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Inhibition of dipeptidyl peptidase I in the human mast cell line HMC-1: blocked activation of tryptase, but not of the predominant chymotryptic activity

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Abstract

The mast cell proteases tryptase and chymase are synthesised as inactive precursors, but are stored and secreted as active enzymes. The cysteinyl protease dipeptidyl peptidase I (DPPI, cathepsin C) can activate the corresponding proenzymes in cell-free systems, but it is unknown whether it fulfils this role within the intact cell. We, therefore, tested the effect the DPPI-selective inhibitor Gly-Phe diazomethyl ketone (Gly-Phe-CHN_2) on the tryptic and chymotryptic activity of the human mast cell-like cell line, HMC-1, and monitored any changes in the amount of immunodetectable enzymes by flow cytometry. Culture in Gly-Phe-CHN_2 produced a significant decrease in tryptase activity in cell lysates within 24 hr and further decreases during continued culturing to 216 hr with periodic replenishment of Gly-Phe-CHN_2-containing media. Flow cytometry showed no significant change in the levels of immunoreactive tryptase. In contrast, chymotryptic activity in treated cells did not differ significantly from untreated cells at any time point. Treatment of 216 hr cell lysates with DPPI revealed significant amounts of activatable protryptase in Gly-Phe-CHN_2-treated cells, but not in controls, whereas activatable prochymotryptic activity was found in both treated and control cells. Chymase was detected immunologically, though small differences in substrate specificity and molecular mass were observed. These results strongly suggest that DPPI plays a role in the activation of tryptase, but not of the predominant chymotryptic activity of HMC-1 cells. As inhibitors of tryptase have proven efficacious in models of allergic disease, these results also indicate that inhibitors of DPPI might provide an additional point of therapeutic control.

Keywords: Tryptase; Chymase; Cathepsin G; Dipeptidyl peptidase I; Proenzyme activation; Gly-Phe diazomethyl ketone

1. Introduction

Mast cells are phenotypically and functionally versatile tissue-dwelling cells capable of secreting a wide variety of mediators [1]. Their role in provoking the immediate hypersensitivity response of allergic reactions is well established and there is increasing evidence for their involvement in chronic inflammation and tissue remodelling, including fibrosis, development of atherosclerotic plaques and angiogenesis in tumours. They are characteristically activated through allergen-dependent cross-linking of high-affinity receptors for IgE (FcεRI), but can also be activated by superoxide, complement proteins, opioids, neuropeptides and lipoproteins. Of particular importance amongst the products secreted by activated mast cells are a number of proteases including tryptase, chymase, carboxypeptidase and cathepsin G, which can rapidly process a variety of biologically active peptides and proteins or their precursors [2].

Tryptase (EC 3.4.21.59), a serine protease with trypsin-like specificity, and chymase (EC 3.4.21.39), a serine protease with chymotrypsin-like specificity, are particularly

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Abbreviations: AAPF-*S*-Bzl, succinyl-Ala-Ala-Pro-Phe thiobenzyl ester; DPPI, dipeptidyl peptidase I; GEpNA, Gly-Glu-*p*-nitroanilide; GFpNA, Gly-Phe-*p*-nitroanilide; Gly-Phe-CHN_2, Gly-Phe diazomethyl ketone; IMDM5, Iscove's modified Dulbecco's essential medium supplemented with foetal bovine serum (5%, v/v), antibiotics, and Glutamax I; <EPRpNA, pyroglutamyl-Pro-Arg-*p*-nitroanilide; Y-40018, 2-[5-amino-6-oxo-2-(3-chlorophenyl)-1,6-dihydro-1-pyrimidinyl]-*N*-[1-benzyl-3,3-difluoro-3-(*N*-benzylcarbamoyl)-2-oxo-propyl]acetamide; Y-40613, 2-[5-amino-6-oxo-2-(4-fluorophenyl)-1,6-dihydro-1-pyrimidinyl]-*N*-[1-(5-methoxycarbonylbenzoxazol-2-yl)carbonyl-2-phenylethyl]acetamide.

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abundant in the mast cell granule, with up to 30 and 5 pg per cell, respectively, compared with 2 pg per cell histamine [2]. Cathepsin G (EC 3.4.21.20), which, like chymase, is also a serine protease with chymotrypsin-like specificity, is less abundant, being present at about one-tenth the levels of chymase [3]. These three proteases have been shown to act on specific extracellular proteins and peptides, as well as to alter the behaviour of various cell types [2,4–6]. Tryptase inhibitors can block allergen-induced airway inflammatory responses in allergic sheep [7,8], and one such inhibitor, APC-366, was able to reduce late phase bronchoconstriction in a Phase II clinical trial with asthmatic patients [9].

An alternative to therapeutic regulation of an enzyme by its inhibitors is to block the processing of an inactive precursor form of that enzyme. Tryptase, chymase and cathepsin G are synthesised as inactive precursors, but stored and released in the fully active mature forms [2]. Chymase [10,11] and cathepsin G [12,13] are synthesised as proenzymes with a two-amino acid extension at the N terminus, similar to other granulocyte proteases including granzyme A, granzyme B, and neutrophil elastase [12–14]. The proforms of these proteases are catalytically inactive, but removal of the N-terminal dipeptide with the cysteinyl protease DPPI (also known as cathepsin C or dipeptidylaminopeptidase I) (EC 3.4.14.1) has been shown to restore full catalytic activity. DPPI is a ubiquitous granule/lysosomal protein that sequentially removes dipeptides from the amino terminus of proteins and is particularly abundant in cells that are rich in granulocyte proteases [15]. It is unique amongst members of the papain family of cysteine proteases in that it exists as a tetramer with each monomeric unit composed of three chains: the pro-chain, the heavy chain and the light chain. The association with the pro-chain confers the dipeptidyl aminopeptidase specificity on the catalytic chains [16,17].

Tryptase is not a single enzyme, but a family of closely related proteases. On the basis of amino acid sequence homology, these have been identified as α-tryptase and three very closely related β-tryptases, βI, βII, and βIII [2,18]. The prosequence for all tryptases is substantially longer than that of chymase (14 amino acids). In the presence of heparin, recombinant βII-tryptase was found to process itself autocatalytically to remove the first 12 amino acids [18]. The resulting pro′enzyme was inactive but removal of the remaining dipeptide with dipeptidyl peptidase I yielded an active product. Recombinant α-tryptase was unable to carry out this autocatalytic step, and so doubts have been expressed as to whether it is activated *in vivo* [18].

The ability of an enzyme to catalyse a reaction in a cell-free system does not necessarily indicate that it has this role in the intact cell. Indeed, in a previous study we have shown that heparin and histamine, compounds present in the mast cell granule along with chymase, strongly inhibit the activation of recombinant prochymase by dipeptidyl peptidase I from a commercially available bovine source [11]. The availability of recombinant human DPPI [19] has permitted a re-examination in the present study as to whether any species-specific factors might be involved. Furthermore, we have been able to follow the expression and activation of both tryptase and chymase in an *ex vivo* cell culture system utilising the human mast cell line HMC-1. This cell line, derived from a patient with mast cell leukaemia, expresses several mast cell-related markers, including tryptase [20–22] and chymase [22]. RT-PCR studies have identified the tryptase expressed as a β-tryptase [22], consistent with the recent finding that HMC-1 cells lack the gene for α-tryptase [23]. We have tested the effects on these cells of the DPPI-selective inhibitor Gly-Phe-CHN_2 [12–14,24], and here present evidence for the first time that DPPI is responsible for processing mast cell tryptase within the mast cell. In contrast, the detectable chymotryptic activity displayed a different response to Gly-Phe-CHN_2, which raises the possibility that it might be activated by an alternative pathway.

