

Human monoclonal IgG antibodies derived from a patient allergic to birch pollen as tools to study the in situ localization of the major birch pollen allergen, Bet v 1, by immunogold electron microscopy

Monika Grote, PhD,^a Petra Wiedemann, MSc,^b Serge Lebecque, MD,^c and Rudolf Valenta, MD^b Münster, Germany, Vienna, Austria, and Dardilly, France

Background: Bet v 1, the major birch pollen allergen, and related allergens present in various tree pollens, fruits, and vegetables represent a family of important cross-reactive allergens. Although the DNA, deduced amino-acid sequence, and structure of Bet v 1 have been determined, little is known regarding its biologic functions.

Objective: Human monoclonal antibodies derived from an individual allergic to birch pollen were used for refined ultrastructural localization of Bet v 1 in birch pollen grains to gain information regarding the allergen distribution and its possible biologic function. These data were to be supplemented by sequence analyses.

Methods: Ultrathin sections of anhydrously prepared birch pollen grains were incubated with human monoclonal antibodies (BAB1, BAB2, and BAB4). The binding sites were visualized in the transmission electron microscope by gold-conjugated anti-human IgG antibodies.

Results: In the cytoplasm of the birch pollen grain, human monoclonal antibodies bound to ribosome-rich areas and to pollen nuclei. Sequence analysis of Bet v 1 and homologous allergens identified a highly conserved p-loop motif in these proteins, which is typically found in nucleotide-binding proteins. **Conclusions:** Immunolocalization of Bet v 1 to ribosome-rich areas and the nucleus would be consistent with a highly conserved biologic function of Bet v 1 and homologous proteins as nucleotide binding proteins and explains why this group of plant proteins represents highly cross-reactive plant allergens against which many type I patients are sensitized. (J Allergy Clin Immunol 1998;101:60-6.)

Key words: Tree pollen allergy, Bet v 1, human monoclonal antibodies, localization, immunogold electron microscopy, protein sequence analysis, p-loop motif

Tree pollen allergens play a major role in the elicitation of hay fever in Middle and Northern Europe and

Abbreviations used

PBS:	Phosphate-buffered saline
SDS-PAGE:	Sodium dodecylsulfate-polyacrylamide gel electrophoresis
BSA:	Bovine serum albumin
p-loop:	Phosphate-binding loop
PR:	Pathogenesis related

Northern America. This is demonstrated by the fact that up to 80% of all patients allergic to pollen are sensitized to allergens from birch, alder, and hazel.¹

The biochemical characterization of pollen allergens has made rapid progress. Thus the major birch pollen allergen, Bet v 1, was isolated, purified, sequenced, and expressed in *Escherichia coli* as a recombinant protein.²⁻⁴ Moreover, it was shown that Bet v 1 is a highly conserved allergen that occurs in a variety of pollen species and vegetable foods and leads to IgE-mediated cross-reactivities in a high percentage of allergic patients.^{5, 6} Although numerous Bet v 1 homologous proteins have been sequenced,⁷ little information has so far been gathered concerning a possible biologic function of this protein. Only two hints have so far been obtained. First, Bet v 1 shows sequence homology with a group of plant proteins referred to as pathogenesis-related (PR) proteins.² Although expression of these proteins is activated by a variety of stimuli (e.g., attack by microorganisms, treatment with salicylic acid, and darkening⁸), the biologic function of these PR proteins has so far remained enigmatic. Second, Bet v 1 was recently found to show ribonuclease-like activity in vitro.^{9, 10}

In this study we wanted to obtain further clues regarding possible biologic functions of this allergen from an improved ultrastructural localization by immunogold electron microscopy. To this end we used human monoclonal antibodies with specificity for the major birch pollen allergen, Bet v 1. These antibodies were obtained by using B cells from a patient allergic to birch pollen. B-cell clones secreting Bet v 1-specific antibodies were established using the anti-CD40 system and subsequent Epstein Barr virus immortalization. Each of these human monoclonal antibodies recognizes a different

From ^aInstitute of Medical Physics and Biophysics, University of Münster, Münster; ^bInstitute of General and Experimental Pathology, AKH, University of Vienna, Vienna; and ^cSchering-Plough, Laboratory for Immunological Research, Dardilly.

Supported in part by grant S06703 of the Austrian Foundation of Science and by a grant from the Austrian Ministry of Science and Transports. Received for publication Apr. 8, 1997; revised June 26, 1997; accepted for publication June 30, 1997.

Reprint requests: Monika Grote, PhD, Institute of Medical Physics and Biophysics, University of Münster, Robert-Koch-Str. 31, D-48149 Münster, Germany.

