

Isolation of cDNA encoding a newly identified major allergenic protein of rye-grass pollen: Intracellular targeting to the amyloplast

(molecular cloning/IgE binding/*Lol pI* allergen/immunogold probes/*Lolium perenne*)

MOHAN B. SINGH*, TERRY HOUGH*, PIYADA THEERAKULPISUT[†], ASIL AVJIOGLU*, SEAN DAVIES*, PENELOPE M. SMITH*, PHILIP TAYLOR*, RICHARD J. SIMPSON[‡], LARRY D. WARD[‡], JAMES MCCLUSKEY[§], ROBERT PUY[§], AND R. BRUCE KNOX*[¶]

*School of Botany, University of Melbourne, Parkville, Victoria 3052, Australia; [†]Joint Protein Structure Laboratory, Ludwig Institute for Cancer Research (Melbourne Branch) and The Walter and Eliza Hall Institute for Medical Research, Parkville, Victoria 3052, Australia; and [§]Department of Pathology and Immunology, Monash University Medical School, Prahran, Victoria 3181, Australia

Communicated by Marshall D. Hatch, November 9, 1990 (received for review September 5, 1990)

ABSTRACT We have identified a major allergenic protein from rye-grass pollen, tentatively designated *Lol pIb* of 31 kDa and with pI 9.0. A cDNA clone encoding *Lol pIb* has been isolated, sequenced, and characterized. *Lol pIb* is located mainly in the starch granules. This is a distinct allergen from *Lol pI*, which is located in the cytosol. *Lol pIb* is synthesized in pollen as a pre-allergen with a transit peptide targeting the allergen to amyloplasts. Epitope mapping of the fusion protein localized the IgE binding determinant in the C-terminal domain.

Inhalation of pollen in the atmosphere provokes IgE-mediated responses of hay fever and allergic asthma in about 20% of humans (1, 2). Key species of pollen implicated in this response include grass, birch, and ragweed. Among the many grasses implicated in clinical responses, rye-grass produces the greatest abundance of pollen (3). In several studies, the major allergen, *Lol pI* (named according to International Union of Immunological Societies nomenclature, ref. 4), is shown to be the major IgE-binding protein and thus of paramount clinical significance (5, 6). There is no consensus about the physicochemical characteristics of *Lol pI*, particularly the apparent molecular mass, which ranges from 28 to 35 kDa (7). Elevated levels of IgE specific for this allergen have been detected in sera of up to 95% of grass pollen-allergic individuals (8). Antigenically cross-reacting proteins corresponding to *Lol pI*, known as the group I allergens, are present in the pollen of other clinically important grasses (9–12).

Although it has been assumed that *Lol pI* is a single protein, we have recently identified two different protein allergens in this region. The most abundant protein is tentatively designated *Lol pIa*, a glycoprotein of 35 kDa and pI 5.5, located in the pollen cytoplasm (13). A cDNA clone encoding *Lol pIa* has been isolated and characterized (14). In this paper, we report the cloning, expression, and sequencing of a full-length cDNA clone encoding a second allergen in the *Lol pI* region, tentatively designated *Lol pIb*.^{||}

MATERIALS AND METHODS

Plant Materials. Pollen of *Lolium perenne* was collected from plants flowering in Melbourne and was stored in liquid nitrogen until used.

Isolation of Pollen Proteins and Immunoblotting. Soluble proteins were extracted from rye-grass pollen by vigorous

shaking in 150 mM NaCl/10 mM phosphate, pH 7.2/1 mM phenylmethylsulfonyl fluoride on ice for 3 hr. Proteins from the leaf, root, and seed were extracted by grinding the tissue in 150 mM NaCl/10 mM phosphate, pH 7.2/1 mM phenylmethylsulfonyl fluoride. Conditions for electrophoresis and immunoblotting were essentially as described (12). Monoclonal antibodies (mAbs) 3.2 and 21.3 bind specifically to the *Lol pI* group of antigens (9); mAb 40.1, although originating in a different laboratory, shows similar specificity (12, 15). mAb 12.3 binds to a 31-kDa component in the *Lol pI* group (15). For IgE antibody binding, blots were incubated in allergic sera collected from at least four patients with high skin sensitivity for grass pollen, pooled and used diluted 1:5 in Tris-buffered saline (TBS: 150 mM NaCl/10 mM Tris-HCl, pH 7.5) containing 0.5% bovine serum albumin. The bound IgE was detected (8) using ¹²⁵I-labeled anti-human IgE (Kallestad Laboratories, Chaska, MN).

cDNA Library and Immunological Screening. Poly(A)⁺ mRNA was isolated essentially as described (16). cDNA was synthesized (17) and cloned into the *EcoRI* site of the vector λ gt11. Immunological screening was done by plating the cDNA library and screening duplicate filters with mAb 40.1, using peroxidase-labeled anti-mouse immunoglobulin as secondary antibody. The plaques that were antibody-positive on both of the duplicate filters were picked off, purified, then replated and tested for binding to other mAbs as well as IgE antibodies.

