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Identification, Recombinant Expression, and Characterization of the 100 kDa High Molecular Weight Hymenoptera Venom Allergens Api m 5 and Ves v 3

Simon Blank,^{*,1} Henning Seismann,^{*,1} Benjamin Bockisch,^{*,1} Ingke Braren,[†] Liliana Cifuentes,[‡] Mareike McIntyre,[‡] Dana Rühl,^{*} Johannes Ring,[‡] Reinhard Bredehorst,^{*} Markus W. Ollert,^{‡,2} Thomas Grunwald,^{†,2} and Edzard Spillner^{*,2}

Insect stings can cause life-threatening IgE-mediated anaphylactic reactions in venom-allergic patients. Although several compounds have already been described as venom allergens, prominent allergen candidates especially in the higher m.w. range have still remained elusive. Tandem mass spectrometry-based sequencing assigned a candidate gene to the most prominent putative high m.w. allergen Api m 5 (allergen C) in honeybee (*Apis mellifera*) venom and also allowed identification of its homologue Ves v 3 in yellow jacket (*Vespula vulgaris*) venom. Both proteins exhibit a pronounced sequence identity to human dipeptidyl peptidase IV or CD26. Reactivity of a human IgE mAb verified the presence of these proteins in the venoms. Both proteins were produced in insect cells and characterized for their enzymatic activity as well as their allergenic potential using sera and basophils from insect venom-allergic patients. Both Api m 5 and Ves v 3 were recognized by specific IgE of the majority of patients even in the absence of cross-reactive carbohydrate determinants. Serologic IgE reactivity closely matched activation of human basophils by Api m 5 or Ves v 3, thus underlining their relevance in functional assays. With Api m 5 and Ves v 3, a new pair of homologous allergens becomes available for future clinical applications in diagnosis and therapy that may also contribute to the understanding of molecular mechanisms of insect venoms. Moreover, the patient IgE reactivity together with the cellular activation demonstrates for the first time the relevance of high m.w. allergens in the context of hymenoptera venom allergy. *The Journal of Immunology*, 2010, 184: 000–000.

Systemic IgE-mediated allergic reactions after insect stings are prevalent causes of life-threatening and sometimes fatal immune-mediated anaphylaxis in humans (1). Among the most common elicitors are stinging insects of the order Hymenoptera, such as honeybee (*Apis mellifera*), yellow jacket (*Vespula* spp.), wasp (*Polistes* spp.), hornet (*Dolichovespula* spp.), and European hornet (*Vespa crabro*). Although venom immunotherapy is effective in the majority of Hymenoptera venom-allergic patients, systemic allergic side effects to immunotherapy injections have been observed in 20–40% of patients. According to sting challenge tests during venom immunotherapy, 10–20% of

patients were not protected by venom immunotherapy and continued to develop generalized allergic symptoms (2, 3). Thus, there is considerable interest in improving safety and efficacy of diagnostic approaches and Hymenoptera venom immunotherapy. The use of recombinant allergens for the development of component-resolved IgE immunoassays and safer and more efficacious immunotherapeutic modalities may provide such an improvement (4), but only a limited number of Hymenoptera venom allergens are available as recombinant proteins (5, 6).

Among the best characterized honeybee venom (HBV) major allergens responsible for IgE-mediated allergic reactions are phospholipase A₂ (Api m 1), hyaluronidase (Api m 2), and acid phosphatase (Api m 3) (7). Melittin (Api m 4), a basic peptide consisting of 26 aas, is considered a minor allergen (8). Phospholipase A₂ and hyaluronidase have been cloned and expressed in bacteria and baculovirus-infected insect cells (9–12), and their structures have been elucidated by x-ray crystallography (13, 14). Acid phosphatase (15, 16) was cloned and recombinantly expressed in insect cells very recently (17). In addition to these major components with known enzymatic function, a panel of high molecular mass proteins exhibits IgE reactivity, the most prominent of which in the range of 100 kDa is termed Api m 5 or allergen C and is supposed to be another major allergen recognized by specific IgE (sIgE) in a majority of HBV-allergic patients. Although present in substantial concentrations, identity and function of this allergen defied elucidation. As determined by gel electrophoretic analysis, Api m 5 has an apparent molecular mass ranging between 102 (18) and 105 kDa (19). In immunodiffusion, it has been demonstrated to be non-cross-reactive with other major bee venom allergens, including Api m 1, Api m 2, Api m 3, and Api m 4 as well as with other minor components (19).

^{*}Institute of Biochemistry and Molecular Biology, Department of Chemistry, University of Hamburg; [†]PLS-Design GmbH, Hamburg; and [‡]Clinical Research Division of Molecular and Clinical Allergotoxicology, Department of Dermatology and Allergy, Biederstein, Technische Universität München, München, Germany

¹S.B., H.S., and B.B. contributed equally to this work.

²M.W.O., T.G., and E.S. contributed equally to this work.

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Address correspondence and reprint requests to Dr. Edzard Spillner, Institute of Biochemistry and Molecular Biology, University of Hamburg, Martin-Luther-King-Platz 6, 20146 Hamburg, Germany. E-mail address: spillner@chemie.uni-hamburg.de

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Abbreviations used in this paper: b, honeybee; CCD, cross-reactive carbohydrate determinant; DPPIV, dipeptidyl peptidase IV; HBV, honeybee venom; LLR, large local reaction; MBP, maltose binding protein; n.r., nonreactive; sIgE, specific IgE; w, yellow jacket; YJV, yellow jacket venom.

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In this study, we report the identification and molecular cloning of the cDNA of the high m.w. allergen Api m 5 and the homologue from yellow jacket venom (YJV), the expression of the genes in eukaryotic insect cells, and the biochemical and immunological characterization of the purified recombinant molecules. We employed tandem mass spectrometry-based strategies for the identification of Api m 5 and used the obtained sequence information to scan the published honeybee genome (20). The cDNA of Api m 5 could be amplified from HBV glands, and on the basis of this information, its homologue in *Vespula* species could be identified. Subsequently, both proteins were expressed in insect cells, yielding proteins that exhibited the expected m.w. in SDS-PAGE and characteristic enzymatic activity. Both allergens, which are dipeptidyl peptidase IV (DPPIV) enzymes, are recognized by a specific mAb and IgE from venom-sensitized patient sera and are capable of activating human basophils from venom-allergic patients. Due to these characteristics, the presence DPPIV enzymes in Hymenoptera venoms may shed light on molecular mechanisms of insect venom allergy and potential physiological and pathophysiological implications. Moreover, the relevance of Api m 5 and its vespid homologue suggests a more complex picture of Hymenoptera venom allergy on the molecular level and renders the recombinant molecules valuable tools for the improvement of current immunodiagnostic tests and venom immunotherapy.

Materials and Methods

Materials

Whole HBV collected by electrostimulation was purchased from Latoxan (Valence, France). YJV (*Vespula* spp.) of immunotherapeutic grade (Venomil), which is obtained by venom sac extraction, was purchased from Bencard (Munich, Germany). Anti-V5 Ab was purchased from Invitrogen (Karlsruhe, Germany). Polyclonal rabbit anti-HRP serum was obtained from Sigma-Aldrich (St. Louis, MO). AlABLOTs were obtained from Siemens Healthcare Diagnostics (Los Angeles, CA). The alkaline phosphatase-conjugated anti-IgE mAb was purchased from BD Biosciences (San Diego, CA).

Characterization of venom-allergic patients

For the initial identification and further immunochemical characterization of Api m 5 and Ves v 3, three groups of sera were obtained without further preselection from the institutional serum bank: 1) sera with a positive sIgE test to HBV extract (i1, sIgE ≥ 0.35 kU/l) ($n = 35$), 2) sera with a positive sIgE test to YJV extract (i3, sIgE ≥ 0.35 kU/l) ($n = 35$), 3) control sera with a negative sIgE test to either HBV or YJV extract (i1 and i3, sIgE < 0.35 kU/l). All of the sera of the first two groups were derived from patients with a history of a systemic allergic reaction after a stinging event. sIgE tests for HBV or YJV were performed in all of the patients on the automated immunoassay systems UniCAP250 (Phadia, Uppsala, Sweden) or Immulite2000 (Siemens Healthcare Diagnostics) as described in detail elsewhere (21). For further investigation of Api m 5/Ves v 3 serologic IgE reactivity and for basophil activation tests with recombinant venom allergens, patients with a suspected history of an allergic reaction to Hymenoptera stings were consecutively recruited during routine allergy diagnostic workup ($n = 25$). Intradermal skin testing with venom extracts was performed as described previously (22). All of the patients had given their informed written consent for additional blood samples to be drawn.