Copyright © 1998 by Mosby, Inc.
0091-6749/98 \$5.00 + 0 1/1/85513

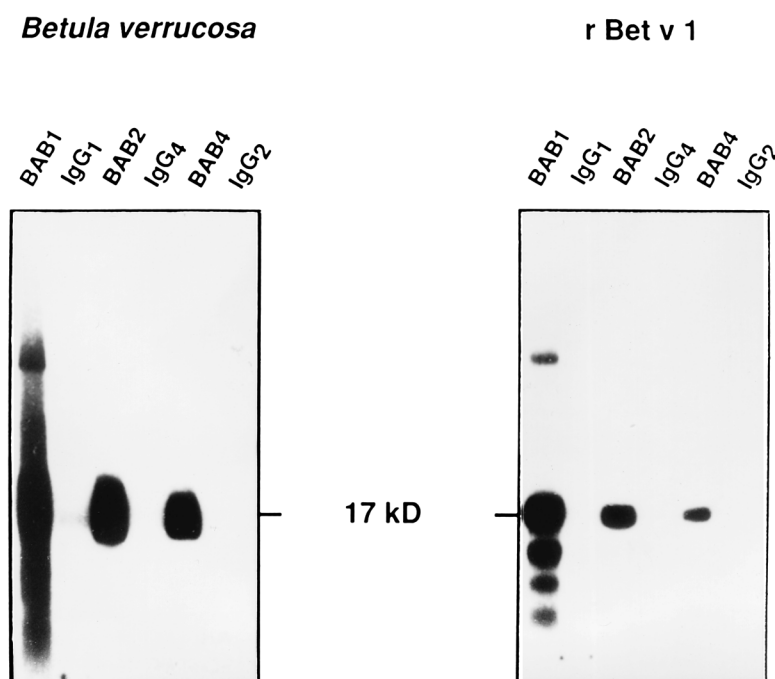


FIG. 1. Detection of nitrocellulose-blotted natural birch pollen extract (*Betula verrucosa*) and recombinant Bet v 1 (r Bet v 1) with human monoclonal antibodies BAB1, BAB2, BAB4, and isotype controls (IgG1, IgG2, IgG4).

epitope on the Bet v 1 molecule. One of them was shown to inhibit the binding of Bet v 1-specific IgE antibodies on the allergen molecule, thus creating the possibility of its use as a potential drug in clinical allergen therapy.¹¹

METHODS

Preparation of natural birch pollen extract

White birch (*Betula verrucosa*) pollen was purchased from Allergon, Valinge, Sweden. Protein extracts were prepared by shaking 1 gm pollen in 25 ml distilled water for 2 hours at room temperature. The aqueous extract was then centrifuged at 18,000 rpm to remove debris and lyophilized in 1 ml portions. The amount and quality of the extracts were estimated by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE)¹² and Coomassie blue staining.^{13, 14}

Production of recombinant Bet v 1

Recombinant Bet v 1 was expressed as a nonfusion protein by using plasmid pET (Novagene, Madison, Wis.), which was transformed into *E. coli* BL21 (DE3).¹⁵ The protein was purified by conventional chromatography.³

Human monoclonal antibodies

Human B cells producing Bet v 1-specific monoclonal antibodies (BAB1, BAB2, and BAB4)¹¹ were cultivated in RPMI 1640 medium supplemented with 2 mmol/L l-glutamine, 50 μ mol/L β -mercaptoethanol, 100 μ g/ml gentamicin (Gibco, Gaithersburg, Md.), and 10% fetal calf serum (Sera Lab, Crawley Down, U.K.). Human monoclonal antibodies were purified from the culture supernatant by protein-G affinity (Pharmacia, Uppsala, Sweden). Antibodies were checked for purity by SDS-PAGE, and the protein contents were adjusted according to photometry at 280 nm.

Binding and specificity of the purified monoclonal antibodies

were checked by immunoblotting and ELISA, with purified natural and recombinant Bet v 1 and recombinant birch profilin (Bet v 2)^{16, 17} as a negative control.

Human immunoglobulin isotype controls

Immunoglobulin isotype controls were purchased from Sigma (St. Louis, Mo.) and adjusted to the same protein concentration as the purified Bet v 1-specific monoclonal antibodies according to photometry (280 nm).

SDS-PAGE and immunoblotting

Birch pollen extract (approximately 150 μ g/cm gel) and recombinant Bet v 1 (2 μ g/cm gel) were separated in a 12.5% preparative SDS-PAGE according to the method of Fling and Gregerson¹² and electroblotted onto nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany).¹⁸ Nitrocellulose strips were blocked in buffer A (50 mmol/L Na-phosphate, pH 7.5, 0.5% wt/vol bovine serum albumin [BSA], 0.5% Tween 20, 0.05% NaN₃) incubated with purified human Bet v 1-specific monoclonal antibodies diluted 1:100 in buffer A or with purified immunoglobulin isotype controls overnight at 4°C. Strips were washed and probed with a 1:1000 diluted rabbit anti-human IgG antiserum (Dako, Glostrup, Denmark) for 2 hours at room temperature. After washing, bound antibodies were detected with a 1:2000 diluted iodine 125-labeled donkey anti-rabbit antiserum (Amersham, Buckinghamshire, U.K.). Washed strips were exposed to Kodak XOMAT films using intensifying screens at -70°C overnight.