Nucleotide Sequencing. cDNA clone 12R was isolated from the phage, subcloned into pGEM-3Z vectors (Promega), mapped for restriction sites, and further subcloned as various-sized restriction fragments into pGEM vectors. DNA sequence was determined by double-stranded sequencing carried out by the dideoxy chain-termination method (18) using Sequenase (United States Biochemical) and T7 DNA polymerase (Pharmacia).

RNA Blotting. Samples (20 μ g) of total RNA from the various tissues were electrophoresed in the presence of formamide (19) in a 1.2% agarose/formaldehyde gel, the separated RNAs were transferred to Hybond-C Extra (Amersham), and the filters were baked at 80°C for 2 hr. The 1.2-kilobase (kb) 12R cDNA probe was oligolabeled with ³²P and hybridized with the nitrocellulose filter at 45°C in the presence of 50% formamide. The membrane was washed with 2 $\times$ SSC (1 $\times$ is 150 mM NaCl/15 mM sodium citrate, pH 7)

Abbreviation: mAb, monoclonal antibody.

[†]Present address: Department of Biology, Faculty of Science, Khon Kaen University, Khon Kaen, Thailand.

[¶]To whom reprint requests should be addressed.

^{||}The sequence reported in this paper has been deposited in the GenBank data base (accession no. M59163).

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

containing 0.1% SDS at room temperature (15 min) and then in $0.2\times$ SSC containing 1% SDS at 65°C (15 min). After a final rinse in $0.5\times$ SSC containing 0.1% SDS, the blots were autoradiographed.

Expression of Truncated Fragments. The full-length cDNA 12R and the restriction fragments 1H and 2P were subcloned into plasmid expression vector pGEX. The procedure for inducing fusion proteins and the preparation of bacterial lysates have been described (20). The lysates obtained were subjected to reducing SDS/15% PAGE followed by transfer to nitrocellulose membranes. These Western blots were probed with IgE antibodies and mAbs 40.1 and 12.3.

Affinity Purification of IgE Antibodies. For the preparation of affinity-purified IgE, a protein extract of *Escherichia coli* transformed with pGEX-12R was prepared and the proteins were immobilized on a 2.5-cm^2 nitrocellulose membrane strip. This strip was then incubated in pooled sera (as above), washed twice with TBS containing 0.1% Tween 20 and then with TBS only. The bound IgE antibodies were eluted with 0.1 M glycine hydrochloride, pH 2.6/1% bovine serum albumin and used to probe Western blots. Binding of IgE was visualized using ^{125}I -labeled goat anti-human IgE (Kallestad) followed by autoradiography (8).

Purification of Allergens for N-Terminal Sequencing. *Lol pI* allergens were isolated by preparative isoelectric focusing (Rotofor; Bio-Rad) followed by preparative SDS/PAGE (A.A. and M.B.S., unpublished data). Allergens were recovered from SDS/polyacrylamide gels by electrotransfer (90 V for 2 hr at 4°C) onto poly(vinylidene difluoride) (PVDF) membrane (Immobilon; Millipore) (21). Proteins were visualized on the membranes by staining with Coomassie brilliant blue R250. The membranes were washed extensively with deionized water (22) and subjected to sequence analysis (21, 22).

Immunoelectron Microscopy. Mature anthers were fixed under anhydrous conditions [0.1% (vol/vol) glutaraldehyde and 1% (wt/vol) paraformaldehyde in 2,2-dimethoxypropane] at 4°C for 2 hr and processed for transmission electron microscopy and immunolabeling (13). Label was silver-enhanced (23) to $\approx 60\text{-nm}$ particle size by using the silver enhancement kit (Amersham). Double immunolabeling was performed on sections incubated with mAb 12.3 followed by goat anti-mouse IgG adsorbed to colloidal gold (15-nm particle size, silver-enhanced to 40 nm) and then with a mixture of three mAbs, 3.2, 21.3, and 40.1, followed by goat-anti-mouse IgG adsorbed to colloidal gold (15-nm particle size).

RESULTS

Isolation of cDNA Clones. The cDNA library was screened initially with mAb 40.1. Eighteen positive clones were plaque-purified and were tested for IgE binding by using sera from grass pollen-allergic subjects. Four clones were selected as encoding proteins recognized by both *Lol pI*-specific mAbs and IgE antibodies.