Protein biochemistry

Api m 5 was enriched from venom via chromatographic methods and subjected to sequencing by tandem mass spectrometry. Therefore, 200 mg lyophilized HBV was dissolved in 10 ml 30 mM sodium citrate buffer (pH 4.5). Following removal of insoluble components by centrifugation at $4000 \times g$ for 30 min, the supernatant was incubated overnight with 5 ml Sephadex C-25 ion exchange resin (GE Healthcare, Munich, Germany) preswollen in the same buffer. After settling of the resin by centrifugation, the supernatant was recovered and reduced to 800 μ l by lyophilization, dialyzed against 3 mM Tris-HCl buffer (pH 7), and further reduced to 300 μ l. This step enriched the ~ 100 kDa Api m 5 in relation to the abundant lower m.w. protein fraction containing melittin and phospholipase A₂. The enriched protein sample was subjected to fractionation by SDS-PAGE. Because in *Vespula vulgaris* venom no high-abundance

peptidic compounds interfere with sequencing approaches, loading of substantial amounts of highly concentrated venom was sufficient for Ves v 3. Proteolytic in-gel fragmentation was performed using trypsin.

Cloning of cDNA

Total RNA was isolated from the separated stinger with attached venom sack and additional glands of honeybee (*A. mellifera*) and yellow jacket (*V. vulgaris*) using peqGold TriFast (Peqlab Biotechnologie, Erlangen, Germany). SuperScript III Reverse Transcriptase (Invitrogen) was used to synthesize cDNA from the isolated total RNA. RNaseOUT recombinant RNase inhibitor (1 μ l) (Invitrogen) was added to the standard 20 μ l reaction mix containing 5 μ l venom gland RNA. Reverse transcription was performed at 50°C for 60 min. First-strand cDNA was used as a template for PCR amplification of Api m 5 and Ves v 3 DNA sequences. Full-length Api m 5 was amplified from *A. mellifera* venom gland cDNA with Pfu DNA polymerase (Fermentas, St. Leon-Rot, Germany) using the primers 5'-ATG GAG GTA CTG GTG CAG CTG GCG CTG CTG CTG-3' and 5'-TCA GTG GGA GTA TCC CAG ACA-3'. DNA from the PCR reaction was isolated from 1% agarose gels (peqGOLD Universal Agarose; Peqlab Biotechnologie) using the peqGOLD Gel Extraction Kit (Peqlab Biotechnologie). Subcloning for sequencing was done using the Zero Blunt TOPO PCR Cloning Kit (Invitrogen) with the pCR-Blunt II-TOPO vector (Invitrogen). The ligated DNA was used to transform *Escherichia coli* of the strain XL1-Blue (Stratagene, La Jolla, CA) by electroporation (2-mm cuvettes, EasyJect+; Eurogentec, Seraing, Belgium) and selected on ampicillin agar plates. After sequencing of selected subcloned cDNA clones and verification of the sequence, the clone was used for secondary amplification of the mature chain coding region with Pfu DNA polymerase in two consecutive PCR reactions adding an N-terminal 10-fold His tag and V5 epitope using the primers 5'-AAG CCT ATC CCT AAC CCT CTC CTC GGT CTC GAT TCT ACG TGG CTC GAG TCT AGA AAA TCC GTT CCA CGA GTG ATC G-3', 5'-GAT CGG ATC CCA TCA CCA CCA CCA TCA TCA CCA CCA CCA TTC TTC TGG TGG TAA GCC TAT CCC TAA CCC TCT CCT CGG-3', and 5'-GAT CGC GGC CGC TCA GTG GGA GTA TCC CAG ACA ATT GGC-3'. The PCR product was subcloned into the BamHI and NotI digested baculovirus transfer vector pAcGP67-B (BD Biosciences) after restriction digest with BamHI and NotI.

Due to the lack of genomic data for *V. vulgaris* a C-terminal fragment of Ves v 3 was amplified from venom gland cDNA as described previously using the oligonucleotides 5'-CCN ATG CTN GTN AAY GTN TAY GCN-3' as deduced from peptides obtained by tandem mass spectrometry sequencing and oligo-deoxythymidine back. After sequence determination of subcloned cDNA fragments, the oligonucleotide 5'-GTG AGC GTG AGA CAG ACT G-3' was deduced from the sequence and used for reverse transcription as described previously. The cDNA was used for 5'RACE employing the 5'/3'RACE Kit, Second Generation (Roche, Basel, Switzerland) according to the recommendations of the manufacturer. Subsequent nested PCR was performed using the primers 5'-CAC GCT ACG ATT CGT TGT C-3' and 5'-CAA CGT ATT TGG TCC AGC G-3'. The obtained cDNA fragments were used as a basis for further sequence determination. Full-length cDNA was then amplified using the forward primer 5'-ATG GTT CCA CTA CGA AGT TTC G-3' and the reverse primer 5'-GTG AGC GTG AGA CAG ACT G-3'. After the selected full-length cDNA clone was sequenced and the sequence was verified, the clone was used for secondary amplification of the mature chain coding region with Pfu DNA polymerase using primers incorporating 5' BamHI and 3' NotI restriction sites as well as a 3' V5 epitope and a 10-fold His tag. The PCR product was subcloned into the BamHI and NotI digested baculovirus transfer vector pACGP67-B after digest with the respective enzymes.

Recombinant bacterial expression and purification of allergens

For expression of Api m 5 and Ves v 3 in *E. coli*, the cDNA was cloned into the prokaryotic expression vectors pGEX-4T (GE Healthcare) or, alternatively, pMAL-c2X (NEB, Beverly, MA). Expression in *E. coli* XL1-Blue cells and purification of the fusion protein were performed according to the recommendations of the manufacturers.

Recombinant baculovirus production

Spodoptera frugiperda Sf9 cells (Invitrogen) were grown at 27°C in serum-free medium (Express Five SFM, containing 16.5 mM glutamine and 10 μ g/ml gentamycin; Invitrogen). Cell density was determined by hemocytometer counts; cell viability was evaluated by staining with trypan blue. Recombinant baculovirus was generated by cotransfection of Sf9 cells with BaculoGold Bright Baculovirus DNA (BD Biosciences) and the baculovirus transfer vector pACGP67-B for Api m 5 or Ves v 3, respectively, according to the recommendations of the manufacturer. High

titer stocks were produced by three rounds of virus amplification, and optimal multiplicity of infection for recombinant protein expression was determined empirically by infection of Sf9 cells in 100 ml suspension flask ($1.5\text{--}2 \times 10^6$ cells per milliliter in 20 ml suspension culture) with serial dilutions of high titer virus stock.

Expression in baculovirus-infected insect cells

High titer stocks of recombinant baculovirus containing the Api m 5- or Ves v 3-coding DNA were used to infect Sf9 or High Five cells (Invitrogen) ($1.5\text{--}2.0 \times 10^6$ cells per milliliter) in a 2000 ml suspension flask (400 ml suspension culture). For protein production, the cells were incubated at 27°C and 110 rpm for 72 h.

Protein purification

The supernatant of baculovirus-infected cells was collected, adjusted to pH 8, and centrifuged at $4000 \times g$ for 5 min. Supernatants were applied to a nickel-chelating affinity matrix (Ni-NTA-agarose; Qiagen, Valencia, CA). The column was washed with binding buffer (50 mM sodium phosphate [pH 7.6] and 500 mM NaCl) and pre-eluted with NTA binding buffer containing 20 mM imidazole. The recombinant protein was eluted from the matrix with NTA-binding buffer containing 300 mM imidazole. Purification was confirmed by SDS-PAGE.

Enzymatic activity of the recombinant Api m 5 and Ves v 3

The DPPIV activity of the native and recombinant enzymes was assessed as follows. Recombinant Api m 5 and Ves v 3 from baculovirus expression at a concentration of 200 ng/ml in NTA binding buffer containing 300 mM imidazole and HBV or YJV at a concentration of 100 µg/ml in 50 mM sodium phosphate (pH 7.6) and 100 mM NaCl were used for activity testing. The synthetic DPPIV substrate glycine-proline-*p*-nitroanilide hydrochloride (Sigma-Aldrich) was applied at a final concentration of 0.5 mM, and DPPIV activity was assayed at 405 nm using a spectrophotometer. For determination of the IC₅₀ values, activity assays were repeated using serial dilutions of the specific DPPIV inhibitor Diprotin A (Sigma-Aldrich) at final concentrations ranging from 2.5 mM to 1.25 µM. All of the values were measured in triplicate. Nonlinear regression curves and inhibition values were calculated using Prism 3.0 (GraphPad, San Diego, CA).

