

Immunogold Electron Microscopic Localization of the Cross-Reactive Two-EF-Hand Calcium-Binding Birch Pollen Allergen Bet v 4 in Dry and Rehydrated Birch Pollen

Monika Grote^a Brigitte Hayek^b Rudolf Reichelt^a Dietrich Kraft^b
Rudolf Valenta^b

^aInstitute of Medical Physics and Biophysics, University of Münster, Münster, Germany, and ^bInstitute of General and Experimental Pathology, University of Vienna, Vienna, Austria

Key Words

Birch pollen · Anhydrous fixation · Immunogold electron microscopy · In situ localization · Bet v 4 · Two-EF-hand calcium-binding allergen · Cross-reactivity · Allergen · Hydration · Immunoblotting

Abstract

Background: Recently, a novel family of low-molecular-weight (8–9 kD), two-EF-hand calcium-binding proteins has been described as allergens in plant pollens. Approximately 10% of pollen-allergic patients have IgE antibodies which cross-react with the two-EF-hand allergens in tree, grass and weed pollens. The aim of the present study was to localize Bet v 4, the two-EF-hand allergen from birch, in mature, dry pollen and to study the release of this allergen after hydration of the pollen by immunogold electron microscopy. **Methods:** Using completely anhydrous fixation techniques in combination with immunogold electron microscopy, we localized Bet v 4 and, for control purposes, the major birch pollen allergen Bet v 1, in dry birch pollen as well as in pollen grains after different periods of hydration. Parallel with these morphological studies, we monitored the release of Bet v 4 and Bet v 1 into aqueous supernatants of hydrated birch pollen grains by immunoblotting.

Results: Bet v 4 was found in the electron-dense cytosol, in particular between the vesicles and cisternae of the endoplasmic reticulum, inside mitochondria and in the vegetative as well as in the generative nucleus. Bet v 1 was localized in similar cellular compartments except for the mitochondria. After 30 s to 1 min of hydration, Bet v 4 migrated into the pollen exine and into the aqueous supernatants. Bet v 1 also moved out of the pollen grain, though not as quickly as Bet v 4. **Conclusion:** Bet v 4 represents an intracellular pollen protein which, following hydration of pollen grains, rapidly migrates to the pollen surface (exine) and is washed out. This behavior explains how Bet v 4, being primarily an intracellular pollen protein, becomes available to sensitize patients.

Copyright © 1999 S. Karger AG, Basel

Introduction

Birch pollen represents one of the most potent allergen sources in early spring in the middle and northern parts of Europe as well as North America [1]. Almost 95% of birch pollen-allergic patients display IgE reactivity to the major birch pollen allergen, Bet v 1, a 17 kD protein [2], which cross-reacts with the major allergens of other *Fagales* pol-

lens (alder, hazel, hornbeam and oak) [3] and plant-derived food (fruits, vegetables, spices) [4–6]. Three other birch pollen allergens have been identified so far: Bet v 2, birch profilin, which is an actin-binding protein and probably one of the most cross-reactive allergens [7–9]; Bet v 3, a three-EF-hand calcium-binding allergen [10], and the recently discovered two-EF-hand birch pollen allergen Bet v 4 [11, 12]. Bet v 4 belongs to a novel family of calcium-binding allergens which contain two calcium-binding domains, termed EF hands [13]. It shares IgE epitopes with the structurally related two-EF-hand calcium-binding allergens present in grass pollens (Bermuda grass, Cyn d 7 [14, 15]; timothy grass, Phl p 7 [16]), weed pollens (oilseed rape, Bra r 1, and Bra r 2 [17]), and tree pollens (alder, Aln g 4 [18]; olive, Ole e 3 [9]). All of these two-EF-hand allergens are primarily expressed in mature pollen, contain two calcium-binding domains, cross-react with IgE antibodies in approximately 10–20% of pollen-allergic individuals and thus constitute a family of pollen ‘panallergens’. Two-EF-hand allergens such as Bet v 4 can occur in two conformational states, the calcium-bound form and the calcium-free form (i.e. apoform) which differ substantially in their conformation and IgE-binding capacity [13, 16, 18]. While the calcium-bound form strongly reacts with IgE antibodies, the apoform has a greatly reduced IgE-binding capacity. Although Bet v 4 represents an allergen less frequently recognized than Bet v 1, the major birch pollen allergen, it is clinically relevant for at least two reasons: (1) patients who are sensitized to Bet v 4 show IgE cross-reactivity and clinical symptoms to the Bet v 4-related allergens in tree, grass and weed pollens and thus display allergic symptoms on contact with pollens of botanically unrelated plants [16, 18], and (2) biological testing (basophil histamine release experiments, skin prick testing) has revealed a strong allergenic activity of the two-EF-hand allergens [16, 18]. In the present investigation, we were interested in determining the *in situ* localization of Bet v 4 by immunogold electron microscopy and to investigate, by both histochemical techniques and immunoblotting, the mode and kinetics of Bet v 4 release after hydration of birch pollen. The results obtained are discussed in the context of the sensitization capacity of this allergen and its possible biological properties.

Materials and Methods

Pollen, Sera and Antibodies

Pollen from birch (*Betula verrucosa*) was obtained from Sigma (St. Louis, Calif., USA). Bip1, a mouse monoclonal anti-Bet v 1 IgG1 antibody, has been described elsewhere [20]. A rabbit antiserum that cross-reacts with two-EF-hand allergens from trees, grasses and

weeds was raised against purified recombinant Aln g 4 [18]. A ¹²⁵I-labeled sheep anti-mouse Ig antiserum and a ¹²⁵I-labeled donkey anti-rabbit Ig antiserum were purchased from Amersham (Little Chalfont, Bucks., UK). Goat anti-rabbit IgG antibodies coupled to 10-nm colloidal gold particles (GAR IgG 10) and goat anti-mouse IgG antibodies coupled to 20-nm colloidal gold particles (GAM IgG 20) were obtained from BritishBioCell (Plano, Wetzlar, Germany).