Clone 12R was selected for further characterization and sequencing because of its size, 1.2 kb, and strong IgE binding. The cDNA- β -galactosidase fusion protein produced by the induction of lysogenic cultures of λ -12R was characterized by immunoblot analysis. The $\approx 146\text{-kDa}$ fusion protein was assumed to be composed of the 116-kDa β -galactosidase protein and a 30-kDa putative allergen fusion protein (data not shown).

Identity of Cloned Allergen 12R. Since the recombinant clone was obtained by use of mAbs, the specificity of these mAbs should provide evidence of its relationship to the native allergens. All the mAbs used in this study bound strongly to the *Lol pI* reference standard provided by the National Institute of Allergy and Infectious Diseases (National Institutes of Health, Bethesda, MD). Not all mAbs

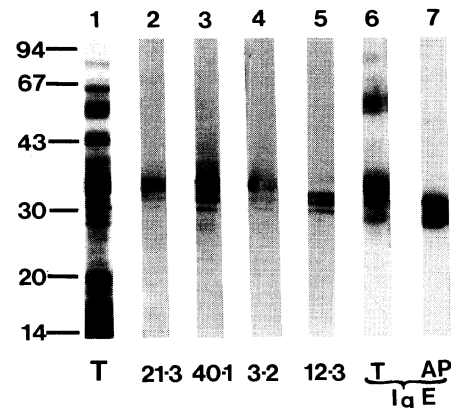


FIG. 1. Immunoblot analysis of mAbs and IgE binding to *Lol pI* antigens from rye-grass pollen. Molecular mass (kDa) is denoted at left. In all cases, $150\text{ }\mu\text{g}$ of protein was loaded per lane. Lane 1 shows SDS/PAGE separation of total rye-grass pollen proteins (Coomassie blue R250 staining); lanes 2-7 show binding of mAbs or IgE on Western blots of total rye-grass pollen proteins, detected using peroxidase-labeled secondary antibody except where indicated. Lane 2, mAb 21.3 (9); lane 3, mAb 40.1 (15); lane 4, mAb 3.2 (9); lane 5, mAb 12.3 (15); lane 6, total (T) IgE antibodies from pooled sera of grass pollen-allergic patients; and lane 7, IgE antibodies affinity-purified (AP) from recombinant allergen pGEX-12R, detected with ^{125}I -labeled anti-human IgE.

showed the same binding pattern in Western blots of rye-grass proteins (Fig. 1). mAbs 3.2, 21.3, and 40.1 showed reactivity with proteins in the 28- to 35-kDa region but reacted most strongly with the 35-kDa band. mAb 12.3 exhibited no binding to the 35-kDa band but bound strongly to the lower molecular mass bands. Because the cloned allergen bound to all the mAbs, including mAb 12.3, we predicted that it corresponds to a protein of lower molecular mass and not to the 35-kDa protein. To further confirm the identity of the cloned allergen, we used an immunological approach that involved the binding of specific IgE antibodies to the cloned allergen 12R immobilized on nitrocellulose membrane. Bound antibodies were eluted and used to probe a Western blot of rye-grass pollen proteins. Highly specific and reproducible patterns of binding to protein components in the lower part of the *Lol pI* range, $\approx 31\text{ kDa}$ (Fig. 1, lane 7), were consistently obtained in several experiments.

These experiments show that clone 12R represents a cDNA encoding an allergen that is smaller than the 35-kDa protein. This allergen is specifically recognized by mAb 12.3 and in Western blots this antibody binds to a doublet band, $31\text{--}33\text{ kDa}$ (Fig. 1). The nature of this multiple band is not clear, but it does not appear to be due to differential N-glycosylation. Enzymatic treatment of pollen antigen with either N-glycanase or endoglycosidase F did not alter the mobility of the proteins in SDS/PAGE (data not shown). These treatments did not affect IgE binding to the 31-kDa component.

Lol pIa and *Lol pIb* were purified by two-dimensional gel electrophoresis involving preparative isoelectric focusing in the first dimension, followed by SDS/PAGE of the individual fractions. Isoelectric focusing showed that the 31- and 35-kDa allergens differ in terms of their isoelectric points, pI 9.0 and 5.5, respectively (data not shown). To test whether these represent structurally different allergens, we obtained the N-terminal sequences as follows.