IgE immunoreactivity of patient sera with recombinant Api m 5 and Ves v 3

For assessment of sIgE immunoreactivity of human sera with purified recombinant Api m 5 and Ves v 3 in ELISA, 384-well microtiter plates (Greiner, Frickenhausen, Germany) were coated with 20 µl of purified recombinant Api m 5 or Ves v 3 (20 µg/ml) at 4°C overnight and blocked with 40 mg/ml skimmed milk powder in PBS at room temperature. Afterwards, human sera were diluted 1:1 with 5 mg/ml BSA in PBS and incubated in a final volume of 20 µl for 4 h at room temperature. Wells were washed four times with PBS before bound IgE was detected with an alkaline phosphatase-conjugated mouse anti-human IgE mAb (BD Biosciences) diluted 1:1000. Wells were again washed four times with PBS, and 50 µl of substrate solution (5 mg/ml 4-nitrophenylphosphate; AppliChem, Darmstadt, Germany) per well was added. After 30 min, the plates were read at 405 nm. The cutoff was calculated as the mean of the negative controls plus two SDs. This resulted in a working range of OD_{405nm} $\geq 0.32\text{--}3$. For reasons of precision, reactivities only slightly higher than the cutoff value were excluded for both allergens (OD_{405nm} $\geq 0.32 < 0.40$). Thus, an effective working range of OD_{405nm} $\geq 0.40\text{--}3$ was applied in all IgE ELISAs used for allergen identification. A second ELISA technique was used to determine sIgE against Api m 5 and Ves v 3 from the sera of venom-allergic patients recruited for further IgE reactivity studies and basophil activation tests and was performed exactly as described recently for Api m 3 (17). The latter ELISA uses total human IgE for internal standardization and has a lower end cutoff of OD_{450nm} ≥ 0.10 . For immunoblot procedures, human sera were diluted 1:10 with 5 mg/ml BSA in PBS and applied to the corresponding AlaBLOTs (1, *A. mellifera* venom; i3, *V. vulgaris* venom; Siemens Healthcare Diagnostics) or to the purified recombinant allergens, separated by SDS-PAGE, and immobilized onto nitrocellulose membranes. Visualization of bound IgE was then performed with anti-human IgE mAb conjugated to alkaline phosphatase and nitroterazolium blue chloride/5-bromo-4-chloro-3-indoyl phosphate according to recommendations of the manufacturer.

Inhibition ELISA

For assessment of sIgE immunoreactivity of patient sera with purified recombinant Api m 5 and Ves v 3 in ELISA, 384-well microtiter plates (Greiner) were coated with 20 µl of purified recombinant Api m 5 or Ves v 3 (20 µg/ml)

at 4°C overnight and blocked with 40 mg/ml skimmed milk powder in PBS at room temperature. Human sera were diluted 1:1 with 5 mg/ml BSA in PBS and incubated in a final volume of 20 µl for 4 h at room temperature. For inhibition, sera were diluted 1:1 with either HBV or YJV at a concentration of 1 mg/ml in PBS and preincubated for 2 h at room temperature prior to incubation as described above. Wells were rinsed four times with PBS before bound IgE was detected as described above. Inhibition values were calculated after subtraction of negative controls and are given in percentages.

Basophil activation test

The basophil activation test was performed as described previously (22) with modifications as recommended by the manufacturer of the assay (Flow-CAST; Bühlmann Laboratories, Basel, Switzerland). In brief, within 3 h after sampling of patient blood in endotoxin-free EDTA tubes, aliquots of 50 µl whole blood were preincubated for 10 min at 37°C with stimulation buffer containing IL-3. Subsequently, basophils were activated for 40 min at 37°C in a water bath with either native or recombinant allergens at a volume of 100 µl. Thereby, HBV or YJV (in a concentration of 50 ng/ml) as well as the allergens (Ves v 3 and Api m 5, for comparison the established major allergens Api m 1 and Ves v 5, all in a concentration range of 0.08–1000 ng/ml) were applied in concentrations according to those reported in the literature (23–25). A murine mAb against the human high-affinity FcεR (Bühlmann Laboratories) served as a positive stimulation control. Plain stimulation buffer was used as a negative stimulation control. The optimal stimulation time and temperature were determined in preliminary experiments. To quantify activated basophils, cells were stained with 20 µl reagent containing a mixture of mAbs to human CD63 labeled with PE (anti-CD63-PE) and human IgE labeled with FITC (anti-IgE-FITC) for 30 min on ice. RBCs were lysed, and WBCs were fixed (BD FACS Lysing Solution; BD Biosciences, San Jose, CA) for 5 min at room temperature. After centrifugation (5 min at $1200 \times g$), cells were resuspended in 500 µl of stop solution. Flow cytometric analysis of basophil activation was performed on a FACScan flow cytometer (BD Biosciences). IgE-staining and side scatter were employed to gate on at least 500 basophils that expressed a high density of surface IgE. Subsequently, within this gate, the percentage of activated basophils (i.e., coexpressing CD63) was measured.

Other methods

SDS-PAGE, Western blotting, and ELISA as well as standard procedures in molecular biology were performed according to established protocols (26). The recombinant venom allergens Ves v 5 were cloned, expressed, and purified according to established procedures (17). The chimeric human IgE Ab against Api m 5 was generated essentially as described recently (27).

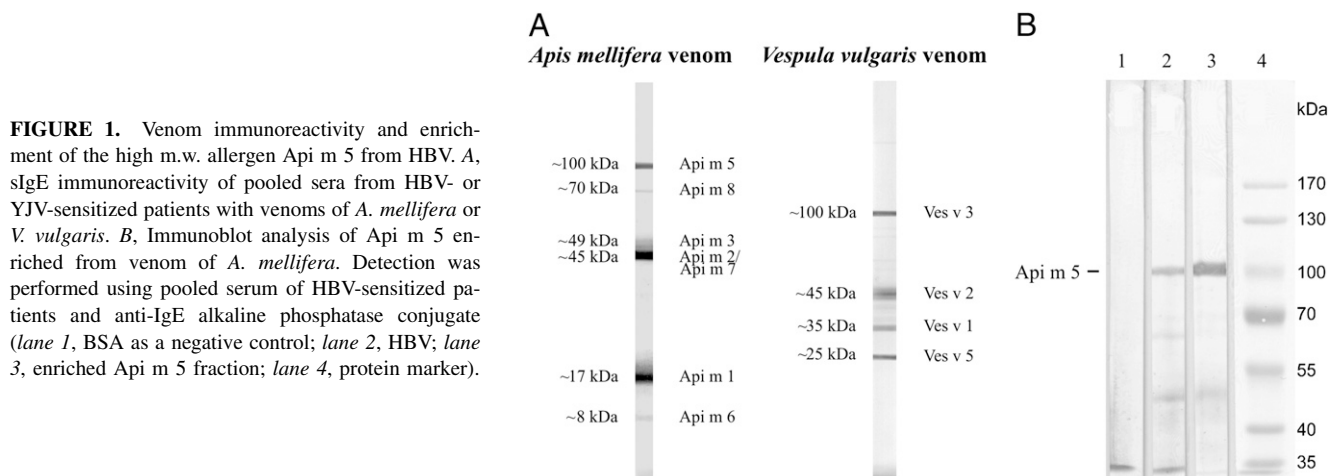
Results

Identification of Api m 5

Major sIgE reactivities with proteins of higher m.w. were evident in immunoblots of Hymenoptera venoms employing pooled sera of venom-sensitized patients as shown in Fig. 1A. Thereby, the most remarkable reactivity in both HBV and YJV was detected with high molecular mass allergens at ~100 kDa, putatively corresponding to Api m 5 or allergen C in HBV. After enrichment by chromatographic procedures, the IgE-reactive putative Api m 5 with an apparent molecular mass of 105 kDa (Fig. 1B) was subjected to sequencing by tandem mass spectrometry. Four peptide sequences (shown in Fig. 2) could be identified, three of which yielded hits in a database search of the *A. mellifera* genome with bioinformatic tools (28, 29). According to the automated gene prediction program GNOMON (National Center for Biotechnology Information, Rockville, MD), a putative gene (XP_393818) codes for the isolated Api m 5. A basic local alignment search tool search for short, nearly exact matches yielded a corresponding result with the fourth peptide sequence. Although, low probability scanning for a potential signal peptide cleavage site (30) indicated a putative N terminus for the Api m 5 protein.

cDNA cloning and sequence analysis

First attempts to amplify the gene from bee venom gland cDNA failed. However, reevaluation of the genomic sequence using the alternative automated gene prediction program GeneMark (Georgia Institute of Technology, Atlanta, GA) suggested a variant



N-terminal splicing, thus unambiguously providing a signal peptide. On the basis of this information, a DNA fragment of 2328 bp in length could be amplified.