Hydration of the Pollen

Fifty micrograms of birch pollen were divided into aliquoted portions in 1.5-ml Eppendorf vials. One milliliter of H₂O double distilled was added to each vial. The contents were mixed immediately and incubated at room temperature for different periods (30 s, 1, 5, 10 and 30 min, 1 and 2 h). The aqueous supernatants were separated from the pollen grains by centrifugation in a bench centrifuge (12,000 rpm, room temperature, 30 s). Then the aqueous supernatants were immediately transferred to new Eppendorf tubes, frozen in liquid nitrogen and stored at –20°C until use.

SDS-PAGE and Immunoblotting

Fifty-microliter aliquots of each supernatant were separated by 12.5% analytical SDS-PAGE [21] and blotted onto nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany) as described previously [22]. Blots were blocked in buffer A (50 mM Na-phosphate, pH 7.5, 0.5% w/v BSA, 0.5% v/v Tween 20, 0.05% NaN₃) and incubated with the mouse monoclonal antibody Bip 1 or rabbit anti-rAln g 4 antiserum overnight at 4°C. Bound mouse and rabbit antibodies were detected with a ¹²⁵I-labeled sheep anti-mouse and donkey anti-rabbit antiserum, respectively.

Preparation of Dry and Hydrated Birch Pollen for Electron Microscopy

Dry pollen was dusted onto a clean glass slide to give a monolayer. Pollen grains hydrated for 30 s, 1, 5 and 10 min (see above) were put onto a glass slide immediately after centrifugation and distributed with a spatula to give a thin layer. All glass slides (with dry and hydrated pollens) were put into a desiccator and fixed in the vapor of *p*-formaldehyde powder for 48 h. Dehydration, embedding in Lowicryl resin and polymerization were carried out based on the technique described previously [23]. Ultrathin sections were cut on a Reichert Ultracut S ultramicrotome (Leitz, Bensheim, Germany) and collected on nickel grids for immunocytochemistry.

Immunogold Labeling Procedure

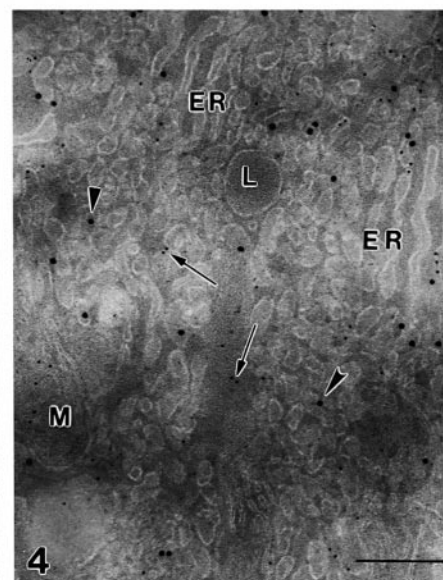
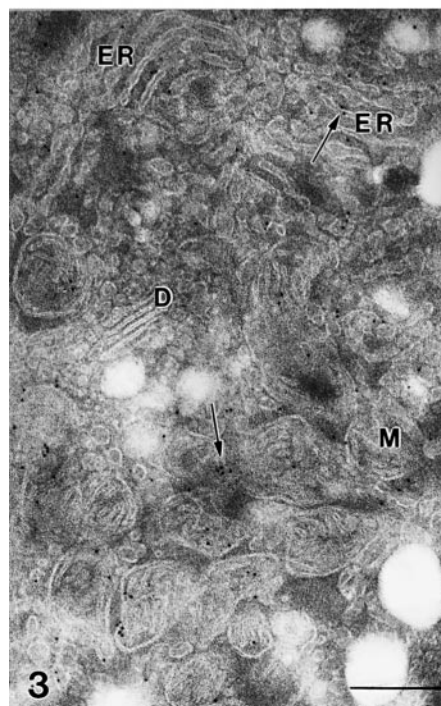
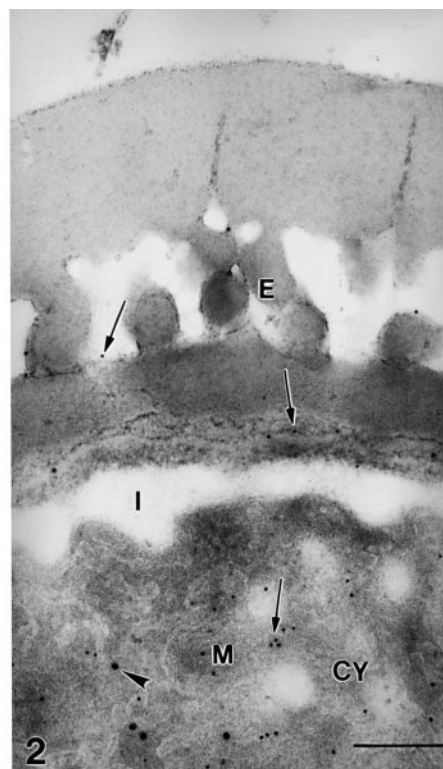
The grids were incubated with the following solutions (face down in a moist chamber, with PBS buffer +1% BSA at pH 7.4): (1) 5% (w/v) BSA in PBS for 15 min; (2) rabbit anti-rAln g 4 antibody diluted 1:25 in PBS+BSA, for 2 h; (3) PBS+BSA for 3×5 min; (4) GAR IgG 10, diluted 1:20 in PBS+BSA, for 1 h, and (5) PBS+BSA for 1×5 min, then distilled water for 2×5 min. In some experiments, double labeling for Bet v 4 and Bet v 1 was performed. It was carried out in the following way: after step (4) of the above protocol, the sections were washed in (5) PBS+BSA for 3×5 min; (6) mouse anti-Bet v 1 antibody (Bip 1), undiluted, for 2 h; (7) PBS+BSA for 3×5 min; (8) GAM IgG 20, diluted 1:10 in PBS+BSA, for 1 h, and (9) PBS+BSA for 2×5 min, then distilled water for 1×5 min. The following controls were run: (1) the anti-rAln g 4 antibody was replaced by rabbit preimmune antiserum; (2) the anti-rAln g 4 antibody was omitted; (3) the anti-Bet v 1 antibody was replaced by a monoclonal antibody of irrelevant specificity, and (4) the anti-Bet v 1 antibody was omitted. After

