

Mast Cell α -Chymase Reduces IgE Recognition of Birch Pollen Profilin by Cleaving Antibody-Binding Epitopes¹

Matthew B. Mellon,* Brendon T. Frank,* and Kenneth C. Fang^{2*†}

In sensitized individuals birch pollen induces an allergic response characterized by IgE-dependent mast cell degranulation of mediators, such as α -chymase and other serine proteases. In birch and other plant pollens, a major allergen is profilin. In mammals, profilin homologues are found in an intracellular form bound to cytoskeletal or cytosolic proteins or in a secreted form that may initiate signal transduction. IgE specific to birch profilin also binds human profilin I. This cross-reactivity between airborne and endogenous proteins may help to sustain allergy symptoms. The current work demonstrates that cultured mast cells constitutively secrete profilin I, which is susceptible to degranulation-dependent proteolysis. Coincubation of chymase-rich BR mastocytoma cells with Ala-Ala-Pro-Phe-chloromethylketone (a chymase inhibitor) blocks profilin cleavage, which does not occur in degranulated HMC-1 mast cells, which are rich in tryptase, but chymase deficient. These data implicate chymase as the serine protease cleaving secreted mast cell profilin. Sequencing of chymase-cleaved profilins reveals hydrolysis at Tyr⁶-Val⁷ and Trp³⁵-Ala³⁶ in birch profilin and at Trp³²-Ala³³ in human profilin, with all sites lying within IgE-reactive epitopes. IgE immunoblotting studies with sera from birch pollen-allergic individuals demonstrate that cleavage by chymase attenuates binding of birch profilin to IgE. Thus, destruction of IgE-binding epitopes by exocytosed chymase may limit further mast cell activation by this class of common plant allergens, thereby limiting the allergic responses in sensitized individuals. *The Journal of Immunology*, 2002, 168: 290–297.

Birch pollen allergens induce IgE Abs and atopic symptoms, such as rhinitis, conjunctivitis, and asthma, via mechanisms mediated in part by degranulation of mast cells (1–4). Individuals exhibiting allergic responses to birch pollen very frequently raise IgE Abs to a specific protein, *Bet v 2*, otherwise known as profilin. These Abs tend to cross-react extensively with a broad range of other plant profilins (5). Challenge of allergic patients with birch pollen redistributes mast cells toward the nasal mucosa (2), induces mast cell chemotactic activity (6), and increases histamine and tryptase levels in nasal lavage fluid (1). However, the role of degranulated mast cell serine proteases in the allergic response to airborne profilins remains unknown. Birch profilin-specific IgE in the sera of allergic individuals also cross-react with human profilin I (a structural homologue of birch profilin) (7). Human profilin induces histamine release from basophils isolated from those with elevated IgE Ab titers. Therefore, endogenous human profilin I may not only exacerbate or prolong symptoms caused by sensitization of mast cells to pollen profilin, but also sustain serum IgE levels via a booster effect even outside the birch pollen season (8).

Intracellular profilins play roles in microfilament dynamics. The roles of secreted profilins are less clear, but data suggest that they can activate extracellular signaling pathways. Plant and mammalian profilins bind actin monomer, phosphatidylinositol-4,5-bisphosphate, poly(L-proline), formin, and vasodilator-stimulated phosphoprotein via different sites, which enables them to participate in a variety of processes, such as cytoskeletal organization and cell signaling. Two isoforms of mammalian profilins demonstrate similar binding affinities, but have distinct expression patterns and specific ligand interactions, suggesting unique functions for each isoform *in vivo*. Death of profilin I-null murine embryos at the two-cell stage and reduced survival of heterozygotes suggest a critical role for profilin I early in development (9, 10). Extracellular accumulation and overexpression of profilins may also occur in tissues surrounding glomerular mesangial cells in rats with anti-Thy-1.1-induced glomerulonephritis. Secreted profilins may initiate signal transduction and regulate cell growth, as demonstrated by studies of cultured rat mesangial cells, in which profilin increases [³H]thymidine incorporation, enhances binding of AP-1 to DNA, and activates protein kinase C (11). One pathway for secretion of profilins occurs via release of exosomes by dendritic cells (12). Thus, secretion of profilin by mesenchymal cells may regulate local cell-cell interactions and cell signaling.

A shared topology and tertiary structure is the presumed basis for extensive IgE cross-reactivity among mammalian and plant profilins despite the low degree of primary sequence homology. Whereas plant profilins are <25% identical with mammalian profilins in amino acid sequence, all are thought to adopt a similar fold consisting of anti-parallel β -sheets associated with α helices. Epitopes clustered at the NH₂- and COOH-terminal α helices and in a two-stranded segment (composed of residues 30–50) provide the basis for profilin immunogenicity and cross-reactivity. This distribution of reactive epitopes permits simultaneous binding of multiple IgE Abs necessary for receptor aggregation. Such characterization of the three epitopes explains why 20% of pollen-allergic individuals demonstrate cross-sensitization toward pollen

*Cardiovascular Research Institute and [†]Department of Medicine, University of California, San Francisco, CA 94143

Received for publication August 24, 2001. Accepted for publication October 29, 2001.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¹ This work was supported by National Institutes of Health Grants HL64897 and HL03345, a research award from the American Lung Association of California, and a research grant from the Research Evaluation and Allocation Committee of the University of California, San Francisco School of Medicine. K.C.F. is the recipient of a Mentored Clinical Scientist Development Award from the National Institutes of Health.

² Address correspondence and reprint requests to Dr. Kenneth C. Fang, Box 0911, Cardiovascular Research Institute, University of California, San Francisco, CA 94143-0911. E-mail address: kfang@itsa.ucsf.edu

profilins from distantly related trees, grasses, and weeds. Cross-reactivity of birch pollen profilin-specific IgE toward human profilin I probably results from epitopes located in the NH₂-terminal α helix and the two-stranded segment (7). In the present work we explore mast cell release of profilin and identify sites in profilin cleaved by α -chymase, a chymotryptic mast cell serine protease.

Materials and Methods

Cell culture

BR mastocytoma cells (a canine mast cell line obtained from S. Lazarus, University of California, San Francisco, CA) or HMC-1 cells (a human mast cell line obtained from J. Butterfield, Mayo Clinic, Rochester, MN) were cultured in DMEM-H16 supplemented with 2% bovine calf serum or IMEM with 10% FCS, respectively, as previously described (13). Cells were maintained at a final concentration of 1×10^6 cells/ml and incubated at 37°C in humidified 5% CO₂ and 95% air. Cells were harvested by centrifuging at $800 \times g$ for 5 min, washing three times in Ca²⁺- and Mg²⁺-free PBS, and resuspending in serum-free culture medium to a final concentration of $4\text{--}5 \times 10^6$ cells/ml.

