Mesotrypsin Signature Mutation in a Chymotrypsin C (CTRC) Variant Associated with Chronic Pancreatitis*

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Background: Mutations in human chymotrypsin C (CTRC) compromise protective trypsinogen degradation and increase risk for pancreatitis.

Results: CTRC variant p.G214R degraded trypsinogen poorly; was resistant to inhibitors; and cleaved their reactive sites.

Pathogenic variant p.G214R is a functional paralog of mesotrypsin, an inhibitor-degrading trypsin isoform.

Significance: The same mutation that evolved a new function in mesotrypsin causes pathology in the context of CTRC.

Human chymotrypsin C (CTRC) protects against pancreatitis by degrading trypsinogen and thereby curtailing harmful intrapancreatic trypsinogen activation. Loss-of-function mutations in CTRC increase the risk for chronic pancreatitis. Here we describe functional analysis of eight previously uncharacterized natural CTRC variants tested for potential defects in secretion, proteolytic stability, and catalytic activity. We found that all variants were secreted from transfected cells normally, and none suffered proteolytic degradation by trypsin. Five variants had normal enzymatic activity, whereas variant p.R29Q was catalytically inactive due to loss of activation by trypsin and variant p.S239C exhibited impaired activity possibly caused by disulfide mispairing. Surprisingly, variant p.G214R had increased activity on a small chromogenic peptide substrate but was markedly defective in cleaving bovine β-casein or the natural CTRC substrates human cationic trypsinogen and procarboxypeptidase A1. Mutation p.G214R is analogous to the evolutionary mutation in human mesotrypsin, which rendered this trypsin isoform resistant to proteinaceous inhibitors and conferred its ability to cleave these inhibitors. Similarly to the mesotrypsin phenotype, CTRC variant p.G214R was inhibited poorly by eglin C, ecotin, or a CTRC-specific variant of SGPI-2, and it readily cleaved the reactive-site peptide bonds in eglin C and ecotin. We conclude that CTRC variants p.R29Q, p.G214R, and p.S239C are risk factors for chronic pancreatitis. Furthermore, the mesotrypsin-like CTRC variant highlights how the same natural mutation in homologous pancreatic serine proteases can evolve a new physiological role or lead to pathology, determined by the biological context of protease function.

Human chymotrypsin C (CTRC)3 is a digestive serine protease synthesized and secreted by the pancreas as inactive chymotrypsinogen C, which becomes activated in the duodenum by trypsin. CTRC exhibits typical chymotryptic activity and cleaves after phenylalanine, tyrosine, leucine, and methionine residues in protein substrates, with higher activity toward leucyl peptide bonds than other chymotrypsins (1–4). Besides its relatively nonspecific digestive function, human CTRC also regulates activation of trypsinogens and procarboxypeptidases through specific cleavages (4–10). Among trypsinogens, the CTRC-sensitive regulatory nick sites have been best characterized in human cationic trypsinogen, the major trypsinogen isoform in human pancreatic juice (4–9). Conversion of inactive trypsinogen to active trypsin is controlled by CTRC via two independent and seemingly conflicting mechanisms: cleavage of the trypsinogen activation peptide at the Phe19–Asp19 peptide bond accelerates autoactivation of trypsinogen (5, 7, 8), whereas cleavage of the Leu81–Glu82 peptide bond in the calcium binding loop promotes degradation of trypsinogen (4, 6, 7, 9). In the intestinal milieu, cleavage of the calcium binding loop is blocked by high calcium concentrations and trypsinogen degradation is minimal. However, in the presence of submillimolar calcium concentrations prevailing in pancreatic secretions, the dominant effect of CTRC is trypsinogen degradation, which is responsible for protecting the pancreas against premature, intra-pancreatic activation of trypsinogen. A number of human genetic studies indicate that loss-of-function mutations in CTRC increase the risk for chronic pancreatitis (11–15). Similarly, mutations in human cationic trypsinogen that inhibit CTRC-mediated degradation or accelerate CTRC-mediated cleavage of the activation peptide cause hereditary chronic pancreatitis (7). Thus, CTRC is an important failsafe in the pancreas against proteolytic enzyme activation, and inborn errors in this mechanism can result in pancreatic pathology.