Fig. 1. Low-magnification micrograph of an ultrathin section through a dry birch pollen grain anhydrously prepared for electron microscopy. The pollen wall surrounding the cytoplasm consists of an outer exine and an inner intine. It is partially detached from the cytoplasm. Inside the cytoplasm, reserve materials (starch, lipid) and the two pollen nuclei (vegetative nucleus and generative nucleus inside the generative cell) are prominent. Details for immunolocalization studies of allergens were taken from the pollen areas indicated by rectangles 1 and 2. $\times 6,620$. Bar = $1\ \mu\text{m}$.

Fig. 2. Detail from the pollen wall and peripheral cytoplasm of dry birch pollen after double immunogold labeling for Bet v 4 allergens (small gold particles, arrows) and Bet v 1 allergens (large gold particles, arrowhead). Bet v 4 is found inside the exine and in the cytoplasm, while Bet v 1 is located only in the cytoplasm. $\times 46,580$. Bar = $0.25\ \mu\text{m}$.

Fig. 3. High-magnification micrograph of dry birch pollen cytoplasm after immunogold labeling for Bet v 4. Cytoplasmic organelles are clearly preserved, with membranes appearing in negative contrast due to lack of osmium impregnation. Most gold particles (arrows) are located in the electron-dense matrix (cytosol) between the numerous vesicles and cisternae. Mitochondria, too, are often decorated with gold particles. $\times 47,700$. Bar = $0.25\ \mu\text{m}$.

Fig. 4. Details from dry birch pollen cytoplasm after double immunogold labeling for Bet v 4 allergens (small gold particles, arrows) and Bet v 1 allergens (large gold particles, arrowheads). Most allergens are located in the cytosol; however, some gold particles of both sizes are also found inside membrane-bound vesicles and cisternae or on the membranes of these organelles. Mitochondria only label for Bet v 4. $\times 46,200$. Bar = $0.25\ \mu\text{m}$.



labeling, the sections were stained with uranyl acetate for 3 min and lead citrate for 5 s.

Electron Microscopy and Documentation

The sections were analyzed in a Hitachi H500 transmission electron microscope operated at 75 kV. For image recording, Agfa-Gevaert 23D56 films were used.

Abbreviations mentioned in figures 1–13

CY = Cytoplasm; D = dictyosome; E = exine; ER = endoplasmic reticulum; GCM = generative cell membrane; GN = generative nucleus; I = intine; L = lipid; M = mitochondrion; R = ribosomes; ST = starch; V = vacuole; VN = vegetative nucleus; W = wall.