35-kDa protein: IAKVPPGPWITA EYGDKWLDAKXT

31-kDa protein: ADAGYTPAAXXPATPAXT

The sequence of the 35-kDa protein is similar to that reported for *Lol pI* (10) and shows no identity with the N-terminal

sequence of the 31-kDa protein. The 31-kDa protein consistently showed a doublet at 31–33 kDa by SDS/PAGE but yielded only a single N-terminal sequence. Accordingly, we

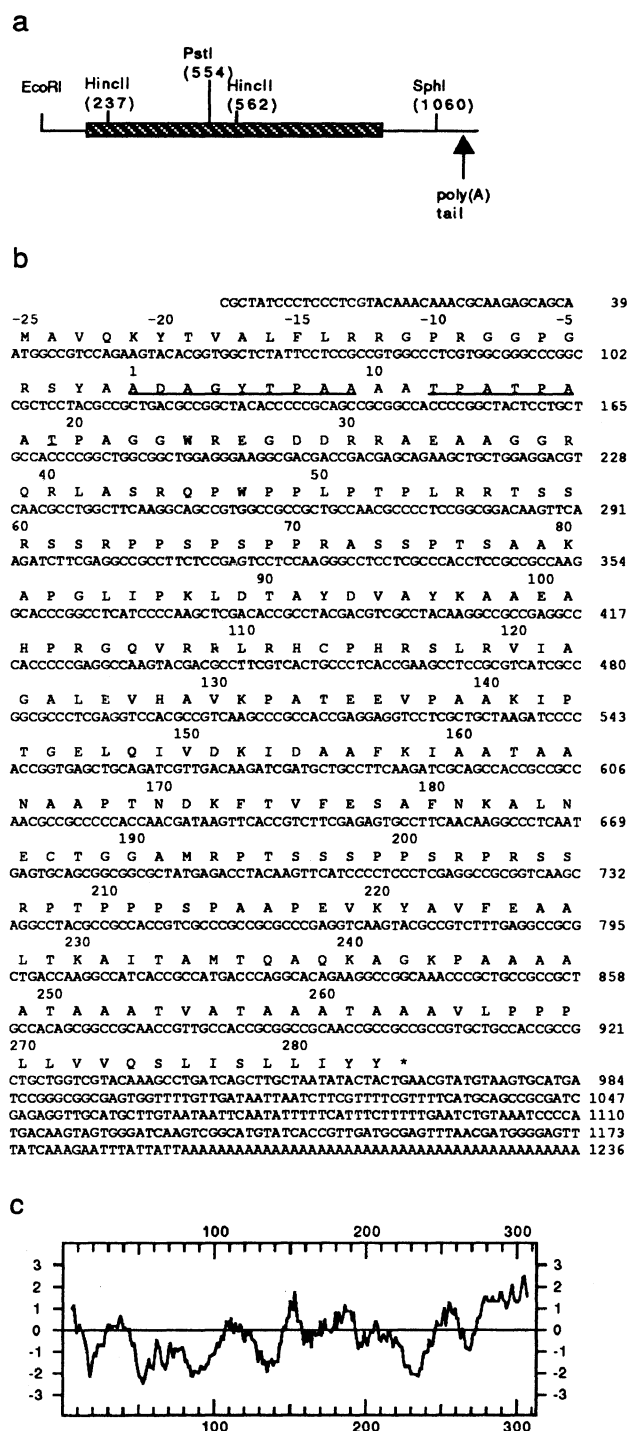


FIG. 2. cDNA, predicted amino acid sequence, and hydrophobicity profile of rye-grass pollen allergen *Lol pIb* (clone 12R). (a) Restriction map of λ-12R cDNA. Hatched box represents the predicted translation reading frame. (b) Nucleotide and deduced amino acid sequence of the 1238-nucleotide *EcoRI* cDNA insert λ-12R. The deduced amino acid sequence begins at the first potential in-frame initiation codon, at nucleotide 40. One uninterrupted open reading frame continues for 283 amino acids (number above the DNA sequence) and ends with the TGA stop codon denoted by the asterisk. The putative transit peptide is indicated by negative numbers. Underlined amino acids were identified by Edman degradation. (c) Hydrophobicity profile of predicted amino acid sequence based on method of Kyte and Doolittle (24), with a window of seven amino acids.

tentatively designate the 35-kDa component as *Lol pIa* (a for acidic pI) and the 31-kDa component as *Lol pIb* (b for basic pI). We conclude from all this evidence that clone 12R encodes *Lol pIb*.

The nucleotide sequence of the *Lol pIb* cDNA clone is G+C-rich (68% G+C; Fig. 2a and b). The open reading frame (924 bases) potentially encodes a protein of 308 amino acids, 34.1 kDa. The predicted protein sequence, rich in alanine (23%) and proline (13%), contains the N-terminal sequence defined by protein microsequencing. This suggests the presence of a putative signal peptide of 25 amino acids and a mature processed protein of 31.3 kDa.

