specific protein substrates such as protease-activated receptors, where resistance to inhibitors could enable prolonged signaling activity (28, 58, 59). Future studies are called for to address the prevalence of similar trypsins in diverse organisms, identify the molecular adaptations responsible for their function, and elucidate their roles in biology.

**Author Contributions**—A. P. A. and E. S. R. designed the study, analyzed and interpreted data, and wrote the paper. A. P. A. performed mutagenesis, purified proteins, performed enzyme kinetics experiments, and crystallized protein complexes. O. K. performed enzyme kinetics experiments and refined, validated, and deposited crystal structures. R. W. solved and refined crystal structures. A. H. performed mutagenesis, purified proteins, and measured hydrolysis rates for p23 peptidase. A. S. S. collected and processed x-ray diffraction data. All authors reviewed the results and approved the final version of the manuscript.

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6 A. Hockla and E. S. Radisky, unpublished data.
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