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Processing of Human Protryptase in Mast Cells Involves Cathepsins L, B, and C

Quang T. Le,* Gregorio Gomez,* Wei Zhao,† Jiang Hu,* Han-Zhang Xia,* Yoshihiro Fukuoka,* Nobuhiko Katunuma,‡ and Lawrence B. Schwartz*

Human β-tryptase is stored in secretory granules of human mast cells as a heparin-stabilized tetramer. β-Protryptase in solution can be directly processed to the mature enzyme by cathepsin (CTS) L and CTSB, and sequentially processed by autocatalysis at R→G, followed by CTSC proteolysis. However, it is uncertain which CTS is involved in protryptase processing inside human mast cells, because murine bone marrow-derived mast cells from CTSC-deficient mice convert protryptase (pro–mouse mast cell protease-6) to mature mouse mast cell protease-6. This finding suggests that other proteases are important for processing human β-protryptase. In the current study, reduction of either CTSB or CTSL activity inside HMC-1 cells by short hairpin RNA silencing or CTS-specific pharmacologic inhibitors substantially reduced mature β-tryptase formation. Similar reductions of tryptase levels in primary skin-derived mast cells were observed with these pharmacologic inhibitors. In contrast, protryptase processing was minimally reduced by short hairpin RNA silencing of CTSC. A putative pharmacologic inhibitor of CTSC markedly reduced tryptase levels, suggesting an off-target effect. Skin mast cells contain substantially greater amounts of CTSL and CTSB than do HMC-1 cells, the opposite being found for CTSC. Both CTSL and CTSB colocalize to the secretory granule compartment of skin mast cells. Thus, CTSL and CTSB are central to the processing of protryptase(s) in human mast cells and are potential targets for attenuating production of mature tryptase in vivo. The Journal of Immunology, 2011, 187: 1912–1918.

The serine protease, β-tryptase (EC 3.4.21.59), is the major protein component of the secretory granules of human mast cells (1, 2). Processing of β-protryptase to mature, enzymatically active β-tryptase occurs in solution by the autocatalytic-cathepsin (CTS) C processing pathway. Experiments with HMC-1 cells—a human mast cell leukemia cell line in which Gly-Phe-CHN2, an inhibitor of CTSC, attenuates the formation of β-tryptase activity (3, 4)—appeared to support the biological relevance of this processing pathway. However, mast cells from mice that are CTSC deficient express mature mouse mast cell protease-6, a murine tryptase, albeit at cellular levels ∼75% lower than in mast cells from wild-type mice (5), indicating the presence of alternative protryptase processing pathway(s), at least in mice and possibly in humans. In fact, direct removal of the propeptides from both α- and β-protryptases by CTSB and CTSL may play a biologically important role in mast cells based on the identification of these enzymes in HMC-1 cells and the demonstration in solution of their protryptase processing activities (6). Together with CTSC, CTSB and CTSL account for nearly all of the protryptase processing activity in HMC-1 cells.

Even though CTSs are classically considered acidic proteases involved in the degradation of proteins in lysosomes, there is ample precedent for their subcellular localization to other compartments where they are involved in cell-specific processing. By removing dipeptides, CTSC removes two amino acid propeptides from several protease zymogens in the secretory granules of several cell types, including progranzymes A, B, and K in cytotoxic T lymphocytes and NK cells (7–10); proCTSG (11), proelastase, and proteoprotease-3 in neutrophils (12); and prochymases in murine mast cells (5). In murine mast cells, CTSE also has been localized to secretory granules, where it binds to heparin proteoglycan and processes mast cell procarboxypeptidase (13). This article shows that CTSB and CTSL colocalize to the secretory granules of primary human skin-derived mast cells and are required for optimal processing of protryptase to active mature tryptase inside human mast cells.

Materials and Methods

Materials

Anti-tryptase mAbs for Western blotting were as follows: G3 (which detects pro and mature forms of α- and β-tryptases, or total tryptase) and G5 (which detects mature forms of α- and β-tryptases) (14), B12 for ELISA capture (which captures total tryptase), G4-biotin for total tryptase ELISA detection, and G5-biotin for mature tryptase ELISA detection; these were used as described (3, 15). Western blot bands were detected with IRDye-conjugated anti-mouse IgG (Odyssey Infrared Imaging System; LiCor Biotecnology, Lincoln, NE). The human mast cell leukemia cell line HMC-1 was provided by Dr. G. Gleich and Dr. J. Butterfield (Mayo Clinic, Rochester, MN) (16), CLIK-148 (CTS L inhibitor), CLIK-060 (CTSB inhibitor), and CA-074 (CTSB inhibitor) (17, 18) were provided by Professor Nobuhiko Katunuma. Primer synthesis and DNA sequencing were performed by the Virginia Commonwealth University Nucleic Acids Core Laboratory (Richmond, VA). Human recombinant stem cell factor (SCF) was provided by Swedish Orphan Biovitrum (Stockholm, Sweden).
Human skin mast cells