Degranulation studies

HMC-1 or BR cells were resuspended in phenol red-free culture medium in the absence or the presence of serum to a final concentration of 2 or 8×10^6 cells/ml, respectively. Cells were incubated at 37°C for different time periods alone, with 2 μ M calcium ionophore A23187 (Sigma, St. Louis, MO), or with ionophore in combination with a protease inhibitor cocktail (Sigma), 10 mM PMSF (Sigma), or 25 μ M Ala-Ala-Pro-Phe-chloromethylketone (AAPF-CMK³; Enzyme Systems Products, Livermore, CA). Aliquots removed at specified intervals were centrifuged at $800 \times g$ to pellet cells. Cell supernatants were stored at -20°C before analysis by immunoblotting. Cytotoxicity of degranulation studies was determined by assaying the amount of lactic dehydrogenase (LDH) present in supernatants using the In Vitro Toxicology Assay Kit (Sigma) according to the manufacturer's protocol. The tryptase content of HMC-1 cells is 175 ± 15 ng/ 10^6 cells based on hydrolysis of 0.1 mM tosyl-L-Gly-Pro-Lys-p-Na in 50 mM Tris-HCl buffer (pH 7.6) containing 0.12 M NaCl and 20 μ g/ml bovine lung heparin (Sigma) at 37°C (14).

Immunoblotting

Aliquots of mast cell-conditioned medium, cell lysates or degranulation supernatants, partially purified proteins, or purified recombinant birch pollen (BIOMAY, Linz, Austria) or purified human platelet (Cytoskeleton, Denver, CO) profilins were electrophoresed onto a 12% bis-Tris gel and blotted onto Polyscreen polyvinylidene difluoride membrane (NEN Life Science Products, Boston, MA). Membranes were washed in 10 mM Tris-HCl (pH 7.5) buffer containing 150 mM NaCl and 0.3% Tween 20 and incubated with rabbit polyclonal anti-human profilin I (which cross-reacts with profilin I of other mammalian species in Cytoskeleton's quality control assays) at 22°C for 30 min. Membranes were then incubated with HRP-linked anti-rabbit Ig for 30 min and washed three times in 10 mM Tris-HCl (pH 7.5) buffer containing 150 mM NaCl and 0.3% Tween 20. Immunoreactive proteins were detected by chemiluminescence using Luminol and peroxidase reagents in the Phototope-HRP Western Blot Detection kit (New England Biolabs, Beverly, MA) according to the manufacturer's protocols and visualized on Hyperfilm ECL (Amersham Pharmacia Biotech, Piscataway, NJ).

Purification

HMC-1 cells (4.5×10^9) were harvested by centrifuging at $800 \times g$ for 5 min and washing three times in Ca²⁺- and Mg²⁺-free PBS. Cells were then incubated in 50 mM HEPES (pH 7.4) and 150 mM NaCl with 10% glycerol, 1 mM PMSF, 25 μ M leupeptin, 1.5 μ M aprotinin, and 10 mM 1,10-phenanthroline to inhibit protease activity before sonication. Following centrifugation at $36,000 \times g$ to remove cells and debris, cell lysate supernatants were stored at -20°C before use. Supernatants equilibrated to 0.5 M NaCl were loaded onto a DEAE-cellulose (Sigma) column equilibrated with 10 mM Tris-HCl buffer (pH 7.5) containing 0.5 M NaCl. Unbound proteins in flow-through fractions were diluted in 20 mM Tris-HCl buffer (pH 7.5) to a final concentration of 0.12 M NaCl and loaded onto a heparin-Sepharose CL-6B (Amersham Pharmacia Biotech) column equilibrated in

20 mM Tris-HCl buffer (pH 7.5). Aliquots of the heparin column loading flow-through were exchanged into 10 mM bis-Tris buffer (pH 6.1) containing 20% glycerol and concentrated using a Centricon Plus-20 centrifugal filter device (Millipore, Bedford, MA) with a 10 kDa *M_r* cut-off before loading onto a Mono S fine performance liquid chromatography column (Amersham Pharmacia). Proteins in flow-through fractions were concentrated as described above and loaded onto a Mono Q fine performance liquid chromatography column (Amersham Pharmacia). Unbound proteins in the flow-through were subjected to continuous elution electrophoresis using a Mini Prep Cell (Bio-Rad, Hercules, CA). Proteins were electrophoresed through a vertical 5-cm $\times$ 1-cm 14% Tris-glycine polyacrylamide cylindrical gel with migration off the bottom of the gel into a collection tube, which enabled isolation of proteins of different sizes in individual fractions. Eluted protein fractions were stored at -20°C before use.

NH₂-terminal sequencing

Purified protein was electrophoresed onto a 16% Tris-glycine polyacrylamide gel and blotted onto sequencing grade polyvinylidene difluoride (PVDF) membrane (Bio-Rad) in 3-[cyclohexylamino]-1-propanesulfonic acid buffer containing 10% methanol. To prepare peptide fragments for internal sequencing by chemical digestion at internal tryptophan residues, *in situ* cleavage of PVDF-bound proteins was performed by incubating membrane fragments in 75% acetic acid containing 1 μ g/ μ l 3-bromo-3-methyl-2-(2'-nitrophenylmercapto)-3H-indole (BNPS-skatole) at 47°C for 1 h (15). Proteins bound to dried membrane strips were eluted by incubation in 70% isopropyl alcohol containing 5% trifluoroacetic acid at 22°C for 16 h. Peptide fragments from BNPS-skatole digestion were electrophoresed onto a 16% Tris-glycine polyacrylamide gel and blotted onto sequencing grade PVDF membrane as described above. Cleavage products resulting from α -chymase hydrolysis were electrophoresed onto a 12% bis-Tris polyacrylamide gel and blotted onto sequencing grade PVDF membrane in 25 mM bicine buffer (pH 7.2) containing 25 mM bis-Tris, 1.0 mM EDTA, 0.05 mM chlorobutanol, and 15% methanol. Protein bands identified by Coomassie blue staining of the membrane were excised and subjected to Edman degradation for determination of amino acid sequence by Midwest Analytical (St. Louis, MO), using a model 477A protein sequencer (Applied Biosystems, Foster City, CA). Protein sequence alignments were performed using MacVector software (Oxford Molecular Group, Hunt Valley, MD).

Substrate cleavage

Purified human platelet profilin I (Cytoskeleton) or recombinant birch pollen profilin (BIOMAY) was incubated with various concentrations of purified recombinant human α -chymase (16) (gift from G. Caughey) or purified recombinant human β -tryptase (Promega, Madison, WI) at 37°C for 18 h. Reaction products were electrophoresed onto 12% bis-Tris polyacrylamide gels and subjected to staining with Coomassie blue or immunoblotting.

IgE immunoblotting

Detection of binding of birch pollen profilin-specific IgE to reactive epitopes in PVDF membrane-bound recombinant birch profilin was performed as previously described (5, 17–19). Briefly, native or α -chymase-cleaved purified recombinant birch profilin (BIOMAY) was electrophoresed onto 12% bis-Tris polyacrylamide gels and transferred to PVDF as described above. Ninety percent of protein in either reaction was allocated for immobilization on PVDF membranes for immunoblotting, while 10% was reserved for SDS-PAGE with detection of proteins by Coomassie blue staining. Membranes were blocked by incubation in 5% nonfat dry milk in PBS at 37°C for 1 h, washed in 10 mM Tris-HCl (pH 7.5) buffer containing 150 mM NaCl (TBS), and incubated in human serum (diluted 1/1 in PBS) from either individuals allergic to birch pollen (National Institute for Biological Standards and Control, Potters Bar, U.K.) or nonallergic individuals (Sigma) at 4°C for 16 h. After washing three times in TBS, detection of bound IgE was performed by incubating membranes with HRP-linked mouse anti-human IgE (Zymed, South San Francisco, CA) in TBS containing 0.3% Tween 20 at 22°C for 30 min, followed by chemiluminescent detection as described above. To determine the sensitivity of detection of native birch profilin, various dilutions of protein were analyzed by IgE immunoblotting with the establishment of a lower limit of detection threshold of approximately 200 ng protein/lane.