Anhydrous preparation of birch pollen for electron microscopy

Anhydrous preparation of birch pollen for electron microscopy is crucial for retaining the highly water-soluble allergens in the pollen grain. It was performed as described earlier.¹⁹ In

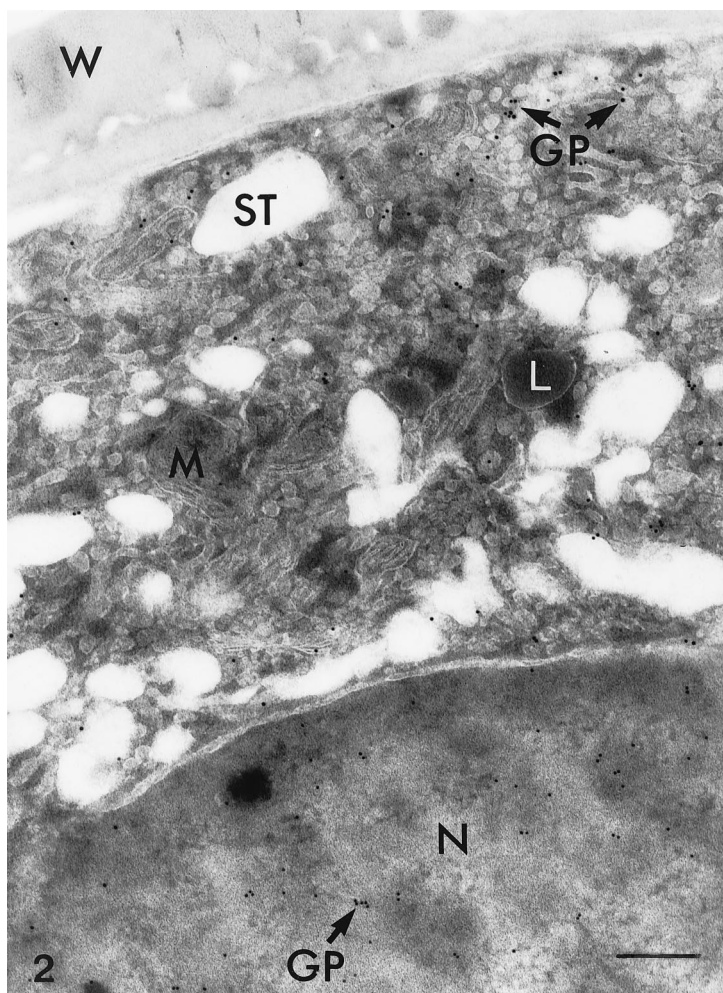


FIG. 2. Survey of pollen grain showing part of pollen wall, peripheral cytoplasm, and nucleus after mapping of Bet v 1 with human monoclonal antibody BAB4 and goat anti-human IgG/gold. Gold particles are distributed in cytoplasm and nucleus, whereas there is no labeling in pollen wall (magnification $\times 40,000$; bar: $0.25 \mu\text{m}$). GP, Gold particle; ST, starch; L, lipid; M, mitochondrion; N, nucleus; W, wall.

short, native birch pollen was fixed in *p*-formaldehyde vapor, dehydrated in dimethoxypropane and ethanol, and embedded in Lowicryl K4M (Plano, Wetzlar, Germany) at -35°C . Ultrathin sections (silver color) were cut on an Ultracut S (Leica, Bensheim, Germany).

In another approach birch pollen grains were fixed in an anhydrous liquid consisting of 6% *p*-formaldehyde dissolved in water-free glycerol, dehydrated in dimethoxypropane and ethanol, and embedded in Lowicryl K4M as above.²⁰

Postembedding immunogold labeling procedure using vapor-fixed birch pollen grains

Sections were incubated face down in a moist chamber on the following solutions: (1) 5% BSA in PBS, pH 7.4, for 15 minutes; (2) monoclonal human antibodies (either BAB1, diluted 1:5 in PBS, pH 7.4; BAB2, diluted 1:5 in PBS, pH 7.4; or BAB4, diluted 1:2 in PBS, pH 7.4, for 2 hours); (3) washing in PBS, pH 7.4, (1×5 minutes), Tris-BSA, pH 8.2, (2×5 minutes); (4) goat anti-human IgG (heavy and light chains), coupled to 10 nm

colloidal gold particles (British BioCell, Cardiff, U.K.), diluted 1:10 in Tris-BSA, pH 8.2, for 1 hour; (5) washing in Tris-BSA, pH 8.2 (1×5 minutes) and distilled water (2×5 min).

Controls

Controls were performed by replacing the primary monoclonal human antibody with normal human immunoglobulin isotypes appropriately diluted in PBS: BAB1 with IgG1 (kappa), BAB2 to IgG4 (kappa), and BAB4 with IgG2 (kappa).

Staining and documentation

The labeled sections were stained for 3 minutes in uranyl acetate and 5 seconds in lead citrate. They were analyzed in a Hitachi H500 (Nissei Sangyor, Tokyo, Japan) or Philips EM410 transmission electron microscope (Philips Electron Optics, Eindhoven, The Netherlands) operated at 75 kV and 80 kV, respectively. Agfa 23D56 films (Agfa, Leverkusen, Germany) were used for documentation.