Skin-derived mast cells were obtained as described (19). Briefly, cells were dispersed from fresh surgical skin using collagenase and hyaluronidase, partially purified by Percoll density-dependent sedimentation, and placed into culture in serum-free AIM-V medium containing 100 ng/ml recombinant human SCF (gift from Amgen, Thousand Oaks, CA). Mast cells were studied after 6 wk of culture, by which time they were >99% pure and >97% viable.

Skin mast cells (105 cells/ml) were activated with 1 μg/ml anti-FCεRi IgG2a Ab (22E7), as described (19). After the activation period, cells and medium were separated by centrifugation. The cells were lysed with 1% Triton X-100, and percent degranulation was calculated from the β-hexosaminidase activity detected in the releasates and retentates by measuring the cleavage of p-nitrophenyl N-acetyl-β-D-glucosaminide, as described (2). Absorbance values were read at 405 nm with a SpectraMax Plus 384 UV-visible plate reader (Molecular Devices, Chicago, IL). Lactate dehydrogenase (LDH) activity, a cytoplasmic marker, was measured to assess cell damage (LDH-Toxocytotoxicity Assay Kit; BioVision, Mountain View, CA). Percent release values were calculated using the following formula:

\[
\text{Percent release} = \text{Net percentage released} \times \frac{\text{Net percentage retained}}{1 - \text{Net percentage released}}
\]

Net percent release was calculated by subtracting spontaneous release from both the numerator and the denominator.

Inhibition of CTSL, CTSB, and CTSC activity in cultured HMC-1 cells and human skin mast cells

HMC-1 and human skin mast cells were cultured starting at 5 × 104 and 1 × 105 cells/ml, respectively. CLIK-148, CA-074 Me, GF-CHN 2 (1–10 μM), or the corresponding diluent alone (0.01–0.025% DMSO) was added at day 0, and cells were cultured up to 4 d for HMC-1 and 6 d for human skin mast cells. Every 2 d the cells were harvested, washed, and counted, and then frozen at −70°C. To maintain the effect of inhibitors, the old medium was replaced every 2 d with fresh medium containing the inhibitor. To analyze protease activities, cells were thawed, sonicated on ice, and assayed.

CTSL, CTSB, and CTSC short hairpin RNA

DNA oligonucleotides encoding short hairpin RNA (shRNA) sequences targeting CTSL and CTSB with loop sequences 5′-CGAA-3′ were cloned into pLentiv6/BLOCK-IT-DEST vector. The specific shRNA sequences were as follows: CTSL-98 (L98) (5′-CACCGGCAAGTGCAACACATTAGGCG-3′); CTSL-102 (L102) (5′-CACCGTGCTGTTGTTGAGGCTGAC-3′); CTSC-171 (L171) (5′-CACCCTCCGTGGTGGAGGCTGAC-3′); CTSC-147 (5′-CACCGGATATATATGTTGACCCACACCAATGGCCAAGA-3′); CTSL (5′-AACATCATATATCCC-3′); CTSL-98 (L98) (5′-CACCCTCCGTGGTGGAGGCTGAC-3′) and CTSC silencing has no substantial effect (6). As shown in Fig. 1B, CLIK-148, CA-074 Me, GF-CHN 2 (1–10 μM), or the corresponding diluent alone (0.01–0.025% DMSO) was added at day 0, and cells were cultured up to 4 d for HMC-1 and 6 d for human skin mast cells. Every 2 d the cells were harvested, washed, and counted, and then frozen at −70°C. To maintain the effect of inhibitors, the old medium was replaced every 2 d with fresh medium containing the inhibitor. To analyze protease activities, cells were thawed, sonicated on ice, and assayed.

Analytic techniques

β-Tryptase enzymatic activity was monitored in 1 ml plastic cuvettes or 96-well microtiter plates, using tosyl-Gly-Pro-Lys-p-nitroanilide (TGPK) as the substrate, as described (20), except that soybean trypsin inhibitor (SBI) (10 μg/ml) was included when impure preparations, such as cell extracts, were used to inhibit trypsin serine proteases other than tetratryptase (21). The increase in p-nitroanilide was measured at 405 nm in a Cary 3 UV-visible spectrophotometer or in a Bio-Tek EL312 Kinetic plate reader (Bio-Tek Instruments, Winooski, VT). One unit of enzyme activity was defined as the amount that cleaves 1 μmol synthetic substrate per minute.