2. Materials and methods

2.1. Materials

All cell culture media and supplements, Hank's balanced salt solution (HBSS) (10×), and phosphate buffered saline (PBS) (10×) were obtained from Invitrogen. The chromogenic substrates succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide, AAPF-*S*-Bzl, succinyl-Ala-Ala-Pro-Lys-*p*-nitroanilide, succinyl-Ala-Ala-Pro-Arg-*p*-nitroanilide, GFpNA, GEpNA were obtained from Bachem. <EPRpNA, pyroglutamyl-Gly-Arg-*p*-nitroanilide, D-Phe-piperyl-Arg-*p*-nitroanilide, D-Pro-Phe-Arg-*p*-nitroanilide, and *Z*-D-Arg-Gly-Arg-*p*-nitroanilide were purchased from Quadratech. *N*-Benzoyl Arg-*p*-nitroanilide and tosyl-Gly-Pro-Lys-*p*-nitroanilide were obtained from Sigma–Aldrich, tosyl-Gly-Pro-Arg-*p*-nitroanilide and methoxycarbonyl-D-Nle-Gly-Arg-*p*-nitroanilide from Boehringer, and Gly-Phe-CHN_2 from Enzyme Systems Products. The chymase inhibitors Y-40018 [25] and Y-40613 [26] were generous gifts of Mitsubishi Pharma. Antipain, leupeptin, benzamidine, aprotinin (from bovine lung), and soy bean trypsin inhibitor (SBTI) were purchased from Sigma. Anti-CD117/c-*kit* monoclonal antibody (AB-4, cocktail of three clones) was obtained from Neomarkers and FITC-conjugated goat anti-mouse antibodies from Becton Dickinson. Recombinant human (rh)-βI tryptase and rh-βII tryptase were generous gifts of Promega. The anti-tryptase mouse monoclonal antibody AA5 [27], the anti-tryptase rabbit polyclonal antibody E2 [28], and the anti-chymase monoclonal antibody CC1 [29] were produced as previously described. The mouse monoclonal antibody to human cathepsin G was purchased from Chemicon. The specificity of this monoclonal antibody was validated by immunohistochemistry against a polyclonal antibody which we had previously found did not cross-react with human

chymase [30] (results not shown). Human lung tryptase [31], human skin chymase [32], and rh-prochymase [11] were purified following previously published protocols. All other chemicals were obtained from Sigma–Aldrich or BDH/Merck.

2.2. rh-DPPI

Recombinant human pro-DPPI fused to the signal peptide from rat DPPI was expressed as a secreted protein in a baculovirus/High Five cell system and purified as described earlier [19]. Briefly, the cultures were harvested 5 days postinfection, and rh-DPPI was purified from the medium on butyl-Sepharose 4 Fast Flow (Pharmacia) at pH 4.5. The pool obtained was incubated for 40 hr at pH 4.5 and 4–6° to complete the proteolytic maturation process. After incubation, rh-DPPI was desalted and subjected to anion exchange chromatography on Q-Sepharose Fast Flow® (Pharmacia) to further purify and concentrate the preparation. The batch of rh-DPPI used in the present study had a specific activity of 13 U/mg with GFpNA as substrate [19] where 1 U is defined as 1 μmol substrate transformed/min. Furthermore, the same batch has been shown to be very similar to purified natural human DPPI with respect to glycosylation, enzymatic processing, oligomeric structure and catalytic activity [19].

2.3. Production of antiserum to rh-DPPI

White New Zealand rabbits were immunised with 25 μg rh-DPPI in Freund's complete adjuvant, followed by successive boosts in Freund's incomplete adjuvant or in saline. Blood was collected from the marginal ear vein and the specificity for human DPPI was confirmed by Western blotting.

2.4. Enzyme assays

Tryptase activity was determined with 0.5 mM <EPRpNA in 1.0 M glycerol, 100 mM Tris, adjusted to pH 8.0 with HCl [33]. Chymotryptic activity was determined with 0.5 mM AAPF-*S*-Bzl in 1.5 M NaCl, 0.125 mM 5,5′-dithio-bis (2-nitrobenzoic acid), 100 mM Tris–HCl, pH 8.0 [30]. Other tryptic and chymotryptic substrates were assayed in the relevant buffers at a concentration of 0.5 mM. Inhibitors were preincubated with the purified enzyme or cell lysate for 10 min on ice prior to assay. Tryptase concentration was determined by active site titration with 4-methylumbelliferyl 4-guanidinobenzoate and expressed as moles of active site [34]. The effects of pH, heparin, and histamine on the activity of rh-DPPI towards 5.0 μg/mL rh-prochymase, 1 mM GEpNA, and 1 mM GFpNA were determined as previously described [11]. Inhibition studies of rh-DPPI with Gly-Phe-CHN_2 were conducted in 143 mM NaCl, 2 mM dithiotreitol, 50 mM Aces, adjusted to pH 6.0 with NaOH.

2.5. Cell culture

The human mast cell line HMC-1 (generously provided by the Mayo Clinic, Rochester, MN, USA) was cultured in suspension at 37° and 5% CO_2 in IMDM5. Cultures were seeded at 0.5×10^5 cells/mL and split 1:5 after every 4 days. The cells were harvested by centrifugation at 300 *g* for 5 min at 20°. The cell pellets were washed twice with PBS before further analysis.

2.6. Western blot analysis

The cell lysates were subjected to SDS–PAGE under reducing conditions on 10% gels and the proteins transferred electophoretically to a nitrocellulose membrane. Endogenous avidin binding activity was blocked with the avidin–biotin blocking kit supplied by Vector Laboratories, the membrane was blocked with 3% gelatin, 0.2% Tween-20 in PBS and probed with the appropriate biotinylated antibody. Bands were visualised by subsequent treatment with Extravidin® peroxidase conjugate and SuperSignal West Pico® chemiluminescent substrate.

2.7. Studies with DPPI-selective inhibitor Gly-Phe-CHN_2

Cells were cultured in the presence of Gly-Phe-CHN_2 at concentrations of 10 or 20 μM in IMDM5. Cells cultured in IMDM5 medium alone and in IMDM5 containing 0.15% (v/v) dimethyl sulphoxide served as controls. The cells were harvested at intervals from 2 to 216 hr. The media were replaced every 48 hr with fresh medium containing the appropriate concentration of Gly-Phe-CHN_2. The harvested cells from each treatment and time point were counted and then apportioned for analysis: two million cells were washed twice with PBS and stored at −70° for enzymatic and protein assays, and approximately the same number were processed for flow cytometry.