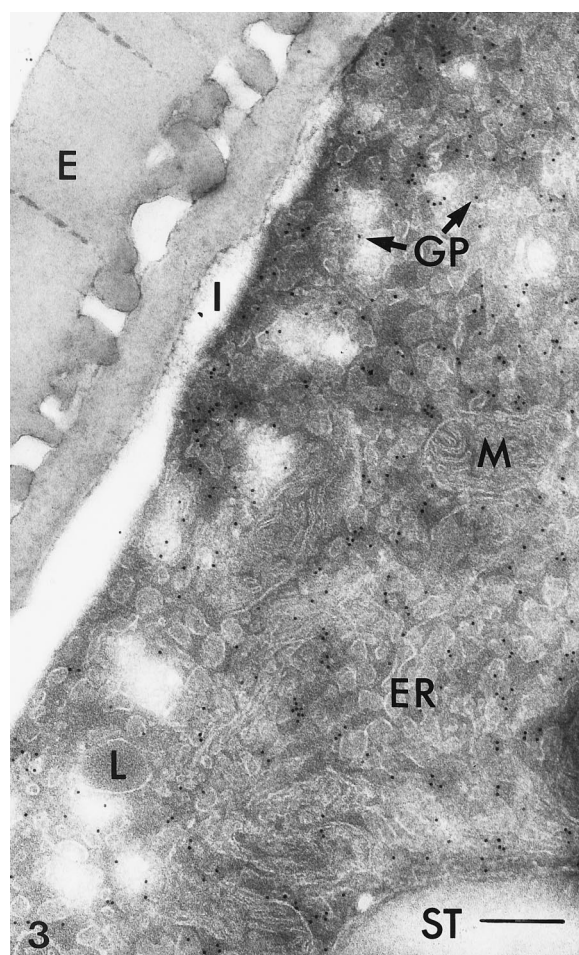


FIG. 3. Part of pollen grain showing wall and cytoplasm after probing with human monoclonal antibody BAB1 followed by goat anti-human IgG/gold. Gold markers predominantly associate with electron-dense matrix between various cell organelles like mitochondria, dictyosomes, endoplasmic reticulum, and vesicles (magnification $\times 46,000$; bar: $0.25 \mu\text{m}$). E, Exine; ER, endoplasmic reticulum; GP, gold particle; I, intine; L, lipid; M, mitochondrion; ST, starch.

Sequence analysis of Bet v 1 and homologous proteins

Bet v 1 homologous proteins were searched in the databases using the BLASTP program and retrieved from the National Institute of Health database. The Bet v 1 amino acid sequence was analyzed for subsequence patterns using the MacVector Program (Kodak, Rochester, N.Y.). Bet v 1 and homologous protein sequences retrieved from the databases were aligned by hand and gaps were introduced to allow maximal fit. The following representative proteins were compared: the major birch pollen allergen, Bet v 1 (MPAV_BETVE); the major alder allergen, Aln g 1 (MPAG_ALNGL); the major allergens from hornbeam pollen, Car b 1 (MPA1_CARBE), and hazel pollen, Cor a 1 (MPAA_CORAV); homologous allergens from apple, Mal d 1 (MAL1_MALDO), and celery, Api g 1 (MPAG_APIGR); and PR proteins from *Phaseolus vulgaris* (PR1_PHAVU), soybean (SAM2_SOYBN), *Lupinus luteus* (L1R18A), *Vigna unguiculata* (VUPR42), pea (DRR1_PEA), potato (PRS1_SOLTU), parsley (PR11_PETCR), and aspara-

gus (PR1_ASPOF). Additionally, the Bet v 1-derived polypeptide ENIEGNGG PGTIKKISFP containing the putative phosphate-binding loop (p-loop) motif was compared with the databases using the BLASTP program.

RESULTS

Purified human monoclonal antibodies (BAB1, BAB2, and BAB4) bound specifically to nitrocellulose-blotted natural and recombinant Bet v 1 (Fig. 1). BAB1 displayed binding to a 34 kd band and to several bands below 17 kd in both Bet v 1 preparations (from birch pollen extract and from recombinant preparation). The 34 kd component represents a Bet v 1 dimer, whereas the low molecular weight bands most likely represent proteolytic fragments of Bet v 1. BAB2 and BAB4 strongly bound to Bet v 1 at 17 kd. No reactivity of the purified immunoglobulin isotypes (IgG1, IgG2, and IgG4) with any of the nitrocellulose blotted proteins was observed (negative control).

Figs. 2 through 4 present immunogold labeling results in ultrathin sections of anhydrously fixed birch pollen grains after use of three different human monoclonal antibodies for localization of Bet v 1.

For a survey of birch pollen morphology, Fig. 2 shows a large segment of the whole birch pollen grain after fixation in p-formaldehyde vapor. Part of the pollen wall (exine and intine), part of the peripheral cytoplasm with well-preserved cell organelles (membranes appearing in negative contrast because of a lack of osmium fixation), and part of the (vegetative) nucleus are shown. In contrast to cell organelles like mitochondria, dictyosomes, and endoplasmic reticulum, ribosomes are not preserved during fixation of birch pollen in p-formaldehyde vapor. Instead, amorphous electron-dense material fills the spaces between the cell organelles (Figs. 2 through 5).

After incubation of the ultrathin section with monoclonal antibody BAB4 (Fig. 2), there are no gold particles in the pollen wall. However, gold labels are distributed throughout the cytoplasm and the nucleus.