Gelatin zymography to detect tryptase was performed with samples that had been incubated in 1% SDS, 125 mM Tris, pH 6.8, and 10% glycerol in the absence of a reducing agent for 10 min at room temperature, and then immediately subjected to SDS-PAGE in a polyacrylamide gel (10%) that had been copolymerized with 0.1% gelatin (22). After electrophoresis the gel was washed twice in 2.5% Triton X-100 for 20 min to remove SDS, and then incubated overnight at 37°C before staining with Coomassie blue.

Chymase activity was measured using Suc-Ala-Ala-Pro-Phe-MCA, as described (5). CTSC activity was determined using Gly-Phε-p-nitroanilide (100 μM) as substrate in 50 mM sodium acetate buffer, pH 4.5, 1 mM EDTA, 4 mM DTT (23). CTSL and CTSB were measured using Z-Phe-Arg-MCA and Z-Arg-Arg-MCA, respectively (24). In cell extracts, unless stated otherwise, CTSL measurements were performed in the presence of 1 μM CA-074, and CTSM measurements in the presence of 1 μM CLIK-148. For inhibition studies, the enzymes were preincubated with various inhibitors at room temperature for 15 min before measurement of activity. Protein concentrations were measured by the bicinchoninic acid method, using BSA as a standard.

The HMC-1 cell RNA from resting mast cells was isolated and DNase treated, respectively, with the RNaseasy Minirep kit and RNase-Free DNase Set (Qiagen, Hilden, Germany). cDNA was synthesized (5 min at 65°C, 60 min at 37°C) from 500 ng total RNA, using the Ready-To-Go T-Primed First-Strand Kit (Amersham Biosciences, Piscataway, NJ) with an Eppendorf Master Gradient amplifier. Primers (sense; antisense; product size; GenBank accession number available at http://www.ncbi.nlm.nih.gov/nuccore/) were designed with primer express 3.0 software (Applied Biosystems, Carlsbad, CA), as follows: CTSL (5′-GATCGTGCACCCACAATG-3′; 5′-GGAGGAGGATGGATGCCTTCACT-3′; 198; BC095408); CTSL (5′-GCTAAATGACCGCGCTTTGTTG-3′; 5′-TTTCAATCTGTTAGA-GCCAACC-3′; 202; M20496.1); tryptase (5′-CTTGGACTGCTACCATCCTAATGGA-3′; 203; M37488); β-actin (5′-GCCCCATCCTCCACTCTAACTA-3′; 5′-GGGGTGTGAGAGGTCTCAA-3′; 203; NM_001101.3). For quantitative PCR, 2 μl cDNA was combined with 1 μl sense and antisense primers (10 μM each) and 12.5 μl SYBR Green Supermix from the iScript SYBR Green RT-PCR kit (Bio-Rad, Hercules, CA) in a final volume of 25 μl. PCR reaction conditions included a hot start at 95°C for 5 min, followed by 35 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s. Ct values for each mRNA were obtained and analyzed using the 2−△△Ct method. mRNA expression levels of CTSL, CTSB, and tryptase were each normalized to that of β-actin.

All reactions were run and analyzed with the CFX96 Real-Time PCR Bio-Rad Detection System and the CFX Manager software 1.0. A melting curve was analyzed at the end of each experiment to assess primer–dimer formation.

Results

CTSL is not essential for the activation of human tryptase in HMC-1 cells

Although the Gly-Phε-CHN2 inhibitor of CTSL activity inhibited the maturation of tryptase by intact and disrupted human mast cell leukemia cells (HMC-1) (3, 4), mature tryptase was detected in mast cells derived from CTSC knockout mice (5). To examine the dependence of the maturation of human β-tryptase on CTSL, an shRNA lentivirus construct was prepared and used to transfect HMC-1 cells that were then selected with blastcidin. Knockdown of CTSL activity by 78% (Fig. 1A) and protein by 77% (Fig. 1B, 1C) in stably transfected HMC-1 cell lines diminished tryptase peptidolytic activity by only ∼23% in cell extracts (Fig. 1D). The tryptase activity assay using TGPK as substrate is made more specific for tryptase by including SBI, which inhibits most serine proteases with tryptic activity, such as CTSL, but does not affect the activity of β-tryptase tetramers (25, 26). As shown in Fig. 1E, using comparable amounts of CTSB, CTSL, and CTSG and of β-tryptase to cleave TGPK, the sp. act. of CTSL is ∼1200-fold less than that of β-tryptase, whereas that of CTSL, CTSB, and CTSC is not detectable (<10,000-fold lower than that of β-tryptase).