³ Abbreviations used in this paper: AAPF-CMK, Ala-Ala-Pro-Phe-chloromethylketone; BNPS-skatole, 3-bromo-3-methyl-2-(2'-nitrophenylmercapto)-3H-indole; LDH, lactic dehydrogenase; PVDF, polyvinylidene difluoride.

Results

Mast cells secrete profilin

Accumulation of profilin in the extracellular matrix and its secretion by stromal cells (11, 12) suggested the likelihood that mast cells (which are noncirculating inflammatory cells resident in tissues) may also secrete profilin. Our prior work has established that cultured HMC-1 cells and BR mastocytoma cells phenotypically resemble human mast cells in their complement of stored and secreted mediators (13, 16, 20–22). To determine whether mast cells may release endogenous profilins, cells were cultured in medium without serum for various periods. As shown in Fig. 1, unconcentrated medium conditioned by HMC-1 or BR mastocytoma cells incubated for 5 min contains an approximately 15-kDa band immunoreactive for profilin, suggesting constitutive release of the protein. Incubation of cells with calcium ionophore A23187 increases the intensity of the immunoreactive band present in HMC-1 or BR supernatants within 5 min, suggesting that rapid degranulation induces release of profilin. Since levels of LDH released by cells incubated in the presence of ionophore were similar to those from cells incubated alone, differences in cell cytotoxicity do not account for increased amounts of profilin in degranulation supernatants. Profilin-immunoreactive bands were absent in serum-containing cell culture medium (data not shown). Thus, neither cell lysis nor contamination by serum proteins explains the presence of profilin in mast cell supernatants. These data suggest that mast cells, like other stromal cells such as dendritic (12) or mesangial cells (11), may release profilins into the extracellular milieu.

Profilin purification

Identification of protein immunoreactive for profilin I in medium conditioned by cultured mast cells suggested both constitutive and degranulation-induced release of stored protein. Although bound to cytoskeletal and signaling proteins, profilin is also secreted in uncomplexed form by stromal cells (12). Immunoreactive protein present in membrane-free HMC-1 cell lysates was purified to isolate the unbound form and to establish its identity as profilin. Potential interference of abundant proteoglycan stored in secretory granules required similar chromatographic approaches previously used successfully in the purification of other mast cell proteins in our laboratory (20, 23). As shown in Fig. 2, purification required sequential anion and cation exchange chromatography to remove contaminants. While some immunoreactive protein bound to each

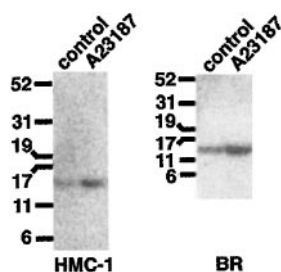


FIGURE 1. Effects of degranulation on mast cell profilin secretion. HMC-1 or BR mastocytoma cells were incubated at a concentration of 2 or 8×10^6 cells/ml, respectively, with protease inhibitors. Cells were incubated without (control) or with $2 \mu\text{M}$ calcium ionophore A23187 at 37°C for 5 min. Cell-free supernatants were subjected to SDS-PAGE on 12% gels under reducing conditions and transferred to PVDF membrane with detection of immunoreactive bands using polyclonal anti-human profilin I Ab. Note the increase in signal intensity in degranulation supernatants vs that in controls. Data shown are representative of experiments performed in triplicate.

of the chromatographic columns (data not shown), the majority of the immunoreactive protein flowed through each column. Immunoblot analysis identified profilin in fractions from each of the chromatographic steps, as shown in Fig. 2A. Coomassie staining of fractions collected during continuous elution electrophoresis identified fractions containing profilin-immunoreactive protein free of contaminants, with an electrophoretic profile identical with that of purified human profilin I and the immunoreactive band present in serum-free HMC-1-conditioned medium, as shown in Fig. 2B.

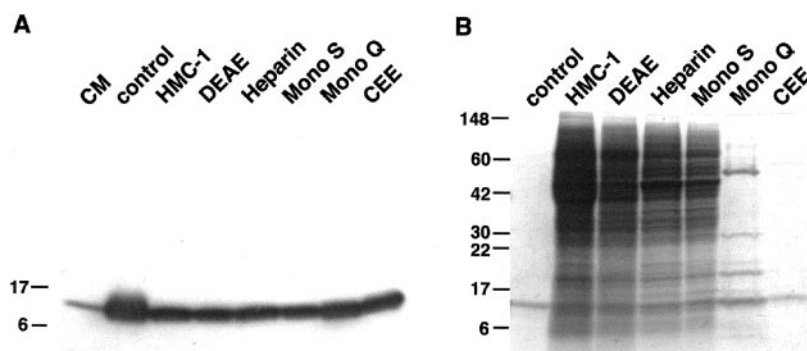
Protein sequencing

To establish the identity of the immunoreactive band, aliquots of protein purified from HMC-1 cell lysates were subjected to Edman degradation, which revealed a blocked NH_2 -terminal residue. To obtain internal sequence data, purified protein was digested with BNPS-skatole, which cleaves at tryptophan residues (15). Edman degradation of protein fragments resulting from digestion with BNPS-skatole yielded two overlapping sequences, AAVPG and AAVPGKTFVN, and an additional sequence, XAYID, as shown in Fig. 3. No residue was assigned to cycle 1 of the last sequence due to insufficient discrimination of chromatographic peak amplitudes from the subsequent cycle. Analysis of these fragments by searching BLAST databases suggested the identity of the protein as human profilin I. Alignment of the first two sequences with the primary sequence of human profilin I identifies cleavage at Trp^{32} , while alignment of the third sequence identifies additional hydrolysis at Trp^4 . Thus, these data establish the identity of the purified mast cell protein as profilin I. The presence of a blocked NH_2 -terminal residue in purified native protein is consistent with prior work demonstrating blockage of the NH_2 -terminus of both mammalian and amoeba profilins (24). Thus, the purification scheme enables successful isolation of uncomplexed profilin I from mast cells whose complement of cytosolic and granular proteins, including proteoglycans, often requires a succession of chromatographic steps to isolate protein from extracts (20–23).