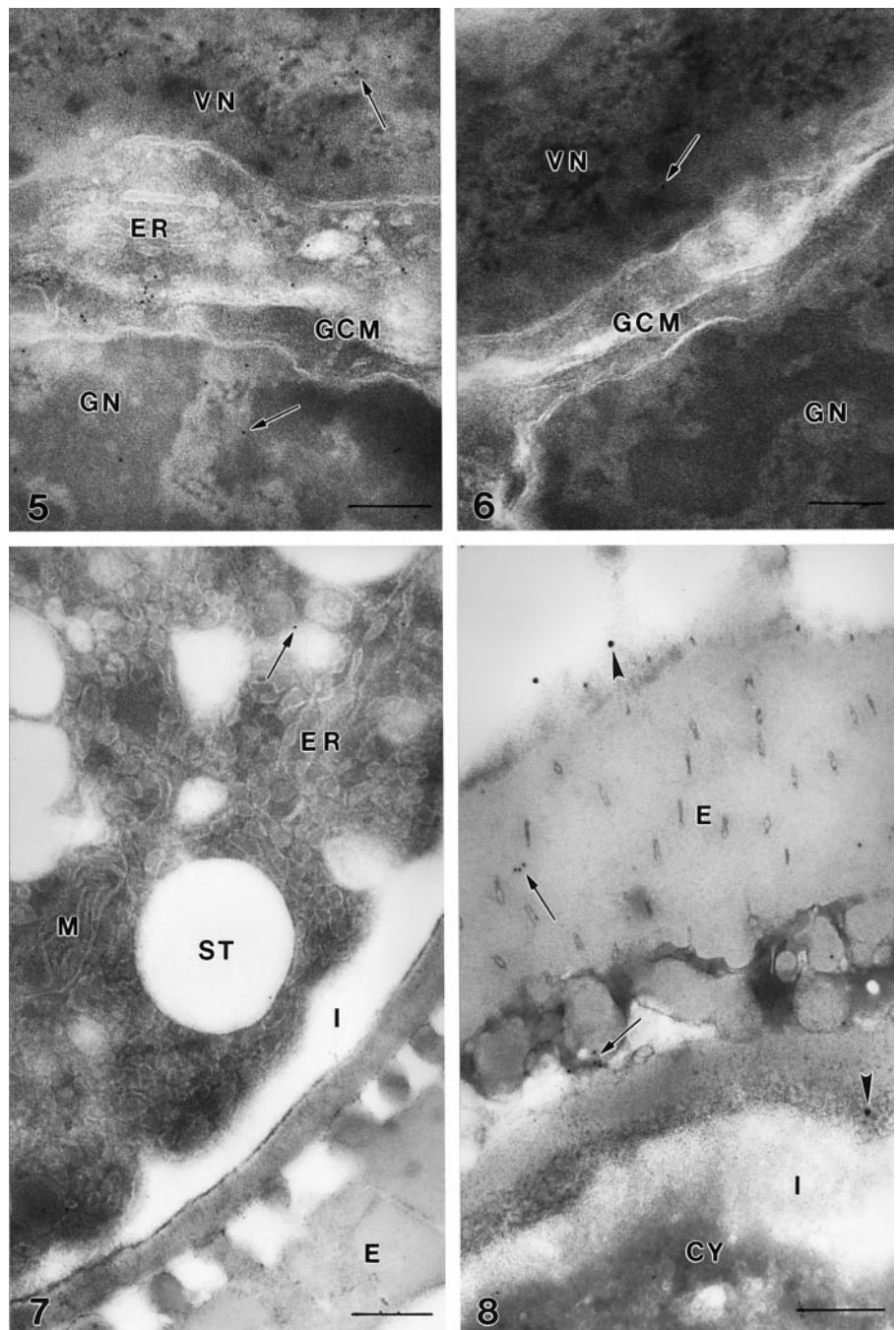


Fig. 5. Detail from the vegetative and generative nuclei of dry birch pollen after immunogold labeling for Bet v 4. The allergens (arrows) are found inside the generative cell and nucleus and inside the vegetative cytoplasm and nucleus. $\times 42,800$. Bar = $0.25 \mu\text{m}$.

Fig. 6. Control experiment. Dry birch pollen nuclei after incubation with rabbit preimmune antibodies. A small number of gold particles (arrow) indicate a low degree of background labeling. $\times 40,650$. Bar = $0.25 \mu\text{m}$.

Fig. 7. Control experiment. Dry birch pollen wall and cytoplasm after incubation with rabbit preimmune antibodies. Both pollen compartments are almost completely free from gold particles (arrow). $\times 40,650$. Bar = $0.25 \mu\text{m}$.

Fig. 8. Detail from the wall and peripheral cytoplasm of birch pollen rehydrated for 30 s. Double immunogold labeling for Bet v 1 allergens and Bet v 4 allergens. Bet v 1 (large gold particles, arrowheads) and Bet v 4 (small gold particles, arrows) are found in the pollen wall. The cytoplasm shows few gold particles. $\times 46,500$. Bar = $0.25 \mu\text{m}$.

Results

Localization of Bet v 4 in Dry Birch Pollen

Figures 1–7 document the localization of Bet v 4 in dry pollen. Figure 1 gives a view of a dry birch pollen grain fixed anhydrously for electron microscopy. The grain exhibits a shrunken shape, which is characteristic for pollen grains at the time of their dispersal from the catkin and in-

dicates their dehydrated state [23]. In figure 1, the pollen wall, consisting of the outer exine layer and the inner intine layer, is partially separated from the cytoplasm which probably must be considered a preparation artifact. Within the cytoplasm, reserve materials (starch and lipid) are prominent. The big vegetative nucleus has a rugged appearance. The generative cell, which is largely filled with the generative nucleus, is much smaller than the vegetative nucleus. It