Another approach to examine the effect of CTSL silencing on tryptase is by gelatin zymography. β-Tryptase tetramers have been shown to migrate considerably slower than inactive tryptase monomers, and only the tetramer exhibited gelatinolytic activity (6). As shown in Fig. 1F, CTSL silencing has no substantial effect on tryptase activity by zymography. The ability of CTSL shRNA to silence CTSL without substantially affecting tryptase matura-
HMC-1 tryptase bands, whereas monomeric recombinant CTSG (G), and tryptase (T) are shown in Fig. 2E of SBTI (10 μM concentration). The CTSC, CTSB, and CTSL inhibitors can be used to further assess and promote the specificity of the CTSB and CTSL assays when both of these enzymes are present. Indeed, the measurement of CTSL activity is performed in the presence of CA-074 (CTSB inhibitor) because CTSB weakly cleaves Z-Phe-Arg-MCA under the conditions used to measure CTSB activity. This is evident in extracts of human primary skin mast cells (Fig. 2D) and HMC-1 cells (Fig. 2E) in which 20% of the Z-Phe-Arg-MCA cleaving activity remains after addition of CLIK-148 (CTSL inhibitor). In the presence of CA-074 (CTSB inhibitor), however, all remaining Z-Phe-Arg-MCA cleavage activity is inhibited by CLIK-148. In contrast, the measurement of CTSB activity by cleavage of Z-Arg-Arg-MCA activity is almost completely inhibited by CA-074 in the skin mast cell (Fig. 2C) and HMC-1 cell (Fig. 2D) extracts. Furthermore, the ability of CTSB and CTSL inhibitors and the cysteine protease inhibitor E64 or its cell permeable analog, E64d, to completely inhibit cleavage of these substrates indicates that noncysteine proteases, including tryptase and CTSB (serine proteases), do not contribute to CTSB and CTSL activity measurements under the conditions used.

**CTSL and CTSB colocalize with tryptase to human mast cell secretory granules**

For CTSB and CTSB to process protryptase in cells, they would need to colocalize with protryptase in an acidic compartment. To determine whether CTSB and CTSB functionally localize to secretory granules, their release by primary human skin mast cells activated with anti-FcεRIα mAb to degranulate was assessed. As shown in Fig. 3, the net percent of CTSB released was only 10% lower than that of tryptase, whereas the net percent release of CTSB was 54% lower. This finding suggests that most of the CTSB and about half of the CTSB in primary skin mast cells colocalize with tryptase to secretory granules, and presumably traverse the same intracellular pathway.

**CTSL and CTSB silencing with shRNA demonstrates their involvement in protryptase processing in HMC-1 cells**

Fig. 4 shows the cellular content of CTSB and tryptase activities in HMC-1 cells that had been stably transfected with CTSB and CTSB shRNA lentivirus constructs. Marked decreases in CTSB activity occur in response to B55, B57, B59, and Bmix constructs, and smaller decrements occur in response to B56 and B58 constructs (Fig. 4A). In cells transfected with CTSB shRNA, tryptase expression is attenuated in proportion to that of CTSB, whereas smaller decrements occur with CTSB activity and no change or a slight increase occurs in CTSL activity. This relationship is shown more clearly in Fig. 4B, where CTSB shRNA is associated with a 90% loss of CTSB activity, a 30% loss of CTSB activity, a 90% loss of tryptase activity, and a 10% increase in CTSL activity. In separate experiments shown in Fig. 4C, the impact of CTSB shRNA on protease mRNA, as well as activity levels in HMC-1 cells, is assessed. Comparative analyses of mRNA expression were done by quantitative real-time RT-PCR, using the $2^{-\Delta\Delta C(t)}$ method. CTSB shRNA causes comparable decreases in cellular levels of CTSB activity and mRNA, as well as a comparable decrease in tryptase activity, whereas tryptase mRNA is activity of the other CTSs at a 10 μM concentration. Further, neither of these inhibitors affects β-tryptase activity (Fig. 2B). Fig. 2C shows the abilities of purified β-tryptase and CTSB to cleave these substrates. Equimolar amounts of β-tryptase and CTSB, respectively, exhibit 0.4% and 0.5% of the activity of CTSB to cleave Z-Phe-Arg-MCA, and β-tryptase and CTSB, respectively, exhibit 0.3% and 0.1% of the activity of CTSB to cleave Z-Arg-Arg-MCA.