CTRC is also a physiological co-activator of procarboxypeptidase A1 (CPA1) and A2 (CPA2) in humans (10). These zymo-

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3 The abbreviations used are: CTRC, chymotrypsin C; Suc, succinyl.
 gens possess 94–96-amino acid-long propeptides, which act as tethered, tight-binding inhibitors of the active enzymes. Trypsin initiates procarboxypeptidase activation by cleaving the propeptide at its C terminus. The trypsin-cleaved propeptide stays bound to the enzyme, and only low levels of carboxypeptidase activity appear. Full enzyme activity develops after CTRC-mediated degradation of the propeptide, primarily driven by cleavage of the Leu⁵⁶-Leu⁹⁷ peptide bond (10).

The regulatory functions of CTRC are highly specific, and other pancreatic chymotrypsins or elastases do not cleave the CTRC-sensitive nick sites (4, 6, 8). Several studies attempted to clarify the determinants of this specificity. Phage display-selected variants of the locust-derived chymotrypsin inhibitor Schistocerca gregaria proteinase inhibitor-2 (SGPI-2) indicated that negatively charged amino acids on the primed side of the scissile peptide bond are important for CTRC recognition (16). This notion seemed in agreement with the natural preponderance of such residues in the regulatory nick sites. However, a subsequent study in which negatively charged residues around the Leu⁴⁶-Glu⁹⁷ peptide bond in human cationic trypsinogen were mutated found only small effects on cleavage by CTRC (4). More recently, the crystal structure of human CTRC was solved in complex with eglin C (see Fig. 1A), and modeling the CTRC cleavage sites in trypsinogen onto this structure indicated that long-range electrostatic complementarity determines CTRC recognition of its regulatory proteolytic sites (17).

Since 2008 when the first CTRC mutations were described (11), our laboratory has characterized natural variants of CTRC to identify pathogenic mechanisms that increase pancreatitis risk (11, 18, 19). In our recent comprehensive study, we analyzed 32 natural CTRC variants and identified three different but mutually non-exclusive loss-of-function mechanisms associated with pancreatitis: diminished secretion, loss of catalytic activity, and degradation by trypsin (18). CTRC variants with reduced secretion also elicited endoplasmic reticulum stress in pancreatic acinar cells, but the significance of this mechanism remains indeterminate (18, 19). In the present study, we extended these findings by functionally analyzing eight previously uncharacterized CTRC variants, including two novel variants reported here for the first time. Surprisingly, one of these variants, p.G214R, turned out to be the functional paralog of human mesotrypsin, a fascinating trypsin isoform with genetic testing because of recurrent acute pancreatitis in Slovakia. No other variants were detected in exons 2 and 3 of CTRC or in the PRSS1 and SPINK1 genes commonly associated with chronic pancreatitis.

CTRC Expression Plasmids and Mutagenesis—The pcDNA3.1(−) expression plasmids harboring the coding DNA for human CTRC with or without a His₁₀ affinity tag were constructed previously (6, 7). CTRC mutants were generated by overlap extension PCR and ligated into the pcDNA3.1(−) vector using XhoI and EcoRI restriction sites. The His-tagged versions of the constructs were used for purifications.

Cell Culture and Transfection—HEK 293T cells were cultured at a density of 1.5 × 10⁶ cells/well in DMEM supplemented with 10% fetal bovine serum, 4 mM glutamine, and 1% penicillin/streptomycin, at 37 °C, in 6-well tissue culture plates. Transfections were carried out using 2 μg of expression plasmid with 5 μl of Lipofectamine 2000 (Invitrogen) in 2 ml of DMEM. After overnight incubation, cells were rinsed and covered with 2 ml of Opti-MEM. The conditioned Opti-MEM medium was harvested after 24 or 48 h, as indicated.