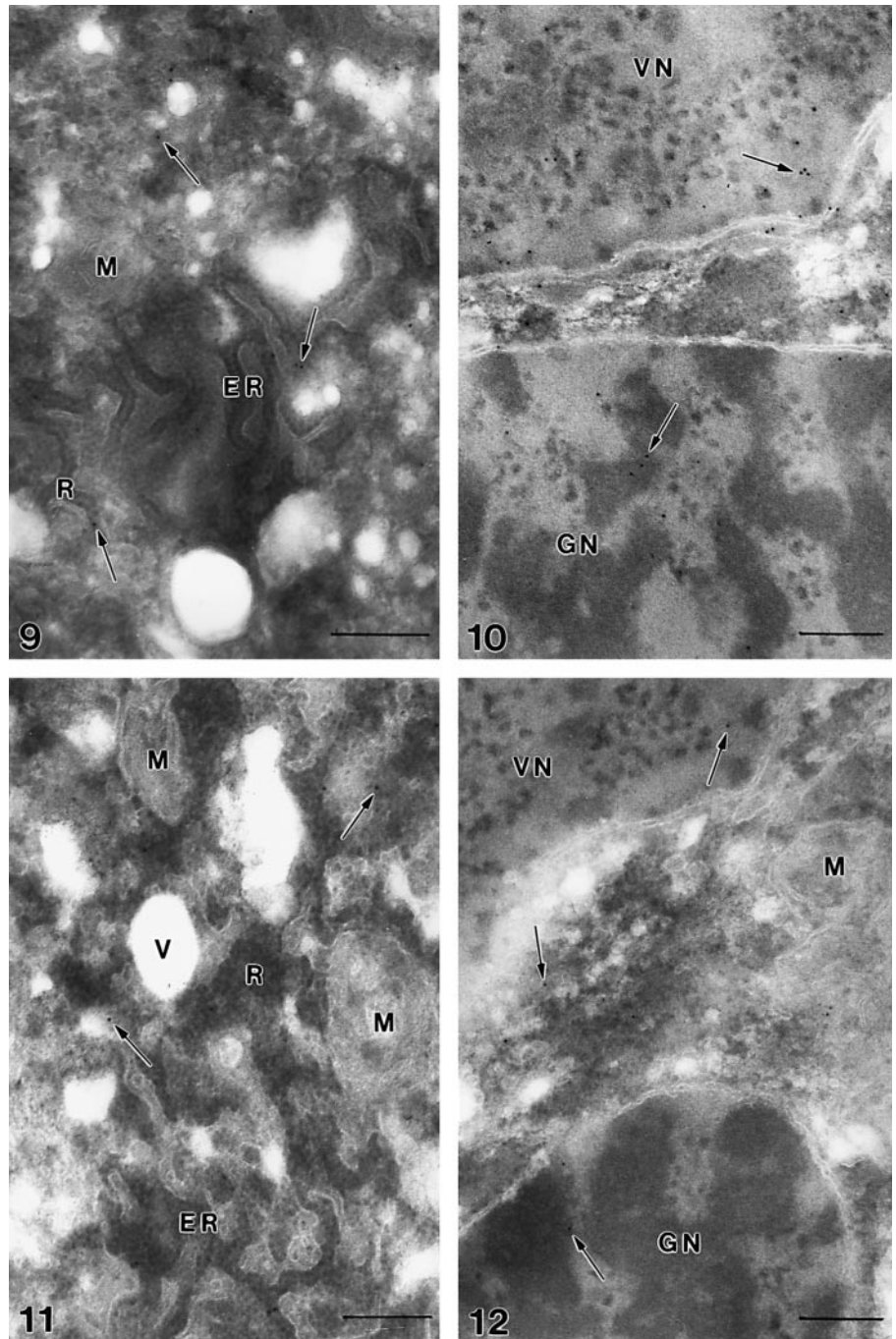


Fig. 9. High-magnification micrograph from the cytoplasm of birch pollen rehydrated for 30 s. Distinct signs of rehydration are visible (swelling of endoplasmic cisternae, vacuoles). After immunogold labeling for Bet v 4, few gold particles (arrows) are detected in the cytoplasm. $\times 52,820$. Bar = $0.25 \mu\text{m}$.

Fig. 10. Within pollen rehydrated for 30 s, both pollen nuclei (generative nucleus and vegetative nucleus) are distinctly immunogold labeled for Bet v 4 allergens (arrows). $\times 43,120$. Bar = $0.25 \mu\text{m}$.

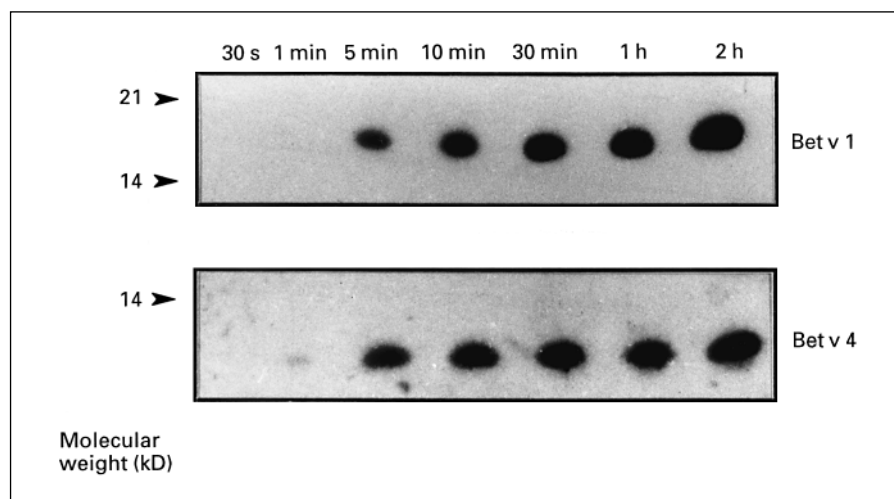
Fig. 11. Birch pollen grain rehydrated for 1 min. After incubation with anti-rAln g 4 antibodies, very few gold particles (arrows) occur within the cytoplasm. $\times 43,900$. Bar = $0.25 \mu\text{m}$.

Fig. 12. Birch pollen rehydrated for 1 min. Generative and vegetative nuclei are largely free from gold particles (arrows), indicating the presence of Bet v 4 allergens. $\times 42,900$. Bar = $0.25 \mu\text{m}$.

is roundish and often found juxtaposed to the vegetative nucleus. Details for the immunolocalization of allergens were taken from the pollen wall and/or the peripheral pollen cytoplasm (rectangle 1) and from the two pollen nuclei (rectangle 2). After double immunogold labeling for Bet v 4 and Bet v 1 (fig. 2), the pollen wall (exine) is sparsely but exclusively labelled with small gold particles, indicating the presence of Bet v 4. There are not Bet v 4 or Bet v 1 aller-

gens in the intine. The cytoplasm, however, contains small and large gold particles pointing to the presence of Bet v 4 and Bet v 1 allergens. Figure 3 shows details of the cytoplasmic location of Bet v 4 allergens at high magnification. Gold particles are preferentially found in the electron-dense cytosol, especially between the vesicles and cisternae of the endoplasmic reticulum and in the mitochondria. In comparison, figure 4 shows a detail from pollen cytoplasm after