The wall and peripheral part of the cytoplasm of the birch pollen grain are shown in Fig. 3 at a higher magnification. After incubation with monoclonal human antibody BAB1, gold particles are found to be mostly associated with the electron-dense matrix between cellular organelles like mitochondria, cisternae of endoplasmic reticulum, and vesicles. Fig. 4 shows a highly magnified detail from the cytoplasm of a birch pollen grain after incubation with monoclonal antibody BAB2. Again, gold markers are predominantly localized in the electron-dense cytoplasmic matrix near vesicles and cisternae. The inset of Fig. 4 shows detail of the rough endoplasmic reticulum of a birch pollen grain after anhydrous-liquid fixation in p-formaldehyde/glycerol. It becomes obvious that in contrast to the vapor technique this fixation preserves ribosomes that closely line the cisternae of the reticulum and fill the cytoplasmic matrix. Otherwise the ultrastructural morphology of the pollen grain appears much the same as after fixation in p-

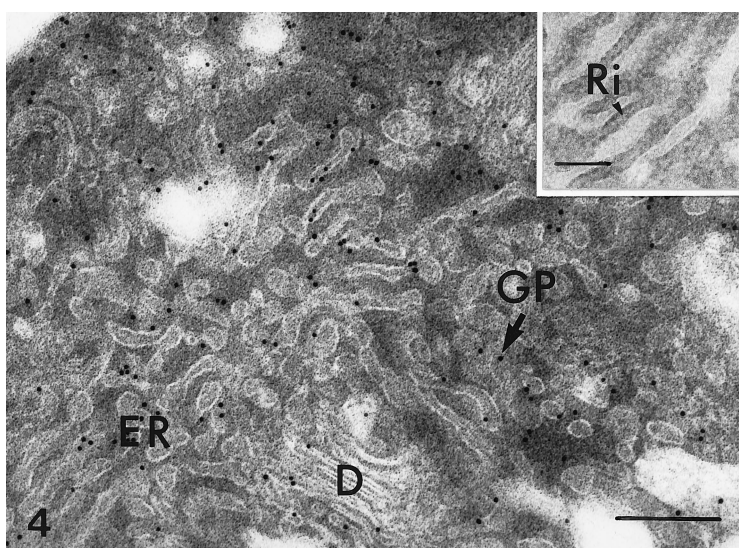


FIG. 4. Detail of pollen grain cytoplasm after probing with human monoclonal antibody BAB2 followed by goat anti-human IgG/gold. Most gold particles are localized between numerous cisternae and vesicles (magnification $\times 54,000$; bar: $0.25 \mu\text{m}$). **Inset.** Detail of birch pollen cytoplasm after anhydrous-liquid fixation in p-formaldehyde/glycerol. Well-preserved ribosomes line endoplasmic membranes (magnification $\times 56,000$; bar: $0.125 \mu\text{m}$). *D*, dictyosome; *ER*, endoplasmic reticulum; *GP*, gold particle; *Ri*, ribosome.

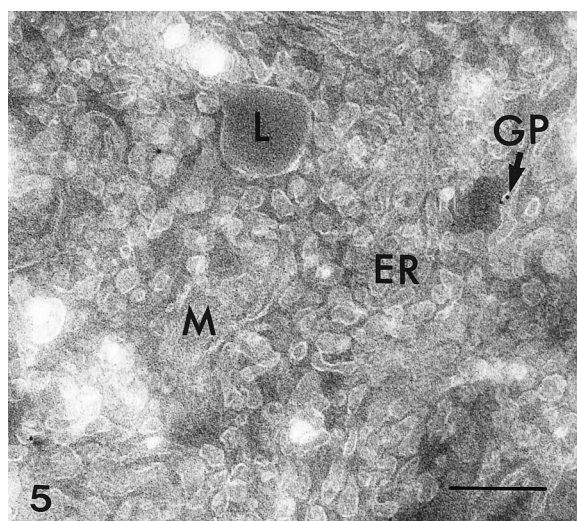


FIG. 5. Control experiment for BAB2. Section was labeled with normal human IgG (subclass 4, kappa) followed by goat anti-human IgG/gold. Detail from cytoplasm shows very few nonspecifically adsorbed gold particles (magnification $\times 54,000$; bar: $0.25 \mu\text{m}$). *ER*, Endoplasmic reticulum; *GP*, gold particle; *L*, lipid; *M*, mitochondrion.

formaldehyde vapor (not shown). Unfortunately, antigenicity is greatly reduced after this fixation; therefore it cannot be applied for use in the immune localization of pollen allergens.²⁰

The result of a control experiment is presented in Fig. 5. After replacing BAB2 with normal human IgG4 isotype, a very low degree of nonspecific adsorption of colloidal gold particles is seen.

In Fig. 6, the multiple sequence alignment of Bet v 1 and Bet v 1-homologous proteins is shown. Starting with aa 47, a p-loop-like motif consisting of GXGGXG followed by a lysine, serine, and/or threonine was identified. With the exception of a putative PR protein from potato, the portion containing the p-loop was highly conserved in all Bet v 1-homologous proteins. The high degree of conservation of the p-loop-containing motif was also shown by comparing the Bet v 1-derived polypeptide ENIEGNGGPGTIKKISFP with the databases. The loop-core motif (GNGGPG) was conserved in all sequences of Bet v 1 isovariants and in all Bet v 1-homologous allergens submitted to GenBank to date.