**Specificities of inhibitors and assays of CTs**

To measure activity levels of CTSB and CTSB in mast cell releasates or extracts, the specificities of the inhibitors and assays used were examined as shown in Fig. 2. GF-CHN2 targets CTSC, CLIK-148 targets CTSL, and CA-074Me targets CTSB (Fig. 2A) in solution with purified CTs with apparent specificity because each inhibitor essentially abrogates the activity of its intended target at a 1 μM concentration without appreciably affecting the activity of the other CTSs at a 10 μM concentration. Further, neither of these inhibitors affects β-tryptase activity (Fig. 2B). Fig. 2C shows the abilities of purified β-tryptase and CTSB to cleave these substrates. Equimolar amounts of β-tryptase and CTSB, respectively, exhibit 0.4% and 0.5% of the activity of CTSB to cleave Z-Phe-Arg-MCA, and β-tryptase and CTSB, respectively, exhibit 0.3% and 0.1% of the activity of CTSB to cleave Z-Arg-Arg-MCA.

**Processing of human protryptase in mast cells**

FIGURE 1. CTSC is not essential for the activation of human tryptase in HMC-1 cells. CTSC shRNA suppresses the expression of CTSC activity (A) and protein (B, C) to a greater extent than tryptase activity (D, F) in HMC-1 cells. Control and CTSC-specific shRNA constructs were transfected into HMC-1 cells. CTSC1 and CTSC2 represent two different stably transfected cell lines using the same CTSC-targeted construct. CTSC activity and protein from extracts of HMC-1 cells, prepared from equivalent cell numbers 15 d postblasticidin (6 μg/ml) selection, were measured, respectively, with Gly-L-Phe-p-nitroanilide and by Western blotting with anti-CTSC polyclonal Ab (1:1000 dilution) and anti–β-actin polyclonal Ab (1:2000 dilution), respectively. A representative Western blot is shown in B. Western blot band intensities, calculated from the infrared fluorescence data captured by the Odyssey system, for CTSC were normalized to those of β-actin and set to 100% in the vector control (C). Tryptase enzyme activity in the HMC-1 extracts was measured with TGPK in the presence of SBTI (10 μg/ml) and by gelatin zymography in F. TGPK tryptase assay results, when performed in the presence and absence of SBTI (10 μg/ml) with comparable amounts of CTSL (L), CTSB (B), CTSB (C), CTSG (G), and tryptase (T) are shown in E. In F, the migrations of β-galactosidase (β-Gal hatch mark, 132,000 kDa, Kaleidoscope Prestained Standards; Bio-Rad) and purified lung-derived β-tryptase tetramer (last lane) used as a zymography control, migrate to the same position as the HMC-1 tryptase bands, whereas monomeric recombinant β-tryptase runs with a faster mobility (not shown) (6). *p ≤ 0.002 compared to Control (A, C, D) and to T (E).
FIGURE 2. Specificities of CTS inhibitors and assays. Inhibitory activities of GF-CNHN₂ (GF), CA-074 (CA), and CLIK-148 (CLIK) for CTSC, CTSB, and CTSL (A) and tryptase (B). Each CTS was assayed with synthetic substrates (see Materials and Methods) in the presence and absence of CTS-specific inhibitors and, for CTSL and CTSB, of an inhibitor of cysteine proteases, E64, and expressed as a percentage of uninhibited activity. The TGPK cleaving activity of lung tryptase was tested with CTS-specific inhibitors and expressed as a percentage of uninhibited activity. The activities of β-tryptase (T) and CTSG (G) against Z-Phe-Arg-MCA, relative to an equimolar amount of CTSL (L), and of β-tryptase and CTSG against Z-Arg-Arg-MCA, relative to an equimolar amount of CTSB (B), are shown in C. Heparin was included with tryptase and CTSG. Extracts of skin mast cells (D) and HMC-1 cells (E) were assessed for Z-Phe-Arg-MCA and Z-Arg-Arg-MCA cleaving activities (U/ml) in the absence and presence of CA-074, CLIK-148, E64, and E64d, the cell-permeable ethyl ester of E64. Black bars, control; and 1 μM inhibitor concentrations; gray bars, control and 5 μM inhibitor concentrations.