Degranulation-induced profilin cleavage

Mast cell degranulation during allergy and inflammatory responses releases a variety of mediators, including tryptase and α -chymase, which are tryptic and chymotryptic serine proteases, respectively. Since proteolysis may affect the stability of allergens (25), we explored the effect of degranulation on mast cell expression of profilin. Degranulation altered the electrophoretic profile of immunoreactive bands in supernatants of BR mastocytoma cells incubated in the presence of calcium ionophore A23187. In addition to revealing the 15-kDa native profilin band, immunoblotting of degranulation supernatants detected a band at about 10 kDa whose intensity increases with prolonged incubation, as shown in Fig. 4A. Similar degranulation experiments performed with HMC-1 cells do not yield the lower band (data not shown), suggesting that their lack of α -chymase expression (26) may explain the absence of profilin processing. The lack of the approximately 10-kDa band in the supernatants of cells incubated alone, even after 4 h, further supports a requirement for degranulation for its appearance. To identify the protease(s) involved in degranulation-associated profilin processing, cells were incubated with calcium ionophore alone or in combination with PMSF, a general serine protease inhibitor, or AAPF-CMK, a specific inhibitor of α -chymase. Coincubation of cells with calcium ionophore and either PMSF or AAPF-CMK blocks the appearance of the approximately 10-kDa band. By contrast, coincubation in the presence of E64 or 1,10-phenanthroline did not block its appearance, suggesting the lack of hydrolysis by cysteine or metalloproteinases, respectively (data not

FIGURE 2. Profilin purification. Aliquots from HMC-1-conditioned medium (CM), purified human platelet profilin (control), crude HMC-1 cell lysate supernatants (HMC-1), or aliquots from sequential chromatographic purification steps were subjected to either immunoblotting with antisera to profilin (A) or SDS-PAGE on 16% gels under reducing conditions stained with Coomassie blue (B). Note the isolation of a single protein in aliquots obtained by continuous elution electrophoresis (CEE).



shown). As shown in Fig. 4B, degranulation-induced chymase-dependent cleavage of profilin also occurs in the presence of serum proteins. Although abundant in mast cells (27), tryptase appears to have a minimal effect on profilin processing, as demonstrated by the minor cleavage of birch profilin by tryptase compared with that by α -chymase, as shown in Fig. 4C, by the ability of AAPF-CMK (which inhibits α -chymase, but not tryptase) alone to block the appearance of the lower band, and by the inability of bis(5-amidino-2-benzimidazolyl) methane (a specific inhibitor of tryptase) to block the appearance of the approximately 10-kDa band (data not shown). No significant differences in LDH release were observed between cells incubated alone or in the presence of calcium ionophore (without or with inhibitors), suggesting no increase in cytotoxicity in degranulating cells. Thus, these data identify α -chymase as the serine protease responsible for cleavage of mast cell profilin during degranulation.

α -Chymase cleaves profilin

To determine whether mast cell α -chymase cleaves profilins generally, we characterized the electrophoretic profile of birch pollen profilin, human profilin I, or purified mast cell profilin following incubation with purified human α -chymase. As shown in Fig. 5A, incubation of α -chymase with birch profilin yields three bands at approximately 10, 6, and 3 kDa. By contrast, cleavage of human profilin I results in the appearance of two bands at approximately 10 and 6 kDa, with a similar electrophoretic profile identified for cleaved mast cell profilin. Incubation of α -chymase with birch profilin at a molar ratio of 1:20,000 yields a faint 10-kDa product, with generation of the 6- and 3-kDa products with increasing protease concentration. By contrast, similar molar ratios of α -chymase cleave human profilin to a lesser degree, as shown in Fig. 5B. NH_2 -terminal sequencing of the 10-kDa birch profilin cleavage product (band a) yields two sequences (VDEHL and AQSSX₁F). Similar analysis of the 6-kDa band yields the same pair of sequences, suggesting truncation due to cleavage in the COOH-terminal domain. Edman degradation of the 3-kDa band (band c)

yields two sequences (MVIQG and AQSSX₁F) in approximately a 4:1 molar ratio. As shown in Fig. 5C, alignment of these sequences with the primary sequence of birch pollen profilin (7) reveals that α -chymase cleaves the Tyr⁶-Val⁷, Trp³⁵-Ala³⁶, and Tyr⁷⁴-Met⁷⁵ bonds. NH_2 -terminal sequencing of the 10- and 6-kDa cleavage products (bands d and e, respectively) of human profilin I yields two sequences (AAVPGKX₂FV and VNGLT) whose alignment to the primary sequence (28) identifies Trp³²-Ala³³ and Tyr⁵⁹-Val⁶⁰ as the bonds cleaved by α -chymase. No residues were assigned to position X₁ or X₂ due to insufficient discrimination of chromatographic peak amplitudes in successive cycles. The P1 residues of each of the scissile bonds is aromatic (Tyr or Trp), as preferred by chymotryptic enzymes (29). Whereas the Tyr⁶-Val⁷ bond is situated in the NH_2 -terminal α helix of birch profilin, the Trp³⁵-Ala³⁶ or Trp³²-Ala³³ bonds are located in the two-stranded segments (approximately residues 30–50) of birch or human profilin, respectively. These scissile bonds cleaved by α -chymase are situated in regions identified to be IgE-reactive epitopes and localized to the surface of profilins, as shown in Fig. 6B. By contrast, cleavage at the Tyr⁷⁴-Met⁷⁵ or Tyr⁵⁹-Val⁶⁰ bonds of birch or human profilin, respectively, occurs in a nonepitopic β strand (based on a structure-based sequence alignment), which is also exposed at the surface (data not shown) (7).

IgE immunoblot

Immunoblotting of pollen extracts or purified allergen proteins using sera from sensitized individuals enables detection of allergen-specific IgE Ab bound to reactive epitopes. The use of control serum from nonallergic individuals establishes the specificity of the binding of IgE present in allergic serum, while preadsorption of allergic sera with purified allergens in IgE inhibition experiments may identify cross-sensitization of individuals to allergens in unrelated trees, weeds, or grasses (5, 17–19). To determine the effect of α -chymase hydrolysis of birch profilin on binding of allergen-specific IgE, immunoblotting of native or α -chymase-cleaved birch profilin was performed using serum from individuals allergic

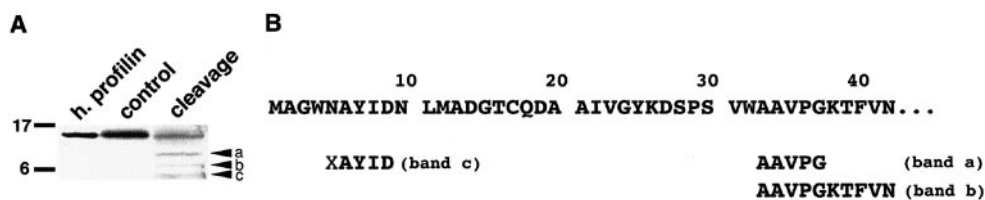


FIGURE 3. Internal NH_2 -terminal amino acid analysis. A, Aliquots of purified HMC-1 protein were incubated in 75% acetic acid alone (control) or BNPS-skatole (cleavage) to obtain peptide fragments for internal sequencing (cleavage). Samples were analyzed by SDS-PAGE under reducing conditions with proteins detected by Coomassie blue. BNPS-skatole digestion yields three products at approximately 11, 7, and 4 kDa (bands a, b, and c, respectively). B, After transfer to PVDF membrane, proteins identified by Coomassie staining were excised and subjected to automated peptide sequencing. Sequences were aligned to the amino-terminal portion of the human profilin I amino acid sequence (GenBank P07737).