Fig. 13. Aqueous supernatants obtained after different time periods of birch pollen hydration (top panel: 30 s, 1, 5, 10 and 30 min, 1 and 2 h). The allergen Bet v 1 was detected with the mouse monoclonal antibody Bip 1 and Bet v 4 was detected with a rabbit anti-rAln g 4 antiserum.



double labeling for Bet v 4 and Bet v 1. Most gold particles of both sizes are found in the cytoplasmic matrix, often close to the membranes of cisternae and vesicles. Some gold particles, however, occur inside these vesicles. In contrast, mitochondria are marked only with small gold particles, indicating the presence of Bet v 4. Figure 5 shows a detail from the vegetative nucleus and the juxtaposed generative cell and nucleus. As can be seen in this figure, Bet v 4 allergens are also found in both nuclei. In figures 6 and 7, the results of two control labeling experiments are shown. After incubating the pollen grain sections with rabbit preimmune serum instead of anti-Bet v 4 antibodies, both pollen nuclei (fig. 6) show very few nonspecifically adsorbed gold particles. Similarly, almost no gold particles can be detected in the pollen wall and pollen cytoplasm (fig. 7).

Bet v 4 Migrates Rapidly out of the Pollen Grain after Hydration

Figures 8–10 show the results of allergen localization in pollen grains hydrated for 30 s. In figure 8, Bet v 4 and Bet v 1 are found in the pollen wall (exine) and on the surface of the grain. Figure 9 gives a detail from the cytoplasm of briefly hydrated pollen grains after labeling for Bet v 4. First of all, this micrograph shows that the morphology of the cytoplasm has changed drastically even after such a short rehydration period as 30 s. Signs of hydration are the dilatation of the cisternae of the endoplasmic reticulum and the formation of many small vacuoles as a result of swelling [23, 24]. As figure 9 demonstrates, only a few gold markers labeling Bet v 4 molecules are observed in the cytoplasm of birch pollen after 30 s of rehydration. The pollen nuclei, however, are still labeled with gold particles (fig. 10), indi-

cating the presence of Bet v 4 allergens. Figures 11 and 12 show Bet v 4 localization in pollen grains rehydrated for 1 min. Not only the cytoplasm, but also the vegetative and generative nuclei, are now largely free from gold particles. This is also the case with the pollen wall (micrograph not shown). Pollen hydrated for 5 and 10 min, respectively, give much the same result as far as Bet v 4 localization is concerned. Bet v 1 allergens also move quickly out of the pollen grain, but not as fast as Bet v 4 allergens, so that in the case of this allergen, activity could still be detected in the pollen grain after 5 min, though greatly reduced. After 10 min of rehydration, birch pollen grains are largely free from Bet v 1 (micrographs not shown) [for a detailed analysis of Bet v 1 and profilin migration from birch pollen, see ref. 24].

Bet v 4 Can Be Detected in Aqueous Supernatants after a few Minutes of Pollen Hydration

In order to determine the release kinetics of Bet v 4 after pollen hydration, equal amounts of pollen were extracted in water for various time periods (30 s to 2 h). Exposure of equal amounts of nitrocellulose-blotted supernatant fractions to a rabbit anti-Aln g 4 antiserum which fully cross-reacts with Bet v 4 revealed by immunoblotting that this allergen can be faintly detected already 1 min after pollen hydration in the aqueous supernatant (fig. 13, α -rAln g 4). Likewise, the major birch pollen allergen, Bet v 1, was detected in the supernatant fractions after a few minutes of pollen hydration by the monoclonal anti-Bet v 1 antibody Bip 1 (fig. 13; Bip 1). Analysis of the pollen grain-containing pellet fractions showed that Bet v 4 was drained faster from the pollen grains than Bet v 1 after hydration (data not shown).

Discussion

By immunogold electron microscopy, we localized Bet v 4 in resting and hydrated pollen in order to obtain clues to its biological role. In the resting pollen, Bet v 4 accumulated between the vesicles and cisternae of the endoplasmic reticulum, in the mitochondria, in the vegetative nucleus and inside the generative cell and nucleus. Sparse labeling occurred also in the pollen wall. In regard to its cytoplasmic distribution, we could not definitely determine in each case if the Bet v 4 allergen was located inside or outside membrane-bound vesicles or cisternae, since on the section the labeled epitope may be located up to 20–30 nm (i.e. the length of two antibodies plus the radius of the gold particle) away from the gold marker. However, most of the gold markers were located in the cytosol, which in resting birch pollen is closely packed with ribosomes [23]. In summary, Bet v 4 is found in those areas in the pollen grain that are known to represent sites of protein metabolism and energy supply. Here, rapid metabolic and structural changes appear as soon as the pollen grain begins to germinate. Its distribution pattern distinguishes Bet v 4 from the other two birch pollen allergens hitherto localized *in situ*: Bet v 1, the birch pollen major allergen, and Bet v 2 (profilin). Both these allergens are confined to the cytosolic areas and the pollen nuclei [23, 24]. The broad intracellular distribution of Bet v 4 is not surprising considering its calcium-binding properties. Calcium-binding proteins either represent intracellular 'calcium buffers' which regulate the calcium homeostasis of the cell and provide calcium as a cofactor for a series of processes (e.g. cytoskeletal changes, signal transduction, muscle contraction) or they function as 'calcium sensor proteins' which act as calcium-sensing ligands for other partner proteins [25–28]. The main feature of the calcium sensor proteins is that calcium binding induces conformational changes in the structure of these proteins to allow binding of a ligand that cannot attach to the calcium-free form of the protein. In this context it is of note that the members of the Bet v 4 family can also occur in different conformations, i.e. the calcium-bound form and the apoform [28], which notably are recognized differently by IgE antibodies of sensitized patients. Most sensitized patients show more IgE antibodies directed against the calcium-bound form than against the apoform [14]. This is probably explained by the fact that during early sensitization a patient's immune system primarily comes into contact with the calcium-bound form of the allergen.