Measurement of CTRC Protein Secretion—Aliquots (200 μl) of the conditioned medium were precipitated with 10% trichloroacetic acid (final concentration), and the proteins were collected by centrifugation, resuspended in 15 μl of Laemmli sample buffer containing 100 mM dithiothreitol, heat-denatured at 95 °C for 5 min, and electrophoresed on 15% SDS-polyacrylamide gels. Gels were stained with Coomassie Blue. Densitometric quantitation of bands was carried out with the Gel Doc XR+ gel documentation system and Image Lab software (Bio-Rad).

Expression and Purification of CTRC—His-tagged forms of CTRC variants were purified from 200 ml of conditioned media using nickel affinity chromatography, as described previously (7). Purified CTRC zymogen was activated with 50 μl of immobilized trypsin (20230, Thermo Scientific) in 3-ml final volume. CTRC activity was then measured by adding 150 μl of 200 μM Suc-Ala-Ala-Pro-Phe-nitroanilide substrate at 22 °C. Release of the yellow p-nitroaniline was followed at 405 nm for 1 min in a SpectraMax Plus 384 microplate reader (Molecular Devices), and the rate of substrate cleavage was determined from the linear portion of the curves.

Experimental Procedures

Nomenclature—Nucleotide numbering of the coding DNA for CTRC starts with the first nucleotide of the ATG translation initiation codon. Amino acid residues in human CTRC are numbered starting with the initiator methionine of the primary translation product (chymotrypsinogen C precursor or prechymotrypsinogen C). By convention, eglin C and ecotin amino acid residues are numbered according to the sequence of the native, secreted protein (20, 21).

Novel CTRC Variants—A heterozygous CTRC variant c.239G>A (p.R80Q) was found in a human pancreatic cDNA sample from a de-identified subject of unknown origin and clinical status. Heterozygous variant c.640G>A (p.G214R) was identified in exon 7 of the CTRC gene in an 18-year-old male referred for
Mesotrypsin-like CTRC Variant

Michaelis-Menten hyperbolic equation. To determine the apparent inhibitory constant (K_I) of chymotrypsin inhibitors against variant p.G214R and mutant p.G214M, kinetic measurements were performed with 5 nM enzyme in the presence of increasing inhibitor concentrations (0–2 μM). The concentration of the Suc-Ala-Ala-Pro-Phe-p-nitroanilide substrate was varied between 5 and 640 μM. Apparent K_m values were plotted as a function of inhibitor concentration, and apparent K_I was derived from the negative intercept (−K_I) of the linear fit on the horizontal axis.

Measurement of CPA1 Activity—The assay mixture contained 10 μl of CPA1 sample and 70 μl of assay buffer (0.1 M Tris-HCl (pH 8.0), 1 mM CaCl_2, and 0.05% Tween 20), and the reaction was started by adding 20 μl of 600 μM N-[4-methoxyphenylazoformyl]-1-phenylalanine substrate (22). The decrease in absorbance was followed at 350 nm for 2 min at 22 °C. Rates of substrate cleavage were calculated from fits to the initial linear portion of the curves and were expressed in milliOD-min^-1 units (where OD indicates optical density).

Expression and Purification of Ecotin, Eglin C, and SGPI-2-C4—Ecotin was overexpressed in Escherichia coli BL21 (DE3) and purified on a trypsin affinity column (23). The expression plasmids for eglin C and SGPI-2-C4 were kind gifts from Evette Radisky (Mayo Clinic Cancer Center, Jacksonville Florida) and Gábor Pál (Eotvos University, Budapest, Hungary), respectively. SGPI-2-C4 is a phage display-selected CTRC-specific variant of the locust-derived chymotrypsin inhibitor SGPI-2 (16). Purification of these inhibitors followed published protocols (16, 24). Inhibitor concentrations were determined by titration against active-site titrated bovine trypsin (ecotin) or wild-type human CTRC (eglin C and SGPI-2-C4).