A search of existing data bases (GenBank, Swiss-Prot, EMBL; November 1990) showed no similarity between the deduced amino acid sequence of *Lol pIb* and any other known protein. Also, there are no potential N-glycosylation sites as depicted by the absence of the characteristic Asn-Xaa-(Ser/Thr) motif. We conclude that the allergen encoded by clone 12R represents a major newly identified allergen, *Lol pIb*.

Pollen-Specific Expression of Allergens. Northern blot analysis of RNA prepared from pollen showed high levels of the 1.2-kb transcripts hybridizing to the cloned allergen gene. Similar hybridizing transcripts were not detectable in RNA from vegetative tissues (Fig. 3a). Pollen-specific RNA expression correlated with pollen-specific expression of antigens recognized by mAbs 40.1 and 12.3 and by IgE antibodies (Fig. 3b). Specific antibody binding occurred only when pollen and floral tissues (containing pollen) were used as protein source.

Delineation of IgE- and mAb-Reacting Epitopes. To localize antigenic and allergenic determinants, an *E. coli* recombinant expression system, pGEX 1–3, was employed (20). These vectors are designed to direct the synthesis of the 1.2-kb *Lol pIb* cDNA insert in *E. coli* fused with the C terminus of S₂₆, a 26-kDa glutathione *S*-transferase of the parasitic helminth *Schistosoma japonicum*. In-frame subcloning of full-length cDNA into pGEX resulted in expression of a 61-kDa fusion protein recognized both by IgE and by mAbs 40.1 and 12.3. Immunoblot analysis showed that most of the fusion protein produced was cleaved by bacterial proteases near its fusion site with glutathione-*S* transferase, generating breakdown products that were recognized by IgE antibodies (Fig. 4).

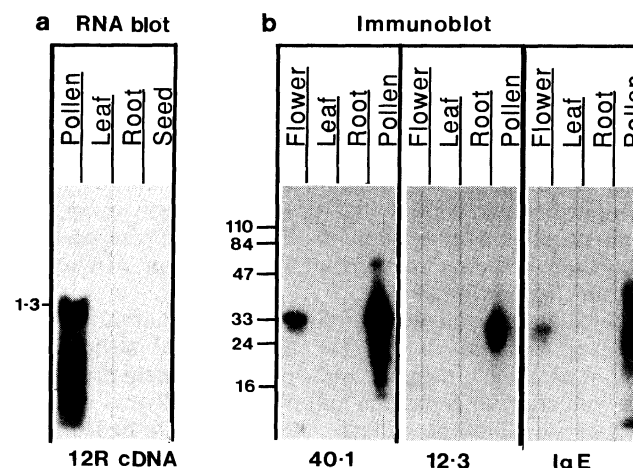


FIG. 3. Tissue-specific expression of *Lol pIb* allergen. (a) Northern blot analysis of total RNA from rye-grass pollen, leaves, roots, and seeds with 12R cDNA as a probe. (b) Immunoblot analysis of proteins showing tissue-specific distribution of *Lol pI* antigens. Twenty micrograms of soluble protein was loaded per lane. Probes were mAbs 40.1 (preferential binding for *Lol pIa*) and 12.3 (specific for *Lol pIb*), detected with ¹²⁵I-labeled anti-mouse immunoglobulin (Amersham), and IgE from pooled grass pollen-allergic patients' sera, detected with ¹²⁵I-labeled anti-human IgE.

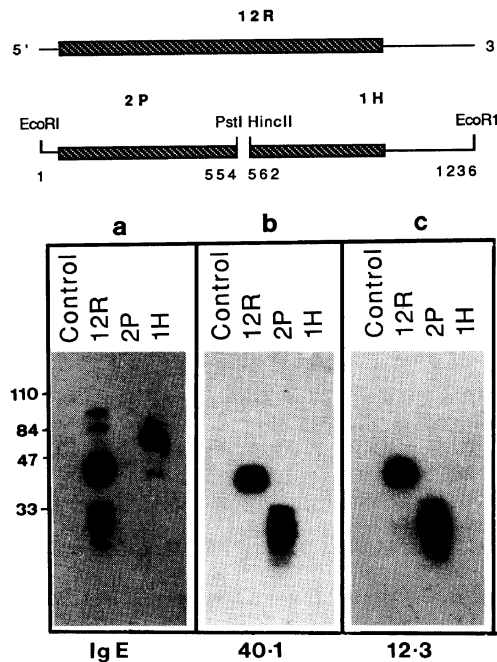


FIG. 4. Delineation of IgE- and mAb-reacting epitopes by immunoblotting of fusion proteins encoded by full-length clone 12R and truncated clones 1H and 2P expressed in pGEX vector (illustrated schematically at top). The size of the truncated fragments is indicated in base pairs. Twenty micrograms of bacterial lysate protein was loaded per lane. Detection used ^{125} I-labeled secondary antibodies. Probes were IgE from grass pollen-allergic patients' sera (a), mAb 40.1 (b), and mAb 12.3 (c). Control lanes contained lysates of bacterial cells transformed with nonrecombinant plasmids.