DISCUSSION

In this study we wanted to obtain more information about the ultrastructural localization of the Bet v 1 allergen and its possible biologic functions. Previous Bet v 1 immunocytochemistry used either mouse monoclonal antibodies, which recognize only one single epitope on the Bet v 1 molecule,¹⁹ or polyclonal human IgE antibodies, which may show specificities for allergenic proteins besides Bet v 1.²¹ Here we present the labeling patterns obtained with defined human monoclonal antibodies specific for Bet v 1, which were generated from an individual allergic to birch pollen. These antibodies are unrivaled in their ability to exactly trace the allergenic structures that are recognized by the patient's immune system. Although binding to different epitopes of Bet v 1, each antibody used showed a consistent association of the gold markers with two pollen compartments, cytoplasm and nuclei. In the cytoplasm it is the electron-dense "structureless" matrix that is preferentially labeled by gold particles (Figs. 2 through 4). However,

MPAV_BETVE	*GVFNYYETETTSVIPAAARLFKAFILDDGNLFKVPAPQAISSVENIE	GNGGPGTILK	KISFP*EGFPFKVKDRVDEVHDHN	80
MPAG_ALNGL	*-----A-P-----K-F-----E-V-----		---T-*--S-----E-----RV-	
MPA1_CARBE	*-----A-----SYV---K-I-----V---VG		---N-T-A*-I---F-E-----NA-	
MPAA_CORAV	*-----V-P-----SYV---K-I-----T---V-		---N-T-G*-SRY-----E-----N-	
PR1_PHAVU	*---TF-DQ---PVAP-T-Y---VAK-A-TI---AL-DSFK---IV		---V*-DGET-F-LHKIEST-EA-	
SAM2_SOYBN	M---TF-D-IN-PVAP-T-Y---LVT-A-VI---AL*DSFK---V		---T-L*-DGET-F-LHKIEST-EA-	
L1R18A	M-I-AF-N-QS-TVAP-K-Y---LTK-S-EIV---IEPIQ*-IV		---IAI*HDGHTSF-LHKL-AI-EA-	
VUPR42	MA---T*-DQ---PVAP-T-YN-LAK-A---II---AV*DSFK---V		---V*-DGET-F-LHKIETI-EA-	
MAL1_MALDO	*C-YTF-N-F-SE-PS-----V-A---I---I---KQA-IL		---T-G*-SQYG---H-I-SI-EAS	
DRR1_PEA	M---V-D-I---VAP-I-Y---LVT-A-T-T---I*D-K-I-IV		---A---LT-V*-DGET-H-LHK-EL---VA-	
PRS1_SOLTU	M-TS-TL---TPVAPT---LVV-S---I-IM-*VKNI-A*		---D***-S---MT-V*-S-I---L-HKIHV---DK-	
PR11_PETCR	M-QKS-V---SVS-EK---GLC---I-T-L-Q-L-G-K-S-TL		---D-V-V---LVHLG*DAS---TM-QK---AI-KAT	
MPAG_APIGR	*---QTHVL-L---SVS-EKI-QG-VI-V-TVL---A-G-YK---IK*		---D---L---I-TL-*D-G-IITMITL-I-G-NKEA	
PR1_ASPOF	MSSGSWSH-VAVNVA-G-M---AM-WH-G---IV-DF-AGGSVVS		---D-SV---R-E-KINNPAT---S---E-L-F---DK	

MPAV_BETVE	FKYNYSVIEGGPIGDILEKISNEIKIVATPDGGSILKISNKYHTKGDHEV*KAEQVKASKEMGETLLRAVESYLLAHSDAYN	162
MPAG_ALNGL	---SF---AV-A---VC-----A-----F-----I*N---I-IE---KAVG-K	
MPA1_CARBE	---T---DVL-K---V-H-L---A-G---V---S-F-A-Y---*N-KM-GA---K-----TAE---	
MPAA_CORAV	-T-S-T---DVL-K---VCH-L---A-G-----S-F-A---I*N-EM-GA---A-K-----T---AE---	
PR1_PHAVU	LG-S---IVG-VALPE-A---TFDS-LSDG-N---L-L-IT---S---APP*NEDEL---G-AKSDS-FK---A---NP****	
SAM2_SOYBN	LG-S---VG-AALP-A---TFDS-L-G-N---AG-LTV-E---A-P*NODEL-TG-AKADA-FK-I-A---P*-	
L1R18R	IT---I-G-EGIDES---Y-S---LPG---G-NV-F---VLS*ETVRDQ*-FK-LG-FK-I-G-V---P*-*	
VUPR42	-G-S---IVG-VGLPGEC---TIDT-LS-G---LI-L-IS-S---APP*NEDEL---G-AKSDA-FK---A---NA****	
MAL1_MALDO	YS-S-TL---DALT-I---Y-T-L---*CGS-TI-SISH---NI-I*-E-H-VG---KAHG-FKLI---KD-P---	
DRR1_PEA	LA---IVG-VGFP-V---F-A-LS-G-N---A-L-V-Y---AAAPTE---L-SD-AK-DG-FK-L-R-C---P*-	
PRS1_SOLTU	LVIK-M---DVL-K-S---YDL-FE-HGN-CVC-SIAE---YVL*-D-DHNEG-KQ-ME-FKI-A---NPSV-A	
PR11_PETCR	-T-S---I-D-DILLGFI-S-N-HFTA-PNA---CIV-STIIFN---AV*PE-NI-FANDQNL-IFK---A---I-N*****	
MPAG_APIGR	LTFD---D-DILLGFI-S-E-HVVL-P-A---C-TTAIF---AV*PE-NI-YAN-QNIA-FK-L-A---I-N*****	
PR1_ASPOF	-EVKQTLV---GL-KMF-CATTHE-FEPSSN-CLV-VTAS-***-ILPGV-DESAKA---GITNHMK-T-A---NPT-V	