2.8. Processing of cells for enzyme and protein assays

Washed cells were disrupted by hypotonic lysis in 6 μM Gly-Phe-CHN_2 followed by the addition of Triton X-100 to a final concentration of 0.3% (w/v). The released proteases were stabilised by the addition of an equal volume of lysis buffer (40 mM Mops, adjusted to pH 6.8 with NaOH, 2.0 M NaCl, 2 mM EDTA, 20% glycerol). The lysate was centrifuged at 3000 *g* at 4° for 10 min, and the supernatant taken for further analysis. The protein concentration in cell lysates was measured by the bicinchoninic acid (BCA) method (Pierce) according to manufacturer's instructions, but modified for a microtitre plate format. BSA was used as standard.

2.9. Flow cytometry (FACS)

Cells were fixed with 1% paraformaldehyde and formaldehyde-activated sites were blocked with 0.1 M

glycine. Permeabilization and blocking were carried out with 0.5% saponin and 0.1% Tween-20 in PBS containing 10% goat serum and 3% BSA, on ice for 45 min. Cells were washed and resuspended in PBS containing 2% goat serum and 1% BSA and 0.2% Tween-20. Cells were aliquoted and incubated for 45 min on ice with the primary antibodies at a concentration of 1.0 μg/mL for the monoclonal antibodies and a dilution of 1:1000 for the polyclonal antisera. The isotype controls were mouse IgG_1, mouse IgG_{2a}, and rabbit IgG. The secondary antibodies used were PE-conjugated goat anti-rabbit antibodies (Sigma) and FITC-conjugated goat anti-mouse antibodies (Becton Dickinson). The stained cells were counted on a FACScan® flow cytometer (Becton Dickinson) using an argon laser at 488 nm for excitation. Emission was measured in log mode. To enable quantitative comparison of the different Gly-Phe-CHN_2 treatments at different time points, the relative net median value of each histogram was calculated by subtracting the median value of the isotype control from the median value for each experimental sample and expressing it as a percentage of the IMDM5 control for that antibody and that particular time point.

2.10. Activation of proenzymes

The presence of the proenzymes of tryptase and chymase in HMC-1 cell lysates was detected by activation with rh-DPPI. Briefly, 200 μL of HMC-1 cell lysates were incubated with 150 μg of rh-DPPI in 0.15 M NaCl, 1 mM EDTA, 5 mM dithiothreitol, 20 mM Aces, pH 6.0, in a final volume of 500 μL at 37° for 90 min. A duplicate activation mix containing 10 μM Gly-Phe-CHN_2 served as control. As tryptase and chymase had little activity in the buffer used for activation, it was replaced with 1.0 M NaCl, 1 mM EDTA, 10% glycerol, 20 mM Mops, adjusted to pH 6.8 with NaOH, by diafiltration using Centricon C-10® centrifugal concentrators (Millipore). The volumes were adjusted to the original assay volume (500 μL) and samples were taken for enzyme assay with the appropriate chromogenic substrate. The activity of samples was expressed as a percentage of the corresponding controls.

2.11. Statistics

Paired *t*-tests were performed using SPSS® v 10 software for Windows®.

3. Results

3.1. Characterisation of HMC-1 tryptic activity

The HMC-1 cell lysate showed a tryptic activity with a substrate preference very similar to that seen with purified recombinant tryptases (Fig. 1A) and with different preparations of tryptase purified from human lung and skin

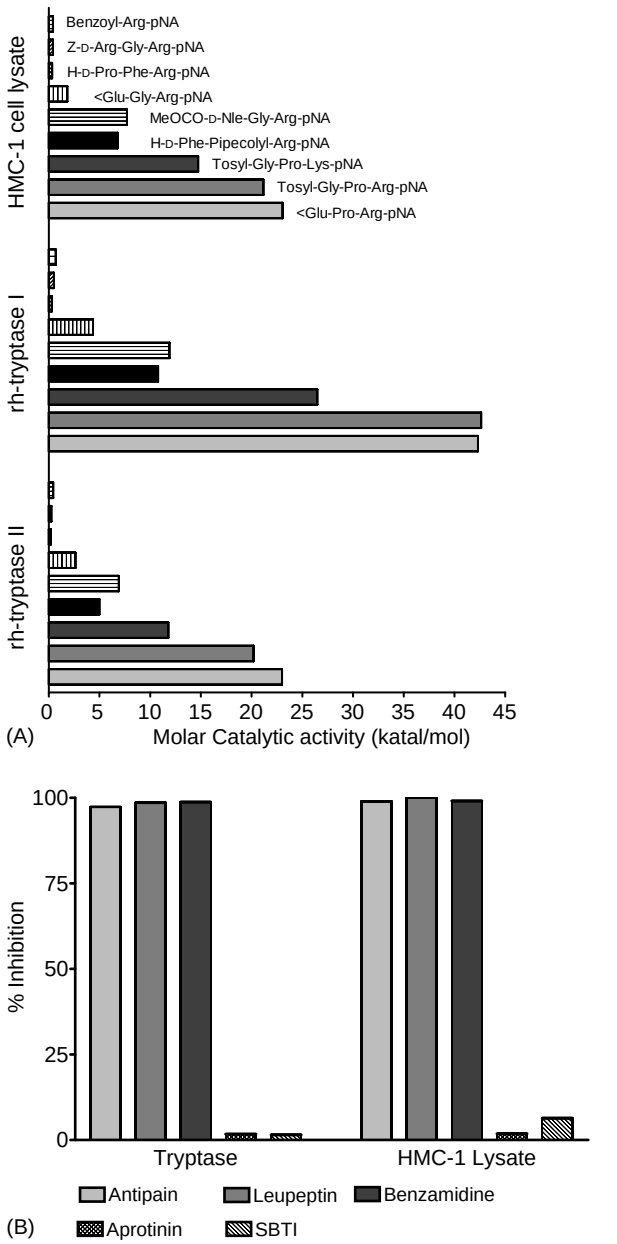

Fig. 1. Characterisation of the tryptase activity in HMC-1 cell lysates. (A) Comparison of tryptase activity in HMC-1 cell lysates with the purified preparations of rh-tryptase I and rh-tryptase II. In a representative experiment, the activity of enzymes was assayed against a panel of chromogenic substrates (0.5 mM) each in 1 M glycerol, 0.1 M NaCl, 0.1 M Tris–HCl, pH 8.0. Enzyme concentration was determined by active site titration with 4-methylumbelliferyl 4-guanidinobenzoate. All assays were performed in triplicate. (B) Inhibitor profile of the tryptic activity in HMC-1 cell lysates. In a representative experiment, cell lysates or purified human lung tryptase were pre-incubated on ice for 10 min with appropriate amounts of inhibitors and the activity determined with the tryptic substrate <EPRpNA. The uninhibited activities of the purified human lung tryptase and in the HMC-1 lysate were 43.3 and 38.4 mU/mL, respectively. All assays were performed in triplicate.

[35]. Like human tryptase, this <EPRpNA hydrolytic activity was inhibited by antipain (500 μg/mL), leupeptin (10 μM) and benzamidine (500 μM), but not by aprotinin (10 μM) or soybean trypsin inhibitor (200 μg/mL) (Fig. 1B). It was therefore concluded for the purposes of

this study that <EPRpNA-cleavage activity was synonymous with tryptase. The presence of immunoreactive tryptase was confirmed by flow cytometry (Fig. 2A) and by Western blot (Fig. 2C).