The Api m 5 nucleotide sequence (Genbank accession EU564832) encodes a 775-aa polypeptide with a calculated molecular mass of 87.9 kDa, which is compatible with the apparent molecular mass (~105 kDa) of native Api m 5 (Figs. 1, 2). The discrepancy between the deduced molecular mass of Api m 5 and its apparent molecular mass of 105 kDa in SDS-PAGE is most likely due to posttranslational modification by glycosylation as suggested by the presence of six predicted sites for N-glycosylation. The amino acid sequence shows significant homology to DPPIV proteins known to cleave dipeptide units from the N termini of growth factors and other peptidic compounds. The enzyme is composed of an N-terminal dipeptidyl peptidase domain and

a C-terminal prolyl peptidase domain. Sequence identity to human DPPIV (CD26) is in the range of 32% (10% on DNA level) and to a DPPIV from the venom of the snake *Gloydus blomhoffi brevicaudus* in the range of 32% (11% on DNA level).

Identification and molecular cloning of the vespid homologue *Ves v 3*

Because venoms of both *A. mellifera* and *V. vulgaris* contain a prominent 100 kDa band detected by sIgE in individual and pooled sera of sensitized patients (Fig. 1A), we aimed for identification of the Api m 5 homologue in *Vesputula* spp. venom. Tandem mass spectrometry approaches yielded peptide sequences with a high degree of identity to Api m 5. On the basis of this information, oligonucleotides were employed to amplify fragments from cDNA of *V. vulgaris* venom glands. Subsequent RACE



FIGURE 2. Alignment of DPPIV amino acid sequences. Alignment of Api m 5 and Ves v 3 with other related proteins. Alignment with database-derived sequences revealed homologies to peptidases from other species. Shown are Api m 5, Ves v 3, DPPIV of the snake *G. blomhoffi brevicaudus* (e.g., Genbank accession AB158224) and human DPPIV (e.g., Genbank accession BC65265). Peptides identified by mass spectrometry are underlined. Signal sequences are italicized, the residues involved in the conserved active center of the enzymes are represented boxed, and putative glycosylation sites are in gray.

approaches finally led to the cloning of the full-length open reading frame, and contiguity was verified by amplification of the entire cDNA. The nucleotide sequence (Genbank accession EU420987) encodes a 776-aa polypeptide with a calculated molecular mass of 88.9 kDa, which is in accordance with the apparent molecular mass (~105 kDa) and that of Api m 5 (Fig. 2) considering the presence of six predicted sites for N-glycosylation. Sequence identity to Api m 5 is in the range of 53% (59% on DNA level, data not shown), to human DPPIV in the range of 31% (11% on DNA level), and to the *G. blomhoffi brevicaudus* DPPIV in the range of 32% (17% on DNA level). According to its presence in the venom and the International Union of Immunological Societies allergen nomenclature, we termed the protein Ves v 3.

Recombinant expression of Api m 5 and Ves v 3 in insect cells

To provide recombinant proteins for subsequent functional and immunological studies and to verify the presence of the identified proteins in the venom, we produced full-length Api m 5 and Ves v 3 as secreted proteins in insect cells. The cDNA of Api m 5 and Ves v 3 was cloned into the particular expression vector for baculovirus-based infection of *Trichoplusia ni* (High Five) or *S. frugiperda* (Sf9) insect cells. Culture supernatants of High Five cells were subjected to Ni-NTA-agarose chromatography, and resulting proteins were analyzed by SDS-PAGE and IgE immunoblotting. The epitope-tagged recombinant proteins (yield of ~0.2 µg per milliliter of supernatant) exhibited an apparent molecular mass of ~105 kDa, corresponding to the natural allergens in HBV or YJV, and, additionally, were reactive with sIgE from pooled sera of venom-sensitized patients (Fig. 3A, 3B).

A recombinant human IgE Ab was generated and produced in mammalian cells by employing a prokaryotically expressed Api m 5 fusion protein. This Api m 5-specific IgE mAb showed reactivity with insect cell-derived recombinant proteins Api m 5 and Ves v 3 (Fig. 3B). Moreover, it detected the corresponding natural forms of Api m 5 and Ves v 3 in the venoms of *A. mellifera* and *V. vulgaris* (Fig. 3A). Furthermore, as shown in Fig. 3C, three exemplary sera of HBV-sensitized patients that were selected by

sIgE immunoreactivity with Api m 5 in immunoblots demonstrated comparable IgE reactivity in ELISA to enriched native Api m 5 (containing residual amounts of mellitin) and recombinant Api m 5 produced in High Five insect cells. Together, these data verified the identity of the recombinantly produced allergens with the IgE-immunoreactive 100 kDa allergens in the native venoms.

Enzymatic activity of recombinant Api m 5 and Ves v 3

Their sequences render Api m 5 and Ves v 3 putative homologues of DPPIV from humans and other species. Using glycine-proline-p-nitroanilide hydrochloride, a synthetic substrate of human DPPIV, specific DPPIV activity could be detected in the venom of both *A. mellifera* and *Vespula* spp. (Fig. 4A, 4B). Moreover, this activity could be abolished by Diprotin A, a highly specific inhibitor of human DPPIV. Purified recombinant Api m 5 and Ves v 3 both proved to exhibit significant DPPIV activity, demonstrating their DPPIV nature and, thereby, correct folding of the insect cell-produced proteins (Fig. 4C, 4D). The inhibition constants of Diprotin A for the DPPIV activities in both venoms and the recombinant allergens Api m 5 and Ves v 3 matched the reported activity of the human enzyme very closely, suggesting mechanistic conservation. Together these data suggest that the DPPIV-like activity contributes to the diverse panel of enzymatic activities exhibited by Hymenoptera venoms and that this activity relies on Api m 5 and its homologues.

IgE immunoreactivity of the recombinant allergens Api m 5 and Ves v 3

To evaluate the IgE immunoreactivity of Api m 5 and Ves v 3 produced in Sf9 insect cells, individual sera of patients with a clinical history of an allergic reaction after a stinging event were assayed by ELISA for sIgE Abs. The production in Sf9 cells resulted in the absence or strongly reduced presence of cross-reactive carbohydrate determinants (CCDs) as supported by ELISA analyses (Supplemental Fig. 1). Fig. 5 shows the reactivity of patients who were characterized by a positive sIgE test to HBV or YJV (Supplemental Table I). Of the 35 HBV-positive sera, nine

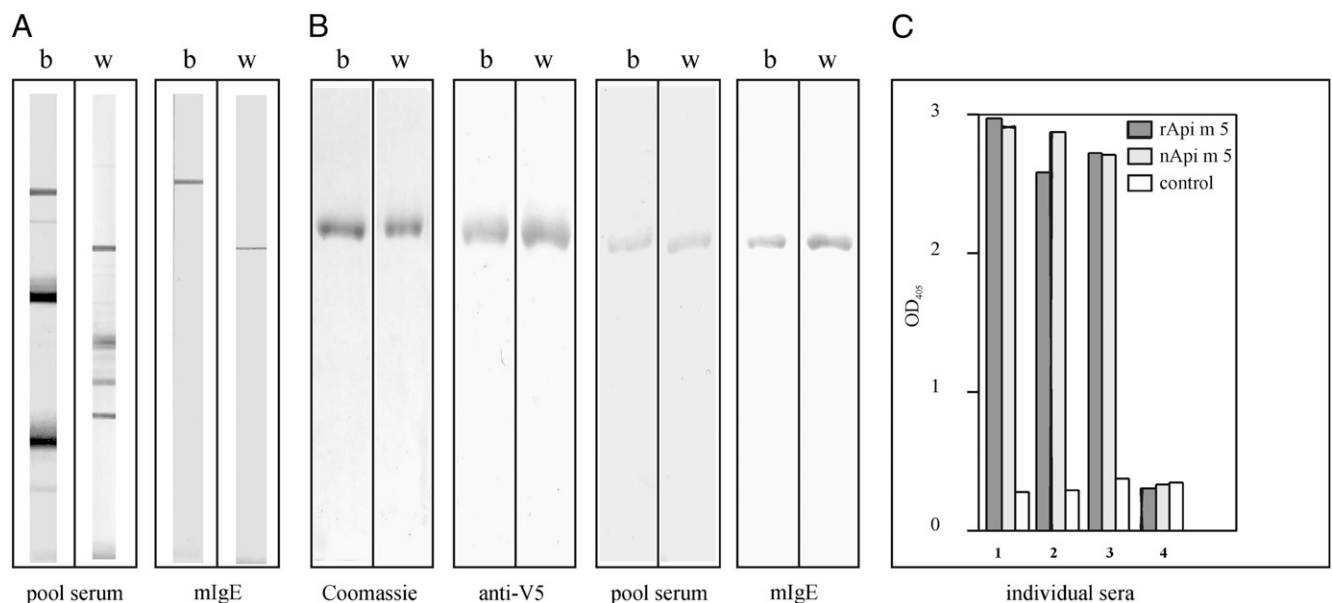


FIGURE 3. Immunoreactivity of native and recombinant Api m 5 and Ves v 3. *A*, Immunoblot of honeybee (b) and yellow jacket (w) venom with pooled HBV- or YJV-allergic patient sera (left) and a human IgE mAb specific for Api m 5 (right). *B*, SDS-PAGE and immunoblot analysis of purified Api m 5 (b) and Ves v 3 (w) expressed in insect cells, visualized by either Coomassie brilliant blue staining or anti-V5 epitope Ab, pooled HBV- or YJV-allergic patient sera and a human anti-API m 5 IgE mAb. *C*, Immunoreactivity of individual sera of HBV-allergic patients (1–3) and a control serum of a nonallergic individual (4) with recombinant and native Api m 5 in ELISA. Api m 5 was expressed in High Five insect cells.