FIG. 6. Multiple sequence alignment of Bet v 1² with Bet v 1 homologous proteins and allergens. Amino-acid sequence of Bet v 1 was aligned by hand with homologous proteins according to their database entry. Sources of proteins and sequence identities with Bet v 1 (MPAV_BETVE) are as follows: MPAG_ALNGL: major alder allergen, Aln g 1 (79.6%); MPA1_CARBE: major hornbeam allergen, Car b 1 (72.8%); MPAA_CORAV: major hazel allergen, Cor a 1 (71%); PR1_PHAVU: PR protein from *Phaseolus vulgaris* (43.8%); SAM2_SOYBN: PR protein from soybean (46.3%); L1R18R: PR protein from *Lupinus luteus* (45.1%); VUPR42: PR protein from *Vigna unguiculata* (43.2%); MAL1_MALDO: major apple allergen, Mal d 1 (53.1%); DRR1_PEA: PR protein from pea (51.2%); PRS1_SOLTU: PR protein from potato (43.2%); PR11_PETCR: PR protein from parsley (39.5%); MPAG_APIGR: major celery allergen, Api g 1 (38.9%); PR1_ASPOF: PR protein from asparagus (32.7%). Identical amino acids are indicated by dashes and blanks represent gaps introduced for maximal fit.

these electron-dense cytoplasmic areas can be characterized in more depth by alternative fixation and preparation procedures. For comparison, in the inset of Fig. 4 a detail from the cytoplasm of a birch pollen grain is presented that shows well-preserved ribosomes along the membranes of cisternae and between vesicles. From a comparison of the pollen morphologies obtained by the two different fixation techniques and the labeling pattern shown in Fig. 4, it can be concluded that birch pollen allergen Bet v 1 is predominantly associated with ribosome-rich areas. Even gold particles located in cell organelles, cisternae, and vesicles (Figs. 3 and 4) may well label an epitope outside these organelles, as simple measurements and calculations show when considering a spatial immunocytochemical resolution of 20 to 30 nm (distance between epitope and gold particle, "worst case resolution"²²) in our indirect antibody/gold technique.

Colocalization of allergens and ribosomes was also observed in grass pollen (*Phleum pratense*²³ and *Lolium perenne*²⁴).

The second pollen compartment distinctly labeled by

all anti-Bet v 1 antibodies is the pollen nucleus, which was also reported for grass pollen.²⁴ One explanation for this finding may be the fact that Bet v 1, which is a low molecular-weight (17 kd) protein, might passively diffuse through the peripheral channels of the nuclear pores²⁵ into the nucleoplasm.

However, recent observations regarding the biochemical behavior and molecular structure of the Bet v 1 molecule would fit well to the localization pattern observed. Reports showing RNase-like activity of Bet v 1^{9, 10} would be in agreement with ribosomal localization. In this investigation, sequence analysis of Bet v 1, homologous allergens, and PR proteins revealed a highly conserved p-loop-like motif that is typically found in nucleotide-binding proteins.²⁶ Among these proteins are elongation factors,²⁷ signal recognition particles²⁸ involved in protein biosynthesis, proteins constituting the nuclear matrix (e.g. CENP-F),²⁹ a terminase from a bacteriophage,³⁰ ras proteins, and chaperonins.³¹

Recently, Gajhede et al.³² investigated the three-dimensional structure and epitopes of Bet v 1 by x-ray

crystallography and nuclear magnetic resonance spectroscopy. The authors report the presence of a glycine-rich loop similar to those found in nucleotide-binding proteins. A possible function of the Bet v 1 protein as a nucleotide-binding protein would be in accordance with the immunolocalization of the allergen to ribosome-rich cell areas and the nucleus, and it may explain why many atopic individuals are cross-sensitized against these conserved proteins.

We thank Ms. Ursula Malkus and Ms. Gudrun Kiefermann for their technical assistance.