3.2. Characterisation of HMC-1 chymotryptic activity

Significant activity was detected with the highly sensitive chymotryptic substrate AAPF-*S*-Bzl. Concomitantly, both chymase and cathepsin G were detected immunologically by flow cytometry, with the relative signal intensity of tryptase > chymase > cathepsin G (Fig. 2A). Both chymase and cathepsin G can cleave the substrate AAPF-*S*-Bzl [30], but chymase has a 20-fold greater specific activity towards it [36], so it was anticipated that most of the observed activity could be attributed to chymase. To confirm this hypothesis, its susceptibility to a panel of inhibitors was tested (Fig. 2B). This panel included two selective reversible competitive inhibitors of chymase, Y-40018 and Y-40613, whose published K_i values for human chymase are 530- and 32-fold lower, respectively, than for human cathepsin G [25,26]. A range of concentrations of these inhibitors was tested with purified chymase and cathepsin G and the concentrations chosen (20 μM for each) gave optimal discrimination between the two enzymes when AAPF-*S*-Bzl was used as substrate (results not shown). As anticipated, the inhibitor profile of the chymotryptic activity in the HMC-1 lysates was similar to that of chymase, but distinct from that of cathepsin G.

However, the substrate specificity of the chymotryptic activity of HMC-1 cells was found to differ from that of chymase, or any other chymotryptic protease tested. Whereas chymase purified from different sources consistently displays a 4- to 8-fold lower activity towards the *p*-nitroanilide analogue of AAPF-*S*-Bzl, chymotrypsin a 3.6-fold lower activity, and cathepsin G a 90-fold lower activity [11,30], the activity of the lysate of HMC-1 cells towards the *p*-nitroanilide was less than 1/1000th of that towards the thioester. To test whether effectors present in the cell lysate might modulate the specificity of chymase towards these two substrates, purified human skin chymase was incubated with HMC-1 lysate for 30 min in ice. Analysis showed 87% recovery of the added *p*-nitroanilide activity and 74% of the added thioester activity. On Western blots, the HMC-1 lysate reacted with the chymase-specific monoclonal antibody CC1 to reveal a distinct band at 28 kDa and a broad dark band at the top of the gel (Fig. 2C, lane 2). The 28 kDa band was not seen when HMC-1 lysate was probed with an isotype-matched control antibody (Fig. 2C, lane 4) whereas the high molecular mass band stained intently. These results indicate that the 28 kDa band represents a specific interaction with the test antibody, while the high molecular mass band is a product of non-specific interaction of components of the lysate with the detection system used. This interpretation is supported by the detection of only a single band in purified chymase with CC1 (Fig. 2C, lane 1) and by the absence of any reaction product between chymase and the isotype-matched control antibody (Fig. 2C, lane 3). The CC1-specific band in the HMC-1 lysate differed from chymase by having a lower molecular mass (28 kDa vs. 32 kDa), and by being present in lower amounts relative to the AAPF-*S*-Bzl-lytic activity. To obtain the comparable intensities between the CC1-specific bands in lanes 1 and 2 (Fig 2C), the loadings were adjusted so that the amount of purified chymase applied to lane 1 had just 1% of the chymotryptic activity of the sample of HMC-1 lysate applied to lane 2. Whether the unusual substrate specificity is linked to the lower molecular mass, and thus still attributable to the expressed chymase, remains unknown. Therefore, for the remainder of this report, the activity detected with AAPF-*S*-Bzl shall be referred to as chymotryptic activity and not necessarily equated with chymase. In comparison, purified human lung tryptase and the tryptase in HMC-1 lysate, when loaded on the gel with equal enzymatic activities, showed nearly equal intensities and molecular mass for the monomer (34 kDa), although the HMC-1 lysate showed an additional band at the expected molecular mass of a dimer (Fig. 2C, lanes 5 and 6).

3.3. Characterisation of rh-DPPI

The levels of DPPI activity detected in HMC-1 cell lysates with GFpNA as substrate were very close to baseline, although this enzyme could be detected immunologically in flow cytometry (*vide infra*). However, studies with purified rh-DPPI showed that although the limit of detection was 0.1 μg/mL with 1 mM GFpNA or 1 mM GEpNA as substrates, significant rates of prochymase activation were obtained at rh-DPPI concentrations of 0.01 μg/mL or less. The pH optimum for the activation by human DPPI of prochymase was higher than that for the hydrolysis of GFpNA (Fig. 3), and was strongly inhibited by heparin and histamine, whereas the hydrolysis of GFpNA was much less affected. As prochymase has a glutamyl residue in the P1 position, the hydrolysis of GEpNA was also examined as a model of the GE-propeptide. The pH optimum was even more acidic than that of GFpNA, nor was the hydrolysis of GEpNA inhibited by heparin or histamine, suggesting factors other than the identity of the P1 residue are responsible for the action of pH, histamine and heparin on the activation of prochymase.

3.4. Characterisation of Gly-Phe-CHN_2

The action of the selective inhibitor Gly-Phe-CHN_2 on purified rh-DPPI was tested with both chromogenic dipeptide substrates and with rh-prochymase. At a concentration of 3 μM, Gly-Phe-CHN_2 inhibited the hydrolysis of