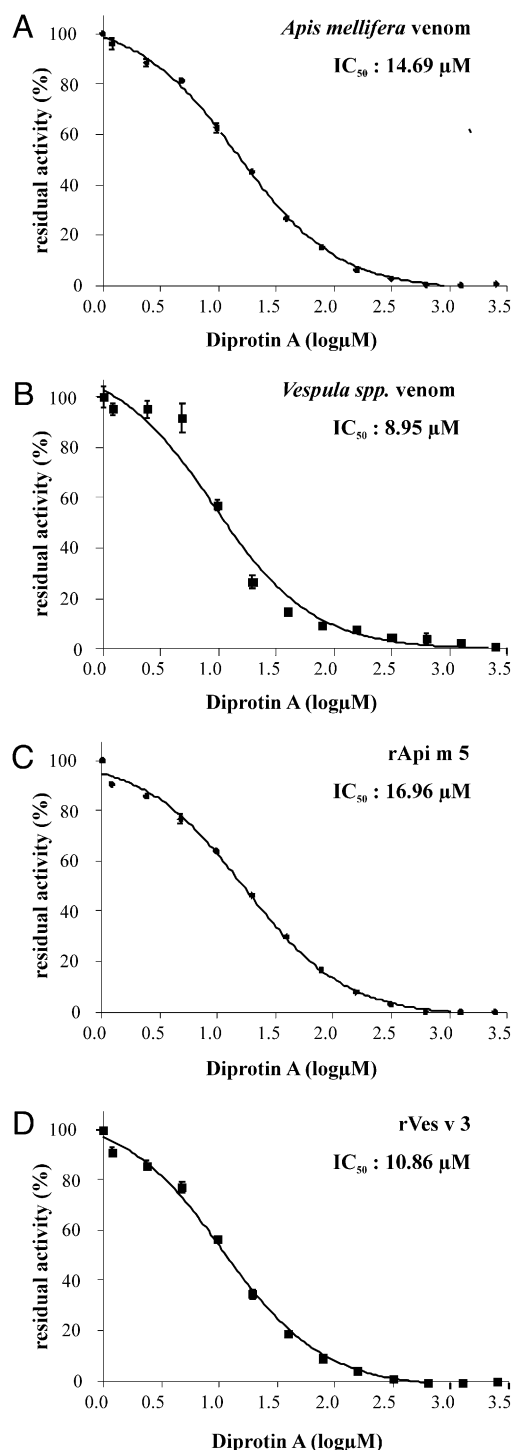


FIGURE 4. Kinetic analyses of DPPIV activity. Inhibition of DPPIV activity by the inhibitor Diprotin A (Ile-Pro-Ile) of *A. mellifera* venom (A), *Vespula* spp. venom (B), purified recombinant Api m 5 (C), and purified recombinant Ves v 3 (D) was analyzed as described in *Materials and Methods*. The y-axis shows the residual activity (%) of cleavage of the chromogenic substrate glycine–proline–*p*-nitroanilide hydrochloride for serial dilutions of the specific DPPIV inhibitor Diprotin A (log μM). IC_{50} values are depicted on each plot.

showed high sIgE reactivity ($\text{OD}_{450\text{nm}} \geq 1.0$), whereas an additional 12 sera showed positive sIgE reactivity to a medium to a lower degree ($\text{OD}_{450\text{nm}} \geq 0.4 < 1.0$). Overall, 21 of 35 (60%) patient sera had detectable sIgE to recombinant Api m 5 (Fig. 5A). Most of these sera also showed various degrees of sIgE ELISA

reactivity to the major allergen native Api m 1, which is in accordance with the high prevalence of allergen-specific IgE to Api m 1 in bee venom allergy (31) (data not shown). Of the 35 YJV-positive sera, 16 showed high sIgE reactivity ($\text{OD}_{450\text{nm}} \geq 1.0$), whereas an additional four sera showed positive sIgE reactivity to a medium to a lower degree ($\text{OD}_{450\text{nm}} \geq 0.4 < 1.0$; cutoff value of 0.35). Overall, 20 of 35 (57%) of the patient sera had detectable sIgE to recombinant Ves v 3 (Fig. 5B).

To verify the obtained reactivities, we used sera employed in Fig. 5 and further compared eukaryotically produced, posttranslationally modified, and enzymatically active proteins with prokaryotically produced Api m 5 and Ves v 3 maltose binding protein (MBP) fusion proteins in ELISA (Fig. 6). The fact that a significant proportion of the reactive sera were also reactive with the prokaryotically produced counterparts (Api m 5, 10 (45%) of overall 22 tested sera; Ves v 3, 10 (52%) of overall 19 tested sera) corroborates the IgE reactivity of the insect cell-produced proteins and further supports the conclusion that the recombinant high m.w. proteins exhibit IgE reactivity beyond CCD reactivity.

Activation of basophils from venom-allergic patients by Ves v 3 and Api m 5

The capability of Ves v 3 and Api m 5 produced in Sf9 cells for activation of human basophils derived from YJV-allergic patients was assessed by a flow cytometry-based basophil activation test employing CD63 as an activation marker. To ensure the maximum of validity of the basophil activation tests, a broad concentration range (0.08, 0.8, 8, 40, 200, and 1000 ng/ml) was covered, and the extent of basophil activation was related to those of established major allergens, Api m 1 and Ves v 5. All of the blood samples were obtained from consecutively selected patients ($n = 25$) with a clinical history of a severe reaction after a stinging event. Of these 25 patients, four were excluded because of a lack of basophil reactivity to HBV or YJV extract, and one patient was excluded due to spontaneous maximal basophil activation in mock-treated cells (negative buffer control). The remaining 20 patients (15 with YJV and five with HBV allergy) were further analyzed (for characterization of the 20 patients, see Table I) (32).

All of the patients had positive intradermal skin and sIgE tests for YJV or HBV extract, or both. All of the patients were tested for serological IgE reactivity against Api m 5, Ves v 3, Api m 1, and Ves v 5. Eight of 20 patients had positive sIgE titers for Api m 5 (patients 3, 4, 8, 9, 10, 12, 16, and 17), and with Ves v 3, 11 of 20 sera showed positive sIgE titers (patients 3, 4, 6, 8, 10, 12, 13, 14, 15, 16, and 20), some of which with strongly elevated sIgE, others with lower sIgE titers. Overall, 12 of the 20 patients (patients 1, 3, 4, 5, 6, 9, 10, 12, 15, 16, 17, and 18) had positive basophil activation with the DPPIV allergens Api m 5 or Ves v 3, and 18 of 20 patients with the known species-specific major allergens Api m 1 or Ves v 5. Two YJV-allergic patients (patients 12 and 18) were negative in the basophil activation test with Ves v 5 but were both strongly positive with Ves v 3.