Immunogold electron microscopy and immunoblotting were used to monitor the kinetics of Bet v 4 allergen release from the pollen grain during hydration. Both methods

showed rapid allergen depletion from the pollen grains and the appearance of allergen in the supernatant. However, as far as the immunogold electron microscopic experiments are concerned, the actual period of rehydration of each pollen sample is not precisely known. The handling of the rehydrated pollen before exposure to the fixating aldehyde vapor and the chemical fixation process itself necessarily take some additional seconds during which allergen migration might have continued. While neither immunoblot nor immunogold electron microscopy represent quantitative methods, immunoblot analysis of supernatants allowed a semiquantitative comparison of the amounts of eluted Bet v 1 and Bet v 4. Bet v 4 was detected in the supernatants after 1 min of pollen hydration. Maximum release of Bet v 4 was found after 5 min of pollen hydration whereas maximum release of Bet v 1 occurred after 10 min. Consistent with the data obtained by immunogold electron microscopy, these findings indicate that Bet v 4 elutes more rapidly than Bet v 1. Our findings are in agreement with previous elution studies showing that prominent pollen allergens from trees (Bet v 2) and grasses (Phl p 1, Phl p 2, Phl p 5) elute within minutes from pollen grains [24, 29]. Thus, rapid elution represents a common feature of relevant pollen allergens.

In any case, the present investigations have clearly shown that Bet v 4, like many other pollen allergens, is found inside the pollen grain [30] and that it rapidly elutes from the pollen after hydration, e.g. under physiological conditions, when the pollen germinates, or under pathological conditions, when pollen enters the respiratory mucosa of the patient. The rapid elution of Bet v 4 out of the hydrated pollen may therefore be of twofold relevance: firstly, it may be responsible for the changes in intracellular calcium levels within the pollen grain which are known to dictate pollen germination and tip growth of the pollen tube [31–37]. Secondly, it also explains how this allergen, although primarily an intracellular protein, becomes available for the induction of IgE antibody responses in allergic individuals.

Acknowledgements

We thank Ms. Ursula Malkus and Ms. Gudrun Kieffermann for their skillful technical assistance. This study was supported in part by grant Y078GEN of the Austrian Science Fund and by the ICP program of the Austrian Ministry of Science and Transport.