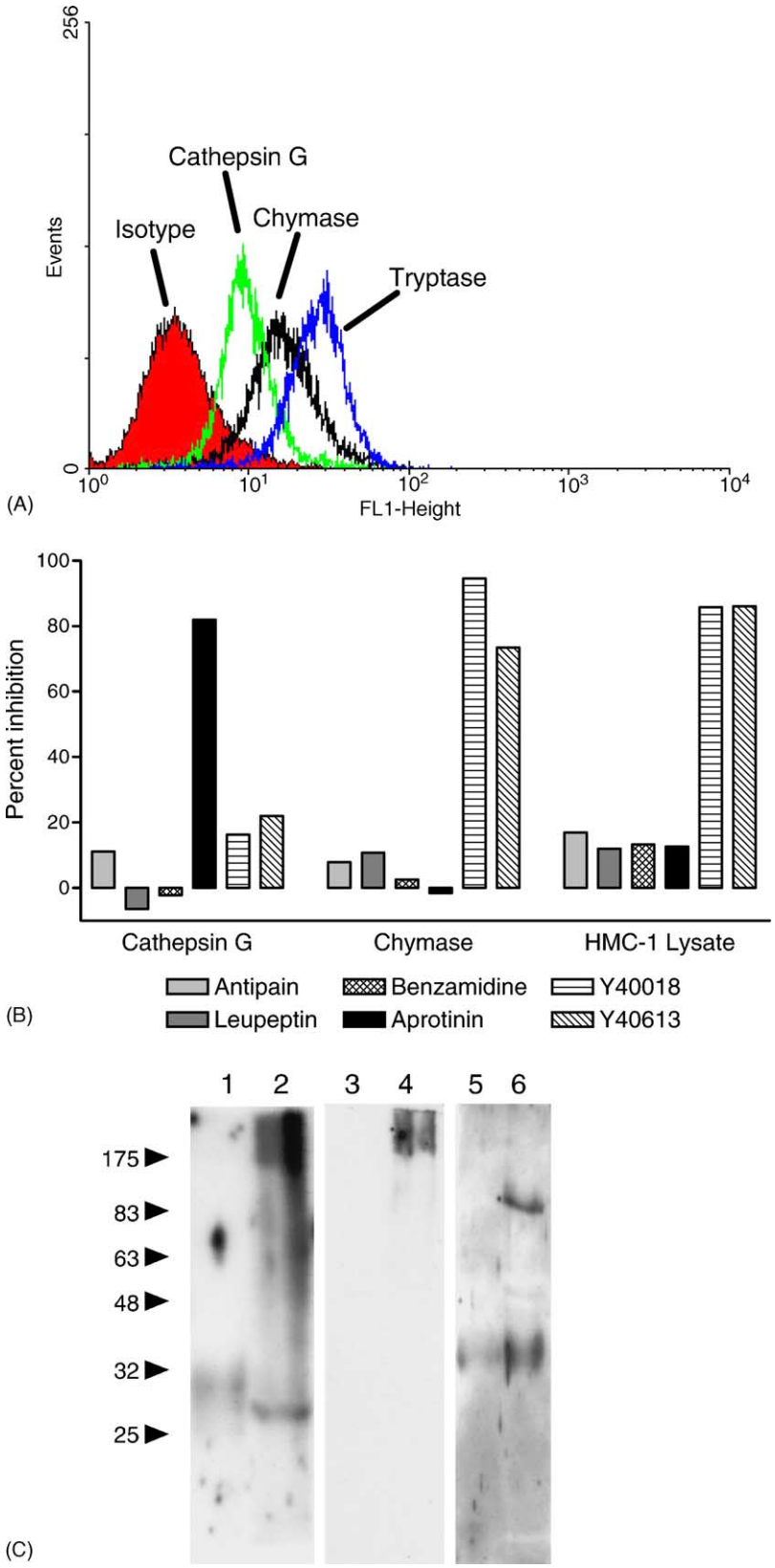

Fig. 2. Characterisation of the chymotryptic activity and immunodetectable proteases in HMC-1 cells. (A) Flow cytometry of HMC-1 cells grown in IMDM5, fixed, permeabilised, and stained with mouse monoclonal antibodies to tryptase (AA5), chymase (CC1) cathepsin G (Chemicon) or a matching isotype control. (B) Inhibitor profile. In a representative experiment, cell lysates or purified enzymes were pre-incubated on ice for 10 min with appropriate amounts of inhibitors and the activity determined with the chymotryptic substrate succinyl-Ala-Ala-Pro-Phe-thiobenzyl ester. Purified preparations of human neutrophil cathepsin G and human skin chymase were used for comparison. The uninhibited activities of the purified cathepsin G and chymase, and in the HMC-1 lysate were 23, 24, and 22 mU/mL, respectively. All assays were performed in triplicate. (C) Western blot. Lanes 1 and 3: purified human skin

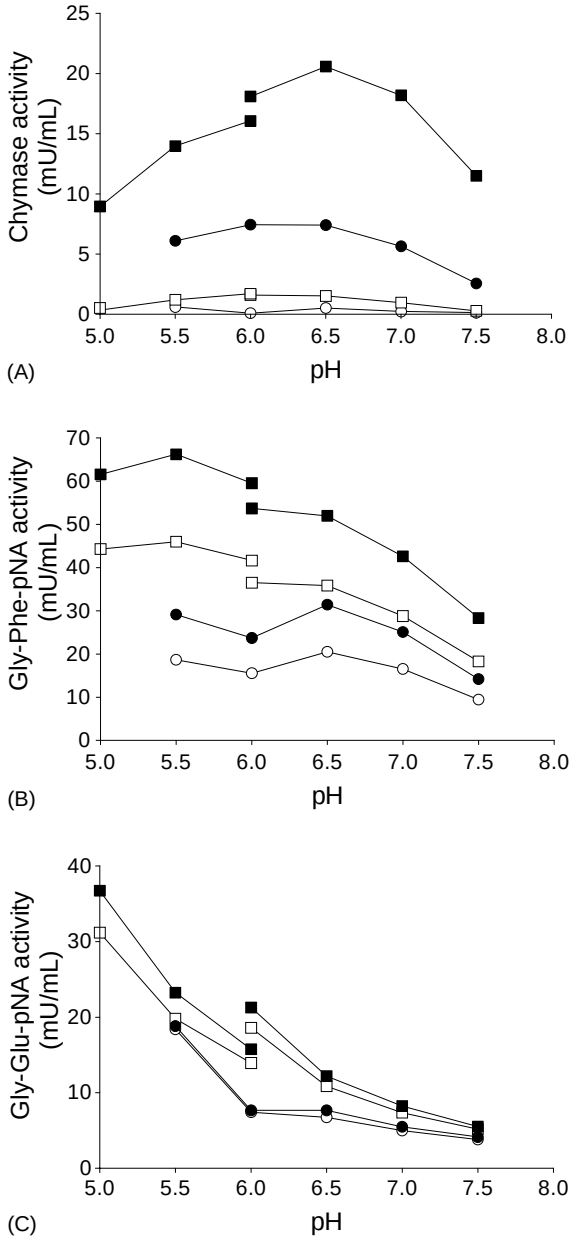

Fig. 3. Effect of pH, heparin, and histamine on the activity of rh-DPPI. Substrates used were (A) rh-prochymase (B) Gly-Phe-*p*-nitroanilide and (C) Gly-Glu-*p*-nitroanilide. Reaction mixtures contained 0.5 μg/mL (A) or 1.0 μg/mL (B and C) rh-DPPI in 2 mM dithiothreitol and (■) 25 mM citrate (pH 5.0–6.0) or 50 mM Aces (pH 6.0–7.5), no heparin; (●) 50 mM histamine, no heparin; (□) 25 mM citrate (pH 5.0–6.0) or 50 mM Aces (pH 6.0–7.5), 100 μg/mL heparin; (○) 50 mM histamine, 100 μg/mL heparin. Ionic strength was adjusted to 0.15 M by the addition of NaCl for each buffer. All reactions were at 37°. All assays were performed in triplicate. SEM values are not shown as most error bars would be smaller than the symbol size.

GFpNA and GEpNA by >95%, but the activation of rh-prochymase by only 80%. Even in the presence of 10 or 20 μM Gly-Phe-CHN_2, which inhibited the hydrolysis of both dipeptide substrates by >99%, significant activation of prochymase occurred (12 and 5% of control, respectively). These results are consistent with a 1000- to 10,000-fold lower K_m for rh-prochymase than for the dipeptides (preliminary estimates of 1 μM and 5–10 mM, respectively). Therefore, for experiments with cell cultures, Gly-Phe-CHN_2 was added at two concentrations, 10 and 20 μM. Gly-Phe-CHN_2 did not inhibit tryptase or chymase at either of these two concentrations.