The recombinant fusion protein expressed by fragment 2P (corresponding to the N-terminal portion of the allergen), although strongly reactive with both mAbs, was not recognized by IgE antibodies in pooled allergic sera. However, the fragment 1H (corresponding to the C-terminal portion of the allergen) was not recognized by either of the mAbs but was highly reactive with the IgE antibodies.

These data suggest that there are two distinct domains of *Lol pIb* antigen: (i) the N-terminal fragment 2P, possessing recognition sites for mAbs 12.3 and 40.1, (ii) the C-terminal fragment 1H, which revealed strong IgE binding and thus carries the allergenic determinant(s). Because the two mAbs have differential reactivity with *Lol pIa* and *pIb* (Fig. 1), the recognition sites for the two mAbs are likely to be different, even though they both map to the same fragment.

Intracellular Targeting of *Lol pIb* in Rye-Grass Pollen. *Lol pIa* was located in the cytosol (Fig. 5) by using immunogold probes with mAbs 3.2, 21.3, and 40.1 (13). In contrast, mAb 12.3, specific for *Lol pIb*, bound mostly to the starch granules (Fig. 5). Mature grass pollen (Fig. 5) is filled with starch granules that are up to 2.5 μ m in size and originate in the amyloplasts. The mixed mAbs preferentially recognized native *Lol pIa* *in situ*, even though they crossreacted with denatured *Lol pIb* in immunoblots. Presumably this reflects a difference in the affinity of these mAbs for *Lol pIa* compared with *Lol pIb*.

The localization of *Lol pIb* in the starch granules implies that this protein should be transported from the cytosol to the lumen of the amyloplasts during development. For transport to plastids, the proteins that are synthesized in the cytosol are synthesized as precursors containing a transit peptide sequence that is cleaved after transport into the organelle.

The transit peptide sequence of *Lol pIb* (Fig. 2b) has features that are typical of other eukaryotic transit peptide sequences (25); namely, both N and C termini are flanked by short relatively hydrophilic sequences. The transit peptide of

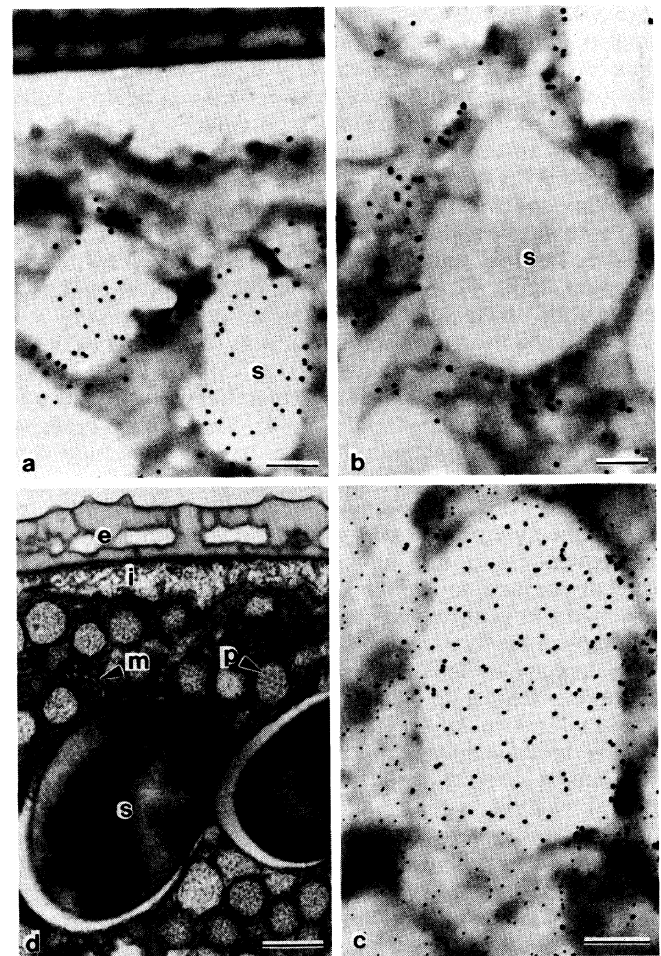


FIG. 5. Detection of *Lol pIa* and *Lol pIb* in mature pollen of rye-grass by anhydrous fixation (13) followed by specific mAbs and immunogold probes. (a-c) Localization of allergens by use of immunogold probes on unstained thin sections: a, typical binding of mAb 12.3, specific for *Lol pIb*, predominantly to starch granules (s); b, typical binding of mixed mAbs 3.2, 21.3, and 40.1, specific for *Lol pIa*, predominantly to the cytosol (electron-opaque regions of cytoplasm); c, double labeling to detect cellular sites of *Lol pIa* and *Lol pIb* on the same section. The large gold particles show binding of mAb 12.3 to *Lol pIb*, while smaller particles indicate binding to *Lol pIa*. (d) Transmission electron micrograph of thin section of rye-grass pollen grain poststained with uranyl acetate and lead citrate, showing structural features: starch granules (s), polysaccharide particles (p), mitochondria (m), exine (e), and intine (i). (Bar = 0.5 μ m in a-d.)