Ten out of 12 patients with positive basophil activation by the respective allergen Api m 5 or Ves v 3 had detectable serum DPPIV-specific IgE. Two patients (patients 1 and 5) with a negative sIgE result for Ves v 3 showed a basophil activation test result just slightly above the lower end cutoff of 15% (20% for patient 1 and 17% for patient 5). Fig. 7A–F depicts representative results for six patients (patients 6, 12, 15, and 16 for yellow jacket and 4 and 10 for honeybee as the relevant insect) with positive basophil activation. Notably, patient 12 (Fig. 7B) exhibited pronounced sIgE titers for Ves v 3 and low titers for Ves v 5, a picture fully reflected in the basophil activation. Of the other five depicted patients, two showed identical basophil activation (patients 6 and 10;

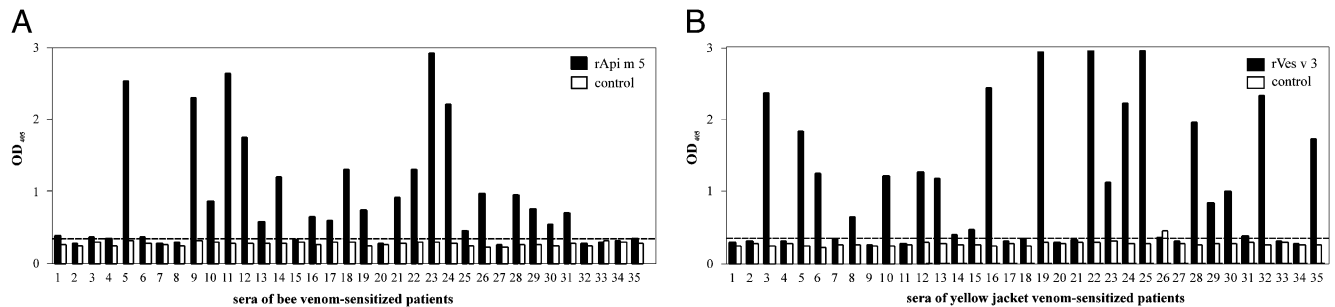


FIGURE 5. IgE immunoreactivity of Api m 5 and Ves v 3. Immunoreactivity of individual patient sera with recombinant Api m 5 and Ves v 3. The IgE reactivity was assessed by ELISA as described in *Materials and Methods* with sera of HBV-sensitized patients ($n = 35$) for recombinant Api m 5 (A) or YJV-sensitized patients ($n = 35$) for recombinant Ves v 3 (B). Controls were performed by omission of Ag. The lower end functional cutoff of the ELISA was $OD_{405nm} \approx 0.40$ as represented by a dashed line in A and B.

Fig. 7A, 7F) for Api m 5/Ves v 3 and Api m 1/Ves v 5, whereas two showed comparable maximal activation but a higher allergen concentration was necessary for Api m 5/Ves v 3 (patients 4 and 15; Fig. 7C, 7E), and in patient 16, Ves v 3 was clearly less active than Ves v 5 (Fig. 7D). Stimulation controls, whole venom and a anti-FcεRI mAb, gave positive results for all of the patients, whereas plain stimulation buffer used as a negative control did not induce reactivity. Overall, 10 of 15 patients with yellow jacket and two of five patients with honeybee as the relevant insect exhibited positive basophil activation to Ves v 3 and Api m 5, respectively. We also used the sera from the six patients shown in Fig. 7 and performed IgE inhibition to Sf9-derived DPPIV allergens Api m 5 and Ves v 3 by preincubation with the respective native venom. As shown in Fig. 7G, the reactivity of the sera used in basophil activation testing could be largely inhibited, thus suggesting that the natural forms of the allergens provide the epitopes present on the recombinant counterparts.

Together, these data suggest that the high m.w. venom allergens Ves v 3 and Api m 5 are able to induce relevant effector cell activation in venom-allergic patients.

Discussion

In this study, we have identified and characterized the 100 kDa high molecular mass allergens in the venoms of the Hymenoptera species *A. mellifera* and *V. vulgaris*. Using advanced sequencing strategies to overcome quantity limitations, detrimental abundance of the major component in HBV, the cytolytic peptide melittin (55% of dry venom weight), and potential N-terminal mod-

ifications, we obtained sequence information of enriched Api m 5, allowing an assignment to a predicted open reading frame on the basis of available genomic sequence information. Finally, we could successfully amplify the full-length cDNA, providing an alternative N-terminal exon from venom gland cDNA. The cDNA of another HBV protein of 94 kDa recently proposed to correspond to allergen C (33) could not be amplified from venom gland cDNA. Moreover, Api m 5 is reported to exhibit an apparent molecular mass ranging between 102 (18) and 105 kDa (19). On the basis of the obtained sequence information of Api m 5, we additionally were able to identify the corresponding protein Ves v 3 as a new allergen in the venom of *V. vulgaris*. This protein corresponds to Api m 5 regarding m.w., amino acid sequence, enzymatic function, IgE immunoreactivity using a broad range of YJV-sensitized individuals, and functional allergenic capability using basophils from venom-allergic patients. Although a protein of this size has been reported as a putative IgE-reactive protein in another *Vespula* species, no attribution of a name or function in *V. vulgaris* has been made so far (34).

Insect cells appeared to be the most appropriate system for expression of the putative Api m 5 and its vespid venom homologue. The yields of the insect cell-based expressions mentioned here have to be considered preliminary and definitely can be increased by routine optimization steps, including evaluation of multiplicity of infection, time courses, and culture conditions. A subsequent scale-up of production can generate substantial amounts for use in structural studies as well as component-resolved diagnostics on a clinical scale level.

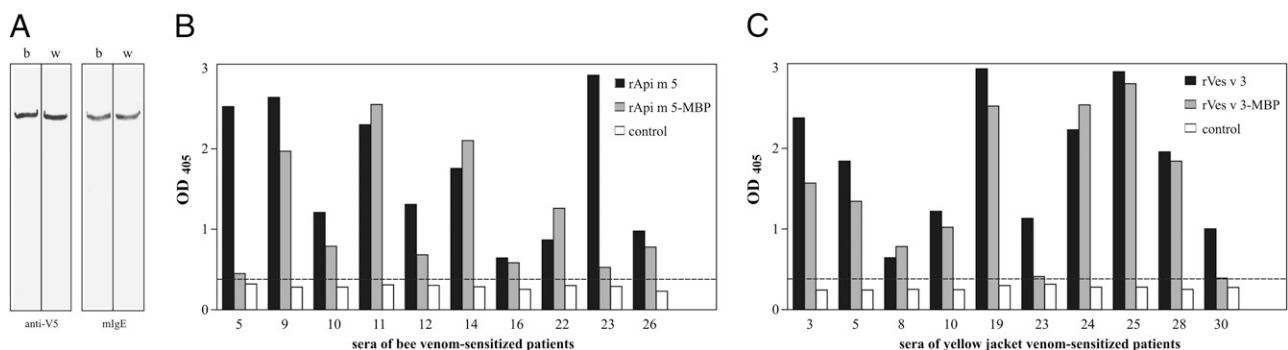


FIGURE 6. Comparative analyses of the immunoreactivity of prokaryotically versus eukaryotically produced Api m 5 and Ves v 3. For SDS-PAGE and immunoblot analysis (A) of Api m 5 and Ves v 3 expressed as MBP fusion proteins in *E. coli*, the proteins were visualized by either anti-V5 epitope Ab or a human anti-Api m 5 IgE mAb. Immunoreactivity of individual patient sera with recombinant Api m 5 and Ves v 3 was assessed by ELISA as described in *Materials and Methods* using sera of HBV-sensitized patients for recombinant Api m 5 and Api m 5-MBP (B) or YJV-sensitized patients for recombinant Ves v 3 and Ves v 3-MBP (C) (exemplary $n = 10$ each) found reactive in Fig. 5. Controls were performed by omission of Ag. The lower end functional cutoff of the ELISA was $OD_{405nm} > 0.40$ as represented by a dashed line in B and C.

Table I. Characterization of the venom-allergic patients selected for basophil activation tests

Patient	Relevant Insect	Anaphylaxis Grade ^a	Skin Test ^b		sIgE Extract ^c		sIgE Allergens ^d			
			HBV (μg/ml)	VV (μg/ml)	i1 (kU/l)	i3 (kU/l)	Api m 5 (OD _{450nm})	Api m 1 (OD _{450nm})	Ves v 3 (OD _{450nm})	Ves v 5 (OD _{450nm})
1	YJ	2	0.01	0.001	3.15	4.46	<0.1	0.135	<0.1	0.201
2	YJ	3	Negative	0.001	<0.1	2	<0.1	<0.1	<0.1	0.142
3	YJ (HB) ^e	2	0.01	0.001	32.9	14.4	0.483	1.192	0.417	0.381
4	HB	1	0.0001	0.1	9.48	0.33	0.224	2.426	0.290	<0.1
5	YJ	1	Negative	0.001	0.15	10.8	<0.1	0.27	<0.1	0.214
6	YJ	LLR	Negative	0.01	1.16	19	<0.1	<0.1	0.536	0.719
7	YJ	1	0.1	0.0001	1.76	16.4	<0.1	0.517	<0.1	0.403
8	YJ (HB) ^e	2	0.01	0.01	1.09	35.1	0.118	1.129	0.207	2.712
9	YJ	2	Negative	0.0001	0.568	>100	0.294	0.262	<0.1	2.735
10	HB	1	0.0001	0.01	38.7	59.7	0.893	1.639	0.337	<0.1
11	HB	2	0.1	Negative	0.963	<0.1	<0.1	<0.1	<0.1	<0.1
12	YJ	2	0.1	0.0001	14.1	20	0.121	1.259	2.13	0.202
13	HB	3	0.0001	0.001	13.8	17.8	<0.1	0.937	0.186	1.194
14	HB	3	0.0001	0.01	>100	0.472	<0.1	1.096	0.189	<0.1
15	YJ	3	0.001	0.0001	1.44	6.04	<0.1	<0.1	0.254	1.462
16	YJ	2	0.01	0.001	25.9	>100	0.180	2.623	0.385	1.397
17	YJ	1	0.1	0.01	0.163	4.34	0.102	0.140	<0.1	0.139
18	YJ	3	0.0001	0.001	1.47	7.06	<0.1	<0.1	0.1	0.214
19	YJ	2	0.1	0.01	<0.1	0.775	<0.1	<0.1	<0.1	<0.1
20	YJ	2	Negative	0.1	<0.1	1.1	<0.1	0.105	0.185	0.441

^aAccording to Ring and Messmer (32).