Equilibrium Binding Assays—Tight-binding inhibition of wild-type CTRC by eglin C, ecotin, and SGPI-2-C4 was assessed by measuring the apparent dissociation constant (K_d) values in equilibrium, as reported previously (16). CTRC (0.5 nM) and inhibitor (0–1 nM range) were incubated in 0.1 M Tris-HCl (pH 8.0), 1 mM CaCl_2, and 0.05% Tween 20 for 1 h (ecotin C, SGPI-2-C4) or 16 h (ecotin) at 22 °C in black 96-well plates in 200-μl volume. Free CTRC concentrations were determined with spectrofluorometry after the addition of 5 μl of 6 mM Suc-Ala-Ala-Pro-Phe-AMC substrate and measuring the rate of substrate cleavage using excitation and emission wavelengths of 380 and 460 nm, respectively. Apparent K_d values were calculated by plotting the free CTRC concentration as a function of the total inhibitor concentration and fitting the data points to the following equation: y = E − (E + x + K − v/(E + x + K)^2 − 4Ex))/2, where the independent variable x represents the total inhibitor concentration, the dependent variable y is the free protease concentration in equilibrium, K is K_d, and E designates the total protease concentration.

Results

CTRC Variants—We studied the functional properties of eight previously uncharacterized natural CTRC variants (Table 1). Five of these were reported in 2013 by a genetic study from Japan; variants p.R29Q, p.S239A, p.S239C, and p.K247E were found in individuals with chronic pancreatitis, whereas variant p.I209M was detected in a control subject (15). Variant p.R80W was identified during the cDNA cloning of human CTRC, described then as a “serum calcium decreasing protein,” caldecrin, by Japanese authors in 1996 (25). Two novel variants were also included in the present study; variant p.R80Q was found in a pancreatic cDNA sample, whereas variant p.G214R was discovered in a subject with chronic pancreatitis in Slovakia. When positions of the amino acid residues affected by these variants were examined in the CTRC structure, Arg-80, Gly-214, and Ser-239 appeared to be in proximity of the bound substrate-like inhibitor, suggesting that changes at these positions might affect substrate binding and cleavage (Fig. 1A). Furthermore, variant p.R29Q eliminated the activating trypsin cleavage site; therefore, this change was expected to abolish activation.

Secretion of CTRC Variants from Transfected Cells—Mutation-induced misfolding may cause intracellular retention and degradation resulting in diminished secretion of CTRC protein to the growth medium. We previously observed this loss-of-function phenotype with several clinically relevant CTRC variants (18, 19). When secreted CTRC protein levels were measured in the conditioned medium of transiently transfected HEK 293T cells by SDS-PAGE and densitometry, all eight variants exhibited levels similar to wild-type CTRC (range 85–125%, Fig. 1, B and C). Thus, none of the studied variants appeared to undergo significant misfolding, as judged by their normal secretion.

Activation and Degradation of CTRC Zymogens by Trypsin—The catalytically inactive CTRC precursor is converted to its active form by trypsin, which cleaves the Arg^29^-Val^30 peptide bond in the CTRC activation peptide. The cleaved activation peptide remains attached to CTRC via a disulfide bond (Fig. 1A), but it becomes released under the reducing conditions utilized during SDS-PAGE, resulting in a small shift in the migration of active CTRC relative to its zymogen form. When the eight CTRC variants were treated with trypsin, all exhibited a small gel shift, with the exception of p.R29Q (not shown). Resistance to cleavage by trypsin is consistent with the predicted effect of the p.R29Q variant, which destroys the activating cleavage site. Normally, trypsin does not cleave CTRC at any other site; however, mutations may render CTRC proteolytically unstable, and some of the pathogenic CTRC variants were previously shown to be degraded by trypsin (18). In contrast, none of the variants tested here was degraded by trypsin (not shown).