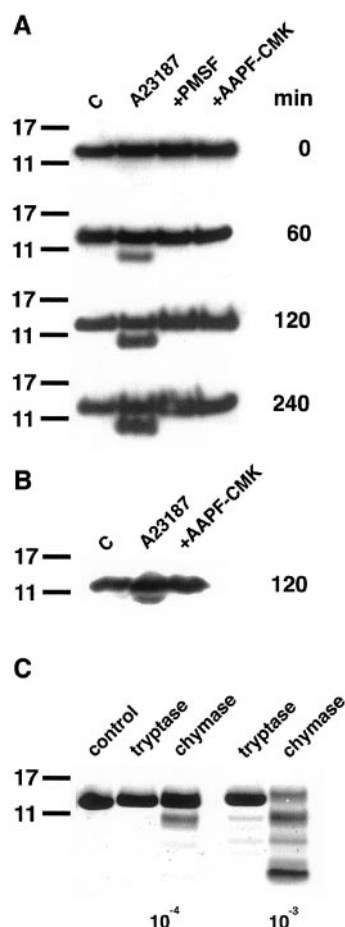


FIGURE 4. Mast cell α -chymase cleaves profilin. **A**, Degranulation supernatants. To identify the specific mast cell serine protease involved in degranulation-dependent profilin processing, BR cells were incubated alone (C) or with 2 μ M calcium ionophore A23187 alone (A23187) or in combination with either 10 mM PMSF or 25 μ M AAPF-CMK at 37°C for the indicated time periods. Proteins in cell-free supernatants were subjected to SDS-PAGE on 12% polyacrylamide gels under reducing conditions and transferred to PVDF membrane with detection of immunoreactive bands using polyclonal anti-profilin Ab. Note the increase in signal intensity of the profilin cleavage product with time and the lack of cleavage products in the absence of degranulation with ionophore or coincubation of degranulation supernatants with PMSF or AAPF-CMK, a specific inhibitor of α -chymase. Similar experiments performed with HMC-1 cells do not yield profilin cleavage products due to their lack of α -chymase production (26) (data not shown). Data shown are representative of experiments performed in triplicate. **B**, Degranulation in the presence of serum. Experiments with BR cells incubated in the presence of serum-containing culture medium demonstrate results similar to those obtained from cells incubated in serum-free medium. Data shown are representative of experiments performed in triplicate. **C**, Mast cell serine protease cleavage of profilin. Purified recombinant human trypsin monomer (which exists in tetrameric form in solution) or purified human α -chymase was incubated with purified recombinant human profilin I at the indicated molar ratio at 37°C for 16 h. Reaction products were subjected to SDS-PAGE on 12% bis-Tris polyacrylamide gels under reducing conditions and stained with Coomassie blue. Note the more extensive cleavage of profilin by α -chymase at either ratio relative to trypsin. (Differences in the cleavage product electrophoretic profile visualized between the degranulation supernatants and the purified human profilin reflect are probably due to detection sensitivity or extent of cleavage.)

to birch pollen. As shown in Fig. 7A, incubation of α -chymase with birch profilin in a 1:2000 molar ratio yields three cleavage products detectable by Coomassie blue staining. Immunoblotting

using allergic serum identified an approximately 14-kDa immunoreactive band in the lane containing uncleaved, native birch profilin, as shown in Fig. 7B. By contrast, no immunoreactive bands were seen in the lane containing α -chymase-cleaved profilin. In addition, no immunoreactive bands were detected in either lane containing native or cleaved profilin in immunoblots incubated with control human serum. To ensure that immunoblotting was performed under conditions of an excess of membrane-bound allergen protein, native and α -chymase-cleaved profilin were apportioned to SDS-PAGE or immunoblotting unevenly, with the bulk of protein in each reaction allocated for immobilization to membrane. Moreover, quantities of Coomassie blue-detectable cleavage products allocated for immunoblotting far exceeded the immunoblot detection sensitivity of the native profilin protein. Thus, the lack of sufficient quantities of α -chymase-cleaved profilin products does not explain the absence of immunoreactive bands in the lane containing cleavage products. Therefore, these data suggest that cleavage of birch profilin by α -chymase attenuates binding of allergen-specific IgE.

Discussion

Profilin proteins regulate mammalian cell integrity, signaling, and survival, yet also threaten atopic individuals as airborne allergens when released in pollens from a broad variety of plants and trees. Despite low primary sequence identity, all profilins share a common tertiary structure that enables cross-reactivity of profilin-specific IgE not only to unrelated plant pollen profilins, but also to human profilin. Recognition of shared epitopes on human profilin I by birch profilin-specific IgE represents the first instance of an autoreactive IgE Ab that may prolong or exacerbate allergic pollinosis despite the seasonal absence of environmental allergen (8). Although previously considered restricted to the intracellular milieu in terms of expression and function, mammalian profilins also accumulate in the extracellular matrix, bind to cell surface receptors, and induce cytosolic signaling. Therefore, stromal cells may be potential sources of secreted profilins (11, 12). Mast cells are inflammatory cells residing in the connective tissue of the lungs, skin, and gut, which are organs interfacing directly with the environment and potential allergens. Activated mast cells degranulate and release a variety of newly formed or stored mediators, such as histamine, heparin, and proteases, which may exacerbate or attenuate allergic or inflammatory responses (4). The current work demonstrates that degranulating mast cells secrete profilin and describes a mechanism by which concomitant release of α -chymase, a chymotryptic serine protease, may preferentially cleave birch pollen profilin, thus altering its surface display of IgE-reactive epitopes.

Degranulation of mast cells induces the release of profilin and synchronous proteolysis of the secreted protein. Cultured mast cells such as HMC-1 or BR mastocytoma cells secrete profilin constitutively, yet increase their release of profilin upon degranulation with calcium ionophore. Prolonged degranulation results in profilin cleavage, which is blocked by specific inhibitors of α -chymase (a chymotryptic mast cell serine protease), thus identifying it as the protease responsible for extracellular cleavage of secreted profilin. The absence of similar profiles in supernatants of α -chymase-deficient HMC-1 cells (26) substantiates the observed mechanism of extracellular profilin processing. These data suggest that quiescent or activated mast cells may contribute and process profilins in normal or inflamed tissues. Immunohistochemical analysis of nasal mucosa biopsies from subjects with birch pollen allergy show a seasonal diminution in the quantity of detectable α -chymase-expressing mast cells, providing clinical evidence that asynchronous secretion (3) or selective degranulation of α -chymase