most chloroplast-targeted proteins possesses a loosely defined cleavage-site consensus sequence: (Val/Ile)-Xaa-(Ala/Cys) $\downarrow$ Ala (25). Arginine is also found in positions -6 to -10. The corresponding motif in the *Lol pIb* transit peptide sequence is Ser-Tyr-Ala $\downarrow$ Ala in this position, and there are two arginine residues at positions -4 and -9 (Fig. 2b). We conclude that the *Lol pIb* molecule first is synthesized as a pre-allergen in the cytosol and then is transported to the amyloplast for posttranslational modification.

DISCUSSION

As a result of molecular cloning of *Lol pI*, we have identified a major new allergenic protein, tentatively designated as *Lol pIb*, a 31-kDa protein, pI 9.0. A cDNA clone encoding *Lol pIb* (12R) has been isolated, sequenced, and characterized. *Lol pIb* is located mainly in the starch granules. This is a distinct allergen from that previously reported as *Lol pI* (i.e., *Lol pIa*), which is located in the cytosol (13).

Lol pIb is synthesized in pollen as a pre-allergen with a transit peptide that targets the allergen to amyloplasts. The only previously reported amyloplast-targeting transit peptide is that of *waxy* protein in maize (26). This peptide mediated protein transport into chloroplasts *in vitro*.

Lol pIa is an allergen of major clinical importance because sera from 95% of grass pollen-allergic patients have IgE antibodies specific for this allergen (8). In a similar study using both native and recombinant *Lol pIb*, >93% of patients had specific IgE antibodies to this allergen (P.M.S., C. Suphioglu, R.P., N. Siemensma, and M.B.S., unpublished data). On this basis, *Lol pIb* is a major allergen.

We have reported the complete amino acid sequence of *Lol pIa* (14). The complete sequences of *Lol pII* and *Lol pIII* (each 97 residues; ref. 27) show a high degree of identity to the C-terminal domain of *Lol pIa* (14). These may represent a family of related proteins. *Lol pIb* has no homology with any of these three allergens. A possibly analogous case occurs in Kentucky blue grass, where *Poa pIb*, 33 kDa, pI 9.1, has been identified using mAbs (28). Although no amino acid sequence data are available for *Poa pIb*, its physicochemical characteristics appear similar to those of *Lol pIb*. These two allergens may possibly represent a conserved class of allergens in related grasses.

We emphasize that the nomenclature for *Lol pIa* and *pIb* used here is tentative, based on the physicochemical nature of these allergens and on their immunochemical relatedness. The N-terminal sequence of a newly identified major allergen from timothy grass, *Phl pV* (29), shows 50% similarity (20% identity) with the N-terminal sequence of *Lol pIb*.

The location of *Lol pIb* in the starch granules is intriguing. When rye-grass pollen encounters water, the grains burst, releasing ≈ 1000 starch granules from each grain. We speculate that these starch granules may be the micronic fraction, reported as asthma-triggering particles containing grass pollen allergen whose nature and origin are unknown (30). This mode of presentation of grass pollen allergen to the immune system represents a massive amplification of the environmental allergen signal.

There is as yet no clue to the natural role of *Lol pIb* in pollen. The deduced amino acid sequence of *Lol pIb* has no known identity with other sequenced proteins. This allergen shows pollen-specific expression and accumulates in the starch granules of mature pollen, suggesting that its role may be in starch mobilization during pollen germination and tube growth. The structure-function relationships of grass pollen allergens can now be approached using molecular cloning. This will advance our understanding of how these environmental molecules provoke the "number one environmental disease" (1).