| CTRC region | Nucleotide change | Amino acid change | References |
|-------------|------------------|------------------|-----------|
| Exon 2      | c.86G>A          | p.R29Q           | 15        |
| Exon 4      | c.238C>T         | p.R80W           | 25        |
| Exon 4      | c.239G>A         | p.R80Q           | This work|
| Exon 6      | c.627C>G         | p.I209M          | 15        |
| Exon 7      | c.640G>A         | p.G214R          | This work|
| Exon 7      | c.715T>G         | p.S239A          | 15        |
| Exon 7      | c.716C>G         | p.S239C          | 15        |
| Exon 7      | c.739A>G         | p.K247E          | 15        |
Catalytic Activity of CTRC Variants on a Small Peptide Substrate—After activation with trypsin, enzymatic activity of CTRC in the conditioned medium was determined with the small chromogenic peptide substrate Suc-Ala-Ala-Pro-Phe-p-nitroanilide (Fig. 1B). Activity of CTRC variants p.R80Q, p.R80W, p.I209M, p.S239A, and p.K247E was comparable with that of wild-type CTRC within experimental error. In contrast, variant p.G214R showed a nearly 2-fold higher specificity constant, which was primarily due to a 2.7-fold increased $k_{cat}$ value. Because activity measurements in the conditioned medium (Fig. 1B) were carried out with near saturating substrate concentrations, the elevated $k_{cat}$ explains the high activity of the p.G214R variant observed under those conditions. Finally, variant p.R29Q had no measurable activity whatsoever, whereas variant p.S239C was catalytically impaired, with a $k_{cat}/K_m$ value that was 56-fold lower than that of wild type. A possible explanation for the low activity of variant p.S239C is conformational distortion caused by spurious disulfides between the newly introduced Cys239 and the adjacent Cys212 and/or Cys243 residues. This notion remains speculative, however, as we were unable to demonstrate such disulfides so far.

Catalytic Activity of CTRC Variants on β-Casein—The activity of all purified CTRC variants was also tested on a larger protein substrate where the enzyme can make extended subsite contacts during substrate binding (Fig. 2). Consistent with the assays using the peptide substrate, five variants readily cleaved β-casein, whereas variants p.R29Q and p.S239C were inactive on this protein substrate. Surprisingly, variant p.G214R, which exhibited exceptionally high activity on the peptide substrate (Fig. 1B and Table 2), digested β-casein poorly (Fig. 2).

**TABLE 2**

| Enzyme kinetic parameters of purified CTRC variants and mutants on the Suc-Ala-Ala-Pro-Phe-p-nitroanilide substrate |
|---------------------------------------------------------------|
| $K_m$ $k_{cat}$ $k_{cat}/K_m$ |
| Wild type | $15.3 \pm 0.6$ | $15.4 \pm 0.1$ | $1.0 \times 10^6$ |
| p.R29Q | ND | ND | ND |
| p.R80Q | $22.6 \pm 0.7$ | $16.5 \pm 0.1$ | $7.3 \times 10^5$ |
| p.R80W | $19.4 \pm 0.8$ | $15.1 \pm 0.2$ | $7.8 \times 10^5$ |
| p.I209M | $15.7 \pm 0.4$ | $10.3 \pm 0.1$ | $6.6 \times 10^4$ |
| p.G214R | $22.1 \pm 1.2$ | $42.2 \pm 0.6$ | $1.9 \times 10^5$ |
| p.S239A | $19.0 \pm 0.7$ | $11.7 \pm 0.1$ | $6.2 \times 10^4$ |
| p.S239C | $33.1 \pm 4.1$ | $0.6$ | $1.8 \times 10^4$ |
| p.K247E | $16.7 \pm 0.9$ | $14.7 \pm 0.2$ | $8.8 \times 10^3$ |
| p.G214A | $47.2 \pm 4.2$ | $14.1 \pm 0.4$ | $3.0 \times 10^5$ |
| p.G214M | $22.1 \pm 1.2$ | $23.6 \pm 0.3$ | $1.1 \times 10^6$ |

*Note:* $K_m$ and $k_{cat}$ values are in micromolar and second, respectively. The error of the fit is indicated.
Catalytic Activity of CTRC Variants on Human Cationic Trypsinogen—The protective trypsinogen-degrading activity of CTRC involves cleavage of the Leu$^{81}$-Glu$^{82}$ peptide bond in the calcium binding loop of cationic trypsinogen, which can be followed on SDS-PAGE (Fig. 3A). Similarly to the digestion pattern observed with the β-casein substrate, five CTRC variants cleaved trypsinogen rapidly, whereas variant p.R29Q was inactive and variants p.G214R and p.S239C exhibited diminished activity. When rates of digestion were determined by densitometry (Fig. 3B), variants p.G214R and p.S239C cleaved trypsinogen at least 30-fold slower than wild-type CTRC.