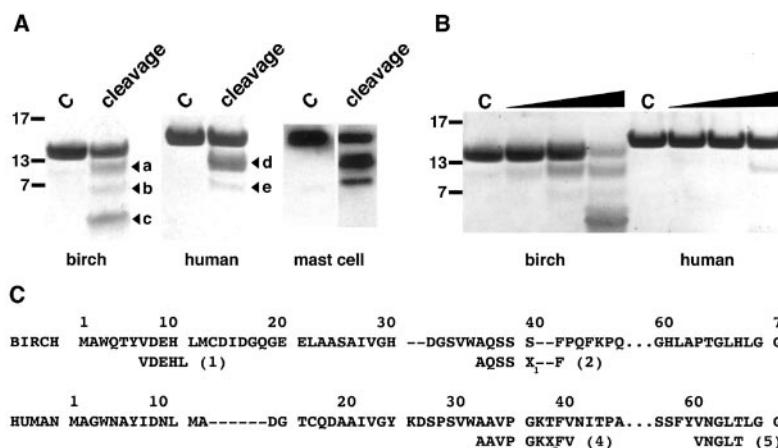


FIGURE 5. Sites of α -chymase cleavage in profilin. **A**, α -Chymase cleaves profilins. Purified human α -chymase and recombinant birch pollen profilin (14 kDa), purified human profilin I (15 kDa), or purified mast cell profilin were incubated alone or together at a molar ratio of 1:40 at 37°C for 30 min. Samples were analyzed by SDS-PAGE under reducing conditions, with birch or human proteins detected by Coomassie blue and mast cell profilin detected by immunoblotting with polyclonal anti-human profilin I Ab. Cleavage generates multiple birch profilin products at approximately 10, 6, and 3 kDa (bands a, b and c, respectively) not seen in the preparations of either α -chymase or purified profilins. By contrast, α -chymase hydrolysis of human profilin yields only 10- and 6-kDa products (bands d and e, respectively). **B**, Preferential cleavage of birch profilin. Purified human α -chymase and recombinant birch pollen profilin or purified human profilin I were incubated alone or together at increasing molar ratios (1:20,000, 1:2,000, or 1:200) at 37°C for 18 h. Samples were analyzed by SDS-PAGE under reducing conditions with proteins detected by Coomassie blue. **C**, Identification of scissile bonds cleaved by human α -chymase. α -Chymase-cleaved birch or human profilins were transferred to PVDF membrane, identified by Coomassie blue staining, and excised for automated peptide sequencing. Sequences were aligned to the primary sequence of birch pollen or human profilin I (GenBank accession no. 1CQA or PO7737), respectively. Birch and human sequences were aligned based on structure-based alignment, showing amino acids occupying equivalent positions after superimposition of refined structure models. Dashes represent amino acids absent in one protein, but present in the other (7). Sequences 1 and 2 identify bands a and b as doublets resulting from two cleavages in birch profilin at scissile bonds containing Tyr⁶ and Trp³⁵ as P1 residues; sequence 3 identifies cleavage at Tyr⁷⁴ in band c (which is also a doublet due to additional hydrolysis at Trp³⁵). Sequence 4 of band d identifies Trp³², and sequence 5 of band e reveals Tyr⁵⁹ as the P1 residues in human profilin I.

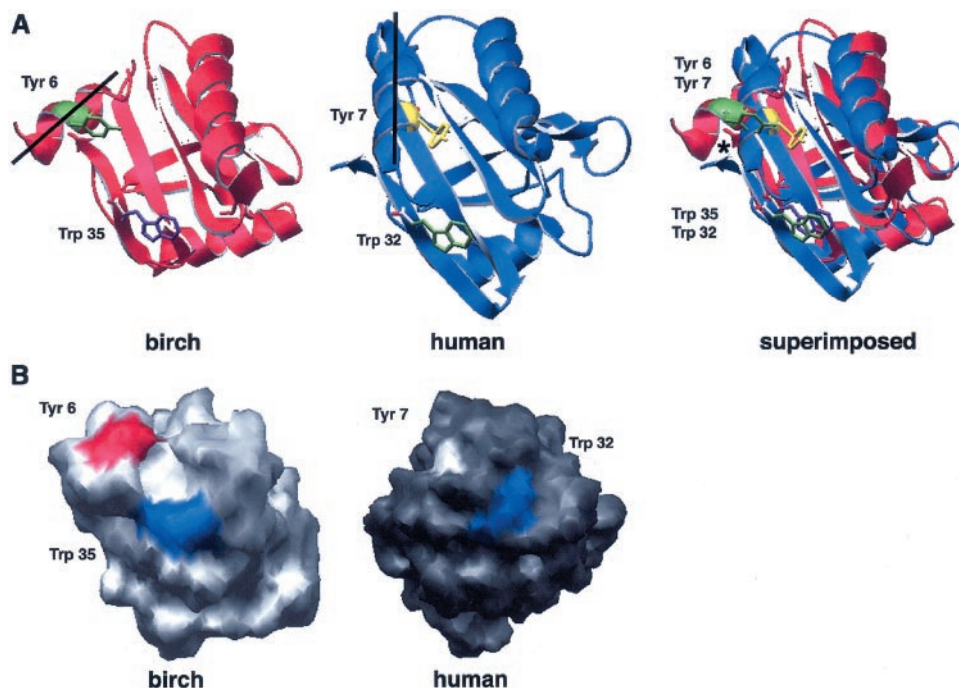


FIGURE 6. Localization of aromatic P1 residues targeted by α -chymase. **A**, Ribbon diagram of birch (red) or human (blue) profilin showing locations of scissile bonds in epitopic regions cleaved by α -chymase. Whereas Tyr⁶ is located in the NH₂-terminal α helix, Trp³⁵ or Trp³² (which occupy equivalent positions based upon superimposition of refined models) are located in a two-stranded segment (residues 30–50); both of these regions contain IgE binding sites (7). Note the orientation of NH₂-terminal α helix relative to the central β sheet and COOH-terminal helix, as shown by black lines. Superimposition of structures illustrates relative displacement of birch profilin Tyr⁶ (green), as shown in the region indicated by the asterisk. Human α -chymase also cleaves birch and human profilin at Tyr⁷⁴ and Tyr⁵⁹, respectively, in a non-epitopic β strand (data not shown). **B**, Surface localization of aromatic P1 residues. Scissile bonds containing Trp (blue) residues at the P1 position are exposed on the surface of birch pollen (light gray) or human (dark gray) profilins. Cleavage by α -chymase occurs at birch Tyr⁶ (red), but not at human Tyr⁷ (white), despite similar surface localization. Tyr⁷⁴ and Tyr⁵⁹ in birch and human profilin, respectively, are also exposed on the surface (data not shown). Figures were drawn and rendered with SwissPdbViewer (version 3.5) based on crystal structures of birch pollen profilin or human profilin I (birch, MMDB Id: 5186 PDB Id: 1CQA; human, MMDB Id: 5748 PDB Id: 1FIK) and profilin structural alignments (7).

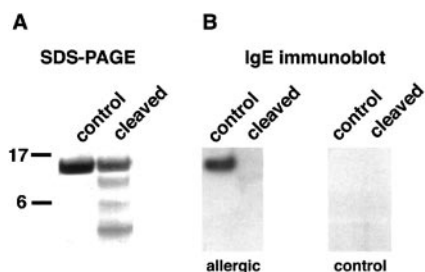


FIGURE 7. Effect of α -chymase on binding of birch profilin-specific IgE. To detect birch profilin-specific IgE bound to reactive epitopes, purified recombinant birch profilin (15 μ g) was incubated alone or in the presence of a 1/2000 molar ratio of purified human α -chymase at 37°C for 16 h. Ten percent of the reaction products was subjected to SDS-PAGE on 16% gels under denaturing conditions and staining with Coomassie blue (A), while 90% was subjected to SDS-PAGE and transferred to PVDF membrane for IgE immunoblotting with sera from birch pollen-allergic or nonallergic (control) individuals (B). Note the detection of IgE bound to native birch profilin, while none binds to cleaved birch profilin. No bound IgE is detected when native or cleaved birch profilin is incubated with control human sera. Data shown are representative of experiments performed in triplicate.

may occur, relative to tryptase. Reductions in α -chymase-positive cells (without changes in the numbers of tryptase-positive cells) also occur in patients with seasonal rhinitis following challenge with grass pollen, further supporting the preferential release of α -chymase as part of the allergic response (30). The release of profilin via secretion of membrane vesicles known as exosomes may be a mechanism that enables mast cells to release profilin. In addition to binding to intracellular cytoskeletal proteins or signaling factors, profilin is present in exosomes secreted constitutively by murine dendritic cells (12). Evidence of similar secretion of exosomes by cultured mast cell lines P815 and MC/9 and IL-4-treated murine bone marrow-derived mast cells (31) suggests that profilin release from HMC-1 or BR cells may also occur via exosomes. Therefore, stromal mast cells may contribute profilins to the extracellular milieu, while subpopulations of allergen-sensitized mast cells may degranulate α -chymase, which may result in cleavage of stromal profilin.