We thank Dr. Ian Smart, Prof. John Bradley, and colleagues at Flinders Medical Centre for generous donation of mAbs 12.3 and 40.1; Prof. David Marsh of Johns Hopkins University, Baltimore, for a generous gift of *Lol pI*-specific mAbs 3.2 and 21.3; the National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD, for a gift of the reference preparation of *Lol pI*; Dr. David Hill of the Children's Allergy Centre, Royal Children's Hospital, for a generous gift of grass pollen-allergic patient sera; Dr. Graham Mitchell, Dr. Barbara Howlett, Shao-Lim Mau, Dr. Chen Chaoguang, and Dr. Ian Staff for advice at various stages of the

project; and the Australian National Health and Medical Research Council, Australian Research Council, Asthma Foundation of Victoria, Immulogic Pharmaceutical Corporation, Baker Shaw Trust, Wenkart Foundation, and Ian Potter Foundation for financial support.

- Wuthrich, B. (1989) *Int. Arch. Allergy Appl. Immunol.* **90**, 3–10.
- Fleming, D. M. & Crombie, D. L. (1987) *Br. Med. J.* **294**, 279–283.
- Smart, I. J., Tuddenham, W. G. & Knox, R. B. (1979) *Aust. J. Bot.* **27**, 333–342.
- Marsh, D. G., Goodfriend, L., King, T. P., Lowenstein, H. & Platts-Mills, T. A. E. (1988) *Clin. Allergy* **18**, 201–209.
- Johnson, P. & Marsh, D. G. (1966) *Immunochemistry* **3**, 101–110.
- Marsh, D. G. (1975) in *The Antigens*, ed. Sela, M. (Academic, London), Vol. 3, pp. 271–359.
- Singh, M. B., Smith, P. M. & Knox, R. B. (1990) *Monogr. Allergy* **28**, 101–120.
- Ford, D. & Baldo, B. A. (1986) *Int. Arch. Allergy Appl. Immunol.* **81**, 193–203.
- Kahn, C. R. & Marsh, D. G. (1986) *Mol. Immunol.* **23**, 1281–1288.
- Cottam, G. P., Moran, D. M. & Standring, R. (1986) *Biochem. J.* **234**, 305–310.
- Esch, R. E. & Klapper, D. G. (1987) *J. Allergy Clin. Immunol.* **79**, 489–495.
- Singh, M. B. & Knox, R. B. (1985) *Int. Arch. Allergy Appl. Immunol.* **78**, 300–304.
- Staff, I. A., Taylor, P. E., Smith, P. M., Singh, M. B. & Knox, R. B. (1990) *Histochem. J.* **22**, 276–290.
- Griffith, I. J., Smith, P. M., Pollock, J., Theerakulpisut, P., Avcioglu, A., Davies, S., Hough, T., Singh, M. B., Simpson, R. J., Ward, L. D. & Knox, R. B. (1991) *FEBS Lett.*, in press.
- Smart, I. J., Hedde, R. J., Zola, M. & Bradley, J. (1983) *Int. Arch. Allergy Appl. Immunol.* **72**, 243–248.
- Herrin, D. & Michaels, A. (1984) *Plant Mol. Biol. Rep.* **2**, 24–29.
- Gubler, U. & Hoffman, B. J. (1983) *Gene* **25**, 263–269.
- Sanger, F., Nicklen, S. & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 5463–5468.
- Maniatis, T., Fritsch, E. G. & Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab., Cold Spring Harbor, NY).
- Smith, D. B. & Johnson, K. S. (1988) *Gene* **67**, 31–40.
- Ward, L. D., Reid, G. E., Moritz, R. L. & Simpson, R. J. (1990) in *Current Research in Protein Chemistry*, ed. Villafraña, J. J. (Academic, New York), pp. 179–193.
- Ward, L. D., Hong, J., Whitehead, R. H. & Simpson, R. J. (1991) *Electrophoresis*, in press.
- Danscher, G. & Norgaard, J. O. R. (1983) *J. Histochem. Cytochem.* **31**, 1394–1398.
- Kyte, J. & Doolittle, R. F. (1982) *J. Mol. Biol.* **157**, 105–132.
- Gavel, Y. & von Heijne, G. (1990) *FEBS Lett.* **261**, 455–458.
- Klößen, R. B., Saedler, H. & Weil, J.-H. (1989) *Mol. Gen. Genet.* **217**, 155–161.
- Ansari, A. A., Shenbagamurthi, P. & Marsh, D. G. (1989) *J. Biol. Chem.* **264**, 11181–11185.
- Lin, Z., Ekramoddoullah, A. K. M., Kisil, F. T., Hebert, J. & Mourat, W. (1988) *Int. Arch. Allergy Appl. Immunol.* **87**, 294–300.
- Klysner, S., Matthiessen, F. & Lowenstein, H. (1989) *Allergologie Suppl.*, 31.
- Spiekma, F. T. M., Kramps, J. A., Van der Linden, A. C., Nikkels, B. H., Plomp, A., Koerten, H. K. & Dijkman, J. H. (1990) *Clin. Exp. Allergy* **20**, 273–280.