FIGURE 3. CTSL and CTSB reside in the secretory granules of skin-derived human mast cells. Skin mast cells were prepared and stimulated with anti-FcεRIα mAb (22E7, 1 μg/ml) for 30 min at 37°C, as described (19). Cell extract and media were assayed for tryptase with TGPK, CTSL, with Z-Phe-Arg-MCA, CTSB with Z-Arg-Arg-MCA, and LDH in the medium and cell extracts. LDH percent release values with unstimulated and 22E7-stimulated cells were <5%, and tryptase, CTSL, and CTSB release values were <5% with unstimulated cells. Net percent release values shown represent the mean ± SD for three independent experiments. Net percent release values of CTSL and of CTSB were significantly lower than those of tryptase by ANOVA. *p = 0.04, #p < 0.001.

unchanged, and no significant changes in cellular levels of CTSL activity and mRNA are noted. Marked decreases in CTSL activity occurred in response to L662 and L98 CTSL shRNA constructs (Fig. 4D). Tryptase activity also diminished, but to a lesser magnitude. Inhibition of CTSL activity by 85% caused a 60% decrease in tryptase activity, but no change in CTSB or CTSC activities. Thus, silencing either CTSL or CTSB has a substantially greater impact on lowering levels of tryptase activity than does silencing CTSC.

The effects of these CTS shRNAs on β-(pro)tryptase in HMC-1 cells were further examined by an immunoassay that detects both mature and pro forms of tryptase. Silencing CTSL with L662 and L98 (Fig. 5A) significantly decreases cellular levels of total tryptase by 51% and 57%, respectively, whereas L662 (but not L98) significantly increases total tryptase levels in the medium by 22%. Silencing CTSB with B55–59 and Bmix constructs (Fig. 5B) significantly decreases cellular levels of total tryptase by 35–46% in B57–59 transfected HMC-1 cells, whereas all constructs except B58 significantly and substantially increase total tryptase levels in the medium by 1.5- to 2.6-fold. Nearly all of the spontaneously secreted tryptase is protryptase because mature tryptase is below the limit of detection (<10 ng/10⁶ HMC-1 cell-equivalents in each condition) with a mature tryptase-specific ELISA. Of note, B58 also fails to significantly diminish tryptase activity in HMC-1 cells (Fig. 4A, bar 4). Thus, attenuating protryptase processing in HMC-1 cells by lowering levels of CTSB or CTSL significantly increases the amounts of protryptase being spontaneously secreted by these cells, particularly if CTSB is targeted.

Pharmacologic inhibition of either CTSB or CTSL activity in HMC-1 and primary skin mast cells shows that both CTSs are needed to process protryptase

HMC-1 cells were used to assess the involvement of CTSC and CTSL in the intracellular processing of β-protryptase using pharmacologic inhibitors. The inhibitor of CTSC, of CTSL, and of CTSB incubated with HMC-1 cells each results in a dose-dependent (Fig. 6A) and time-dependent (Fig. 6B) decrease in cell-associated β-tryptase activity by 50% of control or greater. To validate the findings with HMC-1 cells in tissue-derived mast cells, primary mast cells obtained from human skin (19) were studied with CTSL, CTSB, and CTSC pharmacologic inhibitors. To better detect the effects of these inhibitors on protryptase processing, skin mast cells were first partially depleted of mature tryptase and other secretory granule components by activating these cells to degranulate with anti-FcεRIα mAb. Washed degranulated cells were then placed in culture with SCF alone or together with CTS inhibitors for up to 6 d. Compared with a medium control, treatment for 6 d with CLIK-148 decreased CTSL activity by 88% and CTSB activity by 20%. Treatment with CA-074Me decreased CTSL levels by 83% and CTSB levels by 20%. Treatment with GF-CNHN₂ had no detectable effect on cellular levels of CTSB and CTSL activities. As seen in Fig. 6C, tryptase activity declines to 39–48% of control after 6 d with the CTS inhibitors, reflected in the representative Western blots in Fig. 6D, which show an apparent decline after 6 d of treatment with CTSL, CTSB, and CTSC inhibitors of mature tryptase (G5 mAb label), but not of total tryptase (G3 mAb label). In contrast to mature
tryptase activity, that of β-hexosaminidase (Fig. 6E), another secretory granule component, is not significantly affected by any of these inhibitors. Moreover, chymase activity (Fig. 6F) declines to ~45% of control in the presence of the CTSC inhibitor but is unchanged in the presence of CTSB and CTSL inhibitors. Of further possible interest is that the ratios of protease activities in skin mast cells to HMC-1 cells were 240 for tryptase, 9.7 for CTSB, 5.8 for CTSL, and 0.2 for CTSC. The smaller amount of CTSC activity in skin mast cells relative to that in HMC-1 cells, compared with the higher amounts of tryptase and the other CTSs, has been confirmed by Western blotting of CTSC protein normalized to that of β-actin, which indicates a CTSC protein ratio in skin mast cells to HMC-1 cells of 0.5:1.