3.5. Effect of Gly-Phe-CHN_2 on cell viability and cell parameters

Cells cultured with Gly-Phe-CHN_2 at concentrations of 10 and 20 μM showed no decrease relative to controls in viability, as measured by dye exclusion, or in rate of replication, as measured by increase in cell number. Flow cytometry indicated there was no change in the granularity of the cells, as assessed by the sideways scatter of light of the same wavelength as the incident beam, in response to the different treatments with Gly-Phe-CHN_2 or dimethyl sulphoxide. Similarly, flow cytometry did not detect any changes in the levels of immunodetectable c-*kit*, tryptase, chymase, DPPI or cathepsin G (Fig. 4). Comparisons between time points are less robust due to limitations inherent in flow cytometric methodology, but the use of relative net median fluorescence values showed little variation between treatments at the different time points. However, in all cases, a decrease in cell size was observed over the duration of the 216 hr incubation, as indicated by a decrease in forward scatter in flow cytometry and a decrease in protein content per cell. As there was a 10-fold increase in cell number over the course of the experiment, this decrease might have been a consequence of increased competition for the available nutrients together with a possible negative feedback mechanism between increasingly crowded cells.

3.6. Effect of Gly-Phe-CHN_2 on the expression of active tryptase

At 2 and 4 hr, treatment with Gly-Phe-CHN_2 had negligible effect on tryptase activity ($P > 0.05$), but an effect became apparent at 24 hr (Fig. 5). Cells treated with 10 and 20 μM Gly-Phe-CHN_2 showed a decrease in tryptase activity to 72% ($P < 0.003$) and 47% ($P < 0.002$) of the controls, respectively ($N = 6$). The activity continued to decrease relative to time-matched controls at 48 hr (63 and

chymase; lanes 2, 4, and 6: HMC-1 lysate; and lane 5: purified human lung tryptase. Lanes 1 and 2 were probed with CC1 anti-chymase monoclonal antibody, lanes 3 and 4 were probed with an isotype control monoclonal antibody, and lanes 5 and 6 were probed with AA5 anti-tryptase monoclonal antibody. Loadings were adjusted so that the chymase in lanes 1 and 3 had 1/100th the activity towards the substrate Ala-Ala-Pro-Phe thiobenzyl ester as did the lysate in lanes 2 and 4. Lanes 5 and 6 contained equal amounts of activity towards the substrate <Glu-Pro-Arg-*p*-nitroanilide. The amount of lysate in lane 6 was 1/300th that in lanes 2 and 4.

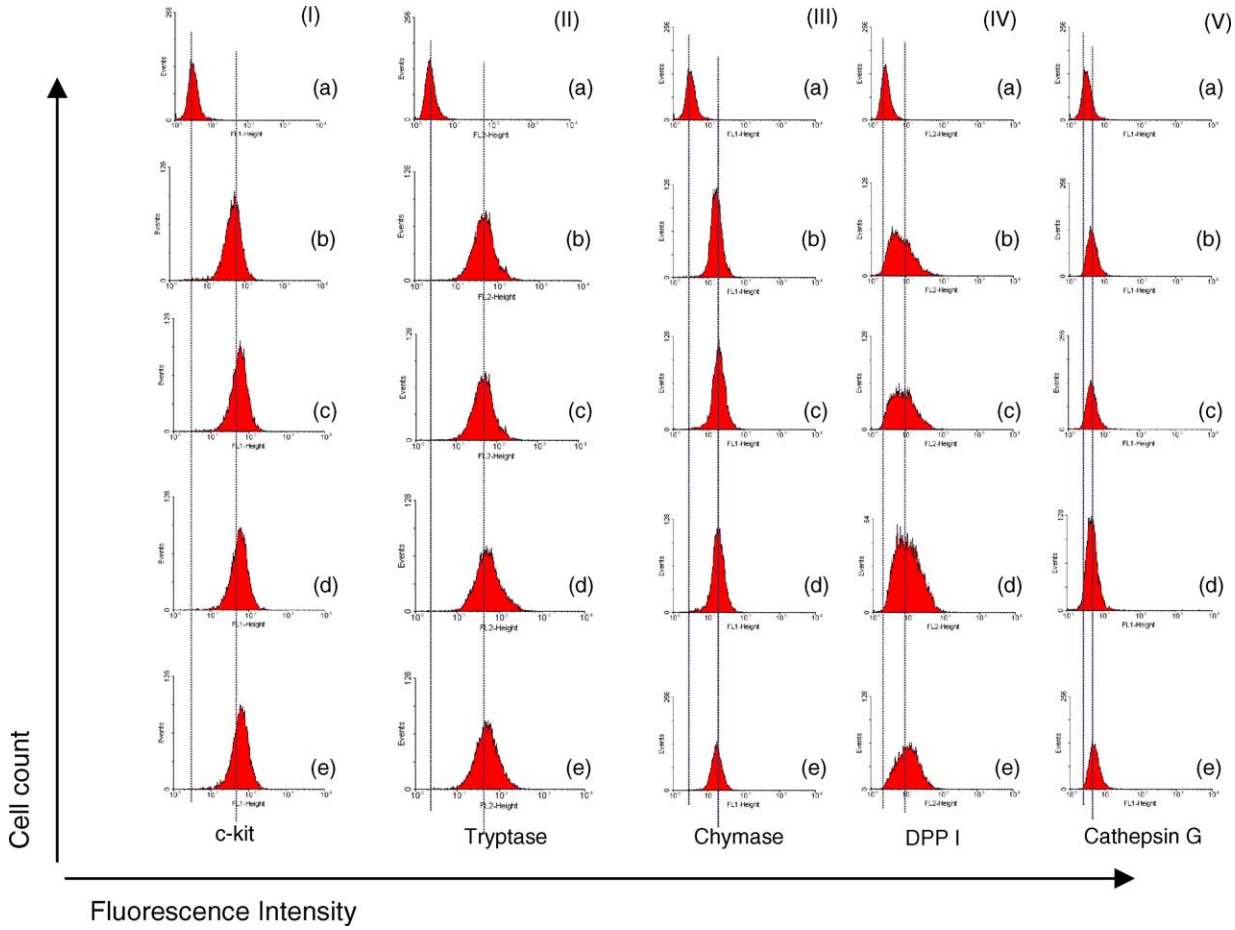

Fig. 4. Representative data for flow cytometric analysis of intracellular staining of proteases. The primary antibodies used were against c-*kit* (CD117) (I), tryptase (II), chymase (III), DPPI (IV) and cathepsin G (V). The cells were cultured for 96 hr in IMDM5 (b) or in IMDM5 supplemented with 0.15% dimethyl sulphoxide (c), 10 μM Gly-Phe-CHN_2 (d) or 20 μM Gly-Phe-CHN_2 (e). Samples of cells from all four treatments (b–e) were pooled and stained with the appropriate isotype immunoglobulin as control (a). All five antibodies gave signals that were well resolved from the isotype controls (I(a)–V(a)).