Activity of Variant p.G214R on Human Procarboxypeptidase A1—CTRC acts as a co-activator for human procarboxypeptidase A1 through catalyzing degradation of the propeptide after the initial trypsin-mediated cleavage (10). Cleavage of the CPA1 propeptide by CTRC was monitored by activity assay (Fig. 4). The CPA1 propeptide by CTRC was monitored by activity assay (Fig. 4). The CPA1 precursor was first partially activated with trypsin, and then 5 nM CTRC was added to induce complete degradation of the propeptide. The increase in CPA1 activity was followed. Similarly to the cleavage of trypsinogen, variant p.G214R activated CPA1 at a significantly reduced rate, about 10-fold more slowly than wild-type CTRC.

Variant p.G214R Is Resistant to Tight-binding CTRC Inhibitors—The catalytic properties of CTRC variant p.G214R are highly reminiscent of those of human mesotrypsin, an exceptional trypsin isoform that exhibits increased activity on small peptide substrates yet cleaves most protein substrates poorly. Furthermore, mutation p.G214R is analogous to the evolutionary mutation p.G198R in mesotrypsin, which appears to be largely responsible for the unique properties of this trypsin isoform. Modeling an Arg side chain at position 214 in the CTRC structure indicated steric clash with the P$_2$ side chain of the bound eglin C (Fig. 5A), a situation analogous to the effect of Arg$^{198}$ in mesotrypsin(26). Because a hallmark feature of mesotrypsin is its resistance to proteinaceous trypsin inhibitors, we investigated whether CTRC variant p.G214R can be inhibited by CTRC inhibitors. Three inhibitors were tested: eglin C, ecotin, and the C4 variant of SGPI-2. The ~8-kDa eglin C (MEROPS I13.001) is derived from the medicinal leech (20), and we previously identified it as a CTRC inhibitor and determined the structure of its complex with CTRC (17). Ecotin (MEROPS I11.001) is a pan-serine-protease inhibitor found in the periplasmic space of E. coli (21, 27). The active molecule is a homodimer of two ~16-kDa subunits, which can bind two protease molecules. In this heterotetrameric complex, ecotin inhibits the protease via a primary substrate-like interaction and a smaller secondary binding site (28). SGPI-2-C4 is a phage display-selected CTRC-specific variant of the ~4-kDa locust-derived chymotrypsin inhibitor SGPI-2 (an ortholog of MEROPS I19.011) (16). Eglin C, ecotin, and SGPI-2-C4 inhibited human CTRC as tight-binding inhibitors, and we determined apparent equilibrium dissociation constant (K$_D$) values of 52,
In contrast to wild-type CTRC, variant p.G214R was poorly inhibited by these inhibitors, and we were unable to determine an apparent $K_D$ in equilibrium binding assays. Therefore, using a small peptide substrate, Michaelis-Menten parameters were measured in the presence of increasing inhibitor concentrations, and the $K_m$ values were plotted as a function of the inhibitor concentration. The slope of the linear fits yielded apparent $K_i$ values of 149, 54, and 224 nM, respectively, indicating that CTRC variant p.G214R binds proteinaceous inhibitors more than 3 orders of magnitude weaker relative to wild-type CTRC (Fig. 5 C).