**Discussion**

The current study identifies CTSB and CTSL as the two principal processing proteases for β-protryptase in human mast cells. Genes for β-tryptase, but not for α-tryptase, are present in the HMC-1 cell genome, and mature β-tryptase accounts for essentially all of the tryptase enzymatic activity in both primary skin mast cells and HMC-1 cells because mature α-tryptase has minimal enzymatic activity. CTSC, based on CTSC gene-specific RNA silencing, may account for a minor portion of β-protryptase processing but is not critical for mature β-tryptase formation in HMC-1 cells. The prior and current finding that Gly-Phe-CHN 2, a putative inhibitor of CTSC, inhibits β-tryptase formation in HMC-1 cells and extracts (3, 4) suggests an off-target effect of this molecule. In skin mast cells, this CTSC inhibitor also causes a decline in chymase activity, probably because prochymase processing depends upon CTSC (5). However, β-hexosaminidase levels are unchanged by Gly-Phe-CHN2, which argues against a general effect on granule enzymes.

These targeted depletion experiments in HMC-1 cells, along with the low level of CTSC activity and protein in primary human skin mast cells, are consistent with the finding that CTSC-deficient murine mast cells do produce mature tryptase (5). CTSB and CTSL, which process β and α protryptases to their mature forms in solution (6), appear to process at least β-protryptase while inside human mast cells because when these enzymes are either silenced with gene-specific shRNA or inhibited by selective pharmacologic agents in situ, cellular levels of mature enzymatically active β-tryptase are markedly diminished and levels of

**FIGURE 4.** ShRNA constructs targeting CTSL and CTSB reduce β-trypase activity in HMC-1 cells. A. Effect of CTSB shRNA on the activities of trypase, CTSL, CTSB, and CTSC in HMC-1 cells. HMC-1 cells were stably transfected with different CTSB (lanes 1–6, B55, B56, B57, B58, B59, and a mixture of these constructs [Bmix], respectively) and control (lanes C) shRNA constructs and assessed for protease activities. *p < 0.05 by ANOVA for a significant difference from the corresponding control. B. Correspondence of percent CTSB enzymatic activity to those of CTSC, CTSL, and trypase from A. Linear regression lines (black) and 95% confidence intervals (gray) are shown. The regression equations and r² values are as follows: 115 – 0.2x and 0.36 for CTSB, 64 + 0.4x and 0.93 for CTSL, and −1.7 + 1.1x and 0.98 for trypase, where x = CTSB cellular activity. C. Effects of CTSB shRNA on protein and mRNA levels of CTSB, CTSL, and trypase (lane numbering corresponds to that in A). mRNA levels are expressed as *p < 0.05 by ANOVA for a significant difference from the corresponding control. D. Effect of CTSL shRNA on the enzymatic activities of trypase, CTSB, CTSL, and CTSC in HMC-1 cells. HMC-1 cells were stably transfected with different CTSL (lane 1 [L662] and lane 2 [L98]) and control (lanes C) shRNA constructs and assessed for protease activities. *p < 0.05 by ANOVA for a significant difference from the corresponding control.

**FIGURE 5.** Immunoreactive levels of trypase in HMC-1 cells stably transfected with CTSL (A), CTSB (B), and control shRNA constructs. Levels of mature and total trypase (ELISAs) in cell extracts and in media collected over 4 d were determined in three independent experiments. *p < 0.05, †p < 0.01, ‡p < 0.001 when compared with the corresponding control.
spontaneously secreted protryptase are increased. Furthermore, CTSL and CTSB functionally colocalize with β-trypstat in the secretory granules of primary human skin mast cells, positioning these CTSs to process protryptase during their intracellular migration or upon their arrival in secretory granules.

CTSL and CTSB have been linked to secretory granule pathways and intracellular processing of proenzymes and other proteins in a variety of different cell types (27–29). In chromaffin cells, CTSL processes proenkephalin to [Met]enkephalin (29). CTSL activates trypsinogen to trypsin in the pancreatic exocrine pathway (28). CTSL and CTSB, but not CTSB, process proCTSC to its active tetrameric form (30). CTSL plays a critical role in processing invariant chain (Ii) in cortical thymic epithelial cells (31), whereas the p41 alternative splice variant of Ii binds to and inhibits CTSL (32). CTSL processes Ag in cortical thymic epithelial cells and thereby influences positive selection of developing CD4+ lymphocytes (33). CTSL deficiency introduced into NOD mice diminishes production of CD4+ T cells and attenuates the development of autoimmune diabetes (34). Thymocyte expression of CTSL also is critical for the development of NKT cells, perhaps through processing of putative natural CD1d ligands needed for NKT cell selection (35).