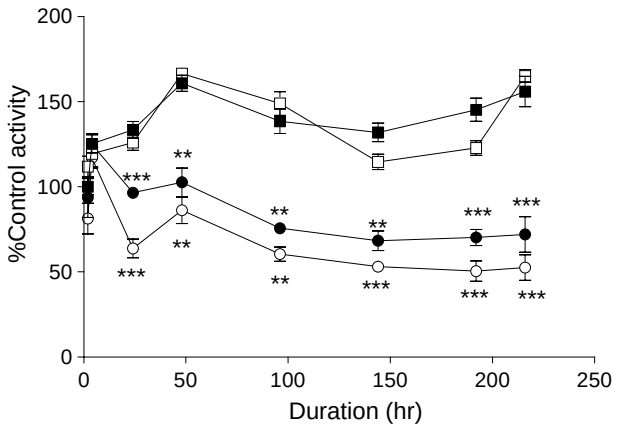

Fig. 5. Effect of DPPI inhibitor Gly-Phe-CHN_2 on the activity of tryptase in HMC-1 cells cultured for 2–216 hr. Cells were cultured in IMDM5 medium (■) or in IMDM5 supplemented with 0.15% dimethyl sulphoxide (□), 10 μM Gly-Phe-CHN_2 (●) or 20 μM Gly-Phe-CHN_2 (○). Culture medium with the appropriate addition was replenished at 48 hr intervals. The tryptase activity was calculated as percent of IMDM5 control at 2 hr time point (24.3 ± 4.7 nU per cell, mean ± SEM). Values plotted are mean ± SEM (N = 6). $^{**}P < 0.01$; $^{***}P < 0.001$.

53% of control activity in 10 and 20 μM Gly-Phe-CHN_2-treated cells, respectively). In preliminary experiments, it was observed that if the growth media were not replenished, then a partial recovery was observed by 120 hr in Gly-Phe-CHN_2-treated cells (results not shown). However, if the growth medium containing the indicated concentration of Gly-Phe-CHN_2 was replenished at 48 hr intervals, there was a steady decrease in tryptase activity in treated cells (Fig. 5). There was also a concomitant increase in tryptase activity in untreated cells, so that relative to time-matched controls, at 216 hr the tryptase activity was 17 and 12% in 10 and 20 μM Gly-Phe-CHN_2-treated cells, respectively. The increase in activity in the controls was not significant ($P = 0.679$, N = 6), but the decrease in Gly-Phe-CHN_2-treated cells was ($P < 0.001$, N = 6).

3.7. *Effect of Gly-Phe-CHN_2 on the expression of chymotryptic activity*

In marked contrast to tryptase, there was no apparent decrease in the chymotryptic activity in either of the Gly-Phe-CHN_2-treated cultures relative to the two control

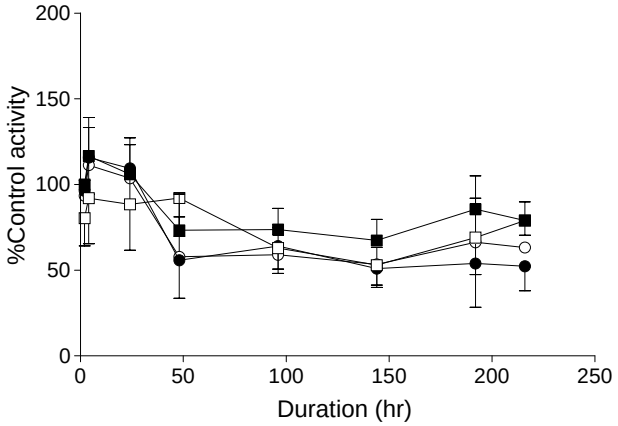

Fig. 6. Effect of DPPI inhibitor Gly-Phe-CHN_2 on chymotryptic activity in HMC-1 cells cultured for 2–216 hr. Cells were cultured in IMDM5 medium (■) or in IMDM5 supplemented with 0.15% dimethyl sulphoxide (□), 10 μM Gly-Phe-CHN_2 (●) or 20 μM Gly-Phe-CHN_2 (○). Culture medium with the appropriate addition was replenished at 48 hr intervals. The chymotryptic activity was calculated as percent of IMDM5 control at 2 hr time point (20.4 ± 3.4 nU per cell, mean ± SEM). Values plotted are mean ± SEM (N = 6).

culture conditions throughout the 216 hr incubation (Fig. 6, $P > 0.1$). In all cultures, AAPF-*S*-Bzl hydrolytic activity showed a steady to moderate decline between 2 and 24 hr, followed by a sudden drop at 48 hr, followed, in turn, by a steady level almost unaltered throughout the rest of the experiment (Fig. 6). When measured against the protein content of the cells, this drop in cellular chymotryptic activity (nU per cell) was matched by a decrease in specific activity (U/mg). It, therefore, cannot be attributed solely to the decrease in cell size reported above (results not shown). A marginal increase in chymotryptic specific activity was observed from 48 to 216 hr.

3.8. Proenzyme activation with rh-DPPI

The presence of proenzyme in lysates of HMC-1 cells grown for 216 hr either in IMDM5 or in 20 μM Gly-Phe-CHN_2 was detected by activation with rh-DPPI (Fig. 7). There was no net increase in activity of tryptase in the control group whereas tryptase activity increased by 43% in the lysates of Gly-Phe-CHN_2-treated cells (N = 11, $P < 0.001$). In the same lysates, the chymotryptic activity was increased after incubation with rh-DPPI in *both* the IMDM5 control and the Gly-Phe-CHN_2-treated cells (by 23 and 45%, respectively). The difference between treatment and control was not significant ($P < 0.078$).

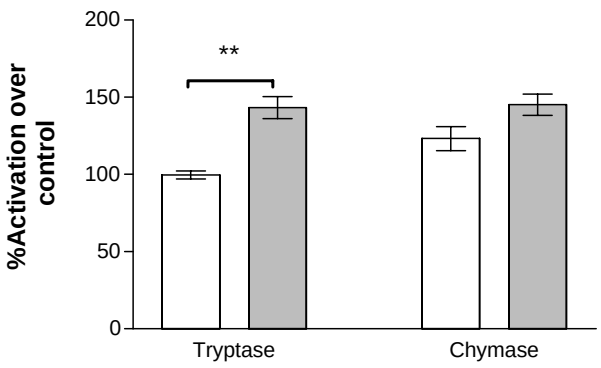

Fig. 7. Effect of Gly-Phe-CHN_2 on accumulation of proenzyme in HMC-1 cells. The proforms of tryptase and chymase present in cell lysate were activated *in vitro* with rh-DPPI. Any increment in tryptase or chymotryptic activity in 20 μM Gly-Phe-CHN_2-treated cells (solid bars) was calculated as activation over control cultured in plain IMDM5 medium (hollow bars). Values are mean ± SEM (N = 11 experiments). $^{**}P < 0.01$.

4. Discussion

In the present study, use of the mast cell-like cell line HMC-1 together with the selective DPPI inhibitor Gly-Phe-CHN_2 has provided evidence that DPPI is involved in the processing of mast cell tryptase. Culturing the cells together with this inhibitor resulted in a significant decrease, relative to controls, in tryptase activity, but not in immunodetectable tryptase. Treatment of cell lysates with rh-DPPI confirmed the presence of unprocessed precursor in inhibitor-treated cells, but not in untreated cells. The absence of observable toxic effects supports the contention that Gly-Phe-CHN_2 is selective for DPPI. These results are consistent with the two-step model of tryptase activation proposed by Sakai *et al*. [18] whereby an autocatalytic step in the presence of heparin removes the first 12 amino acids residues, after which dipeptidyl peptidase I removes the remaining dipeptide to give the mature active enzyme. However, the present evidence does not rule out an alternative model in which DPPI sequentially removes seven dipeptides from the unprocessed proenzyme to generate the mature N terminus [15,19].