Similarities in the amino acid sequences of IgE-reactive epitopes hydrolyzed by α -chymase suggest that its differential cleavage of birch pollen profilin and human profilin I may be explained by a difference in their otherwise highly homologous tertiary structure. Since both profilins undergo cleavage in a surface-accessible β strand, increased susceptibility of birch profilin to α -chymase cleavage probably results from the accessibility of both the Tyr⁶-Val⁷ and Trp³⁵-Ala³⁶ scissile bonds compared with Trp³²-Ala³³ alone in human profilin I. Although the human protein contains the homologous Tyr⁷-Ile⁸ scissile bond, peptide sequencing of α -chymase-cleaved products did not yield evidence of such hydrolysis. Substitution of Ile for Val in the human sequence in the P1' position is not expected to alter the preference of α -chymase for the P1 Tyr residue (29). Moreover, like birch profilin Tyr⁶, human profilin Tyr⁷ localizes to the molecular surface. Thus, neither the primary sequences nor surface localization explain the preferential cleavage of birch profilin by α -chymase. By contrast, selective cleavage of the Tyr⁶-Val⁷ bond of birch profilin may occur due to its location in the NH₂-terminal IgE-reactive region. Crystal structure analysis of the birch protein reveals displacement and increased flexibility of the loop connecting the NH₂-terminal α helix to the first β strand, compared with that present in mammalian or *acanthamoeba* profilins. The different orientation of the

NH₂-terminal α helix disrupts a solvent-accessible hydrophobic patch (which includes the Tyr and Trp residues of the scissile bonds) and leads to an open configuration, as shown in Fig. 6A (7). The absence of a similar displacement in human profilin I suggests that the homologous Tyr⁷-Ile⁸ bond is sterically unavailable to α -chymase hydrolysis. Thus, displacement of the NH₂-terminal α helix in birch profilin may favor α -chymase cleavage of either or both the Tyr⁶-Val⁷ and Trp³⁵-Ala³⁶ bonds.

Susceptibility to proteases may be an important determinant of allergenicity, since hydrolysis may alter the physical and immunologic properties of airborne proteins. Cleavage of domains containing IgE-reactive epitopes in birch profilin may affect not only its immunogenicity, but also IgE-dependent regulation of mast cell effector functions during the allergic response. The capacity of proteins to induce IgE production, bind IgE Ab, and trigger allergic symptoms in sensitized individuals depends on properties such as affinity, epitope valence, fold compactness, stability, proteolysis, solubility, and size (25). Three prominent birch pollen profilin IgE-reactive epitopes map to the NH₂- and COOH-terminal regions and an interposed two-strand region in a tertiary structure highly conserved among plant and mammalian profilins despite low primary sequence identity. The NH₂-terminal α -helical region and the two-stranded segment contain the two epitopes probably responsible for the cross-reactivity exhibited by serum IgE of birch pollen-allergic individuals to endogenous human profilin I (7, 32). Hydrolysis by mast cell α -chymase occurs in these two regions and attenuates the ability of IgE in the serum of allergic individuals to bind to cleaved birch profilin. By contrast, the IgE-reactive epitope in the NH₂-terminal α -helical region of the human protein appears to be sterically unavailable to α -chymase, although cleavage does occur in its two-strand epitope region. Whether hydrolysis also occurs in the third, COOH-terminal epitope (7) is unclear, since neither sequencing nor electrophoresis reveals evidence of such cleavage. α -Chymase hydrolysis of two epitopic areas in birch profilin may not only alter the critical properties of the native allergen, but may also expose previously unavailable molecular surfaces containing new potential epitopes. Decreased binding of IgE may also influence the activation of effector cells such as mast cells. Binding of multiple IgE Abs to polyvalent allergens such as birch profilin (7) induces clustering of cell surface high IgE affinity Fc ϵ RI receptors and the subsequent activation and degranulation of mast cells. Cleavage-induced attenuation of IgE binding to allergen may not only diminish mast cell activation, but may also diminish the sensitivity and the magnitude of the effector cell response, since IgE up-regulates the surface expression of Fc ϵ RI receptors via a positive feedback mechanism. Therefore, attenuation of binding of IgE to allergens such as birch profilin may regulate mast cell participation in acute, late phase, and chronic allergic responses or disrupt interactions with other leukocytes and cytokines via putative cascades that may promote or propagate the inflammatory response (33).

In summary, our data demonstrate that degranulation enhances the secretion of profilin from cultured mast cells, suggesting that activated mast cells responding to allergens may contribute endogenous profilins, perhaps via the release of exosomes. α -Chymase released by degranulating mast cells cleaves birch pollen profilin at two scissile bonds containing aromatic residues located in regions containing IgE-reactive epitopes. Hydrolysis in these regions attenuates binding of IgE, suggesting that the release of α -chymase may not only attenuate the allergenicity of airborne profilins, but also alter the temporal response of mast cells in allergic inflammation.

Acknowledgments

We thank George Caughey, Wilfred W. Raymond, and David McCourt for helpful discussions, and acknowledge the excellent technical assistance of Anthony C. Cruz and Alyn Kim.