^bShown is the lowest venom extract concentration that gave a positive result in intradermal skin testing applying a 10-fold concentration range from 0.1 to 0.0001 μg/ml for each of the venoms (21).

^cAs tested in either UniCAP250 or Immulite2000 sIgE assays using a lower end cutoff of 0.35 kU/l.

^dAs tested in an IgE ELISA using an internal total human IgE standard and a lower end cutoff of 0.1 as described elsewhere (17).

^ePatient 3 successfully completed specific immunotherapy with HBV 10 y ago and now presented with de novo acquired allergy to YJV. Patient 8 reported anaphylactic episodes to both honeybee and yellow jacket stings. Specific immunotherapy in this patient was initiated with vespid venom extract at first.

LLR, large local reaction.

In contrast to mammalian systems, insect cells most likely will provide similar glycosylation to that found in the natural isoforms, a fact that is supported by the apparent molecular masses of the expressed recombinant allergens (35). The identity of the expressed open reading frames with the venom proteins was further proven by a recombinant human IgE mAb specific for Api m 5 selected by phage display (27). This anti-Api m 5 IgE mAb reacted to a similar extent with the natural venom isoforms and the insect cell-expressed isoforms of Api m 5 and Ves v 3, suggesting the presence of a conserved protein epitope in Ves v 3 and Api m 5 (Fig. 3). Such an epitope hints at the possible occurrence of cross-reactive protein epitope-specific IgE also in venom-allergic patients. Indeed, the immunoreactivity of recombinant Api m 5 and Ves v 3 in immunoblot and ELISA analyses with IgE from >50% of HBV- or YJV-sensitized patients demonstrated the general presence of human IgE epitopes on the two high m.w. allergens.

However, it is well known that Api m 5 and the corresponding Ves v 3 are glycoproteins (20, 28) containing multiple potential glycosylation sites. Moreover, most of the proteins in Hymenoptera venom provide carbohydrates carrying α-1,3-linked core fucose residues that form a type of human IgE epitope also known as CCD (36). IgE with specificity for CCD are considered to play a key role in allergen cross-reactivity, representing a major concern for the specificity of diagnostic approaches in Hymenoptera venom allergy (37). In this context, it was postulated in a recent immunoblot study that the immunoreactivity of high m.w. Hymenoptera venom allergens including Api m 5 and Ves v 3 reflects almost exclusively CCD-specific sIgE reactivity in patient sera (38), thus rendering them allergens of potentially minor interest.

In contrast to these findings, our study points to a more important role of Api m 5 and Ves v 3 as allergens recognized by patient sIgE beyond CCD reactivity. Further support for their relevance in venom allergy stems from the fact that Sf9 insect cells were employed for production of the enzymatically active recombinant

proteins. In contrast to High Five cells, Sf9 cells are considered to exhibit significantly reduced α-1,3 core fucosyltransferase activity (39, 40), resulting in proteins without immunologically detectable amounts of CCDs (41). Very recently, we were able to demonstrate the validity of these findings in the context of Hymenoptera venom allergy and to develop a strategy for defining the presence or absence of CCD reactivities on insect cell-derived proteins (42). Moreover, as shown in Supplemental Fig. 1, we could adopt this strategy for the DPPIV enzymes. With recombinant Sf9-produced allergens, >50% of not preselected HBV or YJV sIgE-positive sera were reactive with recombinant Api m 5 or Ves v 3, respectively. Considering the absence of CCDs on Sf9-derived proteins, these results render both 100 kDa proteins major allergens containing protein IgE epitopes of putative clinical relevance. Additional evidence in this direction is derived from the facts that even prokaryotically expressed Api m 5 and Ves v 3 devoid of both glycosylation and, most likely, proper folding exhibited significant IgE reactivity with most patient sera found to be reactive with the eukaryotically produced Api m 5 and Ves v 3. This also fits into the context of the basophil activation, in which only clinically relevant IgE reactivities (not including IgE directed against CCDs) are documented. Both findings support the conclusion that the recombinant high m.w. proteins exhibit a clear IgE reactivity beyond CCD reactivity. Additionally, previous in-depth cross-reactivity studies demonstrated that Api m 5 is not cross-reactive with other HBV allergens (19).

As mentioned, the best evidence that high m.w. Hymenoptera venom allergens could be of clinical relevance in venom allergy was provided by the activation of human patient-derived basophils through recombinant Ves v 3 or Api m 5. The basophil activation pattern closely matched the serologic sIgE reactivity in each of the patients. Thus, the finding that a significant portion of the consecutively recruited patients is susceptible to basophil activation further strengthens the concept of the relevance of the venom DPPIV