To validate the relevance of these CTS-dependent processing pathways to what happens inside mast cells, shRNA constructs targeting specific CTSs were employed. Decrements in the cellular activity levels of the targeted CTSB and CTSL of >80% correlated with substantial decrements in mature, enzymatically active tryptase levels within HMC-1 cells, along with increases in the spontaneous secretion of protryptase. The magnitude of the decline in intracellular tryptase levels was almost equal to the decline in CTBS when CTBS shRNA was used, but was not as great as the decline in CTSL levels when CTSL shRNA was used. Why CTSL silencing appears to be more potent than CTSB silencing at lowering cellular levels of active tryptase is not obvious, but might be explained in part by two of our observations. First, CTSL silencing potentiates the spontaneous secretion of protryptase better than does CTSB silencing. Second, CTSB silencing also causes a modest reduction in CTSL levels, whereas silencing CTSL has no effect on CTSL levels. In contrast, inhibition of CTSL and CTSB with specific pharmacologic antagonists results in comparable declines in β-trypstat activity in both HMC-1 cells and primary human skin mast cells.

Localization of CTSL and CTSB to mast cell secretory granules indicates they will be released into tissues in vivo when mast cells are activated to degranulate, probably bound to proteoglycan, and thereby extend the protease repertoire of the protease:proteoglycan complex of human mast cell secretory granules. CTSL and CTSB act optimally at the acidic pH found in mast cell secretory granules as well as in lysosomes, and are irreversibly inactivated at pH ≈ 7 (36), which should restrict their extracellular activities to sites where the pH is acidic. Such sites might include those where inflammation occurs, such as the asthmatic airway, or where poor vascularity exists, such as regions associated with solid tumors and healing wounds. Heparin and chondroitin sulfate, which colocalize to mast cell secretory granules, bind to and promote the processing of proCTSL (37, 38) and bind even better to mature CTSL and CTSB (39), stabilizing these enzymes at neutral pH.

Why CTSL cannot compensate for CTSB when the latter is silenced and vice versa is not clear. Purified CTSL and CTSB each readily process α/β-protrypstat to maturity in solution (6). Within mast cells, however, silencing or inhibiting either CTSL or CTSB impairs protryptase processing. Silencing CTSL has a modest effect on lowering CTSL levels, whereas silencing CTSL has no discernible effect on CTSL levels. Perhaps these CTSs do more than just remove the propeptide and play additional non-redundant roles in the production of mature tryptase or its trafficking to, and storage in, secretory granules.

In summary, CTSL and CTSB play novel nonredundant roles in the processing of human β-trypstat and perhaps of α-protrypstat. These CTSs both with (pro)tryptases and heparin to human mast cell secretory granules, act at the acidic pH present either before or after entry into this organelle, and are secreted when these cells degranulate, raising an opportunity for them also to act in the extracellular milieu. Furthermore, pharmaceutical agents that enter mast cells and target these CTSs in vivo are likely to attenuate protryptase processing, increase the spontaneous se-

FIGURE 6. HMC-1 cells (A, B) and primary skin mast cells (C–E) treated with pharmacologic CTS inhibitors. Dose–response (A) and time–course (B) effects of CTSL, CTSB, and CTSC pharmacologic inhibitors on β-trypstat activity in HMC-1 cells. HMC-1 cells in culture were incubated with different concentrations of CLIK-148, CA-074Me, and GF-CHN2 for 3 d (A) or with each inhibitor (10 μM) for various times (B) and then assessed for tryptase activity in the presence of STBI with TGPK. Viable cell percentages were always >90%. The average from two independent experiments along with range (error bars) are shown. C–F. Primary skin mast cells. Mast cells derived from human skin that had been activated with anti-FcεRI mAb (22E7; 0.1 μg/ml for 30 min) (19) were washed and placed into culture in the presence or absence of CLIK-148, CA-074Me, or GF-CHN2 for up to 6 d; medium (SCF ± CTS inhibitors) was replenished on days 2 and 4. Tryptase activity (C), tryptase and β-actin Western blots (D), β-hexosaminidase activity (E), and chymase activity (F) were measured or detected in cell extracts. Activities: mean ± SD, n = 3 to 4. †p < 0.001 when compared with the buffer control value at the same time point. The Western blot detects mature (G5 mAb) and total (G3 mAb) tryptase in identical numbers of skin mast cells treated for 6 d as in C and is representative of three experiments. Arrowhead, β-actin; arrow, tryptase.
cretion of protryptases, and lead to higher extracellular levels of protryptases in vivo.

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