Variant p.G214R Cleaves the Reactive-site Peptide Bond of Inhibitors—Human mesotrypsin cleaves the reactive-site peptide bond of canonical trypsin inhibitors and thereby promotes their inactivation and further degradation. To test whether CTRC variant p.G214R also exhibited inhibitor-degrading activity, eglin C and ecotin were incubated with wild-type and p.G214R CTRC, and the reactions were analyzed by SDS-PAGE. Due to its small size, cleavage of SGPI-2-C4 could not be evaluated by this method. Over the time courses studied, wild-type CTRC did not cleave eglin C or ecotin to any extent whatsoever, whereas variant p.G214R almost completely cleaved...
both inhibitors (Fig. 6). As these reactions were performed with nearly saturating inhibitor concentrations ($4 \times 10^{-2}$), we could estimate $k_{cat}$ values from the rates of cleavage and obtained 1.3 and 2 $s^{-1}$ for eglin C and ecotin, respectively. N-terminal protein sequencing by Edman degradation confirmed that CTRC-mediated cleavage took place at the Leu45-Asp46 and Met84-Met85 reactive-site peptide bonds in eglin C and ecotin, respectively (Fig. 6). The results also indicate that the hydrolysis equilibrium of the reactive-site peptide bonds in both inhibitors is shifted toward the cleaved form.

Side-chain Bulk Is Responsible for the Mesotrypsin-like Properties of CTRC Variant p.G214R—Crystal structure of mesotrypsin harboring the evolutionary p.G198R mutation (32) and modeling of CTRC variant p.G214R (Fig. 5A) both indicate that the bulky Arg side chain is responsible for weakened inhibitor binding and enhanced inhibitor cleavage. To test this notion experimentally and to assess the possible contribution of the guanidinium positive charge, we constructed CTRC mutants p.G214A and p.G214M. When compared with variant p.G214R, the catalytic properties of mutant p.G214M were highly similar. Thus, mutant p.G214M cleaved a small peptide substrate with an increased $k_{cat}$ (Table 2), but it was defective in cleaving $\beta$-casein and human cationic trypsinogen (Fig. 7). Furthermore, mutant p.G214M bound eglin C poorly ($K_D$ 169 nM) and rapidly cleaved the reactive-site peptide bond of eglin C (Fig. 8). In contrast, mutant p.G214A cleaved the small peptide substrate with an unchanged $k_{cat}$ but with an elevated $K_m$ (Table 2) and digested both $\beta$-casein and human cationic trypsinogen, although the rate of trypsinogen cleavage was 3.5-fold slower relative to wild-type CTRC. Interestingly, mutant p.G214A exhibited inhibitor resistance and bound to eglin C more than 200-fold weaker than wild-type CTRC, yet at least 20-fold stronger than variant p.G214R or mutant p.G214M. We note that the measured $K_D$ value of 11 nM is a higher estimate determined after 10 min of incubation when full equilibrium may not have been reached. The short incubation time was
necessary because in the low inhibitor concentration range employed, mutant p.G214A slowly inactivated eglin C. However, when inhibitor cleavage was tested at 4 μM concentration with wild-type CTRC or the p.G214R variant (100 nM with eglin C and 200 nM with ecotin) in 0.1 M Tris-HCl (pH 8.0), and 1 mM CaCl₂ at 37 °C. At the indicated time points, aliquots (100 μl) were precipitated with trichloroacetic acid (10% final concentration) and analyzed by SDS-PAGE and Coomassie Blue staining. The bands marked by asterisks were subjected to Edman degradation. The N-terminal sequences determined are indicated below the gels. Note that the cleaved eglin C band contains two co-migrating bands of similar size. Representative experiments from three experiments are shown. The amino acid sequences of the inhibitors are also shown with the sequenced regions bolded and underlined. Note that in recombinant eglin C, the native N-terminal sequence of TEF was replaced with MSM and the initiator methionine was removed by methionine aminopeptidase during expression in E. coli.

**Discussion**

Loss-of-function CTRC variants increase the risk for chronic pancreatitis by compromising the protective trypsinogen-degrading activity of CTRC in the pancreas. Because most CTRC variants are rare, genetic analysis cannot determine or rule out disease association, and classification of clinical significance must rely on functional analysis (18). Here we characterized eight CTRC variants, which were not included in our latest comprehensive study that analyzed 32 CTRC variants (18). Two of the eight variants, p.R80Q and p.G214R, were reported here for the first time. Our results identified three CTRC variants with significant catalytic defects due to unique mechanisms not observed before with other CTRC variants. These variants, p.R29Q, p.G214R, and p.S239C, are hereby categorized as risk factors for chronic pancreatitis. In agreement with our assessment, all three variants were identified in patients with chronic pancreatitis. In contrast, two variants (p.S239A...
and p.K247E) originally identified in subjects with chronic pancreatitis were fully functional, indicating that these are not pathogenic and were only coincidentally found in patients rather than controls.