References

- Valenta R, Niederberger V, Fischer S, Jäger S, Spitzauer S, Kraft D: Tree pollen allergens; in Lockey RF (ed): Allergen Immunotherapy, ed2. New York, Marcel Dekker, 1998, pp 85–102.
- Breiteneder H, Pettenburger K, Bito A, Valenta R, Kraft D, Rumpold H, Scheiner O, Breitenbach M: 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;8:1935–1938.
- Niederberger V, Pauli G, Grönlund H, Fröschl R, Rumpold H, Kraft D, Valenta R, Spitzauer S: 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* 1998;102:579–591.
- Ebner C, Hirschwehr R, Bauer L, Breiteneder H, Valenta R, Ebner H, Kraft D, Scheiner O: 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;95:962–969.
- 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;97:893–895.
- Jensen-Jarolim E, Leitner A, Hirschwehr R, Kraft D, Wüthrich B, Scheiner O, Graf J, Ebner C: Characterization of allergens in *Apiaceae* spices: Anise, fennel, coriander and cummin. *Clin Exp Allergy* 1997;27:1299–1306.
- Valenta R, Duchêne M, Pettenburger K, Sillaber C, Valent P, Bettelheim P, Breitenbach M, Rumpold H, Kraft D, Scheiner O: Identification of profilin as a novel pollen allergen; IgE autoreactivity in sensitized individuals. *Science* 1991;253:557–560.
- Valenta R, Duchêne M, Ebner C, Valent P, Sillaber C, Deviller P, Ferreira F, Tejkl M, Edelman H, Kraft D, Scheiner O: Profilins constitute a novel family of functional plant pan-allergens. *J Exp Med* 1992;175:377–385.
- Valenta R, Ferreira F, Grote M, Swoboda I, Vrtala S, Duchêne M, Deviller P, Meagher RB, McKinney E, Heberle-Bors E, Kraft D, Scheiner O: Identification of profilin as an actin-binding protein in higher plants. *J Biol Chem* 1993;268:22777–22781.
- Seiberler S, Scheiner O, Kraft D, Lonsdale D, Valenta R: Characterization of a birch pollen allergen, Bet v 3, representing a novel class of Ca²⁺ binding proteins: Specific expression in mature pollen and dependence of patients' IgE binding on protein-bound Ca²⁺. *EMBO J* 1994;13:3481–3486.
- Twardosz A, Hayek B, Seiberler S, Vangelista L, Elfman L, Grönlund H, Kraft D, Valenta R: Molecular characterization, expression in *Escherichia coli*, and epitope analysis of a two EF-hand calcium-binding birch pollen allergen, Bet v 4. *Biochem Biophys Res Commun* 1997;239:197–204.
- Engel E, Richter K, Obermeyer G, Briza P, Kungl AJ, Simon B, Auer M, Ebner C, Rheinberger H, Breitenbach M, Ferreira F: Immunological and biological properties of Bet v 4, a novel birch pollen allergen with two EF-hand calcium-binding domains. *J Biol Chem* 1997;272:28630–28637.
- Valenta R, Hayek B, Seiberler S, Bugajska-Schretter A, Niederberger V, Twardosz A, Natter S, Vangelista L, Pastore A, Spitzauer S, Kraft D: Calcium-binding allergens: From plants to man. *Int Arch Allergy Immunol* 1998;117:160–166.
- Smith PM, Xu H, Swoboda I, Singh MB: Identification of a Ca²⁺ binding protein as a new Bermuda grass pollen allergen Cyn d 7: IgE cross-reactivity with oilseed rape pollen allergen Bra r 1. *Int Arch Allergy Immunol* 1997;114:265–271.
- Suphioglu C, Ferreira F, Knox RB: Molecular cloning and immunological characterization of Cyn d 7, a novel calcium-binding allergen from Bermuda grass pollen. *FEBS Lett* 1997;402:167–172.
- Niederberger V, Hayek B, Vrtala S, Laffer S, Vangelista L, Sperr WR, Valent P, Rumpold H, Kraft D, Ehrenberger K, Valenta R: Calcium-dependent immunoglobulin E recognition of the apo- and calcium-bound form of a cross-reactive two EF-hand timothy grass pollen allergen, Phl p 7. *FASEB J*, in press.
- Toriyama K, Okada T, Watanabe M, Ide T, Ashida T, Xu H, Singh MB: A cDNA clone encoding an IgE-binding protein from *Brassica* anther has significant sequence similarity to Ca²⁺-binding proteins. *Plant Mol Biol* 1995;29:1157–1165.
- Hayek B, Vangelista L, Pastore A, Sperr WR, Valent P, Vrtala S, Niederberger V, Twardosz A, Kraft D, Valenta R: Molecular and immunological characterization of a highly cross-reactive two EF-hand calcium-binding alder pollen allergen, Aln g 4. Structural basis for calcium-modulated IgE recognition. *J Immunol* 1998;161:7031–7039.
- Batanero E, Villalba M, Ledesma A, Puente XS, Rodriguez R: Ole e 3, an olive-tree allergen, belongs to a widespread family of pollen proteins. *Eur J Biochem* 1996;241:772–778.
- Jarolim E, Tejkl M, Rohac M, Schlerka G, Scheiner O, Kraft D, Breitenbach M, Rumpold H: Monoclonal antibodies against birch pollen allergens: Characterization by immunoblotting and use for single-step affinity purification of the major allergen Bet v 1. *Int Arch Allergy Appl Immunol* 1989;90:54–60.
- Fling SP, Gregerson DJ: Peptide and protein molecular weight determination by electrophoresis using a high molarity Tris buffer without urea. *Anal Biochem* 1986;155:83–88.
- 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;76:4350–4354.
- Grote M: Ultrastructural morphology and allergen detection in birch pollen after aqueous, anhydrous-liquid, and vapor fixation techniques. *Microsc Res Tech* 1992;21:242–248.
- Grote M, Vrtala S, Valenta R: Monitoring of two allergens, Bet v 1 and profilin, in dry and rehydrated birch pollen by immunogold electron microscopy. *J Histochem Cytochem* 1993;41:745–750.
- Kawasaki H, Kretsinger RH: Calcium-binding proteins; in Kawasaki H, Kretsinger RH (eds): Protein Profile 1. San Diego, Academic Press, 1994, pp 343–390.
- Heizmann CW, Hunziker W: Intracellular calcium-binding proteins: More sights than insights. *Trends Biochem Sci* 1991;16:98–103.
- Ikura M: Calcium binding and conformational response in EF-hand proteins. *Trends Biochem Sci* 1996;21:14–17.
- Strynadka NC, James MN: Towards an understanding of the effects of calcium on protein structure and function. *Curr Opin Struct Biol* 1991;1:905–914.
- Vrtala S, Grote M, Duchêne M, van Ree R, Kraft D, Scheiner O, Valenta R: Properties of tree and grass pollen allergens: Reinvestigation of the linkage between solubility and allergenicity. *Int Arch Allergy Immunol* 1993;102:160–169.
- Grote M: In situ localization of pollen allergens by immunogold electron microscopy: Allergens at unexpected sites. *Int Arch Allergy Immunol* 1999;118:1–5.
- Brewbaker JL, Kwack BH: The essential role of calcium ion in pollen germination and pollen tube growth. *Am J Bot* 1963;50:859–865.
- Malho R, Read ND, Pais MS, Trewavas AJ: Role of cytosolic free calcium in the reorientation of pollen tube growth. *Plant J* 1994;5:331–341.
- Franklin-Tong VE, Ride JP, Read ND, Trewavas AJ, Franklin FC: The self-incompatibility response in *Papaver rhoeas* is regulated by cytosolic free calcium. *Plant J* 1993;4:163–177.
- Rathore KS, Cork RJ, Robinson KR: A cytoplasmic gradient of Ca²⁺ is correlated with the growth of lily pollen tubes. *Dev Biol* 1991;148:612–619.
- Miller DD, Callahan DA, Gross DJ, Hepler PK: Free Ca²⁺ gradient in growing pollen tubes of *Lilium*. *J Cell Sci* 1992;101:7–12.
- Pierson ES, Miller DD, Callahan DA, van Aken J, Hackett G, Hepler PK: Tip-localized calcium entry fluctuates during pollen tube growth. *Dev Biol* 1996;174:160–173.
- Messeri M, Robinson KR: Tip localized Ca²⁺ pulses are coincident with peak pulsatile growth rates in pollen tubes of *Lilium longiflorum*. *J Cell Sci* 1997;110:1269–1278.

Copyright: S. Karger AG, Basel 1999. Reproduced with the permission of S. Karger AG, Basel. Further reproduction or distribution (electronic or otherwise) is prohibited without permission from the copyright holder.