References

- Jacobi, H. H., P. S. Skov, L. K. Poulsen, H. J. Malling, and N. Mygind. 1999. Histamine and tryptase in nasal lavage fluid after allergen challenge: effect of 1 week of pretreatment with intranasal azelastine or systemic cetirizine. *J. Allergy Clin. Immunol.* 103:768.
- Enerback, L., G. Karlsson, and U. Pipkorn. 1989. Nasal mast cell response to natural allergen exposure. *Int Arch Allergy Appl Immunol.* 88:209.
- Juliusson, S., F. Aldenborg, and L. Enerback. 1995. Proteinase content of mast cells of nasal mucosa; effects of natural allergen exposure and of local corticosteroid treatment. *Allergy* 50:15.
- Schulman, E. S. 1993. The role of mast cells in inflammatory responses in the lung. *Crit. Rev. Immunol.* 13:35.
- Niederberger, V., G. Pauli, H. Gronlund, R. Froschl, H. Rumpold, D. Kraft, R. Valenta, and S. Spitzauer. 1998. Recombinant birch pollen allergens (*rBet v 1* and *rBet v 2*) contain most of the IgE epitopes present in birch, alder, hornbeam, hazel, and oak pollen: a quantitative IgE inhibition study with sera from different populations. *J. Allergy Clin. Immunol.* 102:579.
- Nilsson, G., M. Hjertson, M. Andersson, L. Greiff, C. Svensson, K. Nilsson, and A. Siegbahn. 1998. Demonstration of mast-cell chemotactic activity in nasal lavage fluid: characterization of one chemotaxin as *c-kit* ligand, stem cell factor. *Allergy* 53:874.
- Fedorov, A. A., T. Ball, N. M. Mahoney, R. Valenta, and S. C. Almo. 1997. The molecular basis for allergen cross-reactivity: crystal structure and IgE-epitope mapping of birch pollen profilin. *Structure* 5:33.
- Valenta, R., M. Duchene, K. Pettenburger, C. Sillaber, P. Valent, P. Bettelheim, M. Breitenbach, H. Rumpold, D. Kraft, and O. Scheiner. 1991. Identification of profilin as a novel pollen allergen; IgE autoreactivity in sensitized individuals. *Science* 253:557.
- Gieselmann, R., D. J. Kwiatkowski, P. A. Janmey, and W. Witke. 1995. Distinct biochemical characteristics of the two human profilin isoforms. *Eur. J. Biochem.* 229:621.
- Witke, W., J. D. Sutherland, A. Sharpe, M. Arai, and D. J. Kwiatkowski. 2001. Profilin I is essential for cell survival and cell division in early mouse development. *Proc. Natl. Acad. Sci. USA* 98:3832.
- Tamura, M., N. Yanagihara, H. Tanaka, A. Osajima, T. Hirano, K. Higashi, K. M. Yamada, Y. Nakashima, and H. Hirano. 2000. Activation of DNA synthesis and AP-1 by profilin, an actin-binding protein, via binding to a cell surface receptor in cultured rat mesangial cells. *J. Am. Soc. Nephrol.* 11:1620.
- Thery, C., M. Boussac, P. Veron, P. Ricciardi-Castagnoli, G. Raposo, J. Garin, and S. Amigorena. 2001. Proteomic analysis of dendritic cell-derived exosomes: a secreted subcellular compartment distinct from apoptotic vesicles. *J. Immunol.* 166:7309.
- Frank, B. T., J. C. Rossall, G. H. Caughey, and K. C. Fang. 2001. Mast cell tissue inhibitor of metalloproteinase-1 is cleaved and inactivated extracellularly by α -chymase. *J. Immunol.* 166:2783.
- Schwartz, L. B., and T. R. Bradford. 1986. Regulation of tryptase from human lung mast cells by heparin: stabilization of the active tetramer. *J. Biol. Chem.* 261:7372.
- Crimmins, D. L., D. W. McCourt, R. S. Thoma, M. G. Scott, K. Macke, and B. D. Schwartz. 1990. In situ chemical cleavage of proteins immobilized to glass-fiber and polyvinylidene difluoride membranes: cleavage at tryptophan residues with 2-(2'-nitrophenylsulfenyl)-3-methyl-3'-bromoindolenine to obtain internal amino acid sequence. *Anal. Biochem.* 187:27.
- Caughey, G. H., W. W. Raymond, and P. J. Wolters. 2000. Angiotensin II generation by mast cell α - and β -chymases. *Biochim. Biophys. Acta.* 1480:245.
- Diez-Gomez, M. L., S. Quirce, M. Cuevas, C. Sanchez-Fernandez, G. Baz, F. J. Moradillos, and A. Martinez. 1999. Fruit-pollen-latex cross-reactivity: implication of profilin (*Bet v 2*). *Allergy* 54:951.
- Scheurer, S., A. Wangorsch, D. Hausteiner, and S. Vieths. 2000. Cloning of the minor allergen *Api g 4* profilin from celery (*Apium graveolens*) and its cross-reactivity with birch pollen profilin *Bet v 2*. *Clin. Exp. Allergy* 30:962.
- Kazemi-Shirazi, L., G. Pauli, A. Purohit, S. Spitzauer, R. Froschl, K. Hoffmann-Sommergruber, H. Breiteneder, O. Scheiner, D. Kraft, and R. Valenta. 2000. Quantitative IgE inhibition experiments with purified recombinant allergens indicate pollen-derived allergens as the sensitizing agents responsible for many forms of plant food allergy. *J. Allergy Clin. Immunol.* 105:116.
- Fang, K. C., W. W. Raymond, S. C. Lazarus, and G. H. Caughey. 1996. Dog mastocytoma cells secrete a 92-kD gelatinase activated extracellularly by mast cell chymase. *J. Clin. Invest.* 97:1589.
- Caughey, G. H., N. F. Viro, S. C. Lazarus, and J. A. Nadel. 1988. Purification and characterization of dog mastocytoma chymase: identification of an octapeptide conserved in chymotryptic leukocyte proteinases. *Biochim. Biophys. Acta* 952:142-9.
- Caughey, G. H., N. F. Viro, J. Ramachandran, S. C. Lazarus, D. B. Borson, and J. A. Nadel. 1987. Dog mastocytoma tryptase: affinity purification, characterization, and amino-terminal sequence. *Arch. Biochem. Biophys.* 258:555.
- Raymond, W. W., E. K. Tam, J. L. Blount, and G. H. Caughey. 1995. Purification and characterization of dog mast cell protease-3, an oligomeric relative of tryptases. *J. Biol. Chem.* 270:13164.
- Aspenstrom, P., I. Lassing, and R. Karlsson. 1991. Production, isolation and characterization of human profilin from *Saccharomyces cerevisiae*. *J. Muscle Res. Cell Motil.* 12:201.
- Aalberse, R. C. 2000. Structural biology of allergens. *J. Allergy Clin. Immunol.* 106:228.
- Xia, H. Z., C. L. Kepley, K. Sakai, J. Chelliah, A. M. Irani, and L. B. Schwartz. 1995. Quantitation of tryptase, chymase, Fc ϵ RI α , and Fc ϵ RI γ mRNAs in human mast cells and basophils by competitive reverse transcription-polymerase chain reaction. *J. Immunol.* 154:5472.
- Caughey, G. H. 1991. The structure and airway biology of mast cell proteinases. *Am. J. Respir. Cell Mol. Biol.* 4:387.
- Kwiatkowski, D. J., and G. A. Bruns. 1988. Human profilin. Molecular cloning, sequence comparison, and chromosomal analysis. *J. Biol. Chem.* 263:5910.
- Powers, J. C., T. Tanaka, J. W. Harper, Y. Minematsu, L. Barker, D. Lincoln, K. V. Crumley, J. E. Fraki, N. M. Schechter, G. G. Lazarus, et al. 1985. Mammalian chymotrypsin-like enzymes: comparative reactivities of rat mast cell proteases, human and dog skin chymases, and human cathepsin G with peptide 4-nitroanilide substrates and with peptide chloromethyl ketone and sulfonyl fluoride inhibitors. *Biochemistry* 24:2048.
- KleinJan, A., A. R. McEuen, M. D. Dijkstra, M. G. Buckley, A. F. Walls, and W. J. Fokkens. 2000. Basophil and eosinophil accumulation and mast cell degranulation in the nasal mucosa of patients with hay fever after local allergen provocation. *J. Allergy Clin. Immunol.* 106:677.
- Skokos, D., S. Le Panse, I. Villa, J. C. Rousselle, R. Peronet, B. David, A. Namane, and S. Mecheri. 2001. Mast cell-dependent B and T lymphocyte activation is mediated by the secretion of immunologically active exosomes. *J. Immunol.* 166:868.
- Fedorov, A. A., T. Ball, R. Valenta, and S. C. Almo. 1997. X-ray crystal structures of birch pollen profilin and *Phl p 2*. *Int. Arch. Allergy Immunol.* 113:109.
- Williams, C. M., and S. J. Galli. 2000. The diverse potential effector and immunoregulatory roles of mast cells in allergic disease. *J. Allergy Clin. Immunol.* 105:847.