Undoubtedly, the most exciting finding of the present study was variant p.G214R, which exhibited exceptional catalytic properties. Thus, the variant was highly active on a small peptide substrate, which does not present extensive prime side contacts for binding, but it exhibited low activity on large protein substrates such as β-casein, human cationic trypsinogen, or procarboxypeptidase A1. Structural modeling indicated that the Arg side chain occupied the S2 subsite and sterically clashed with the P2 side chain of substrates and inhibitors. In agreement with these predictions, CTRC inhibitors eglin C, ecotin, and SGPI-2-C4 inhibited variant p.G214R with several thousand-fold diminished affinity relative to wild-type CTRC. Furthermore, variant p.G214R readily cleaved the reactive-site peptide bonds in eglin C and ecotin. Taken together, properties of the p.G214R variant were essentially identical to those of human mesotrypsin, an inhibitor-resistant trypsin isoform capable of degrading trypsin inhibitors (26, 29–35). Mesotrypsin poorly cleaves most proteins but has high activity on small chromogenic peptide substrates with limited prime side contacts. Canonical trypsin inhibitors such as bovine pancreatic trypsin inhibitor (MEROPS I02.001), soybean Kunitz trypsin inhibitor (MEROPS I03.001), and pancreatic secretory trypsin inhibitor (MEROPS I01.011) inhibit mesotrypsin with greatly reduced affinity relative to other human trypsins. Mesotrypsin, however, efficiently cleaves the reactive-site peptide bond of these inhibitors, and this function appears to be the most plausible physiological role for this protease. Mesotrypsin consti-
tutes only a few percent of human trypsins secreted by the pancreas (29, 36, 37), which seems to support the idea for such a highly specialized function. Remarkably, essentially all unique properties of mesotrypsin are due to an evolutionary mutation that replaced a Gly residue at position 198 (position 193 in conventional chymotrypsin numbering) with an Arg. This mesotrypsin signature mutation p.G198R is analogous to the p.G214R variant in CTRC; both affect the same conserved Gly residue. Mutation of Arg198 in mesotrypsin to Gly converts mesotrypsin to a normal trypsin, demonstrating that this single evolutionary change is the critical determinant of mesotrypsin function (31). This notion agrees with our findings as $k_\text{cat}$ values determined for the cleavage of eglin C and ecotin by CTRC variant p.G214R fall in the same range as turnover numbers measured for inhibitor cleavage by mesotrypsin (see Table 2 in Ref. 38). Interestingly, however, recent work from the Radisky laboratory (39) demonstrated that mesotrypsin may have accumulated other evolutionary mutations that, to a lesser degree than Arg198, further reduced binding affinity and increased cleavage rates toward inhibitors. The unique catalytic properties of the p.G214R CTRC variant were almost entirely mimicked by mutant p.G214M but not by mutant p.G214A, confirming predictions that side-chain bulk at position 214 is responsible for inhibitor resistance and inhibitor cleavage, whereas the positive charge of the Arg side chain is unimportant.

Finally, it is intriguing to observe that the same natural mutation in two highly homologous digestive serine proteases has led to dramatically different biological outcomes: evolutionary selection of a new physiological function versus pancreatic pathology. In the case of mesotrypsin, the newly acquired inhibitor-cleaving capability has been advantageous for the organism, whereas the low digestive activity on protein substrates was immaterial due to the abundance of other trypsin isofoms. In contrast, the diminished catalytic activity of CTRC variant p.G214R toward protein substrates compromised an essential protective function and resulted in higher disease risk. Although carriers of the p.G214R CTRC variant may be protected from dietary chymotrypsin inhibitors that may compromise protein digestion, the harmful pathogenic effect clearly outweighs this potential digestive benefit.

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