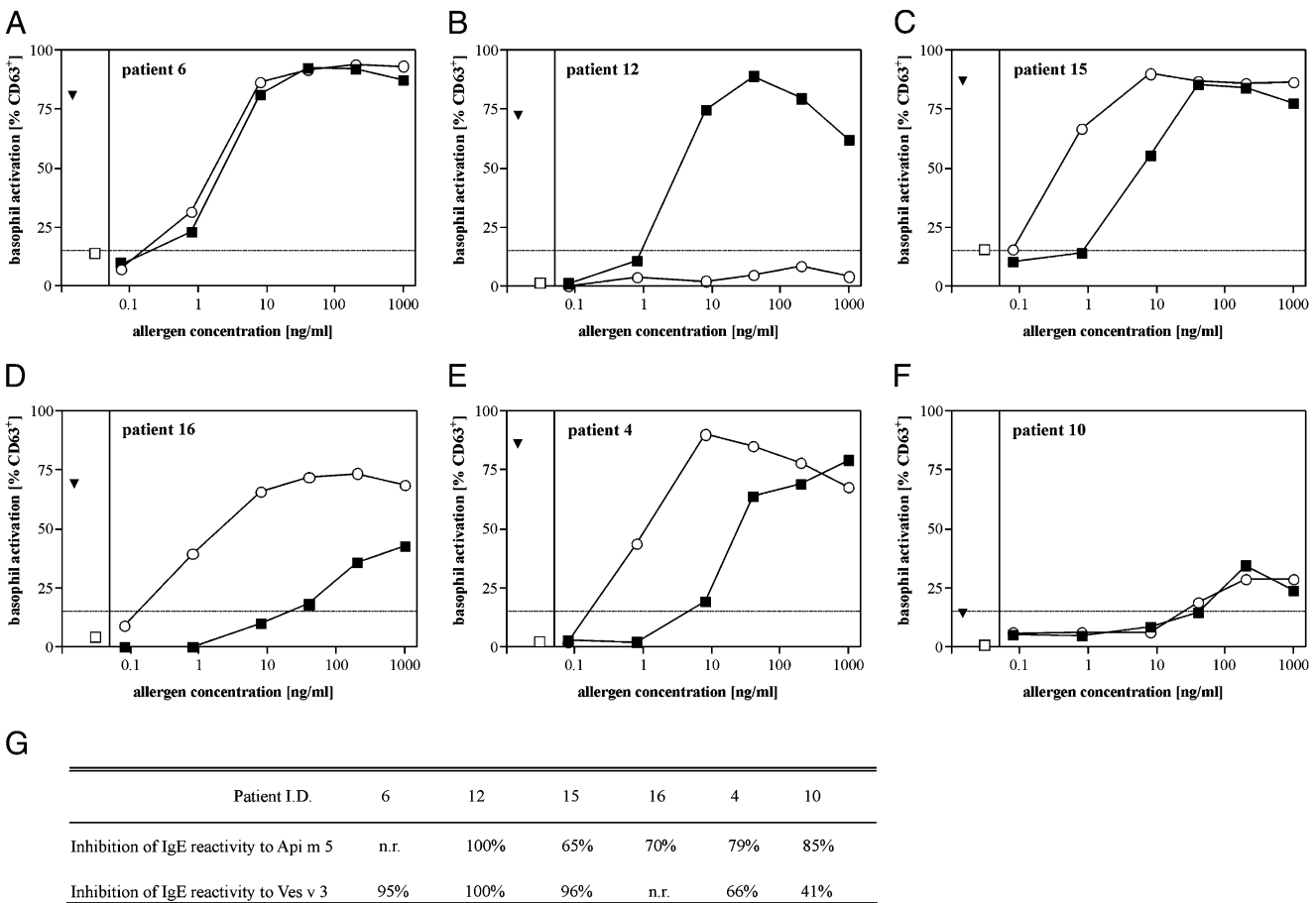


FIGURE 7. Basophil activation tests with recombinant Api m 5 and Ves v 3. Human basophils from six exemplary YJV- or HBV-sensitized patients were exposed to serial dilutions of the DPPIV enzyme (filled squares) and an established reference allergen (open circles). Incubations with an anti-FcεRI mAb (triangles) or with plain stimulation buffer (open squares) were used as positive or negative stimulation controls. *A–D*, Shown is the percentage of activated basophils on incubation with recombinant Ves v 3 and Ves v 5 (patients 6, 12, 15, and 16). *E* and *F*, Shown are the percentages of activated basophils on incubation with recombinant Api m 5 and Api m 1 (patients 4 and 10). Activation was assessed by flow cytometric analysis using anti-CD63 and anti-human IgE Abs as described in *Materials and Methods* and is shown as percentage of CD63⁺ cells. The dashed line in *A–C* represents the lower end functional cutoff of the assay (15% CD63⁺ cells). *G*, Inhibition of IgE binding to recombinant Api m 5 by HBV or Ves v 3 by YJV of the patient sera used in *A–F*. Given is the relative decrease assessed in ELISA as described in *Materials and Methods*. n.r., nonreactive.

enzymes. Thereby, the concentrations required for efficient activation of basophils with maxima in some patients at concentrations of 8–40 ng/ml protein corresponding to those of the control proteins are entirely in the range employed throughout a plethora of studies regarding allergenic potential of proteins from different sources including venom, pollen, food, or animal (23–25).

Moreover, our data provide for the first time evidence for the presence of homologous DPPIV glycoproteins of substantial sequence identity in Hymenoptera venom allergy. So far, double-positivity in venom-allergic patients had been largely attributed to IgE directed against either hyaluronidases (Api m 2 or Ves v 2) or against CCD (38, 43). Apart from CCD reactivity and although the primary structures and the reactivity of a mAb suggest the presence of conserved epitopes, the IgE cross-reactivity remains to be verified. With the identification, characterization, and recombinant expression of Ves v 3 and Api m 5, a new pair of potentially cross-reactive homologous allergens becomes available for future clinical applications in diagnosis and therapy.

Sequence analysis and characterization of the enzymatic activities provide clear evidence that Api m 5 and Ves v 3 belong to the class of DPPIV enzymes. In general, proline-specific dipeptidyl peptidases are emerging as a protease family with important implications for the regulation of signaling by peptide hormones.

Human DPPIV is a membrane-anchored 110 kDa serine protease expressed on various cell types (44–46). The extracellular domain of DPPIV encodes an ectopeptidase and is able to cleave N-terminal dipeptides from polypeptides with either proline or alanine at the penultimate position (47, 48). Therefore, DPPIV shows a variety of functions, including regulation of inflammatory and immunological responses, signal transduction, and apoptosis by degrading physiological substances, such as chorionic gonadotropin and substance P, TNF-α, IL-2, and various chemokines including CCL5 (RANTES) (46, 49, 50). Originally characterized as a T cell differentiation Ag (CD26), human DPPIV plays a role in tumor progression, such as cell adhesion, invasion, and cell cycle arrest (51–54). Interestingly, soluble DPPIV activity is found in human serum and supposed to exert similar effects (55).

DPPIV activity has also been reported in snake venoms of different species (56), and a corresponding cDNA of a snake DPPIV has been cloned. However, the presence of DPPIV in Hymenoptera venoms has remained obscure. A single report of DPPIV activity suggested the presence of the enzyme in venom gland extracts of queen bees (57); nevertheless, neither in *A. mellifera* nor *V. vulgaris* venom such an activity has been reported. Insect venom DPPIV may function through the conversion of venom components into their active forms in the venom gland on

the one hand and the enhancement or decrease of the chemotactic activity of immune cells after the insect sting on the other hand. The former may be confirmed by the hypothesis that promelittin is processed into its active form in a stepwise manner by enzymes of the DPPIV type in vitro (57). Given a relative protein content of 1% Api m 5 in native HBV and an amount of 50–140 µg protein delivered per honeybee sting (58), 0.5–1.5 µg DPPIV is injected into the skin per stinging event. However, further studies will be necessary to prove whether the amounts of enzyme injected are actually sufficient to modulate local immune responses in humans.

In summary, we have identified Api m 5 and Ves v 3 as DPPIV homologues in the venom of *A. mellifera* and *V. vulgaris* as major allergens in insect venom allergy. The characterization of DPPIV homologues may provide further insights into potential immunomodulatory functions of different Hymenoptera venoms. Additionally, our results demonstrate for the first time the clinical relevance of Api m 5 and Ves v 3 as high m.w. allergens in IgE-mediated Hymenoptera venom allergy. The recombinant allergens Api m 5 and Ves v 3 will represent valuable tools for the improvement of current diagnostic tests and immunotherapy of insect venom allergy. These findings provide a novel view on the molecular patterns of allergic IgE sensitization in venom allergy that requires reconsideration of current and future concepts for component-resolved diagnosis and specific immunotherapy of Hymenoptera venom allergy.

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Disclosures

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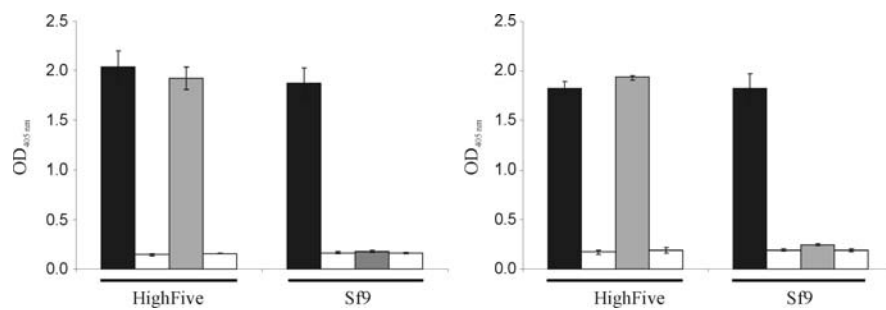
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Supplementary data Figure 1:

Figure 1: ELISA analyses of the immunoreactivity of recombinant proteins. Assessment of recombinant Api m 5 (left panel), and Ves v 3 (right panel) derived from either Sf9 or HighFive insect cells for presence of α -1,3-core fucosylation was performed using the anti-HRP-antibody and anti-rabbit-IgG-AP conjugate (grey bars). Normalisation of the amounts of immobilised proteins was performed employing an anti-V5 epitope antibody and anti-mouse IgG-AP conjugate (dark bars), for controls antigen was omitted (white bars).



Supplementary data Table I: IgE binding to honeybee (i1) or yellow jacket (i3) venom for the patient sera used in ELISA analyses as tested in either UniCAP250 or Immulite2000 sIgE assays.

Patient I.D.	sIgE Honeybee venom (i1) (kU/L)	Patient I.D.	sIgE Yellow jacket venom (i3) (kU/L)
1	7.84	1	0.62
2	2.32	2	0.35
3	11.8	3	43.7
4	2.83	4	2.85
5	39	5	24
6	52.8	6	1.04
7	0.35	7	27
8	0.45	8	3.24
9	26.8	9	19.2
10	4.74	10	>100
11	5.48	11	1.81
12	14.8	12	4.12
13	4.99	13	15.5
14	21	14	8.69
15	3.61	15	>100
16	19.3	16	41.9
17	n.d. (class 4)	17	10.3
18	>100	18	>100
19	8.32	19	14.6
20	14.2	20	9
21	20.5	21	3.67
22	19.6	22	13.7
23	5.33	23	n.d.
24	4.01	24	n.d.
25	8.52	25	6.41
26	1.49	26	1.67
27	20.6	27	14.8
28	12.6	28	42.6
29	14.1	29	4.23
30	16.1	30	>100
31	5.2	31	>100
32	21	32	17.3
33	6.23	33	1.36
34	5.04	34	n.d.
35	1.44	35	14.5