The present findings suggest a scenario for HMC-1 chymotryptic activity quite different from that of tryptase. Although the amount of chymotryptic activity per cell decreased during prolonged culture with Gly-Phe-CHN_2 with the concomitant appearance of unprocessed proenzyme, as was seen with tryptase, similar results were consistently observed in the control cells. Any effect the DPPI inhibitor might be having is masked by another, as yet unidentified, consequence of prolonged culture. The dimethyl sulphoxide used to dissolve the inhibitor is unlikely to be responsible as a similar effect was seen with the control lacking any trace of this solvent.

Whether or not the chymotryptic activity may be attributable to chymase has not been conclusively resolved by the present study. We have shown by flow cytometry and Western blotting that chymase is expressed in HMC-1 cells. Cathepsin G is also expressed, but at what appears to be a much lower level. Its 20-fold lower specific activity for AAPF-*S*-Bzl [36] would be expected to reduce its contribution to the observed activity even further. In addition, the chymotryptic activity of HMC-1 is inhibited by Y-40018 and Y-40613 at concentrations that inhibit chymase but not cathepsin G, and, conversely, is not inhibited

by aprotinin which inhibits cathepsin G but not chymase. However, the very low activity of the HMC-1 chymotryptic enzyme towards the *p*-nitroanilide homologue of AAPF-*S*-Bzl is out of character with any previous isolate of chymase in this laboratory, native or recombinant. Two possibilities present themselves: either the activity is attributable to the chymase observed immunologically, or it is attributable to an as yet unidentified chymotryptic protease distinct from either chymase or cathepsin G. For the former possibility to be true, there must be something intrinsically different about chymase from this cell source, either a genetic variant [37], alternative mRNA splicing [38], or unique post-translational modifications. The expressed chymase in HMC-1 cells did have a lower than usual molecular mass, similar to that expected for the non-glycosylated peptide chain [39]. However, this aglycosylation would have had to have been highly specific, as HMC-1 tryptase was the same mass as normal (i.e. glycosylated) lung tryptase (Fig. 2C). Lack of glycosylation alone is unlikely to account for the altered specificity as rh-chymase expressed in a bacterial system was found to have a substrate specificity very similar to that of native human skin chymase.[1] A lower mass for the HMC-1 chymase might also have arisen from a nonsense mutation or alternative splicing. The alternative explanation, that a novel chymotryptic enzyme is responsible for the observed activity, would require the novel protease to be present at several hundred fold greater levels of activity to mask the activity of chymase. It would also have to have an inhibitor profile virtually identical to chymase, a degree of similarity that would be highly unusual for two different enzymes. On the present evidence, the first possibility, that of an aberrant chymase, would appear to be more plausible, but further investigation is required.

The observation that the processing of prochymase by human DPPI is inhibited by compounds abundant in the developing secretory granule combines with the above data to suggest that there might be an alternative pathway for the processing of the principal chymotryptic protease present in this cell line. In an analogous situation with neutrophil proteases expressed in the myelomonocytic cell line U937, Gly-Phe-CHN_2 was found to block cathepsin G processing, but not that of neutrophil elastase or proteinase 3 [13]. The authors concluded that another cysteinyl protease was responsible for removing the dipeptide prosequence from these two proteases. The same or similar enzyme might be acting in HMC-1 in the present study, a view supported by the observation that rh-proteinase 3 transfected and expressed in this cell line was processed correctly [40]. An alternative explanation for the differential effects of Gly-Phe-CHN_2 on tryptase and chymotryptic activation without invoking a second processing enzyme is that the residual DPPI activity even in the presence of the highest concentration of inhibitor was still sufficient to activate prochymase as it was synthesised, but not protryptase because of a significantly higher K_m for the latter. This hypothesis could be tested were recombinant protryptase available. The pharmacological consequences if this were true would be that selective DPPI inhibitors with the affinity of Gly-Phe-CHN_2 would selectively impede tryptase activation, but more potent inhibitors would block the activation of both proteolytic activities.

The inhibition by histamine and heparin of the processing of prochymase by human DPPI confirms our previous observations with the bovine enzyme [11]. Thus, the effects of these compounds cannot be attributed to artefacts arising from the use of a heterologous system. Neither can the differences observed in the effects of heparin, histamine, and pH on prochymase processing and GFpNA hydrolysis be attributed to the choice of amino acid in the P1 position as GEpNA behaved similarly to GFpNA. Indeed, GEpNA had an even more acidic pH optimum than GFpNA. If a preference for uncharged residues at P1 explains such an acidic optimum for GEpNA by DPPI, it does not explain the more neutral pH optimum for prochymase which has the same N-terminal amino acid sequence. The available evidence suggests that these factors (pH, heparin, histamine) exert their effects through the prochymase molecule as a whole.

The role of DPPI in the processing of human mast cell proteases differs from that reported for murine mast cell proteases. In the murine mast cell-like cell line KiSV-MC14, Gly-Phe-CHN_2 inhibited the processing of chymotryptic activity, but not that of tryptic activity [41]. In another murine mast cell-like cell line, P815, which lacked chymotryptic activity, treatment with Gly-Phe-CHN_2 greatly reduced tryptic activity [12]. Although these reports appear to be contradictory, mice produce two tryptases, mMCP-6 and mMCP-7, and neither report sought to identify which was present in the cell line under investigation. DPPI null mice had normal immunodetectable levels of the chymases mMCP-4 and mMCP-5, but no chymotryptic activity, either by enzyme histochemistry or in lysates of bone marrow-derived mast cells [42]. The parent strain of these knock-out mice does not express mMCP-7, but normal levels of immunoreactive mMCP-6 were detected, along with reduced levels of tryptic activity. The authors conclude that DPPI is essential for chymase processing in mice, but not for tryptase, although it may make an important contribution to its activation in normal mice. The present study indicates that what may be true for mice is not necessarily true for humans.

Although gene knock-out studies to confirm the present results with Gly-Phe-CHN_2 are not possible with humans, it has been reported that patients with Papillon–Lefevre syndrome [43,44], Haim–Munk syndrome [45], and prepubertal periodotitis [46] possess lack-of-function mutations in DPPI. Both nonsense and missense mutations have been identified. Nine missense mutations have been mapped on to the three-dimensional crystal structure of DDP I: some have

[1] A.R. McEuen, unpublished results.

been found to interfere with substrate binding, others with the proper folding of the enzyme [17]. It remains to be seen what enzyme histochemical and immunohistochemical studies on biopsy specimens from these patients would reveal about the processing of mast cell proteases. It would also be informative to know more about the patients' clinical history, e.g. whether they have reduced incidence of allergic diseases, increased susceptibility to helminthic parasites, decreased incidence of atherosclerotic plaques, etc.

Inhibitors of tryptase have proved efficacious in the treatment of asthma, both in animal models [7,8], and in human clinical trials [9]. A possible alternative to administering inhibitors of tryptase is to administer inhibitors of enzymes required for tryptase activity. The present results indicate that DPPI is required for the conversion of the inactive precursor of tryptase to the fully active mature form of this key enzyme in the pathophysiology of asthma and other allergic diseases. Further studies are required to explore and confirm the feasibility of targeting DPPI in the treatment of these diseases.

Acknowledgments

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