REFERENCES

1. Jarolim E, Rumpold H, Endler AT, Ebner H, Breitenbach M, Scheiner O, et al. IgE and IgG antibodies of patients with allergy to birch pollen as tools to define the allergen profile of *Betula verrucosa*. *Allergy* 1989;44:385-95.
2. Breiteneder H, Pettenburger K, Bito A, Valenta R, Kraft D, Rumpold H, et al. The gene coding for the major birch pollen allergen, Bet v 1, is highly homologous to a pea disease resistance response gene. *EMBO J* 1989;8:1935-8.
3. Ferreira F, Hoffmann-Sommergruber K, Breiteneder H, Pettenburger K, Ebner C, Sommergruber W, et al. Purification and characterization of recombinant Bet v 1, the major birch pollen allergen. Immunological equivalence to natural Bet v 1. *J Biol Chem* 1993;268:19574-80.
4. Valenta R, Duchêne M, Vrtala S, Birkner T, Ebner C, Hirschwehr R, et al. Recombinant allergens for immunoblot diagnosis of tree pollen allergy. *J Allergy Clin Immunol* 1991;88:889-94.
5. Ebner C, Hirschwehr R, Bauer L, Breiteneder M, Valenta R, Ebner H, et al. Identification of allergens in fruits and vegetables: IgE cross reactivities with the important birch pollen allergens Bet v 1 and Bet v 2 (birch profilin). *J Allergy Clin Immunol* 1995;95:962-9.
6. Valenta R, Kraft D. Type I allergic reactions to plant-derived food: a consequence of primary sensitization to pollen allergens. *J Allergy Clin Immunol* 1996;97:893-5.
7. Scheiner O, Kraft D. Basic and practical aspects of recombinant allergens. *Allergy* 1995;50:384-91.
8. Bowles DJ. Defense-related proteins in higher plants. *Annu Rev Biochem* 1990;59:873-907.
9. Bufer A, Spangfort MD, Kahlert H, Schlaak M, Becker WM. Major birch pollen allergen Bet v 1 shows ribonuclease activity. *Planta* 1996;199:413-5.
10. Swoboda I, Hoffmann-Sommergruber K, O'Riordáin G, Scheiner O, Heberle-Bors E, Vicente O. Bet v 1 proteins, the major birch pollen allergens and members of a family of conserved pathogenesis-related proteins, show ribonuclease activity in vitro. *Physiologia Plantarum* 1996;96:433-8.
11. Visco V, Dolecek C, Denépoux S, Le Mao J, Guret C, Rousset F, et al. Human IgG monoclonal antibodies that modulate the binding of specific IgE to birch pollen Bet v 1. *J Immunol* 1996;157:956-62.
12. Fling SP, Gregerson DS. Peptide and protein molecular weight determination by electrophoresis using a high molarity Tris buffer system without urea. *Anal Biochem* 1986;55:83-8.
13. Bradford MM. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye-binding. *Anal Biochem* 1976;72:248-54.
14. Vrtala S, Grote M, Duchêne M, Van Ree R, Kraft D, Scheiner O, et al. Properties of tree and grass pollen allergens: reinvestigation of the linkage between solubility and allergenicity. *Int Arch Allergy Clin Immunol* 1993;102:160-9.
15. Studier FW, Rosenberg AH, Dunn JJ, Dubendorf JW. Use of T7 RNA polymerase to direct expression of cloned genes. *Methods Enzymol* 1990;185:60-89.
16. Valenta R, Duchêne M, Pettenburger K, Sillaber C, Valent P, Bettelheim P, et al. Identification of profilin as a novel pollen allergen; IgE autoreactivity in sensitized individuals. *Science* 1991;253:557-60.
17. Susani M, Jertschin P, Dolecek C, Sperr WR, Valent P, Ebner C, et al. High level expression of birch profilin (Bet v 2) in *Escherichia coli*: purification and characterization of the recombinant allergen. *Biochem Biophys Res Commun* 1995;215:250-63.
18. Towbin H, Staehelin T, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci USA* 1979;76:4350-4.
19. Grote M. Immunogold electron microscopy of soluble proteins: localization of Bet v I major allergen in ultra-thin sections of birch pollen after anhydrous fixation techniques. *J Histochem Cytochem* 1991;39:1395-401.
20. Grote M. Ultrastructural morphology and allergen detection in birch pollen after aqueous, anhydrous-liquid, and vapor fixation techniques. *Microsc Res Tech* 1992;21:242-8.
21. Grote M, Valenta R. Über die Darstellung von Allergenen mit Hilfe der Elektronenmikroskopie. *Allergo Journal* 1994;3:144-7.
22. Griffiths G. Fine structure immunocytochemistry. Berlin: Springer; 1993. p. 271-5.
23. Grote M, Dolecek C, Van Ree R, Valenta R. Immunogold electron microscopic localization of timothy grass (*Phleum pratense*) pollen major allergens Phl p I and Phl p V after anhydrous fixation in acrolein vapor. *J Histochem Cytochem* 1994;42:427-31.
24. Staff JA, Taylor PE, Smith P, Singh MB, Knox RB. Cellular localization of water-soluble, allergenic proteins in rye-grass (*Lolium perenne*) pollen using monoclonal antibodies with immunogold probes. *Histochem J* 1990;22:276-90.
25. Jinshaw JE, Carragher BO, Milligan RA. Architecture and design of the nuclear pore complex. *Cell* 1992;69:1133-41.
26. Saraste M, Sibbald PR, Wittinghofer A. The p-loop: a common motif in ATP- and GTP-binding proteins. *Trends Biochem Sci* 1990;15:430-4.
27. Kjeldgaard M, Nissen P, Thirup S, Nyborg J. The crystal structure of elongation factor EF-Tu from *Thermus aquaticus* in the GTP conformation. *Structure* 1993;1:35-50.
28. Miller JD, Wilhelm H, Gierasch L, Gilmore R, Walter P. GTP binding and hydrolysis by the signal recognition particle during initiation of protein translocation. *Nature* 1993;366:351-4.
29. Nilsson L, Nygard O. Localization of the sites of ADP-ribosylation and GTP binding in the eukaryotic elongation factor EF-2. *Eur J Biochem* 1985;148:299-304.
30. Hwang Y, Catalano CE, Feiss M. Kinetic and mutational dissection of the two ATPase activities of terminase, the DNA packaging enzyme of bacteriophage Chi. *Biochemistry* 1996;35:2796-803.
31. Hartman DJ, Hoogenraad NJ, Condron R, Hoj PB. The complete primary structure of rat chaperonin 10 reveals a putative beta alpha beta nucleotide-binding domain with homology to p21 ras. *Biochim Biophys Acta* 1993;1164:219-24.
32. Gajhede M, Osmark P, Poulsen FM, Ipsen H, Larsen JN, Jost van Neerven RJ, et al. X-ray and NMR structure of Bet v 1, the origin of birch pollen allergy. *Nat Struct Biol* 1996;3:1040-5.